Determination of *Pseudomonas aeruginosa* MexXY-OprM substrate profile in a major efflux knockout system reveals distinct antibiotic substrate classes

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ABSTRACT

Defining the substrates of Resistance-Nodulation-Division (RND) systems is often complicated by the presence of multiple systems in a single bacterial species. Using a major efflux knockout strain, we developed a *Pseudomonas aeruginosa* system that enables controlled expression of MexXY-OprM in the absence of background efflux complexes with overlapping substrate profiles. This system identified three groups of potential substrates: substrates, partial substrates, and nonsubstrates, and defined trimethoprim as a new substrate for MexXY-OprM.

Pseudomonas aeruginosa is a Gram-negative, multidrug resistant (MDR) opportunistic pathogen associated with acute and chronic infections, linked to ventilators and catheters in nosocomial settings and, for example, localized in the lungs of individuals with cystic fibrosis and chronic obstructive pulmonary disease, respectively[1-3]. In 2019, MDR *P. aeruginosa* contributed to >300,000 deaths worldwide[1], with the occurrence of hospital-associated infections increasing by 32% in 2020 due to the COVID-19 pandemic driving longer hospital stays and increasing the prevalence of secondary infections[2]. A major contributor to resistance in *P. aeruginosa* is active removal of antimicrobials via efflux, particularly by members of the Resistance-Nodulation-Division (RND) efflux family[4-7].

RND efflux pumps are tripartite complexes comprising a trimeric outer membrane protein (OMP), a hexameric periplasmic adaptor protein (PAP), and a trimeric inner membrane (IM) transporter[8], e.g., OprM, MexX and MexY, respectively (**Fig. 1A**). MexXY-OprM is recognized for its unique ability in *P. aeruginosa* to efflux aminoglycosides, but its substrate profile is also proposed to include ethidium bromide (EtBr), tetracyclines, macrolides, chloramphenicol (Chl), and some β-lactams[5, 9-16]. Prior studies have examined substrate preference in a limited efflux knockout background (e.g., Δ*mexAB,* Δ*mexC*D, and/or Δ*mexXY*), through heterologous expression (e.g., in *E. coli*), and/or with uncontrolled induction systems (e.g., knockout of the native *mexXY* repressor, MexZ)[5, 9-16]. As such, these systems do not account for compensating effects from the remaining RND pumps and determining the specific contribution of MexXY can be challenging.

To overcome these limitations, we aimed to identify substrate preference for PAO1 MexXY through its controlled induction from a genomic chromosomal insertion in a multi-deletion *P. aeruginosa* background, PΔ6 (PAO1 Δ*mexAB-oprM, mexCD-oprJ, mexEF-oprN, mexJKL, mexXY, triABC*)[17, 18]. PΔ6 was selected for these studies as the six deleted pumps are the major contributors to intrinsic antibiotic resistance in *P. aeruginosa* and have overlapping substrate profiles which would otherwise complicate MexXY-OprM substrate identification. Additionally, in the absence of these additional efflux systems, measurements of minimum inhibitory concentrations (MIC) can reliably serve as a proxy for MexXY-OprM efflux activity for each potential substrate. The native *mexXY* operon and ribosome binding site (rbs) were PCR amplified from *P. aeruginosa* PAO1 genomic DNA and cloned into the miniTn7 vector, pJM220[19], under a rhamnose inducible promoter (resulting in pTn7XY), and a FLAG tag-encoding

sequence subsequently appended 3' of *mexY* (resulting in pTn7XY-FLAG; **Tables S1** and **S2**). Using transposon insertion, pTn7XY-FLAG and helper plasmid, pTNS3, were electroporated into PΔ6. As outlined previously[20], insertion of rbs-*mexXY* and removal of the gentamicin resistance marker using pFLP were confirmed using colony PCR (resulting in strain LK17). To complement the absent OMP in LK17 (due to deletion of *mexAB-oprM* in PΔ6), *oprM* was amplified from PAO1 genomic DNA and cloned into the arabinose-inducible complementation plasmid, pHERD20T[21], and electroporated into both PΔ6 and LK17 resulting in strains LK36 and LK21, respectively (**Fig. 1A**, and **Tables S1** and **S2**). This approach allows for MexXY overexpression in the same isogenic genetic background and without the need for non-native chimeric operon construction with the OMP, which may result in polar effects on transcription.

Optimal inducer concentrations for MexXY (rhamnose) and OprM (arabinose) were empirically determined, with 1% wt/vol of each inducer determined to show the largest increase in resistance without affecting cell growth (**Fig. S1** and **Table S3**). To quantify efflux-mediated resistance for a panel of chemically and functionally diverse antimicrobials, we measured the MIC of each potential substrate as previously described[22, 23], in the absence or presence of inducer (i.e., rhamnose and/or arabinose) in all strains (**Fig. 2** and **Table S4**). Western blot analysis was performed on whole cell lysate after seven hours of rhamnose induction to confirm MexY expression in LK21 (**Fig. S2**).

Deletion of the six efflux systems in PΔ6 resulted in increased sensitivity to all antibiotics tested with the exception of tobramycin (Tob), ceftazidime (Caz), imipenem (Imi), and polymyxin B (Pmb), which are weak or non-substrates for RND-type efflux pumps in *Pseudomonas*[24, 25]. Substrates with the highest change in susceptibility (up to 64-fold) were Ery, Chl, and trimethoprim (Tmp), confirming that the deleted RND efflux systems are collectively a major contributor to resistance to these antimicrobials in *P. aeruginosa* PAO1 (**Fig. 2**). Interestingly, the use of arabinose and rhamnose sugars antagonized action of aminoglycosides in both PAO1 and PΔ6, as well as Ery, cefepime (Fep), and Chl in PAO1 (**Table S4**). We speculate that because aminoglycosides are modified sugars, the presence of additional sugars in the media may regulate other aminoglycoside entry/efflux transporters[26].

Overexpression of MexXY-OprM in PΔ6 allowed for robust efflux of aminoglycosides, ciprofloxacin (Cip), Fep, tigecycline (Tig), Ery, meropenem (Mem), and Tmp, for which MICs were restored to or exceeded wild-type PAO1 levels. We refer to these antimicrobials as substrates of MexXY-OprM. These analyses also revealed Tmp to be a previously undefined substrate for MexXY-OprM (**Fig. 1B, Fig. 2** and **Table S4**). We further classified the tested antibiotics into two additional categories: 'partial substrates' or 'nonsubstrates'. We define partial substrates as either effluxed antimicrobials for which resistance levels did not change in PΔ6 compared to PAO1 but for which resistance increased moderately in the presence of MexXY-OprM expression, or when resistance levels were decreased in PΔ6 but overexpression of MexXY alone could not restore the resistance observed in PAO1 (**Fig. 1B**). For example, resistance to EtBr and Chl increased but was not restored to the level observed for PAO1

upon MexXY-OprM overexpression, suggesting that additional RND systems or mechanisms are partly or primarily responsible for resistance to these substrates. Additionally, the MICs for Tob, Caz, and Imi did not change upon deletion of the six efflux systems in P∆6 but were increased upon overexpression of MexXY-OprM (**Table S4**). These data suggest that these antimicrobials are not preferred MexXY-OprM substrates but may be exported when the pump is overexpressed, or that the overexpression of MexXY-OprM itself leads to physiological changes that impact resistance to these antimicrobials. Furthermore, as these compounds are members of antibiotic families with substrates, it is possible that the small differences in chemical substituents may result in loss of efflux recognition (**Fig. 2**). The only antibiotic tested whose resistance was unaffected by the deletion or complementation of MexXY-OprM was Pmb which is thus classified as a nonsubstrate.

Overall, these studies identified Tmp as a MexXY-OprM substrate and the potential for MexXY-OprM to contribute to Caz and Imi resistance when overexpressed, a phenomenon typically observed in CF clinical isolates[27, 28]. As our system allows for the overexpression of MexXY-OprM, without significantly impacting growth, the increased threshold allows identification of subtle differences within substrate classes (e.g., aminoglycoside, cephalosporins, and carbapenems). Furthermore, this system can enable future studies on transporter-adaptor pairs with exchangeable plasmid-encoded OMPs allowing for detailed analyses of OMP impact on substrate selectivity. As efflux-mediated resistance is a major contributor to multidrug resistance, and therefore therapeutic failures, such approaches to better understand efflux substrate specificity as well as the impact of overexpression on 'nonsubstrate' antibiotics are of vital importance.

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References

1. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. Lancet. 2022;399(10325):629-55. Epub 20220119. doi: 10.1016/s0140-6736(21)02724-0. PubMed PMID: 35065702; PMCID: PMC8841637.
2. COVID-19 : U.S. impact on antimicrobial resistance, special report 20222022. PubMed PMID: cdc:119025.
3. Reynolds D, Kollef M. The Epidemiology and Pathogenesis and Treatment of Pseudomonas aeruginosa Infections: An Update. Drugs. 2021;81(18):2117-31. Epub 20211107. doi: 10.1007/s40265-021-01635-6. PubMed PMID: 34743315; PMCID: PMC8572145.
4. Lorusso AB, Carrara JA, Barroso CDN, Tuon FF, Faoro H. Role of Efflux Pumps on Antimicrobial Resistance in Pseudomonas aeruginosa. Int J Mol Sci. 2022;23(24). Epub 20221213. doi: 10.3390/ijms232415779. PubMed PMID: 36555423; PMCID: PMC9779380.

5. Masuda N, Sakagawa E, Ohya S, Gotoh N, Tsujimoto H, Nishino T. Substrate specificities of MexAB-OprM, MexCD-OprJ, and MexXY-oprM efflux pumps in Pseudomonas aeruginosa. Antimicrob Agents Chemother. 2000;44(12):3322-7. Epub 2000/11/18. doi: 10.1128/aac.44.12.3322-3327.2000. PubMed PMID: 11083635; PMCID: PMC90200.

6. Okamoto K, Gotoh N, Nishino T. Alterations of susceptibility of Pseudomonas aeruginosa by overproduction of multidrug efflux systems, MexAB-OprM, MexCD-OprJ, and MexXY/OprM to carbapenems: Substrate specificities of the efflux systems. Journal of Infection and Chemotherapy. 2002;8(4):371-3. doi: 10.1007/s10156-002-0193-7.

7. Okamoto K, Gotoh N, Nishino T. Extrusion of penem antibiotics by multicomponent efflux systems MexAB-OprM, MexCD-OprJ, and MexXY-OprM of Pseudomonas aeruginosa. Antimicrob Agents Chemother. 2002;46(8):2696-9. Epub 2002/07/18. doi: 10.1128/aac.46.8.2696-2699.2002. PubMed PMID: 12121960; PMCID: PMC127373.

8. Kavanaugh Logan G, Dey D, Shafer William M, Conn Graeme L. Structural and functional diversity of Resistance-Nodulation-Division (RND) efflux pump transporters with implications for antimicrobial resistance. Microbiology and Molecular Biology Reviews. 2024;88(3):e00089-23. doi: 10.1128/mmbr.00089-23.

9. Chuanchuen R, Wannaprasat W, Ajariyakhajorn K, Schweizer HP. Role of the MexXY multidrug efflux pump in moderate aminoglycoside resistance in Pseudomonas aeruginosa isolates from Pseudomonas mastitis. Microbiol Immunol. 2008;52(8):392-8. Epub 2008/08/01. doi: 10.1111/j.1348-0421.2008.00051.x. PubMed PMID: 18667038.

10. Sobel ML, McKay GA, Poole K. Contribution of the MexXY Multidrug Transporter to Aminoglycoside Resistance in Pseudomonas aeruginosa Clinical Isolates. Antimicrobial Agents and Chemotherapy. 2003;47(10):3202-7. doi: 10.1128/AAC.47.10.3202-3207.2003.

11. Masuda N, Sakagawa E, Ohya S, Gotoh N, Tsujimoto H, Nishino T. Contribution of the MexX-MexY-oprM efflux system to intrinsic resistance in Pseudomonas aeruginosa. Antimicrob Agents Chemother. 2000;44(9):2242-6. Epub 2000/08/22. doi: 10.1128/aac.44.9.2242-2246.2000. PubMed PMID: 10952562; PMCID: PMC90052.

12. Llanes C, Hocquet D, Vogne C, Benali-Baitich D, Neuwirth C, Plésiat P. Clinical strains of Pseudomonas aeruginosa overproducing MexAB-OprM and MexXY efflux pumps simultaneously. Antimicrob Agents Chemother. 2004;48(5):1797-802. Epub 2004/04/24. doi: 10.1128/aac.48.5.1797-1802.2004. PubMed PMID: 15105137; PMCID: PMC400543.

13. Aires JR, Köhler T, Nikaido H, Plésiat P. Involvement of an active efflux system in the natural resistance of Pseudomonas aeruginosa to aminoglycosides. Antimicrob Agents Chemother. 1999;43(11):2624-8. Epub 1999/10/30. doi: 10.1128/aac.43.11.2624. PubMed PMID: 10543738; PMCID: PMC89534.

14. Mine T, Morita Y, Kataoka A, Mizushima T, Tsuchiya T. Expression in Escherichia coli of a New Multidrug Efflux Pump, MexXY, from Pseudomonas aeruginosa. Antimicrobial Agents and Chemotherapy. 1999;43(2):415-7. doi: 10.1128/AAC.43.2.415.

15. Westbrock-Wadman S, Sherman DR, Hickey MJ, Coulter SN, Zhu YQ, Warrener P, Nguyen LY, Shawar RM, Folger KR, Stover CK. Characterization of a Pseudomonas aeruginosa efflux pump contributing to aminoglycoside impermeability. Antimicrob Agents Chemother. 1999;43(12):2975-83. Epub 1999/12/03. doi: 10.1128/AAC.43.12.2975. PubMed PMID: 10582892; PMCID: PMC89597.

16. Morita Y, Tomida J, Kawamura Y. Primary mechanisms mediating aminoglycoside resistance in the multidrug-resistant Pseudomonas aeruginosa clinical isolate PA7. Microbiology (Reading). 2012;158(Pt 4):1071-83. Epub 2012/01/28. doi: 10.1099/mic.0.054320-0. PubMed PMID: 22282519.

17. Wolloscheck D, Krishnamoorthy G, Nguyen J, Zgurskaya HI. Kinetic Control of Quorum Sensing in Pseudomonas aeruginosa by Multidrug Efflux Pumps. ACS Infect Dis. 2018;4(2):185-95. Epub 20171110. doi: 10.1021/acsinfecdis.7b00160. PubMed PMID: 29115136; PMCID: PMC5807214.

18. Cooper CJ, Krishnamoorthy G, Wolloscheck D, Walker JK, Rybenkov VV, Parks JM, Zgurskaya HI. Molecular Properties That Define the Activities of Antibiotics in Escherichia coli and Pseudomonas aeruginosa. ACS Infect Dis. 2018;4(8):1223-34. Epub 20180525. doi: 10.1021/acsinfecdis.8b00036. PubMed PMID: 29756762; PMCID: PMC6449051.

19. Meisner J, Goldberg JB. The Escherichia coli rhaSR-PrhaBAD Inducible Promoter System Allows Tightly Controlled Gene Expression over a Wide Range in Pseudomonas aeruginosa. Appl Environ Microbiol. 2016;82(22):6715-27. Epub 2016/10/30. doi: 10.1128/aem.02041-16. PubMed PMID: 27613678; PMCID: PMC5086565.

20. Choi KH, Schweizer HP. mini-Tn7 insertion in bacteria with single attTn7 sites: example Pseudomonas aeruginosa. Nat Protoc. 2006;1(1):153-61. doi: 10.1038/nprot.2006.24. PubMed PMID: 17406227.

21. Qiu D, Damron FH, Mima T, Schweizer HP, Yu HD. PBAD-based shuttle vectors for functional analysis of toxic and highly regulated genes in Pseudomonas and Burkholderia spp. and other bacteria. Appl Environ Microbiol. 2008;74(23):7422-6. Epub 20081010. doi: 10.1128/aem.01369-08. PubMed PMID: 18849445; PMCID: PMC2592904.

22. Kavanaugh LG, Mahoney AR, Dey D, Wuest WM, Conn GL. Di-berberine conjugates as chemical probes of Pseudomonas aeruginosa MexXY-OprM efflux function and inhibition. npj Antimicrobials and Resistance. 2023;1(1):12. doi: 10.1038/s44259-023-00013-4.

23. Wiegand I, Hilpert K, Hancock RE. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat Protoc. 2008;3(2):163-75. doi: 10.1038/nprot.2007.521. PubMed PMID: 18274517.

24. Masuda N, Sakagawa E, Ohya S, Gotoh N, Tsujimoto H, Nishino T. Substrate Specificities of MexAB-OprM, MexCD-OprJ, and MexXY-OprM Efflux Pumps in Pseudomonas aeruginosa. Antimicrobial Agents and Chemotherapy. 2000;44(12):3322-7.

25. Dey D, Kavanaugh LG, Conn GL. Antibiotic Substrate Selectivity of MexY and MexB Efflux Systems Is Determined by a Goldilocks Affinity. Antimicrobial Agents and Chemotherapy. 2020;64(8):e00496-20. doi: 10.1128/AAC.00496-20.

26. Kotra LP, Haddad J, Mobashery S. Aminoglycosides: perspectives on mechanisms of action and resistance and strategies to counter resistance. Antimicrob Agents Chemother. 2000;44(12):3249-56. doi: 10.1128/aac.44.12.3249-3256.2000. PubMed PMID: 11083623; PMCID: PMC90188.

27. Singh M, Yau YCW, Wang S, Waters V, Kumar A. MexXY efflux pump overexpression and aminoglycoside resistance in cystic fibrosis isolates of Pseudomonas aeruginosa from chronic infections. Canadian Journal of Microbiology. 2017;63(12):929-38. doi: 10.1139/cjm-2017-0380.

28. Vogne C, Aires JR, Bailly C, Hocquet D, Plésiat P. Role of the multidrug efflux system MexXY in the emergence of moderate resistance to aminoglycosides among Pseudomonas aeruginosa isolates from patients with cystic fibrosis. Antimicrob Agents Chemother. 2004;48(5):1676-80. Epub 2004/04/24. doi: 10.1128/aac.48.5.1676-1680.2004. PubMed PMID: 15105120; PMCID: PMC400545.

Figure Legends

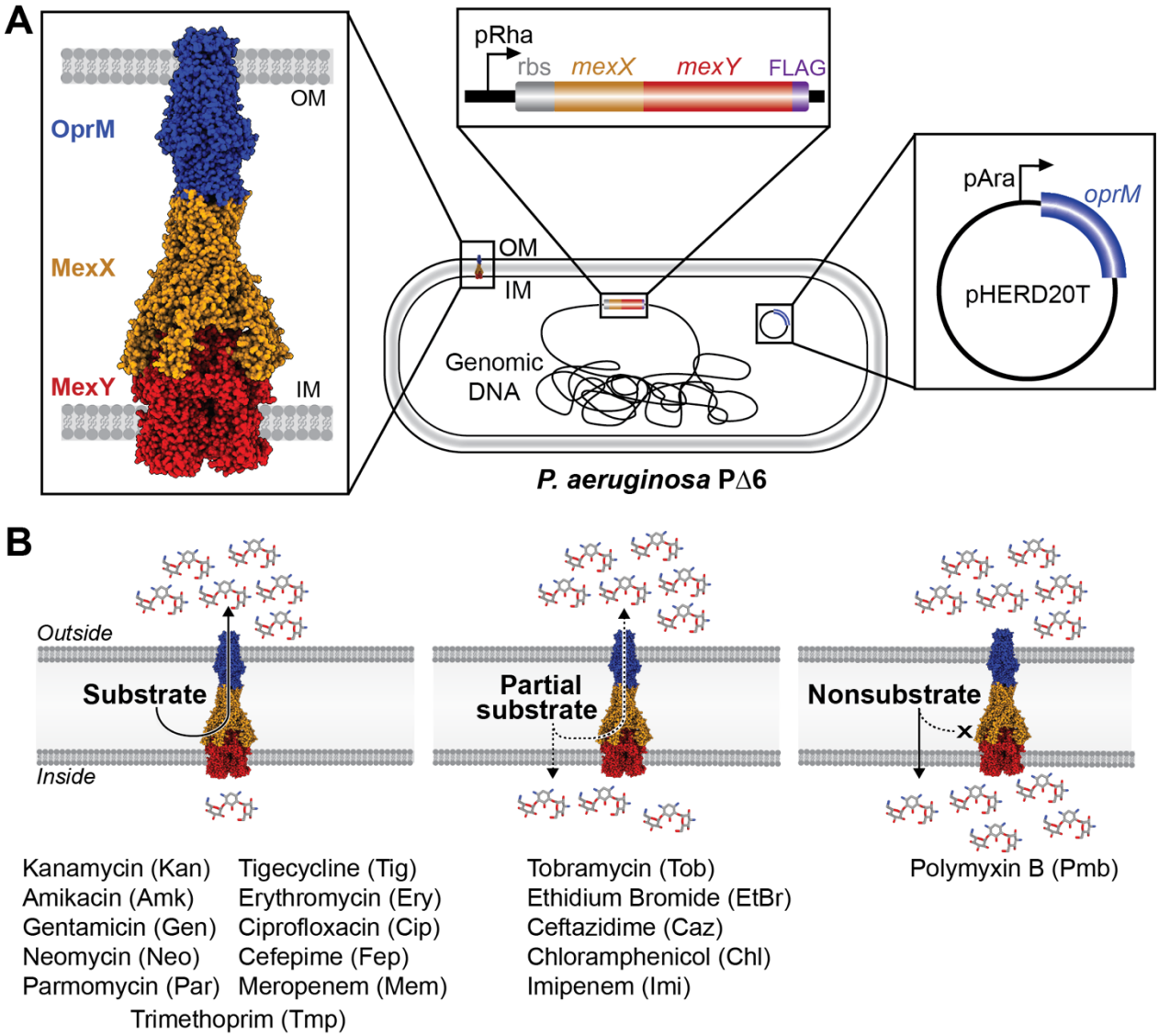

Fig. 1. Overview of the MexXY-OprM induction system and substrate classes. *A*, Homology model *(left*) of the tripartite complex containing outer membrane (OM) protein OprM (blue), periplasmic adaptor protein MexX (orange), and inner membrane (IM) transporter MexY (red). Cartoon schematic of the transposon system containing the rbs and PAO1 *mexXY* with FLAG-tag (purple) under the control of a rhamnose promoter in the PΔ6 genomic DNA (*center*) and *oprM* under an arabinose promoter located on the pHERD20T plasmid (*right*). ***B***, Schematic representation of the three substrate classes (substrate, partial substrate, and nonsubstrate) with MexXY-OprM located in the cell envelope. Antibiotics identified in each class are given below.

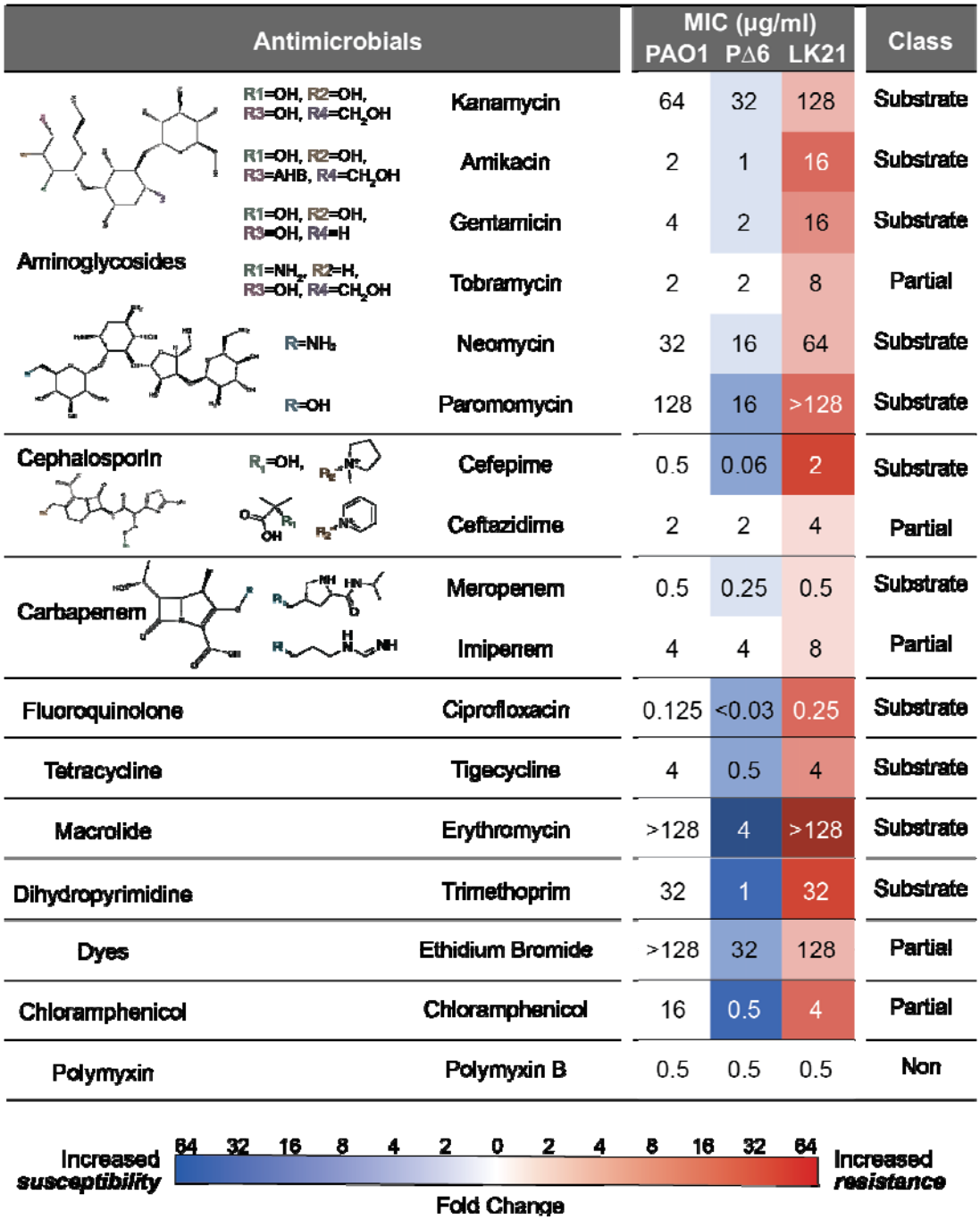

Fig. 2. Substrate profile of the MexXY-OprM efflux pump in the *P. aeruginosa* PΔ6 expression system. *Left*, antibiotic families and the individual antibiotics tested; where two or more antibiotics within the same family were tested, differences in their chemical structure are indicated. *Center*, average MICs (µg/mL) for 10 classes of antibiotics in *P. aeruginosa* PAO1, PΔ6, and LK21 (PΔ6::mexXY^{PAO1},pHERD-oprM^{PAO1}) in the presence of 1% wt/vol arabinose and rhamnose are shown numerically overlayed on a heatmap indicating fold-change compared to parent strain (PΔ6 is compared to PAO1 and LK21 is compared to PΔ6). Blue is increase in susceptibility (reduced efflux) and red is increase in resistance (increased efflux). *Right*, the resulting substrate classification for each antibiotic is indicated as substrate (Substrate), partial substrate (Partial) or nonsubstrate (Non).