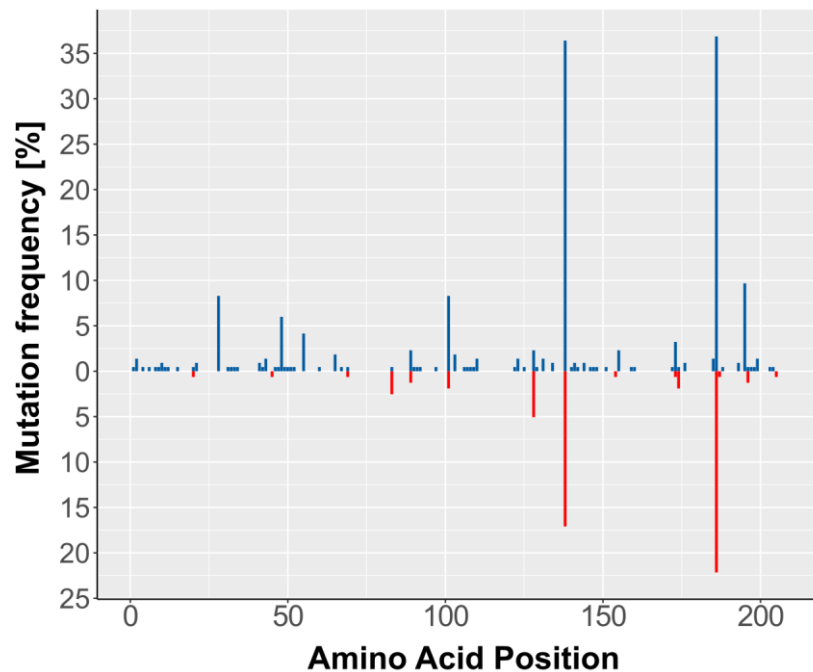**Supplementary Material**



**Figure S 1: *mexZ* mutation distribution of *mexY* high and low expressing clinical *P. aeruginosa* isolates.** The frequency and localization within the amino acid sequence of non- synonymous mutations of the gene *mexZ* is shownrelative to the number of *mexY* high (blue) and low expressing (red) isolates. Mapping was accomplished using stampy and variant calling was performed using SAMtools (version 0.1.19) with UCBPP-PA14 or PAO1.
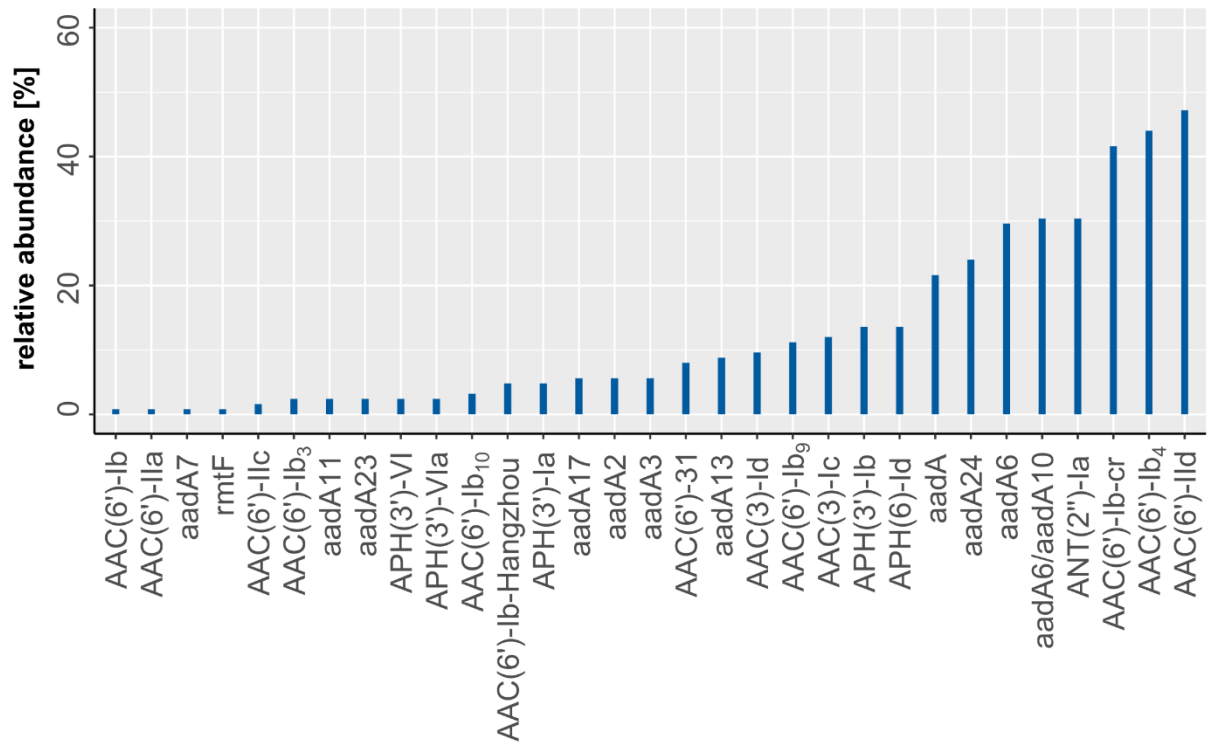
**Figure S2: Distribution of detected aminoglycoside modifying enzymes (AME) in our data-set.** The resistance genotyping tool ARIBA (version 2.10.2) using the CARD (3) as resistance factor data-base was employed to detect AMEs in the whole genome sequencing data of the analyzed strains. Results were screened for resistance factor descriptions containing "aminoglycosides" and hits for mexZ as well as the chromosomally encoded APH(3')-II phosphotransferase were excluded.
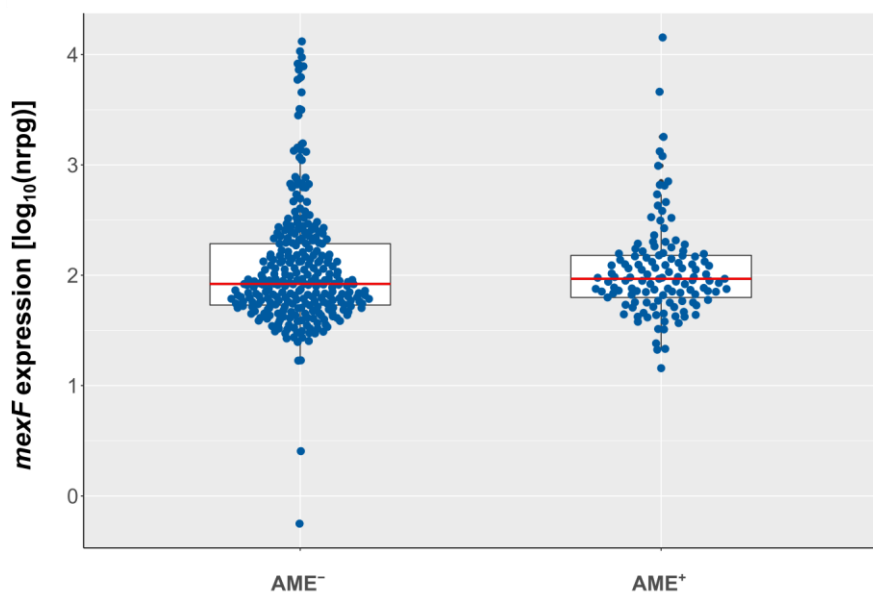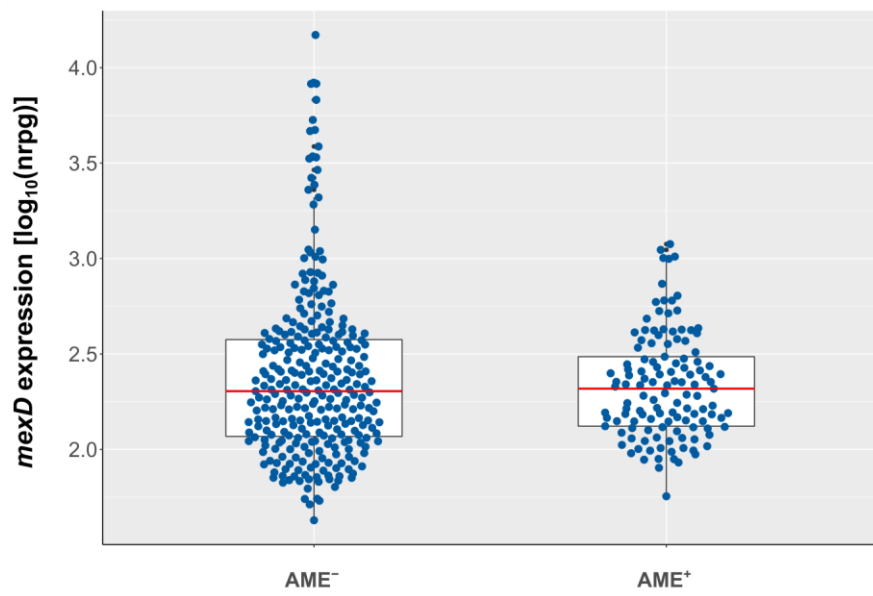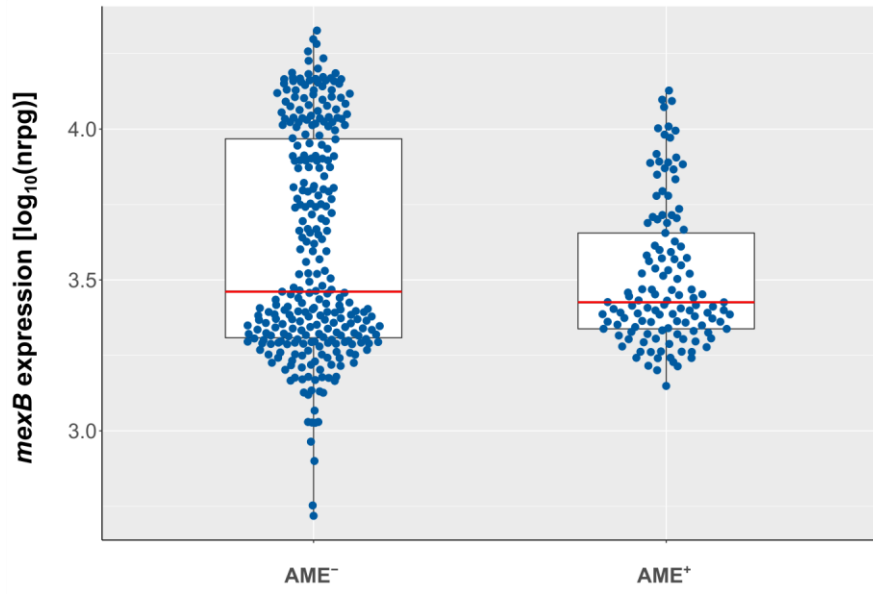
**Figure S3: AME possession and efflux pump expression.** Normalized expression values ($\log_{10}$(NRPG)) of substrate specificity-providing efflux pump components of clinical *P. aeruginosa* isolates that were identified as AME containing (AME$^+$) and non AME containing (AME$^-$). Boxes comprise 50% of the values, red line = median.



**Figure S4: AME induced gentamicin resistance is impacted by the presence of the MexXY-OprM efflux pump.** Gentamicin resistance levels of *P. aeruginosa* PA14 and mutants thereof. All strains were transformed with the pSEVA634::oprM vector, harboring a gentamicin acetyl-transferase (AME+) (4). 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.

## Table S1: Strains used in this study.

| Strain | Plasmid | Resistance | Reference |
|---|---|---|---|
| ***Pseudomonas aeruginosa*** | | | |
| PA14 WT | - | - | (1) |
| PA14ΔmexAB | - | - | this study |
| PA14ΔmexXY | - | - | this study |
| PA14ΔmexCD | - | - | this study |
| PA14ΔmexEF | - | - | this study |
| PA14ΔmexZ | - | - | (2) |
| PA14ΔmexR | - | - | (2) |
| PA14ΔmexZ/ΔmexR | - | - | (2) |
| PA14 WT AME⁺ | pSEVA621 | Gm$^R$ | this study |
| PA14Δ*mexXY*AME⁺ | pSEVA621 | Gm$^R$ | this study |
| PA14 ΔmexZ AME⁺ | pSEVA621 | Gm$^R$ | this study |
| PA14 ΔmexCD AME⁺ | pSEVA621 | Gm$^R$ | this study |
| PA14 ΔmexEF AME⁺ | pSEVA621 | Gm$^R$ | this study |
| PA14 WT AME⁺/+OprM | pSEVA634::oprM | Gm$^R$ | this study |
| PA14Δ*mexXY* AME+/+OprM | pSEVA634::oprM | Gm$^R$ | this study |
| PA14 ΔmexZ AME+/+OprM | pSEVA634::oprM | Gm$^R$ | this study |
| PA14 ΔmexAB AME+/+OprM | pSEVA634::oprM | Gm$^R$ | this study |

Gm$^R$ = Gentamicin resistance

# Table S2: Primers used in this study.

| Target Region | Primer | Sequence | Goal |
|---|---|---|---|
| *mexAB* | mexAB_FW3-HindIII | GATAAGCTTCAATACATGGACG | knockout of target region |
| *mexAB* | mexAB_RW3-BAmHI | TTAGGATCCAAAGGGCCGAAATCG | knockout of target region |
| *mexAB* | mexAB_RW2-ECoR | AGCGAATTCTGATGTCCTTGGTCAGTT | knockout of target region |
| *mexAB* | mexAB_FW2-BAmHI | TTCGGATCCAGCATATGAAACGGTCCTTC | knockout of target region |
| *mexCD* | mexCD_FP1_ECoRI | ATGAATTCTATTCCAGGTAGGACTGCCAGC | knockout of target region |
| *mexCD* | mexCD_RP1+20bp | GTTTGCGCATCTCGCTCAGACACACCCGACCGTTGATT | knockout of target region |
| *mexCD* | mexCD_FP2+20bp | AATCAACGGTCGGGTGTGTCTGAGCGAGATGCGCAAAC | knockout of target region |
| *mexCD* | mexCD_RP2_XbAl | AATTCTAGAACCAGGGCGATCCGTG | knockout of target region |
| *mexEF* | mexEF_FP1_ECoRI | AATGAATTCATTCGACGAGGAACTGGAGAAA | knockout of target region |
| *mexEF* | mexEF_RP1+20bp | ATCGACTGCGCGTGAATCAGCTTGACTCCGCCAGTCG | knockout of target region |
| *mexEF* | mexEF_FP2+20bp | CGACTGGCGGAGTCAAGCTGATTCACGCGCAGTCGAT | knockout of target region |
| *mexEF* | mexEF_RP2_XbAl | AATTCTAGAATCAGGCTGACCTGCAGTTG | knockout of target region |
| *mexXY* | mexXY_FP1_XbAl | ATCATCTAGATAGCTCGTTCTCGTCGCTGC | knockout of target region |
| *mexXY* | mexXY_RP1+20bp | GTTTCGCTAGGGGCATCAGGTGGGTGTCCCTCGATTCGT | knockout of target region |
| *mexXY* | mexXY_FP2+20bp | ACGAATCGAGGGACACCCACCTGATGCCCCTAGCGAAAC | knockout of target region |
| *mexXY* | mexXY_RP2_HindIII | ACATAAGCTTAGCCTCTACTACGACAAGGCCAG | knockout of target region |
| *oprM* | oprM_RBS_EcoRI_L | ACCGAATTCTTTGTTTAACTTTAAGAAGGAGATATACCATGAAACGGTCCTTCCTTTC | amplification of target region |
| *oprM* | oprM_clon_HindIII | ACCAAGCTTTCAGGCCTGCGGATCTTCCT | amplification of target region |

**References**

1.    Liberati NT, Urbach JM, Miyata S, Lee DG, Drenkard E, Wu G, Villanueva J, Wei T, Ausubel FM. 2006. An ordered, nonredundant library of *Pseudomonas aeruginosa* strain PA14 transposon insertion mutants. Proc Natl Acad Sci U S A 103:2833–2838.

2.    Bruchmann S, Dotsch A, Nouri B, Chaberny IF, Haussler S. 2013. Quantitative Contributions of Target Alteration and Decreased Drug Accumulation to *Pseudomonas aeruginosa* Fluoroquinolone Resistance. Antimicrob Agents Chemother 57:1361–1368.

3.    Jia B, Raphenya AR, Alcock B, Waglechner N, Guo P, Tsang KK, Lago BA, Dave BM, Pereira S, Sharma N, Doshi S, Lo R, Williams LE, Frye G, Elsayegh T, Sardar D, Westman EL, Pawlowski AC, Johnson A, Brinkman FSL, Wright GD, Mcarthur AG. 2017. CARD 2017 : expansion and model-centric curation of the comprehensive antibiotic resistance database. Nucleic Acids Res 45:D566–D573.

4.    Martínez-García E, Aparicio T, Goñi-Moreno A, Fraile S, De Lorenzo V. 2015. SEVA 2.0: An update of the Standard European Vector Architecture for de-/re-construction of bacterial functionalities. Nucleic Acids Res 43:D1183–D1189.