A Small Multidrug Resistance Transporter in *Pseudomonas aeruginosa* Confers Substrate-Specific Resistance or Susceptibility

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ABSTRACT

Small Multidrug Resistance (SMR) transporters are key players in bacterial defense against toxins, providing resistance to antibiotics, antiseptics, and other homeostasis-perturbing compounds in multidrug-resistant pathogens. However, recent evidence demonstrates that EmrE, an SMR from *Escherichia coli* and a model for understanding transport, can also induce susceptibility to some compounds by drug-gated uncontrolled proton leak. Proton Motive Force (PMF) rundown is a relatively unexplored drug target, and is orthogonal to the targets of most known antibiotics. This requires an SMR to be merely present, rather than be the primary resistance mechanism, and dissipates the energy source for many other efflux pumps. PAsmr, an EmrE homolog from *P. aeruginosa*, confers resistance to some EmrE substrates in cells and in purified systems. We

hypothesized that PAsmr, like EmrE, could confer susceptibility to some compounds. Growth assays of *E. coli* expressing PAsmr displayed substrate-dependent resistance and susceptibility phenotypes, and *in vitro* solid-supported membrane electrophysiology experiments revealed that PAsmr performs both antiport and substrate-gated proton uniport, confirming functional homology between PAsmr and EmrE. Growth assays of *P. aeruginosa* strain PA14 demonstrated that PAsmr contributes resistance to some antimicrobial compounds, consistent with prior data, but confers *susceptibility* to at least one aminoglycoside antibiotic. Phenotypic differences between *P. aeruginosa* and *E. coli* expressing PAsmr point to differential impacts of proton leak, constituting an advance in our understanding of underlying resistance mechanisms in *P. aeruginosa* and prompting further investigation into the role that SMRs play in antibiotic resistance in pathogens.

IMPORTANCE

Small multidrug resistance transporters are a class of efflux pumps found in many pathogens, but whose contributions to antibiotic resistance are poorly understood. We hypothesize that in addition to conferring resistance to toxic compounds, these transporters may also confer susceptibility by dissipating the proton-motive force. This means that an SMR merely needs to be present in the pathogen in order to be targeted (as opposed to being the primary resistance mechanism). Here, we test this hypothesis with an SMR found in the pathogen *Pseudomonas aeruginosa* and find that it can confer both resistance and susceptibility depending upon the compounds it interacts with.

INTRODUCTION

Multidrug-resistant *Pseudomonas aeruginosa* is responsible for at least 30,000 healthcare-associated infections every year (1), and some strains appear resistant to all known antibiotics (2). Due to the high degree of resistance exhibited, *P. aeruginosa* is a compelling and clinically relevant system to investigate mechanisms of drug resistance in general. One such mechanism is secondary active transport of toxic compounds by efflux pumps. The Small Multidrug Resistance (SMR)

53 transporters are a family of efflux pumps that have been identified in many high priority pathogens,
54 but their contributions to antibiotic resistance are minimally understood (3–10). The SMR family may
55 be broadly divided into four subfamilies, with the QAC subfamily representing the most promiscuous
56 transporters (10).

57 Historically, SMRs were understood as antiporters, tightly coupling proton import to drug export
58 (11, 12). However, *in vitro* biophysical investigation of the model QAC SMR EmrE has demonstrated
59 that EmrE is also theoretically capable of symport and drug or proton uniport, with transporter
60 behavior determined by substrate identity and kinetic parameters (13–16). This differential transport
61 behavior will ultimately lead to the transporter conferring susceptibility to some chemicals instead of
62 resistance. Substrate-gated proton uniport has been experimentally demonstrated for EmrE in *E. coli*,
63 leading to disruption of the ΔpH component of the proton-motive force (PMF), impacting cellular ATP
64 production and other PMF-dependent processes (14). PMF disruption has been validated as a
65 potential antibiotic and/or adjuvant strategy (17–21). Should other SMRs or even other families of
66 efflux pumps confer opposing biological outcomes in a substrate-dependent manner, this may
67 broaden our understanding of how to target pathogens, inducing susceptibility instead of merely
68 disabling specific bacterial resistance processes.

69 PAsmr, also known as $EmrE_{Pae}$, is an SMR expressed by *P. aeruginosa,* originally identified on
70 the basis of sequence homology to EmrE and conferring resistance to ethidium bromide, methyl
71 viologen, and other quaternary ammonium compounds (3, 6, 22). *smrPA* (PAO1_4990,
72 PA14_65990), the gene expressing PAsmr, has been identified in clinically isolated strains of
73 multidrug resistant *P. aeruginosa* (19), and expression in strain PAO1 confirmed by RT-PCR (6).
74 PAsmr has also been investigated as a potential target and model for efflux pump inhibiting peptides
75 (22).

76 Here, we hypothesize that PAsmr may be used to induce susceptibility to toxic compounds in *P.*
77 *aeruginosa,* based on the observations that EmrE can perform ligand-gated proton uniport that
78 causes enhanced susceptibility in its native organism, *E. coli* (13, 14, 16). We test a *P. aeruginosa*

79 strain lacking PAsmr for differences in resistance and susceptibility to both known and novel SMR
80 substrates (Fig. 1A), as well as how this phenotype corresponds to those observed in *E. coli*
81 expressing PAsmr. We also test the function of PAsmr in a purified system, finding that resistance
82 phenotypes are due to antiport, while substrate-triggered proton uniport does occur in the presence of
83 some substrates and contributes to susceptibility in *E. coli*, but a range of phenotypes in *P.*
84 *aeruginosa*.
85

86 **MATERIALS AND METHODS**

87 **smrPA *(*PA14_65990*) Deletion in* P. aeruginosa *PA14***

88 *smrPA* was deleted in PA14 using *sacB* counterselection (24, 25). Briefly, a plasmid vector
89 (pJMP3262) containing an origin of replication that replicates in *E. coli* but not *P. aeruginosa*, a
90 gentamicin resistance cassette, and the *sacB* gene was used as the backbone. The regions 1000bp
91 upstream and downstream of *smrPA* were cloned into the plasmid vector via Gibson Assembly.

92 Following extraction of genomic DNA from PA14, the regions 1000bp upstream and
93 downstream of *smrPA* were amplified via PCR using primers oJMP 1832 and 1833 for the upstream
94 region and oJMP1834 and 1835 for the downstream region (Table 1). The resulting products were gel
95 purified and cloned into pJMP 3262 via Gibson Assembly. The plasmid was then transformed into *E.*
96 *coli* (sJMP 2630). The plasmid sequence was confirmed by Sanger sequencing using primers oJMP
97 1339,1340,1836, and 1837 (Table 1). The plasmid was then transformed into a conjugative *E. coli*
98 strain (sJMP3257).

99 The newly constructed plasmid donor (sJMP3257) and recipient (WT PA14 sJMP744) were
100 grown overnight on plates and resuspended in 1mL LB to $OD_{600} \approx 3$. Three tubes were prepared, one
101 for mating containing 800uL LB, 100uL of the recipient, and 100uL of the donor strain, and two
102 controls (900uL LB and 100 uL of either donor or recipient strains alone). Cells were pelleted at 4000
103 x g for 3 minutes. Media was removed and the pellet was resuspended in 25uL of LB and spotted on

104 a filter on a LB + diaminopimelic acid (DAP) plate. Following incubation for 3.5 hours at 37°C, the
105 filter was placed in a 1.5mL Eppendorf tube with 200uL LB and vortexed for 30 seconds. The cells
106 were further diluted in LB at 1:10, 1:100, 1:1000, 1:10000, and 1:100000. 90uL of each dilution was
107 plated onto LB + gent (30µg/mL) plates to select for *P. aeruginosa* transconjugants containing the
108 plasmid integrated into the genome at the *smrPA* (PA14_65990) locus by single crossover. Donor
109 and recipient controls were also plated on LB + gent(30µg/mL) plates.

110 Several isolated colonies from the LB + gent(30µg/mL) plates were grown in 10mL LB
111 overnight. The resulting overnight cultures were plated on LB + sucrose 15% plates and incubated for
112 48-72 hours at room temperature. Incubation at room temperature enhanced recovery of correct,
113 double-crossover clones. To confirm the selection of Δ*smrPA*, 40 isolated colonies from the LB
114 +sucrose 15% plates were patched on LB + gent(30 µg/mL) + sucrose 15%, LB + gent(30µg/mL),
115 and LB plates. Any colonies growing on the plates containing gent (30 µg/mL) were discarded. To
116 confirm the deletion of *PAsmr,* the gDNA was extracted from a few samples, and the primers
117 oJMP1881 and oJMP 1882 were used to amplify the region including *smrPA.* Gel electrophoresis of
118 the PCR product was used to confirm the deletion of the 333bp gene product.(Fig 1B).

119

120 ***Growth Assays in* Pseudomonas aeruginosa**

121 WT PA14 and PA14 Δ*smrPA* were grown in Nutrient Broth (Difco 234000) from single colonies
122 to an OD of 1 at 37 ºC. Cultures were diluted to a final OD of 0.01 in microplates containing select
123 concentration values of ethidium bromide, chelerythrine chloride, streptomycin, harmane, and 18-
124 crown-6 ether. Plates were incubated in a microplate reader (TECAN) at 37 ºC. OD_{600} (or OD_{700} for
125 ethidium) were measured every 15 minutes for 18 hours. Experiments were performed in at least
126 biological triplicate. Data were analyzed by normalizing WT growth and standard deviation by growth
127 of the knock-out strain.

128

129 ***Growth assays in* E. coli**

130 MG1655 Δ*emrE E. coli* cells were transformed with either WT or non-functional (E14Q) PAsmr
131 (PAO1-PA4990) cloned into the pWB plasmid, a low copy, leaky-expression vector(26). Cells were
132 grown in Nutrient Broth (Difco 234000),100 µg/mL carbenicillin, from a single colony to an OD of 0.2
133 at 37 ˚C. Cultures were diluted to a final OD of 0.01 in microplates (Corning, REF: 351172) containing
134 select concentration values of ethidium bromide, methyltriphenylphosphonium bromide (MeTPP+),
135 chelerythrine chloride, streptomycin, harmane, and 18-crown-6 ether. The plates were incubated in a
136 microplate reader (BMG-Labtech or TECAN) at 37 °C. OD_{600} (or OD_{700} for ethidium) were measured
137 every 5 minutes for 18 hours. Experiments were performed in biological triplicate. Data were analyzed
138 by normalizing WT growth and standard deviation by growth of non-functional (E14Q) mutant.
139

140 ***PAsmr Expression and Purification***

141 PAsmr was expressed as a maltose binding protein (MBP) fusion construct, using pet28-TEV-
142 MBP, a gift from Zita Balklava & Thomas Wassmer (27). Following TEV protease cleavage of the N-
143 terminal MBP, the N-terminal 6xHis-tag may be used to purify PaSMR via affinity chromatography,
144 and then removed with thrombin leaving an extra N-terminal GSHGS. BL21(DE3) Gold cells
145 transformed with pet28-MBP-PaSMR were grown in M9 minimal media (28), with protein expression
146 induced by 0.33 mM IPTG at an OD_{600} of 0.9-1 at 17ºC. Cells were harvested after 17 hours and cell
147 pellets were resuspended in lysis buffer (250 mM sucrose, 100mM NaCl, 2.5 mM MgSO4, 20mM tris
148 pH 7.5, 5mM β-mercaptoethanol, 1 mg/mL lysozyme, DNAse, 1 µg/mL pepstatin, 10 µM leupeptin,
149 and 100 µM PMSF), and lysed by sonication. 0.25 mg/mL TEV protease was added, and following
150 incubation for 16-48 hours at RT or 48-72 hours at 4ºC, the membrane fraction was separated by a
151 high-speed spin (30,000 × g for 1 hour), resuspended in the same buffer, and solubilized with 40 mM
152 DM (decylmaltoside, Anatrace) at RT for 2 hours. After a second high-speed spin, 6x-His tagged
153 PAsmr was purified via NiNTA affinity chromatography, thrombin cleavage, and size-exclusion
154 chromatography as previously described for EmrE (28). When more complete removal of MBP was
155 required for downstream experiments, 50-100mM maltose was included in all steps of purification

156 through NiNTA column elution and an additional desalting step was added to the end of the
157 purification.
158
159 ***Solid-supported membrane electrophysiology***
160 Purified WT PAsmr was reconstituted into 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
161 (POPC) liposomes at a lipid-to-protein ratio of 1:250 in pH 7 assay buffer (100 mM Potassium
162 Phosphate, 100 mM Sodium Acetate, 200 mM NaCl, 4 mM MgCl2). Liposome preparation was
163 performed as described in (14). All SSME data was acquired using a Nanion SURFE2R N1
164 instrument as described in (29). Liposome aliquots were thawed, diluted 2-fold, and briefly sonicated.
165 10 µL of liposomes were used to prepare 3 mm sensors as previously described. Before experiments,
166 sensor capacitance and conductance values were obtained to ensure sensor quality. All experiments
167 used assay buffer with internal pH values of 7.0 and external pH values of 6.7. Drug concentrations
168 used are in Table S1. Sensors were rinsed with at least 500 µL of internal buffer before each
169 measurement to set the internal buffer, pH, and drug concentrations as described in (29). Data
170 acquisition occurred in three stages. First, sensors were perfused with an internal buffer, then
171 transport was initiated by perfusion of the external buffer, and finally, perfusion of the internal buffer
172 re-equilibrated the sensors. Signals were obtained by integrating the current during perfusion of the
173 external buffer, with the final 100 ms of the initial internal buffer perfusion used as the baseline.
174 Reported data are average values of at least three sensors, with error bars representing the standard
175 deviation.
176
177 ***Proton Leak***
178 PAsmr was reconstituted into 3:1 POPC:POPG liposomes, LPR 1:400. Proton leak was
179 monitored by diluting PaSMR liposomes in inside buffer (50 mM sodium phosphate, 100 mM KCl, pH
180 7) into weakly buffered outside buffer (75 µM Phenol Red, 99 mM NaCl, 1 mM KCl, pH 7) to 0.8 µM
181 final PaSMR concentration. Valinomycin diluted in inside buffer was added to final concentration of 1

182 μg/mL to create a negative inside membrane potential. FCCP diluted in inside buffer was added to
183 final concentration of 1 μg/mL as a positive control. At the end of the assay, HCl (50 nM final) was
184 added to return to resting potential.
185
186 **RESULTS**
187 **smrPA *deletion phenotype in* P. aeruginosa *PA14 is substrate-dependent***
188 To test the function of PAsmr in the native organism, we created a deletion strain in PA14, a
189 clinically derived virulent strain of *Pseudomonas aeruginosa*. We deleted the gene encoding PAsmr,
190 *smrPA*, from the PA14 genome using *sacB* counterselection to generate an unmarked strain (Fig. 1B).
191 We used Nutrient Broth (NB), a low-ionic-strength medium, for these experiments because we
192 hypothesized that it would result in more dramatic and easily detectable phenotypes, especially for
193 susceptibility substrates, due to decreased ability of the bacteria to compensate for ∆pH dissipation
194 with an increase in ∆Ψ compared to Lysogeny Broth (LB). There was no significant growth defect in
195 the Δ*smrPA* strain compared to wild type when cultured in NB alone (Fig 1C). We then tested several
196 known and recently discovered PAsmr substrates (Fig. 1A) in PA14 and the ∆*smrPA* strain. Li *et al.*
197 previously demonstrated that deletion of *smrPA* (previously known as $EmrE_{Pae}$) decreased resistance
198 to ethidium bromide, acriflavine, and several aminoglycoside antibiotics in PAO1, a less virulent strain
199 of *P. aeruginosa* (6). Deletion of *smrPA* from PA14 substantially reduced resistance to ethidium bromide
200 at the tested concentration (Fig. 2A), consistent with previous results. Previously, we performed an
201 unbiased screen to identify potential substrates of PAsmr using heterologous expression of the
202 transporter in *Escherichia coil.* That experiment identified several novel substrates, including the
203 antibiotics chelerythrine chloride and streptomycin, and the small molecules harmane and 18-crown-6
204 ether. The ∆*smrPA* strain showed decreased resistance to chelerythrine chloride and harmane (Fig.
205 2B, 2C). However, deletion of *smrPA* improved tolerance to the aminoglycoside streptomycin, indicating
206 that WT PAsmr likely confers susceptibility, which has not been previously reported for any SMR *in vivo*
207 besides EmrE. We tested but did not find growth differences in the presence of tobramycin, methyl

208 viologen, or acriflavine near their minimum inhibitory concentrations (MICs), consistent with the results
209 of Li et al (6). Finally, PA14 showed a slight but reproducible growth defect in high concentrations of
210 18-crown-6 ether starting near the transition to stationary phase (Fig 2D). Based on the high
211 concentrations we tested, 18-crown-6 ether has limited impact on growth of *P. aeruginosa*.
212
213 ***PAsmr phenotype is accentuated but sometimes opposite in* E. coli**
214 E14Q is a known transport-dead variant of EmrE, and has been shown to abolish resistance
215 due to PAsmr expression in *E. coli* as well (26). Therefore, the E14Q variant is a valuable negative
216 control for phenotyping *E. coli* strains that heterologously express SMRs. *E. coli* expressing WT
217 PAsmr or E14Q-PAsmr were grown in NB in the presence of both known and novel substrates.
218 Expression of WT PAsmr conferred resistance to ethidium bromide (Fig. 3A),
219 methyltriphenylphosphonimum bromide (MeTPP+), which is closely related to known substrate TPP+
220 (Fig 3B)(3), and chelerythrine chloride (Fig 3C). Resistance to ethidium bromide and chelerythrine
221 chloride was consistent with the phenotype observed in PA14. However, PAsmr expressed in *E. coli*
222 conferred susceptibility to harmane (Fig 3D), opposite the resistance phenotype observed in PA14.
223 PAsmr also conferred susceptibility to 18-crown-6 ether, (Fig 3E), and streptomycin (Fig 3F) as
224 expected based on growth in PA14. Overall, phenotypic results were also consistent with growth in *E.*
225 *coli* in higher ionic strength media (26), and confirmed that PAsmr confers resistance to the known
226 EmrE substrate, MeTPP. Susceptibility to streptomycin was a novel phenotype of PAsmr in *E. coli*,
227 consistent with the observed susceptibility phenotype observed in *P. aeruginosa* above. Compared to
228 previously tested growth in higher ionic strength media (26), growth in nutrient broth also resulted in
229 earlier and more pronounced phenotypic differences between WT and E14Q (Fig 3, S1, S2). This
230 confirms that PAsmr phenotypes in low-ionic strength media are consistent but more pronounced
231 than those in Muller-Hinton Broth, supporting our hypothesis that decreased ability to compensate for
232 ΔpH dissipation may occur in low-ionic strength media.
233

234

235 ***PAsmr substrate phenotype corresponds to transport mode***

236 Next, we investigated how PAsmr can confer opposing phenotypes depending upon substrate.
237 We hypothesized that PAsmr may confer resistance via antiport but susceptibility via proton uniport,
238 drug uniport, or proton/drug symport (Figure 4A). To test if PAsmr performs only antiport or may also
239 use other transport modes, we tested four substrates in a purified system using solid-supported
240 membrane electrophysiology (SSME) (29).

241 PAsmr was reconstituted into liposomes, which were adsorbed onto a gold electrode sensor.
242 In SSME, signal is detected as net charge moves in or out of the liposomes when different gradient
243 conditions are applied. A twofold outward pH gradient is established across the liposome in each
244 assay, and the drug gradient is varied: Equal drug on both sides, greater drug concentration outside,
245 or greater drug concentration inside (Figure 4B). Net charge transport in response to establishment of
246 these gradients distinguishes between antiport, symport, and drug/proton uniport (Figure 4C).

247 In all cases, empty liposomes were used as a control, and background signal was not
248 significantly different in PAsmr or empty liposomes, indicating that the presence of PAsmr alone does
249 not induce proton transport and that there is minimal proton leak through the liposomes. For all
250 compounds, addition of drug in equal concentration inside and outside of the liposome resulted in a
251 significant increase in transport compared to empty liposomes (Fig 4D-G). For $MeTPP^+$ and
252 chelerythrine chloride, addition of an outwardly directed 32x drug gradient resulted in the largest net
253 charge transport (Fig. 4C), and an inwardly directed 32x gradient resulted in reversal of charge, a
254 hallmark of coupled antiport (29) (Fig 4D, E).

255 Harmane and 18-crown-6 ether, however, induced net positive transport under all conditions,
256 with transported charge being largest under those conditions that included high drug concentration
257 inside the liposome (Fig 4F,G). This is consistent with harmane and 18-crown-6 ether not being
258 transported, but instead triggering proton uniport down the 2-fold proton gradient (Fig 4C).
259 Streptomycin did not show activity via SSME, potentially due to activity at much lower concentrations

260 biologically than other substrates. Further experimentation will be needed to determine transport
261 mode or specific binding of streptomycin, although preliminary NMR experiments suggest that
262 streptomycin is interacting directly with PAsmr (Fig 5).
263
264 ***Substrate is required to trigger proton leak***
265 There was no evidence of proton leak in PAsmr liposomes with a proton gradient but no drug
266 present; however, to confirm that the proton uniport observed in the SSME experiments was induced
267 by drug and not simply an intrinsic property of PAsmr we performed an additional voltage-driven
268 proton leak assay as previously performed for EmrE (16). PAsmr was reconstituted in tight liposomes
269 with a high internal potassium concentration at pH 7. These liposomes were then resuspended in a
270 weakly buffered solution with a low potassium concentration and phenol red, which has pH-sensitive
271 absorbance at 559 nm (Fig 6A). Addition of valinomycin creates a negative inside membrane
272 potential as potassium moves according to its concentration gradient. If PAsmr leaks protons, this
273 negative inside membrane potential would trigger proton transport and change the external pH
274 leading to change in Abs_{559}. This does not occur, and only after addition of a protonophore (FCCP)
275 allowing direct proton transport across the membrane does the external pH shift, indicating that
276 PAsmr does not leak under a voltage gradient and substrate is required to trigger transport (Fig 6B).
277 Therefore, substrate-induced proton uniport is at least one pathway by which PAsmr confers
278 susceptibility to some compounds.
279
280 **DISCUSSION**
281 Our model of SMR function predicts that these efflux pumps act as double-edge swords for
282 bacteria by conferring resistance to some compounds and susceptibility to others. While resistance
283 behavior is expected, the ability to confer susceptibility to some substrates has previously only been
284 observed in EmrE in *E. coli.* Here we show that this paradigm also plays out in the antibiotic resistant
285 bacterium *P. aeruginosa*, raising the possibility that SMRs could be therapeutically targeted in this

286 deadly pathogen by developing small molecules that trigger the alternative proton leak activity. While
287 PAsmr was identified based on sequence homology to EmrE, there has been minimal investigation
288 into the substrate profile of PAsmr, particularly in light of the discovery of the “free-exchange model”
289 and the ability of EmrE to confer susceptibility rather than resistance to some substrates (13, 14, 16).
290 Here, we expanded the PAsmr substrate profile, and determined that PAsmr may also act via
291 substrate-induced proton uniport, therefore allowing it to confer susceptibility in *P. aeruginosa.* Using
292 PAsmr to induce susceptibility or impact resistance phenotype is appealing as it does not require
293 PAsmr to be the primary resistance mechanism, but simply be present and expressed in a particular
294 *P. aeruginosa* strain or isolate. Given that SMR orthologs are generally conserved in *P. aeruginosa*
295 isolates (nearly 300 *P. aeruginosa* strains in the pseudomonas.com database contain SMR orthologs
296 [Winsor: 26578582]), we suggest that SMR targeting could be broadly applicable in *P. aeruginosa*.
297 We discovered that *smrPA* deletion has an impact on resistance phenotypes in *P. aeruginosa*
298 strain PA14, a virulent model strain. While Li *et al.* demonstrated that *smrPA* deletion results in a
299 reduced minimum inhibitory concentration to some substrates, only traditional transport substrates
300 and aminoglycosides were tested, and full growth curves were not examined (6). We demonstrated
301 that deletion of *smrPA* from PA14 resulted in loss of resistance to ethidium bromide compared to WT,
302 which is surprising given the numerous additional efflux mechanisms present in *P. aeruginosa* (30,
303 31). Complete growth curves of WT and Δ*smrPA* PA14 revealed differences even among resistance
304 and susceptibility phenotypes, with ethidium bromide and harmane completely killing Δ*smrPA* and
305 chelerythrine extending the initial lag phase (Fig. 2). In contrast, loss of PAsmr provided resistance to
306 streptomycin. This is consistent with the various resistance mechanisms displayed by *P. aeruginosa*
307 and the interplay between SMR activity and other mechanisms. This supports the potential for
308 combination therapy and adjuvant approaches to overcome resistance due to efflux pump activity.
309 Comparison to growth of *E. coli* expressing PAsmr is therefore valuable not only to compare
310 directly to other SMRs without the complicating factors of additional resistance mechanisms in the
311 native organism, but also to elucidate those mechanisms and the role of bacterial metabolism. In

312 particular, phenotypic differences between *P. aeruginosa* and *smrPA-E. coli* in "susceptibility
313 substrates" are useful to investigate further. Harmane, originally identified as an EmrE substrate,
314 triggers proton uniport through PAsmr *in vitro.* Growth with harmane results in susceptibility in *E. coli*,
315 but resistance in *P. aeruginosa*. This may indicate that, if proton uniport is the mechanism of PAsmr
316 when exposed to harmane in *P. aeruginosa*, it may result in overcompensation and eventual
317 resistance to harmane. In contrast, streptomycin results in a greater susceptibility phenotype in *P.*
318 *aeruginosa* than in *E. coli,* with *smrPA-E. coli* eventually recovering growth but not WT PA14. This
319 may be due to the relationship between aminoglycoside action and the proton-motive force: that is, if
320 streptomycin triggers proton uniport, the resultant rundown of the proton-motive force may decrease
321 streptomycin transport into the cell resulting in lack of antibacterial action by streptomycin (32, 33).
322 While Δ*smrPA* PA14 is a whole gene deletion and E14Q-PAsmr *E. coli* is a plasmid-expressed single
323 point mutant, differences in phenotype between PAsmr expression in PA14 and *E. coli* and their
324 respective controls are likely also related to the physiology of the organisms and additional resistance
325 mechanisms in PA14, and understanding action of PAsmr may improve our understanding of
326 resistance in *P. aeruginosa* more broadly. Future directions will include constructing and expressing
327 the E14Q mutant in PA14, as well as understanding when PAsmr expression is induced in PA14.
328 Inducing susceptibility via efflux pumps may be possible in other organisms with SMRs or
329 similar transporters. This investigation serves as a starting point for the further characterization of
330 PAsmr as well as the molecular determinants of substrate profile and phenotype for SMRs more
331 broadly. Known PAsmr substrates, phenotypes, and transport modes are summarized in Table 2. A
332 robust expression protocol supports this goal, as does the testing of additional substrates. While
333 binding of substrates to PAsmr was not quantified here, Ninio *et al*. confirmed that TPP+ and ethidium
334 bromide interact directly with PAsmr (3), and future studies will use NMR to define specific binding
335 interactions that contribute to distinct phenotypes. Solid-supported membrane electrophysiology is the
336 first direct confirmation that proton uniport occurs in an SMR other than EmrE, and other transport
337 modes may still be possible given the very small set of substrates we have tested so far. As efforts

338 are made to expand the substrate profile, understanding what makes an SMR substrate may help
339 with potential therapeutic development to induce susceptibility via proton-motive force targeting.
340

341 **FIGURES AND TABLES**

342 **Figure 1. Testing known and novel PAsmr substrates in PA14**

343 **Figure 2. Phenotypes of *P. aeruginosa* Δ*smrPA* are substrate-dependent**

344 **Figure 3. PAsmr expression in *E. coli* shows a substrate-dependent phenotype, often but not**
345 **always consistent with that in *P. aeruginosa***

346 **Figure 4. PAsmr performs both antiport and proton uniport depending on substrate**

347 **Figure 5. Streptomycin has selective interactions with PAsmr**

348 **Figure 6. PAsmr does not leak proton at neutral pH**

349 **Table 1. Primers used in PAsmr deletion**

350 **Table 2. Known PAsmr Substrates and Phenotypes**

351

352 **CONFLICTS OF INTEREST**

353 The authors declare no conflicts of interest with this work.

354

355 **SUPPORTING INFORMATION (ATTACHED)**

356 Supplemental Methods

357 Fig. S1-S4, Table S1

358 Data repository: MendeleyData

359

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372

373 **AUTHOR CONTRIBUTIONS**

374 Conceptualization-KAHW, JMP, AKW; Methodology-KAHW, JMP, WJH, AWK; Investigation-AKW,
375 WJH, SPD, MSM; Data Curation-AKW, WJH, SPD, MSM; Writing-Original Draft-AKW, MSM; Writing-
376 Review & Editing-AKW, KAHW, JMP, WJH; Visualization-AKW, MSM; Supervision-KAHW, JMP;
377 Funding acquisition-KAHW, AKW

378

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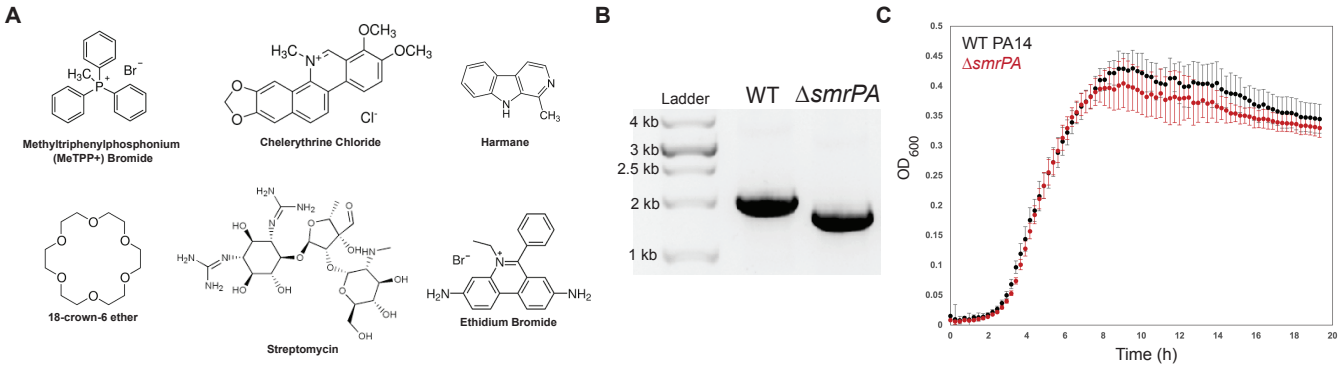

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467 **Figure 1. Known and novel PAsmr substrates and construction of a PAsmr deletion strain.** A)
468 Compounds used in this study. B) Confirmation of the *smrPA* deletion in PA14 by PCR with flanking
469 primers. C) *smrPA* deletion does not affect PA14 growth in the absence of drug.

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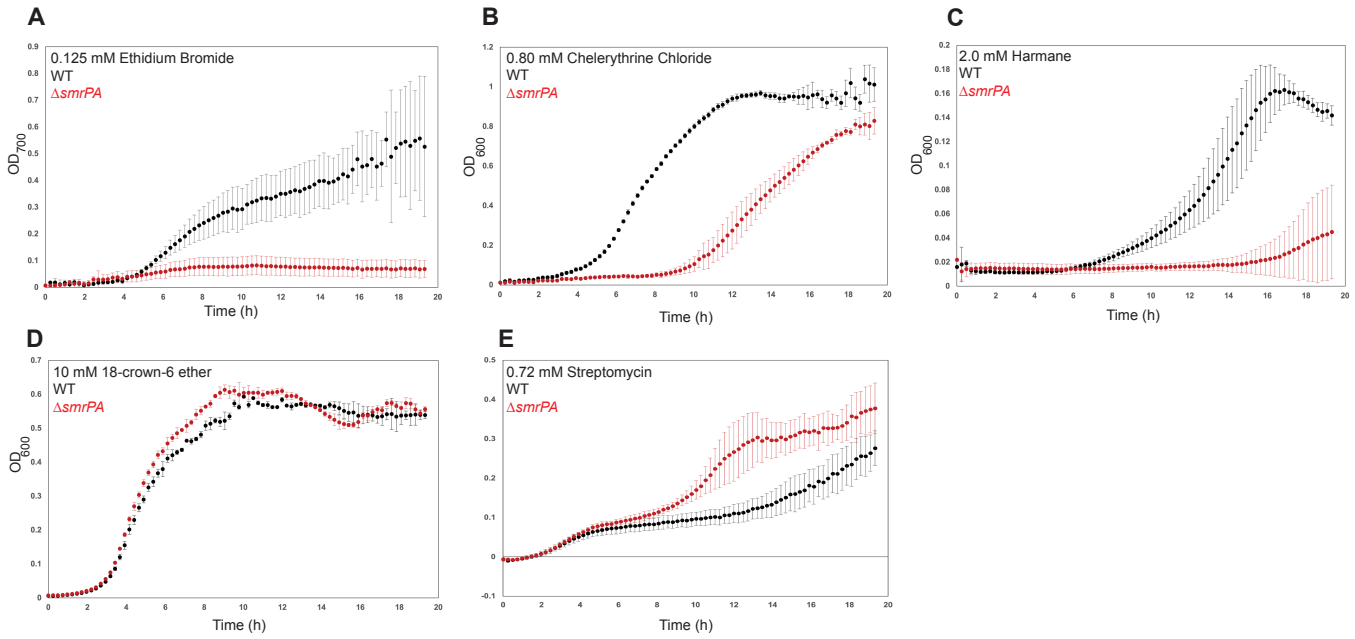

Figure 2. Phenotypes of *P. aeruginosa* Δ*smrPA* are substrate-dependent. A-C) Δ*smrPA* shows reduced growth relative to WT PA14 in the presence of ethidium, chelerythrine, and harmane, suggesting that PAsmr confers resistance to these compounds. D-E) Δ*smrPA* growth in the presence of these compounds suggests that PAsmr confers susceptibility to 18-crown-6 ether and streptomycin.

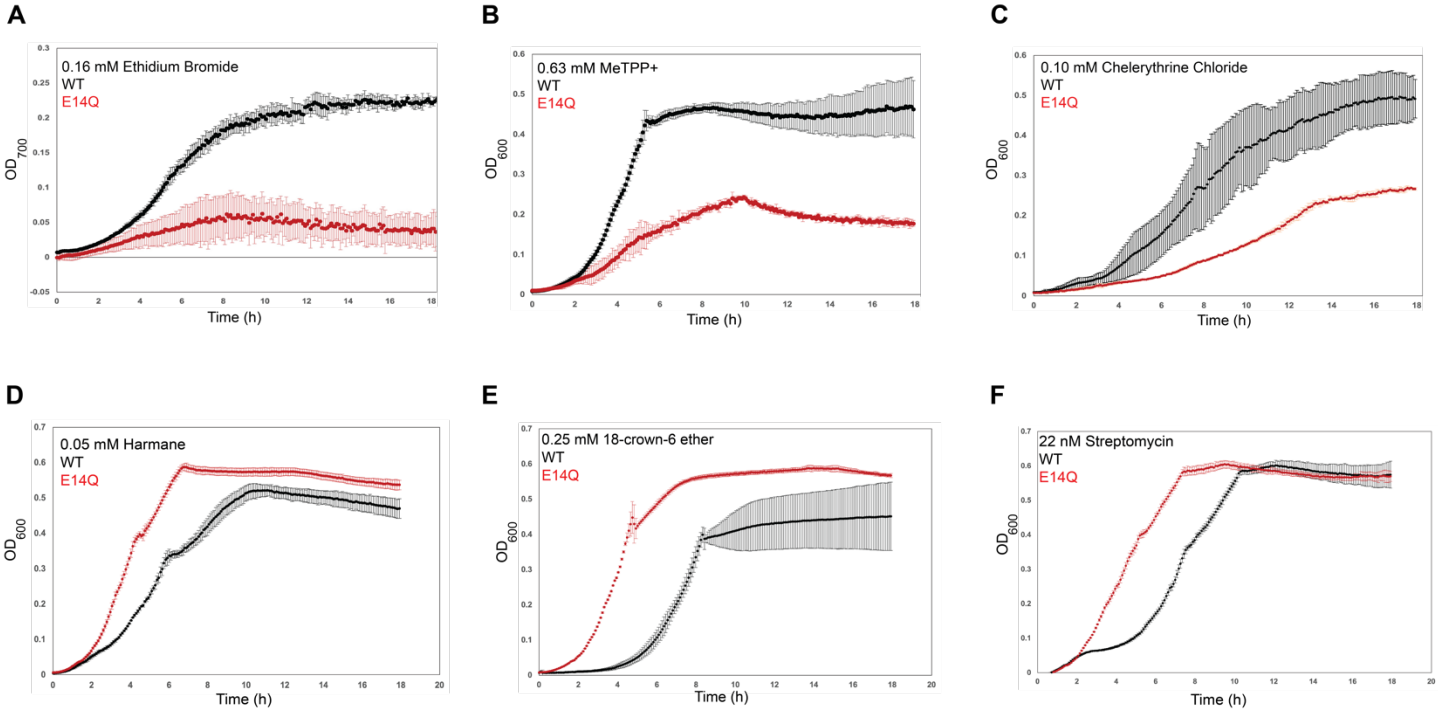

Figure 3. PAsmr expression in *E. coli* shows a substrate-dependent phenotype, often but not always consistent with that in *P. aeruginosa* A-C) *E. coli* expressing WT PAsmr shows improved growth relative to the E14Q variant in the presence of ethidium, MeTPP+, and chelerythrine, suggesting that PAsmr confers resistance to these compounds. D-E) E14Q-PAsmr- *E. coli* shows improved growth relative to WT PAsmr in the presence of harmane, 18-crown-6 ether, and streptomycin, suggesting that functional PAsmr confers susceptibility to these compounds in *E. coli*.

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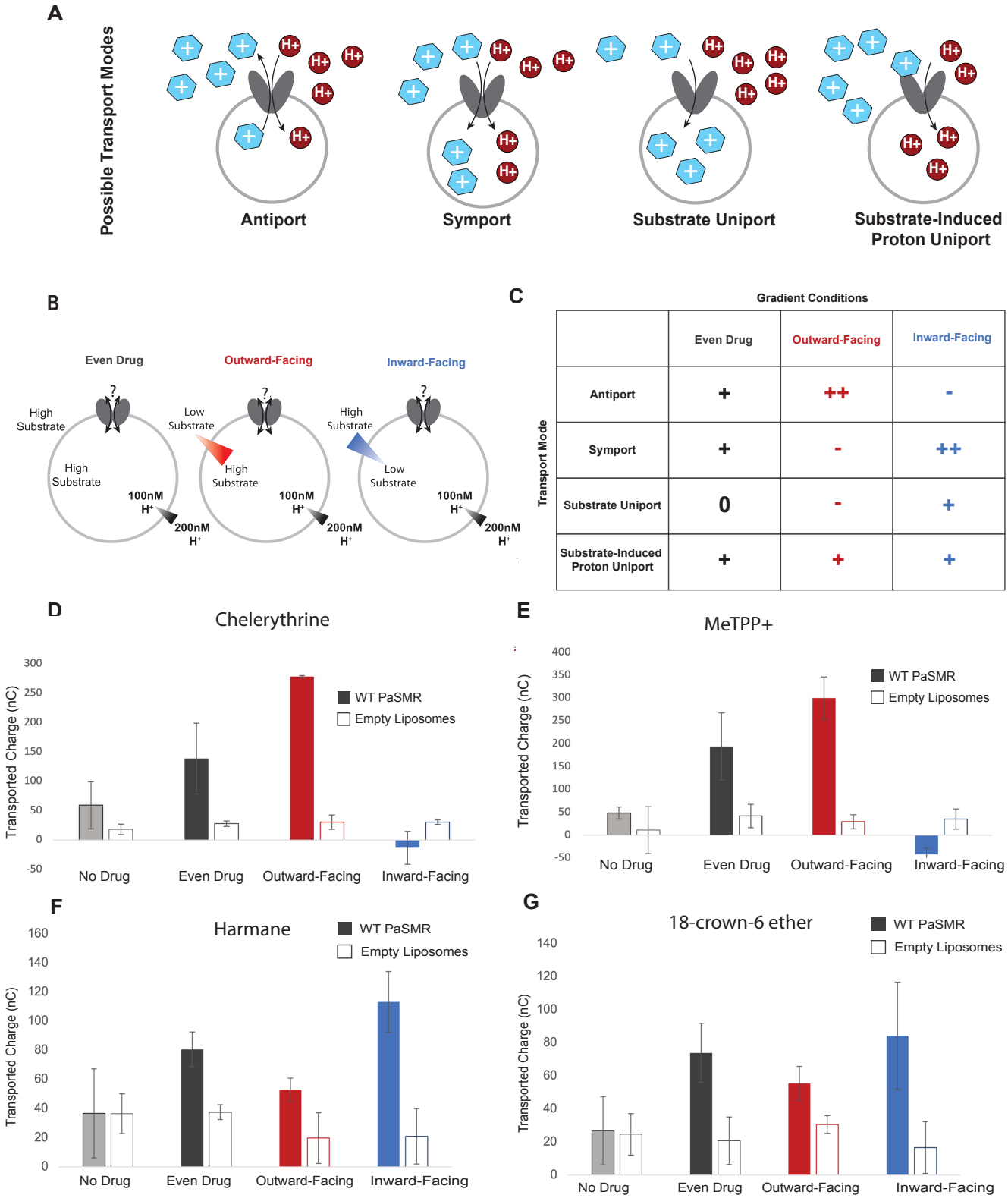

A
Possible Transport Modes
Antiport
Symport
Substrate Uniport
Substrate-Induced Proton Uniport
B
Even Drug
Outward-Facing
Inward-Facing
High Substrate
Low Substrate
100nM H+
200nM H+
C
Gradient Conditions
Even Drug
Outward-Facing
Inward-Facing
Transport Mode
Antiport + ++ -
Symport + - ++
Substrate Uniport 0 - +
Substrate-Induced Proton Uniport + + +
D
Chelerythrine
E
MeTPP+
F
Harmane
G
18-crown-6 ether
Transported Charge (nC)
WT PaSMR
Empty Liposomes
No Drug
Even Drug
Outward-Facing
Inward-Facing

490

491 **Figure 4. PAsmr performs both antiport and proton uniport depending on substrate.** A) Based
492 on the free-exchange model of EmrE, four transport modes are theoretically possible for PAsmr (16)
493 B) Solid-supported membrane electrophysiology was performed using three different drug gradient
494 conditions and a consistent 2-fold proton gradient. C) Resulting ion transport for each condition allows
495 discrimination between four different transport modes. D) PAsmr transports Chelerythrine and E)
496 MeTPP+ via antiport. F) Harmane and G) 18-crown-6 ether trigger increased proton transport
497 regardless of drug gradient.
498

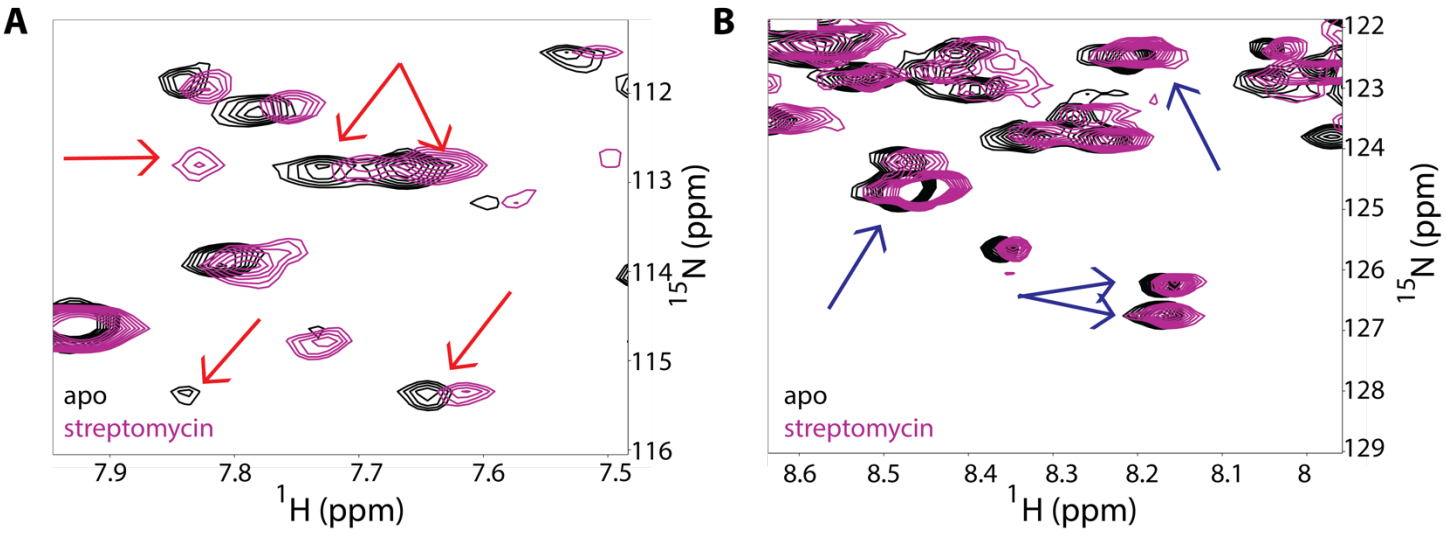
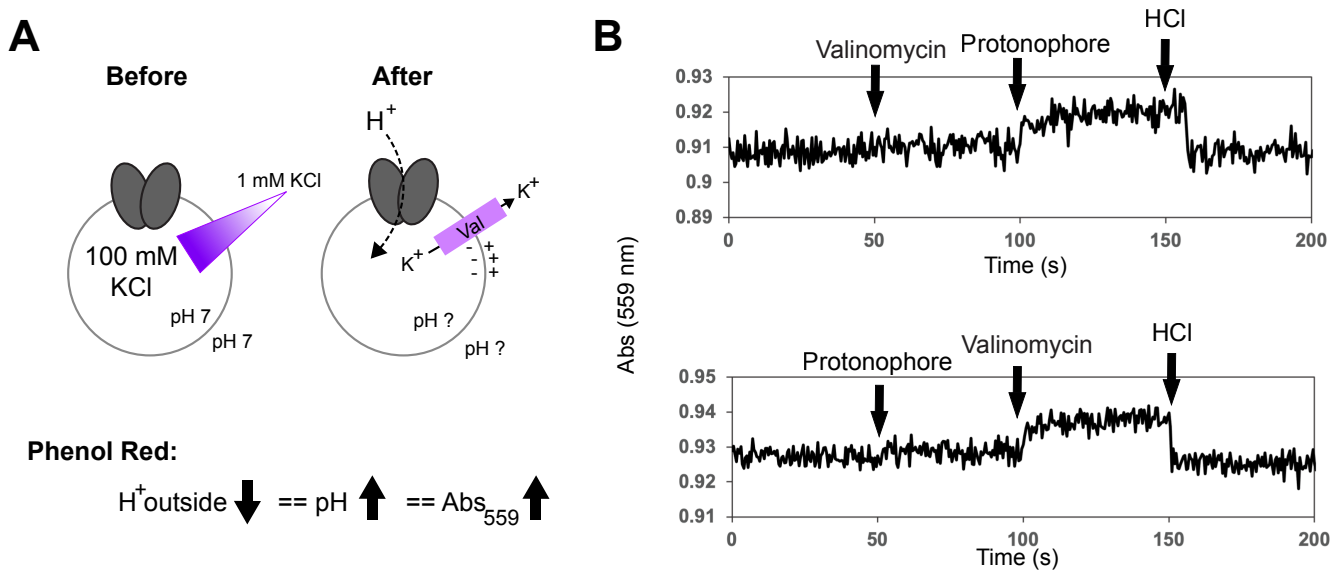


Figure 5. Streptomycin has selective interactions with PAsmr 1H-15N TROSY spectra of S64V PaSMR with (purple) and without (black) streptomycin indicate that streptomycin is likely interacting with the protein due to A) sizeable chemical shift perturbations and peak differences in some portions of the spectrum. B) Some regions of the spectra are overlaid completely, indicating backbone residues where streptomycin does not have an impact, indicating a specific interaction.



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508 **Figure 6. PAsmr does not leak proton at neutral pH**. A) PAsmr was reconstituted into liposomes
509 and resuspended with a 100-fold potassium gradient in neutral pH and phenol red. Addition of
510 valinomycin dissipates the potassium gradient and establishes a negative-inside membrane potential,
511 favoring proton transport into the liposome if it is possible. B) Phenol red absorbance at 559 nm
512 reveals no change upon valinomycin addition (top), indicating that PAsmr does not leak proton under
513 these conditions. Protonophore addition increases phenol red absorbance and HCl addition
514 decreases absorbance, confirming establishment of a membrane potential. Protonophore addition
515 first also does not result in an increase of absorbance, indicating that both membrane potential and
516 protonophore are necessary for proton transport through liposomes (bottom).

517

518 **Table 1. Primers used in PAsmr deletion**

oJMP Primer	Sequence (5'-3')
1832	CTAGAACTAGTGGATCCCCCCGGGCAAGGACAGGGAAAA
1833	TTCAGTGCCCATTGGTCATGGCGGCGTC
1834	CATGACCAATGGGCACTGAACGACCGTC
1835	ATATCGAATTCCTGCAGCCCCCTGTTCGGCGAGGAAGG
1881	GCTCGGTTTCCATGATCACC
1882	GGATGCCGTCTTCTTCCAGG

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523 **Table 2. PAsmr Substrates and Phenotypes**

Substrate/Compound	Interaction
Tetraphenylphosphonium	Inhibits methyl viologen transport *in vitro* (3)
Benzalkonium	Inhibits methyl viologen transport *in vitro* (3); PAsmr inhibition re-sensitizes PAsmr-E. coli (22)
Acriflavine	Inhibits methyl viologen transport *in vitro* (3); *smrPA* deletion from PAO1 decreases MIC (6)
Ethidium	Inhibits methyl viologen transport *in vitro* (3); Transports/effluxes (22); *smrPA* deletion from PAO1 decreases MIC (6); *smrPA* deletion from PA14 decreases resistance; WT expression in E. coli confers resistance; transported *in vitro* via antiport
Methyl viologen	Transported *in vitro* (3)
Cetylpyridinium	PAsmr inhibition re-sensitizes PAsmr-E. coli (22)

Cetyltrimethylammonium	PAsmr inhibition re-sensitizes PAsmr-E. coli (22)
Gentamicin	*smrPA* deletion from PAO1 decreases MIC (6)
Kanamycin	*smrPA* deletion from PAO1 decreases MIC (6)
Neomycin	*smrPA* deletion from PAO1 decreases MIC (6)
Nalidixic acid	*smrPA* deletion from PAO1 decreases MIC (6)
Chelerythrine	*smrPA* deletion from PA14 decreases resistance; WT expression in E. coli confers resistance; transported *in vitro* via antiport
Harmane	*smrPA* deletion from PA14 decreases resistance; WT expression in E. coli confers susceptibility; triggers proton uniport
18-crown-6 ether	*smrPA* deletion from PA14 increases resistance; WT expression in E. coli confers susceptibility; triggers proton uniport
Streptomycin	*smrPA* deletion from PA14 increases resistance; WT expression in E. coli confers susceptibility
Methyltriphenylphosphonium	*smrPA* deletion from PA14 decreases resistance; WT expression in E. coli confers resistance; transported *in vitro* via antiport

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