1 **Structural basis of inhibition of a putative drug efflux transporter NorC,**
2 **through a single-domain camelid antibody**
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15 **Abstract**

16 **Multi-drug efflux is a major mechanism of acquiring antimicrobial resistance**
 17 **among superbugs. In this study, we report the X-ray structure of NorC, a 14**
 18 **transmembrane major facilitator superfamily member that is implicated in**
 19 **fluoroquinolone resistance in drug-resistant *Staphylococcus aureus* strains, at a**
 20 **resolution of 3.6 Å. The NorC structure was determined in complex with a**
 21 **single-domain camelid antibody that interacts at the extracellular face of the**
 22 **transporter and stabilizes it in an outward-open conformation. The**
 23 **complementarity determining regions of the antibody enter and block solvent**
 24 **access to the interior of the vestibule, thereby inhibiting alternating-access. NorC**
 25 **specifically interacts with an organic cation, tetraphenylphosphonium, although**
 26 **it does not demonstrate an ability to transport it. The interaction is compromised**
 27 **in the presence of NorC-antibody complex, consequently establishing a strategy**
 28 **to detect and block NorC and related efflux pumps through the use of single-**
 29 **domain camelid antibodies.**

30 **Keywords:** NorC, major facilitator superfamily, Drug:H⁺ antiporter, single-domain
 31 camelid antibody, multi-drug efflux

32 Introduction

33 Antimicrobial resistance in superbugs acquired through multi-drug efflux facilitates
 34 the survival of pathogens against antibacterial compounds, either through direct efflux
 35 or through enhanced fitness or persistence(Blair et al., 2015; Piddock, 2006). The
 36 Gram positive superbug, methicillin resistant *Staphylococcus aureus* (MRSA) is
 37 known to cause mild skin and soft-tissue infections to severe infections like
 38 endocarditis, bacteremia, sepsis, and pneumonia(Tong et al., 2015). In the presence of
 39 penicillin, staphylococcal cultures are known to contain sub-populations of persisters
 40 that can survive the toxic effect of the antibiotic(Bigger, 1944). A major factor that
 41 aids in persistence is the enhanced activity of efflux pumps(Pu et al., 2016). Among
 42 multidrug efflux pumps, the major facilitator superfamily (MFS) constitutes an
 43 extensive array of efflux transporters with a promiscuous ability to transport a diverse
 44 array of substrates in both Gram positive and negative pathogens(Kumar et al., 2016;
 45 Reddy et al., 2012). Drug-resistant strains of *S. aureus* employ a diverse set of
 46 chromosomal and plasmid encoded MFS transporters to gain antibiotic
 47 resistance(Costa et al., 2013). Transporters like NorA, NorB, and NorC are
 48 chromosomally encoded and protect *S. aureus* against fluoroquinolones(Costa et al.,
 49 2013; Truong-Bolduc et al., 2005; Truong-Bolduc et al., 2006). QacA and QacB are
 50 plasmid encoded and provide resistance to monovalent and divalent quaternary
 51 ammonium compounds(Chitsaz and Brown, 2017; Paulsen et al., 1996a).

52 MFS transporters involved in multi-drug efflux primarily act by coupling
 53 efflux to proton gradients across the bacterial membrane(Paulsen et al., 1996b). MFS
 54 transporters are multi-pass integral membrane proteins comprising 12 or 14
 55 transmembrane (TM) helices and includes drug:H⁺ antiporters (DHA) family
 56 classified as DHA1 and DHA2 depending on the presence of 12 or 14 TM helices,
 57 respectively(Reddy et al., 2012). Multiple structures of DHA1 members including
 58 MdfA, LmrP and EmrD have been solved in different conformational states that
 59 facilitate an understanding of the alternating-access in DHA members through the
 60 rocker-switch mechanism(Debruycker et al., 2020; Nagarathinam et al., 2018; Yin et
 61 al., 2006). However, there is no representative structure for the DHA2 members that
 62 comprise well-studied transporters including QacA/B, Tet38, NorB & NorC. Besides
 63 *S. aureus*, numerous pathogens express DHA2 members to effectively counter
 64 antibacterial stress. Inhibitors that block antimicrobial efflux could enhance the

65 efficacy of existing antibiotics, thereby serving as antibiotic adjuvants (Stavri et al.,
66 2007) (Wright, 2016).

67 In this study, we report the X-ray structure of NorC, a 14 TM putative efflux
68 transporter that represents a unique subset among MFS transporters, in complex with
69 a Zn²⁺-bound single-domain Indian camelid antibody (ICab). The structure of the
70 ICab-NorC complex was solved at a resolution of 3.6 Å. Originally isolated as a
71 crystallization chaperone for NorC, the ICab interacts with NorC through the insertion
72 of CDR loops into the vestibule to effectively lock the transporter in an outward-open
73 state. The transporter does not display a direct ability to transport fluoroquinolone or
74 monovalent cations but has an ability to specifically interact with
75 tetraphenylphosphonium (TPP), a monovalent cationic antibacterial whose
76 interactions are blocked by the presence of the ICab. We anticipate that the structure
77 of the DHA2 member NorC would facilitate the investigation of other DHA2
78 members in diverse pathogens and the identification of single-domain antibodies that
79 block efflux can be explored as a novel paradigm for efflux pump detection and
80 inhibition.

81 **Results and Discussion**

82 **NorC represents a unique subset among DHA2 members.** Phylogenetic analysis
83 reveals that NorC and related sequences form a separate clade among MFS
84 transporters implicated in multi-drug efflux (Figure 1A). Despite a clear prediction of
85 14 TM helices, NorC/NorB-like transporters differ substantially in comparison to the
86 DHA2 members of the QacA-like transporters and the 14 TM proton-coupled
87 oligopeptide symporters (POTs). Among the members of this subset, a high degree of
88 sequence conservation is observed (~58-88%) (Supplemental Figure 1S-1). Given the
89 ~70% sequence identity of NorC with NorB, it is highly likely that they would be
90 performing very similar roles to aid in antimicrobial resistance. It is observed that
91 NorB can aid in the survival of *S. aureus* in an abscess environment and is also
92 overexpressed in persister populations of antibiotic resistant *S. aureus* strains (Dawan
93 et al., 2020; Ding et al., 2008; Truong-Bolduc et al., 2006). Interestingly, we
94 discovered that NorC/NorB-like transporters lack the typical protonation and
95 conserved motif C residues that are a characteristic of drug:H⁺ antiporters (Varela et
96 al., 1995). While conventional DHA2 members retain one or more negatively charged

97 residues for protonation driven efflux, NorB and NorC lack negative charges facing
98 the transport vestibule. For instance, Asp34 (TM1), a conserved residue amongst all
99 the known DHA2 members for substrate recognition and protonation, is replaced by a
100 glutamine in the NorB/NorC clade. These changes may have significant
101 consequences on the transport properties of NorC. Also, most members related to
102 NorC retain an amidohydrolase in the open reading frame alongside the transporter
103 gene, which could be involved in processing the substrates transported by NorC.

104 **Crystallization and structure determination.** The WT (wild-type) NorC protein
105 was heterologously expressed and purified from *E. coli* membranes and crystallized in
106 complex with a Zn²⁺-bound ICab that was identified and isolated in an earlier
107 study (Kumar et al., 2020) (Supplemental Figure 2S-1). Despite obtaining single
108 crystals, only a minor fraction would diffract and were susceptible to radiation
109 damage. Multiple datasets were merged and scaled together to obtain a complete
110 dataset to a resolution of 3.7 Å. The phases were estimated through Se-SAD phasing
111 (described in methods). The resulting electron density was subjected to density
112 modification, followed by manual model building and refinement. All the 14 TM
113 helices could be modeled, and the main chain was traced with the help of selenium
114 peaks resulting in the correct assignment of amino acids into the density. Multiple
115 rounds of model building and refinement iteratively led to a significantly improved
116 electron density map (Supplemental Figure 2S-2). A mutation in TM13, K398A
117 allowed a marginal improvement of data quality to 3.6 Å with improved side-chain
118 densities in the molecule leading to the refinement of the structure to acceptable R-
119 factors (Table 1).

120 **X-ray structure of NorC bound to single-domain camelid antibody.** The structure
121 of NorC retains a typical MFS fold and all the 14 TM helices of NorC could be
122 modeled unambiguously in the electron density, although loop regions connecting
123 TM6 with TM7 (189-198), TM7 with TM8 (223-226) and TM8 with TM9 (247-257)
124 were missing due to inherent disorder (Supplemental Figure 2S-2). The TM helices
125 are organized in two six-helix bundles, a conserved feature of MFS transporters, with
126 the linker between the helical domains forming two additional TM helices leading to a
127 6+2+6 arrangement (Figure 1B). The helices 1 to 6 and 9 to 14 are related by a
128 pseudo-two fold symmetry that facilitates conformational transitions through rocker-
129 switch mechanism (Drew and Boudker, 2016). The arrangement of the six-helix

130 bundles is consistent with the organization in MdfA and LmrP structures in the
131 outward-open state (Debruycker et al., 2020; Nagarathinam et al., 2018). The MFS
132 members, proton-coupled oligopeptide transporters (POTs) that are involved in
133 symport of di or tri-peptides, also comprise 14 TM helices (Doki et al., 2013; Solcan
134 et al., 2012). However, all the POT structures known thus far were elucidated in the
135 inward-open conformation unlike NorC, which is in the outward-open conformation.
136 The relative positions of the additional TM helices 7 and 8 differ substantially in their
137 orientation in NorC, in comparison to equivalent helices in POTs (Figure 1C). Despite
138 these differences, the presence of two additional helices as an insertion in the linker
139 connecting the six-helix bundles among distantly related NorC and POTs makes this a
140 consistent organization among 14TM MFS members. The TMs 7 and 8, in NorC, are
141 observed to occlude a wide opening between the helical bundles towards the outer
142 leaflet of the membrane lined by TMs2 and 13 (Supplemental Figure 2S-3A). When
143 compared to the Hoechst bound LmrP structure(Debruycker et al., 2020), the position
144 of TMs7 and 8 clashes with Hoechst, thereby indicating the ability of TMs 7 and 8 to
145 regulate lateral entry and binding of substrates in the vestibule (Supplemental Figure
146 2S-3B).

147 The structure of NorC is in an outward-open conformation with entry into the
148 vestibule blocked by the ICab that was used as a crystallization chaperone (Figure
149 2A). Both aromatic and polar residues line the vestibule creating hydrophobic pockets
150 as well as regions that can allow interactions with polar or charged substrates (Figure
151 1D). Unlike QacA, NorC lacks any negatively charged residues within the vestibule
152 although a single cationic charge at Lys398, is observed in the cytosolic half of TM13
153 whose side chain is positioned facing the vestibule (Figure 1B). Incidentally, lysines
154 can also couple protonation-driven transport as observed in Na^+/H^+ antiporters and
155 aminoacid, polyamine and organocation (ApcT) transporters(Shaffer et al., 2009;
156 Uzdavinyas et al., 2017). The location of K398 coincides with the position of E407 in
157 TM13 of QacA homology model where it was observed to be vital as both a general
158 protonation site and substrate recognition site for certain substrates(Majumder et al.,
159 2019). The vestibule also has a substitution of Q27 instead of acidic residues (D34)
160 commonly observed in DHA members that transport cations including QacA and
161 MdfA.

Most MFS transporters are characterized by the presence of conserved motifs that are characteristic of MFS members despite weak sequence identities. In NorC, the motif A with a consensus sequence G₆₆X₃D₇₀(K/R)XGR₇₄X(K/R) is well-conserved and consistent with other MFS transporters (Supplemental Figure 1S-1). The Asp70 is involved in a salt bridge interaction with Arg74 of the same motif (Supplemental Figure 2S-4). Arg105 in TM4 forms the motif B and interacts with the main chain carbonyl groups of A26 (TM1) and occupies a similar position in comparison to its equivalent residue Arg112 in MdfA. The TM5 helix comprises motif C, which is characteristically present in antiporters and includes a stretch of glycine residues interspersed with a GP dipeptide (GX₈GX₃GPX₂GG) (Varela et al., 1995). This motif is consistently present across numerous antiporter sequences and is absent in the case of symporters and uniporters (Varela et al., 1995). Incidentally, NorC retains a bulk of the glycine residues although the GP dipeptide is substituted by -CS- dipeptide that can have profound implications on its transport characteristics.

ICab interacts with NorC in the outward-open state. The vestibule of NorC is open to the extracellular side although solvent access is limited through the interactions with ICab (Figure 2A). Nanobodies derived from llamas have proven to be powerful tools in studying integral membrane protein structures (Manglik et al., 2017). A few transporters like LacY have been crystallized in complex with nanobodies that bind to the extracellular face of the transporter. However, the nanobody bound LacY retains sugar interactions in the binding pocket (Jiang et al., 2016; Kumar et al., 2018a). Interestingly, the ICab has a longer CDR1 loop compared to other camelid and llama antibodies and the CDR3 loop harbors a unique Zn²⁺-binding site. Disturbing the Zn²⁺-binding site led to a loss of interactions to NorC suggesting that it is vital to retain interactions with NorC (Kumar et al., 2020). The CDR1 loop has an additional -STYS- motif that forms a β-turn and inserts deep into the vestibule of NorC, to nearly half its depth (Figure 2A) forming multiple polar and hydrophobic interactions with NorC (Figure 2B). The motif wedges between symmetry-related helices TM5 from the N-terminal domain and TM 9 and TM 10 from the C-terminal domain. TM5 of MdfA undergoes angular shifts of 15° and a clockwise twist of 45° while changing from inward-open to outward-open conformation to facilitate alternating-access (Nagarathinam et al., 2018). The TM5 of NorC is blocked by the presence of ICab whose CDR1 as well as CDR3 interacts with

195 it. The presence of ICab CDR1 loop in the NorC vestibule further prevents the TM9
196 from curving as observed in the equivalent helix in MdfA(TM7) and restrains it as a
197 linear helix (Supplemental Figure 2S-5). The CDR3 also interacts closely with
198 residues in TMs 1, 2 and 5 towards the extracellular face (Figure 2D), which undergo
199 substantial shifts during the rocker-switch motion. While the epitope residues do not
200 display any interaction with the Zn^{2+} ion directly, it is evident from the NorC-ICab
201 complex that Zn^{2+} -binding allows the CDR3 to have a conformation that facilitates
202 high-affinity interactions with NorC. The residues Phe111 and Leu112 of ICab also
203 wedge in the gap between TMs 5 and 10 (Figure 2C).

204 The ICab used in this study displays a high affinity of 38 nM (Figure 2E) and
205 interacts specifically to NorC without any cross-reactivity to NorB, despite their close
206 similarity, as observed in FSEC experiments (Figure 3A, B). A comparison of the
207 ICab crystal structures in the NorC-bound and free forms reveals subtle
208 conformational changes in the CDR3 loops when it interacts with the antigen. The β -
209 turn undergoes a straightening in its position and the Thr35 undergoes a 3.0 Å shift in
210 its C α position. Similarly, the Tyr36 in the β -turn undergoes a displacement of its
211 phenol side chain by 3.3 Å to facilitate its wedging between TMs 5 and 10 (Figure
212 2F). The CDR3 largely retains a conformation in the bound state similar to that of the
213 free ICab with a minor displacement of about 1.5 Å in the region between 109 and
214 115. However, the side chain of Phe111 undergoes a massive shift in the χ_1 torsion
215 angle with 148° rotation to interact with NorC residues Leu152 and Leu298.

216 **ICab blocks access to antibacterial compounds.** The ability of ICab to interact with
217 the vestibule results in a massive reduction of solvent accessibility within the
218 vestibule of NorC, likely compromising substrate interactions (Figure 4A). The
219 natural substrates of NorB/NorC-like transporters are unknown. As NorC is reported
220 to be involved in efflux of fluoroquinolones, we performed survival assay of *E. coli*
221 expressing NorC in presence of fluoroquinolones. To our surprise, we did not observe
222 a direct efflux of fluoroquinolones with NorC (Supplemental Figure 1S-2). While
223 screening for potential substrates that can interact with NorC, we discovered specific
224 interactions between NorC and TPP with a K_d value of 4 μ M (Figure 4B). Although
225 TPP interactions with both NorB and NorC K398A led to a nominal enhancement of
226 stability ($\Delta T_m = +1.5^\circ\text{C}$ and $+2.9^\circ\text{C}$ respectively) (Supplemental Figure 4S-1), WT

227 NorC lacks the ability to transport TPP (Supplemental Figure 1S-2). This is plausible
 228 in the context of other MFS transporter structures like LmrP, which has a detergent
 229 molecule, a phospholipid and a cationic dye (Hoechst) bound in distinct subsites
 230 within the vestibule in LmrP(Debruycker et al., 2020). We suggest that TPP, given its
 231 multiple phenyl groups could stack and interact with the aromatic side chains within
 232 the NorC vestibule. The interactions of TPP with NorC are clearly blocked in the
 233 presence of ICab causing a complete loss of interactions of the antibacterial
 234 compound with NorC (Figure 4B). In doing so, the ICab-NorC complex, resembles a
 235 ‘bottle-cork’ that prevents substrate interactions and enforces a lock on the
 236 conformational changes within the transporter.

237 **Conclusions.** NorC structure is the first representative structure of the DHA2 family
 238 and reveals the architecture of this distinct class of transporters within the MFS fold.
 239 While the true substrate of NorC is not known, both NorC and its closely related
 240 homologue, NorB are observed to protect the superbug against fluoroquinolones,
 241 which are broad-spectrum antibiotics. The lack of direct transport of fluoroquinolones
 242 through NorC suggests that the transporter could be aiding in reducing antibiotic
 243 stress through alternate mechanisms including improved fitness and persistence, as
 244 observed in the case of NorB (Ding et al., 2008) (Dawan et al., 2020). The ability to
 245 detect and block efflux transporters is proposed as a promising strategy to aid and
 246 improve the efficacy of existing antibiotics. The ICab characterized in this study
 247 provides a high fidelity detection tool to observe the presence of NorC in *S. aureus*
 248 populations. Its ability to wedge deeply in the NorC vestibule and lock it in an
 249 outward-open conformation serves as a proof-of-principle to use ICabs or nanobodies
 250 as efflux pump inhibitors and a potential strategy to overcome antimicrobial
 251 resistance.

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275 **Author contributions.** SK and AA purified NorC and ICab. SK and AA optimized
276 the crystallization of NorC-Icab complex and selmet derivatization of NorC. SK
277 solved and built the structure of NorC with inputs from AP. AA, SK performed the
278 microbial growth assays and binding assays with TPP, respectively. AG and RMN
279 cloned, isolated and optimized the behavior of NorC. AP designed the project. SK and
280 AP wrote the manuscript with inputs from all authors.

281 **Data availability.** The atomic coordinates and structure factors of WT NorC and
282 NorC-K398A are deposited in the Protein Data Bank with PDB IDs 7D5P and 7D5Q,
283 respectively.

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286 **Competing interests.** Authors declare no competing interests.

287 **Methods**

288 **Sequence alignment and Phylogenetic analysis**

289 NorC (Uniprot ID A0A0E1ACG1) was used to search for closely related homologous
290 sequences in non-redundant sequence database. To search for more homologous
291 sequences amongst the known DHA family members, transporter classification
292 database was used. The alignment was carried out using clustalw program with
293 iterative HMM clustering. The output properly aligned the TMs of all the query
294 proteins –a benchmark we used to check the alignment accuracy for this highly

divergent dataset. The alignment was used further for functional and phylogenetic analysis. The evolutionary history was inferred by using the maximum likelihood method and JTT matrix-based model (Jones et al., 1992). The tree with the highest log likelihood (-35519.34) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (2 categories (+G, parameter = 3.0280)). This analysis involved 39 amino acid sequences. There were a total of 611 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018b).

Survival Assays

To check for phenotype (resistance against antibacterials), C41 cells (transformed with IPTG inducible *pET16b-norC* or *pET16b-norA*) and JD838 cells (transformed with L-arabinose inducible *pBAD-norC*, *pBAD-empty* or *pBAD-qacA*) were grown as primary culture overnight. A secondary culture was inoculated using this and split into two parts: one treated for induction of protein expression (using 0.2mM IPTG or 0.05% (w/v) L-arabinose) and the other left untreated. The induction was done when the O.D._{600nm} reached 0.4 A.U. The cultures were further grown till all of them had an O.D._{600nm} between 1.5-2.0 A.U.; the cultures were then diluted accordingly to bring their O.D.s to 1.0 and spotted in 10 fold serial dilutions on 1.5% (w/v) agar plate containing 2% (w/v) luria bertani (LB) broth and 100 µg/ml Ampicillin with/without inducer in the media. Control plates containing none of the antibacterials but ampicillin were spotted similarly with/without inducer. The cells were allowed to grow overnight at 37°C. These assays were replicated independently with n=5.

Cloning, expression and purification of NorC

Full length *norC* gene was cloned into pET-16b vector between restriction sites NcoI and KpnI with 8X-His tag at its C-terminus. The *E. coli* BL21-C41 cells were transformed with the cloned vector containing ampicillin resistance cassette. A primary culture was grown in LB-broth (HiMedia) for 10-12 hours at 37 °C. Primary culture was used to inoculate large scale culture (2-3 l) that were grown at 37 °C until the OD₆₀₀ reached 0.4-0.5 following which protein expression was induced with 0.2 mM Isopropyl-β-D-1-thiogalactopyranoside (IPTG). The cells were further grown for 12-15 hrs at 20°C. Cells were harvested by centrifugation and flash frozen in liquid N₂ for storage or processed immediately. The cell pellet was dissolved in HBS (20 mM Hepes pH 7.0, and 200 mM NaCl). The cell suspension was lysed at high pressure of about 800 bar using a homogenizer (GEA Niro Soavi PandaPlus 1000 Homogenizer) and spun at ~100000g for 1 hour at 4 °C. The pelleted membranes were resuspended in HBS and 1mM PMSF using a rotor-stator homogenizer, following which DDM (n-Dodecyl-β-D-maltopyranoside, Anatrace) was added to a final concentration of 20mM. The solution was nutated for 2 hours at 4°C to extract NorC into micelles, followed by ultracentrifugation at 100000 g for 1 hour at to remove the insoluble debris. The supernatant was incubated with Ni-NTA beads pre-equilibrated with buffer A (1mM DDM in HBS) at 4°C for 1 hour. The solution containing beads were transferred into gravity columns (Bio-rad) and washed with 50 column volumes of buffer A having 30mM imidazole. The protein was eluted in

buffer A containing 300 mM imidazole and concentrated using a 30 kDa cut-off centrifugal filter (Amicon, Merck Millipore). It was further purified by size exclusion chromatography using Superdex S-200 increase 10/300 GL column (GE Healthcare) in HBS containing 4 mM n-Decyl- β -D-maltopyranoside (DM, Anatrace). The purity of the protein was analyzed by SDS-PAGE. The mutant NorC K398A protein was purified in a similar manner.

WT NorC was labelled with L-selenomethionine by growing *E. coli* BL21-C41 cells, transformed with NorC-containing vector, in M9 medium supplemented with 50 mg/L of L-selenomethionine. The purification of SeMet-labeled WT NorC was similar to that of the native protein.

ICab generation and purification

Generation and purification of ICab was carried out in a manner described previously (Kumar et al., 2020). The ICab isolated through yeast-display screening was subcloned into pET-22b vector. *E. coli* Rosetta cells were transformed with *vhh*-containing vector. Large scale cultures were grown in LB broth 37 °C until OD₆₀₀ reached 0.6 and protein expression was induced by adding IPTG to a final concentration of 0.5 mM; cells were further grown at 37 °C for 5-6 hours. The cells were harvested and the protein was extracted from inclusion bodies using urea denaturation followed by refolding. The refolding was done using step-wise dialysis at 4°C. The protein was further purified by size-exclusion chromatography using Superdex S-75 10/300 GL column (GE Healthcare) in HBS. The purity and integrity of the protein was checked by SDS-PAGE and MALDI-TOF.

Site-directed mutagenesis

To perform site-directed mutagenesis of NorC, primers were designed to amplify entire plasmid carrying *norC* gene in a single step with desired point mutation. The amplicons were treated with DpnI at 37°C for 2-3 hours to digest the wild-type plasmid. *E. coli* Top10 cells were transformed with the mutant amplicon and plated on LB-agar plate containing 100 μ g/ml ampicillin and grown overnight at 37°C. The positive clones were confirmed by DNA sequencing. The mutant proteins were purified in a manner similar to that of the wild type protein.

Fluorescence-detection size exclusion chromatography (FSEC)

Briefly, FSEC was done using a high-performance liquid chromatography system attached to an autosampler and a multi-wavelength fluorescence detector (Shimadzu). 10 μ M each of WT NorC, NorC-K398A and NorB purified in DM buffer were incubated with 1:1.2 molar ratios of ICab and shifts in their elutions (measured at λ_{ex} = 395nm, λ_{em} = 340nm) in the same DM buffer were used to qualitatively determine whether or not ICab binds to them. A superdex 200 HR column was used for analyzing elution times.

Crystallization and structure determination

Purified WT NorC (native and SeMet-derivative) and NorCK398A (native only) proteins were concentrated to 3 mg/ml using 50 kDa cut-off centrifugal filter (Amicon, Merck Millipore) and mixed with ICab in a molar ratio of 1:1.2. The crystallization was carried out by hanging drop vapor diffusion method at 20 °C where protein and crystallization conditions were mixed in a ratio of 3:2 v/v. WT

NorC and NorC-K398A crystallized in a condition containing 0.1 M MES pH 6.0, 50 mM NaCl, 35.7 % PEG 600, 57.1 mM CaCl₂, 10 mM YCl₃ and 6.0 mM CHAPSO. The crystals appeared within 3-4 days but took about 2 weeks to attain full size. The crystals were washed in the crystallization condition and flash frozen in liquid N₂. The X-ray diffraction data sets were collected at Advanced Photon Source (APS) Synchrotron, USA. The crystals diffracted X-rays to a resolution of about 3.6-4Å.

All the data sets were indexed and integrated with XDS (Kabsch, 2010). Eight data sets were merged and scaled for SeMet-derivative WT NorC using AIMLESS (Evans, 2006) resulting in a highest resolution of 3.65Å. The space group was determined to be P2₁ with unit cell dimensions of a=71.2 Å, b=139.9 Å, c=118.0 Å and $\alpha=\gamma=90^\circ$ and $\beta=106^\circ$ (Extended Data Table 1). The Matthews coefficient suggested two NorC-ICab complexes in the asymmetric unit with a solvent content of about 70 percent. SAD phasing was carried out using CRANK2 (Skubak and Pannu, 2013) in CCP4i2 (Winn et al., 2011). For the substructure determination, the highest resolution was set at 5.0 Å as anomalous signal was very weak at higher resolution. SHELEX C and D (Schneider and Sheldrick, 2002) in CRANK2 were used for substructure determination and refinement. The resulting phases were extended to a resolution of 3.65 Å and the electron density was subjected to density modification using PARROT (Cowtan, 2010). Manual model building was carried out in the density modified map using Coot (Emsley and Cowtan, 2004). Selenium positions were used as markers to trace the main chain and place amino acids.

Multiple X-ray diffraction data sets were collected for the native NorCK398A crystals. Six best data sets were merged and scaled using AIMLESS to a resolution of 3.6 Å. The structure was determined by molecular replacement using PHASER (McCoy et al., 2007) with WT NorC structure as a model. The refinement of both the structures were carried out using REFMAC (Murshudov et al., 2011) until the refinement converged. The resulting structures were validated using MOLPROBITY (Williams et al., 2018). The data collection and refinement statistics are presented in the Extended Data Table 1.

NorC interaction with TPP

Quantification of NorC's interaction with TPP was carried out using microscale thermophoresis (Nanotemper) (Seidel et al., 2013). The protein was labelled with red Tris-NTA dye (Nanotemper) by mixing both in an equimolar proportion. The labelled protein was mixed with TPP such that the concentration of labelled protein and TPP were 50 nM and 4 mM respectively. A total of 16 two-fold serial dilutions of TPP were prepared keeping the protein concentration constant at 50 nM. The experiment was carried out with Monolith™ NT.115 MST premium-coated capillaries. For NorC-ICab complex titrations against TPP, 50 nM of NorC was mixed with ICab in 1:3 molar ratio and the starting concentration of TPP was kept at 4 mM. For ICab titrations against NorC, 10 nM of NorC was used with a starting concentration of 5 µM for the ICab. The curves were fitted with single-site binding model.

TPP's propensity to interact with NorB and NorC-K398A was similarly measured using differential scanning fluorimetry (DSF) using Prometheus NT.48 instrument (NanoTemper Technologies) (Kotov et al., 2019). A temperature scan was carried out between 20 and 90 °C with a scan rate of 1 °C/min. All measurements were carried out in duplicates. The first derivative of the ratio of F350/330 was plotted against

432 temperature, and shift in T_m was used to determine whether or not TPP binds to the
433 proteins.

434 Isothermal Titration Calorimetry

435 The binding affinity of NorC with ICab was determined by ITC using Microcal
436 PEAQ-ITC (Malvern Panalytical). Both NorC and ICab were purified in a buffer
437 containing 20 mM Hepes, pH 7.0, 200 mM NaCl, and 4 mM decyl- β -D-
438 maltopyranoside detergent. The titration consisted of 18 injections of 4s duration
439 each, with first injection of 0.4 μ l and all the subsequent injections of 2 μ l. The time
440 between two consecutive injections was kept at 150 s, and the sample in the cell was
441 stirred at 750 rpm during the entire run. The concentration of NorC in the cell was
442 kept at 25 μ M and the concentration of ICab in the syringe was kept at 250 μ M. The
443 titration was carried out at a constant temperature of 25 °C. For blank, ICab was
444 titrated against buffer, and the heat of mixing and dilution were subtracted from the
445 titration data against NorC. All of the data were fit using Microcal PEAQ-ITC
446 analysis software with one set of sites model(Kumar et al., 2020).

447 References

- 448 Bigger, J.W. (1944). Treatment of Staphylococcal infections with Penicillin. *The Lancet* 2,
449 4.
450 Blair, J.M., Webber, M.A., Baylay, A.J., Ogbolu, D.O., and Piddock, L.J. (2015). Molecular
451 mechanisms of antibiotic resistance. *Nat Rev Microbiol* 13, 42-51.
452 Chitsaz, M., and Brown, M.H. (2017). The role played by drug efflux pumps in bacterial
453 multidrug resistance. *Essays Biochem* 61, 127-139.
454 Costa, S.S., Viveiros, M., Amaral, L., and Couto, I. (2013). Multidrug Efflux Pumps in
455 *Staphylococcus aureus*: an Update. *Open Microbiol J* 7, 59-71.
456 Cowtan, K. (2010). Recent developments in classical density modification. *Acta*
457 *Crystallogr D Biol Crystallogr* 66, 470-478.
458 Dawan, J., Wei, S., and Ahn, J. (2020). Role of antibiotic stress in phenotypic switching to
459 persister cells of antibiotic-resistant *Staphylococcus aureus*. *Annals of Microbiology* 70.
460 Debruycker, V., Hutchin, A., Masureel, M., Ficici, E., Martens, C., Legrand, P., Stein, R.A.,
461 McHaourab, H.S., Faraldo-Gomez, J.D., Remaut, H., *et al.* (2020). An embedded lipid in the
462 multidrug transporter LmrP suggests a mechanism for polyspecificity. *Nat Struct Mol*
463 *Biol*.
464 Ding, Y., Onodera, Y., Lee, J.C., and Hooper, D.C. (2008). NorB, an efflux pump in
465 *Staphylococcus aureus* strain MW2, contributes to bacterial fitness in abscesses. *J*
466 *Bacteriol* 190, 7123-7129.
467 Doki, S., Kato, H.E., Solcan, N., Iwaki, M., Koyama, M., Hattori, M., Iwase, N., Tsukazaki, T.,
468 Sugita, Y., Kandori, H., *et al.* (2013). Structural basis for dynamic mechanism of proton-
469 coupled symport by the peptide transporter POT. *Proc Natl Acad Sci U S A* 110, 11343-
470 11348.
471 Drew, D., and Boudker, O. (2016). Shared Molecular Mechanisms of Membrane
472 Transporters. *Annu Rev Biochem* 85, 543-572.
473 Emsley, P., and Cowtan, K. (2004). Coot: model-building tools for molecular graphics.
474 *Acta Crystallogr D Biol Crystallogr* 60, 2126-2132.
475 Evans, P. (2006). Scaling and assessment of data quality. *Acta Crystallogr D Biol*
476 *Crystallogr* 62, 72-82.
477 Jiang, X., Smirnova, I., Kasho, V., Wu, J., Hirata, K., Ke, M., Pardon, E., Steyaert, J., Yan, N.,
478 and Kaback, H.R. (2016). Crystal structure of a LacY-nanobody complex in a periplasmic-
479 open conformation. *Proc Natl Acad Sci U S A* 113, 12420-12425.

480 Jones, D.T., Taylor, W.R., and Thornton, J.M. (1992). The rapid generation of mutation
481 data matrices from protein sequences. *Comput Appl Biosci* 8, 275-282.
482 Kabsch, W. (2010). Xds. *Acta Crystallogr D Biol Crystallogr* 66, 125-132.
483 Kotov, V., Bartels, K., Veith, K., Josts, I., Subhramanyam, U.K.T., Gunther, C., Labahn, J.,
484 Marlovits, T.C., Moraes, I., Tidow, H., *et al.* (2019). High-throughput stability screening
485 for detergent-solubilized membrane proteins. *Sci Rep* 9, 10379.
486 Kumar, H., Finer-Moore, J.S., Jiang, X., Smirnova, I., Kasho, V., Pardon, E., Steyaert, J.,
487 Kaback, H.R., and Stroud, R.M. (2018a). Crystal Structure of a ligand-bound LacY-
488 Nanobody Complex. *Proc Natl Acad Sci U S A* 115, 8769-8774.
489 Kumar, S., He, G., Kakarla, P., Shrestha, U., Ranjana, K.C., Ranaweera, I., Willmon, T.M.,
490 Barr, S.R., Hernandez, A.J., and Varela, M.F. (2016). Bacterial Multidrug Efflux Pumps of
491 the Major Facilitator Superfamily as Targets for Modulation. *Infect Disord Drug Targets*
492 16, 28-43.
493 Kumar, S., Mahendran, I., Athreya, A., Ranjan, R., and Penmatsa, A. (2020). Isolation and
494 structural characterization of a Zn(2+)-bound single-domain antibody against NorC, a
495 putative multidrug efflux transporter in bacteria. *J Biol Chem* 295, 55-68.
496 Kumar, S., Stecher, G., Li, M., Knyaz, C., and Tamura, K. (2018b). MEGA X: Molecular
497 Evolutionary Genetics Analysis across Computing Platforms. *Mol Biol Evol* 35, 1547-
498 1549.
499 Majumder, P., Khare, S., Athreya, A., Hussain, N., Gulati, A., and Penmatsa, A. (2019).
500 Dissection of Protonation Sites for Antibacterial Recognition and Transport in QacA, a
501 Multi-Drug Efflux Transporter. *J Mol Biol* 431, 2163-2179.
502 Manglik, A., Kobilka, B.K., and Steyaert, J. (2017). Nanobodies to Study G Protein-
503 Coupled Receptor Structure and Function. *Annu Rev Pharmacol Toxicol* 57, 19-37.
504 McCoy, A.J., Grosse-Kunstleve, R.W., Adams, P.D., Winn, M.D., Storoni, L.C., and Read, R.J.
505 (2007). Phaser crystallographic software. *J Appl Crystallogr* 40, 658-674.
506 Murshudov, G.N., Skubak, P., Lebedev, A.A., Pannu, N.S., Steiner, R.A., Nicholls, R.A., Winn,
507 M.D., Long, F., and Vagin, A.A. (2011). REFMAC5 for the refinement of macromolecular
508 crystal structures. *Acta Crystallogr D Biol Crystallogr* 67, 355-367.
509 Nagarathinam, K., Nakada-Nakura, Y., Parthier, C., Terada, T., Juge, N., Jaenecke, F., Liu,
510 K., Hotta, Y., Miyaji, T., Omote, H., *et al.* (2018). Outward open conformation of a Major
511 Facilitator Superfamily multidrug/H(+) antiporter provides insights into switching
512 mechanism. *Nat Commun* 9, 4005.
513 Paulsen, I.T., Brown, M.H., Littlejohn, T.G., Mitchell, B.A., and Skurray, R.A. (1996a).
514 Multidrug resistance proteins QacA and QacB from *Staphylococcus aureus*: membrane
515 topology and identification of residues involved in substrate specificity. *Proc Natl Acad*
516 *Sci U S A* 93, 3630-3635.
517 Paulsen, I.T., Brown, M.H., and Skurray, R.A. (1996b). Proton-dependent multidrug efflux
518 systems. *Microbiol Rev* 60, 575-608.
519 Piddock, L.J. (2006). Multidrug-resistance efflux pumps - not just for resistance. *Nat Rev*
520 *Microbiol* 4, 629-636.
521 Pu, Y., Zhao, Z., Li, Y., Zou, J., Ma, Q., Zhao, Y., Ke, Y., Zhu, Y., Chen, H., Baker, M.A.B., *et al.*
522 (2016). Enhanced Efflux Activity Facilitates Drug Tolerance in Dormant Bacterial Cells.
523 *Mol Cell* 62, 284-294.
524 Reddy, V.S., Shlykov, M.A., Castillo, R., Sun, E.I., and Saier, M.H., Jr. (2012). The major
525 facilitator superfamily (MFS) revisited. *FEBS J* 279, 2022-2035.
526 Schneider, T.R., and Sheldrick, G.M. (2002). Substructure solution with SHELXD. *Acta*
527 *Crystallogr D Biol Crystallogr* 58, 1772-1779.
528 Seidel, S.A., Dijkman, P.M., Lea, W.A., van den Bogaart, G., Jerabek-Willemsen, M., Lazic,
529 A., Joseph, J.S., Srinivasan, P., Baaske, P., Simeonov, A., *et al.* (2013). Microscale
530 thermophoresis quantifies biomolecular interactions under previously challenging
531 conditions. *Methods* 59, 301-315.
532 Shaffer, P.L., Goehring, A., Shankaranarayanan, A., and Gouaux, E. (2009). Structure and
533 mechanism of a Na⁺-independent amino acid transporter. *Science* 325, 1010-1014.

534 Skubak, P., and Pannu, N.S. (2013). Automatic protein structure solution from weak X-
535 ray data. *Nat Commun* 4, 2777.
536 Solcan, N., Kwok, J., Fowler, P.W., Cameron, A.D., Drew, D., Iwata, S., and Newstead, S.
537 (2012). Alternating access mechanism in the POT family of oligopeptide transporters.
538 *EMBO J* 31, 3411-3421.
539 Stavri, M., Piddock, L.J., and Gibbons, S. (2007). Bacterial efflux pump inhibitors from
540 natural sources. *J Antimicrob Chemother* 59, 1247-1260.
541 Tong, S.Y., Davis, J.S., Eichenberger, E., Holland, T.L., and Fowler, V.G., Jr. (2015).
542 *Staphylococcus aureus* infections: epidemiology, pathophysiology, clinical
543 manifestations, and management. *Clin Microbiol Rev* 28, 603-661.
544 Truong-Bolduc, Q.C., Dunman, P.M., Strahilevitz, J., Projan, S.J., and Hooper, D.C. (2005).
545 MgrA is a multiple regulator of two new efflux pumps in *Staphylococcus aureus*. *J*
546 *Bacteriol* 187, 2395-2405.
547 Truong-Bolduc, Q.C., Strahilevitz, J., and Hooper, D.C. (2006). NorC, a new efflux pump
548 regulated by MgrA of *Staphylococcus aureus*. *Antimicrob Agents Chemother* 50, 1104-
549 1107.
550 Uzdaviny, P., Coincon, M., Nji, E., Ndi, M., Winkelmann, I., von Ballmoos, C., and Drew, D.
551 (2017). Dissecting the proton transport pathway in electrogenic Na(+)/H(+) antiporters.
552 *Proc Natl Acad Sci U S A* 114, E1101-E1110.
553 Varela, M.F., Sansom, C.E., and Griffith, J.K. (1995). Mutational analysis and molecular
554 modelling of an amino acid sequence motif conserved in antiporters but not symporters
555 in a transporter superfamily. *Mol Membr Biol* 12, 313-319.
556 Williams, C.J., Headd, J.J., Moriarty, N.W., Prisant, M.G., Videau, L.L., Deis, L.N., Verma, V.,
557 Keedy, D.A., Hintze, B.J., Chen, V.B., *et al.* (2018). MolProbity: More and better reference
558 data for improved all-atom structure validation. *Protein Sci* 27, 293-315.
559 Winn, M.D., Ballard, C.C., Cowtan, K.D., Dodson, E.J., Emsley, P., Evans, P.R., Keegan, R.M.,
560 Krissinel, E.B., Leslie, A.G., McCoy, A., *et al.* (2011). Overview of the CCP4 suite and
561 current developments. *Acta Crystallogr D Biol Crystallogr* 67, 235-242.
562 Wright, G.D. (2016). Antibiotic Adjuvants: Rescuing Antibiotics from Resistance. *Trends*
563 *Microbiol* 24, 862-871.
564 Yin, Y., He, X., Szewczyk, P., Nguyen, T., and Chang, G. (2006). Structure of the multidrug
565 transporter EmrD from *Escherichia coli*. *Science* 312, 741-744.

566

567 **Figure Legends**

568 **Figure 1| Phylogeny and X-ray structure of NorC. A.** (top) Radial phenogram of
569 DHA1 and DHA2 transporters. Characterized DHA2 transporters and their close
570 homologs have been categorized with QacA/EmrB and their homologs for their
571 sequentially coherent residues. **B.** (top-left) Side view of NorC bound to the camelid
572 antibody ICab in outward-open conformation. The transmembrane helices have been
573 grouped in repeats of three to depict pseudo-two fold symmetry; vestibule lining TM
574 helices 1, 4, 9 and 12 are in blue, 2, 5, 10 and 13 lining the domain interface in
575 orange, 3, 6, 11 and 14 in green, and helices 7 and 8 are in grey. (top right) APBS
576 electrostatic representation showing positively charged region of the vestibule near
577 the cytoplasmic end of NorC, owing to K398 (shown as sticks). (bottom) Note that
578 TM7 and TM8 lie outside the primary helical bundles that surround the vestibule. **C.**
579 Structural superposition of LmrP (blue, RMSD = 3.0 Å), MdfA (brown, RMSD = 2.5
580 Å), GkPOT (olive, RMSD = 4.4 Å) and XcPOT (cyan, RMSD = 4.2 Å) with NorC;
581 angular differences between TMs 7 and 8 of NorC and those of POTs are shown in
582 the same superposition. **D.** Solid section of NorC showing vestibular environment.

583 **Figure 2 | ICab interacts with NorC in an outward-open state. a.** Solid section of
 584 NorC (light blue) in complex with ICab (purple). CDRs 1-3 are shown in red, green
 585 and cyan loops respectively, with Zn^{2+} as blue sphere. ROI in dashed border is
 586 magnified in **B.** with interchain H-bonds (dashed lines, lengths labelled) and
 587 participating residues (in sticks; lines shown for those not participating via their side
 588 chains, for clarity). NorC helices interacting with ICab through H-bonds are shown as
 589 cartoon. **C.** Tyr36 (CDR1) and Phe111 and Leu112 (CDR3) involved in extensive
 590 hydrophobic interactions at the interface of the N- and C- terminal domain of NorC
 591 are shown with participating residues (sticks) and complementary NorC contour
 592 (transparent surface). **D.** CDR3 (cyan cartoon and surface) forming a near perfect
 593 shape complementary, with interacting residues from NorC (white surface patches)
 594 involved in polar and VdW interactions. **E.** ITC profile (differential power, top panel;
 595 binding isotherms with integrated peaks normalized to moles of injectant and offset
 596 corrected, bottom panel.) of NorC titrated with ICab. $K_d = 38 \pm 5$ nM. **f.** Structural
 597 alignment of ICab in native state (grey) and when bound to NorC (purple). CDR1 and
 598 CDR3 display the conformational changes that residues (sticks and transparent
 599 spheres) undergo during complexation (arrows).

600 **Figure 3 | ICab specifically interacts with NorC. A.** Tryptophan fluorescence trace
 601 of NorC (green traces) in FSEC, shifts to a slightly higher molecular mass when
 602 complexed with ICab. **B.** NorB (red traces) does not display a shift indicating a lack
 603 of interaction with ICab (inset).

604 **Figure 4 | ICab interaction alters solvent accessibility of NorC vestibule. A.** Water
 605 accessible surface (green spheres) in NorC's vestibule starting from Trp139/Lys398
 606 (black spheres), in the absence (left) and presence (right) of ICab. The outward open
 607 conformation completely blocks accessibility from the cytosolic side of NorC. **B.**
 608 Microscale thermophoresis profiles for NorC and tetraphenylphosphonium in the
 609 presence and absence of ICab. $K_d = 4 \pm 0.8$ μ M for NorC and TPP.

Table 1. Crystallographic Data and Refinement Statistics

<i>Data Collection Statistics</i>	WT NorC	NorC K398A
X-ray source	APS NE-CAT 24ID-C	APS NE-CAT 24ID-C
Temperature	100 K	100 K
Datasets merged *	8	6
Space group	$P2_1$	$P2_1$
Cell dimensions a, b, c (Å) α, β, γ (°)	71.21, 139.87, 118.03 90, 106.03, 90	70.07, 139.14, 115.55 90, 105.7, 90
Wavelength (Å)	0.97910	0.97918
Resolution (Å)	49.18-3.65 (3.90-3.65)	139.45-3.60 (3.85-3.60)
Total number of observations	431001 (68355)	426838 (45048)
Unique Reflections	24592 (4248)	24925 (4460)
Multiplicity	17.5 (16.1)	17.1 (10.1)
Data Completeness	99.1 (95.2)	99.9 (99.3)
Mean I/ σ I	8.0 (1.0)	8.5 (1.1)
R _{pim} (%)	4.8 (105.7)	4.3 (87.9)
CC _{1/2}	0.997 (0.563)	0.998 (0.486)
Anomalous completeness (%)	99.1 (95.2)	
Anomalous multiplicity	8.8 (8)	
DelAnomalous CC _{1/2}	0.476 (0.022)	
<i>Refinement Statistics</i>		
Resolution (Å)	48.96 (3.65)	111.68 (3.60)
Unique reflections	23409	23566
R _{work} (%) / R _{free} (%)	29.8/31.4	31.1/31.8
Non-hydrogen atoms/molecules		
Protein	7567	7495
Zn	2	2
Average B-factor (Å ²)		
Protein	160.50	174.72
Zn	112.60	118.12
RMS bond lengths (Å)	0.0143	0.015
RMS bond angles (°)	1.84	1.79
Ramachandran plot		
Favored (%)	91.61	90.97
Allowed (%)	8.39	9.03
Outliers (%)	0.00	0.00

*Data were obtained by scaling together multiple datasets that were collected on different crystals. The highest-resolution shell used in the final refinement is shown in parentheses.

Figure 1 | Phylogeny and X-ray structure of NorC. **A.** (top) Radial phenogram of DHA1 and DHA2 transporters. Characterized DHA2 transporters and their close homologs have been categorized with QacA/EmrB and their homologs for their sequentially coherent residues. **B.** (top-left) Side view of NorC bound to the camelid antibody ICab in outward-open conformation. The transmembrane helices have been grouped in repeats of three to depict pseudo-two fold symmetry; vestibule lining TM helices 1, 4, 9 and 12 are in blue, 2, 5, 10 and 13 lining the domain interface in orange, 3, 6, 11 and 14 in green, and helices 7 and 8 are in grey. (top right) APBS electrostatic representation showing positively charged region of the vestibule near the cytoplasmic end of NorC, owing to K398 (shown as sticks). (bottom) Note that TM7 and TM8 lie outside the primary helical bundles that surround the vestibule. **C.** Structural superposition of LmrP (blue, RMSD = 3.0 Å), MdfA (brown, RMSD = 2.5 Å), GkPOT (olive, RMSD = 4.4 Å) and XcPOT (cyan, RMSD = 4.2 Å) with NorC; angular differences between TMs 7 and 8 of NorC and those of POTs are shown in the same superposition. **D.** Solid Section of NorC showing vestibular environment.

Figure 2 | ICab interacts with NorC in an outward-open state. **A.** Solid section of NorC (light blue) in complex with ICab (purple). CDRs 1-3 are shown in red, green and cyan loops respectively, with Zn²⁺ as blue sphere. ROI in dashed border is magnified in **B.** with interchain H-bonds (dashed lines, lengths labelled) and participating residues (in sticks, light grey for ICab and dark grey for NorC; in lines shown for those not participating via their side chains, for clarity). NorC helices interacting with ICab through H-bonds are shown as cartoon. **C.** Tyr36 (CDR1) and Phe111 and Leu112 (CDR3) involved in extensive hydrophobic interactions at the interface of the N- and C-terminal domain of NorC are shown with participating residues (sticks) and complementary NorC contour (transparent surface). **D.** CDR3 (cyan cartoon and surface) forming a near perfect shape complementary, with interacting residues from NorC (white surface patches) involved in polar and VdW interactions. **E.** ITC profile (differential power, top panel; binding isotherms with integrated peaks normalized to moles of injectant and offset corrected, bottom panel.) of NorC titrated with ICab. $K_d = 38 \pm 5$ nM. **F.** Structural alignment of ICab in native state (grey) and when bound to NorC (purple). CDR1 and CDR3 display the conformational changes that residues (sticks and transparent spheres) undergo during complexation (arrows).

Figure 3 | ICab sepcifically interacts with NorC. **A.** Tryptophan fluorescence trace of NorC in FSEC shifts to a slightly higher molecular mass when complexed with ICab. **B.** NorB does not display a shift indicating a lack of interaction with ICab.

A

Accessible surface
(min. probe size = 0.9 Å)

B

Figure 4 | Icab interaction alters solvent accessibility of NorC vestibule. A. Water accessible surface (green spheres) in NorC's vestibule starting from Trp139/Lys398 (black spheres), in the absence (left) and presence (right) of Icab. The outward open conformation completely blocks accessibility from the cytosolic side of NorC. **B.** Microscale thermophoresis profiles for NorC and tetraphenylphosphonium in the presence and absence of Icab. $K_d = 4 \pm 0.8 \mu\text{M}$ for NorC and TPP.