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Fluoroquinolone Supersusceptibility Mediated by Outer Membrane Protein OprH Overexpression in *Pseudomonas aeruginosa*: Evidence for Involvement of a Nonporin Pathway

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Overexpression of *Pseudomonas aeruginosa* outer membrane protein OprH led to an 8- to 32-fold increase in susceptibility to chloramphenicol and the quinolones nalidixic acid, norfloxacin, ciprofloxacin, and fleroxacin in comparison with the susceptibility of the wild-type strain H103 grown on Mg^{2+}-sufficient medium. This was true regardless of whether OprH overexpression was induced by growth of strain H103 on Mg^{2+}-deficient medium, the addition of 5 mM *m*-toluate to cells containing the cloned *oprH* gene behind the inducible *tol* promoter in plasmid pGB25, or mutation in the polymyxin-resistant derivative strain H181. In contrast, OprH overexpression failed to reverse the quinolone resistance phenotype of a *nalB* mutant. OprH was purified to homogeneity by selective detergent solubilization and fast protein liquid chromatography. The addition of OprH to the solution bathing a black lipid bilayer membrane failed to give rise to an increase in membrane conductance. This suggests that OprH is not a porin but, instead, may cause increased uptake of quinolones and chloramphenicol via a non-porin pathway.

Fluoroquinolones have recently emerged as one of the most effective classes of antibiotics. Their broad spectra of activity as well as their potential to be delivered orally to patients have been important in their clinical use as therapeutic agents. In *Pseudomonas aeruginosa*, the outer membrane has been proposed to act as a major permeability barrier to the antimicrobial activity of fluoroquinolone antibiotics (1, 8). Mutations which lead to decreased drug permeation through the disruption of uptake pathways are prominent. For example, some studies indicated that alterations or deficiencies in the outer membrane porin protein OprF of *P. aeruginosa* (8, 23) are observed in mutants with decreased fluoroquinolone susceptibility. Alternatively, decreases in the amount of OprG (5) or the lack of OprD (9, 19) have been associated with quinolone resistance. Other studies have indicated that the acquisition of a 54-kDa outer membrane protein is associated with reduced fluoroquinolone penetration (9, 14, 25). In contrast, Chamberland et al. (6) could show no correlation between OprF deficiency or 54-kDa protein acquisition and resistance to quinolones. Yamano et al. (26) proposed that outer membrane proteins C, D, and E of *P. aeruginosa* play a role in quinolone resistance. In addition, it has been demonstrated that mutants with lipopolysaccharide (LPS) modifications of the outer membranes of both *Escherichia coli* and *P. aeruginosa* had altered fluoroquinolone permeation (6, 7, 13, 16), whereas quinolones have been reported to penetrate the outer membrane of *E. coli* (15) and *Salmonella typhimurium* (13) through the lipid bilayer. Clearly, these studies have provided conflicting data. Therefore, we considered the possibility that the various phenotypic alterations of the mutants described above were secondary manifestations of a primary mutation(s) affecting a non-porin pathway of uptake across the outer membrane.

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The self-promoted (non-porin) uptake pathway, which is used by polycationic antibiotics such as polymyxin B and gentamicin (3, 12, 20), has also been suggested as a contributing mechanism in fluoroquinolone uptake. Recently, Chapman and Georgopapadakou (7) proposed that fluoroquinolones might promote their own uptake through the chelation of divalent cations which cross-bridge adjacent LPS molecules. This was then proposed to result in destabilization of the outer membrane, thereby promoting uptake of the fluoroquinolones. Previously, Nicas and Hancock (20, 21) demonstrated that outer membrane protein H1 (OprH) overexpression in *P. aeruginosa* correlated with increased resistance to polymyxin B, the aminoglycoside gentamicin, and the divalent cation chelator EDTA. It was proposed that OprH blocks the self-promoted uptake of these antibiotics. More recently, molecular genetic techniques were used to provide a direct correlation between OprH overexpression and EDTA, gentamicin (3, 27), and polymyxin (27) resistance. Therefore, we decided to examine whether blocking the self-promoted uptake pathway through OprH overexpression would lead to quinolone resistance. In contrast, we report here that OprH overproduction in *P. aeruginosa* rendered the cells supersusceptible to the fluoroquinolones ciprofloxacin, norfloxacin, and fleroxacin as well as to chloramphenicol. Therefore, we examined whether this supersusceptibility reflected a role for OprH as a porin for fluoroquinolone uptake across the outer membrane.

MATERIALS AND METHODS

Strains and media. The *P. aeruginosa* strain used was the wild-type PAO1 prototroph strain H103. Strain H181 is a constitutive, OprH-overproducing derivative of H103 which was previously selected in our laboratory as polymyxin resistant (20). The OprH-deficient strain H703 was constructed in our laboratory by gene replacement experiments in which the chromosomal *oprH* gene of strain H103 was

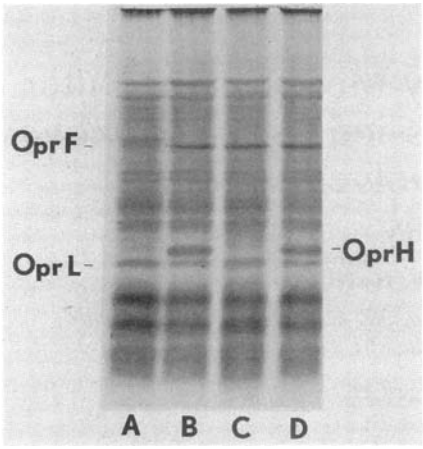

FIG. 1. SDS-PAGE of cell envelope preparations indicating the differential levels of expression of OprH in various strains of *P. aeruginosa* grown in Mg^{2+}-sufficient BM2 glucose minimal medium containing 5 mM *m*-toluate to induce OprH production by plasmid pGB25. Lanes: A, Strain PAO505; B, strain PAO505/pGB25; C, strain PAO6002; D, PAO6002/pGB25.

replaced by a cartridge containing a tetracycline resistance gene inserted within the *oprH* gene (27). As a result, the constructed mutant strain H703 expressed tetracycline resistance and lacked any OprH in the membrane. Strain PAO505 (*met-9011 amiE-200*) and its *nalB4* mutant derivative PAO6002 were obtained from D. Haas (24). Strains H103, H703, PAO6002, and PAO505 were maintained on Luria broth (LB; 1% bacto Tryptone, 0.5% yeast extract, 0.1% NaCl) or LB agar containing, in addition, 1.5% Bacto Agar. To maintain its phenotype, strain H181 was maintained on LB agar containing 8 μg of polymyxin B per ml, whereas strain H703 was maintained on LB agar with 150 μg of tetracycline per ml. The expression vector pNM185 (18) and its derivative pGB25 containing the cloned *oprH* gene under the control of the *m*-toluate-regulated *tol* promoter have been described previously (3).

MIC determination and antibiotics. To determine the MIC, each strain was grown overnight in BM2 minimal medium (20) containing glucose as the carbon source. BM2 glucose agar plates containing serial twofold dilutions of the appropriate antimicrobial agents were inoculated with 10^4 cells in a 10-μl volume. MICs were determined at least three times and were assessed after 18 h of incubation at 37°C. The MIC was taken as the lowest antibiotic concentration at which cell growth was no longer visible. All but one of the antibiotics were obtained from Sigma Chemical Co., St. Louis, Mo.; gentamicin sulfate was purchased from ICN Biomedicals, Inc., St. Laurent, Quebec, Canada.

SDS-PAGE. Cell envelopes were isolated as described previously. The sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) method of Hancock and Carey (11) was used to analyze the outer membrane proteins of *P. aeruginosa*. Solubilization prior to SDS-PAGE involved the dilution of the outer membrane protein sample in a 1:1 ratio with a reduction mix containing 4% (wt/vol) SDS, 20% (vol/vol) glycerol, 40 mM EDTA, and 0.125 M Tris-hydrochloride (pH 6.8). Because OprH is generally observed, after SDS-PAGE, in both its partly unfolded 18-kDa form and its 21-kDa heat-modified form (4), 2% (vol/vol) trichloroacetic acid was often added to the sample prior to heating the samples at 100°C for 10 min to maximize the amount of OprH in the 21-kDa band after SDS-PAGE.

Purification of OprH. The OprH-overproducing strain H181 was used for the isolation of OprH. Cells from mid-logarithmic-phase cultures (optical density at 600 nm, 0.6 to 0.8) were harvested at 7,000 rpm in a Sorvall RC-5B rotor for 15 min. The pellet was resuspended in 20% (wt/vol) sucrose–10 mM Tris-HCl (pH 8.0) with the inclusion of 50 μg of pancreatic DNase I (Sigma) per ml. The cells were broken in a French press at 14,000 lb/in^2, and whole cells were removed by centrifugation at 1,000 × *g* for 10 min. A two-step sucrose gradient (50 and 70%) was set up onto which the sample was applied. Overnight centrifugation at 100,000 × *g* on a Beckman SW27 rotor allowed separation of the outer and inner membranes. The outer membrane fraction was collected and diluted with distilled water to a final sucrose concentration of approximately 5% and was centrifuged at 150,000 × *g* for 1 h. The pellet was resuspended in 10 mM Tris-HCl (pH 8.0)–0.5% octylpolyoxyethylene (octyl-POE; Bachem Bioscience Inc., Philadelphia, Pa.), sonicated three times for 10 s each time by using the Biosonik sonicator (Bronwill Scientific, Inc., Rochester, N.Y.), and centrifuged at 150,000 × *g* for 1 h. The soluble fraction was retained for analysis, and the pellet was resuspended in 10 mM Tris-HCl (pH 8.0)–3% octyl-POE and centrifuged at 150,000 × *g* for 1 h. The soluble fraction was retained and the pellet was subjected to a second solubilization with 3% octyl-POE. The fourth and fifth solubilization steps were carried out in 10 mM Tris-HCl (pH 8.0)–3% octyl-POE–50 mM EDTA. All fractions were tested for the presence of OprH by SDS-PAGE. OprH was primarily solubilized in 10 mM Tris-HCl (pH 8.0)–3% octyl-POE with 50 mM EDTA. Fast-protein liquid chromatography (FPLC) was carried out to further purify OprH from other contaminating proteins within the fraction. The column used was a Mono Q anion exchanger (Laboratory Separation Division, Pharmacia, Uppsala, Sweden). The starting buffer contained 10 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 1% octyl-POE. A sample containing 6 to 8 mg of protein was loaded onto the column, and the proteins were eluted gradually as the salt concentration increased from 0 to 1 M. OprH, a basic protein (4), eluted in apparently pure form in the void volume.

Black lipid bilayer experiments. The black lipid bilayer experiments have previously been described in detail by Hancock and Benz (10). Conductance was measured across a lipid bilayer in the presence of 10^{-12} M FPLC-purified OprH. The lipid membrane was formed by using 1% oxidized cholesterol in *n*-decane. The applied voltage was 10 mV. Purified OprP (10) provided by C. Egli, from our laboratory, was used as a control.

RESULTS

Influence of OprH overexpression on susceptibility to quinolones and chloramphenicol. To test whether quinolones accessed the self-promoted uptake pathway to cross the outer membrane of *P. aeruginosa*, a set of isogenic strains with normal or altered expression of OprH was used. As demonstrated previously OprH expression in wild-type strain H103 is influenced by the level of Mg^{2+} in the medium. In low-level Mg^{2+} (0.02 mM) medium, OprH is overexpressed at least 24-fold (20, 21; confirmed in this study) and cells are 4-fold more resistant to polymyxin and gentamicin compared with the resistance of strain H103 cells grown in Mg^{2+}-sufficient (0.5 mM) medium (20) (Table 1). In strain H703 cells in which the *oprH* gene was mutated by insertion

TABLE 1. Influence of OprH expression on MIC of various antibiotics for the wild-type strain H103, the OprH-overproducing strain H181, and the OprH-deficient strain H703 grown under Mg^{2+}-sufficient or Mg^{2+}-deficient conditions

Strain	Mg^{2+} concn (mM) during growth	OprH level	MIC (μg/ml)[a]							
			PX	GM	NAL	NOR	CIP	FLX	CAP	CFP
H103	0.5	+	2	2	512	4	1	2	256	2
	0.02	+++	8	8	16	0.5	0.12	0.25	12	2
H703	0.5	−	2	2	512	4	1	2	256	2
	0.02	−	2	2	512	4	1	2	256	2
H181	0.5	+++	16	16	32	0.25	0.06	0.12	12	2
	0.02	+++	16	16	32	0.25	0.06	0.12	12	2

[a] Abbreviations: PX, polymyxin B;GM, gentamicin; NAL, nalidixic acid; NOR,norfloxacin;CIP, ciprofloxacin; FLX,fleroxacin;CAP, chloramphenicol; CFP,cefpirome.

of a *tet* gene, OprH was not expressed regardless of the Mg^{2+} concentration in the medium (27), and polymyxin and gentamicin MICs were identical to those observed for H103 expressing low levels of OprH in Mg^{2+}-sufficient medium (27) (Table 1). In strain H181, which constitutively overexpressed OprH (20), cells were resistant to polymyxin and gentamicin at levels similar to those observed for strain H103 grown on Mg^{2+}-deficient medium (27) (Table 1).

We used the strains described above to see whether OprH could also block the uptake of quinolones. In contrast, cells that overexpressed OprH (i.e., strain H181 grown in Mg^{2+}-sufficient or Mg^{2+}-deficient medium or strain H103 grown in Mg^{2+}-deficient medium) were supersusceptible to the quinolones nalidixic acid, norfloxacin, ciprofloxacin, and fleroxacin and to chloramphenicol when compared with cells with normal (strain H103 grown in Mg^{2+}-sufficient medium) or no (strain H703) expression of OprH. The increase in susceptibility varied from 8- to 32-fold in OprH-overexpressing cells. No alteration in susceptibility to the β-lactam cefpirome (Table 1) or rifampin (data not shown) was observed in any of the cells described above. Previous studies also revealed no alteration in the MICs of tetracycline or carbenicillin for strain H103 or H181 (20).

Recently, Bell et al. (3) demonstrated that polymyxin resistance in strain H181 appeared to involve a second change, possibly an LPS alteration. However, the lack of polymyxin resistance in the *oprH::tet* insertion mutant H703 grown in Mg^{2+}-deficient medium indicated a requirement for OprH overexpression for expression of polymyxin resistance (27). Nevertheless, overexpression of OprH from the cloned gene present in the expression plasmid pGB25 did not result in polymyxin resistance, suggesting that there is a factor other than OprH overexpression that is required for polymyxin resistance (3). To test whether OprH overexpression was solely responsible for the supersusceptibility phenotype, the quinolone susceptibility of strain H103 carrying the control plasmid pNM185 was compared with that of H103 carrying the OprH expression plasmid pGB25 after induction with 5 mM *m*-toluate. Again, OprH overexpression was correlated with supersusceptibility to ciprofloxacin, nalidixic acid (Table 2), norfloxacin, and chloramphenicol.

Is OprH a porin? There were at least two possible explanations for the quinolone and chloramphenicol supersusceptibilities caused by OprH overexpression. Either OprH is a porin or OprH mediates in a non-porin uptake pathway. We reasoned that if OprH is a porin, it should be specific for the quinolones and chloramphenicol, since OprH overexpression did not result in supersusceptibility to the other antibiotics tested. Therefore, the possibility that these antibiotics are pyrimidine and purine analogs, respectively (on the basis of consideration of their structures), and that OprH is a base- or nucleotide-specific channel like *E. coli* protein Tsx (17) was considered. However, the addition of 1 or 0.1 mM deoxyadenosine or adenine did not reduce the quinolone susceptibility of strain H103, nor did the addition of 1 or 0.1 mM deoxycytidine reduce the susceptibility of the strain to chloramphenicol. Thus, we concluded that these compounds are unable to bind to a specific nucleotide binding site and block quinolone uptake.

A second test involved an attempt to examine the ability of OprH to reverse the norfloxacin resistance phenotype in the mutant PAO6002 *nalB4*. This mutant is cross-resistant to several quinolones and β-lactams but not to gentamicin (24), and it has been proposed that it contains an outer membrane alteration (14). If OprH is a porin, it should have been able to reverse the resistance phenotype of strain PAO6002. However, overexpression of OprH in PAO6002/pGB25 (Fig. 1) did not affect susceptibility to nalidixic acid, ciprofloxacin (Table 2), or chloramphenicol compared with that of the vector control PAO6002/pNM185 or the plasmidless mutant PAO6002. A similar result was observed when OprH was overexpressed in *E. coli* (data not shown). In contrast, overexpression of OprH in the parent strain PAO505/pGB25 or in H103/pGB25 did result in supersusceptibility (Table 2).

To further confirm that OprH was not acting as a porin for quinolone uptake, OprH was purified by FPLC in the

TABLE 2. Effect of OprH overexpression on the antibiotic susceptibilities of *P. aeruginosa* strains

Strain	Plasmid[a]	OprH level[b]	MIC (μg/ml)[c]		
			NAL	CIP	CFP
H103	None	+	512	1	2
H103	pNM185	+	512	1	2
H103	pGB25	+++	32	0.06	2
PAO505	None	+	64	1	2
PAO505	pNM185	+	64	1	2
PAO505	pGB25	+++	8	0.06	2
PAO6002	None	+	200	0.5	2
PAO6002	pNM185	+	200	0.5	2
PAO6002	pGB25	+++	200	0.5	2
H181	None	+++	32	0.06	2

[a] To induce the plasmids' *tol* promoters, 5 mM *m*-toluate was added.
[b] As determined by SDS-PAGE.
[c] Abbreviations: NAL, nalidixic acid; CIP, ciprofloxacin; CFP, cefpirome.

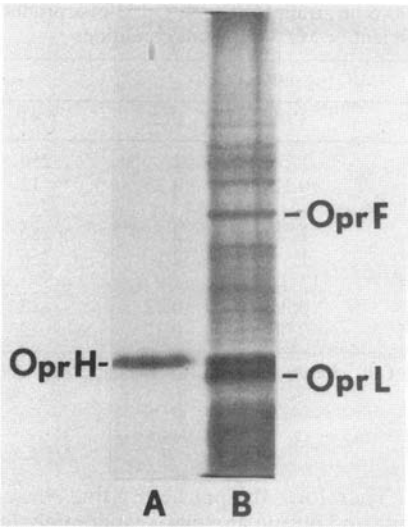

FIG. 2. Purification of outer membrane protein OprH from *P. aeruginosa* H181. Selective solubilization of OprH from outer membrane preparations of H181 grown in BM2-glucose proceeded as indicated in the text. Samples were run on an SDS–14% polyacrylamide gel. Lanes: A, FPLC-purified OprH; B, H181 outer membrane preparation.

presence of the detergent octyl-POE (Fig. 2) and analyzed in black lipid bilayer experiments. The purified protein was present in the heat-unmodified form when run on SDS-polyacrylamide gels without heating prior to electrophoresis, suggesting that it was not denatured during purification. Assuming that OprH retained its normal configuration, it was observed that the addition of FPLC-purified OprH from a stock sample in 1% octyl-POE–0.1% Triton X-100 to a final concentration of 8 or 80 pg/ml of bathing solution resulted in no increase in membrane conductance (Fig. 3). Stepwise increases in membrane conductance of the appropriate size(s) were, however, observed for the control porin OprP (purified by a similar procedure) when added at a concentration of 4 to 8 pg/ml to the chamber.

DISCUSSION

The data presented here suggest that quinolone and chloramphenicol uptake across the outer membrane of OprH-overexpressing *P. aeruginosa* strains involves a non-porin pathway distinct from self-promoted uptake. Previous data

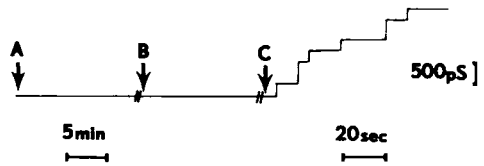

FIG. 3. Black lipid bilayer analysis of FPLC-purified OprH. The membrane was made from 1% cholesterol in *n*-decane. The applied voltage was 10 mV. Three separate sections from a chart recording are shown: arrow A, 8 pg of purified OprH per ml was added to the chamber; arrow B, a further 80 pg of purified OprH per ml was added to the chamber; arrow C, 8 pg of purified OprP per ml was added to the chamber, leading to stepwise increases in conductance. The time scale on the left refers to the chart sections between arrows A and C; the time scale on the right refers to the section of chart recording after arrow C.

have indicated that OprH overexpression, either in wild-type *P. aeruginosa* H103 grown on Mg^{2+}-deficient medium or in the constitutive mutant H181, results in resistance to polymyxin, gentamicin, and EDTA (9, 20, 27). Despite the coincident requirement of a second alteration for polymyxin resistance (3, 27), the data published to date are consistent with the hypothesis that OprH blocks the self-promoted uptake of cationic antibiotics by interacting with LPS and reducing the formation of sites at which divalent cations cross-bridge adjacent LPS molecules (20). Since these sites are involved in aminoglycoside uptake and EDTA susceptibility, OprH-overexpressing cells become resistant to these agents (3, 12, 20).

Using the strains from which the conclusions given above arose, we tested the hypothesis of Chapman and Georgopapadakou (7) that fluoroquinolones can penetrate the outer membrane via the self-promoted uptake pathway. We confirmed their observations (7) that exogenous Mg^{2+} increases the MICs of fluoroquinolones (data not shown), as has been shown previously (20) for other agents that access the self-promoted uptake pathway, including polymyxin and gentamicin. However, we were unable to demonstrate that fluoroquinolones are capable of increasing the permeability of the outer membrane to the hydrophobic fluorophor, 1-*N*-phenyl-naphthylamine the β-lactam nitrocefin or the protein lysozyme (data not shown), in contrast to results obtained for polymyxin, gentamicin, and other polycations that access self-promoted uptake (20). Furthermore, overexpression of OprH correlated with supersusceptibility to quinolones (and chloramphenicol), not resistance, as observed for gentamicin and polymyxin (Table 1). Thus, we can conclude that the quinolones and chloramphenicol are not taken up by self-promoted uptake in *P. aeruginosa*.

The results obtained with cells in which OprH was overexpressed from the cloned gene confirmed that the supersusceptibility phenotype is entirely due to the presence of OprH in the outer membrane. However, three lines of evidence that were inconsistent with the fact that OprH forms a channel for the quinolone antibiotics and chloramphenicol were obtained. Thus, we hypothesize that OprH increases the rate of non-porin uptake of quinolones and chloramphenicol across the outer membrane of *P. aeruginosa*. Although we are not in a position to define the actual mechanism of uptake of fluoroquinolones, it is possible that OprH, a basic protein, might act by partly neutralizing the highly negative surface charge of *P. aeruginosa*, as indicated by phase partitioning experiments with strains H103 and H181 (2). This might then permit quinolones to better access sites involved in uptake via a non-porin pathway. Alternatively, uptake may occur at the interface between OprH and LPS in the outer membrane. Nevertheless, uptake at such sites would not seem to account for the quinolone susceptibility of wild-type *P. aeruginosa*. Consistent with this, the MICs of quinolones and chloramphenicol were not different for strain H103 grown in 0.5 mM Mg^{2+} (which expresses low levels of OprH) and the *oprH::tet* mutant H703 (which cannot produce OprH). We also considered the possibility that OprH overexpression disrupted normal barrier function to create an unphysiological route of entry. However, the lack of change in β-lactam MIC (Table 1) (20) and unaltered outer membrane permeability toward the β-lactam nitrocefin of strain H181 (22) seem to argue against this possibility.

The information in the literature regarding the route of uptake of quinolones across the outer membrane of *P. aeruginosa* appears contradictory. Thus, OprC, -D, -E, -F, and -G as well as LPS (5–8, 13, 19, 26) have been variously

suggested to be involved in fluoroquinolone uptake across the outer membrane of *P. aeruginosa* on the basis of crude compositional analyses of quinolone-resistant mutants. Thus, it is possible that the variety of phenotypic alterations in resistant mutants reflects a chemically more subtle alteration to a non-porin pathway of quinolone uptake. It is known that LPS alterations can influence outer membrane protein composition (11), and LPS chemistry is so complex that changes to LPS can evade definition by simple chemical analyses. Several investigators have suggested that LPS may be involved in some way in fluoroquinolone uptake in *P. aeruginosa* (26) and *E. coli* (7, 13). Thus, unless a thorough analysis of LPS is performed, it seems inappropriate to conclude that a protein is a porin which mediates fluoroquinolone uptake solely on the basis of the lack of expression of this specific outer membrane protein in a fluoroquinolone-resistant mutant. Results with the *nalB4* mutant PAO6002 were consistent with the possibility of a non-porin pathway for fluoroquinolone uptake in *P. aeruginosa*.

Our results indicate that OprH overexpression alone renders strains supersusceptible to quinolones and chloramphenicol. We also presented evidence that OprH is not a porin. We therefore propose a model of quinolone uptake which accounts for these findings in which quinolones penetrate the outer membrane of OprH-overexpressing strains via a non-porin pathway and further propose that this general mode of uptake is also used in wild-type *P. aeruginosa* cells.

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