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Role of Mutations in DNA Gyrase Genes in Ciprofloxacin Resistance of *Pseudomonas aeruginosa* Susceptible or Resistant to Imipenem

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In *Pseudomonas aeruginosa*, resistance to imipenem is mainly related to a lack of protein OprD and resistance to fluoroquinolones is mainly related to alterations in DNA gyrase. However, strains cross resistant to fluoroquinolones and imipenem have been selected in vitro and in vivo with fluoroquinolones. We investigated the mechanisms of resistance to fluoroquinolones in 30 clinical strains of *P. aeruginosa* resistant to ciprofloxacin (mean MIC, >8 μg/ml), 20 of which were also resistant to imipenem (mean MIC, >16 μg/ml). By immunoblotting, OprD levels were markedly decreased in all of the imipenem-resistant strains. Plasmids carrying the wild-type *gyrA* gene (pPAW207) or *gyrB* gene (pPBW801) of *Escherichia coli* were introduced into each strain by transformation. MICs of imipenem did not change after transformation, whereas those of ciprofloxacin and sparfloxacin dramatically decreased (25- to 70-fold) for all of the strains. For 28 of them (8 susceptible and 20 resistant to imipenem), complementation was obtained with pPAW207 but not with pPBW801. After complementation, the geometric mean MICs of ciprofloxacin and sparfloxacin (MICs of 0.3 μg/ml and 0.5 μg/ml, respectively) were as low as those for wild-type strains. Complementation was obtained only with pPBW801 for one strain and with pPAW207 and pPBW801 for one strain highly resistant to fluoroquinolones. These results demonstrate that in clinical practice, *gyrA* mutations are the major mechanism of resistance to fluoroquinolones even in the strains of *P. aeruginosa* resistant to imipenem and lacking OprD, concomitant resistance to these drugs being the result of the addition of at least two independent mechanisms.

Fluoroquinolones and carbapenems represent major progress in antimicrobial therapy for *Pseudomonas aeruginosa* infections. Because of good antibacterial activity, tissue diffusion, and oral bioavailability, fluoroquinolones have been widely used. However, the intensive use of fluoroquinolones has led to the selection of quinolone-resistant strains of *P. aeruginosa* (30, 39). Imipenem, a carbapenem, has also been widely used in hospitals, and imipenem-resistant strains of *P. aeruginosa* have been readily selected (23, 32). Since there is generally no cross-resistance between fluoroquinolones and other antibiotics, fluoroquinolones have been used for the treatment of infections caused by strains of *P. aeruginosa* already resistant to β-lactams, leading to the emergence of strains with associated resistance to quinolones and β-lactams, including imipenem. As an example, 22% of clinical strains of *P. aeruginosa* isolated in 1991 in French intensive care units were resistant to imipenem and half of them were also resistant to ciprofloxacin (19).

Three mechanisms of resistance are known to cause quinolone resistance in *P. aeruginosa* (3, 39): alteration in DNA gyrase by mutations in *gyrA* or *gyrB* genes, decreased drug accumulation by decreased permeability of the cell wall, and enhanced efflux (22). *gyrA* mutations appear to be the most prominent cause of resistance in clinical strains (40, 41). Resistance of *P. aeruginosa* to imipenem is mainly due to decreased expression of OprD, an outer membrane protein (OMP) known to facilitate specifically the permeation of several carbapenems (2, 16, 38). Quinolone resistance and carbapenem resistance resulting from the two mutational events described above are unlikely to occur simultaneously. However, recently, some one-step in vitro mutants of *P. aeruginosa* selected for quinolone resistance were also found to be resistant to imipenem (1, 10, 25, 26, 28, 33), raising the question of a common mechanism of resistance. It has been suggested that OprD could facilitate the diffusion of some fluoroquinolones (27), a suggestion that has given rise to controversies by other authors on the basis of genetic studies (16).

The question of the mechanism involved in fluoroquinolone resistance in the strains of *P. aeruginosa* also resistant to carbapenems is of clinical importance. Indeed, concomitant gyrase alteration and lack of OprD would demonstrate the accumulation of distinct mechanisms of resistance, whereas absence of the former mechanism would suggest the possibility of a single event leading to both resistances. In the present study we investigated the mechanism of acquired resistance to fluoroquinolones in clinical strains of *P. aeruginosa* susceptible or resistant to imipenem. By using complementation tests with wild-type *gyrA* and *gyrB* genes, we detected mutations in DNA gyrase genes in all of the strains studied, including those resistant to imipenem which lacked OprD, demonstrating that in clinical practice, concomitant resistance to fluoroquinolones and to imipenem in *P. aeruginosa* is the result of at least two independent mechanisms.

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MATERIALS AND METHODS

Strains. Thirty strains of *P. aeruginosa* resistant to ciprofloxacin (MIC, >1 μg/ml), collected between 1990 and 1992 from clinical specimens in our hospital,

TABLE 1. Distribution of ciprofloxacin and sparfloxacin MICs for the 32 clinical strains of *P. aeruginosa* used in this study

Relevant phenotype	No. of strains for which the MIC (μg/ml) was:									
	0.25	0.5	1	2	4	8	16	32	64	128
Imipenem susceptible (MIC range, 0.5–4 μg/ml)										
Ciprofloxacin	2[a]	0		1	3	2		1	3	
Sparfloxacin	1[a]	0	1[a]			1	2	3	1	3
Imipenem resistant (MIC range, 8–64 μg/ml)										
Ciprofloxacin		0		2	2	1	4	6	5	
Sparfloxacin		0			1	3	1	10	3	2

[a] Control strain(s) susceptible to ciprofloxacin.

were included in the study. For technical reasons (see "Transformation"), these strains were chosen for their susceptibility to ticarcillin. The strains were either susceptible ($n = 10$) or resistant (MIC, >8 μg/ml) ($n = 20$) to imipenem. Two wild-type clinical strains were included as controls. Identification was performed by standard methods with the API 20 NE identification system (bioMérieux, La Balme les Grottes, France), a positive oxidase test, and pigment production.

Transformation. Plasmids pPAW207 (40), carrying the wild-type *gyrA* gene of *Escherichia coli* K-12, and pPBW801 (40), carrying the wild-type *gyrB* gene of *E. coli* KL-16, were kindly given by Hiroaki Yoshida. Each of these plasmids, which carry a ticarcillin resistance gene, was introduced into each of the 32 strains of *P. aeruginosa* by transformation by the $CaCl_2$ method (12). Briefly, 20 ml of bacteria cultured in brain heart infusion broth (4-h incubation at 37°C with shaking) was centrifuged (4,000 × *g*) for 10 min at 4°C. The pellet was suspended in an equal volume of cold $CaCl_2$ (50 mM) and incubated for 30 min at 4°C. After centrifugation (4,000 × *g*) for 10 min at 4°C, cells were suspended in 1/10 volume of cold $CaCl_2$, and incubated overnight at 4°C to become competent. Two hundred microliters of competent cells was mixed with 25 to 100 ng of plasmid pPAW207 or pPBW801 DNA and incubated for 30 min at 4°C before heat shock (2 min at 42°C). After 90 min of incubation at 37°C, cells were spread onto brain heart infusion agar plates containing ticarcillin (1,000 μg/ml). Transformants obtained after 24 to 48 h of incubation at 37°C were subcultured on brain heart infusion agar containing ticarcillin (1,000 μg/ml).

MIC determination. MICs of ciprofloxacin (Bayer Pharma, Puteaux, France), sparfloxacin (Specia, Paris, France), imipenem (Merck Sharp & Dohme-Chibret, Paris, France), and meropenem (Zeneca, Cergy, France) for the 32 strains and the 64 transformants were determined by the dilution method on Mueller-Hinton agar (Difco) with an inoculum of ca. 10^4 bacteria per spot. To determine the MICs for transformants, ticarcillin (1,000 μg/ml) was added to the agar in order to maintain the plasmids.

Immunoblotting. For all of the strains resistant to imipenem, the presence of protein OprD was determined by immunoblotting as previously described (8, 38). Briefly, OMP preparations underwent sodium dodecyl sulfate-polyacrylamide gel electrophoresis (11% polyacrylamide). Proteins were transferred to a nitrocellulose membrane with Trans-blot (Bio-Rad). The membranes were probed with polyclonal anti-OprD antibodies obtained from rabbits (38), and the bound antibodies were detected with goat anti-rabbit antibodies conjugated to alkaline phosphatase.

RESULTS

Antimicrobial susceptibility. As can be calculated from Table 1, the levels of resistance (geometric mean MICs) in the 10 imipenem-susceptible and 20 imipenem-resistant strains were 10 and 19 μg/ml, respectively, for ciprofloxacin and 30 μg/ml for sparfloxacin. The range of imipenem and meropenem MICs was 8 to 64 μg/ml (mean, 19 μg/ml) for the carbapenem-resistant strains.

Immunoblotting with anti-OprD antibodies. OprD was undetectable in all of the imipenem-resistant strains except for one strain (MICs of imipenem and meropenem, 8 μg/ml) for which a small amount of OprD was observed (data not shown).

Complementation tests with pPAW207 (*gyrA*$^+$) and pPBW801 (*gyrB*$^+$). The 32 strains for which transformants were obtained with both plasmids represented a third of the 100 clinical strains subjected to transformation. Geometric mean imipenem and meropenem MICs were similar for the strains and their respective transformants bearing plasmid pPAW207 or pPBW801 (Table 2 for pPAW207 and results not shown for pPBW801), and only minor changes (≤2-fold dilution) in MICs were observed for individual strains.

In contrast, the MICs of quinolones dramatically decreased after transformation with the pPAW207 or pPBW801 plasmid for all of the strains (Table 2). For 28 of them, 8 susceptible and 20 resistant to imipenem, quinolone MICs decreased after introduction of pPAW207 but not after introduction of pPBW801. For these 28 strains, the mean decreases in quinolone MICs were comparable for the strains susceptible to imipenem (24- and 69-fold decreases in ciprofloxacin and sparfloxacin MICs, respectively) and for those resistant (58- and 60-fold decreases). For one strain susceptible to imipenem, MICs of ciprofloxacin and sparfloxacin decreased only after introduction of pPBW801. For the remaining strain, also susceptible to imipenem but with a very high level of resistance to quinolones, the same 1,000-fold decrease in the MICs of ciprofloxacin and sparfloxacin was observed after introduction of either pPAW207 or pPBW801 (Table 2). All of the strains, whether imipenem resistant or susceptible, were fully complemented by the *gyrA* (or *gyrB*) allele; i.e., the transformants became as susceptible as the wild-type strains. Finally, the susceptibility to ciprofloxacin and sparfloxacin of the two control strains remained unchanged after transformation. Geometric mean MICs of ciprofloxacin and sparfloxacin for transformants bearing pPAW207 were similar to those for strains susceptible and strains resistant to imipenem (Table 2). However, the difference between ciprofloxacin MICs (but not between sparfloxacin MICs) for the strains before and after transformation was larger for imipenem-resistant strains than

TABLE 2. Geometric mean MICs of carbapenems and fluoroquinolones for the 30 quinolone-resistant strains of *P. aeruginosa* and their transformants carrying wild-type *gyrA* or *gyrB* alleles

Relevant phenotype (*n*)	Allele yielding complementation (*n*)	MIC (μg/ml) before and after introduction of plasmid[a]							
		Imipenem		Meropenem		Ciprofloxacin		Sparfloxacin	
		Before	After	Before	After	Before	After	Before	After
Imipenem susceptible (10); MIC range, 0.5–4 μg/ml	*gyrA* (8)[b]	2	1.6	2.2	1.6	7.3	0.3	29.4	0.5
	gyrB (1)	1	1	2	2	8	0.12	32	0.12
	gyrA or *gyrB* (1)	0.5	1	2	1	128	0.12	>128	0.12
Imipenem resistant (20); MIC range, 8–64 μg/ml	*gyrA* (20)[b]	19	16	19	16.6	19	0.3	29.9	0.5

[a] Before and after introduction of the plasmid carrying wild-type *gyrA* (pPAW207) or *gyrB* (pPBW801) alleles.
[b] Geometric mean.

for imipenem-susceptible strains, because of the two- to threefold higher initial level of resistance to ciprofloxacin of the former strains.

DISCUSSION

Complementation tests, widely used for studying quinolone resistance in gram-negative bacilli, including *P. aeruginosa* (14, 29, 34, 40, 41), rely upon the dominance of the wild-type allele of DNA gyrase genes initially described with a diploid *gyrA* strain (13). Complementation is also observed when a multicopy plasmid bearing the wild-type allele is introduced in resistant strains (14, 34, 40). When quinolone-resistant strains with mutations involving genes other than *gyrA* or *gyrB*, e.g., *cfxB* (*nalB*) (34, 40) or *nfxB* (21, 40, 41), are transformed by DNA gyrase genes, reversion to susceptibility is not observed. It has been suggested that DNA gyrase genes could also complement mutations in topoisomerase IV (second target of quinolones) genes in *E. coli* (36), which could be explained by the homology between the two proteins. When susceptible DNA gyrase genes of *E. coli* are introduced into wild-type strains of *P. aeruginosa*, the level of susceptibility to ciprofloxacin remains unchanged (40). This fact was recently confirmed by Kureishi et al. (21), who demonstrated that *gyrA* genes of *E. coli* or *P. aeruginosa* can complement in a similar manner *E. coli* or *P. aeruginosa gyrA* mutants.

In order to investigate the mechanisms of quinolone resistance in *P. aeruginosa*, Yoshida et al. constructed and successfully used plasmids carrying *gyrA* or *gyrB* alleles of *E. coli* and replicating in *P. aeruginosa* (40). By using these plasmids, we transformed ciprofloxacin-resistant strains of *P. aeruginosa*, either imipenem susceptible or resistant. The relatively low efficiency of transformation obtained was in fact comparable to results obtained by others working with *P. aeruginosa* with the same or similar plasmids, even when they used methods other than $CaCl_2$ ($MgCl_2$ or electroporation) (18, 41). It could be suspected that strains which could not be transformed had surface alterations impeding transformation and causing quinolone resistance, resulting in a bias in the strain selection. However, results by others do not support such a supposition, since quinolone-resistant strains having *nfxB* or *nfxC* phenotypes, and thus likely to be permeability mutants, have been successfully transformed with $CaCl_2$ (40) or by another method (18, 41). Therefore, limited efficiency of transformation is probably the result of incompatibility between the new plasmid and plasmids already present in clinical strains or, alternatively, of the relative incompetence of *P. aeruginosa* cells (e.g., we obtained only 1 to 50 transformants per 100 ng of DNA plasmid).

Despite these limitations, complementation techniques are very useful for studying large numbers of strains. By using these techniques, we showed that mutations in DNA gyrase genes were the main mechanism of resistance in clinical strains of *P. aeruginosa* resistant to ciprofloxacin even when imipenem resistance was associated. Indeed, all of the 30 ciprofloxacin-resistant strains of *P. aeruginosa* studied were complemented by *gyrA* or *gyrB* wild-type alleles, demonstrating that mutations in these genes were present in those strains. Moreover, 29 of the 30 strains were complemented by the *gyrA* wild-type allele, showing the prominence of *gyrA* mutations in the clinical strains of *P. aeruginosa* resistant to fluoroquinolones that we studied.

Previous studies of quinolone-resistant clinical strains of *P. aeruginosa* clearly showed the prominence of DNA gyrase mutations (20, 24, 40, 41) and the rarity of other mechanisms (1, 11, 27, 31). Moreover, as in the present study, *gyrA* appears to be much more often involved than *gyrB* in quinolone-resistant clinical strains. Indeed, by using the same complementation tests as those in the present study, Yoshida et al. found, among 13 resistant clinical strains, 12 strains with *gyrA* mutations and only 1 strain with a *gyrB* mutation (40), and others found that 9 of 9 resistant clinical strains had *gyrA* mutations (41). *gyrA* mutations have also been prominent in most of the quinolone-resistant clinical strains in other bacterial species such as *E. coli* (29), *Staphylococcus aureus* (9), and *Mycobacterium tuberculosis* (37), whereas *gyrB* mutations have been rarely found in the two former species (17, 29) and have never been found in the latter species (37).

In contrast, studies of in vitro-selected mutants of *P. aeruginosa* rarely found DNA gyrase mutations (42) but emphasized the prominence of other mechanisms of resistance such as those involving the *cfxB* (or *nalB*) (5, 35), *nfxB* (15, 18), and *nfxC* (10, 18) genes. More recently, Kureishi et al. found by sequencing a small part of the *gyrA* gene only three strains carrying a *gyrA* mutation among nine resistant clinical strains isolated from cystic fibrosis patients (21). The relatively low proportion of *gyrA* mutants in these clinical strains could be explained by the fact that they exhibited alterations in outer membrane composition (lipopolysaccharide [LPS] and OMP) consistent with permeability alterations, which are common in strains isolated from such patients.

Decreased drug accumulation has been described as a mechanism of quinolone resistance in different *P. aeruginosa* mutants. In these mutants, quinolone resistance was always associated with modifications of OMPs (5–7, 10, 11, 15, 20, 24–26, 28, 31, 42) or LPS (6, 27). In *cfxB* (5, 26, 35) and *nfxC* (10, 11, 19, 25, 26) mutants, an increase in the levels of OMPs with sizes of 49 and 51 kDa, corresponding to OprM and OprN, respectively (26), has been reported. In *nfxB* mutants, an increase in the level of an OMP with a size of 54 kDa, thought to be OprJ (26), has been reported. Finally, in other mutants (7, 20, 31, 42), a decrease in the level of an OMP with a size of 31 to 34 kDa which corresponds to OprF (31, 42) was associated with quinolone resistance. In all of these mutants with OMP modifications, cross-resistance to chloramphenicol, tetracycline, carbenicillin, cephalosporins, and gentamicin was inconstant and depended on the mutation involved. The *nfxC* mutants (10, 11) are resistant to both quinolones and imipenem and lack an OMP with a size of 46 kDa, probably OprD. Conversely it has to be emphasized that isogenic mutants of *P. aeruginosa* lacking OprD did not show increases in the MICs of quinolones but only in those of carbapenems (16).

It has been suggested on the basis of the results of competition experiments carried out in vivo (27) that fluoroquinolones could diffuse by using OprD; these results were criticized by others who by genetic experiments did not find increased susceptibility to fluoroquinolones when OprD was overexpressed (16). So far, it seems clear that if some of the in vitro mutants of *P. aeruginosa* selected for quinolone resistance were also imipenem resistant (1, 10, 18, 25–27, 33), the in vitro mutants selected for imipenem resistance were not resistant to quinolones (16, 33), except for one strain described by Michea et al. which exhibited a fourfold increase in the quinolone MIC (27). However, the relevance and prevalence in hospitals of cross-resistance to quinolones and imipenem remained unclear.

In our study, a sharp decrease in the level of OprD or a lack of OprD was observed in all of the strains of *P. aeruginosa* resistant to imipenem (and to meropenem) as in our previous studies (8, 38). As expected, introduction of plasmids carrying *gyrA* or *gyrB* genes in imipenem-resistant strains did not modify the level of resistance to carbapenems, showing that mecha-

nisms of resistance to these drugs were distinct from those of resistance to quinolones. In contrast, introduction of a wild-type *gyrA* allele resulted in a sharp decrease in the MICs of ciprofloxacin and sparfloxacin for all of these strains. Finally, the fluoroquinolone MICs obtained after complementation were the same for imipenem-resistant and imipenem-susceptible strains and were similar to those usually obtained for wild-type strains, such as the two control strains used in this study.

These results demonstrate that even in the strains of *P. aeruginosa* resistant to carbapenems and lacking OprD, *gyrA* mutations are the major mechanism of resistance to fluoroquinolones. These results also suggest that no other significant mechanism of resistance is responsible for quinolone resistance in the strains studied. Indeed, it has been shown that only partial complementation was observed when double mutants such as *gyrA-nfxC* (11), *gyrA-nfxB* (41), *gyrA-cfxB* (34), and *gyrA-marA* (42) mutants have been transformed by a plasmid carrying *gyrA*, resulting in MICs higher for transformants than for fully susceptible strains. Thus, other mechanisms decreasing drug accumulation such as *cfxB*, *nfxB*, or *nfxC*, if present, are likely marginal for determining quinolone resistance in the strains studied in the present work. However, we suspect that the permeability barrier to ciprofloxacin (but not to sparfloxacin) could be higher in imipenem-resistant than in imipenem-susceptible strains of *P. aeruginosa*, leading to a higher level of resistance when gyrase mutations are present.

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