Supplementary Materials

The Transmembrane Domain of a Bicomponent ABC Transporter Exhibits Channel-forming Activity

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1. Wzm protein was purified as a mixture of different-size oligomers. Expression of Wzm was accomplished using a pBAD vector fused to an M13 signal peptide (**Experimental procedures**). We used *E. coli* TOP10 strain as an expression host that is deficient in LPS production. This approach enabled the purification of LPS contamination-free Wzm proteins. They were solubilized in 3% *n*-dodecyl-*β*-D-maltopyranoside (DDM) and purified by His^+ tag affinity chromatography. Remarkably, the SDS-PAGE analysis revealed that the purified Wzm protein features four bands with the apparent molecular masses of ~95, ~63, ~48 and 28 kDa, named hereafter bands 1, 2, 3 and 4, respectively (**Fig. S1A**).

Bands 3 and 4 are predominant. It is likely that the band 4 corresponds to the Wzm monomer. Calculated molecular mass for Wzm is ~29.5 kDa,[1] not surprisingly because some membrane proteins run differently than their expected molecular-weight standard.[2, 3] Specific anti-His^+ tag staining predominately reacted with bands 3 and 4 (**Fig. S1B**). Band 2 is faintly stained, whereas band 1 is not stained due to limited staining sensitivity of ~25 ng. However, the treatment of the purified Wzm protein sample with dithiothreitol (DTT), a reducing agent, resulted in mostly protein band 4 (**Fig. S1C**), indicating that all bands in the lane reverted in part to band 4. Therefore, all protein bands contained the monomer. It is likely that the higher-molecular weight bands represent a consequence of SDS-resistant interactions between monomers (e.g., one or more unexposed disulfide bonds coupled with other conserved motifs responsible for tight TM helix-helix packing). As the Wzm proteins were not boiled due to their aggregation, this fact presumably hindered the accessibility of DTT to disulfide bridges. In addition, DDM surrounding the Wzm protein might have precluded the DTT access to the targeted disulfides, so a full conversion of the oligomers to the reduced monomers was not accomplished. Taken together, we conclude that all bands are Wzm proteins.

The observation that the purified Wzm proteins show several bands with distinctive molecular weight in SDS-PAGE analysis (**Fig. S1A**) suggests that these bands belong to either one or more oligomers. These oligomers should have SDS-sensitive and SDS-resistant interactions. In this case, the SDS-resistant interactions might be caused by disulfide bonds between cysteines located in the TMD (**Fig. 1B**; **Fig. S1C**). Therefore, we conducted crosslinking experiments with the DTT-cleavable dithiobis(succinimidylpropionate) (DSP) cross-linker, whose spacer arm length is 12 Å.[4] DSP is a cross-linker that reacts with the amine group of the lysine residues. The crosslinking reaction can take place both intra- and inter-molecularly, but only between lysines that are located within a distance of 12 Å.

The DSP-treated Wzm proteins migrated as a smeary pattern on the SDS gel at higher molecular weights (**Fig. S1D, lanes 1 and 3**). In the presence of the reducing agent DTT, this smeary pattern of bands reverted to that closely similar to DTT-treated Wzm (**Fig. S1C**; **Fig. S1D, lanes 2 and 4**). This finding indicates that some bands might result from greater molecular weight Wzm complexes. In accord with this finding, when the purified Wzm proteins were separated by gel filtration chromatography, each band of Wzm identified by SDS-PAGE analysis eluted as part of four different sizes of complexes (**Fig. S1E**). The estimated molecular weight of these complexes were 350 (band 1), 230 (band 2), 120 (band 3) and 60 (band 4) kDa, corresponding to dodecamers, octamers, tetramers, and dimers, respectively. Taken together, these results lead to the information that the purified Wzm proteins exist as a heterogeneous mixture of oligomers, whose predominant species are the dimers and tetramers (**Fig. S1E**). In addition, the gel filtration chromatography analysis enabled us to identify that the Wzm oligomer with the lowest molecular weight is the dimer. It is worth mentioning that, intriguingly, these bands did not run as exact multiple of the monomer unit on the lanes of the SDS-PAGE gel.

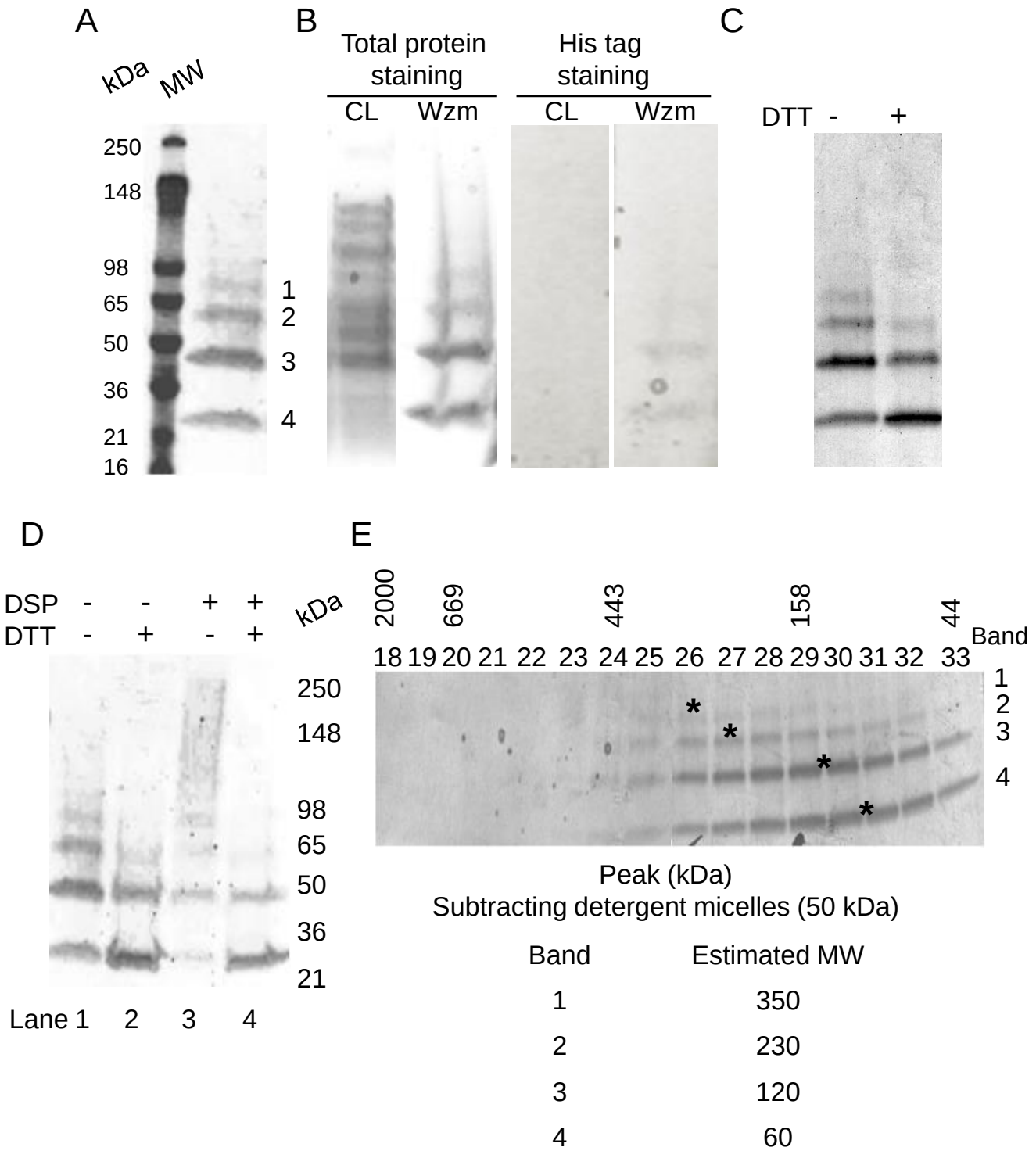

Figure S1

<u>Figure S1:</u> Detection of the oligomerization of the Wzm protein. (**A**) The SDS-PAGE gel analysis of the membrane, DDM-extracted, and purified TMD Wzm protein. 7 μg of protein was loaded onto the SDS-PAGE gel, which was stained by the GelCode Blue Stain Reagent, whose sensitivity is 25 ng. The molecular-weight markers are indicated on the left side. Purified Wzm protein has four bands, designated from the top to the bottom as bands 1, 2, 3 and 4; (**B**) The identification of Wzm proteins. Cell lysates

(CL) and purified Wzm were stained by the GelCode Blue Stain Reagent (the first two panels on the left side). The proteins were identified by specific His^+ tag staining, on the first two panels of the right side. Therefore, in this case the staining was specific His^+ tagged proteins, showing the absence of bands on CL lane on the right side of panel **B**; (**C**) Treatment of the purified Wzm protein with 30 mM dithiothreitol (DTT). The SDS-PAGE gel shows the Wzm protein in the presence (+) or absence (-) of DTT; (**D**) Dithiobis[succinimidylpropionate] (DSP) cross linking analysis of the purified Wzm protein in the presence (+) or the absence (-) of DTT. The molecular-weight markers are indicated on the right side; (**E**) Gel-filtration chromatography analysis of the purified Wzm protein based on their native sizes. Fractionated proteins were run on an SDS-PAGE gel (separating proteins based on their sizes) and visualized as in **A**. In other words, the SDS-PAGE gel shows which polypeptides contribute to the native Wzm protein complexes. Protein molecular weight markers, which were used to calibrate the column, are indicated on the top of the gel. Asterisks present the peak fractions of the different Wzm protein bands. The estimated molecular sizes belonging to each Wzm band are illustrated below the SDS-PAGE gel. All protein samples were not heated up prior to running on the SDS-PAGE gel, as heating up caused the aggregation of the protein samples and smeared patterns on the SDS-PAGE gel lanes. In this figure, the SDS-PAGE gel images are representative of two independent experiments.

2. *In vitro* coupled transcription and translation (IVTT) of polypeptides. Protein expression was accomplished by IVTT reaction supplemented with [^{35}S]methionine, as previously described (**Fig. S2** and **Fig. S3**).[5, 6] The total plasmid amount was 140 ng in a 25 µl reaction. In the case of the Wzm protein expression, rRBCM or microsomes (Promega, Madison, WI) were included before the protein synthesis. The reactions were centrifuged and pellets containing membrane-incorporated proteins were washed three times with MBSA buffer (150 mM NaCl, 0.1% BSA, 10 mM MOPS-Na, pH 7.4). In the case of the Wzt protein expression, the lipids were excluded and the IVTT reaction was used directly for analysis. [^{35}S]methionine-labeled polypeptides were separated on an SDS-PAGE gel and visualized by autoradiography.

3. Extended N terminus of the Wzt protein is implicated in the Wzt-Wzm coupling interaction. *In vivo* complementation experiments between the *E. coli 09* and *E. coli 08* PS ABC transporters indicated that the N terminus of the Wzt protein is involved in the coupling interaction between Wzt and Wzm.[7] Therefore, we wanted to examine whether this mechanism exists for the Wzt/Wzm proteins in *P. aeruginosa* as well. We deleted 118 residues from the N terminus of the Wzt protein (Wzt Δ13-131) to probe the Wzt-Wzm interaction using a closely similar approach as that mentioned above**.** This deletion includes Walker A as well as the Q loop, which is implicated in the contact interface between NBD and TMD.[8-12] Unfortunately, this deletion Wzt mutant was not expressed in the inclusion bodies. Hence, we decided to employ the *in vitro* coupled transcription and translation (IVTT) expression system [5, 6] for the synthesis of Wzt Δ13-131. We found that this deletion Wzt mutant did not interact with Wzm (**Fig. S2**), indicating that the extended N terminus of the Wzt protein is indeed required for the Wzt-Wzm coupling interaction in *P. aeruginosa*. Moreover, we provide direct experimental evidence that Wzm and Wzt interact *in vitro* (**Fig. S3**).

4. Supplementary evidence for the interactions of Wzm and Wzt. The interaction of the Wzt protein with its cognate Wzm protein is mediated by the N terminus of the Wzt protein and is required for efficient PS export.[7] Further, both the N and C termini of the Wzt proteins are required for the A-band PS export in *E. coli* O9a and *E. coli* O8.[13] Interestingly, although conserved in Wzt proteins, the N termini are not functionally interchangeable among bacteria. For example, the N terminus of Wzt of *E. coli* O8 could not facilitate the PS export when it was expressed with the C terminus of Wzt from *E. coli* O9a,[13] indicating that specific interactions between Wzm and the extended N terminus of Wzt lead to the A-band

PS efflux. In *P. aeruginosa*, all prior biochemical studies were focused on the synthesis and ligation of PS (**Fig. 1A**),[14-16] leaving the export mechanisms of the ABC transporter poorly understood.

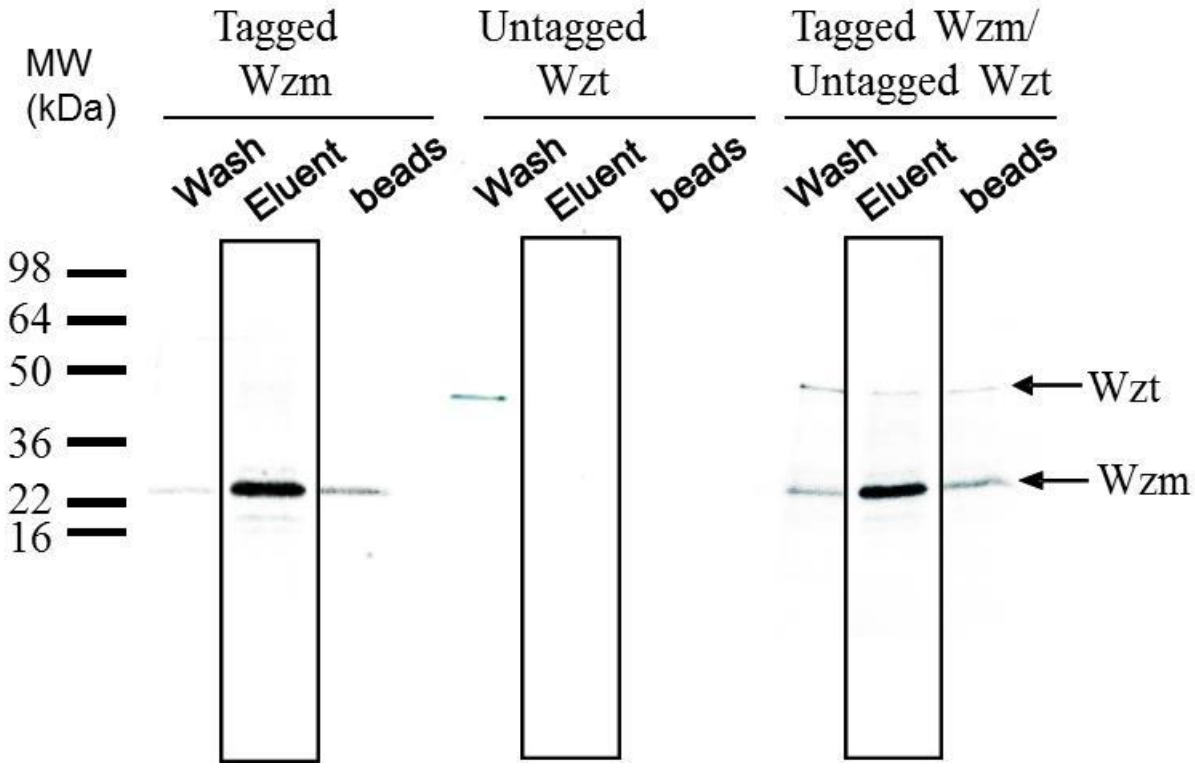

Figure S2

Figure S2: The subunits of the A-band PS ABC transporter interact *in vitro*. This SDS-PAGE gel analysis shows the interaction between Wzm and Wzt. 10×His^{+}-tagged Wzm was detergent-extracted and incubated with Ni^{+2} nitrilotriacetic acid-charged (Ni-NTA) beads (the left panel) or when mixed with Wzt protein (the right panel). The untagged Wzt protein did not bind to the Ni-NTA beads (the middle panel). Wzm was prepared by IVTT in the presence of rabbit red blood cell membranes (rRBCM) and the pellet was incubated with 3% DDM to release the Wzm protein. Wzt was prepared using IVTT as well, but in the absence of rRBCM and detergent. All reactions were mixed with the Ni-NTA beads in 1 ml of 500 mM NaCl, 50 mM NaH_2PO, 0.2% DDM, pH8.0 and 2.5 mM ATP for one hour and washed with the same buffer (Wash 1), and with 25 mM imidazole (Wash 2). The elution was accomplished with 250 mM imidazole within the same buffer (Eluent). After elution, the Ni-NTA beads were incubated with 1 ml SDS loading buffer for 5 minutes, and spun down. The supernatant was used for further analysis (Beads). 50 µl of each sample was run on a 12% SDS-PAGE gel and visualized by autoradiography. In this figure, the SDS-PAGE gel images are representative of five independent experiments.

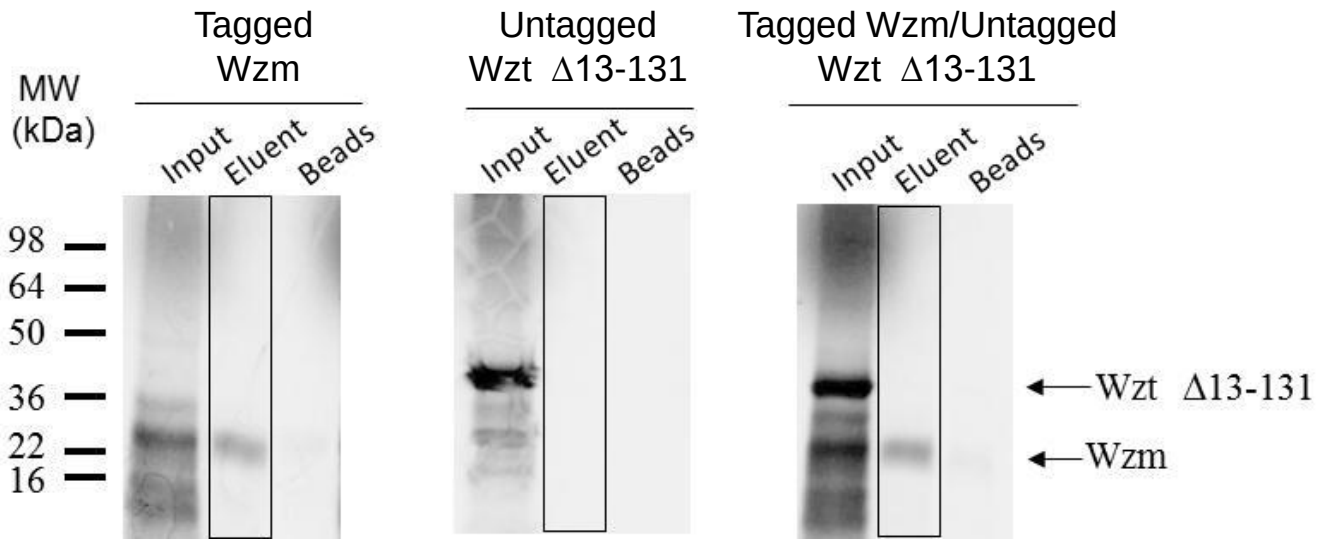

Figure S3

<u>Figure S3:</u> The Wzt protein interacts with Wzm through its extended N terminus. This SDS-PAGE gel analysis illustrates the interaction between Wzm and Wzt Δ13-131. 10×His^{+}-tagged Wzm was detergent-extracted and incubated with Ni^{+2} nitrilotriacetic acid-charged (Ni-NTA) beads (the left panel) or mixed with Wzt Δ13-131 (the right panel). The untagged Wzt Δ13-131 protein did not bind to the Ni-NTA beads (the middle panel). Wzm and Wzt Δ13-131 proteins were synthesized in a 25 μl *in vitro* coupled transcription and translation (IVTT) reaction. In the case of Wzm, the reactions were centrifuged and the pellets were washed three times with 1 ml MBSA buffer (150 mM NaCl, 1% BSA, 10 mM MOPS, pH 7.4). The pellet was incubated with 3% DDM to release the Wzm protein. The untagged Wzt was prepared as Wzm, but without rRBCM. The reactions were centrifuged at 16,000×*g* for 15 minutes. The supernatants were used for further experimentation. All reactions were mixed with the Ni-NTA beads in 1 ml of 500 mM NaCl, 50 mM NaH_2PO, 0.2% DDM, pH8.0 and 2.5 mM ATP for one hour and washed with the same buffer (Wash 1), and with 25 mM imidazole (Wash 2). They were eluted with 250 mM imidazole in the same buffer (Eluent). After elution, the Ni-NTA beads were incubated with 1 ml SDS loading buffer for 5 minutes, and spun down. The supernatant was used for further analysis (Beads). 50 μl of each sample was run on a 12% SDS-PAGE gel and visualized by autoradiography. In this figure, the SDS-PAGE gel images are representative of three independent experiments.

5. Comparison between *P. aeruginosa* Wzm and *E. coli K1* KspM1 proteins.

A

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Wzm     MLLGLSRSLWGYRGFVLGS-VKREFQSRYRGSLFGALWTVLNPLSMIVVYTVIFSQVMRA 59
KspM1   ----MARSGFEVQKVTVEALFLREIRTRFGKFRLGYLWAILEPSAHLLILLGILGYVMHR 56
            ::** :  : ..: : . **:::*:    :* **::*:* : :::   *:. **:

Wzm     RLPGVDDGLAYSVYLCAGLLTWGLFAEITSRSQSMFIENANLLKKISFPRICLPVIVLLN 119
KspM1   TMP----DISFPVFLLNGLIPFFIFSSISKRSIGAIEANQGLFNYRPVKPIDTIIARALL 112
         :*    .:::.*:*  **:.: :*:.*:.** . :  * .*::  ..  *   :   *

Wzm     AGVNFAIILALFLGFLALSGR-LPGAALLALVPLLAIQVLFAAGLGMILGVLNVFFRDVG 178
KspM1   ETLIYVAVYILLMLIVWMTGEYFEITNFLQLVLTWSLLIILSCGVGLIFMVVGKTFPEMQ 172
          : :. :  *:: :: ::*. :  : :* **   :: ::::.*:*:*: *:.  * ::

Wzm     QLFGICLQFWFWLTPIVYPIGILPEGIRSLIELNPMTALMRSYQQLFLHGQWPDWPSLLP 238
KspM1   KVLPILLKPLYFISCIMFPLHSIPKQYWSYLLWNPLVHVVELSREAVMPGYISEGVSLNY 232
        ::: * *:  :::: *::*:  :*:   * :  **:. ::.  :: .: *  .:  **

Wzm     ITLLALLLCALGLRLFRQRVGEMVDEL 265
KspM1   LAMFTLVTLFIGLALYRTREEAMLTS- 258
        :::::*:   :** *:* *   *: .
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B

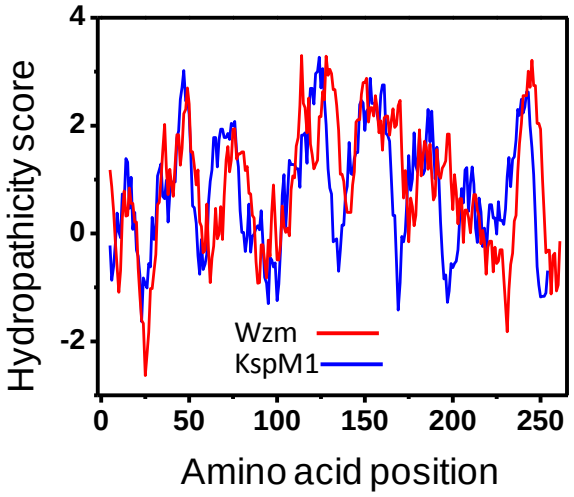

Figure S4: Comparison between *P. aeruginosa* Wzm and *E. coli K1* KspM1 proteins. (**A**) Alignment of the transmembrane domains (TMD) of ABC transporter of *P. aeruginosa* Wzm and *E. coli* K1 KspM1 using the ClustalW 1.74 software.[17] Asterisks, colons and dots denote identical, highly similar and similar residues, respectively; (**B**) The Wzm and KspM1 proteins have nearly identical hydrophobicity plots. Plots were generated using the ProtScale software.

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