Functional insights of two MATE transporters from *Vibrio fluvialis*

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Running title: MATE-type efflux pumps from Vibrio fluvialis

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Summary

Functional characterization of H- and D-MATE (Multidrug and Toxin Extrusion) transporters from clinical isolates of *Vibrio fluvialis* revealed H-type conferred resistance to fluoroquinolones, ethidium bromide and safranin whereas D-type exhibited marginal resistance towards ethidium bromide only. Both H-/D-type transporters were inhibited by reserpine resulting in increased intracellular norfloxacin concentration. The efflux was facilitated by both Na^+/K^+ ions, suggesting that these efflux pumps were ion-dependent. In presence of various classes of EPIs, there was decrease in MIC exhibited by H-/D-type efflux pumps towards norfloxacin which didn't translate into transport inhibition. But reserpine presented a conclusive pattern with decrease in MIC towards norfloxacin and increased norfloxacin accumulation inferring maximum inhibition.. Substrate binding and electrostatic charge distribution of both the transporters was similar to other known MATE transporters. The H-type exhibited 10 transmembranes and D-type exhibited 11 TMs which was different from other MATE transporters known to have 12 TMs (Transmembranes). Data derived from molecular docking and ion binding studies revealed that Aspartic Acid residue in 1st TM acts as ion binding site with transport mechanism similar to

NorM. Electrostatic potential map of both the transporters revealed that there is a cavity formation within the transporters surrounded by charged electronegative amino acid residues.. Interestingly, surface models of both transporters revealed that 1st TM forms covalent bond with 7th TM towards extracellular space. Docking studies also revealed that reserpine covalently binds to central pocket of both transporters and serves as excellent EPI against these transporters as evidenced by MIC and drug accumulation assays.

Introduction

Resistance mediated by efflux pumps is one of the non-specific mechanisms that pathogenic microbes employ to lower intracellular drug concentration (Bhardwaj & Mohanty, 2012, Van Bambeke *et al.*, 2003, Poole, 2000, Poole, 2004, Poole, 2005). Efflux pumps could be primary transporters utilizing ATP for transport or could be secondary transporters that utilize sodium-/proton-motive force for transport of substrates. Till date six families of transporters are known for their involvement in multidrug resistance (MDR) in prokaryotes (Bhardwaj & Mohanty, 2012, Van Bambeke *et al.*, 2003, Hassan *et al.*, 2015). Multidrug and toxic compound extrusion (MATE) family of efflux pumps is one of the newest and least characterized family of transporters that are proven to be involved in resistance to various antibiotics and dyes including quinolones (Mohanty *et al.*, 2012, Omote *et al.*, 2006). NorM, the prototype member was discovered from *Vibrio parahaemolyticus* (Morita *et al.*, 1998) and subsequently its homologs and orthologs were identified in *Escherichia coli*, *Neisseria gonorrhoeae* and *V. cholerae* (Long *et al.*, 2008, Morita *et al.*, 2000). The MATE family further consists of three protein clusters : the NorM branch involved in MDR, a branch containing several eukaryotic proteins and a branch containing DinF involved in SOS response and MDR (Otsuka *et al.*, 2005). Growing academic interest in this family of transporters is evident from the fact that structures have been solved for a few within a span of lastfew years from prokaryotic as well as eukaryotic sources(Tanaka *et al.*, 2013, He *et al.*, 2010, Lu *et al.*, 2013b, Lu *et al.*, 2013a, Wong *et al.*, 2014, Tanaka *et al.*, 2017, Miyauchi *et al.*, 2017) . Preliminary *in-silico* and drug transport

59 studies of MATE family of efflux pumps revealed that these efflux pumps are sodium-ion
60 coupled and recognize wide spectrum of substrates but neither of these characteristics have
61 been explicitly explainedat molecular level (He *et al.*, 2004). MATE pumps originated from
62 flippases (Meeske *et al.*, 2015). Both MATE and flippases belong to same family of proteins
63 multidrug/oligosaccharidyl-lipid/polysaccharide (MOP) transporter superfamily. Divergence can
64 be seen as functional role has been attributed to aspartic acid in 1st transmembrane of MATE-
65 pumps, which acts as ion binding site whereas serine at 17 position is known to play functional
66 role in MurJ flippases (Kuk *et al.*, 2017). From our laboratory, two MATE-type members (H- and
67 D-) were first identified in *Vibrio fluvialis* (causes cholera-like illness) (Mohanty *et al.*, 2012). The
68 genes were cloned in pBR322 vector and the protein functions were assessed using MIC and
69 drug transport assays. Both the pumps conferred resistance to fluoroquinolones norfloxacin
70 and ciprofloxacin. In the present study, we cloned both the transporters in arabinose promoter-
71 based pBAD vector which allowed visualization of recombinant protein and better control over
72 protein expression. Functional characterization was performed with assays involving MICs, drug
73 uptake and efflux pump inhibitors (EPIs). Molecular docking, ion binding site predictions and
74 surface electrostatics of these transporters using the known MATE-type transporters as
75 reference proteins was carried out to further characterize the functionality of these transporters.
76

77 **Results**

78 ***Expression of recombinant H- and D- MATE-type efflux pumps in E. coli-*** The sequenced
79 genes of D- and H-type efflux pumps were designated as *vcd*, *vfd*, *vch* and vfh, *where* c stands
80 for *V. cholerae* and *f* stands for *V. fluvialis*. Corresponding to each gene, the proteins were
81 designated as VCH, VCD, VFH and VFD. Further the genes were cloned in arabinose driven
82 pBAD expression system and induced as mentioned in materials in methods. Coomassie blue
83 stained gel of arabinose-induced pBAD-*vfd* samples did not show the band corresponding to

recombinant VFD at the expected position of 55 kDa (Fig. 1A) whereas the expression of positive control Lac-His was detected at 125 kDa as expected.

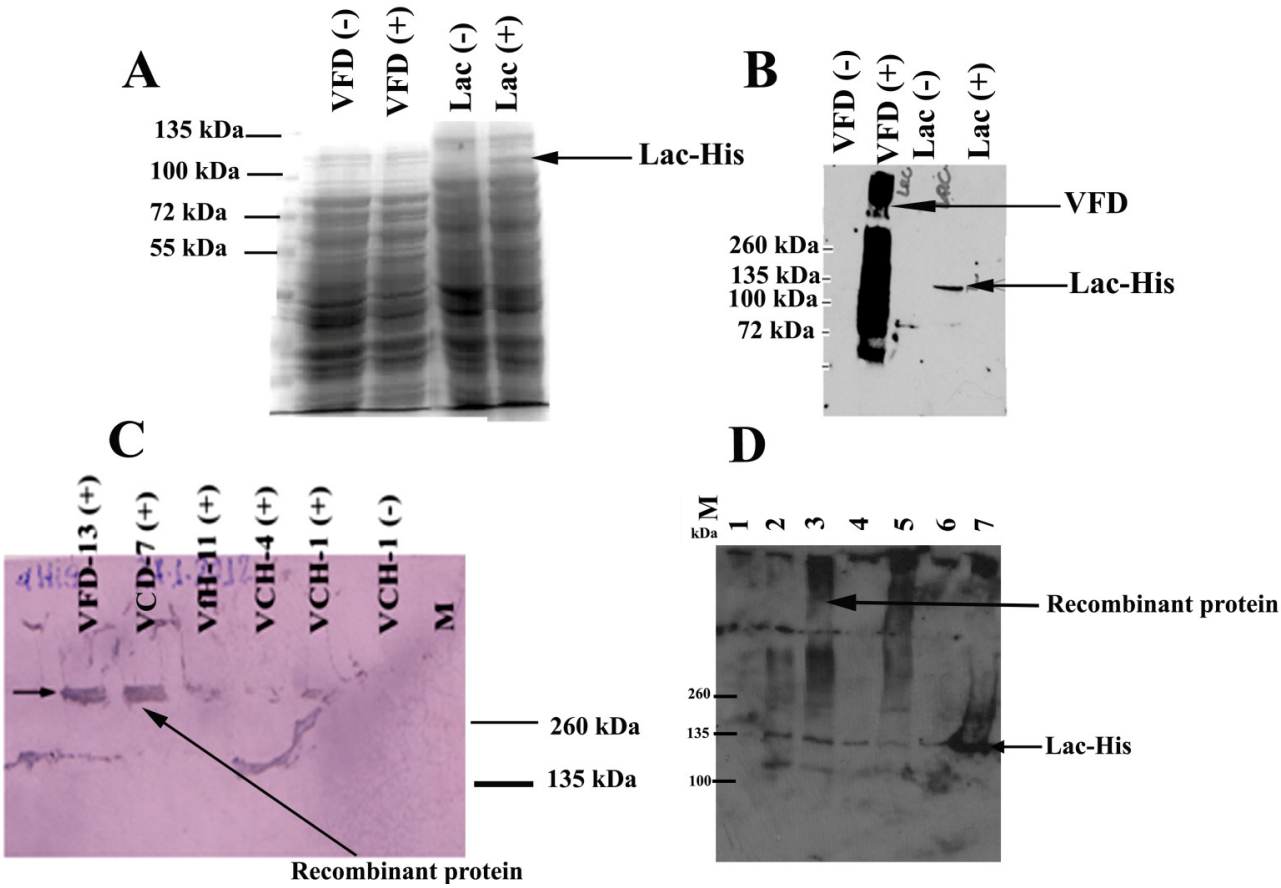


Fig. 1. Total cell expression in induced (+) and uninduced (-) cell lysates of different recombinant pBAD clones. **A.** Coomassie blue stained 10% SDS-PAGE for total cell lysates from pBAD-*vfd* along with pBAD-Lac-His positive control in *E. coli* LMG194; **B.** Western blot of induced (+) and uninduced (-) cell lysates of pBAD-*vfd* along with pBAD-Lac-His positive control. **C.** Western blot of recombinant VCH, VFH, VCD, VFD and Lac-His in LMG194. **D.** Western blot of recombinant proteins in K-12 Δ*tolC*. The cells were induced and developed as described in materials and methods. Lane-1 Prestained Spectramulticolour (Fermentas) molecular weight markers have been indicated on the left of each pane. Lane 2,3,4 and 5 represents VCH, VFH, VCD and VFDFor Panels **B** and **D, the** blots were developed with ECL substrates (Novex, Invitrogen) and for Panel **C**, they were developed with DAB and H_2O_2 Goat Ant-V_5 antibody was used as primary antibody.

Therefore, Western Blot was carried out to determine the expression of recombinant VFD protein with goat anti-V_5 primary antibody and developed with enhanced chemiluminescence substrates. Results revealed the expression of Lac-His positive control at

right molecular weight but interesting observation was made in case of pBAD-*vfd*, which showed diffused pattern of bands in the whole lane (Fig 4.16 B). Subsequently, all the four recombinant clones for H- and D-genes were expressed in *E. coli* LMG194 under same conditions. The development of Western blots with goat anti-His antibody using colorimetry clearly depicted a single major band with the mobility greater than 260 kDa in the lanes with induced cells for all the clones (Fig 1 C) . Therefore, the slow development of the Western blots showed single major bands whereas the fast/more sensitive ECL detection could also depict the degradation pattern for the recombinant proteins.

Further, all the four recombinant clones for H- and D-genes (*vch*, *vfh*, *vcd* and *vfd*) were expressed in *E. coli* LMG194 and Western blots using colorimetry clearly depicted a single major band with the mobility greater than 260 kDa instead of 55 kDa in the lanes with induced cells for all the clones (Fig. 1C). The sensitivity of ECL led to the visualization of diffused band pattern probably indicating either various stages of oligomerisation/recombinant protein degradation whereas the less sensitive colorimetry showed a single band on Western blot. The diffused band pattern of the recombinant efflux pumps observed in the current study could be the final outcome of aberrant mobility due to oligomerisation. The observed aberrant mobility of recombinant transporter proteins due to oligomerisation has also been reported in case of ammonium transporter AmtB of *E. coli* (Blakey *et al.*, 2002) where the 45 kDa protein showed series of bands of varying molecular weights suggesting it to be an oligomer. In our study, the mobility of the H- and D-recombinant effux pump proteins showed similar pattern on Western blots where ~55 kDa proteins migrated at >260 kDa molecular weight suggesting that the recombinant protein existed as oligomers that were stable in the presence of SDS and β-ME. The recombinant efflux pumps formed a series of aggregated species due to the presence of hydrophobhic patches that appeared as a ladder of high molecular weight bands under denaturing and reducing conditions (Blakey *et al.*, 2002). The wild type Na^+/H^+ antiporter of *Methanococcus jannaschii* presents itself as a dimer even in the presence of SDS and

129 undergoes proteolytic degradation (Goswami *et al.*, 2011). The recombinant expression of
130 membrane proteins is tricky where specialized expression systems have been developed to
131 counteract protein degradation and oligomerisation (Wagner *et al.*, 2008). Taken together, the
132 facts from earlier studies and observations from the present study suggested that H- and D-
133 MATE efflux pumps exhibited aberrant mobility possibly be due to oligomerisation similar to
134 AcrB due to intra-molecular van der Waals bonding that resists dissociation in presence of SDS
135 and β-ME.

136 ***pBAD recombinant efflux pumps are translocated to the membrane-*** Upon successful
137 detection of recombinant proteins in the host cells with aberrant mobility, classical traits of
138 membrane proteins, further studies were carried out to determine if the recombinant proteins
139 were actually targeted to the membrane of the *E. coli* host. Two approaches *i.e.* confocal
140 microscopy and membrane fractionation were utilised to investigate the same.

141 Earlier bioinformatics studies of both the efflux pumps had predicted that these pumps
142 were transmembrane proteins (Mohanty *et al.*, 2012). Localization of these efflux pumps in cell
143 membrane was investigated utilizing confocal microscopy where the laser was programmed to
144 detect membrane marker Film Tracer 1-43 and CY3 fluorochrome conjugated secondary
145 antibody from the same pin hole. Results revealed that VFD and VFH were localised in the
146 membrane of *E. coli* LMG194 (Fig. 2 Panel A: A-F). The recombinant proteins generated red
147 signal due to CY3 (Fig. 2 A, panelsB and E) and membrane marker generated green signal for
148 cell membrane (Fig. 2A,panels A and D). DAPI, which was used for nuclear staining was also
149 employed as counterstain but it did not bind with the nucleoid and was not visible by confocal
150 imaging. Change in the colour of membrane to yellow in the superimposed image of
151 recombinant efflux pump protein and membrane marker (Fig. 2A, panels C and F) suggested
152 that the recombinant proteins were localised in the membrane and were detected by the same
153 pinhole of the microscope.

154

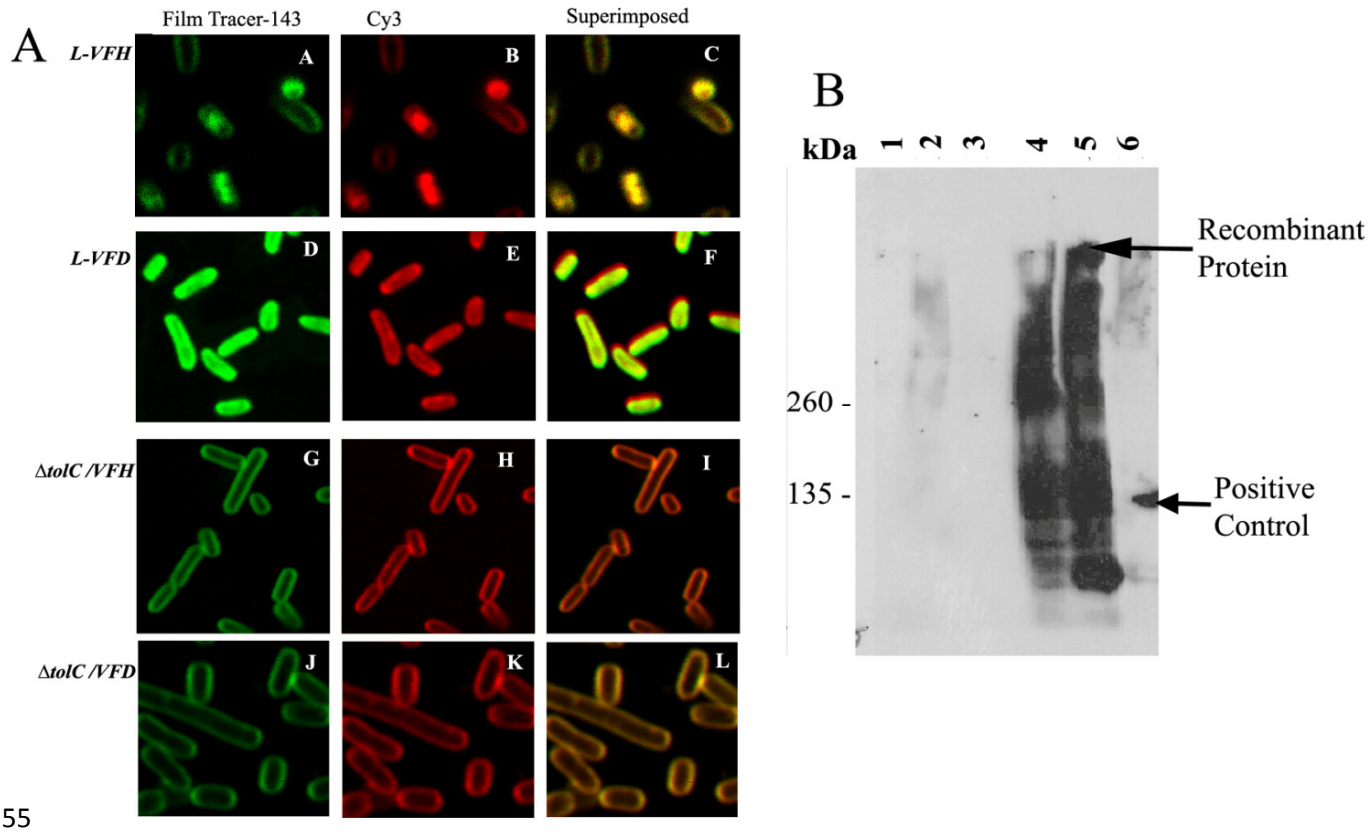


Fig. 2. Membrane localisation of recombinant efflux pumps in *E. coli.* **Panel A.** Confocal imaging of pBAD-recombinants in heterologous *E. coli* host. **A**/**D**:Visualisation of *E. coli* cells harbouring pBAD-*vfh/vfd* stained with membrane marker Film-tracer 1-43; **B**/**E**:Detection of recombinant pBAD-*vfh/vfd* in *E. coli* labelled with Goat anti-Mouse Cy3 secondary antibody; **C**/**F** (63X ZOOM 4): Superimposed/merged images from **A**/**B or D**/**E** of recombinant pBAD-*vfh/vfd* in *E. coli* LMG194; **G**/**J**: Visualisation of *E. coli* cells harbouring pBAD-*vfh/vfd* using membrane marker Film-tracer 1-43; **H**/**K**: Detection of recombinant pBAD-*vfh/vfd* in *E. coli* labelled with Goat anti-Mouse Cy3 secondary antibody; **I**/**L**: Superimposed/merged images from **G**/**H** or **J**/**K** of recombinant pBAD-*vfh/vfd* in *E. coli* K-12 Δ*tolC*2 Δ*tolC*. **Panel B.** Membrane fractionation of *E. coli* harbouring pBAD-*vfd.* 63X Zoom 4 settings were used for obtaining these images. M- Spectramulticolour protein marker (Fermentas). Lane 1- VFD –ve- Total cell lysates for uninduced samples; Lane 2-VFD +ve- Total cell lysates for induced samples; Lane 3- VFD Son (Sup) - total soluble fraction after sonication.; Lane 4- VFD SAR (Sup)- Total soluble proteins after sarkosyl treatment; and Lane 5- VFD SAR Pellet- debris and other subcellular organelles. Lane 6- Lac-HisPositive control. Goat anti-V5 antibody was used as primary antibody. HRP conjugated Mouse Anti-Goat IgG was used as secondary antibody (Jackson Immunoresearch). Prestained Spectramulticolour (Fermentas) molecular weight marker in kDa has been indicated of the left of panel. The blots were developed with Novex ECL substrates (Invitrogen).

To further confirm if the recombinant proteins were localized in the inner membrane, a protocol utilising a combination of membrane fractionation and selective solubilisation of the inner membrane with detergents was employed (Hobb *et al.*, 2009, Leyh, 1990, Frankel *et al.*, 1991).Western blot revealed that upon induction, the recombinant protein was detected in total

178 cell lysate (Fig. 2B, lanes 1 and 2) for VFD. The fraction containing total soluble protein did not
179 show the presence of recombinant protein indicating that the recombinant protein was not
180 cytosolic but probably associated with insoluble fraction (Fig. 2B, lane 3). Sarkosyl solubilised
181 inner membrane fraction (lane 4) revealed the band pattern corresponding to recombinant VFD
182 confirming that the protein was translocated to the inner membrane. This protein was also
183 detected in the sarkosyl treated outer membrane pellet indicating partial solubilisation of the
184 membrane upon sarkosyl treatment (Fig. 2B, lane 5). Apart from the above, partially purified
185 recombinant VFD from inner membrane also showed aberrant migration and diffused band
186 pattern as observed earlier.

187 ***MIC assays for pBAD recombinants in E. coli K-12 ∆tolC indicated different substrate***
188 ***profile for H and D pumps -*** The recombinant constructs for H- and D-type proteins when
189 introduced into *E. coli* LMG194 did not exhibit any change in the MIC against ethidium bromide
190 and norfloxacin although both the compounds had been shown as substrates for these
191 membrane proteins in earlier studies from this laboratory (Mohanty *et al.*, 2012). Earlier studies
192 with pBR322 expression system had utilized *E. coli* host KAM32 with deletion of two major efflux
193 pumps AcrAB and YdhE for functional characterization of the recombinant proteins expressed in
194 this host (Mohanty *et al.*, 2012). In the current study *E. coli* K-12 strains with deletions in genes
195 for major RND efflux systems; ∆*acrA* (deletion of connecting protein), ∆*acrB* (deletion of its
196 inner protein) and ∆*tolC* (deletion of its outer membrane protein) were utilized for functional
197 characterization of pBAD recombinants (Kuroda & Tsuchiya, 2009, Matsuo *et al.*, 2008, Matsuo
198 *et al.*, 2007, Otsuka *et al.*, 2005, Mohanty *et al.*, 2012).

199 Results for MICs of ethidium bromide, ciprofloxacin and norfloxacin showed that though
200 all the three K-12 deletion strains, K-12 ∆*acrA*, K-12 ∆*acrB* and K-12 ∆*tolC* showed reduction in
201 MIC as compared to the control wild type strains K-12 and LMG194, K-12 ∆*tolC* exhibited
202 maximum hypersusceptibilty *i.e.* at least 16-fold decrease in MIC towards ethidium bromide and
203 norfloxacin and more than 8-fold decrease in MIC towards ciprofloxacin (Supplementary Table

204 1). Subsequently, accumulation assays also revealed that *E. coli* K-12Δ*tolC* had the highest
205 drug accumulation in comparison to the other hosts utilised in the study (Supplementary Fig.1).

207 Western blot and confocal microscopy (Fig. 1D and Fig. 2A, panels G to L respectively)
208 revealed that the recombinant proteins were expressed in K-12Δ*tolC* and localized in its inner
209 membrane. Taken together, the results of Western blots, confocal microscopy, MIC and
210 accumulation assays (Supplementary Fig.1) indicated that K-12 Δ*tolC* was a suitable
211 hypersusceptible strain for expression and functional characterization of the recombinant MATE
212 pumps.

213 **Table 1. MIC for recombinant clones carrying MATE-type efflux pump genes from *V.***
214 ***fluvialis* and *V. cholerae* in pBAD expression vector and *E. coli* Δ*tolC* host**

	MIC in µg/mL for the recombinant clones				
Drugs/ Dyes	**Δ*tolC***	**Δ*tolC* /VCH**	**Δ*tolC* /VFH**	**Δ*tolC* /VCD**	**Δ*tolC* /VFD**
Ethidium Bromide	<3.9	**15.6**	**15.6**	**3.9**	**7.8**
Norfloxacin	<0.0156	**0.03125-0.0625**	**0.03125-0.0625**	<0.0156	<0.0156
Ciprofloxacin	<0.00156	**0.003125-0.00625**	**0.003125-0.00625**	<0.00156	<0.00156
Nalidixic Acid	0.3125-0.625	0.3125-0.625	0.3125-0.625	0.3125-0.625	0.3125-0.625
Ofloxacin	0.0125-0.025	0.0125-0.025	0.0125-0.025	0.0125-0.025	0.0125-0.025
Neomycin	>200	200	200	200	200
Gentamicin	3.125	1.56-3.125	3.125	1.56	1.56
Acridine Orange	37.5	37.5	37.5	37.5	37.5
Acriflavin	6.25	**12.5**	**12.5**	6.25	6.25
Safranin	3.125	**12.5**	**12.5**	3.125	3.125

215
216 As kanamycin cassette is inserted in place of the gene of interest (Δ*tolC*) to be disrupted, so MIC against
217 kanamycin could not be evaluated against pBAD recombinants in Δ*tolC* . Increase in MIC is shown as
218 bold.

219 Subsequently, *E. coli* K-12 Δ*tolC* expressing pBAD recombinants for VFH, VCH, VFD
220 and VCD presented an interesting profile in terms of recognition/transport by these two pumps
221 (Table 1). H-type pumps exhibited increase in MIC for ethidium bromide (at least 4-fold),
222 norfloxacin and ciprofloxacin (atleast 2-4-fold), acriflavin (2-fold) and safranin (4-fold). As
223 against this, D-type pumps conferred resistance only towards ethidium bromide with at least 2-

224 fold increase in MIC when compared to the control. Both H- and D-type pumps showed almost
225 similar/slight decrease (2-fold) in MIC for the aminoglycosides (neomycin and gentamicin). Both
226 H- and D-type pumps showed unaltered MIC for nalidixic acid, ofloxacin and acridine orange
227 while D-type pumps additionally showed unaltered MIC for norfloxacin, ciprofloxacin, acriflavin
228 and safranin.

229 NorM, the prototype member of MATE-type efflux pumps from *V. parahaemolyticus* is a
230 broad spectrum efflux pump reported to confer resistance towards basic lipophilic compounds
231 like ethidium bromide and rhodamine, hydrophilic aminoglycosides like kanamycin and
232 neomycin and hydrophilic zwitterionic fluoroquinolones such as norfloxacin and ciprofloxacin,
233 but not to hydrophobic quinolones such as sparfloxacin and nalidixic acid (Morita *et al.*, 1998).
234 In the present study, H-type recombinant seemed to be an efflux pump with primary transport
235 capability towards zwitterionic fluoroquinolones and basic lipophilic compounds. Earlier studies
236 had revealed that H-type pumps (VCH/VFH) exhibited resistance towards norfloxacin and
237 ciprofloxacin only when expressed in pBR322 expression system (Mohanty *et al.*, 2012) but
238 when these pumps were expressed in pBAD/Δ*tolC* system, they exhibited norfloxacin and
239 ciprofloxacin resistance along with extended resistance towards ethidium bromide, acriflavin
240 and safranin. The expression of efflux pumps is generally very low and results in a modest
241 increase of two-four folds in MIC (Long *et al.*, 2008) which was also observed in the current
242 study (Long *et al.*, 2008). KetM from *Klebsiella pneumoniae* also exhibited narrow spectrum
243 resistance to fluoroquinolones with modest change in MIC (Ogawa *et al.*, 2015). Asp32, Glu251
244 and Asp367 from *V. parahaemolyticus* NorM were implicated in sodium-dependent transport of
245 antimicrobials and Asp32 specifically is known for its role in efflux thus conferring resistance
246 towards norfloxacin and ethidium bromide (Otsuka *et al.*, 2005). Even though the efflux pumps
247 in the present study carried only Asp at position 40/38 in TM1 out of three conserved residues
248 they were able to exhibit marginal MDR phenotype. This also gives a notion that apart from
249 these three amino acids, other residues might play important role in transport. It was later

250 proven in MATE transporter from the plant *Camelina sativa* (Tanaka *et al.*, 2017). Glutamic
251 Acid , glutamine, asparagine, arginine along with aspartic acid amino acid residues were
252 implicated for their role in forming hydrophobic cavity formation and transport in MATE
253 transporter from the plant *Camelina sativa* (Tanaka *et al.*, 2017).

254
255 ***Accumulation assays revealed that the recombinant strains were able to efflux***
256 ***drugs/dyes-*** The substrate transport studies revealed that there was an increased
257 accumulation of ethidium bromide by pBAD recombinants expressing either H-/D-type efflux
258 pumps in comparison with control as also observed in earlier studies with their corresponding
259 pBR322 recombinants (Mohanty *et al.*, 2012) (Fig. 3A and 3B). The intracellular ethidium
260 bromide accumulation profiles of both the efflux pumps was in contrast to the results of MIC
261 assays where elevation in MIC was observed towards ethidium bromide (Fig. 3A and 3B; Table
262 2). The absence of correlation between the results of MIC and accumulation assays particularly
263 in ethidium bromide could result due to the time period of both the assays.

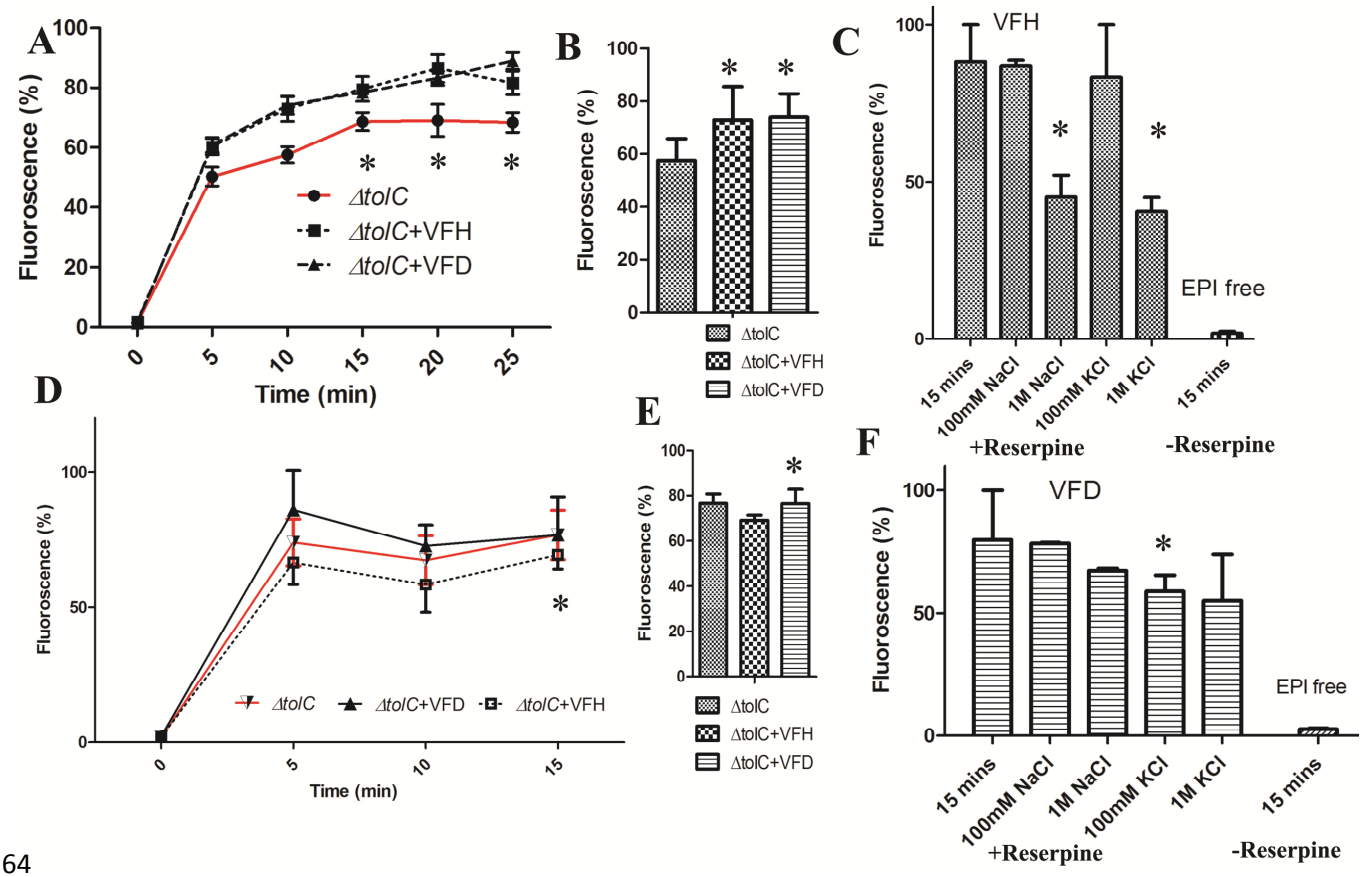


Fig. 3. **A/D.** Intracellular ethidium bromide/norfloxacin accumulation in recombinant K-12 Δ*tolC* harbouring *vfd* and *vfh* genes in pBAD vector. Samples were collected at every 5 mins for processing and fluorescence was measured A/**B.** Intracellular ethidium bromide accumulation in recombinant K-12 ΔtolC harbouring *vfd* and *vfh* genes in pBAD vector after 15 mins. D/**E.** Intracellular norfloxacin accumulation in recombinant K-12 ΔtolC harbouring *vfd* and *vfh* genes in pBAD vector after 15 mins. **C/F.** Ion dependence of pBAD recombinant efflux pumps in the presence of 100 mM or 1 M sodium chloride (NaCl) or 100 mM or 1 M potassium chloride (KCl) in K-12 ΔtolC cells transformed with *pBAD-vfh/vfdVFD*. Data is expressed as mean percentage fluorescence intensity of the maximum fluorescence intensity obtained during the experiment from three independent experiments and * represents significant difference to control ($P < 0.05$)..

However, in case of norfloxacin, there was a correlation between MIC assays and accumulation assays. Results revealed that recombinants with H-type pumps which exhibited increased MIC towards norfloxacin, showed lowering of intracellular norfloxacin accumulation whereas recombinants harbouring D-type efflux pumps that exhibited unaltered susceptibility towards norfloxacin showed almost similar intracellular norfloxacin concentration as compared to the control (Fig. 3D and 3E). Subsequently ion dependence assays were performed for both the transporters to understand their fueling mechanism.

283 ***The Na^+/K^+-ions equally facilitated the efflux of norfloxacin by H- pumps -*** Earlier studies
284 from our laboratory as well as others had revealed that MATE pumps belonging to NorM cluster
285 could transport their substrates in the presence of monovalent ions like Na^+ (Morita *et al.*, 1998,
286 Mohanty *et al.*, 2012). The K-12 Δ*tolC/* pBAD-*vfh* recombinants were loaded with norfloxacin in
287 the presence of reserpine, a compound known to block efflux activity. Subsequently, different
288 concentrations of 0.1 M and 1 M NaCl or KCl were added to the assay mixture and intracellular
289 norfloxacin was monitored to assess the effect of these ions on the efflux activity of these
290 pumps. Results showed that there was an increase in intracellular norfloxacin accumulation in
291 this recombinant in the presence of reserpine compared to reserpine free control (Fig. 3C and
292 3F, columns 1 and 6 respectively). Though the addition of 100 mM KCl/NaCl did not
293 significantly induce the efflux, increase in salt concentration to 1M KCl/NaCl resulted in a
294 greater efflux of the drug from these cells compared to the cells without any salts (Fig. 3C and
295 3F, columns 2-5).
296
297 Earlier studies on MATE- efflux pumps had established that these efflux pumps were
298 fueled either by Na^+/H^+-ions for transport (Bhardwaj & Mohanty, 2012) which was also observed
299 in the current study. There was an increase in intracellular drug concentration in H-
300 recombinants when the Efflux Pump Inhibitor (EPI) reserpine was added. Effluxing commenced
301 on addition of Na^+/ K^+ ions. Newer studies also point out that the transport capability of MATE-
302 type *E. coli* ClbM transporter involved in transport of precolibactin was equally influenced by
303 Na^+, K^+ and Rb^+ ions (Mousa *et al.*, 2017). K^+ ions are known to be congener of Na^+ ions.
304 Another study revealed that NorM from *V. parahaemolyticus* utilises both PMF and Na^+ for
305 transport (Jin *et al.*, 2014). Overall it seems both the transporters are Na^+-ion dependent which
306 has been proved earlier (Mohanty *et al.*, 2012).
307 ***Changes in MIC of norfloxacin in the presence of EPIs-*** Different types of EPIs were utilised
308 to deduce actual transport mechanism of these efflux pumps.. For this, the MIC of these EPIs

309 was deduced against host *E. coli* K-12 Δ*tolC* and sub-lethal concentrations of EPIs (~50% of
310 MIC) were utilized to monitor changes in MIC/drug accumulation patterns for recombinant efflux
311 pumps.

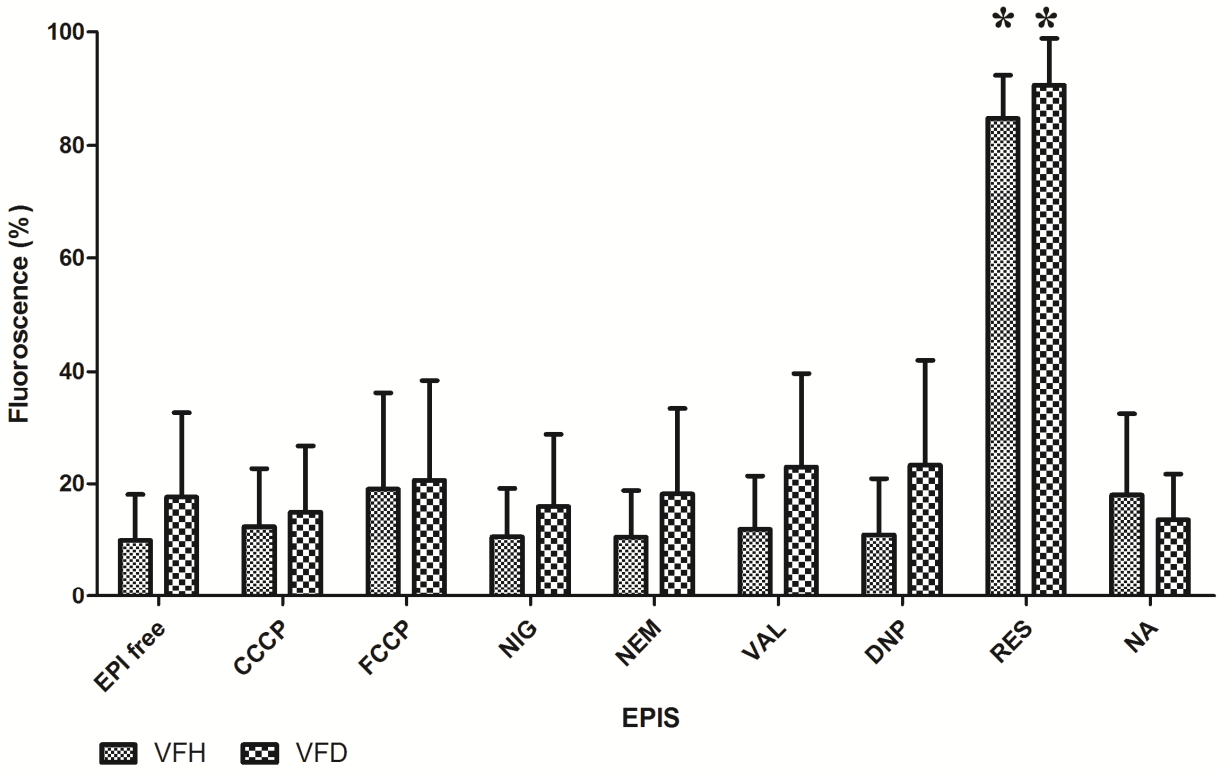
312 **Table 2. Change in MICs of norfloxacin in pBAD recombinant clones carrying MATE-type**
313 **efflux pump genes from *V. fluvialis* and *V. cholerae* with addition of EPIs**

Substrate/EPI	**Strain/ clones**				
Nor	**ΔtolC**	**ΔtolC /VCH**	**ΔtolC /VFH**	**ΔtolC /VCD**	**ΔtolC /VFD**
Nor	<0.0156	0.03125-0.0625	0.03125 -0.0625	<0.0156	<0.0156
+CCCP	**<0.0078**	**<0.0078**	**<0.0078**	**<0.0078**	**<0.0078**
+FCCP	**<0.0078**	**<0.0078**	**<0.0078**	**<0.0078**	**<0.0078**
+RES	**<0.0078**	**<0.0078**	**<0.0078**	**<0.0078**	**<0.0078**
+NEM	**<0.0078**	**<0.0078**	**<0.0078**	**<0.0078**	**<0.0078**
+DNP	**<0.0078**	**<0.0078**	**<0.0078**	**<0.0078**	**<0.0078**
+VAL	0.0625	0.0625	0.0625	0.03125	0.0625
+NIG	**<0.0078**	**<0.0078**	**<0.0078**	**<0.0078**	**<0.0078**
+NA	0.03125-0.0625	0.03125-0.0625	0.03125	0.0156-0.03125	0.0625

314 Carbonyl cyanide-p-trifluoromethoxyphenyl hydrazone (FCCP), Carbonyl cyanide m-chlorophenyl
315 hydrazone (CCCP), Nigericin (NIG), Valinomycin (VAL), N-ethyl maleidamide (NEM), Dintrophenol
316 (DNP), Reserpine (RES), Sodium Ionophore (NA), Norfloxacin (Nor). The concentration of EPIs used in
317 µg/mL are FCCP 3.125, CCCP 1.56, NIG 50, VAL 50, NEM 62.5, DNP 25, RES 125 and NA 50.
318

319 Results revealed that compounds like FCCP and CCCP were effective at low
320 concentrations to host and with norfloxacin a decrease in MIC was observed for H-type (at least
321 8-folds) and for D-type (2 folds) Table 2). Both compounds are known to kill living cells at low
322 concentration as they disrupt PMF, and have been extensively utilised for inhibition of efflux
323 pumps with concentrations varying for each study (Begum *et al.*, 2005, Morita *et al.*, 2000,
324 Morita *et al.*, 1998). Other compounds like DNP which deplete ATP or compounds like NIG, NA
325 and VAL which are known as H^+, Na^+ and K^+ specific ionophores respectively, exhibited high
326 MIC for host. But with norfloxacin, DNP and NIG also decreased the MIC by at least 2-8-folds
327 for norfloxacin. against both the compounds (Table 2). VAL and NA did not inhibit both the

328 pumps for norfloxacin (Table 2). These ionophores mostly increase transferability of ions across
329 membrane involved in generating EMF, which may or may not be important for energy recycling
330 (Castro *et al.*, 2015). RES and NEM, both of which either bind or covalently modify the efflux
331 pumps, also exhibited very high MIC for *E. coli* K-12 ΔtolC but in conjunction with norfloxacin,
332 both compounds decreased the MIC (Table 2). Both NEM and RES decreased the MIC
333 exhibited by both pumps by at least 2-16-folds whereas (Table 2). It was not surprising that
334 reserpine increased sensitivity towards fluoroquinolones. Resperine had been earlier reported to
335 inhibit various efflux pumps resulting in increased susceptibility (Baranova & Neyfakh, 1997,
336 Brenwald *et al.*, 1997, Aeschlimann *et al.*, 1999, Markham, 1999, Markham *et al.*, 1999,
337 Gibbons & Udo, 2000, Mullin *et al.*, 2004).
339
340 ***Accumulation of norfloxacin by H-/D-type pumps in the presence of EPIs-*** The MIC assays
341 had established that some of the EPIs were active in reducing the MIC of norfloxacin for the
342 recombinants expressing efflux pumps. Therefore, norfloxacin accumulation studies were
343 performed to assess the real time effect on drug transport by the H-/D- recombinants in the
344 presence/absence of EPIs.
345 Results revealed that *E. coli* K-12 Δ*tolC* expressing H-type pumps showed a marginal to
346 moderate degree of increased norfloxacin accumulation in presence of CCCP and FCCP in
347 comparison to EPI free control (Fig. 4). These pumps exhibited almost unaltered/marginal
348 increased accumulation of norfloxacin in presence of NIG, NEM, VAL and DNP (Fig. 4).
349 Interestingly, both efflux pumps exhibited very high accumulation of norfloxacin in presence of
350 RES (Fig. 4). MIC studies of norfloxacin against both recombinants in the presence of EPIs had
351 revealed that



352

353 **Fig. 4.** Accumulation of norfloxacin in presence of various EPIs. Accumulation of norfloxacin by *E. coli* K-
354 12 Δ*tolC* expressing H- and D-type pumps in the presence of EPIs. Recombinant *E. coli* K-12 ΔtolC was
355 loaded with norfloxacin and incubated at 37℃ for 15 mins in presence/absence of EPIs. These samples
356 were subsequently processed and analyzed for fluorescence intensity as described in the materials and
357 methods. * represents significant difference to control ($P < 0.05$).. The concentration of EPIs used in
358 µg/mL are FCCP 3.125, CCCP 1.56, NIG 50, VAL 50, NEM 62.5, DNP 25, RES 125 and NA 50.
359

360 compounds like CCCP, RES and FCCP decreased the MICs (Table 4). This was in sync to the
361 norfloxacin accumulation results, where these compounds increased the accumulation of
362 norfloxacin. NA increased the intracellular norfloxacin for H-type of efflux which was in contrast
363 to MIC assays where it did not decrease the MIC for norfloxacin (Table 2). However, for NEM,
364 DNP and NIG this correlation between MIC and accumulation results was missing probably due
365 to the kinetics of inhibition by these EPIs and the difference in the time course for the two type
366 of assays. MATE family of efflux pumps utilise Na^+ ions for transport which was observed in the
367 current study. There was an increased intracellular norfloxacin accumulation with utilisation of
368 H^+/ Na^+/K^+ ion specific ionophores such as NA, CCCP and FCCP. This was in corroboration

369 with our earlier observation in ion dependence experiments where we observed that both the
370 efflux pumps showed greater efflux in presence of both Na^+ and K^+ ions also evidenced in MIC
371 experiments (Table 2) where these EPIs decreased the MIC of norfloxacin. Taken together,
372 there was a relative correlation between drug accumulation, ion dependence and change in MIC
373 patterns in case of some selected EPIs.

374 **Molecular Modeling and docking-** Structural prediction of MATE-type efflux pumps had
375 suggested 12 transmembrane helices in their structure which was further validated with the
376 structure of first Na^+ dependent MATE-type efflux pump from *Pyrococcus furiosus* having 12
377 transmembranes (Lomovskaya & Bostian, 2006, Tanaka *et al.*, 2013). In our earlier attempt in
378 predicting 3D structures of VFH and VFD using I-TASSER server, we had reported that VFD
379 had 11 transmembranes (TMs) whereas VFH had 10 TMs (Mohanty *et al.*, 2012). After 2012,
380 more structures were solved for the members of this family of transporters (Tanaka *et al.*, 2013,
381 He *et al.*, 2010, Lu *et al.*, 2013b, Lu *et al.*, 2013a). Subsequent to these studies, further attempts
382 for protein modeling were made using SwissModel (https://swissmodel.expasy.org/).to
383 understand the topology and mechanism of efflux by these two MATE pumps Both PyMol
384 (https://pymol.org/2/) and Chimera (https://www.cgl.ucsf.edu/chimera/) were used
385 forvisualization of their molecular structures. Results revealed that both the efflux pumps had 10
386 predicted TMs arranged in two bundles of 5 TMs each (Fig. 5.) similar to crystal structures of
387 earlier published MATE transporters (Lu *et al.*, 2013b). They were also outward facing like NorM
388 (Lu *et al.*, 2013b) with distinct internal cavity. It was both in corroboration (for VFH) and contrast
389 (for VFD) to our earlier finding where VFH model was similar to NorM and VFD had different
390 predicted protein model (Mohanty *et al.*, 2012).

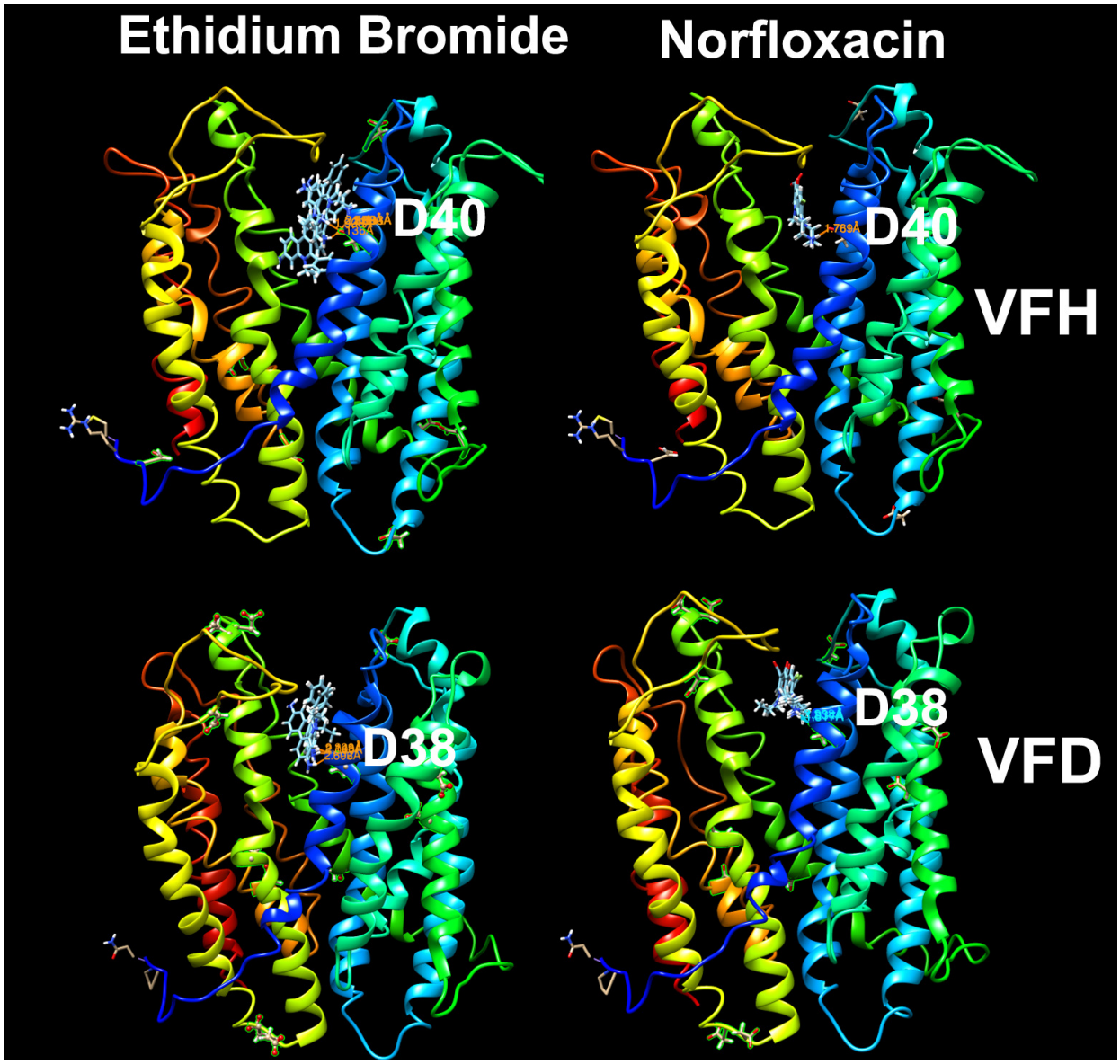


Fig. 5. Docked pose of ethidium bromide and Norfloxacin with *V. fluvialis* MATE efflux pumps (VFH and VFD) forming covalent complex at Asp 38/40 of 1st transmembrane.

Subsequently, molecular docking was done using SwissDock (http://www.swissdock.ch/) to validate binding interactions of both the transporters with ethidium bromide and norfloxacin as both the transporters showed certain degree of transport for these substrates. Classically, a MATE pump has 12 TMs arranged in two bundles of six TMs each (Tanaka *et al.*, 2013, Tanaka *et al.*, 2017, Radchenko *et al.*, 2016, Lu *et al.*, 2013b).

400 The first TM has an ion binding site represented by D32, the Aspartic Acid residue at 32
401 position (Otsuka *et al.*, 2005, Long *et al.*, 2008, Lu *et al.*, 2013a, Lu *et al.*, 2013b). With this
402 input, the protein sequences of both the transporters were submitted to SwissDock ≈server
403 (http://www.swissdock.ch/). The docking was programmed for finding all possible ligand
404 interactions. All aspartic acid residues were first identified from the generated models and
405 hydrogen bonds were calculated. The details have been presented in Table 3 and Fig 5.
406 Interaction between the protein and ligands was optimum in the first TM at D40 for VFH and
407 D38 for VFD which was similar to earlier published findings (Tanaka *et al.*, 2013, He *et al.*,
408 2010, Lu *et al.*, 2013b, Lu *et al.*, 2013a). This also indicated similar transport mechanism for
409 both the transporters. Both the compounds ethidium bromide and norfloxacin fitted well in the
410 central cavity of both the transporters. The only noticed difference was that while norfloxacin
411 fitted in a straight fashion forming bonds with D40/38, Ethidium bromide showed bent fitting from
412 1st to 7th TM (Fig. 5).

413 Table 3 **Ligand-docking of VFH and VFD efflux pumps with various substrates**

Protein	**Substrate**	**Orientation**	**Total Binding sites**	**Total Hydrogen Bonding**	**Most plausible binding site**
VFH	Nor	Outward	35 clusters with 256 binding sites	11 clusters with 65 sites	1 cluster with 6 binding sites (deltag -8.4)
VFH	EtBr	Outward	30 clusters with 256 binding sites	10 clusters with 69 sites	3 clusters with 20 binding sites (deltag -6.9)
VFD	Nor	Outward	35 clusters with 256 binding sites	23 clusters with 139 sites	1 cluster with 8 binding sites (deltag -7.4 to -7.5)
VFD	EtBr	Outward	34 clusters with 256 binding sites	15 clusters with 80 sites	2 clusters with 10 binding sites (deltag -7.19)

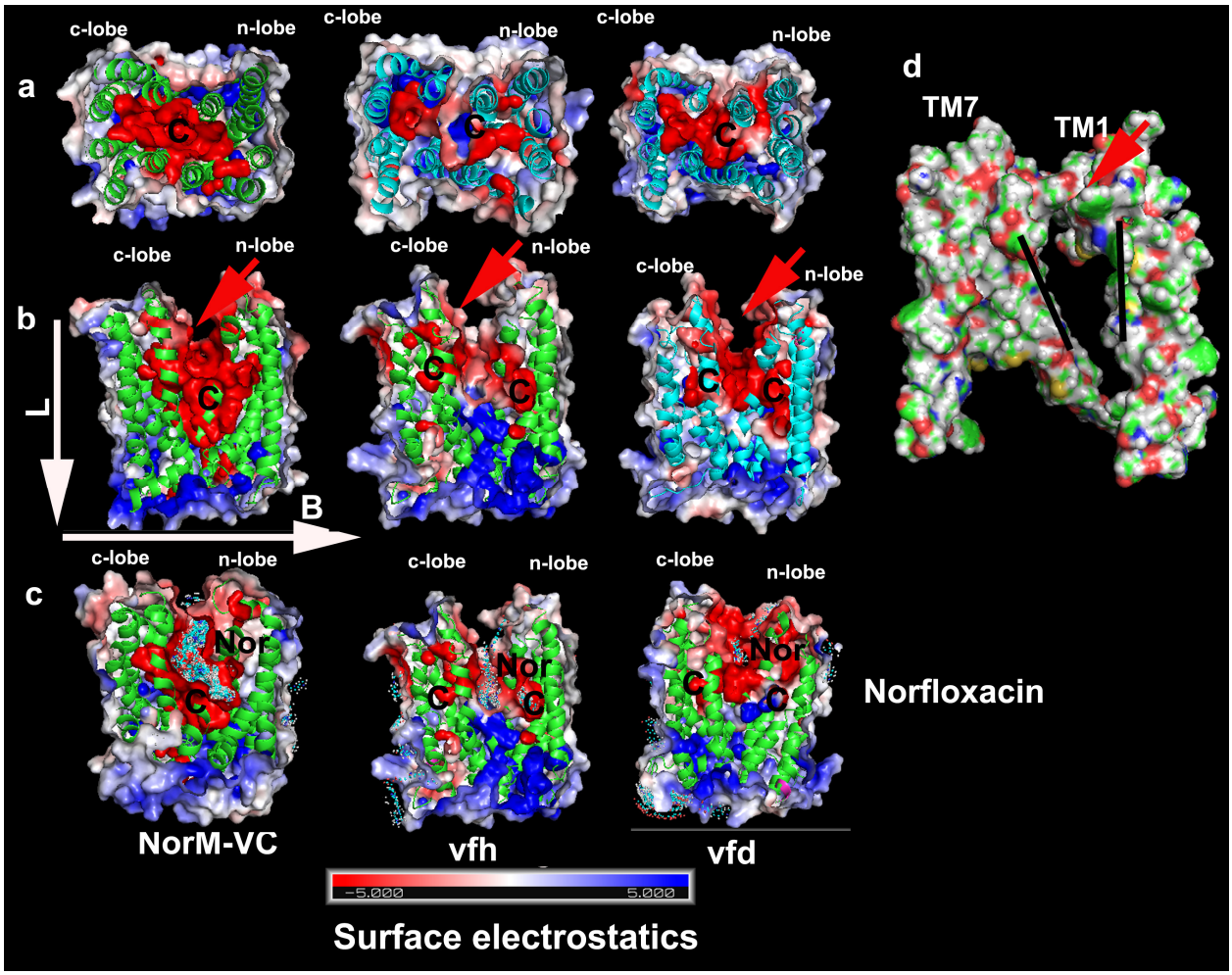
414

415 **Ion binding and Electrostatic potential surface representation-** MATE-type efflux pumps are
416 secondary pumps that utilize Na^+/H^+ for transport (Jin *et al.*, 2014, Mohanty *et al.*, 2012). Earlier
417 studies had proved that these were Na^+ ion-driven transporters (Mohanty *et al.*, 2012). In the
418 ion-dependence assays, we observed the transport capability was equally influenced by both
419 sodium and potassium ions for VFH (reference). For better understanding of ion dependence
420 and amino acid residues involved, we utilized IonCom server
421 (https://zhanglab.ccmb.med.umich.edu/IonCom/) to predict their ion dependence and amino
422 acid residues involved.

423 Results revealed that both the efflux pumps had no binding site detected for the
424 following ions:(CU, FE, CA, MG, MN, NA, K, CO_3, NO_2, SO_4, PO_4). The predicted ion binding
425 amino acid residues were D40, E51, Q82, D88, Y138, H163, N179, N309 and D393 for VFH
426 and D38, H44, W49, Q81, N163 and D204 for VFD. Interestingly, docking studies clearly
427 indicated that D38/40 of both the transporters were involved in binding. So, even though we
428 couldn't predict dependence of ion but D38/40 seems most plausible ion binding site for both
429 the transporters which was similar to NorM from *V. cholerae* (He *et al.*, 2010).

430
431 Electrostatic potential of proteins is caused by charged amino acid residues in side
432 chains, bound or unbound by various ions and play various roles such as protein folding and
433 stability and function of the proteins. APBS pluggin in PyMol was used to calculate the
434 electrostatic potential for both the proteins. It may reflects secondary and tertiary structures of
435 transporters and their affinity to bind to substrates. Results revealed that both VFH and VFD
436 had different hydrophobicity patches similar to other known MATE transporters (Fig 6a) (Zheng
437 *et al.*, 2018). Both VFH and VFD formed a central cavity surrounded by charged electronegative
438 amino acids. VFH also had an electropositive centre at the end of the cavity (Fig 6a).
439 Subsequently, VFH and VFD with all possible interaction with norfloxacin via docking were
440 mapped along and electrostatic potential was calculated, an interesting scenario came out. A

441 sizable norfloxacin molecule was assembled in the cavity of VFH whereas fewer norfloxacin
442 molecules were assembled in the cavity Of VFD. NorM which was taken as control had
443 maximum norfloxacin molecules assembled in the cavity (Fig 6c). This was in sync with our
444 earlier observation where VFH exhibited broader transport capability than VFD in MIC. The
445 arrangement was mostly concentrated towards n-lobe which was similar like NorM (Lu *et al.*,
446 2013a)



447

448 **Fig. 6.** Surface charge differences between VFH, VFD and NorM-VC. Surface electrostatics were
449 calculated by the APBS plugin in PyMOL. Cross-section model of VFH, VFD and NorM-VC surface
450 representation is shown from the membrane region (a) or from the extracellular (b) sides (c) with
451 norfloxacin and (d) Cross-section model of VFH/VFD exposing covalent interaction between TM1 and
452 TM7. C represents cavity. Red arrows represent cavity.
453
454 It has been widely accepted that MATE transporters evolved from genetic duplication of
455 flippases (Lomovskaya & Bostian, 2006, Tanaka *et al.*, 2013). Comparison of both the

transporters with known structures of MATE transporters from *Camelina sativa,* NorM from *Vibrio cholarae* and MurJ flippases from *Thermosipho africanus* reveals interesting similarities and divergence from both VFH and VFD. D38/40 of both the transporters seemed to more conserved in bacterial transporters (Lu *et al.*, 2013b, He *et al.*, 2010) in comparison to their eukaryotic counterparts (Tanaka *et al.*, 2017). When both these transporters were compared to their evolutionary predecessors like MurJ (Kuk *et al.*, 2017), the difference is quite significant. MurJ flippases from *Thermosipho africanus* exhibited presence of 14 TMs arranged in 6+6 similar to bacterial transporters and functional role of last two TMs is unknown. Ser17, Arg18, Arg24, Arg52, and Arg255 in 1st Tm of Murj has been proved important for recognizing the diphosphate and/or sugar moieties of lipid II whereas Asp in 1st Tm of NorM has been proved for ion dependence and substrate transport (He *et al.*, 2010). It seems both VFH and VFD has similar topology like NorM with presence of D38/40 in 1st TM creating a hydrophobic cavity for substrate recognition and transport.

During mapping of electrostatic potential of VFH and VFD, an aberrant architecture was also observed in both the transporters. MATE transporters usually have 12 TMs arranged in two bundles with 6 TMS each. Each forms one lobe and are usually free. The 1st TM upon protonation displaces to 7th TM to facilitate transport. But in case of both VFH/VFD, the loop connecting 1st TM to 2nd TM forms a covalent structure with loop of 7th TM connecting 8th TM as elucidated by surface model (Fig 6d). MATE type transporters are thought to be evolved of internal genetic duplication, resulting in two lobes. VFH/VFD had 10 TMs, still there was greater scope for transport. The formation of covalent bond reduces mobility of 1st TM to be displaced to 6th TM and may overall hinder transport efficiency (Fig 7b).

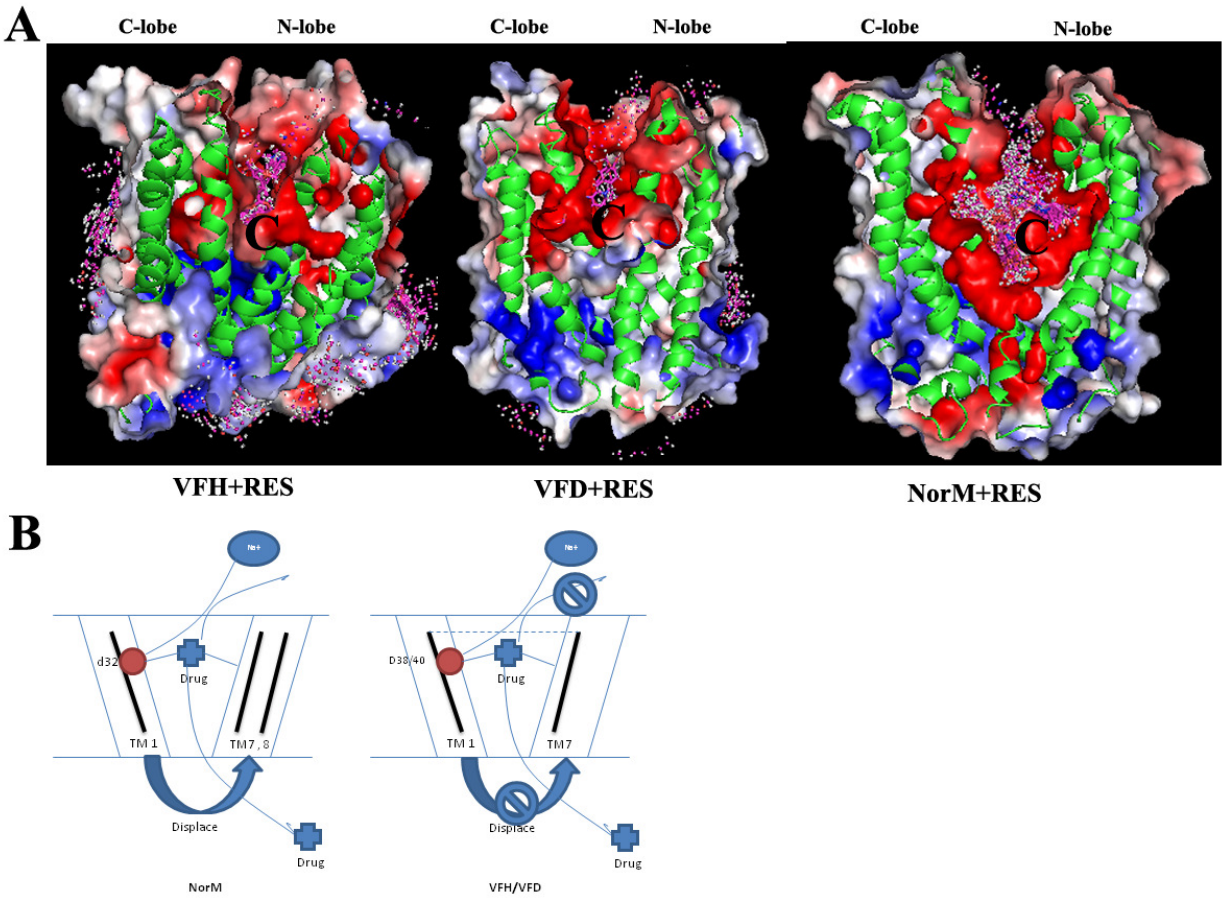
Mechanism of inhibition of both the transporters by reserpine- Our earlier observation with electrostatic mapping and inhibition of both the transporters with various EPIs had led to an indication that the transport of norfloxacin was indeed hampered in presence of these EPIs. Surprisingly, the similar effect couldn't be observed in case of drug transport assay. Compounds

482 like CCCP/FCCP, which are extremely toxic to cell at low concentration showed marginal
483 inhibition. Reserpine which was fairly non-toxic, was able to inhibit both the transporters in real
484 time drug transport assays. In our earlier study, we had observed 20 microgram/mL was
485 sufficient to inhibit both the transporters (Mohanty *et al.*, 2012). Even in the current study, the
486 MIC against the host was fairly high i.e >128 mcg/mL. So, range of concentration was quite
487 enough to inhibit. Even thoughit has been earlier proved in case of Bmr transporters, where
488 certain residues were implicated for inhibition (Klyachko *et al.*, 1997), no direct structural studies
489 have ever proved the actual inhibition by reserpine in MATE efflux pump. To investigate the
490 exact nature, we tried to dock reserpine with both the transporters along with NorM from *V.*
491 *cholerae*.

492 Results revealed that reserpine could conveniently fit into electronegative cavities of
493 both the transporters. VFH had 3 binding clusters with 19 binding sites whereas VFD had 2
494 binding clusters with 15 binding sites. Interestingly, NorM had 26 binding clusters with 190
495 binding sites.

496 In some of the earlier studies, MATE transporters have been co-crystalized along with
497 the inhibitors. (Tanaka *et al.*, 2013) . These studies have delineated the role of hydrogen
498 bonding between transportersand ligand for exact inhibition mechanism. Our own observation
499 with NorMhad hinted the same as there were almost 15 clusters and 96 binding sites forming
500 hydrogen bonds in the cavity of NorM. But in case of VFH there was no predicted hydrogen
501 bond formation and for VFD, 1 cluster and 3 binding site configuration was observed. Previous
502 studies regarding co-crystallization of MATE-transporters with certain inhibitors had shed light
503 on formation of electrostatic bonds for inhibition with verapamil and macrocyclic peptides
504 (Radchenko *et al.*, 2015, Tanaka *et al.*, 2013). Observation from our earlier studies and current
505 study reflect that reserpine acts pretty well at different concentrations also. The exact
506 mechanism of reserpine inhibition is binding to phenylalanine, tryptophan and tyrosine residues
507 of Bmr efflux pumps (Steinfels *et al.*, 2004) and inhibition by reserpine does not involve ion

508 bioenergetics of the cell and seems to be a non-specific inhibition of efflux pumps as was also
509 seen in the current study. This led us to believe three important aspects of transport a. mobility
510 of each lobe plays a vital role in transport b. size and electronegativity of central cavity plays
511 important role for actual substrate recognition c. binding of ligand in electronegative pocket is
512 not always electrostatic but may be covalent as reserpine was able to inhibit without actual
513 hydrogen bond formation in both the transporters.



514

515 **Fig. 7. A.** Docked pose of reserpine with *V. fluvialis* MATE efflux pumps (VFH and VFD) . **B.** Proposed
516 mechanism of transport of NorM-VC and VFD/VFH. Contrasting to NorM-VC, VFH/VFD forms covalent
517 interactions between TM1 and TM7, which along low cavity volume leads to low transport capability of
518 VFH/VFD.
519

520

521

522 **Experimental procedures**

523 ***Cloning in pBAD-TOPO-TA vector-*** The primers used for cloning in pBAD vectors were VFH-
524 BAD-F/VFH-BAD-R and VFD-BAD-F/VFD-BAD-R mentioned in supplementary table 2The
525 amplicons were subsequently purified using PCR purification kit (Qiagen) and ligated in pBAD
526 vector (Invitrogen) according to manufacturer's protocol.

527

528 ***Expression and detection of recombinants harbouring pBAD efflux pump genes-*** pBAD
529 clones for expression of VCH, VFH, VCD and *VFD* (where D- and H- are two MATE-type efflux
530 pumps, VC stands for *V. cholerae* and VF stands for *V. fluvialis*) and Lac-His (positive control)
531 were transformed in *E. coli* LMG194 and *E. coli* K-12 Δ *tolC* (JW5503-1, *E. coli* Genetic
532 Resource Centre, Yale University) for expression as well as functional characterization. For
533 Western blots, *E. coli* LMG194/K12 Δ*tolC* cells transformed with the recombinant vectors were
534 grown in Luria-Bertani (LB) medium to an optical density of 0.5 at 600 nm (OD_{600}) and induced
535 with 0.2% L-arabinose for 4 hours and the total cell lysates prepared in SDS-PAGE lysis buffer
536 were electrophoresed on 8% SDS-PAGE. Western blot was done with 0.45 micron
537 nitrocellulose membrane (Whatman/Pall Life sciences) and blocked with 5% fat free milk
538 powder in Tris buffered saline with 0.05% tween 20 (TBST) for 2 hours at room temperature.
539 The blot was incubated with goat Anti-His/V_5 primary antibody at 1:5000 dilutions in 0.5% milk
540 powder in TBST for 2 hours at room temperature with constant shaking followed by washing in
541 TBST and incubation with anti-Goat IgG-HRP (Horseradish Peroxidase)/Anti-Goat IgG-AP
542 (Alkaline Phosphatase) (Jackson Immunoresearch) secondary antibody. The blots were
543 developed either by using Novex ECL HRP chemiluminiscent development kit (Invitrogen) or by
544 colorimetry using DAB/H_2O_2 (for HRP conjugated secondary antibody) or BCIP/NBT
545 (Calbiochem) (for AP conjugated secondary antibody) according to the manufacturer's
546 instructions. For chemiluminiscence, the blot was dried and developed on X-ray.

547

548 ***Detection of expression of recombinant proteins by confocal imaging-*** Briefly, the cells
549 were grown and induced as discussed in earlier subsection of Experimental Procedures. 200 µL
550 of induced cells were centrifuged at 5000 rpm for 5 min, the cells were resuspended in 50 µL of
551 supernatant and were spotted on coverslips coated with 0.2% L-Poly-lysine (Sigma) in 24-well
552 tissue culture plates (Corning) for 30 min at room temperature. Cells were then fixed using 4%
553 paraformaldehyde (PFA) for 15 min followed by washing twice with 250 µL PBST (PBS
554 containing 0.05% Tween 20) for 5 min without shaking. The cells were blocked with 5% Bovine
555 Serum Albumin (BSA) in PBST for 1 hour at room temperature followed by washing thrice with
556 500 µL PBST for 10 min each and solutions were aspirated gently using a pipette. 50 µL mouse
557 monoclonal anti-6X His tagged primary antibody (Calbiochem) was added at a dilution of 1:150
558 in 0.5 % BSA in PBST in the respective wells and incubated for 2 hours without shaking at room
559 temperature. Washing was done thrice as described above. Goat anti-mouse secondary
560 antibody labeled with Cy3 (Invitrogen) was added at the dilution of 1: 50 in 0.5 % BSA in PBST
561 and incubated for 2 hours at room temperature followed by one washing step. Cells were
562 counterstained with 200 µL of 4', 6-diamidino-2-phenylindole (DAPI) at 1 µg mL^{-1} concentration
563 and 50 µL of Film Tracer 1-43 membrane marker (Invitrogen) at 1 µg mL-1 concentration in
564 DMSO. Confocal imaging was carried out using CTR 6500 confocal microscope (Leica). Diode
565 lamp was utilised for detection of DAPI, UV HeNe lamp for CY3 and Argon lamp was used to
566 detect membrane marker.
567
568 ***Preparation of Membrane fraction-*** The membrane fraction was prepared according to the
569 published protocol (Leyh, 1990). The *E. coli* LMG194/ K12 Δ*tolC* recombinant cells were grown
570 and induced as described earlier. 25 mL of induced cultures were harvested at 5000 rpm, 4℃
571 for 15 min. The supernatant was discarded and the pellet was washed with 5 mL ice cold PBS
572 (phosphate-buffered saline) followed by centrifugation at 5000 rpm, 4℃ for 10 min. The cells
573 were resuspended in 1 mL lysis buffer (50 mM potassium phosphate, pH 7.8) having 1 mM

574 phenylmethanesulfonyl fluoride and incubated in -80℃ deep freezer for 2 hours. The samples
575 were then thawed and sonicated 4 times with 30 sec on and off pulse on ice. The samples were
576 then centrifuged at 8000 rpm, 4℃ for 5 min. The supernatants were saved as soluble fraction
577 and the pellet was treated with 200 µL of 2% sarcosyl solution (Sigma Aldrich) and incubated at
578 room temperature for 30 min. The sample was then centrifuged at 15,000 rpm for 30 min at
579 room temperature to obtain inner membrane proteins as supernatant and the rest of the proteins
580 as pellet. Samples were subsequently subjected to Western blot for detection of recombinant
581 proteins.

582 ***Minimum Inhibitory Concentration (MIC) assays-*** The pBAD expression clones *of vfh*, *vch*,
583 *vcd* and *vfd* were transformed in *E. coli* LMG194/K-12 Δ*tolC* cells, grown in LB broth containing
584 ampicillin (100 µg mL^{-1}) to an OD_{600} of 0.5 and induced with 0.2% L-arabinose for 4 hours.
585 Kanamycin (30 µg mL^{-1}) was additionally used for *E. coli* K-12 Δ*tolC* cells. Drugs/dyes were
586 serially double diluted in LB broth containing 0.2% L-arabinose and 2 mL dilution/well were
587 dispensed in 24 well tissue culture plates (Corning). OD_{600} of the induced sample was adjusted
588 to 0.08-0.13 and 50 µL of this sample was added to each well. Incubation was done at 37℃.
589 Readings were observed visually after 16 hours. The assays were repeated at least three times.

590 ***Drug/Dye accumulation assays-*** For *E. coli* K-12ΔtolC cells harboring either pBAD-*vfh* or
591 pBAD-*vfd* or not harbouring any plasmids were grown and induced as described earlier in
592 Materials and methods. Ethidium bromide (1.9 µg mL^{-1}) and norfloxacin (0.0156 µg mL^{-1}) were
593 used for accumulation assays. This was followed by single washing with 0.1 M Tris.HCl, pH 7.0
594 and resuspension in equal volume of the same buffer. The samples were collected every 15
595 min. The processing of the cells and monitoring of the fluorescent content was carried out
596 exactly as described earlier (Mohanty *et al*., 2012).

597 ***Effect of Na^+/K^+ ions on efflux of drugs/dyes-*** *E. coli* K-12Δ*tolC* cells harboring either *pBAD-*
598 *vfh* or *pBAD-vfd* or not harbouring any plasmids were grown in LB broth containing kanamycin

599 (30 µg mL^{-1}) and induced with 0.02% L-arabinose for 4 hours. Ampicillin was only added to the
600 cells harbouring recombinant plasmids. The recombinants were grown and processed as
601 described above in this section. The samples were then harvested by centrifugation, washed
602 thrice with buffer containing 0.1M Tris.HCl, pH 7.0, and suspended in the same buffer to an
603 OD_{600} of 1.0. The drug/dyes were used at 50% of the MIC values for transport studies.
604 Norfloxacin at a final concentration of 0.0156 µg mL^{-1} (50% MIC concentration)along with 125
605 µg mL^{-1} of reserpine was added to the cell suspension and incubated for 30 min at 37℃. After
606 30 min, 1 mL samples were collected and stored in prechilled microcentrifuge tubes. Negative
607 control (without drug/dyes) and EPI free control were also included in the study. Appropriate
608 volume of 5 M and 0.5 M NaCl/KCl stock solution were added to assay mixture to a final
609 concentration of 1M and 100 mM, incubated at 37℃ for 15 minutes and 1 mL sample was
610 collected. The volume was kept constant irrespective of salt concentration. Samples were then
611 centrifuged at 10,000 rpm for 5 min at 4℃, washed once with 0.1 M Tris.HCl, pH 7.0 buffer and
612 resuspended in 1 mL of 100 mM glycine.HCl, pH 3.0. These samples were subsequently
613 processed and analyzed for fluorescence intensity as described in the previous sections. The
614 maximum fluorescence observed in the experiment was considered to be 100%.

615 ***Assays to determine efficacy of various EPIs on recombinant efflux pumps-*** The MIC of
616 various EPIs was deduced against *E. coli* K-12 Δ*tolC*.Different concentrations of EPIs were
617 prepared by two-fold dilution method in LB broth containing kanamycin (30 µg mL^{-1}) and 100
618 µL/well was dispensed in 96-well tissue culture plates (Corning Inc.). The EPIs were procured
619 from Sigma Aldrich. *E. coli* K-12 Δ*tolC* was grown in LB containing kanamycin (30 µg mL^{-1}) to
620 an OD_{600} of 0.08-0.13. Five microlitre of cells were inoculated in 100 µL medium containing
621 various concentrations of EPIs in a 96 well plate. Observations were made visually after 16
622 hours. The experiments were repeated at least thrice. 50% concentration of MIC of each EPI
623 was utilised in combination with various concentrations of norfloxacin. The *E. coli* K-12 Δ*tolC*
624 carrying recombinant efflux pump plasmids and control *E. coli* K-12 Δ*tolC* were each grown in

625 LB with kanamycin (30 µg mL^{-1}) and ampicillin (100 µg mL^{-1}). Overnight primary cultures were
626 done in same way. Then 10 mL of secondary culture was prepared. Upon reaching OD_{600} of 0.5,
627 the cells were induced with 0.02% L-arabinose for 4 hours with 200 rpm shaking at 37℃. The
628 optical density of induced cells were adjusted to 0.08-0.13. Five microlitre of cells were
629 inoculated in 100 µL medium containing 50% concentration of various EPIs along with various
630 concentrations of norfloxacin in a 96 well plate (Corning Inc). Observations were made visually
631 after 16 hours. The experiments were repeated at least thrice. The change in MIC was noted to
632 observe efficacy of each EPI.

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638 **Author contributions:** Conceived and designed the experiments: AKB and PM. Performed the
639 experiments: PM AS AKB. Analyzed the data: AKB PM. Contributed reagents/materials/analysis
640 tools: AKB. Wrote the paper: AKB PM.

641 **Conflict of interest:** The authors declare that they have no conflicts of interest.

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