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Pseudomonas aeruginosa: the whats, whys, and hows of bacterial efflux

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Abstract

P. aeruginosa is a pathogenic bacteria that causes a range of diseases, including cystic fibrosis and pneumonia in nosocomial communities. Despite existing anti-pseudomonal medications, treatment against this microbe is increasingly difficult due to its antibiotic resistance mechanisms. Efflux pumps are notably concerning due to their ability to pump out a wide range of antibiotics from the *P. aeruginosa* cytosol. This challenge has been escalating due to mutations in antibiotic efflux pumps - specifically, due to mutations in the regulatory genes of such pumps, which could potentially trigger their overexpression and confer greater resistance. This literature review analyzes MexAB-OprM, an RND (resistance-nodulation-division) family, constitutively expressed efflux pump in Pseudomonas. Their regulatory genes nalC, nalD and mexR are points of interest, as they partake in MexAB-OprM repressor pathways which could be inhibited by loss-of-function mutations, and have featured as frequently-mutated sites in previously isolated strains. Concerningly, these mutants have risen from combination therapies with aminoglycosides and beta-lactams - first line treatments against *P. aeruginosa* - which has severe implications toward the development of this superbug. This paper will first cover the regulators of MexAB-OprM to gain a better understanding of how these mutations could contribute to resistance. Specific genetic mutations from post-therapy Pseudomonas strains will then be discussed in detail, and virulence data from bacterial studies will be presented to form an evaluation: establishing a clearer connection between bacterial mutation and antibiotic resistance.

Keywords

Efflux pumps, Antibiotics, P. aeruginosa, Regulatory genes, Mutations, Virulence, Pre- and post therapy strains, Antibiotic resistance, Overexpression, Inducers and repressors

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Introduction

Pseudomonas aeruginosa: bacteria profile

A free-living, aerobic, gram-negative bacillus, *P. aeruginosa* is a versatile pathogen that is responsible for a plethora of community-acquired infections including pneumonia, folliculitis and osteomyelitis (1). Because of its proclivity toward freshwater, the drinking water, taps, and sinks of a hospital may be contaminated and become dangerous breeding grounds. This is especially worrying due to *P. aeruginosa*'s potent virulence against immunocompromised individuals, such as those with burns, cancer, AIDS, cystic fibrosis, transplants, and even those using invasive devices. *P. aeruginosa* are armed with a wide range of virulence factors: flagella for motility, two QS (quorum sensing) systems, proteins for biofilm formation, T3SS (type three secretion systems) capable of exotoxin secretion, and pili for adhesion and gene transfer through conjugation (2). The latter, concerningly, could potentially even confer antibiotic resistance by introducing resistance traits (3). Coupled with the antibiotic-resistant mechanisms harbored within these Pseudomonas, *P. aeruginosa* becomes extremely challenging to treat and threatening to nosocomial communities: found in >7% of all healthcare-associated infections and 23% of ICU-acquired infections, and becoming increasingly prevalent over the last decade (4).

Among immunocompromised patients, ICU admits and those with more severe infections, antibiotics (usually combination therapies) including carbapenems (e.g., meropenem), cephalosporins (e.g., ceftazidime, cefepime), aminoglycosides (e.g., gentamicin, tobramycin, and amikacin), and fluoroquinolones (e.g., ciprofloxacin and levofloxacin) are used as first-line treatments against P. aeruginosa (5). Yet, clinicians are still recommended to minimize antibiotic prescription in order to curb superbug emergence - a testament to the risks of mutation. Not only is this phenomenon aided by horizontal gene transfer, but short generation times, selective pressure from antibiotics, and high populations increase the likelihood of bacterial populations gaining new, advantageous mutations (6). Such mutations may influence the antibiotic defenses possessed by *P. aeruginosa*: ranging from reduced permeability due to intrinsic resistance, to the production of antibiotic-inactivating enzymes like beta-lactamases, to multidrug efflux pumps that eject harmful substances - including antibiotics - out of the cytosol (7). While a majority of these mutations can remain silent or even disbenefit the bacteria, others like efflux pump protein overexpression and gain-of-function mutations can flourish under selective pressure, aggravating superbug resistance (8).

Efflux pumps

Found predominantly in gram-negative bacteria, efflux pumps are transporters that eject recognised toxic compounds - especially antibiotics - from the bacteria's internal environment. However, other substances like detergents, antiseptics, dyes and naturally occurring toxic pollutants may also be effluxed by some pumps (9). To that end, the presence of such pumps even within nonpathogenic bacteria suggest that their evolutionary origins did not concern synthetic antimicrobial agents (10). The lack of a structural relationship between some of these substrates are a testament to the versatility of some efflux pumps, partially due to their structure, which support the molecular recognition of many drugs (11).

Efflux pumps exist as six evolutionarily-sustained families and superfamilies across different organisms. These range from the ATP binding cassette (ABC) family, to others that

make use of an electrochemical gradient driven through secondary active transport, including the major facilitator superfamily (MFS), the multidrug and toxin extrusion (MATE) family, the small multidrug resistance (SMR) family, the proteobacterial antimicrobial compound efflux (PACE) family, and the resistance-nodulation-cell division (RND) superfamily (12). The latter, in particular, forms tripartite structures spanning a gram negative bacteria's membranes; it also includes the MexAB-OprM pump, the focus of our review.

Additional evidence suggests that efflux pumps also cater to a set of other biological roles. For example, within Pseudomonas, many RND efflux pumps play key roles in colony behavior; MexAB-OprM, for example, is known to export AHLs (acyl-homoserine lactones) responsible for QS, as well as its inhibitors; MexGHi and MexEF-OprN export a PQS (Pseudomonas quinolone signal) precursor with QS purposes; MexGHi also exports metabolites for biofilm formation (13). The inactivation of these pumps has been shown to quell virulence due to the diverse roles these exported ligands play in signal transduction pathways. Still, the multidrug efflux (Mex) pumps of *P. aeruginosa* primarily function as antibiotic-extruding channels, such as MexAB-OprM, MexXY, MexEF-OprN and MexCD-OprJ - including resistance to antipseudomonal, first-line drugs (14).

MexAB-OprM: Overview and capabilities

Mex operons in *P. aeruginosa* encode for RND (resistance-nodulation-division) superfamily of efflux pumps, conferring multidrug resistance (MDR). Found exclusively in gram-negative bacteria, each pump consists of a tripartite protein complex, where the absence of even one component renders the pump nonfunctional (15). A resistance-nodulation-cell division transporter (RNDt), entrenched in the inner plasma membrane, works in association with two separate families: a periplasmic membrane fusion protein (PMFP) that is present in the periplasm, and an outer membrane factor (OMF) that acts as a porin on the outer membrane (16). In MexAB-OprM; which this review specifically analyzes; MexB is part of the RNDt family, with MexA and OprM belonging to the PMFP and OMF protein families respectively. Both the MexA and MexB proteins are recognised by the OMF (OprM). The MexB trimer does not directly contact OprM; instead, MexA forms a funnel-like hexamer, linking both MexB and OprM through hydrogen bonding at a number of critical residues. Contact between MexA-OprM and MexA-MexB relies on domains; protein structural units that contain the residues serving as contact points. These residues bind the proteins via hydrogen bonding or hydrophobic interactions, forming the tripartite efflux pump (17).

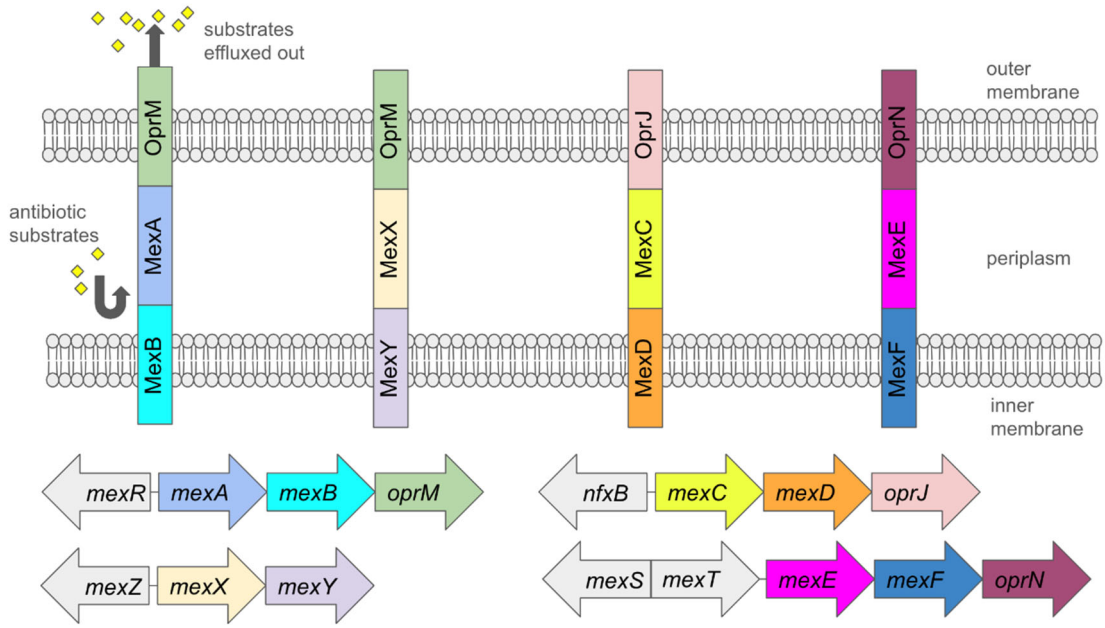

Figure 1. Representations of Mex pumps, notably, MexAB-OprM protein and its genes. a) MexAB-OprM (leftmost) representation on a gram-negative bacterial cell membrane. b) MexAB-OprM genes on mex operon (top left); MexR serves as one of its regulatory genes. Adapted from https://www.mdpi.com/1422-0067/23/24/15779

Mex-type efflux pumps in *P. aeruginosa* are capable of using a proton-motive force to eject substrates from both the periplasm and periplasm-cytoplasmic membrane interface into the extracellular space, even against a concentration gradient (18). This represents a profound strength of the superbug's antimicrobial resistance mechanism, as these substrates would have to re-bypass the outer membrane to interfere with their cytoplasmic components. Efflux pumps are particularly concerning due to their ability to confer MDR, compared to the narrow scope of mere antibiotic-inactivating enzymes. A reason behind this is their structure, where flexible, hydrophobic cavities in the membrane-bound MexB are able to lodge various compounds via hydrogen bonding and electrostatic attraction (19). Because of this, the development and overexpression of Mex-type pumps could greatly reduce the scope of treatments against pathogenic Pseudomonas.

Once formed, MexAB-OprM is responsive to the surrounding concentration of foreign toxicants - including antibiotics. Through PMF, conformational changes occurring within the MexB trimer causes one of its protomers to move to an active binding state, where substances can ingress into MexB into the periplasmic MexA tunnel. Once the particle concentration in the MexA protein exceeds the outside environment, they are effluxed out through the OprM factor. On the same note, a decreased concentration of drugs would shift the MexB factor toward a resting state; preventing the backflow of antibiotics (19).

Middlemiss et al. pointed out that mutations impairing MexB trimerization would lower efflux activity for only some substrates, as it is the component substrates first bind to (20). However, impaired MexB trimerization could also completely compromise the bacteria's efflux activity, insinuating that improper trimer

assembly could impede OprM recruitment. Without it, the efflux pump would not be assembled and no substrate efflux would occur. This assertion was supported by a proteomic study, validating how structural mutations on either component of MexAB-OprM could hinder pump assembly, especially if they were at the domains crucial for complex formation. Failure to properly assemble the pump caused a reduction in antibiotic resistance, in turn, reducing bacterial fitness (19).

Gene regulators of MexAB-OprM

The expression of MexAB-OprM is primarily controlled by three transcriptional regulator genes: mexR, nalC and nalD. Their protein products bind to the promoter and prevent RNA-polymerase (RNAP) in Pseudomonas from producing an mRNA transcript, rendering translation of the MexAB-OprM gene into protein unviable.

A homodimer, mexR is a divergent operon of MexAB-OprM. It encodes for MexR, a protein that binds to two sites in the mexR-mexA intergenic region, which are on overlapping promoters for both mexR and MexAB-OprM. As such, mexR is also negatively autoregulated - where the gene product represses its own transcription - hence forming a feedback loop that keeps expression in a certain range. Additionally, MexR can enter an oxidized state where it no longer binds to the promoter. This occurs due to two Cys residues in MexR's structure that are redox-active - easily oxidized by reactive oxygen species (ROS). Under oxidized conditions, the residues will form an intermonomer disulfide bridge (21). This conformational change inactivates MexR, which dissociates from the promoter, leaving MexAB-OprM to be freely transcribed and expressed. nalC operates indirectly. Also known as PA3721, it belongs to a divergent operon and represses two adjacent genes: PA3720 and PA3719, armR genes. ArmRs are anti-repressors - enzymes that repress a repressor. The genes allosterically inhibit MexR dimerization, preventing MexR from binding to the MexAB-OprM promoter. They also disassociate already-bound proteins. A higher concentration of NalC proteins would be expected to repress ArmR enzyme production; consequently, less ArmR would result in more MexR, repressing MexAB-OprM and reducing efflux (22). Similar to MexR and ROS, NalC is repressed by phenols that are damaging to *P. aeruginosa*, since the high protein affinity of phenolic compounds may interfere with metabolic processes in bacteria (23).

More similar to MexR, but situated on a different operon, nalD functions by binding directly to the MexAB-OprM promoter, preventing transcription. Novobiocin, an antibiotic, negatively modulates NalD, causing its disassociation from the promoter (24).

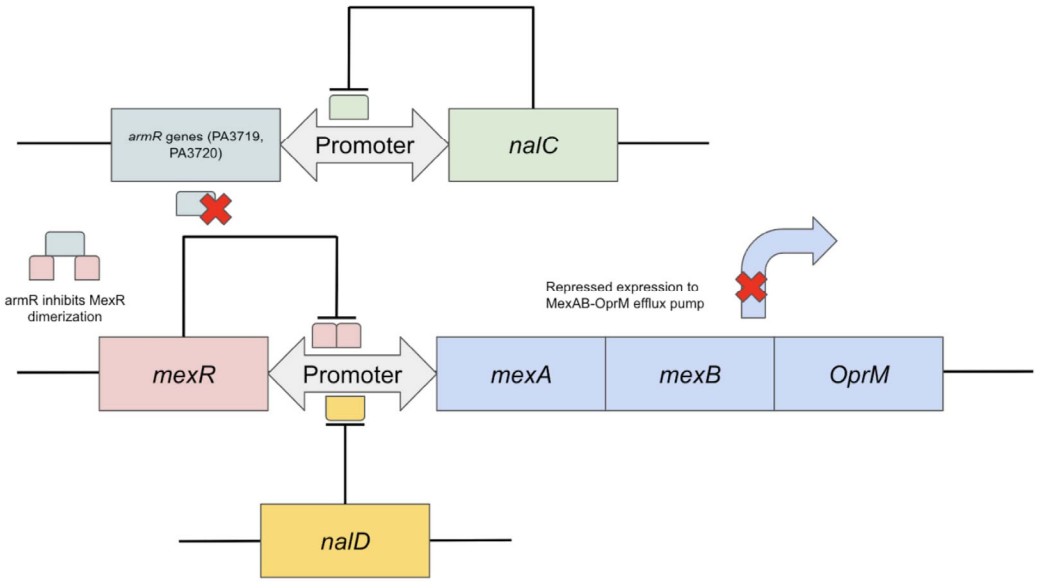

Figure 2. Transcriptional repressors of MexAB-OprM and their mechanisms of action. NalC represses armR expression; ArmR proteins normally cause MexR loss-of-function, but their absence allows MexR to bind to MexAB-OprM operon promoter. NalD does the same, limiting MexAB-OprM expression.

Methods

Information for this literature review, especially the introduction, was primarily sourced from NCBI and searched using Google Scholar. Data surrounding the mutations and their effects were extracted from investigations published in the ASM journal, NCBI and the Oxford University Press. International studies, conducted in Iran, China, and Pakistan, were also surveyed when discussing how varying antibiotic treatments could spawn distinct resistance patterns. One figure was reused from an IJMS under a CC-BY license; others were recreated using PowerPoint.

Results

Mutations affecting MexAB-OprM

Mutations represent changes in the genome of an organism. Whether they are single nucleotide polymorphisms (SNPs)--which represent substitutions of specific DNA bases–deletions, or additions, or such mutations can cause a loss or gain of function in the protein that the gene encodes for. An early peptide termination, for instance, could render a protein nonfunctional. A codon change could yield an amino acid with different properties, affecting the whole protein. An addition could introduce a new, functional domain in an enzyme, or it could cause a frameshift mutation, rendering the protein possibly non functional. While the selective pressure of antibiotics do not affect the mutation rate of pathogenic bacteria, they emphasize the benefit of antibiotic-resistance mutations, increasing the allelic frequency of resistance genes. As susceptible bacteria are killed, it creates an opportunity for resistant bacteria to proliferate. In this context, mutations in *P. aeruginosa*, particularly MexAB-OprM's transcriptional regulators, could be deleterious: a nonfunctional repressor would cause efflux pump overexpression and augment multidrug resistance.

mexR mutants

Researchers have found mutations observed in mexR from clinical isolates that cause MexAB-OprM overexpression. For instance, nonsense

mutations at mexR may lead to premature termination. There are two domains of concern (25): loss-of-function mutations observed between residues 36-97 on each monomer, the DNA-binding domains of the MexR dimer - rendered the protein unable to block MexAB-OprM transcription. Those on the dimerization domains - composed of N- and C-terminal regions (residues 5–17 and 120–139) are equally significant as they potentially hamper the protein's ability to form the MexR homodimer - hence rendering the protein unable to repress efflux pump overexpression and conferring resistance. Choudhury et al. predominantly sourced isolates from surgical and ICU wards (26). Overlaps between the mutation patterns of isolates categorized them into two groups: PMUT-1 (19 point) and PMUT-2 (22 point). In both these groups, the presence of deletions on the binding domain and additions elsewhere caused significant frameshifts. Relative to the wildtype strain (PA01), nearly all of the mutants in this study exhibited MexA overexpression. This led to significant meropenem (up to 256 fold) and ciprofloxacin (up to 128 fold) MIC (minimum inhibitory concentration) increases, which is the lowest drug concentration at which bacterial growth is inhibited. These drugs belong to the classes carbapenems and fluoroquinolones, which are first line treatments for *P. aeruginosa*. Aside from point mutations, frameshifts can also occur in nosocomial strains, resulting in OprM overexpression and consistently higher antibiotic MICs for the bacteria: up to 4-fold for cefepime and ceftazidime (cephalosporins) and up to 8-fold for meropenem (a carbapenem). mexR can also be influenced by transposable gene elements, known as transposons. IS21, a 2131 base pair insertion sequence that performs as a transposon was found in a *P. aeruginosa* strain. It contributed to MexR inactivation, which increased antibiotic MICs over a range of relevant first-line drugs (27). Given that transposons have the ability for horizontal gene transfer to different bacteria, rapidly spreading resistance can occur through these sequences. Ziha-Zarafi et al., however, concluded that the type of mexR mutation and levels of MexAB-OprM overexpression did not have a discernible correlation (28). Other studies investigating strains have documented nalB-type mutants located on the mexR gene. Across an antibiogram of different antibiotics (including carbapenems, cephalosporins, aminoglycosides and fluoroquinolones), nalB-type mutants displayed resistance to first-line quinolones, with the lowest being ciprofloxacin (72.9% resistant) and the highest being amikacin (87.1% resistant). There was also a statistically significant correlation between nalB mutation and ciprofloxacin resistance ($p=0.016$) (29). Even a point mutation on mexR is capable of increasing antibiotic resistance: strain OCR1, a notable nalB-type SNP mutant, is a source of discussion and derivative strains due to its association with efflux component (MexA and OprM) overexpression (30). The subsequent overexpression of MexAB-OprM in these MDR NalB-type mutants has, across different studies, consistently led to higher meropenem, ceftazidime, cefepime, ofloxacin and other drug MICs compared to the wildtype (31,32).

nalC and nalD mutants

nalC (PA3721) mutants in Pseudomonas have demonstrated an ability to confer resistance to aztreonam, a first-line beta-lactam, even without mutations on other regulatory genes, mexR and nalD. Aztreonam MICs for these nalC mutants ranged from 8µg/ml to > 256µg/ml, while wildtype strains demonstrated values of 4µg/ml (22). Literature has cited nalC mutants with MexA overexpression values similar to the wildtype (33), as well as more than quadrupled cefepime and ticarcillin MICs (34).

An earlier paper by Quale et al. confirmed that nalC and nalD mutants had greater resistance toward a range toward first-line cephalosporins and carbapenems, even when mexR was not mutated in the strains (35). nalC mutants are also found in nosocomial studies. Having similar mutations as those from environmental strains, Tafti et al. reported that 37% of the isolates sourced from burn victims had nalC mutant strains resistant to at least two carbapenems (36). Other literature had reported nalC mutation rates > 87.1%, although this disparity in resistance can be attributed to different treatment methods, as outlined in the conclusion (37).

Literature recognizes two of the most common nalC mutations, Gly71Glu and Ser209Arg, to be significantly associated with MexA overexpression (38). There are some noteworthy nalC mutations which warrant further research as they cause spikes in resistance. Ala186Thr (22) and Leu61Pro (33) caused an aztreonam MIC spike to > 256µg/ml compared to 4µg/ml for the wildtype, and overexpressed MexA 11.7-fold. A 500bp deletion also multiplied MexA expression by 17.9 fold relative to PA01, exhibiting the detrimental impact of larger deletions (33).

nalD mutants are much less predictable than nalC, often including more variable deletions and insertions. Generally, the resulting frameshift contributes to higher MexA expression in strains with both nalD and nalC mutations compared to nalC mutations alone (35). For instance, a study producing nalD mutants identified great variance in its mutations. These ranged from deletions, substitutions and silent mutations. When a wildtype nalD gene was reintegrated into a deletion mutant, there was a resulting decrease in MexA expression by nearly 80%.

Additionally, resistance to nalidixic acid, a fluoroquinolone, increased by 8 times; ticarcillin, a beta-lactam, by 8 times; and aztreonam, a beta-lactam, by 16 times; suggesting that mutations in this gene can lead to profound antibiotic resistance (39). A separate paper used an in silico approach, substantiating that nalD protein variation from clinical isolates had a particularly strong association with aztreonam resistance, more so than mexR or nalC (40). This claim was further reinforced by tracking aztreonam resistance of *P. aeruginosa* within a patient. Obtaining isolates in intervals revealed that aztreonam resistance only arose after the bacteria had acquired an SNP: Phe198Leu on nalD. Comparing this with a crystal structure of the NalD protein showed that the mutation occurred on the 10th alpha helix, a crucial part of the NalD protein's secondary structure that enables dimerization (24).

Discussion

This review provided insights into the relationship between MexAB-OprM transcriptional repressor mutations, and subsequent virulence from efflux pump overexpression. The discussion will reflect upon the limitations of the current research, and the consequences these may harbor upon existing interpretations of virulence data from mutant *P. aeruginosa* strains.

Other genes affecting antibiotic resistance

Other genes besides MexAB-OprM also influence the resistance shown by bacterial strains; the proteins they express may be components or regulators for different Mex pumps. This can explain the varying levels of resistance displayed by strains of the same mutation, most commonly seen among strains with the nalC substitutions Gly71Glu and Ser209Arg (38). This could be attributed to

other efflux pumps in Pseudomonas, notably the MexXY-OprM system, which makes use of the same OprM OMF (14). Hence, overexpression of the pleiotropic OprM gene would promote resistance conferred by this other pump. Other genes that play regulatory roles for Mex pumps may also affect antibiotic resistance data among the multidrug-resistant strains of Pseudomonas. Other genes, such as the RocS2-RocA2 system (41) or MexT (42), are also capable of influencing MexAB-OprM, despite this not being their primary function. This contributes to the differences in resistance levels. It is notable that mutations among these regulatory genes are not mutually exclusive: simultaneous coexpression of multiple Mex pumps in Pseudomonas have been found in strains, and often have equal or increased resistance against antibiotics compared to single pump mutants (49). In double efflux strains overexpressing both MexAB-OprM and MexXY due to mutations in mexR and mexZ, the transcriptional regulator for MexXY, Pseudomonas had reduced susceptibility against a wide range of aminoglycosides, cefepime, aztreonam, and other drugs known to be substrates of both pumps (33). Lee et al. validated this conclusion, recording multiplicative effects on antibiotic resistance in combinations of strains overexpressing MexAB-OprM, MexCD-OprJ and MexEF-OprN. Mutant strains were even more resistant when they overexpressed multiple pump families. Simultaneous overexpression of TetA or TetC pumps - limited to extruding tetracycline - and MexAB-OprM led to much higher levels of resistance against tetracycline compared to when MexAB-OprM was knocked out (50). This suggests that the overlapping substrates of different pumps are especially prone to efflux in cases of coexpression.

A first step to resolving these research obstacles would be to develop a more comprehensive resistance profile for different Mex pumps. For instance, it is known that MexAB-OprM overproduction often reduces beta-lactam susceptibility, and MexXY-OprM has the same relationship with aminoglycoside treatments. Identifying the substrates associated with each efflux pump will allow researchers to correlate the antibiotic MIC changes of mutant strains with the mutations affecting a specific pump, strengthening existing analyses of mutations and their effects. A way to enact this would be the use of gene knockout strains to eliminate the antibiotic efflux caused by Mex pumps outside the one being tested. This also eliminates the presence of regulatory genes for Mex pumps that have pleiotropic effects. For example, MexT primarily influences MexEF-OprN but is also known to affect MexAB-OprM expression (51); but it was demonstrated that MexAB-OprM was responsible for effluxing its substrates if MexEF-OprN was knocked out in the strain subject to an antibiotic assay. An equally important facet of efflux pump research is measuring protein overexpression. By measuring the protein expression levels specific to one pump - with minimal effect on other phenotypes - researchers can have greater confidence that a certain efflux pump was responsible for conferring resistance against a specific antibiotic. For instance, a study that measures OprM instead of MexA expression levels to demonstrate MexAB-OprM overexpression, then relates MexAB-OprM overexpression to increased resistance, fails to account that OprM is a component in other efflux pumps. Applying these two measures could reduce misinterpretations in data caused by efflux pump coexpression and pleiotropic regulatory and component genes.

Our evaluation could also be extended to non-efflux-pump resistance. Despite the exceptionally low substrate specificities of efflux pumps, their inability to remove certain compounds, such as MexAB-OprM and imipenem, can be masked by other resistance mechanisms. Previous literature has highlighted the influence of metallo-beta-lactamases and AmpC cephalosporinase for combating antibiotics in *P. aeruginosa* (43,44). Future research could normalize comparing MIC and antibiogram readings of pre-therapy and post-therapy strains where possible, especially among clinical strains, instead of comparing antibiotic resistance results against a wildtype strain. This method would account for undetected, yet accumulated mutations in other resistance genes.

Positions of mutations on MexAB-OprM transcriptional regulator genes

Antibiotic resistance can also be influenced by the positions of mutations. For MexR, crystal structure analyses have identified residues within the protein's binding and dimerization domains, allowing a trend to be recognized between mutations at these sites and efflux pump overexpression as the MexR repressor becomes dysfunctional (25). The same was achieved with NalD, where important DNA-binding, ligand-binding, and dimerization domains were linked with certain alpha helices (24). However, despite the recurring substitutions of NalC - Gly71Glu, Ser209Arg and Arg97Gly - which were all consistently associated with MexA overexpression (22), there is a lack of NalC structural research. Identifying key NalC residues within its relevant domains is crucial to determine whether mutation sites have implications on efflux pump overexpression - after all, some mutations may have been insignificant, which would cause existing results to be misinterpreted. Interestingly, in MexR, even mutations outside known domains can occur and cluster, still conferring antibacterial resistance to Pseudomonas despite their effects on MexR impairment being unclear. Adewoye et al. documented that mexR SNP mutations clustered between residues 59-83 led to negligible MexR protein production (45), and Quale et al. found that MexA expression remained similar between strains with binding site mutations and those without (35): demonstrating that mutations can lie outside important domains and still affect efflux pump expression, and hinting that other residues in the MexR gene also contribute to the protein's stability and function. As such, further investigation - such as crystal structure analyses of mutants - is required to understand the significance of residues outside known domains.

Different sources of bacterial mutants

The source of mutated bacteria is a point of consideration, especially nosocomial strains. Tafti et al. postulated that the usage of varying antibiotics could promote distinct resistance patterns across geographical regions (36). A proven example is with the antibiotic imipenem: in clinical Pseudomonas isolates from Iran, high imipenem resistance among isolates (95%) were attributed to nalC mutations that correlated with the drug's frequent prescription (36), while 81.6% of MDR strains were resistant according to a Pakistani study (46), and 71.5% in China (47). It is worth noting that the articles were published years apart - as the proportion of *P. aeruginosa* isolates resistant to imipenem has risen with time - and that the samples were sourced from different types of infections (48). Yet, the varying antibiotic therapies used across these studies would have played a role in their strains' varying susceptibilities as well. Moreover, certain drugs in combination

therapies have been hypothesized to more frequently trigger specific mutations, such as MexAB-OprM associated resistance following aminoglycoside treatment. A method of research could be tracing the progression of resistance in patients with frequent antibiogram assessments to gain a better understanding of this relationship, as introducing antibiotics can often trigger resistance toward new substrates if the bacteria are not killed. This assessment could be expanded across different patient groups - accounting for the severity of infection, infection caused, and patient demographics. Yan et al., for instance, related a cancer patient's aztreonam treatment with a nalD mutation that conferred resistance to Pseudomonas against that drug days later, leading to sepsis (40). Studies like these provoke debate regarding the antibiotics used for Pseudomonas treatment and their dosage, as the microbe's tendency to accumulate mutations may encourage higher dosages or more potent therapies in order to avoid mutation risks. Ultimately, elucidating a relationship between the antibiotic therapies used by patients, and how this selective pressure could mutate *P. aeruginosa*, is key to developing a treatment standard that limits superbug spread.

Efflux pumps and their implications on virulence

Interestingly, efflux pump overexpression can confer negative effects on bacterial virulence. There is substantial evidence that QS systems, which regulate virulence factor production in Pseudomonas, may be impaired by efflux pump overuse. The broad range of efflux pump substrates often cover QS signaling molecules as well; MexEF-OprN, for instance, reduces intracellular levels of PQS, an AI (autoinducer) that regulates the PQS system, by extruding HHQ, a precursor to PQS (52). Both MexCD-OprJ and MexEF-OprN also efflux out kynurenine, another PQS precursor (53).

MexEF-OprN also affects the las and rhl QS systems (54) by reducing levels of C4-homoserine lactone, a QS signaling molecule (55). The combined effects of reduced AI levels downregulated the expression of AI-dependent virulence factors, including pyocyanin, elastase, and rhamnolipids; leading to reduced swarming and lethality in mice (52) and C. elegans infection models (55). Through impairing QS, efflux pump overexpressing strains could have an evolutionary advantage compared to the WT even without the selective pressure of antibiotics, because they are less likely to trigger the QS response when unneeded due to the reduced level of autoinducers. As such, mutant strains are more resource-efficient and competitive (53). There are other mechanisms behind the dampened virulence of efflux pump overexpressing strains: instead of an impaired QS response, MexCD-OprJ overexpression could reduce virulence via increased C3 opsonization (56), which would clear the pathogen from an infection more efficiently as the complement C3 causes bacteria to be more susceptible to immune attack. Another study cited downregulation of genes encoding for T3SS (57), a key virulence factor that delivers toxins into a host cell's cytosol.

MexAB-OprM is more conflicting. An early study by Evans et al. documented a reduction in the release of virulence factors regulated by the LasI/R QS system - pyocyanin, elastase, and protease - from nalB mutants that overexpress MexAB-OprM (58). Alcalde-Rico et al. later identified that the impaired QS response was caused by reduced extracellular and intracellular HHQ and PQS levels in a MexR mutant (59). This relationship between MexAB-OprM overexpression and reduced virulence was substantiated in an in-vivo experiment, as

WT Pseudomonas strains were shown to kill more infected mice and cause greater lung burden than mutant strains (60). However, there was no MexAB-OprM knockout strain used in this experiment, which weakens the result, as reduced virulence could have been caused by an extraneous gene. Conversely, there is more compelling evidence from opposite findings that argue how MexAB-OprM's overexpression increases virulence instead. Murine infection models involving the lungs (61), kidney cells (62), and gastrointestinal tract (63) have consistently illustrated that MexAB-OprM overexpressing mutants are more lethal and competitive against their WT counterparts. This hypothesis is somewhat supported by known underlying mechanisms, as Zahedani et al. found a strong association between biofilm formation and efflux pumps in nosocomial *Pseudomonas aeruginosa* strains (64). PAβN, an EPI (efflux pump inhibitor), was also reported to cause the downregulation of key virulence genes in a WT strain (65).

Non-antibiotic treatments to target efflux pumps

It is necessary to be cognizant of the interplay between efflux pump overexpression and virulence because this relationship has implications on Efflux Pump Inhibitors or EPIs, a promising yet commercially untapped treatment against efflux pump-expressing bacteria (66). EPIs targeting Mex pumps in Pseudomonas have the advantage of reducing antibiotic resistance by more than 10 fold across different substrates and even reduce virulence. Yet, they come with a slew of challenges - one of which is their risk of increasing virulence and worsening prognosis for patients (67), because their use reduces the extrusion of substrates by pumps - including QS signaling molecules. PAβN use against a murine lung infection caused by PAO1, for instance, was shown to cause rhamnolipid expression; a virulence factor that allows Pseudomonas to penetrate epithelial barriers (61). A solution to this could be the administration of pump-specific or strain-specific EPIs for therapy (68). For instance, knowing that MexAB-OprM overexpression can lead to increased virulence instead of reducing it like other pumps; it is an ideal target. In practice, RP1, which is thought to be specific to only MexAB-OprM, had promising results in the treatment of murine lung infection models when paired with an antibiotic (69). A balance must be struck between addressing the multidrug resistance of Pseudomonas while preventing the pathogen from becoming more virulent. To enforce this, more research is needed to pinpoint the specific QS-signaling molecule substrates of Mex pumps. Virulence factors have different modes of action and are hence specific to infection types, and making sense of the pathway from efflux pump overexpression, QS system impairment and virulence factor downregulation is important to understand what pumps to target in particular infection cases. Other pathways causing reduced or increased virulence could also be explored besides influencing QS, so that we have a better understanding of how to tackle different infection types. The study on MexCD-OprJ overexpression and C3 opsonization (56), for instance, used a lung infection model, and the study relating biofilm formation to MexAB-OprM overexpression used a range of sources including burn wounds, urine, and blood (64). EPIs will equally need to be developed and tested outside of murine infection models as a novel drug against Pseudomonas, so that they can be administered with the appropriate antibiotic(s) when used in combination therapies.

In addition to EPIs, phage therapy offers an alternative approach to treating Pseudomonas infections. Through bacteriophage infection,

this method involves initiating lytic or lysogenic cycles within bacterial hosts, resulting in the lysis of bacteria and the subsequent release of additional phages, which can then infect other bacteria (70). Efflux pumps are relevant because they can be exploited as a surface receptor by using phages that use the pump as binding target to infect the microbe. In Pseudomonas, this was demonstrated by Chan et al. who used lytic phage OMKO1 capable of binding to OprM for treatment. In turn, mutants overexpressing MexXY-OprM and MexAB-OprM were lysed, stripping the strains of their resistance toward ciprofloxacin and ceftazidime. Interestingly, antibiotic resistance is not the only factor here; phage resistance can develop among bacteria, which often comes at the cost of increased antibiotic susceptibility, which was observed with phages targeting TolC of AcrAB-TolC efflux pump system in E. coli. Consistent with this finding, Chan et al. pinpointed that OMKO1 phage-resistant strains also exhibited lower resistance against the antibiotics effluxed by the two Mex pumps (71). However, some bacteria are capable of resisting the trade-off, as the work of Burmeister et al. showed how phage-resistant E. coli still retained the efflux function of AcrAB-TolC when only subject to minor mutations (72). Bacteriophage administration as a treatment method, therefore, will have to undergo rigorous trials before use as therapy. In general, more OMF-targeting phages need to be researched for therapy against *P. aeruginosa* infections for them to be used on a wide scale, because bacteriophages are extremely strain-specific. Knowing the substrates of the targeted pumps provides the added benefit of knowing what phage-antibiotic combination therapies to administer depending on the infection (71).

Conclusion

A threatening pathogen across nosocomial communities, *P. aeruginosa* represents a growing concern in medicine due to its ability to rapidly accumulate mutations. One of its mechanisms - multidrug efflux (Mex) pumps - particularly, the MexAB-OprM tripartite system, confers non-specific resistance to many first-line drugs. When the transcriptional repressor genes for this system mutate, namely mexR, nalC and nalD, there is a consistent trend of increased first-line antibiotic MICs including carbapenems, cephalosporins, fluoroquinolones, and aminoglycosides, rendering treatment especially challenging. The possibility of mutagenesis from patient therapies and even transposable elements aggravates this problem. To develop our understanding of *P. aeruginosa* resistance patterns in-vivo and improve current treatment standards, increased research using nosocomial strains is a priority, diversifying our current knowledge by using bacterial strains from varying sources of infection, subject to different antibiotic therapies, and cultured from different patient profiles. Moreover, more proteomic studies of the Mex systems - not just MexAB-OprM - can help define the substrates each pump is responsible for effluxing. Increased structural genomics research of the MexR, NalC, and NalD transcriptional repressors is equally important to identify which residue mutations are associated with resistance. Developing EPIs and phages, untapped solutions against the efflux pump problem, could also be the next step in the fight against multidrug resistant bacteria.

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