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Kinetic control of quorum sensing in *Pseudomonas aeruginosa* by multidrug efflux pumps

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Abstract

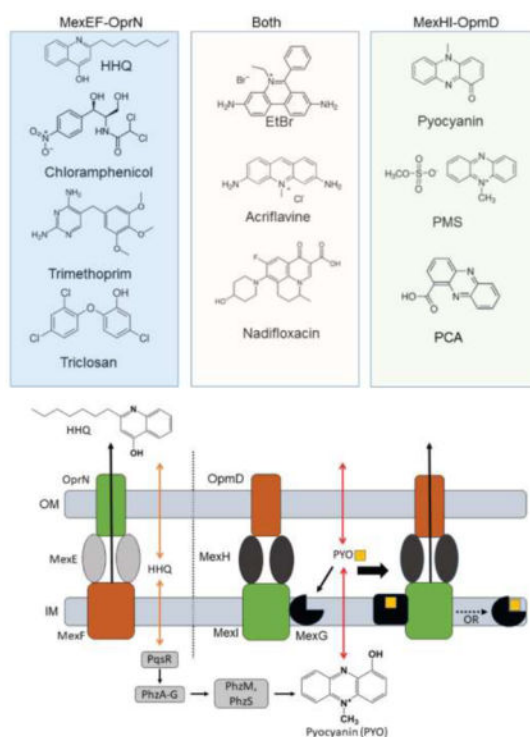
Pseudomonas aeruginosa is an important human pathogen, the physiology and virulence of which are under the control of quorum sensing signals. These signals often have dual roles, functioning as toxins to some cells and as oxidative-stress protectors for their producer cells. Hence, their internal and external concentrations should be tightly controlled. In this study, we analyzed the interplay between the multidrug efflux transporters MexEF-OprN and MexG/HI-OpmD in quorum sensing of *P. aeruginosa*. We found that the two transporters have overlapping substrate specificities but different efficiencies. When overproduced, both MexEF-OprN and MexG/HI-OpmD provide clinical levels of resistance to diverse fluoroquinolones and protect *P. aeruginosa* against toxic phenazines. However, this similarity is enabled by synergistic interactions with the outer membrane. In hyperporinated cells, MexG/HI-OpmD is saturated by much lower concentrations of fluoroquinolones but is more efficient than MexEF-OprN in efflux of phenazines. Unlike MexEF-OprN, mutational inactivation of MexG/HI-OpmD reduces the levels of pyocyanin and makes *P. aeruginosa* cells hypersusceptible to phenazines. Our results further show that MexG binds pyocyanin, physically associates with MexHI and represses the activity of the transporter, revealing a negative regulatory role of this protein. We conclude that differences in kinetic properties of transporters are critical to maintain proper intra- and extra- cellular concentrations of phenazines and other signaling molecules, and that MexG/HI-OpmD controls the steady-state in the synthesis and secretion of phenazines.

Graphical Abstract

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Supporting Information:

Detailed description of Materials and Methods used in the study with references; Tables S1 and S2 containing strains/plasmids and primers constructed in this study, respectively; Supporting Figures S1–S7.



Keywords

Antibiotic Resistance; Outer Membrane Barrier; Permeation; Efflux Constant; Phenazines; Hyperporination

Pseudomonas aeruginosa is a gram-negative, opportunistic human pathogen that is most commonly associated with nosocomial diseases and infections in cystic fibrosis patients¹⁻². The pathogen achieves its high level of drug resistance mainly through the interplay of low outer membrane permeability and active drug efflux across the cell envelope³⁻⁴. In gram-negative bacteria, the most prominent efflux transporters involved in resistance are RND type transporters, largely because of their ability to translocate substrates across the outer membrane and their broad range of substrates⁵. These transporters associate as tripartite complexes consisting of the inner membrane RND transporter, a membrane fusion protein (MFP), and an outer membrane channel (OMF). *P. aeruginosa* has at least twelve RND transporters encoded on its chromosome, which differ from each other in their level of expression and substrate specificity⁶⁻⁷.

The most clinically relevant of these are MexAB-OprM⁸, MexCD-OprJ⁹, MexEF-OprN¹⁰⁻¹¹, and MexXY¹¹. The role these transporters play in resistance is well-documented and their broad substrate specificity highlights some redundancy between them. What is less understood is their role in cell physiology and virulence and why different transporters are overproduced and selected under different environmental conditions. It has been shown that MexAB-OprM translocates 3-oxo-acyl-homoserine lactones and, thus, exerts some control over the quorum sensing network of *P. aeruginosa*¹². HHQ (4-hydroxy-2-heptylquinoline), a

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direct precursor for the quorum sensing regulator PQS (*Pseudomonas* Quinolone Signal), was found to be a substrate of MexEF-OprN¹³ (Figure S1). In fact, overexpression of this RND transporter was shown to decrease the production of PQS, as well as several PQS regulated virulence factors like pyocyanin and rhamnolipids^{13–15}. In addition, MexHI-OpmD was also shown to be involved in virulence of *P. aeruginosa*^{16–17}. A mutation in either *mexI* or *opmD*, the RND transporter or the OMF respectively, resulted in the loss of virulence and a reduction of quorum sensing signaling molecules¹⁶. These phenotypes were suggested to result from MexHI-OpmD transport of a PQS precursor¹⁶. Another study found that a deletion of MexHI-OpmD results in different colony morphology when compared to WT colonies and that the transporter exports the endogenously produced phenazine 5-methylphenazine-1-carboxylate (5-Me-PCA)¹⁸ (Figure S1).

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Homology and clustering analyses showed that MexG/HI-OpmD is the most closely related to MexVW-OpmM (70% sequence similarity between MexI and MexW) and MexEF-OprN (55% sequence similarity between MexI and MexF). Among various RND pumps, MexHI-OpmD is somewhat unusual because of the fourth protein MexG encoded by the first gene in the operon. Sequence analysis indicates that MexG is an inner membrane protein with four transmembrane α -helices along with one long, and one short periplasmic loop. Other transporters containing additional genes in the operons, like MdtABC of *E. coli*¹⁹ or TriABC from *P. aeruginosa*²⁰, contain either an additional RND or MFP subunit, whereas MexG does not share any homology to those components. It is also distinctly different from small peptides like AcrZ of *E. coli*, which changes the substrate specificity of the transporter²¹. Previous studies found that MexG is not required for the antibiotic efflux activity of MexHI-OpmD²², and that MexG binds to PQS²³. However, the function of MexG and whether or not it is a component of the complex is still unknown.

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All RND transporters function in the context of the two-membrane cell envelope and are believed to translocate their substrates through the specific outer membrane channel and across the outer membrane. The synergistic interactions with the low permeability barrier of the outer membrane masks the activities of efflux pumps and complicates the assessment of their kinetic properties and substrate specificities^{24–25}. In kinetic terms, this interaction is described by two constants: the barrier factor (B-factor) that compares maximum fluxes across the outer membrane and via efflux pumps ($B = F/V$, where F is the flux across the barrier and V is the maximal efflux velocity) and the efflux constant (K_E) that relates efficiency of efflux pumps and the diffusion rate across the membrane ($K_E = V/(K_m \cdot 0.5k_2)$, where K_m is a Michaelis constant and k_2 is the rate constant for drug diffusion across the outer membrane)²⁵. Importantly, for some drugs the outer membrane barrier becomes saturated before efflux pumps do ($B > 1$), creating a situation when efflux pumps never reach saturation and appear to be highly efficient, even with very poor substrates. We previously developed a hyperporination approach that enables influx of various compounds across the Gram-negative outer membranes, allowing for the separation of the contributions of active efflux and of the outer membrane in intracellular accumulation of compounds and in antibacterial activities^{24, 25, 26}. In this study, we used this approach to gain insight into the role of RND pumps in quorum sensing.

We analyzed substrate specificities and efflux capacities of MexG/HI-OpmD and MexEF-OprN, the two pumps implicated in efflux of quorum sensing signals. Unexpectedly, the overexpression of MexHI-OpmD provides strong resistance to fluoroquinolones (FQ), at levels comparable to those provided by MexEF-OprN. This MexHI-OpmD efficiency, however, is achieved only if the outer membrane is intact. In hyperporinated cells, MexEF-OprN retains its efflux efficiency, whereas MexG/HI-OpmD is oversaturated by substrates and loses almost all of its ability to protect cells from FQ. In contrast, MexHI-OpmD is more efficient than MexEF-OprN in protection of *P. aeruginosa* against toxic phenazines, as seen not only in strains overexpressing the pumps but also in mutants lacking MexHI-OpmD. The transporter allows for the extracellular accumulation of pyocyanin, while providing resistance from its toxic effects. Our results also show that MexG interacts with the MexHI-OpmD complex and negatively affects its efflux activity. We conclude that the endogenous activity of MexG/HI-OpmD establishes the steady-state concentration of phenazines inside and outside of cells.

Results and Discussion

Physiological responses of *P. aeruginosa* cells to efflux pump inactivation are exaggerated by hyperporination

P. aeruginosa contains a large array of efflux pumps responsible for the intricate interplay of controlling the intracellular concentrations of endo- and exo- toxins. MexAB-OprM is the only efflux pump of this bacterium whose deletion makes cells highly susceptible to antibiotics, suggesting that the expression of other pumps is too low to significantly contribute to intrinsic antibiotic resistance²⁷. We previously showed that hyperporination of *P. aeruginosa* cells allows characterization of efflux pump functions without the contribution of the outer membrane barrier²⁴. To analyze the role of efflux in *P. aeruginosa* physiology, we integrated the gene encoding the pore under control of an IPTG-inducible promoter onto the chromosomes of strains with progressive deletions of operons encoding the efflux pumps: P 4-Pore lacking *mexAB-OprM*, *mexCD-OprJ*, *mexXY* and *mexJKL*, and P 6-Pore, a derivative of P 4-Pore further lacking *mexEF-oprN* and *triABC* pumps (Table S1). The properties of the constructed strains were compared to those of PAO1-Pore with full efflux capacity and P 3-Pore with *mexAB*, *mexCD* and *mexXY* deletions²⁴.

Efflux-proficient and -deficient strains without pores did not have significant differences in growth rates in LB medium (Figure S2). However, the optical densities of stationary P 4 and P 6 cultures were ~30–40% lower compared to those of PAO1 and P 3 strains. Increasing concentrations of the inducer IPTG did not have a notable effect on growth parameters (Figure S2).

In contrast, the growth of the Pore-producing (“Pore”) strains was sensitive to the presence of inducer. Without IPTG, the cell cultures of “Pore” strains were visually similar to their parental strains, including the difference in pigmentation (Figure 1A and 1E). PAO1-Pore produced the largest amounts of the blue-green pigment pyocyanin, whereas P 3-Pore, P 4-Pore, and P 6-Pore cells produced 5–10 times less of this pigment, the amount of which decreased with increasing number of inactivated efflux pumps (Figure 1E). In the presence of IPTG, both the cell densities and the amounts of pyocyanin decreased progressively with

the number of inactivated efflux pumps (Figure 1B). The P₆-Pore cells were the most sensitive to the expression of the pore. Based on OD₆₀₀ measurements, even at low concentrations of the inducer these cells grew slower and entered stationary phase at cell densities 30–50% lower than PAO1-Pore cells (Figure 1B–D). At 0.1 mM IPTG, growth rates and OD₆₀₀ of stationary phase cells decreased for all efflux-deficient constructs suggesting that inactivation of efflux and hyperporination of the outer membrane have a cumulative effect on growth physiology of *P. aeruginosa*.

We noticed that overnight cultures of P₄ and P₆ strains and their hyperporinated variants contained large cell clumps and biofilms (Figure 1E), suggesting that the decreased cell density in growth measurements could be caused by cell aggregation and surface adhesion. Such aggregation of *P. aeruginosa* cells is usually caused by quorum response signaling and a transition from a planktonic to a sessile cell growth²⁸. Indeed, we found that decreased cell densities of the induced “Pore” cultures did not correlate with decreased number of colony forming units (CFUs). In fact, for both efflux-proficient and –deficient cells, the CFUs/ml/OD₆₀₀ remained the same in the presence and absence of the inducer (Figure 1C). This result suggested that the observed differences in cell densities and apparent growth rates are due to cell aggregation and not caused by cell division defects. Since such aggregation is already measurable in P₄ and P₆ cultures (Figure S2), these results further suggest that hyperporination of the outer membrane does not lead to cell death or cell division defects, but exaggerates cell aggregation caused by the loss of active efflux, presumably because of the hypersensitivity of cells to quorum sensing signals.

MexHI-OpmD and MexEF-OprN have overlapping substrate specificities

Pyocyanin is one of the most important virulence factors in *P. aeruginosa* and is required to establish full virulence of the pathogen^{29–30}. It leads to oxidative stress by the formation of reactive oxygen species and reduces ATP levels through the oxidation of NADH in host cells^{30–31}. This phenazine has significant antimicrobial and antifungal activity^{32–34}, but *P. aeruginosa* is intrinsically resistant to its antibiotic activity. Furthermore, pyocyanin was shown to assist in the adaptation of *P. aeruginosa* to anaerobic conditions by acting as an electron mediator between NADH and oxygen³⁵ (Figure S1). Thus, the intracellular and extracellular levels of this compound have to be tightly regulated to ensure that it can act as a signaling molecule and electron mediator without becoming toxic to the cell.

The amounts of extracellular pyocyanin were previously linked to the expression of MexEF-OprN (negative correlation) and MexG/HI-OpmD (positive correlation) efflux pumps^{15–16}. To analyze the efficiency and substrate specificity of MexHI-OpmD and MexEF-OprN, we created three constructs that constitutively overexpress either MexGHI-OpmD, MexHI-OpmD, or MexEF-OprN. The overexpression of these efflux pumps is expected to increase significantly the efflux efficiency for their substrates. We confirmed similar expression levels of these constructs in P₄ cells (Figure 2A) and tested susceptibilities of these cells against selected antibacterials (Table 1). In agreement with previous results, all three constructs provided resistance against the FQ ciprofloxacin and levofloxacin, ethidium bromide, and acriflavine but only the overexpression of MexEF-OprN drastically increased the MICs of chloramphenicol, tetracycline, triclosan, and trimethoprim. Hence, all three pumps are

overproduced and functional. Interestingly, we were unable to find an antibacterial agent that is a substrate of MexG/HI-OpmD but not MexEF-OprN. Thus, in agreement with previous studies^{10, 22}, MexEF-OprN has a broader substrate specificity than MexG/HI-OpmD.

To gain insight into the underlying differences in substrate specificities of the two pumps, we next analyzed MICs of a library of 15 structurally diverse FQ in P₄ cells carrying the three efflux pump constructs (Table 2). In P₄, both MexEF-OprN and MexHI-OpmD transporters provided strong resistance to all FQ tested, indicating that overexpression of either one of the pumps could lead to clinical levels of fluoroquinolone resistance (Figure 2D). Surprisingly, for the majority of tested FQ there was no difference in MICs in cells producing either MexEF-OprN or MexHI-OpmD. In a few cases, the MICs differed only by two-fold (Table 2 and Figure 2D). Furthermore, for most fluoroquinolones the fold MIC change in P₄(pump)/P₄(empty vector) was 64–128 with a few exceptions of 32 fold change in MICs (Figure 2D). Thus, despite the different substrate specificities, MexEF-OprN and MexHI-OpmD seem unable to recognize differences in structures of FQ.

As was previously reported²², MexG was not required for the functionality of MexHI-OpmD. In fact, we observed a reduction of the activity of MexHI-OpmD when MexG is co-expressed (Table 2 and Figure 2D). However, this difference was not the same across all compounds. For example, most of the compounds tested showed 2–4 fold decrease in MIC, whereas the presence of MexG potentiated the activity of moxifloxacin by 8 fold, decreasing the MIC from 2.5 μ M in P₄(pMexHI-OpmD) to an MIC of 0.3 μ M in P₄(pMexGHI-OpmD). Since we did not detect notable differences in expression between the MexHI-OpmD and MexGHI-OpmD constructs (Figure 2A), this result suggests that MexG negatively affects the activity of MexHI-OpmD in a substrate-dependent manner.

Hyperporination of the outer membrane differentially affects activities of MexEF-OprN and MexHI-OpmD

We next analyzed the change in MICs of FQ in the hyperporinated P₄-Pore overproducing efflux pumps, thus reducing the contribution of the outer membrane barrier in antibacterial activities. We first titrated IPTG, the inducer of the pore, to select its optimal concentration (Figure 2B and C). As expected, a decrease in MICs of ciprofloxacin with increasing concentration of inducer was seen in P₄-Pore strains, but not in P₄ strains. We measured a 4-fold decrease in the MIC of ciprofloxacin in P₄-Pore(pBSPII) in the presence of 2 mM IPTG. Similarly, we also observed a decrease in the MIC by 4 fold and 8 fold in the induced P₄-Pore(pMexEF-OprN) and P₄-Pore(pMexHI-OpmD), respectively.

We then tested the remaining FQ in P₄-Pore strains induced by 2 mM IPTG (Table 2 and Figure 2E). In agreement with previous studies²⁴, hyperporinated cells were much more susceptible to the antibiotics as seen by a decrease of the MIC up to 64-fold when comparing P₄ and P₄-Pore(pBSPII). Hyperporination also increased susceptibilities of strains overexpressing the efflux pump constructs, albeit to a different extent. Surprisingly, the hyperporination seemed to affect the activity of MexG/HI-OpmD much stronger than MexEF-OprN. For most of the FQ, the overexpression of MexEF-OprN in P₄-Pore generated the same 64–128 fold increase in MICs. This result suggests that even with the reduced barrier factor and increased influx of FQ, this pump still operates below saturation.

However, some substrate specificity could be deduced from the fold MIC change in MexEF-OprN overproducers with and without the Pore. Norfloxacin, prulifloxacin and pefloxacin are excellent substrates of MexEF-OprN and the efficiency of their efflux is only weakly (4 fold) affected by hyperporination (Table 2). Thus, even in the hyperporinated cells, MexEF-OprN is far from saturation with these FQ.

In contrast, nadifloxacin, sparfloxacin and difloxacin are poor substrates of MexEF-OprN and the pump is effective against these FQ only if the influx is very slow (Table 2). In contrast, in hyperporinated cells MexHI-OpmD could provide the same 64-fold MIC change only for sparfloxacin and nadifloxacin, whereas for all other antibiotics the fold MIC changes were significantly lower with most in the 4–16 fold range. As a result, for such antibiotics as moxifloxacin, norfloxacin, or pefloxacin, the difference in susceptibility between MexHI-OpmD and MexEF-OprN in hyperporinated P₄-Pore was up to 8-fold (Table 2 and Figure 2E). In addition to nadifloxacin, sparfloxacin and difloxacin for which MexEF-OprN is not as effective, MexHI-OpmD also lost its efficiency against moxifloxacin, gatifloxacin and levofloxacin (64-fold MIC change). Thus, MexHI-OpmD is not only less efficient in efflux of FQs than MexEF-OprN, but also less specific to these antibiotics. This result explains why the overproduction of MexEF-OprN but not MexHI-OpmD is selected in clinical isolates exposed to high concentrations of FQs^{10, 36}.

Interestingly, for other non-FQ substrates of MexEF-OprN and MexHI-OpmD, hyperporination dramatically reduced the efficiency of both pumps (Table 1), suggesting that they are effective against these drugs only because of the low permeability barrier of the outer membrane.

The presence of MexG further diminished the ability of MexHI-OpmD to protect against FQ as seen from only a 4–8 fold change in MICs for most of the compounds in P₄-Pore(MexGHI-OpmD) cells. Surprisingly, the efflux of nadifloxacin remained unaffected by hyperporination and MexG, further supporting the conclusion that the negative effect of MexG depends on specific substrates of MexHI-OpmD.

MexEF-OprN and MexHI-OpmD have different efflux efficiencies

To establish that the changes in MICs observed in hyperporinated cells are due to the differences in efflux activities of the overproduced pumps, we utilized a real-time fluorescence uptake assay. For this purpose, we used a fluorescent probe Hoechst 33342 (HT) to carry out the uptake experiment. Like fluoroquinolones, HT is an inhibitor of DNA topoisomerases³⁷. However, HT is also a fluorescent probe, the emission of which is significantly enhanced when it binds to lipids or DNA. This enhancement allows us to monitor its uptake into cells²⁵. The uptake data were fitted to an exponential equation to extract initial rates of uptake (Figure 3 and Figure S3). We observed a clear difference in the initial rates of HT uptake with and without the transporters present, indicating that HT is a substrate of both MexEF-OprN and MexHI-OpmD efflux pumps. Overexpression of MexEF-OprN dramatically (up to 10-fold) decreased the rates of uptake of HT both in the absence and presence of the pore, suggesting that HT is an excellent substrate of this efflux pump. Thus, as with the MICs of FQ, hyperporination does not affect the efficiency of this pump against HT. The overexpression of MexHI-OpmD also decreased the rates of HT

uptake, but only in cells with an intact outer membrane. In P₄(pMexHI-OpmD) cells, the rate of HT uptake decreased by ~2 fold, indicating that HT is also a substrate of MexHI-OpmD, but is expelled from the cells more slowly than from cells overexpressing MexEF-OprN. The co-expression of MexG with MexHI-OpmD further reduced its activity, supporting the conclusion that MexG negatively affects the activity of the efflux pump. Furthermore, neither MexHI-OpmD nor MexGHI-OpmD overexpression was able to reduce the rate of HT uptake in hyperporinated cells, indicating that slow diffusion across the outer membrane is critical for activity of MexG/HI-OpmD.

Taken together with MIC measurements, these results suggest that MexHI-OpmD is a weak efflux pump that heavily relies on the outer membrane barrier for its activity against fluoroquinolones and HT. Alternatively, in hyperporinated cells, MexHI-OpmD is presented with an elevated concentration of an endogenously produced substrate that outcompetes the antibiotics, resulting in increased susceptibility.

Deletion of *mexGHI-opmD* leads to a decrease in the extracellular levels of pyocyanin

We next investigated a possibility that endogenous substrates outcompete FQ in the hyperporinated cells overproducing MexG/HI-OpmD. For this purpose, we constructed a series of strains lacking *mexGHI-opmD*. As described above, the progressive deletion of efflux pumps leads to the progressive loss of pyocyanin secretion due to changes in quorum sensing signaling (Figure 1E). Therefore, the *mexGHI-opmD* operon was deleted from chromosomes of PAO1, P₃, P₄ and P₆ strains. For simplicity reasons, the *mexGHI-opmD* variants will be referred to as PAO1 GHID, P₃ GHID, P₄ GHID, and P₆ GHID respectively.

The constructed mutants did not show an extended lag phase (Figure S4) that was previously reported in *mexI* and *opmD* mutants¹⁶. Similarly, we were unable to see a decrease in the production of PQS (data not shown), but we did notice that the *mexGHI-opmD* mutants secreted significantly less pyocyanin even in already pyocyanin-deficient efflux mutants (Figure 4A). We measured the amounts of this phenazine in supernatants of bacterial cultures and found a noticeable (more than 2-fold) decrease in the amounts of pyocyanin in the cultures of all *mexGHI-opmD* strains when compared to their respective parental strains (Figure 4A).

To confirm that MexGHI-OpmD is linked to the synthesis and excretion of pyocyanin, we measured amounts of this pigment in the cultures of the PAO1 strains overproducing different components of the transporter (Figure 4B). When compared to the wild type, PAO1 overexpressing MexGHI-OpmD or MexHI-OpmD produced twice the amount of pyocyanin. Expression of MexG alone did not change the amounts of pyocyanin in the culture medium, but expression of MexGHI without the outer membrane channel OpmD resulted in a 50% increase. This suggests that MexHI might be able to associate with another outer membrane channel to achieve at least partial activity. MexG does not appear to be required for this functionality of the transporter. In agreement with previous results, overexpression of MexEF-OprN resulted in decreased production of pyocyanin¹⁴. Thus, the amount of secreted pyocyanin positively correlates with the expression of MexG/HI-OpmD in *P.*

aeruginosa cells and this correlation is independent of the presence or absence of other RND pumps.

MexGHI-OpmD provides a self-protection of *P. aeruginosa* to phenazines

Pyocyanin is a powerful toxin that inhibits the electron transport chain and several other pathways^{29, 35}. To test whether pyocyanin is a substrate of MexG/Hi-OpmD, we performed growth spot inhibition assays with PAO1 and its efflux deficient mutants (Figure 4C and E). All strains that contained a chromosomal copy of *mexGHI-opmD* were fully resistant to extracellular pyocyanin. On the other hand, the efflux deficient mutants that lacked *mexGHI-opmD* were all hypersusceptible, as seen from large zones of inhibition. At the tested concentrations, PAO1 GHID was still resistant to pyocyanin, likely indicating that the constitutively expressed MexAB-OprM can provide some level of resistance to extracellular phenazines.

Consistent with the above results and previous studies¹⁸, deletion of *mexGHI-opmD* resulted in hypersusceptibility to PMS, a close analog of 5-Me-PCA, which is an endogenous precursor of pyocyanin (Figure 4D and F). Compared to that of pyocyanin, the zone of inhibition for PAO1 GHID was roughly 2-fold larger than for PAO1, indicating that PMS is more toxic than pyocyanin and that the MexG/Hi-OpmD is indispensable for immunity against PMS even when MexAB-OprM is expressed.

We next inserted the Pore onto the chromosomes of P 4 GHID and P 6 GHID strains, as well as of P 5, a P 4 derivative lacking MexEF-OprN, and analyzed MICs of pyocyanin and PMS in these strains (Table 3). Deletion of *mexGHI-opmD* from P 4 resulted in a 4 fold and a 16 fold decrease of the MICs of exogenous pyocyanin and PMS, respectively. Interestingly, P 4 GHID strain is four times more resistant to chloramphenicol and ciprofloxacin than P 4. This result suggests that upon deletion of *mexGHI-opmD*, another transporter, likely MexEF-OprN as based on the resistance profile, is overexpressed. Indeed, no increase in MICs of these antibiotics could be seen in strains lacking MexEF-OprN (Table 3). Interestingly, hyperporination reduced the MICs of pyocyanin, chloramphenicol and ciprofloxacin, but not the MIC of PMS in P 4 GHID-Pore. This result shows that the expression of MexEF-OprN can provide partial protection from the toxic effects of pyocyanin but not from PMS.

Both P 5 and P 6 strains were still completely resistant to exogenous pyocyanin and PMS, indicating that MexGHI-OpmD alone is sufficient to provide protection against toxic effects of phenazines. In both strains, the activities of phenazines were potentiated by hyperporination, indicating that the protection is mediated by efflux. In contrast, the MIC of pyocyanin decreased by a staggering 64 fold in P 6 GHID and no further decrease was observed in the hyperporinated cells, suggesting that P 6 GHID is depleted of phenazine efflux. Furthermore, MICs of both phenazines were the same in P 4 GHID-Pore, P 6 GHID, and P 6 GHID-Pore cells, suggesting that the MexEF-OprN mediated efflux of pyocyanin in P 4 GHID cells is weak and highly dependent on the outer membrane barrier. On the other hand, hyperporination of cells producing MexGHI-OpmD decreased the MICs of phenazines by only 4–8 fold, indicating that MexGHI-OpmD is more efficient in efflux of both phenazines and can compensate for the increased influx across the outer

membrane. In agreement, even when MexEF-OprN or MexG/HI-OpmD were strongly overproduced from plasmids in strains with and without the Pore, MexG/HI-OpmD was more efficient in protection against phenazines (Table 3).

Taken together, these results show that MexG/HI-OpmD is the major and highly efficient efflux pump of phenazines whereas inactivation of MexEF-OprN has no effect on the MIC of phenazines. The absence of MexG/HI-OpmD and MexAB-OprM upregulates the expression of MexEF-OprN that can provide partial protection against pyocyanin but not PMS.

Antibacterial activities of phenazines and fluoroquinolones are additive

To test whether phenazines are responsible for the decreased activity of MexHI-OpmD against fluoroquinolones in hyperporinated strains, we set up checkerboard assays to analyze possible interactions between ciprofloxacin and extracellular pyocyanin with P₄-Pore cells containing different plasmids (Figure S5). For each strain, we calculated an average fractional inhibitory concentration (FIC) index. The FIC is the fraction of the MIC in combination with a second drug and the MIC by itself. The FIC index is the sum of the two FIC from each drug and is commonly used to describe interactions of two antimicrobials^{38–39}. Generally, an FIC index of 0.5 represents synergy, an FIC index of 1 represents an additive/inconclusive effect, and an FIC of 2 is defined as antagonism between the two antimicrobials^{40–41}. We found the FIC index of ~2.3 for the control P₄-Pore with empty vector, whereas the FIC indices measured for P₄-Pore cells expressing MexEF-OprN, MexHI-OpmD, and MexG/HI-OpmD were 1.6, 1.1, and 1.0 respectively. This suggests that there is no significant interaction between pyocyanin and ciprofloxacin in the hyperporinated strains overproducing efflux pumps, however an antagonistic interaction is possible in the absence of these efflux pumps. The higher FIC indices for MexEF-OprN and the empty vector strain could, presumably, be due to physiological effects of exogenous pyocyanin. Low, non-inhibitory concentrations of pyocyanin could stimulate cell growth in these strains by maintaining redox homeostasis³⁵. These results also show that in the strains overproducing MexG/HI-OpmD, ciprofloxacin and pyocyanin do not interact with each other and the antibacterial activities are simply additive.

Thus, we conclude that the increase in the susceptibility to FQ in hyperporinated cells highlights the low efficiency of the MexG/HI-OpmD transporter for these substrates and the high dependency on synergistic interactions with the outer membrane barrier. In contrast, phenazine efflux by MexG/HI-OpmD is specific and efficient even with increased influx of these toxins in hyperporinated cells.

MexG binds MexHI-OpmD and pyocyanin

The results described above show that MexG/HI-OpmD is highly specific for phenazines and that the presence of MexG has a negative effect on the activity of the transporter. Hence, we tested whether MexG physically associates with MexHI. For this purpose, we created a construct expressing MexG with an N-terminal FLAG tag, and MexH and MexI with a C-terminal His tag. Cells producing the tagged MexGHI were split into two aliquots and the 12 Å crosslinker dithiobis(succinimidylpropionate) (DSP) was added to one of them, before

purification of MexI using His Bind resin. After purification, the MexI-containing fractions were analyzed by immunoblotting with anti-His and anti-FLAG antibodies to detect MexI and MexG, respectively. No MexG was detected by immunoblotting in MexI fractions purified from cells untreated with the cross-linker. In contrast, we could clearly detect MexG in elution fractions purified from the cross-linked cells, even after extensive washes with imidazole. Reduction of the cross-linker yielded a 15 kDa band reacting with anti-FLAG antibody and corresponding by size to MexG (Figure 5A). We repeated the experiment with a plasmid expressing MexG with N-terminal FLAG tag and MexH with a C-terminal His tag, however we were unable to detect any crosslinking of MexG and MexH (Figure 5B). This result suggests that there is a physical interaction between MexG and MexHI-OpmD, and that MexI is required for this interaction.

We next analyzed whether MexG will bind to pyocyanin. To test this, we purified MexG using metal affinity chromatography and analyzed binding to pyocyanin by fluorescence spectroscopy. MexG contains five tryptophan residues that are predicted to be in the transmembrane helices. As tryptophan residues were excited, the emission of MexG fluorescence peaked at around 330 nm. In contrast, pyocyanin has a very low fluorescence at the same excitation and emission wavelengths (Figure S6). However, addition of increasing concentrations of pyocyanin to MexG significantly quenches the fluorescence of MexG and causes a slight red shift in the emission spectra (Figure S6), suggesting that pyocyanin binds MexG and causes a change in the environments of the tryptophan residues. We calculated a K_D of pyocyanin to MexG to be about 0.6 μM (Figure 5C). This suggests that the binding is quite strong, with the K_D is similar to what was previously reported for MexG and PQS²³.

To determine whether pyocyanin binding is specific, we constructed the MexG mutant MexG-HAWA, which contains two substitutions His95Ala and Trp98Ala at the C-terminal end of the transmembrane domain 3 facing the periplasm. We repeated the fluorescence binding experiment with this mutant and found that the affinity of MexG-HAWA to pyocyanin decreased by at least 10 fold when compared to MexG WT to a K_D of about 7 μM (Figure 5D and Figure S7). The lower binding affinity for MexG-HAWA shows that pyocyanin binds specifically to MexG, suggesting a functional relationship between the protein and the MexHI-OpmD transporter.

Based on the described results, we propose that MexG plays a role as a negative regulator of the efflux activity of MexHI-OpmD. We found that it physically associates with the transporter *in vivo*, establishing that at least a fraction of this pump exists as a four component complex MexGHI-OpmD. Antibiotic susceptibility testing showed a decrease in activity of the transporter in respect to some substrates when MexG is co-expressed (Figure 2D and E, Tables 2 and 3). This decrease seems to vary depending on the substrate tested. Norfloxacin, ethidium bromide, and acriflavine did not show any changes in activity, whereas moxifloxacin showed the biggest reduction of the MIC of 8 fold. Some of these compounds might more closely resemble the native binding partner of MexG and can activate the small membrane protein. This could point to a regulatory function of MexG with respect to the activity of MexHI-OpmD. The fluorescence assays showed that MexG binds pyocyanin and that this binding is specific with a K_D of about 0.6 μM . It is possible that MexG could act as a sensor of phenazines and other signaling molecules in the inner

membrane and modulate the activity of MexHI-OpmD by changing the conformation or by dissociating from the complex in the ligand-bound state (Figure 6).

Conclusions

P. aeruginosa is an important human pathogen that utilizes an array of regulatory networks and virulence factors to successfully establish itself and proliferate in the human host. Numerous efflux pumps play an important role in this process but often the assigned role is non-specific. Using different levels of expression of efflux pumps and hyperporination of the outer membrane, we manipulated the barrier factors and efflux constants for antibiotics and phenazines in *P. aeruginosa*. We uncovered a specific role of MexG/HI-OpmD in maintaining the non-toxic intra- and extra-cellular concentrations of pyocyanin, an important virulence factor. Our results show that the low expression of efflux pumps is essential for their specificities, so that only the substrates with high affinities are expelled from cells. Furthermore, MexG provides an additional level of control over the activity of MexHI-OpmD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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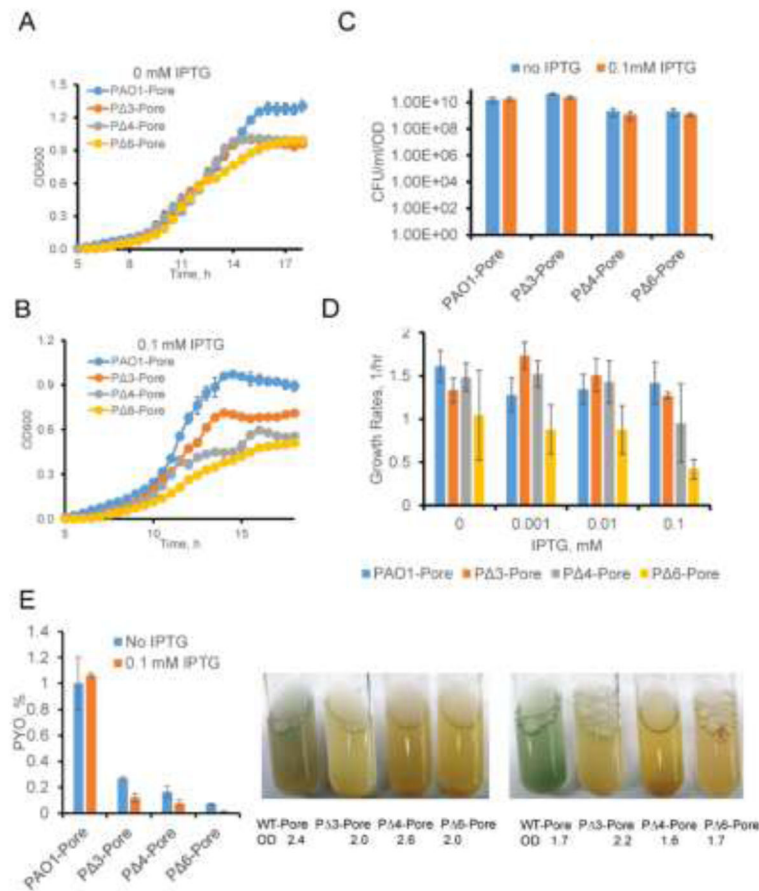


Figure 1. The effect of hyperporination on growth of *P. aeruginosa* cells with different efflux capacities

A. Growth curves of the indicated *P. aeruginosa* strains grown in the absence of the inducer. Overnight cultures were diluted 1:100 into a fresh LB medium; the cells were grown for 18 hours and OD₆₀₀ measured every 30 min. Data shown are the averages of three repeats and the error bars are SD (n=3). **B.** The same as in A but cultures were grown in the presence of 0.1 mM IPTG. **C.** After the growth curves were analyzed as described in A and B, cell aliquots were plated onto LB agar plates and CFUs counted after 24 hrs incubation at 37°C. Averages of three repeats are shown with SD (n=3) as the error bars. **D.** Growth rates of indicated *P. aeruginosa* strains at increasing concentrations of IPTG. Averages of six repeats are shown with SD (n=3) as the error bars. **E.** Amounts of pyocyanin produced by indicated strains in the absence and presence of 0.1 mM IPTG. Test tubes containing overnight cultures of the indicated strains and their OD₆₀₀ readings.

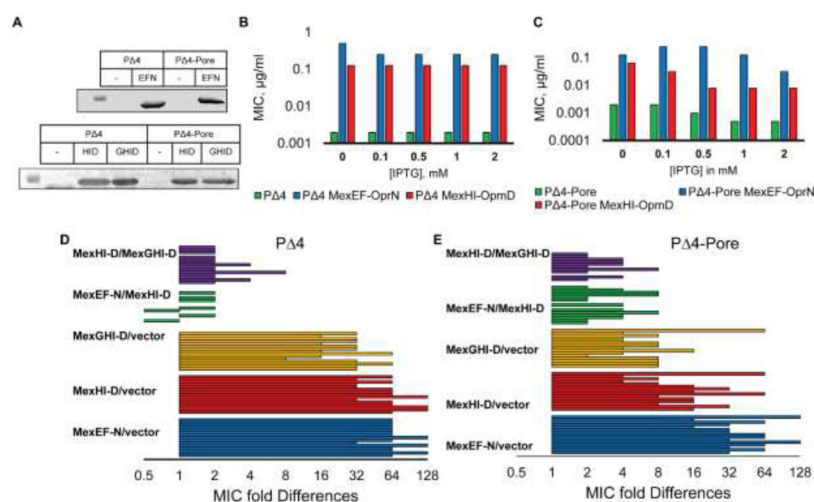


Figure 2. Fluoroquinolone susceptibility testing of cells overexpressing MexHI-OpmD, MexGHI-OpmD, and MexEF-OprN

A. Immunoblotting of membrane fractions from cells harboring the plasmids overproducing the indicated transporters. The respective outer membrane channels (OMF) are tagged with a C-terminal His-tag and visualized with anti-His monoclonal antibody. **B and C.** Effect of IPTG inducer concentration on MICs of ciprofloxacin in P Δ 4 (**B**) and P Δ 4-Pore (**C**) cells expressing indicated transporters. **D and E.** P Δ 4 (**D**) or P Δ 4-Pore (**E**) cells harboring either pMexHI-OpmD, pMexGHI-OpmD, or pMexEF-OprN are tested against a library of FQ. Fold changes in MICs for cells carrying different plasmid constructs are shown. FQ for each ratio are listed from bottom to top: Ciprofloxacin, Enrofloxacin, Levofloxacin, Gatifloxacin, Moxifloxacin, Prulifloxacin, Sparfloxacin, Difloxacin, Lomefloxacin, Ofloxacin, Pazufloxacin, Norfloxacin, Pefloxacin, Sarafloxacin, and Nadifloxacin.

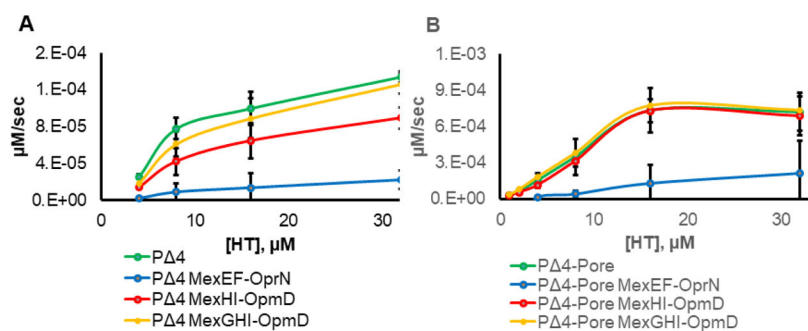


Figure 3. Kinetic uptake measurements

A. The plot of initial rates of HT accumulation in the indicated cells as a function of extracellular HT concentration. **B.** Graph of the initial rates of HT accumulation in pore expressing cells plotted against the extracellular HT concentration. All kinetic measurements were done in triplicate. Error bars are SD (n=3).

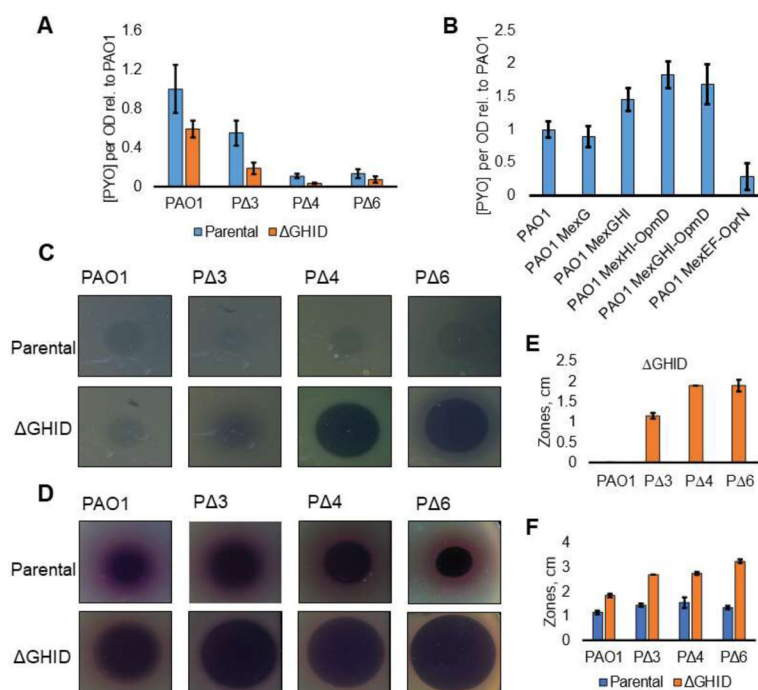


Figure 4. Expression MexG/HI-OpmD correlates with amounts of extracellular pyocyanin and is required for *P. aeruginosa* self-protection against its toxicity

A. Extracellular concentrations of pyocyanin in PAO1, P Δ 3, P Δ 4, and P Δ 6 parental strains (blue) and their derivatives lacking *mexGHI-opmD* (Δ GHID) mutants (orange). **B.** Extracellular concentrations of pyocyanin in cultures of PAO1 strains overexpressing MexEF-OprN or different combinations of MexG/HI-OpmD components. All measurements were done on cultures grown to stationary phase and in triplicate. Error bars are SD (n=3). **C.** Spot assays with pyocyanin showing zones of inhibition in strains with and without *mexGHI-opmD*. **D.** The same as C but with PMS. **E and F.** Quantification of zones of inhibition of pyocyanin (**E**) and PMS (**F**) in indicated strains. Error bars are SD (n=3).

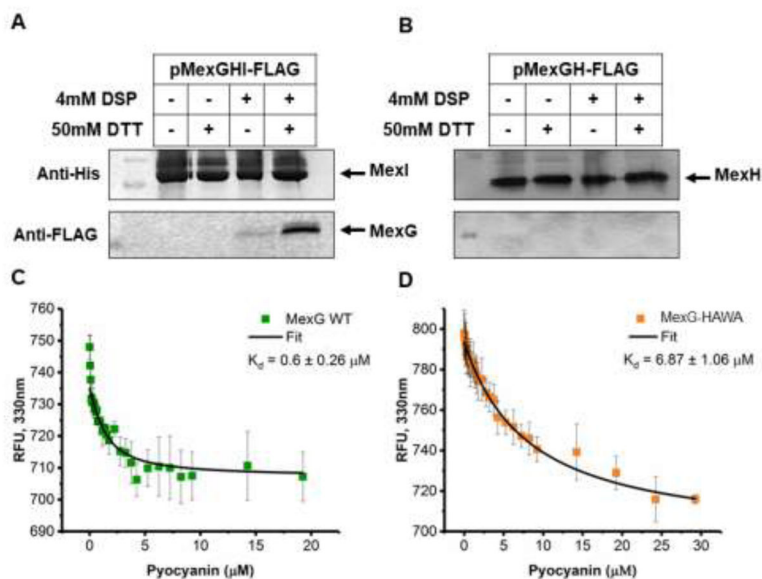


Figure 5. MexG binds MexHI and pyocyanin

A and B. Immunoblotting analyses of MexI^{His} (**A**) and MexH^{His} (**B**) elution fractions purified from P 3 cells harboring pMexGHI-FLAG and pMexGH-Flag respectively. Cells were treated with crosslinker prior to lysis as indicated. The lower panel shows the release of FLAG-tagged MexG from crosslinked samples when elution fractions are treated with a reducing agent (DTT). DSP, dithiobis(succinimidylpropionate); DTT, dithiothreitol. Top panels show the development with anti-His and the lower panels with anti-FLAG primary antibodies. **C and D.** Fluorescence emission at 330 nm (excitation at 290 nm) of the wild type MexG (**C**) and MexG-HAWA (**D**) incubated with increasing concentration of pyocyanin was measured and normalized as described in Methods and is plotted as a function pyocyanin concentration. Fitted line shown in black. Error bars are SD (n=3).

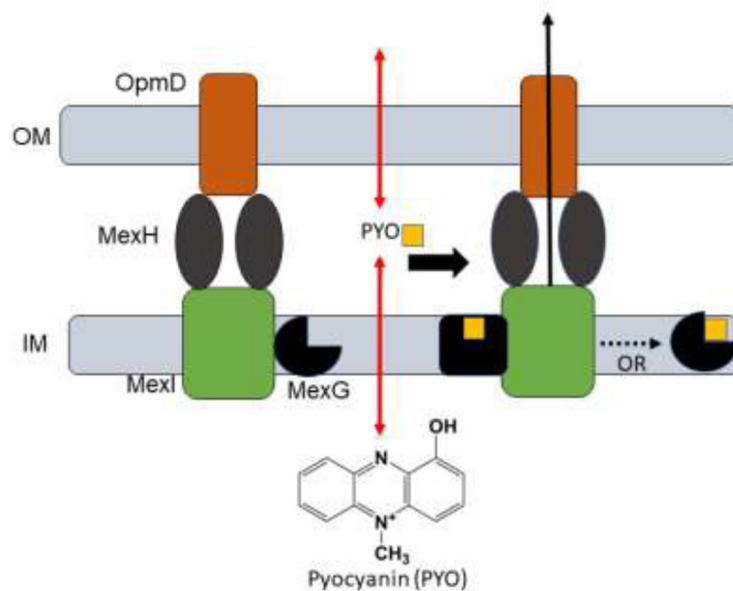


Figure 6. A proposed mechanism of MexG/Hi-OpmD efflux pump

The presence of MexG negatively affects the activity of MexHI-OpmD efflux pump. MexG physically interacts with the pump and its substrates such as pyocyanin. In the ligand-bound state MexG could be in a different conformation or could dissociate from the pump, which in turn could lead to increased efflux of compounds.

Table 1

Minimal inhibitory concentrations of antibiotics in *P. aeruginosa* cells with intact and hyperporinated outer membranes carrying the plasmid borne MexEF-OprN and MexG/HI-OpmD.

PAOI	MICs (µg/ml)									
	P 4					P 4-Pore				
Drugs	-	MexEF-OprN	MexHI-OpmD	MexGHI-OpmD	-	MexEF-OprN	MexHI-OpmD	MexGHI-OpmD	MexGHI-OpmD	MexGHI-OpmD
CIP	0.031	0.007	0.41	0.21	0.002	0.052	0.026	0.013	0.013	0.013
LEV	0.125	0.007	0.9	0.45	0.002	0.056	0.014	0.014	0.014	0.014
EtBr	ND	2048	2048	2048	8	16	32	32	32	32
ACR	>64	32	>1024	>1024	4	8	16	16	16	16
TET	4	0.25	0.25	0.25	0.031	0.063	0.031	0.031	0.031	0.031
CF	8	0.5	>128	0.5	0.25	2	0.25	0.25	0.25	0.25
TRI	1024	128	>256	64	16	128	16	16	16	16
TRM	>256	1	>256	1	1	32	1	1	1	1

CIP, ciprofloxacin; LEV, levofloxacin; EtBr, ethidium bromide; ACR, acriflavine; TET, tetracycline; CF, chloramphenicol; TRI, triclosan; TRM, trimethoprim. ND, no data.

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Table 2

Antibacterial activities of fluoroquinolones in *P. aeruginosa* cells with intact and hyperporinated outer membranes carrying the plasmid borne MexEF-OprN and MexG/Hi-OpmD.

FQs	MICs, μ M											
	PAOI	P 4	P 4MexEF-OprN	P 4 MexHI-OpmD	P 4 MexGHI-OpmD	P 4-Pore	P 4-Pore MexEF-OprN	P 4-Pore MexHI-OpmD	P 4-Pore MexGHI-OpmD	P 4-Pore MexEF-OprN	P 4-Pore MexHI-OpmD	P 4-Pore MexGHI-OpmD
Ciprofloxacin	0.39	0.02	1.25	1.25	0.625	0.005	0.16	0.08	0.04	0.16	0.08	0.04
Enrofloxacin	1	0.04	5	5	1.25	0.005	0.31	0.16	0.04	0.31	0.16	0.04
Levofloxacin	1	0.02	1.25	2.5	1.25	0.005	0.16	0.04	0.04	0.16	0.04	0.04
Gatifloxacin	1	0.04	2.5	2.5	1.25	0.002	0.16	0.04	0.02	0.16	0.04	0.02
Moxifloxacin	6.25	0.04	5	2.5	0.313	0.002	0.31	0.04	0.005	0.31	0.04	0.005
Prulifloxacin	0.25	0.01	0.313	0.313	0.156	0.003	0.08	0.02	0.01	0.08	0.02	0.01
Sparfloxacin	1	0.04	2.5	5	2.5	0.001	0.08	0.08	0.02	0.08	0.08	0.02
Difloxacin	1.56	0.04	5	2.5	0.625	0.002	0.16	0.04	0.02	0.16	0.04	0.02
Lomefloxacin	1.56	0.08	5	5	2.5	0.01	0.31	0.31	0.08	0.31	0.31	0.08
Ofloxacin	1.56	0.08	5	5	2.5	0.01	0.31	0.16	0.08	0.31	0.16	0.08
Pazufloxacin	1	0.04	2.5	1.25	0.625	0.01	0.16	0.08	0.04	0.16	0.08	0.04
Norfloxacin	1	0.04	2.5	1.25	1.25	0.02	0.63	0.08	0.08	0.63	0.08	0.08
Pefloxacin	1	0.09	5	5	2.5	0.02	1.25	0.16	0.16	1.25	0.16	0.16
Sarafloxacin	0.39	0.02	1.25	0.625	0.313	0.005	0.08	0.02	0.02	0.08	0.02	0.02
Nadifloxacin	6.25	0.08	5	5	2.5	0.001	0.16	0.08	0.08	0.16	0.08	0.08

The contributions of MexEF-OprN and MexG/HI-OpmD in *P. aeruginosa* susceptibility to phenazines and antibiotics.

Table 3

MIC, μ M	Genotype	Pyocyanin		PMS		Chloramphenicol		Ciprofloxacin	
		-	Pore	-	Pore	-	Pore	-	Pore
P 4	EF ⁺ , GHI ⁺	1000	250	500	62.5	3.1	1.6	0.001	0.0002
P 4 G	EF ⁺ , GHI ⁻	250	31.25	31.3	31.3	12.4	1.6	0.004	0.0002
P 5	EF ⁻ , GHI ⁺	>1000	250	500	25	3.1	1.6	0.001	0.0002
P 6	EF ⁻ , GHI ⁺	>1000	125	500	125	1.6	1.6	0.001	0.0002
P 6 G	EF ⁻ , GHI ⁻	31.3	31.3	31.3	15.6	1.6	1.6	0.0002	0.0001
P 6 G(pMexEF-OprN)	EF ⁺ , GHI ⁻	>500	125	500	31.25	>256	3.1	0.4	0.012
P 6 G(pMexHI-OpmD)	EF ⁻ , HI ⁺	>500	1000	2000	125	1.6	0.8	0.4	0.012
P 6 G(pMexGHI-OpmD)	EF ⁻ , GHI ⁺	>500	500	1000	62.5	1.6	0.8	0.2	0.012