Expression of the MexXY aminoglycoside efflux pump and presence of an aminoglycoside modifying enzyme in clinical Pseudomonas aeruginosa isolates are highly correlated

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The impact of MexXY efflux pump expression on aminoglycoside resistance in clinical Pseudomonas aeruginosa isolates has been debated. In this study, we found that in general, elevated mexXY gene expression levels in clinical P. aeruginosa isolates confer to slight increases in aminoglycoside MIC levels, however those levels rarely lead to clinically relevant resistance phenotypes. The main driver of resistance in the clinical isolates studied here was the acquisition of aminoglycoside modifying enzymes (AMEs). Nevertheless, acquisition of an AME was strongly associated with mexY overexpression. In line with this observation, we demonstrate that the introduction of a gentamicin acetyltransferase confers to full gentamicin resistance levels in a P. aeruginosa type strain only if the MexXY efflux pump was active. We discuss that increased mexXY activity in clinical AME harboring P. aeruginosa isolates might affect ion fluxes at the bacterial cell membrane and thus might play a role in the establishment of enhanced fitness that extends beyond aminoglycoside resistance.
Introduction

The metabolically versatile bacterium *Pseudomonas aeruginosa* is known to cause a wide range of opportunistic infections and is recognized as one of the leading causes of severe hospital-acquired acute infections (1–6). In cystic fibrosis (CF), *P. aeruginosa* frequently establishes lifelong chronic infections, associated with hyper-inflammation, severe tissue damage and ultimately high mortality rates (7–9). Although antimicrobial therapy is a cornerstone of the management of CF, even intensified treatment is often not able to clear the infection (10–12). The recalcitrance of chronic *P. aeruginosa* infections against antibiotic treatment is only incompletely understood. A known important adaptive resistance trait is the induction of an active efflux of antibiotics into the extracellular space (13–15). Multi-drug efflux pumps recognize a variety of structurally diverse compounds as their substrates, including different clinically relevant antibiotic classes (3, 16). In *P. aeruginosa* there are four efflux pump systems that are of particular interest: MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM (17, 18). Previous studies have shown that mexZ, encoding the negative regulator of MexXY-OprM, is one of the mutational hot-spots in CF-derived *P. aeruginosa* isolates (19–22). *P. aeruginosa* mexZ mutants exhibit constitutive overexpression of MexXY-OprM. This efflux pump was shown to play an important role in the development of resistance against aminoglycosides and fluoroquinolones (23, 24), both being important antibiotic classes in the treatment of CF patients (10).

However, the causal contribution of *P. aeruginosa* MexXY-OprM pump expression to aminoglycoside resistance in the clinic is disputed. Recently published evolutionary studies did not detect the emergence of mexXY overexpressing strains even under...
prolonged aminoglycoside exposure (25, 26). Other studies show only limited aminoglycoside resistance in clinical isolates despite overexpression of the MexXY-OprM efflux pump (27). Nevertheless, the MexXY-OprM overproducing strains seem to have a fitness advantage when grown under sub-MIC tobramycin concentrations, a condition that especially CF-isolates face during chronic lung infections (7).

In this study, we aimed to shed further light on the impact of MexXY efflux pump expression on aminoglycoside resistance in clinical P. aeruginosa isolates. For this purpose, we analyzed the expression of genes encoding the MexXY efflux pump in a recently published collection of 412 clinical P. aeruginosa isolates from a variety of geographical origins and infection sites (28). We found that clinically relevant tobramycin resistance was in general not associated with elevated mexXY gene expression levels, but that the acquisition of aminoglycoside modifying enzymes (AMEs) was the main driver of resistance. However, acquisition of an AME was strongly associated with mexY overexpression, and the full gentamicin acetyl-transferase-mediated gentamicin resistance potential was dependent on an active MexXY efflux pump.

Material and Methods

Bacterial strains and growth conditions

We analyzed efflux pump gene expression in 412 clinical P. aeruginosa isolates, which have been previously collected from different laboratories and clinics and for which transcriptional profiles have been recorded (28). The RNA sequencing (RNA-Seq) data of the clinical isolates have been uploaded to NCBI’s Gene Expression Omnibus.
(GSE122938). Unless otherwise stated, bacteria were grown in standard rich medium culture conditions (LB medium) at 37°C. All strains used in this study are listed in Table S1.

**Generation of deletion mutants**

Markerless deletions of structural efflux pump genes were performed as previously described (29). Primers for the amplification of adjacent regions of the targeted genes from UCBPP-PA14 genomic template DNA are listed in Table S2. Briefly, approximately 500 bp located upstream and downstream of the target regions were amplified and then combined in an overlap extension PCR (to generate PA14ΔmexXY, PA14ΔmexCD, PA14ΔmexEF) (30), or integrated into the pEX18AP cloning vector in a two-step process (to generate ΔmexAB). PA14ΔmexZ, PA14ΔmexR and the double mutant PA14ΔmexZ/ΔmexR have been generated in the frame of a previous study (31).

**Sequence variant calling**

We screened for mutations in mexZ, encoding the negative regulator of MexXY, in previously published whole genome sequencing data (32). Mapping was accomplished using stampy and variant calling was performed using SAMtools (version 0.1.19) with UCBPP-PA14 (see RNA sequencing) or PAO1 (NCBI Assembly: GCA_000006765.1 ASM676v1) as a reference. Strain background was assessed based on the phylogenetic analysis documented in Khaledi et al. 2020 (32). PA7-like isolates were excluded in this study.

**Detection of aminoglycoside modifying enzymes**
The resistance genotyping tool ARIBA (version 2.10.2) (33) using the CARD (34) as resistance factor data-base (downloaded via the ARIBA command ‘getref card’ on 2020/11/09) was employed to detect aminoglycoside modifying enzymes from the DNA sequencing reads directly in its default settings. We screened the results for resistance factor descriptions containing “aminoglycosides” and excluded hits for mexZ as well as the chromosomally encoded APH(3’)-II phosphotransferase from our analysis.

**MexY protein immunodetection**

Overnight grown cultures of clinical isolates (published in (35)) or PA14 deletion mutants (ΔmexZ or ΔmexR/ΔmexZ) were used to inoculate 10 ml LB main cultures (1:100). After growth to early stationary phase (OD_{600}=1.8-2.1), 1 ml of the culture was harvested by centrifugation (6,000×g, 5 min). The pellet was either stored at -70 °C or directly suspended in 100 µl lysis buffer (2 % sodium dodecyl sulfate, 20 mM tris-hydrochloride, pH 8.0) and boiled at 95 °C for 10 min. Residual cells were removed by centrifugation at 21,000 × g for 30 min and the resulting supernatant was sonicated (3 min, setting 100 % ultrasound power, Elma Trasonic waterbath) to shear DNA and reduce viscosity. The protein concentration was measured at an absorbance of 280 nm with a NanoDrop spectrophotometer (Thermo Scientific). In total, 60 µg of whole cell protein solution was mixed 2:1 with SDS sample buffer and heated at 95 °C for 10 min before it was applied to a 7.5 % SDS-polyacrylamide gel (SDS-PAGE). After separation, the gel was subsequently blotted to a PVDF membrane (Immobilon-P, Millipore). MexY protein was detected with purified, polyclonal anti-MexY serum (1:20,000) kindly provided by Katy Jeannot, University of Franche-Comté, Besançon, France, followed by a peroxidase conjugated
secondary antibody (anti-rabbit-PO, Dianova, 1:2000) and Lumilight (chemiluminescent peroxidase substrate, Roche) incubation.

**Determination of gentamicin resistance in PA14 efflux mutants**

In order to compare gentamicin resistance levels among the diverse PA14 efflux pump mutants bacteria were grown for 24 h in Mueller Hinton 2 medium (Sigma Aldrich) containing 2-fold dilutions of gentamicin (Roth, concentration range 50 µg/mL to 12800 µg/mL) in 96-well plates at 37°C and at constant shaking (180 rpm). Each well was inoculated with an optical cell density of OD$_{600}$ = 0.01. The lowest concentration of gentamicin, which prevented growth of the bacteria to an OD$_{600}$ of 0.1 was recorded.

To inhibit efflux pump activity 40 µM, 20µM or 0 µM (control) carbonyl cyanide m-chlorophenyl hydrazine (CCCP; Sigma Aldrich) dissolved in dimethyl sulfoxide (DMSO, Sigma Aldrich) was added to the medium.

**Results**

*mexY overexpression can be frequently found in clinical *P. aeruginosa* isolates*

In this study we took advantage of previously recorded transcriptome data on 412 clinical *P. aeruginosa* isolates grown in LB medium until late logarithmic phase (OD$_{600}$ = 2) (28). Most of the clinical isolates exhibited resistance against at least one class of antibiotics (32), and about one third of the *P. aeruginosa* isolates have been isolated from CF patients. We focused on the expression of the MexXY-OprM efflux pump and extracted gene expression values for *mexY* across all isolates. We defined a cut-off value for high *mexY* gene expression by analyzing the distribution of normalized reads per gene (NRPG)
over all analyzed clinical isolates (Figure 1). We found a biphasic pattern of mexY gene expression and classified an isolate as overexpressing mexY, if at least 575.44 NRPG mapped to mexY (Figure 1; orange line). This resulted in the categorization of 53% (n = 217) of the clinical isolates as mexY overexpressing. 9% (n = 37) of the isolates exhibited intermediate mexY expression values (between 229.09 and 575.44 NRPG) (Figure 1).

Under non-inducing rich medium growth conditions, MexZ acts as a negative regulator of mexXY gene expression. We found at least one non-synonymous mutation in mexZ in 86% (n = 187) of the mexY overexpressing clinical isolates. There was no clear mutational hot spot within the mexZ gene sequence (Figure S1), although isolates with high mexY expression levels tend to be enriched in mutations in the N-terminal region of the gene, in which the DNA binding domain was identified (22). Nevertheless, our results suggest that inactivation of the negative regulator MexZ leads to constitutive efflux pump overexpression in the majority of our clinical isolates. However, there are additional MexXY-OprM expression regulators, which might play a role in our data set. E.g. overexpression of the MexZ anti-repressor AmrZ or activating mutations in the sensor histidine kinase of ParRS have been described to positively control mexXY expression (22).

**Overexpression of mexY correlates with high MexY protein abundance**

To evaluate whether the constitutively high mexY transcript levels are translated into high protein levels, Western blot analysis was performed on a set of 53 clinical *P. aeruginosa* isolates (35). We included a PA14ΔmexZ (31) mutant as a control. Due to the inactivation of the negative regulator, MexZ, PA14ΔmexZ is producing elevated levels of the efflux
In six of the clinical isolates (almost) no MexY protein was detected, despite mexY overexpression (see arrows in Figure 2). We cannot exclude that variations in the protein sequence impedes the detection by the antibody used in the Western blot. Nevertheless, in general the clinical isolates, which exhibited high mexY transcripts, also produced high MexY protein expression levels. For most of the isolates (83%; n = 44), these levels were far above the levels of the PA14 ΔmexZ mutant. In line with the finding that not all clinical isolates harbored mexZ mutations, these results suggest additional levels of MexXY regulation in the clinical isolates studied here. In this respect, it is interesting that in a PA14 strain background with an inactivation of not only mexZ but also mexR, mexY expression was further increased (Figure 2). MexR represses transcription of the mexAB-oprM operon and OprM is an outer membrane factor that is shared between the MexXY and the MexAB efflux systems. Thus, there seems to be a functional link of the two efflux systems that also impacts mexY expression levels.

**Increased mexY expression has a limited impact on resistance against tobramycin**

We next evaluated, whether higher expression of mexXY-oprM was associated with aminoglycoside resistance. Among the group of mexY overexpressing isolates, 51% (n = 110) were classified as tobramycin susceptible according to the standards defined by the Clinical and Laboratory Standards Institute (tobramycin minimal inhibitory concentration (MIC) < 16 µg/mL; Figure 3) (36), indicating that mexY expression is not of major importance for exhibiting clinical relevant resistance levels. Nevertheless, we detected a significant, yet limited, impact of increased mexY expression levels on resistance towards tobramycin. Compared to the tobramycin susceptible isolates with low mexY expression levels...
(n = 151), the 110 tobramycin susceptible isolates with high mexY expression exhibited a
two-fold higher mean MIC against tobramycin (2 µg/mL versus 1 µg/mL respectively,
Figure 4). Our results are in agreement with previously published data demonstrating that
efflux pump expression does play - albeit a minor - role in the development of resistance
against tobramycin (25, 27).

Presence of aminoglycoside modifying enzymes and fusA1 mutations are drivers
of resistance against tobramycin

Besides efflux pump overexpression, the acquisition of genes encoding for
aminoglycoside modifying enzymes (AME) are well known to confer to aminoglycoside
resistance (37). We therefore screened our clinical isolate collection for the presence of
AMEs. Within the group of tobramycin resistant isolates, 87% (n=111) harbored at least
one AME (for AME frequency see Figure S2), while in the group of tobramycin susceptible
isolates only 5% (n = 14) contained at least one AME (Figure 3). The presence of AMEs
was thus significantly overrepresented in the resistant isolates (hypergeometric test for
overrepresentation: p< 0.001) clearly indicating that a main driver for tobramycin-
resistance is the acquisition of an AME.

Recently, mutations in fusA were identified to contribute to elevated resistance levels
following prolonged aminoglycoside treatment (20, 26, 38). Thus, we investigated the
impact of those mutations on the overall resistance pattern in our data set. Of the overall
48 isolates with non-synonymous fusA mutations, six isolates (12.5 %) exhibited clinically
relevant (MIC ≥ 16 µg/mL) tobramycin resistance levels that could not be attributed to the
presence of an AME. This corresponds to 4.72 % of all tobramycin resistant isolates.
The full AME potential is dependent on \textit{mexXY} expression

Strikingly, 85% (n= 94) of the tobramycin resistant AME\textsuperscript{+} isolates also overexpressed \textit{mexY}. Only few (n= 6) tobramycin resistant isolates that harbored an AME exhibited low \textit{mexY} expression values. 11 isolates expressed intermediate \textit{mexY} levels (Figure 3). To test for a possible correlation of the presence of an AME and \textit{mexY} expression, we compared \textit{mexY} expression values of all AME\textsuperscript{+} isolates and all AME\textsuperscript{-} isolates. We found a significant 6-fold increased median \textit{mexY} expression in AME\textsuperscript{+} isolates (Figure 5, p-value < 0.001). Of note, this correlation was specific to \textit{mexY}. No correlation was identified between the presence of an AME and the expression of RND genes encoded in other important efflux systems (\textit{mexB, mexD, mexF}) (Figure S3).

Our results suggest a functional link of \textit{mexXY-oprM} expression and the presence of an acquired AME. To evaluate this further, we constructed clean deletions of structural genes of important ‘mex’ efflux pump systems (PA14\textDelta\textit{mexAB}, PA14\textDelta\textit{mexCD}, PA14\textDelta\textit{mexEF}, PA14\textDelta\textit{mexXY}) and introduced a gentamicin acetyl-transferase on the cloning vector pSEVA621 (39) into the respective mutants, the PA14 WT as well as into PA14\textDelta\textit{mexZ}. The introduction of the AME into all strains conferred high gentamicin resistance levels (Figure 6). The introduction of the AME into \textDelta\textit{mexCD} or \textDelta\textit{mexEF} strains did not lead to resistance levels that were different to those of PA14 AME\textsuperscript{+}, indicating that these pumps do not play the same important role for the full AME-mediated resistance phenotype as MexXY. However, when the AME was introduced into the \textDelta\textit{mexAB} mutant, this mutant showed the same phenotype as the \textDelta\textit{mexXY} AME\textsuperscript{+} strain (Figure 6), indicating that the MexAB-OprM efflux pump is important for the full resistance potential of the AME.
However, this phenotype was lost in the ∆mexAB mutant when complemented with oprM on the pSEVA634 expression vector (Figure S4). This suggests that the lack of OprM in the ∆mexAB AME+ strain, rather than the activity of the MexAB-OprM efflux pump is responsible for the phenotype.

We then added the protonophore carbonylcyanid-m-chlorphenylhydrazon (CCCP), which has been demonstrated to inhibit efflux pumps (40) to PA14 WT AME+, PA14∆mexXY AME+ as well as PA14∆mexZ AME+ and again recorded the resistance profiles. In agreement with a functional contribution of the efflux pump activity to the high AME-mediated resistance levels, we observed a dose-dependent reduction of gentamicin resistance levels in the PA14 WT AME+ and the ∆mexZ AME+ strains, but not in the ∆mexXYAME+ strain (Figure 7). It is important to note, that CCCP can also decrease the uptake of aminoglycosides via the disruption of the proton motive force (41–43). However, we did not detect an increase in overall gentamycin resistance upon CCCP addition, indicating that the experimental conditions used in this study are not interfering with aminoglycoside up-take.

In conclusion, our results show that a functional MexXY-OprM efflux pump is important for the full exploitation of the resistance potential of the horizontally acquired gentamicin acetyl-transferase.

Discussion

Aminoglycosides remain important in the treatment of P. aeruginosa infections, despite their known toxicity. In CF for example, tobramycin inhalation is applied as a means to
control chronic infection, and as a first-line treatment for the eradication of early acquisition of *P. aeruginosa*. Resistance to aminoglycosides is, however, common (37, 44–46). Resistance typically results from drug inactivation by plasmid- or chromosome-encoded aminoglycoside modifying enzymes (AMEs), or by mutation-driven resistance mechanisms (20, 26, 38). Nevertheless, resistance, because of increased efflux is also commonplace, particularly in isolates from CF patients and intensive care units (19, 47–49). Furthermore, aminoglycoside uptake was shown to be inhibited by the presence of mono- and divalent cations (42). Thus, the environment, e.g. in the CF lung might additionally impact aminoglycoside resistance levels.

The *P. aeruginosa* genome encodes several multi-drug efflux pumps. MexXY-OprM is the only pump that has been described to export aminoglycosides (3, 50). In addition to aminoglycosides, various substances are known to induce the expression of *mexXY* such as fluoroquinolones or cephalosporins (51). Here, we correlated the expression of *mexY*, the substrate specificity-providing component of the MexXY-OprM efflux pump, in 412 clinical *P. aeruginosa* isolates from various geographical origins and infection sites with their respective tobramycin minimal inhibitory concentrations (MIC). The high number of constitutively *mexY* overexpressing isolates (53%) in our data set clearly shows the importance of the expression of the MexXY-OprM efflux pump in the clinical context. Furthermore, the high number of isolates in our strain collection that did not acquire an AME provided the unique opportunity to evaluate the contribution of *mexXY* gene expression to tobramycin MIC values across a large number of clinical isolates. Indeed, in agreement with previous studies, increased *mexY* expression positively correlated with a decrease in tobramycin susceptibility (27). This effect, however, was limited and in general, isolates that overexpressed the pump, exhibited MIC values that were only two-
fold higher as compared to isolates that did not overexpress mexXY. In the majority of cases this did not result in clinically relevant resistance phenotypes. Instead, the main driver of clinically relevant resistance against tobramycin in our set of clinical isolates were horizontally acquired AMEs. They act by either acetylating, nucleotidylating or phosphorylating specific residues of the aminoglycosides and thereby decreasing the affinity of the compound towards the ribosome (37, 52).

Importantly, we observed a hitherto undetected high correlation between the presence of an AME and overexpression of mexY, indicating a relationship of pump overexpression and the exploitation of the full AME resistance potential. We showed that a laboratory strain that lacked the structural genes, mexX and mexY, did not gain the same aminoglycoside resistance level when harboring a gentamicin acetyl-transferase as compared to the wild type or an mexXY overexpressing strain. In agreement, inhibition of efflux pumps via the protonophore CCCP (40) led to a concentration-dependent decrease in aminoglycoside resistance levels in mexXY-expressing strains, but had no effect on the strain lacking the efflux pump encoding genes.

Thus, even though the efflux system on its own is not a major resistance determinant, it is obviously important for the development of high-level AME-driven resistance against aminoglycoside antibiotics. These results indicate that MexXY is working synergistically with the AME and could be involved in the export of the AME-modified aminoglycosides that otherwise would accumulate in the cell. Nevertheless, it is also conceivable that not the export of the modified aminoglycoside per se, but the enhanced activity of the efflux pump is important for the full resistance phenotype. In this context two things are interesting. First, in P. aeruginosa low extracellular pH increases resistance towards
aminoglycosides (53, 54) and second, RND efflux pumps operate as drug/proton antiporters and their activity has been shown to lead to intracellular H\(^+\) accumulation (55).

Thus, increased activity of MexXY in the presence of an AME and aminoglycosides, might lead to increased intracellular proton levels due to an elevated MexXY-driven efflux activity. The efflux pump-driven increase in the intracellular protons might further contribute to aminoglycoside resistance and possibly also to enhanced fitness under a variety of different stressful conditions.
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Figure 1: Distribution of *mexY* expression values and cut-off values for high *mexY* expression across clinical *P. aeruginosa* isolates. The distribution of log_{10} expression values (NRPGs) of *mexY* over all analyzed clinical isolates (n = 412 (28)) is depicted. Cut-off values for high (orange line) and low (red line) *mexY* expression were defined as the 30% distance from the lowest-point between the two maxima of the distribution to the two maxima, respectively.

Figure 2: Correlation of MexY protein abundance with *mexY* gene expression level and aminoglycoside resistance. (A) MexY (113 kDa) protein levels were detected by Western blot analysis using a polyclonal anti-MexY antibody in 53 clinical isolates. The results of tobramycin susceptibility testing as reported previously (35) are shown (MIC of tobramycin (MIC TOB) in μg/ml). *mexY* expression (also reported in (35)) is indicated as fold change gene expression as compared to the PA14 reference strain. (B) The PA14 Δ*mexZ* or Δ*mexRZ* mutant strains were used as positive controls on each blotting membrane. Arrows indicate strains with *mexY* overexpression on the mRNA level but missing or only faint MexY protein band. M = PageRuler prestained protein ladder. Black bars indicate borders of single western blot membranes.

Figure 3: Correlation of tobramycin MIC values with *mexY* gene expression. Expression of *mexY* in 412 clinical *P. aeruginosa* isolates relative to the tobramycin minimal inhibitory concentration (MIC) as reported previously (28, 32). Clinical isolates...
exhibiting \( \text{mexY} \) expression levels above the orange line were defined to exhibit intermediate, and above the red line high \( \text{mexY} \) expression levels. The black line delineates the CLSI breakpoint for clinical resistance. The blue color code indicates that the respective clinical isolate does not harbor an aminoglycoside modifying enzyme (AME\(^-\) isolates), while the red color code indicates that the isolates are AME positive (AME\(^+\)).

**Figure 4: High \( \text{mexY} \) expression correlates with elevated MICs against tobramycin.**

MICs of tobramycin susceptible isolates (MIC < 16 \( \mu \text{g/mL} \)) were assigned to high and low \( \text{mexY} \) expression levels, respectively. Boxes comprise the MIC values of 50% of the clinical isolates in the respective group; red line = mean; statistical significance: p-value < 0.05, Wilcoxon rank sum test.

**Figure 5: Presence of an AME correlates with high \( \text{mexY} \) expression.** Normalized expression values (\( \log_{10}(\text{NRPG}) \)) of isolates that were identified as AME containing (AME\(^+\)) and non-AME containing (AME\(^-\)). Boxes comprise 50% of the values, red line = median; statistical significance: p-Value < 0.01, Wilcoxon rank sum test.

**Figure 6: AME induced gentamicin resistance is impacted by the presence of the MexXY-OprM efflux pump.** Gentamicin resistance levels of \( P. \text{aeruginosa} \) PA14 and mutants thereof. All strains were transformed with the pSEVA621 vector, harboring a gentamicin acetyl-transferase (AME\(^+\)) (39). The \( \Delta \text{mexAB AME}^+/+\text{oprM} \) strain was complemented with pSEVA634::oprM. pSEVA634 harbors the same acetyl transferase as
pSEVA621. Red line depicts the median resistance level of at least 4 biological replicates; Test for statistical significance: Wilcoxon rank sum test; P-Value < 0.05.

Figure 7: The protonophore CCCP reduces the gentamicin resistance levels of strains with active MexXY-OprM efflux pumps. Gentamicin resistance levels of *P. aeruginosa* PA14 and mutants thereof. DMSO-dissolved CCCP was added in a concentration of 40µM, 20µM and 0µM. Each box includes 50% of the data with of least 6 biological replicates; the asterisk indicates a p-value < 0.05, Wilcoxon rank sum test.
mexY expression \[\log_{10}(nrpg)\]
