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Biochemical Reconstitution and Characterization of Multicomponent Drug Efflux Transporters

Martin Picard, Elena B. Tikhonova, Isabelle Broutin, Shuo Lu, Alice Verchère, and Helen I. Zgurskaya

Abstract

Efflux pumps are the major determinants in bacterial multidrug resistance. In Gram-negative bacteria, efflux transporters are organized as macromolecular tripartite machineries that span the two-membrane cell envelope of the bacterium. Biochemical data on purified proteins are essential to draw a mechanistic picture of this highly dynamical, multicomponent, efflux system. We describe protocols for the reconstitution and the in vitro study of transporters belonging to RND and ABC superfamilies: the AcrAB–TolC and MacAB–TolC efflux systems from *Escherichia coli* and the MexAB–OprM efflux pump from *Pseudomonas aeruginosa*.

Key words Membrane protein purification, Proteoliposomes, Transport kinetics

1 Introduction

1.1 Multicomponent Drug Transporters

Gram-negative bacteria are notoriously more resistant to antibiotics than Gram-positive bacteria. For a long time, the outer membrane was believed to be the major reason why Gram-negative pathogens are recalcitrant to antibiotic treatment [1]. The seminal finding that *Escherichia coli* cells contain multidrug efflux transporters changed dramatically our view on how these bacteria resist antibiotics [2, 3]. Current models postulate that intrinsic multidrug resistance of Gram-negative bacteria is the result of synergy between reduced uptake of drugs across the outer membrane and active drug efflux from the inner membrane [4–6]. This synergy is possible because multidrug efflux transporters of Gram-negative bacteria function together with proteins belonging to the Membrane Fusion Protein (MFP) and the Outer Membrane Factor (OMF) families [7]. MFPs are located in the periplasm and act on both membranes to enable drug efflux across the whole cell envelope directly into the medium. On the inner membrane (IM), MFPs associate with drug efflux

transporters and stimulate their activities [8–10]. On the outer membrane (OM), they recruit OMF channels that enable expulsion of drugs into the medium [11, 12]. The three components form large multiprotein assemblies that traverse both the inner and outer membranes of Gram-negative bacteria. Working together as a well-coordinated team, they achieve the direct extrusion of substrates from the cytoplasm and/or the periplasm into the medium.

Drug transporters that associate with MFPs and OMF channels can belong to any of the three major superfamilies of proteins: RND (Resistance-Nodulation-Cell Division), ABC (ATP-Binding Cassette) and MF (Major Facilitator) superfamilies [13–16]. These transporters are structurally and mechanistically very diverse. ABC transporters are driven by ATP hydrolysis, whereas drug efflux by RND and MF pumps is coupled to transport of protons. MF transporters are thought to function as monomers, whereas ABC and RND transporters are dimers and trimers, respectively. Surprisingly, MFPs that associate with these transporters are structurally very similar. Furthermore, in *E. coli* and other enterobacteria, multidrug transporters share the same OM channel. How transporters with different architectures and molecular mechanisms assemble into tri-partite complexes with MFPs and OM channels and how transporter activities are coupled to efflux across the OM are the main questions of our research programs.

This chapter is focused on reconstitution and in vitro activities of transporters belonging to RND and ABC superfamilies that include the best-studied RND transporters *Escherichia coli* AcrB and *Pseudomonas aeruginosa* MexB functioning with MFPs AcrA/MexA and the OM channels TolC/OprM, respectively [10, 17] and the ABC transporter MacB with MFP MacA and TolC [18–20].

1.2 Reconstitution of Proteoliposomes and Transport Across One Membrane

Reconstitution of proteoliposomes is a tried-and-tested methodology that requires expertise and skills. It allows incorporation of the purified proteins into lipid membranes of known composition that create a closed compartment necessary for the vectorial transport of substrates. Such reconstituted proteoliposomes enable specific characterization of membrane protein transporters outside of the complexity of their native environment.

Zgurskaya and Nikaido achieved the first successful functional reconstitution of AcrB in 1999 [10], where they showed that proteoliposomes containing AcrB catalyze the extrusion of fluorescent phospholipids to protein-free acceptor vesicles in the presence of a proton gradient. This study provided the first in vitro experimental evidence that AcrB is a proton antiporter and that its activity is catalyzed in the presence of AcrA and Mg^{2+} . Proteoliposomes can also be used to extract dynamic and kinetic information: for example, the functional reconstitution of CzcA allowed characterization of the kinetics of heavy metal extrusion by this transporter, which is responsible for heavy metal resistance in *Ralstonia* sp. [21],

whereas reconstitution of MacAB led to the proposal of a kinetic mechanism of this ATP-dependent transporter, which depends on MFP MacA [18, 20, 22]. Lastly, proteoliposome reconstitution can also be used to address the molecular mechanism of transport. Indeed, upon reconstitution of AcrD from *E. coli*, Aires and Nikaido reported that gentamicin can be transported from either side of the membrane, thereby providing experimental evidence in support of a controversial cytoplasmic access of the drug toward its transport side [8]. At the atomic level, the group of Edward Yu obtained a high-resolution structure of the RND CusA from the efflux pump CusCBA *E. coli* [23, 24]. They designed a functional assay for characterization of the proton transport pathway suggested by their X-ray crystal structure and confirmed that CusA mutants with substitutions at amino acid residues involved in the putative proton pathway were transport defective. More recently, Verchère and colleagues have described the first successful reconstitution of the whole tripartite efflux pump MexA-MexB-OprM from *P. aeruginosa*. This study highlights the mutual interplay between the various partners of the pump [17].

1.3 Energizing the System

Bacterial efflux pumps catalyze the active transport of substrates at the expense of proton counter transport or ATP hydrolysis. In the latter case, the system is energized upon addition of ATP and the activity of ABC transporters can be indirectly investigated by titrating the release of Pi by colorimetry [25] or by ^{32}P γ -phosphate counting [20]. Using such methodologies, it was shown that the MFP MacA does stimulate the basal activity of the macrolide transporter MacB [20]. Note that the use of transition state analogs such as aluminum fluoride, magnesium fluoride, or sodium orthovanadate or nonhydrolyzable nucleotide analogs can provide very useful mechanistic insights [18].

In the case of pmf-dependent transporters, several strategies can be followed to trigger the function of a transporter. Generation of a proton gradient can be achieved simply upon incubation of the proteoliposome suspension in a buffer which pH is different from that used to prepare the liposomes [10] or even more simply by adding HCl to the proteoliposome suspension [17]. However, such procedures are believed to generate gradients that are not very stable. More stable gradients can be obtained with proteoliposomes loaded with NH_4Cl that are subsequently diluted into an iso-osmotic buffer containing KCl, which leads to diffusion of uncharged NH_3 and acidification of the proteoliposome lumen [21]. Proton gradient can also be generated in the other direction (alkaline-inside) by diluting proteoliposomes containing potassium acetate in a potassium acetate-free buffer [26]. The most stable gradients are obtained when the protein under study is co-reconstituted together with bacteriorhodopsin, the famous archaeobacterial proton transporter from *Halobacterium salinarium* [27, 28].

Another classical strategy involves the use of valinomycin, a potassium-selective ionophore. Addition of valinomycin to KCl-containing liposomes that have been diluted into a buffer containing NaCl allows potassium to leak out of the vesicle down its concentration gradient. This creates a membrane potential across the membrane that is spontaneously compensated by an influx of protons, hence a proton gradient [8, 29, 30].

1.4 Fluorescent, Nucleic Acid, and Radioactive Probes

The choice of a quantitative readout for the monitoring of transport is the most critical parameter. In case of amphiphilic and hydrophobic substrates, such as most antibiotics and drugs, assays relying on the transmembrane transport are often difficult to design and interpret because substrates can bind nonspecifically to the membrane or leak out of the vesicles. In such cases, it is possible to deduce the transport activity from the associated variations of the pH inside the liposome or ATP hydrolysis rates. For example, dissipation of a proton gradient is often monitored by using pH-sensitive probes such as pyranine [8, 29, 30]. However in multidrug efflux transporters, proton translocation or ATP hydrolysis processes not always are stoichiometrically coupled to the substrate transfer. Therefore, energy depletion assays may not serve as a quantitative measure of the substrate transport.

The direct transport of substrates can sometimes be deduced from the distribution of the substrate itself. In that case, studies often resort to the use of radioactively labeled substrates such as heavy metals [21], or antibiotics [8] but assays with fluorescent substrates can also be performed [10]. Fluorescently labeled lipids have been demonstrated to be useful reporters of activities of multidrug efflux pumps [10]. In addition, Hoechst 33,342 or ethidium bromide are commonly used because they become highly fluorescent once intercalated into lipid bilayers or into DNA molecules that can be loaded into the proteoliposome lumen [26, 27] or into the lumen of an acceptor vesicle [17]. Note that we demonstrated that incorporation of stable RNA scaffolds is much more efficient than incorporation of DNA [17].

2 Materials

2.1 Purification of Inner Membrane Transporters

2.1.1 *AcrB*^{His} Purification

1. Luria-Bertani (LB) broth or 2×TY (16 g Bacto-tryptone, 10 g Yeast extract, 5 g NaCl for 1 L of medium) were used as growth media.
2. Unless otherwise noticed, 100 µg/ml ampicillin were added to the growth medium for plasmid selection.
3. Syringes and syringe acetate 0.22 µm filters.
4. Rotors JLA8.1, 70 Ti, TLA 100 rotors (Beckman Coulter).
5. Beckman Coulter centrifuges Avanti J-20 XP and Optima LE-80K.

6. AcrB Cell Buffer: 20 mM Tris-HCl (pH 8.0), 0.1 M NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF) in MilliQ water.
7. AcrB Cell Lysis Buffer: AcrB cell buffer supplemented with 1 mM MgCl₂ and 0.1 mg/ml DNase I.
8. French Pressure Cell Press (SLM Instruments, Inc.).
9. 0.5 M disodium ethylenediaminetetraacetate (Na₂EDTA) (pH 8.0) stock solution in MilliQ water.
10. 6 M Urea solution in MilliQ water.
11. 10% Triton X-100 (TX) prepared in buffers as indicated for each protein purification protocol.
12. AcrB Binding Buffer: 20 mM Tris-HCl (pH 8.0), 0.5 M NaCl, 1 mM PMSF, 5 mM imidazole in MilliQ water.
13. Metal-chelating Sepharose resin charged with Cu²⁺.
14. Column buffers: AcrB Binding Buffer supplemented with 0.2% TX and a linear imidazole gradient of 5–200 mM.

2.1.2 *MexB*^{His} Purification

1. Syringes and syringe acetate 0.22 µm filters.
2. Rotors JLA8.1, 45Ti, SW32 rotors (Beckman Coulter).
3. Beckman Coulter high performance centrifuge (Avanti JXN-26), Beckman ultracentrifuge (Optima XPN).
4. MexB Cell Buffer: 20 mM Tris-HCl (pH 7.0), 0.15 M NaCl, supplemented with a protease inhibitor tablet (Roche) in MilliQ water.
5. MexB Membrane Buffer: 20 mM Tris-HCl (pH 7.4), 20% glycerol, 10 mM imidazole, 500 mM NaCl.
6. Cell-disrupter (Cell-D from Constant LTD).
7. 10% dodecyl maltoside (DDM) in MilliQ water.
8. MexB Binding Buffer: 10 mM Bis-Tris 10 (pH 7.4), 0.2% DDM, 20% glycerol, 10 mM imidazole and 500 mM NaCl in MilliQ water.
9. MexB storage buffer: 10 mM Bis-Tris (pH 7.4), 20% glycerol (w/v), 500 mM NaCl, 0.2% DDM (w/v).
10. BCA Reagent A, containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide.
11. BCA Reagent B containing 4% cupric sulfate.
12. A plastic Bio-Rad column for gravity, step purification.
13. Column buffers: MexB Binding Buffer supplemented with imidazole for a concentration step-gradient as follows: 10, 50, 300 mM.
14. A PD-10 column (GE Healthcare).
15. Ni-NTA resin (Macherey Nagel).

2.1.3 *MacB^{His}*

Purification

1. 20% L-arabinose in MilliQ water.
2. MacB Cell Washing Buffer: 10 mM Tris-HCl (pH 8.0), 0.1 M NaCl in Milli Q water.
3. MacB Cell Lysis Buffer: 10 mM Tris-HCl (pH 8.0), 5 mM Na₂EDTA, 1 mM PMSF and 100 µg/ml lysozyme in MilliQ water.
4. Sonifier 450 (Branson).
5. MacB Membrane Buffer: 20 mM Tris-HCl (pH 7.0), 200 mM NaCl, 1 mM PMSF, 0.05 mM β-mercaptoethanol in MilliQ water.
6. Cu²⁺-charged NTA column (Novagen).
7. Column washing and elution buffers: MacB Membrane Buffer supplemented with 0.2% TX and either one of 5, 20, 40, 100 and 250 mM of imidazole.
8. MacB Storage Buffer: 20 mM HEPES-KOH (pH 7.7), 200 mM NaCl, 1 mM PMSF, 0.2% TX, 2 mM DTT, 50% glycerol in MilliQ water.

2.2 Purification of Accessory Proteins

2.2.1 *AcrA^{His} Purification*

1. 1 M isopropyl β-D-1-thiogalactopyranoside (IPTG) stock prepared in MilliQ water.
2. AcrA Binding Buffer: 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM PMSF, 10% glycerol with 5 mM imidazole and lysozyme (100 µg/ml) in MilliQ water.
3. Sonifier 450 (Branson).
4. Cu²⁺-charged His-binding resin (Novagen).
5. Washing and elution buffers: AcrA Binding Buffer supplemented with imidazole for a concentration step-gradient as follows: 5, 20, 50 and 500 mM.
6. AcrA Storage Buffer: 50% glycerol, 20 mM HEPES-KOH, (pH 7.7), 500 mM NaCl, 1 mM PMSF.

2.2.2 *MexA^{His}*

Purification

1. 20% L-arabinose in MilliQ water.
2. Selection marker: 25 µg/ml chloramphenicol in sterile LB medium, filter sterilized.
3. Syringes and syringe acetate 0.22 µm filters.
4. Rotors used in all protocols: JLA8.1, 45Ti, SW32 rotors (Beckman).
5. Beckman high performance Centrifuges (Avanti JXN-26), Beckman ultracentrifuge (Optima XPN).
6. MexA Cell Buffer: 20 mM Tris-HCl (pH 7.0), 0.15 M NaCl, supplemented with a protease inhibitor tablet (Roche) in MilliQ water.

7. Cell-disrupter (Cell-D from Constant LTD).
8. 10% β -octyl glucopyranoside (β -OG) in MilliQ water.
9. 10% N-laurylsarcosine sodium salt solution from Sigma Aldrich.
10. MexA Membrane Buffer: 10 mM Tris-HCl (pH 8.0), 10% glycerol, 15 mM imidazole in MilliQ water.
11. MexA Binding Buffer (B): 10 mM Tris-HCl (pH 8.0), β -OG 0.9%, 10% glycerol, 15 mM imidazole in MilliQ water.
12. MexA storage Buffer: 10 mM Tris-HCl (pH 8.0), 10% glycerol, in MilliQ water.
13. Bicinchoninic acid (BCA) from Sigma Aldrich.
14. Plastic Bio-Rad columns.
15. Column buffers: MexA Binding Buffer supplemented with 0.9% β -OG and increasing imidazole concentrations (10, 50, 300 mM).

2.2.3 *MacA*^{His} Purification

1. MacA Membrane Buffer: 20 mM HEPES-KOH (pH 7.7), 500 mM NaCl, 5 mM imidazole and 1 mM PMSF in MilliQ water.
2. Column washing and elution buffers: MacA Membrane Buffer containing 0.2% TX and 5, 50, 500 mM imidazole.
3. MacA Dialysis Buffer: polyethylene glycol 20,000 in 20 mM HEPES-KOH (pH 7.7), 200 mM NaCl, 1 mM PMSF, 0.2% TX in MilliQ water.
4. MacA Storage Buffer: 20 mM HEPES-KOH (pH 7.7), 200 mM NaCl, 1 mM PMSF, 0.2% TX, 50% glycerol in MilliQ water.

2.3 Purification of Outer Membrane Channels

2.3.1 *TolC*^{His} Purification

1. 1 M IPTG prepared in MilliQ water.
2. TolC Cell Washing Buffer: 20 mM Tris-HCl (pH 7.5) prepared in MilliQ water.
3. TolC Cell Lysis Buffer: 20 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 1 mM PMSF and 0.05 mg/ml DNAase I (Sigma) prepared in MilliQ water.
4. French Pressure Cell Press (SLM Instruments, Inc.).
5. TolC Membrane Buffer: 20 mM Tris-HCl (pH 7.5), 100 mM NaCl and 1 mM PMSF prepared in MilliQ water.
6. Solubilization buffer: 5% TX, 20 mM Tris-HCl (pH 7.5), 1 mM PMSF and 100 mM NaCl prepared in MilliQ water.
7. Protein Assay (Bio-Rad).
8. His-Binding resin (Novagen).

9. TolC Binding Buffer: 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM PMSF, 0.2% TX and 5 mM imidazole prepared in MilliQ water.
10. Column washing buffers: TolC Binding Buffer supplemented with imidazole for a step-gradient of 20, 50, and 100 mM concentrations.
11. TolC^{His} Elution Buffer: TolC Binding Buffer supplemented with 500 mM imidazole.
12. TolC^{His} Storage Buffer: 20 mM Tris-HCl (pH 7.5), 1 mM PMSF, 500 mM NaCl, and 0.2% TX prepared in MilliQ water.

2.3.2 *OprM*^{His} Purification

1. 20% L-arabinose in MilliQ water.
2. Selection marker: 25 µg/ml chloramphenicol in sterile LB medium, filter sterilized.
3. Syringes and syringe acetate 0.22 µm filters.
4. Rotors used in all protocols: JLA8.1, 45Ti, SW32 rotors (Beckman Coulter).
5. Beckman Coulter high performance centrifuge (Avanti JXN-26), Beckman Coulter ultracentrifuge (Optima XPN).
6. *OprM* Cell Buffer (A): 20 mM Tris-HCl (pH 7.0), 0.15 M NaCl, supplemented with a protease inhibitor tablet (Roche) in MilliQ water.
7. Cell-disrupter (Cell-D from Constant LTD).
8. 10 Octyl-polyoxyethylene (C8POE) in MilliQ water.
9. 10% β-OG in MilliQ water.
10. *OprM* Binding Buffer (B): 10 mM Tris-HCl 20 (pH 8), 0.9% β-OG, 10% glycerol, 15 mM imidazole in MilliQ water.
11. BCA Reagent A, containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide.
12. BCA Reagent B containing 4% cupric sulfate.
13. Plastic Bio-Rad columns.
14. Column buffers: *OprM* Binding Buffer supplemented with imidazole for a step-gradient of imidazole (washing step at 25 mM imidazole, elution at 300 mM imidazole).

2.4 Purification of the tRNA Scaffold

1. 1 M IPTG prepared in MilliQ water.
2. Selection marker: 100 µg/ml of ampicillin in sterile LB medium, filter sterilized.
3. Syringes and syringe acetate 0.22 µm filters.
4. Rotors used in all protocols: JLA8.1, 45Ti, SW32 rotors (Beckman).

5. RNA Cell Buffer: 20 mM Tris, 200 mM NaCl pH 7.5.
6. RNA Lysis Buffer: 40 mM of MgSO₄, 50 mM Na₃Citrate pH 5.6.
7. Phenol.
8. Ethanol.
9. Resource Q column (GE Healthcare, 50 ml).

2.5 Preparation of Proteoliposomes

2.5.1 *AcrB*

Reconstitution into Unlabeled and Fluorescent Vesicles

1. Sepharose G-25 prepacked column (GE Healthcare).
2. Prepacked metal-chelating Sepharose column, charged with Cu²⁺ (GE Healthcare).
3. 10% β-OG stock prepared in *AcrB* Binding Buffer (*see* Subheading 2.1.1).
4. Column buffers: 20 mM HEPES-KOH (pH 7.0), 500 mM KCl, 1 mM PMSE, 1.2% β-OG, 10% glycerol, 5 or 100 mM imidazole in MilliQ water.
5. *Escherichia coli* polar lipids (Avanti).
6. 1,2-Dipalmitoyl N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) phosphatidylethanolamine (N-NBD-PE) (Avanti).
7. 1,2-Dipalmitoyl N-(lissamine rhodamine B sulfonyl) phosphatidylethanolamine (N-Rh-PE) (Avanti).
8. Reconstitution Buffer: 25 mM HEPES-KOH (pH 7.0), 1 mM DTT, 100 mM KCl in MilliQ water.
9. Water bath sonicator (Branson, Model 1510).
10. SM-2 Adsorbent Bio-Beads (Bio-Rad).

2.5.2 *MexB*

Reconstitution into Vesicles

1. PD-10 prepacked column (GE Healthcare).
2. 10% β-OG stock prepared in *MexB* Binding Buffer (Subheading 2.1.2).
3. *MexB* Reconstitution Buffer: 25 mM HEPES-KOH (pH 7.0), 100 mM K₂SO₄, 2 mM MgSO₄ in MilliQ water.
4. 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) (Avanti).
5. Mini-extruder (Avanti).
6. Cholesterol, powder (Sigma Aldrich).
7. Pyranine 100 mM (Sigma Aldrich) prepared in MilliQ water.
8. Sonifier (GM 3100 generator, MS73 sonotrode).
9. SM-2 Adsorbent Bio-Beads (Bio-Rad).

2.5.3 Reconstitution of *MacAB* and *TolC* into Vesicles

1. *Escherichia coli* polar lipids (Avanti).
2. *MacAB* reconstitution buffer: 20 mM HEPES-KOH (pH 7.0), 5 mM DTT in MilliQ water.
3. 10% TX stock solution in *MacAB* reconstitution buffer.

4. MacAB reconstitution buffer supplemented with 100 mM KCl.
5. SM-2 Adsorbent Bio-Beads (Bio-Rad).
6. TLA 100 rotor (Beckman Coulter).
7. Proteoliposome Storage Buffer: 20 mM HEPES-KOH (pH 7.0), 5 mM DTT and 50 mM KCl in MilliQ water.
8. Bovine Serum Albumin (BSA) (Sigma Aldrich).

2.5.4 ATP Hydrolysis

1. 100 mM Adenosine-5'-Triphosphate (ATP) (Sigma Aldrich).
2. ^{32}P γ -phosphate-labeled ATP (3000 Ci mmol^{-1} , Amersham).
3. Reaction buffer: 20 mM HEPES-KOH (pH 7.0), 5 mM DTT, 50 mM KCl, 2 mM MgCl_2 in MilliQ water. Chill to 4 °C before use.
4. 10 mM Mg-ATP mix: 10 mM ATP, 10 mM MgCl_2 , 1 μl ^{32}P γ -phosphate-labeled ATP in 20 μl reaction buffer.
5. Stop buffer: 50 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 0.5% SDS, 200 mM NaCl, 0.5 mg/ml proteinase K in MilliQ water.
6. PEI-F cellulose (Millipore).
7. TLC running buffer: 10% formic acid, 0.5 mM LiCl in MilliQ water.
8. Storm PhosphoImager (GE Healthcare).
9. ImageQuant Software (Molecular Dynamics).

2.5.5 Reconstitution of OprM

1. 10% β -OG stock prepared in OprM Binding Buffer (*see* Subheading 2.3.2).
2. OprM Reconstitution Buffer: 25 mM HEPES-KOH (pH 7.0), 100 mM K_2SO_4 , 2 mM MgSO_4 in MilliQ water.
3. L- α -Phosphatidylcholine (Egg-PC) and 1,2-dioleoyl-snglycero-3-phosphoethanolamine (DOPE) (Avanti Polar lipids).
4. Mini-extruder (Avanti).
5. Sonifier (GM 3100 generator, MS73 sonotrode).
6. SM-2 Adsorbent Bio-Beads (Bio-Rad).

2.6 Energy Consumption Assays

1. AcrB, MexB, and MacB proteoliposomes prepared as described in Subheading 2.5.
2. Purified AcrA (sAcrA^{His}), MexA and MacA (*see* Subheading 2.2).
3. Pyranine (Molecular Probes, 100 mM in methanol).
4. Nigericin (Molecular Probes, 1 mM in methanol).
5. Buffer 25 mM HEPES-KOH (pH 7.0), 5 mM DTT and 0.1 M KCl.

6. Buffer 25 mM HEPES-KOH (pH 6.0), 5 mM DTT and 0.1 M NaCl.
7. 25 mM 2-(*N*-morpholino)ethane-sulfonic acid (MES) buffers with pH ranges of 4.5 to 6, 5 mM DTT and 0.1 NaCl.
8. Erythromycin (Sigma Aldrich) 200 mM stock in methanol.
9. Chloramphenicol (Sigma Aldrich) 200 mM stock in methanol.
10. Cloxacillin (Sigma Aldrich) 200 mM stock in methanol.
11. Sodium glycolate (Sigma Aldrich) 200 mM stock in water.
12. Sodium taurocholate (Sigma Aldrich) 200 mM stock in water.
13. Valinomycin (Sigma Aldrich) 10 mM stock in methanol.
14. 1 M MgCl₂ prepared in MilliQ water.
15. 0.5 M Na₂EDTA (pH 8.0) prepared in MilliQ water.
16. 100 mM ATP (Sigma Aldrich).
17. Trypsin (Sigma, 10 mg/ml in water).

2.7 *In Vitro* AcrB and MexB-Dependent Transport Assays

1. AcrB and MexB proteoliposomes prepared as described in Subheading 2.5.
2. Purified AcrA and MexA (*see* Subheading 2.2).
3. Purified tRNA scaffold (*see* Subheading 2.4).
4. Reconstituted TolC and OprM (*see* Subheading 2.5).
5. Buffer 25 mM HEPES-KOH (pH 7.0), 5 mM DTT and 0.1 M KCl.
6. Buffer 25 mM HEPES-KOH (pH 6.0), 5 mM DTT and 0.1 M NaCl.
7. 25 mM 2-(*N*-morpholino)ethane-sulfonic acid (MES) buffers with pH ranges of 4.5–6.0, 5 mM DTT and 0.1 NaCl.
8. Erythromycin (Sigma) 200 mM stock in methanol.
9. Chloramphenicol (Sigma) 200 mM stock in methanol.
10. Cloxacillin (Sigma) 200 mM stock in methanol.
11. Sodium glycolate (Sigma) 200 mM stock in water.
12. Sodium taurocholate (Sigma) 200 mM stock in water.
13. Valinomycin (Sigma) 10 mM stock in DMSO.
14. Ethidium Bromide (Sigma) 2 mM stock in water.
15. 1 M MgCl₂ prepared in MilliQ water.
16. 0.5 M Na₂EDTA (pH 8.0) prepared in MilliQ water.
17. 10% DDM stock prepared in MilliQ water.

3 Methods

3.1 Purification of Inner Membrane Transporters

3.1.1 *AcrB*^{His} Purification

1. Grow *E. coli* DH5 α cells with pUC151A plasmid in LB medium supplemented with ampicillin (100 mkg/ml) to induce until OD₆₀₀ reaches 1.8 (*see Note 1*).
2. Harvest the cells by centrifugation at 3220 $\times g$ for 20 min at 4 °C and wash once in AcrB Cell Washing Buffer.
3. Resuspend the cell pellet in the same buffer supplemented with 1 mM MgCl₂ and 0.1 mg/ml DNase I. Break the cells once by French press at 12,000 psi. After 10 min incubation on ice add 2 mM Na₂EDTA (pH 8.0).
4. Remove unbroken cells by centrifugation at 3220 $\times g$ for 20 min at 4 °C.
5. Isolate membrane fractions by high speed centrifugation at 200,000 $\times g$ for 1 h at 4 °C.
6. Resuspend the membrane pellet in AcrB Cell Washing Buffer. Slowly add an equal volume of 6 M urea at room temperature. Incubate for 10 min and collect the membrane fraction as described above.
7. Resuspend the resulting membrane pellet in AcrB Binding Buffer.
8. Extract AcrB^{His} overnight by slow addition of 10% TX prepared in AcrB Binding Buffer.
9. Remove insoluble fraction by centrifugation and load the supernatant onto a column packed with metal-chelating Sepharose charged with Cu²⁺ and pre-equilibrated with AcrB Binding Buffer supplemented with 0.2% TX (*see Note 2*).
10. Wash the column with AcrB Binding Buffer containing 0.2% TX and then with a linear imidazole gradient (5–200 mM). AcrB was found to elute with 100 mM imidazole. The protein was stable at 4 °C for at least 1 week.

3.1.2 *MexB*^{His} Purification

1. Grow *E. coli* C43 (DE3) Δ AcrB cells with pET22b-MexB^{His} [31] plasmid in 2xTY medium supplemented with 100 μ g/ml ampicillin until OD₆₀₀ reaches 0.6. Induce proteins expression with 1 mM IPTG overnight at 20 °C.
2. Harvest the cells by centrifugation at 9000 $\times g$ for 20 min at room temperature and wash once in ice-cold MexB Cell Buffer.
3. Resuspend the cell pellet in MexB Cell Buffer. Break the cells by a cell-disrupter (Cell-D from Constant LTD) at 4 °C, two passages at 2.4 kbar.
4. Remove unbroken cells by centrifugation at 9000 $\times g$ for 20 min at 4 °C. Repeat the centrifugation step 2–3 times, until no pellet is visible after the centrifugation.

5. Isolate membrane fractions by high speed centrifugation at $100,000 \times g$ for 1 h at 4 °C.
6. Resuspend the membrane pellet in 10 ml MexB Membrane Buffer.
7. Determine the total membrane protein concentration by the BCA test (usually around 15 mg/ml).
8. Gently add a DDM solution to the membrane suspension under agitation. The final DDM concentration should be 2% (w/v) in a final volume, so that the total amount of the detergent is twice of that of the total membrane proteins. Solubilize the membrane fraction overnight at 4 °C under gentle stirring on an end-over-end rotator.
9. Remove insoluble fraction by centrifugation ($100,000 \times g$ for 1 h, 4 °C) and incubate the supernatant for 2 h with 3 ml of Ni-NTA resin (previously equilibrated with MexB Binding Buffer).
10. Transfer the resin into a plastic Bio-Rad column and collect the flow-through (FT).
11. Wash the resin with 50 ml of MexB Binding Buffer supplemented with 10 mM imidazole and then repeat the washing with 50 ml of MexB Binding Buffer supplemented with 50 mM imidazole.
12. Elute MexB with 10 ml of MexB Binding Buffer supplemented with 300 mM imidazole.
13. Remove imidazole by gel filtration on PD-10 column equilibrated with 10 mM Bis-Tris (pH 7.4), 20% glycerol (w/v), 500 mM NaCl, 0.2% DDM (w/v).

3.1.3 MacB^{His} Protein Purification

1. Grow *E. coli* cells carrying pBB^{His} [20] until $OD_{(100 \mu g/ml)} \sim 0.6$ at 37 °C in LB medium supplemented with ampicillin. Induce proteins expression at 37 °C with 0.1% arabinose for 3 h.
2. Collect cells by centrifugation at $3220 \times g$ for 20 min at 4 °C. Wash cells once with the ice-cold MacB Cell Washing Buffer.
3. Resuspend the cell pellet in the ice-cold MacB Cell Lysis Buffer. Incubate cells on ice for 30 min and then sonicate six times 30 s each on ice using Sonifier 450 (Branson).
4. Remove unbroken cells by centrifugation at $3220 \times g$ for 20 min at 4 °C.
5. Pellet the membrane fractions by centrifugation at $200,000 \times g$ for 1.5 h at 4 °C.
6. Resuspend the membrane fraction pellet in MacB Membrane Buffer. Slowly add an equal volume of the same buffer containing 10% TX (*see* **Notes 2** and **3**). Leave the membrane fraction for solubilization overnight at 4 °C with stirring.

7. Remove an insoluble material by centrifugation at $200,000 \times g$ for 40 min at 4 °C and load solubilized membrane fractions onto the Cu^{2+} -charged NTA column pre-equilibrated with MacB Membrane Buffer supplemented with 0.2% TX.
8. Wash the column using a step-gradient of imidazole in the same buffer at 5, 20, 40, 100 and 250 mM of imidazole (*see Note 3*).
9. Concentrate the purified protein if needed against polyethylene glycol 20,000 in MacB Dialysis Buffer following by dialysis in the same buffer containing 50% glycerol instead of polyethylene glycol. Protein can be stored at -20 °C.

3.2 Purification of Membrane Fusion Proteins *sAcrA^{His}*, *MexA^{His}* and *MacA^{His}*

3.2.1 *sAcrA^{His}* Purification

1. Grow *E. coli* BL21 cells harboring pEM13 [30] plasmid in LB medium with ampicillin (100 $\mu\text{g}/\text{ml}$) until OD_{600} reaches 0.3–0.4.
2. Induce the protein expression with 1 mM IPTG for 3 h at 37 °C.
3. Collect the cells at $3220 \times g$ for 15 min at 4 °C.
4. Lyse the cells in AcrA Binding Buffer supplemented with lysozyme (100 $\mu\text{g}/\text{ml}$) for 30 min on ice.
5. Break the cells by 4–6 rounds of sonication on ice using Sonifier 450 (Branson).
6. Remove unbroken cells by centrifugation at $3,220 \times g$ for 15 min at 4 °C.
7. Remove membrane fraction by centrifugation at $200,000 \times g$ for 40 min at 4 °C.
8. Load the supernatant onto a Cu^{2+} -charged His-binding column (Novagen) equilibrated with AcrA Binding Buffer.
9. Wash the column with AcrA Binding Buffer and an imidazole concentration step-gradient of 5, 20 and 50 mM imidazole in AcrA Binding Buffer.
10. Elute the protein by 500 mM imidazole-containing AcrA Binding Buffer.
11. To remove imidazole, dialyze the eluted AcrA first in AcrA Binding Buffer without imidazole.
12. For storage at -20 °C perform dialysis in AcrA Storage Buffer.

3.2.2 *MexA* Purification

1. Grow *E. coli* BL21 cells carrying pBAD33-GFPUv-MexA^{His} [32] plasmid in LB medium supplemented with 25 $\mu\text{g}/\text{ml}$ chloramphenicol until OD_{600} reaches 0.6. Induce the protein expression with 0.02% L-arabinose for 3 h at 20 °C.
2. Harvest the cells by centrifugation at $9000 \times g$ for 20 min at room temperature and wash once with the ice-cold MexA Cell Buffer.

3. Resuspend the cell pellet in the same buffer. Break the cells by a cell-disrupter (Cell-D from Constant LTD) at 4 °C, two passages at 2.4 kbar.
4. Remove unbroken cells by centrifugation at $9000 \times g$ for 20 min at 4 °C. Repeat centrifugation 2–3 times until no pellet is visible upon centrifugation.
5. Isolate membrane fractions by a high speed centrifugation at $100,000 \times g$ for 1 h 4 °C.
6. Resuspend the membrane pellet in 10 ml of MexA Membrane Buffer.
7. Determine the total membrane protein concentration with Bicinchoninic acid (BCA) test (usually around 10 mg/ml).
8. Gently add β -OG to the membrane suspension under agitation. The final β -OG concentration should be 2% (w/v) in a final volume, so that the total amount of the detergent is 40 times that of the membrane proteins. Solubilize the membranes overnight at room temperature under gentle stirring on an end-over-end rotator.
9. Remove insoluble fraction by centrifugation ($50,000 \times g$ for 45 min, 4 °C) and add 0.2% of N-lauryl sarkosyl to minimize binding of contaminants.
10. Incubate the supernatant for 2 h with 3 ml of Ni-NTA resin (previously equilibrated with MexA Binding Buffer supplemented with 0.2% N-lauryl sarkosyl (w/v)).
11. Transfer the resin into a plastic Bio-Rad column and collect the flow-through.
12. Wash the resin with 50 ml of MexA Binding Buffer supplemented with 0.2% N-lauryl sarkosyl (w/v).
13. Elute protein with MexA binding buffer containing 0.2% N-lauryl sarkosyl (w/v) and 300 mM imidazole.
14. Remove imidazole by gel filtration on a PD-10 column equilibrated with MexA Storage Buffer.

3.2.3 *MacA*^{His} Purification

1. Grow *E. coli* cells lacking OmpT protease (*see Note 4*) and harboring pBMA^{His} [20]. To produce MacA^{His} and purify membrane fractions use the same protocol described in Subheading 3.1.3.
2. Resuspend isolated membranes in MacA Membrane Buffer and extract the protein with 5% TX as described in Subheading 3.1.3.
3. Centrifuge the sample at $200,000 \times g$ for 30 min at 4 °C to remove insoluble fraction.

4. Load solubilized membrane proteins onto a Cu^{2+} -charged NTA column prewashed with MacA Membrane Buffer containing 0.2% TX.
5. Wash the column with 10 volumes of MacA Membrane Buffer containing 0.2% TX and then with 10 volumes of the same buffer supplemented with 50 mM imidazole.
6. Elute His-tagged MacA with MacA Membrane Buffer containing 0.2% TX and 500 mM imidazole.
7. Dialyze and concentrate eluted protein against MacA Concentrating Buffer. For storage dialyze protein samples in MacA Storage Buffer containing 50% glycerol. Protein can be stored at $-20\text{ }^{\circ}\text{C}$.

3.3 Purification of Outer Membrane Channels

3.3.1 Purification of TolC^{His}

1. Grow freshly transformed with pTolC^{His} *E. coli* ZK796 cells [20, 33] at $30\text{ }^{\circ}\text{C}$ until OD_{600} reaches 0.4, then induce TolC^{His} expression by 0.2 mM IPTG and continue cell growth overnight.
2. Pellet the cells by centrifugation at $3220 \times g$ for 20 min at $4\text{ }^{\circ}\text{C}$. Wash cell once with TolC Cell Washing Buffer.
3. Resuspend cells in TolC Cell Lysis buffer.
4. Break the cells by passing three times through French Pressure cell at 12,000 psi.
5. Remove unbroken cells by repeating centrifugation at $3220 \times g$ for 20 min at $4\text{ }^{\circ}\text{C}$.
6. Isolate total membrane fractions by ultracentrifugation at $200,000 \times g$ for 1.5 h at $4\text{ }^{\circ}\text{C}$.
7. Resuspend membranes in TolC membrane buffer. Measure the total protein concentration with Bio-Rad Protein Assay.
8. Dilute the membrane fractions with TolC Membrane buffer supplemented with 0.5% TX and 20 mM MgCl_2 to have the final protein concentration of 5 mg/ml. Centrifuge for 1 h as described in **step 6**.
9. Repeat washing of the membrane pellet with the same buffer one more time.
10. Resuspend the pellet, containing mostly the outer membrane proteins, in TolC solubilization buffer. Keep the protein concentration of 5 mg/ml. Stir the membrane sample at $4\text{ }^{\circ}\text{C}$ overnight, then remove insoluble fraction by centrifugation at $200,000 \times g$ for 1 h at $4\text{ }^{\circ}\text{C}$. The supernatant is ready for metal affinity purification.
11. Equilibrate Cu^{2+} -charged His-binding resin with TolC Binding Buffer.
12. Load the solubilized TolC^{His} sample (**step 10**), wash the column with 10 volumes of TolC Binding Buffer and then

with the same buffer containing imidazole step gradient (20, 50 and 100 mM).

13. Elute TolC^{His} with 5 volumes of TolC Binding Buffer containing 500 mM imidazole. At this step, collect one column volume fractions.
14. Remove imidazole and increase NaCl concentration to 500 mM by dialysis in TolC Storage Buffer. Protein will be stable on ice for a few weeks.

3.3.2 Purification of OprM

1. Grow *E. coli* BL21 cells with pBAD33-GFPuv-OprM^{His} [34] plasmid in LB medium supplemented with 25 µg/ml chloramphenicol until OD₆₀₀ reaches 0.6. Induce proteins expression with 0.02% Arabinose 3 h at 20 °C.
2. Harvest the cells by centrifugation at 9000 × *g* for 20 min at 4 °C and wash once in ice-cold OprM Cell Buffer.
3. Resuspend the cell pellet in the same buffer. Break the cells by a cell-disrupter (Cell-D from Constant LTD) at 4 °C, two passages at 2.4 kbar.
4. Remove unbroken cell by centrifugation at 9000 × *g* for 20 min at 4 °C. The suspension is centrifuged 2–3 times, until no pellet is visible after centrifugation.
5. Isolate membrane fractions by high speed centrifugation at 100,000 × *g* for 1 h 4 °C.
6. Resuspend the membrane pellet in 10 ml of 20 mM Tris-HCl (pH 8.0), 10% glycerol, 15 mM imidazole.
7. Add C₈POE (2% final) to the membrane suspension and incubate the mixture for 30 min at 37 °C. C₈POE is a detergent that specifically solubilizes bacterial inner membranes (*see Note 5*).
8. Centrifuge the resulting suspension at 100,000 × *g* for 1 h at 4 °C and resuspend the membranes in 10 ml of OprM Binding Buffer without β-OG.
9. Determine the total membrane protein concentration with Bicinchoninic acid (BCA) test (usually around 12 mg/ml).
10. Gently add β-OG on the membrane suspension under agitation. The final β-OG concentration should be 2% (w/v) in a final volume, so that the total amount of the detergent is 40 times that of the membrane proteins. Solubilize the membranes overnight at room temperature under gentle stirring in a conical flask (200 ml) with a stir bar.
11. Remove insoluble fraction by centrifugation (100,000 × *g* for 1 h, 4 °C).
12. Incubate the supernatant for 2 h with 3 ml of Ni-NTA resin (previously equilibrated with OprM Binding Buffer).

13. Transfer the resin into a plastic Bio-Rad column and collect the flow-through (FT).
14. Wash the resin with 50 ml of 10 mM Tris-HCl (pH 8.0), 10% glycerol (w/v), 25 mM imidazole, 0.9% β -OG (w/v).
15. Elute protein with 10 mM Tris HCl (pH 8.0), 10% glycerol (w/v), 300 mM imidazole, 0.9% β -OG (w/v).
16. Remove imidazole by gel filtration on a PD-10 column equilibrated with 10 mM Bis-Tris (pH 7.4), 20% glycerol (w/v), 500 mM NaCl, 0.2% DDM (w/v).

3.4 Purification of the tRNA Scaffold (Adapted from [35])

1. Grow *E. coli* XL1 cells carrying the pBSTNAV-AtRNA [35] plasmid in LB medium supplemented with 100 μ g/ml ampicillin until OD₆₀₀ reaches 0.6. Induce the protein expression with 1 mM IPTG 3 h at 37 °C.
2. Harvest the cells by centrifugation at 7000 $\times g$ for 20 min at room temperature and wash once in tRNA Cell Buffer (*see* Subheading 2.4).
3. Resuspend the cell pellet in the Cell Lysis Buffer. Extract RNA upon incubation with 40 ml of phenol during 2 h under gentle stirring at room temperature. The solution should turn from green to white.
4. Centrifuge the mixture 15 min at 3200 $\times g$.
5. Incubate the supernatant with two volumes of ethanol and 1/20 volume of NaCl 5 M. A precipitate forms.
6. Centrifuge 5 min at 3200 $\times g$.
7. Dry the pellet under the fume hood for 5 min and add 10 ml of distilled water.
8. Purify the RNA by anion chromatography on a Resource Q column equilibrated in 20 mM potassium phosphate (pH 6.5), wash with potassium phosphate (pH 6.5), 400 mM NaCl, and elute with a gradient of NaCl.
9. Dialyze the eluate against 10 mM potassium phosphate (pH 6.5) and 50 mM KCl.

3.5 Preparation of Proteoliposomes

3.5.1 Reconstitution of AcrB into Proteoliposomes

1. First, remove imidazole by gel filtration on a Sepharose G-25 column equilibrated with AcrB Binding Buffer with 0.2% TX.
2. Bind the purified AcrB on Cu²⁺-column again. To exchange TX with β -OG, wash the column extensively with a buffer containing 20 mM HEPES-KOH (pH 7.0), 500 mM KCl, 5 mM imidazole, 1 mM PMSF, 1.2% β -OG, and 10% glycerol.
3. Elute AcrB in the same buffer containing 100 mM imidazole. Use AcrB for reconstitution into proteoliposomes immediately (*see* Note 6).

4. Resuspend *E. coli* polar lipids in AcrB Reconstitution Buffer to the final concentration 20 mg/ml. Briefly sonicate the mixture using Water Bath Sonicator (Branson 1510).
5. Mix 50 µg of AcrB and 4.5 mg of lipids. Adjust the volume of mixture to 500 µl with reconstitution buffer and 1.1% β-OG.
6. Dilute the protein–lipid mixture with 3 ml of prechilled AcrB Reconstitution Buffer. Remove the detergent by dialysis against 1 L of AcrB Reconstitution Buffer containing 2 g of SM-2 Adsorbent Bio-Beads overnight at 4 °C.
7. To prepare control vesicles, substitute the protein sample with the same volume of AcrB Reconstitution Buffer.

3.5.2 Preparation of Fluorescent AcrB-Containing Donor Vesicles

1. Dissolve *E. coli* phospholipids, N-NBD-PE and N-Rh-PE lipids in chloroform.
2. Mix *E. coli* phospholipids, N-NBD-PE and N-Rh-PE lipids in 99:0.5:0.5 molar ratio to the final concentration of 20 mg/ml. Aliquot 0.25 ml of the lipid mixture into glass tubes, dry it down under nitrogen, and then under reduced pressure for 2 more hours.
3. Rehydrate the mixed lipid pellets in AcrB Reconstitution Buffer.
4. To reconstitute AcrB into fluorescent vesicles follow the procedure described in Subheading 3.5, step 1.
5. To prepare unlabeled vesicles use the same technique without addition of fluorescent phosphatidylethanolamine derivatives.

3.5.3 Reconstitution of MexA and MexB

1. Dissolve 3 mg cholesterol in 800 µl DOPC suspension in chloroform (at 25 mg/ml). Let the chloroform evaporate overnight under a vacuum bell (*see Note 7*).
2. Resuspend the latter in MexB Reconstitution Buffer supplemented with 3 mM pyranine, to the final concentration 20 mg/ml.
3. Incubate for 10 min at 37 °C and subject to sonication for 10 min with 30 s pulse/30 s pause cycles (power: 40 W).
4. Extrude the lipid suspension through 200 and 100 nm membranes (21 pass for each type of membrane), using an Avanti mini-extruder. Before extruding the liposome, make sure the membrane is moisturized by rinsing the whole set-up with the MexB Reconstitution Buffer (*see Note 8*).
5. Aliquot 100 µl of liposomes at 3 mM of lipids into a 96-well plate (cuvettes could be used for a smaller number of samples). Add increasing concentrations of β-OG and measure the optical density at 550 nm in each well. Determine the *R*_{sat}, which corresponds to the maximum optical density at a saturating level (*see Note 9*).

6. Solubilize the liposomes with β -OG at R_{sat} for 30 min at 4 °C.
7. Add the purified MexB at the 2.5:1 lipid to protein ratio w/w and the purified MexA at the 20:1 protein-to-protein ratio.
8. Remove the detergent by addition of Bio-Beads at a Bio-Bead-to-detergent ratio equal to 30 (w/w) and incubation overnight at 4 °C with gentle stirring.
9. Remove the nonencapsulated pyranine using a PD-10 desalting column previously equilibrated with MexB Reconstitution Buffer.
10. To prepare control vesicles, substitute the protein sample with the same volume of a protein buffer (this buffer should have the exact same detergent concentration that the protein, so one should use the buffer used to equilibrate the PD-10 column at the end of the protein purification).
11. Filter the liposomes through a 0.22 μ m filter.

3.5.4 Reconstitution of MacA and MacB Proteins into Proteoliposomes

1. Resuspend *E. coli* polar lipids in MacAB Reconstitution Buffer to the final concentration 20 mg/ml. Briefly sonicate the mixture using Water Bath Sonicator. Dilute 250 μ l of the lipid solution with the same volume of the MacAB Reconstitution Buffer containing 0.45% TX.
2. Dilute 25 μ g of MacB only or mixture of MacA and MacB proteins (with molar ratio MacA to MacB being 3:1 and *see Note 10*) in 500 μ l of MacAB Reconstitution Buffer supplemented with 100 mM KCl. Adjust concentration of TX to 0.45% (*see Note 11*).
3. Slowly add 500 μ l of the protein sample to 500 μ l of the lipid solution and incubate for 30 min at the room temperature.
4. Wash 40 mg of SM-2 Adsorbent Bio-Beads three times in methanol, then in water and MacAB Reconstitution Buffer (*see Note 12*).
5. To remove TX, incubate protein–lipid mix at room temperature for 1 h with prewashed beads. Briefly spin down the beads and transfer the supernatant into another tube with fresh beads. Repeat the procedure two more times with fresh beads for 1 h at room temperature and 1 h at 4 °C.
6. To decrease the residual concentration of TX, dilute the sample with 2 ml of cold MacAB Reconstitution Buffer. Collect the proteoliposomes by ultracentrifugation at $250,000 \times g$ for 1 h at 4 °C using TLA 100 rotor (Beckman).
7. Resuspend the pellet in MacAB Proteoliposome Storage Buffer (*see Note 13*) and briefly sonicate.
8. Determine the protein concentration in the resulting proteoliposomes by a quantitative SDS-PAGE. On average, up to 40% of the protein will be recovered in proteoliposomes.

9. Measure total phosphate concentration of phospholipids according to Ames [36]. The molar protein:lipid ratio varied between 2:1 and 4:1.
10. To prepare control vesicles substitute the protein sample by the same volume of MacAB Reconstitution Buffer.
11. Store the sample on ice. The ATPase activity of reconstituted MacAB complex is stable and reproducible up to 1 week of storage on ice.

3.5.5 Reconstitution of OprM in Proteoliposomes

1. Mix 24.4 mg of Egg PC and 12 mg of DOPE. Let the chloroform evaporate overnight under a vacuum bell.
2. Resuspend the latter in 2 ml of OprM Reconstitution Buffer supplemented with 3.2 mg/ml RNA (*see* Subheading 3.4 and **Note 14**).
3. Incubate 10 min at 37 °C and subject to five freeze–thaw cycles (freeze in liquid nitrogen and thaw in water at 37 °C).
4. Extrude the lipid suspension through 200 and 100 nm membranes (21 pass for each type of membrane), using an Avanti mini-extruder. Before extruding the liposome, make sure the membrane is moisturized by rinsing the whole set-up with the buffer.
5. Aliquot 100 µl of liposomes at 3 mM of lipids into a 96-well plate (cuvettes could be used for a smaller number of samples). Add increasing concentrations of β-OG and measure the optical density at 550 nm in each well. Determine the Rsat, i.e. the optical density reaches a maximum.
6. Solubilize the liposomes with β-OG at Rsat during 30 min at 4 °C.
7. Add OprM at a 20: 1 lipid-to-protein ratio (w/w).
8. Remove the detergent by addition of Bio-Beads at a Bio-Bead-to-detergent ratio equal to 30 (w/w) overnight at room temperature under gentle stirring.
9. Liposomes are concentrated using a Millipore concentrator with the cut off 100,000 kDa to the final volume of 1 ml.
10. Remove the nonencapsulated RNA by anion exchange chromatography. Equilibrate a Sephadex Q column with OprM Reconstitution Buffer and load liposomes.
11. Liposomes loaded with RNA are found in the flow-through, while the nonencapsulated RNA remains bound to the column. It can be subsequently eluted with 1 M NaCl.
12. To prepare control vesicles, substitute the protein sample with the equal volume of the protein buffer.
13. The efficiency of the RNA encapsulation and the removal of the nonencapsulated RNA are analyzed by the agarose-syber safe

gel. Nonencapsulated RNA migrates into the gel during electrophoresis (bands around 40 bp) but encapsulated RNA remains in wells because liposomes are too large to enter the gel matrix. If liposomes are now solubilized with detergent, the encapsulated material migrates into the gel.

3.6 Loading of Fluorescent, Nucleic Acid, and Radioactive Probes into Proteoliposomes

3.6.1 Entrapment of Probes

1. Add a probe to the final concentration of 1 mM in case of pyranine to the reconstituted proteoliposomes. Freeze the sample in a dry ice/ethanol bath and thaw at room temperature twice. Briefly bath-sonicate liposomes between the cycles.
2. To remove untrapped probe, load the sample onto a gel filtration P-40 column (Pharmacia). Use the protein reconstitution buffer to wash the column. Collect the fractions containing proteoliposomes.

3.6.2 Loading of ATP Inside of Vesicles (See Note 15)

1. Mix 1 μl of proteoliposomes and ATP probe in a 10 μl reaction volume on ice. Immediately freeze the tube in liquid N_2 .
2. Thaw proteoliposomes on ice and sonicate briefly in water bath sonicator. Repeat the procedure two more times.
3. Minimize the exposure time of proteoliposomes mixed with ATP to room temperature to delay ATP hydrolysis.

3.7 ATP Hydrolysis by Reconstituted MacAB (See Note 17)

1. Prepare 10 mM unlabeled Mg-ATP solution by mixing equal volumes of 20 mM ATP and 20 mM MgCl_2 diluted in the reaction buffer.
2. Add 5% ^{32}P γ -phosphate labeled Mg-ATP (3000 Ci/mmol, Amersham).
3. Prepare 10 μl reaction mix containing 1–2 μl of MacAB proteoliposomes (0.3–0.4 μM of MacB and 0.9–1.9 μM MacA depending on proteoliposome batch), reaction buffer and ATP in desired concentrations (*see* Note 17). Keep all solutions on ice while mixing.
4. Immediately after addition of ATP, freeze the sample in liquid N_2 . Load the radioactive ATP inside of vesicle as described in Subheading 3.6.2.
5. After the last round of sonication, start the reaction by placing the sample into a designated 37 $^\circ\text{C}$ water bath.
6. To study the kinetics of the ATP hydrolysis perform a time course experiment (Fig. 1). Transfer 1 μl of reaction at different time points into a tube containing 10 μl of Stop Buffer. Immediately place the tube with a time point sample into a designated 50 $^\circ\text{C}$ water bath. To ensure complete digestion of the protein incubate the tube for 20 min at 50 $^\circ\text{C}$.
7. Spot 1 μl of stop reaction on prewashed PEI-F cellulose plate and separate the products of the reaction by thin-layer chromatography (TLC) in 10% formic acid and 0.5 M LiCl [37].

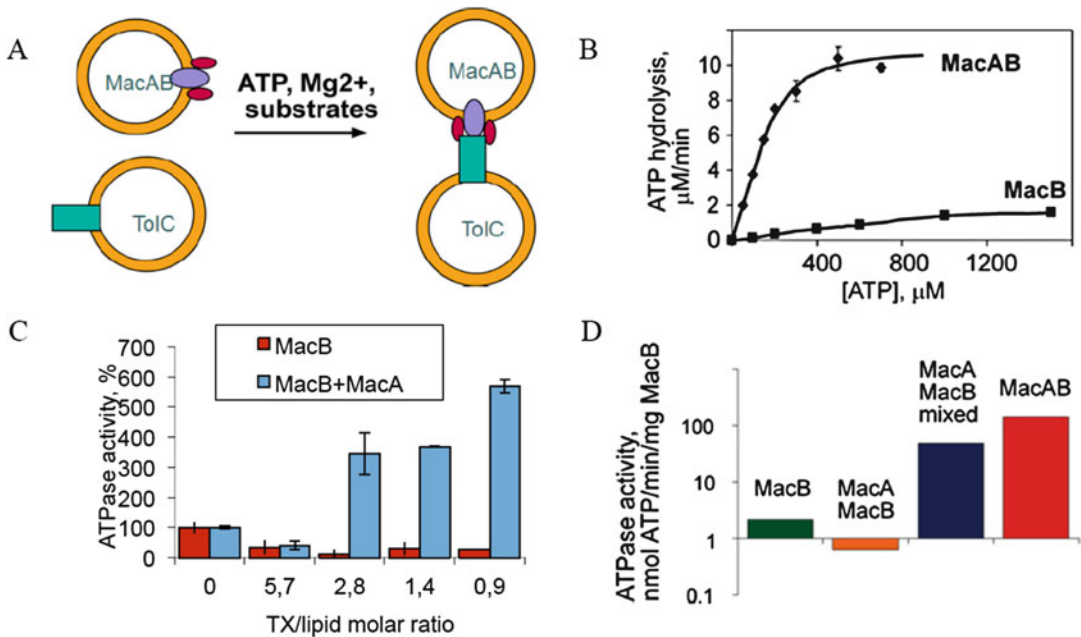


Fig. 1 Assay schematics and reconstitution of the ABC-type efflux pump MacAB. (a) Proteins are reconstituted to mimic the arrangement of the pump within the two-membrane envelope of *E. coli*. For this purpose, MacB alone or in combination with MacA is reconstituted into one population of proteoliposomes, whereas TolC into a different population of proteoliposomes and they are mixed in the presence of cofactors such as ATP and Mg²⁺ ions. (b) ATP hydrolysis rates of MacB-proteoliposomes and MacAB-proteoliposomes. (c) ATPase activities of MacB and MacAB measured at different detergent:lipid molar ratios. (d) ATPase activity of MacB proteoliposomes alone or mixed with MacA-proteoliposomes with and without sonication. The activity of the co-reconstituted MacAB-proteoliposomes is shown for comparison

8. After overnight exposure scan dried TLC plates using Storm PhosphoImager (GE Health Sciences). Quantify the amounts of generated Pi using ImageQuant Software. Based on the concentration of MacB and ATP in the reaction, calculate the specific ATPase activity of MacB in mol ATP/min/mol MacB.
9. To evaluate the effect of substrates, inhibitors or accessory proteins on the ATPase activity of MacB, perform the reaction in the presence of different concentration of additives ([20] and see Note 18).

3.8 Proton Translocation by Reconstituted AcrB and MexB

1. Unload AcrB/MexB proteoliposomes in reconstitution buffer pH 7.0 and 100 mM KCl with 1 mM pyranine and remove the access of the probe as described in Subheading 3.6.1. Use empty liposomes as a negative control.
2. Calibrate the fluorescent response of pyranine trapped inside the vesicles by evaluating the pyranine fluorescence at different pH in the presence of 0.1 μM nigericin (see Note 19). Add 20 μl of AcrB/MexB-pyranine vesicles in 2 ml buffers with

0.1 M NaCl and different pH (*see* Subheading 2.6). Measure pyranine fluorescence with excitation and emission wavelengths as 455 and 509 nm correspondingly and plot it against the Δ pH value.

3. To study the effect of drugs on proton efflux through AcrB/MexB, mix 20 μ l AcrB/MexB vesicles containing pyranine in 25 mM HEPES-KOH (pH 7.0), 5 mM DTT and 0.1 M KCl in 2 ml of iso-osmotic 0.1 M NaCl -buffer, then add substrates at 0.2 mM and allow equilibration for 1 min.
4. To generate Δ pH across the membrane, add 10 μ M valinomycin (*see* Note 20) which causes fast decrease in pyranine fluorescence. Δ pH will be stable for at least 4 min with the half-life time of 5 min. Dissipation of Δ pH will be faster in the presence of AcrB substrates.
5. Perform control measurements with AcrB-free liposomes and in the absence of Δ pH.

3.9 Transport of Fluorescent Lipids by Reconstituted AcrB (See Note 21)

1. Prepare AcrB-containing donor vesicles with fluorescent lipids with the internal pH 7.0 as described in Subheading 3.5, step 2.
2. Mix 5 μ l of donor vesicles (0.36 μ g of AcrB in 40 nmol of total phospholipids) with 10 μ l of unlabeled protein-free acceptor vesicles (650 nmol of phospholipids) (*see* Subheading 3.5.2). Dilute the vesicles into buffer with pH 6.0 to generate Δ pH.
3. Initiate the transport reaction by adding 5 mM MgCl₂. Monitor NBD fluorescence at 30 °C (*see* Note 21). To stop the reaction, add 10 mM EDTA (Fig. 2). Then solubilize the vesicles with 0.5% DDM to completely de-quench N-NBD-PE fluorescence.
4. To exclude nonspecific effect of Mg²⁺ on NBD fluorescence, perform the control reaction in the absence of Δ pH.
5. To evaluate the effect of accessory protein AcrA on the transport of lipids by reconstituted AcrB, add sAcrA^{His} after addition of MgCl₂. To confirm that the effect of AcrA is specific, perform the transport reaction with different AcrA concentrations (from 15 to 90 μ g/ml).
6. To confirm that the observed transport reaction depends on the presence of AcrB in vesicles, digest the AcrB-containing vesicles with 0.1 μ g/ml trypsin for 15 min and then repeat the transport reaction. The treatment should result in 50% decrease of the AcrB-dependent lipid transport.

3.10 Transport of Ethidium Bromide by Reconstituted MexAB-OprM (See Note 22)

1. Mix 125 μ l of OprM-proteoliposomes (Subheading 3.5, step 5) with 125 μ l of MexAB-proteoliposomes (Subheading 3.5, step 3) and incubate for 20 min at room temperature, no stirring.

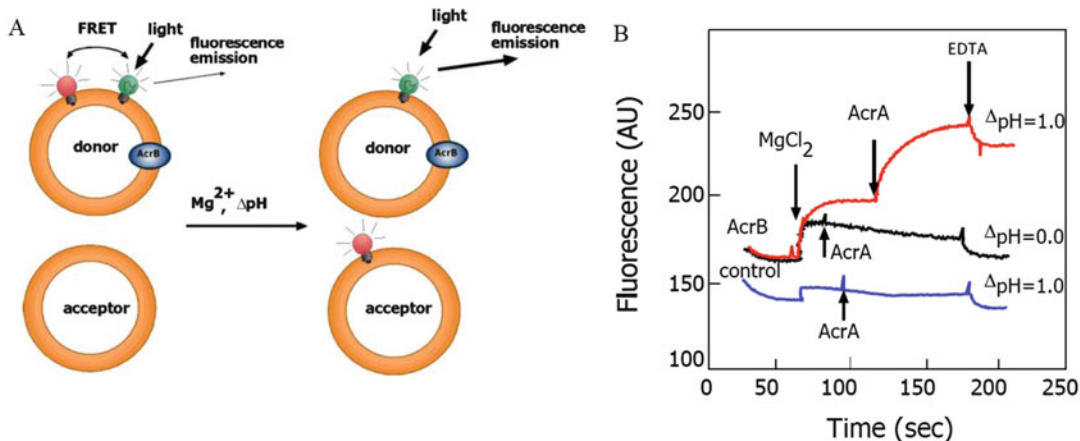


Fig. 2 Assay schematics and reconstitution of the RND-type efflux pump AcrAB. (a) Proteins are reconstituted to mimic their arrangement within the two-membrane cell envelope of *E. coli*. For this purpose, AcrB is reconstituted into proteoliposomes containing two types of fluorescent lipids that constitute a FRET pair (donor proteoliposomes). After mixing of donor proteoliposomes with the empty acceptor liposomes, the transport reaction is initiated by addition of cofactors such as AcrA, Mg^{2+} ions and by imposing a proton gradient. (b) Time-dependent changes in fluorescence intensity of empty donor liposomes mixed with acceptor vesicles (blue trace), and AcrB-containing donor proteoliposomes mixed with acceptor vesicles in the presence (*red trace*) and absence (*black trace*) of a proton gradient. The addition of Mg^{2+} ions and AcrA are shown by arrows. The reaction is stopped by EDTA, which chelates Mg^{2+} and separates the donor and the acceptor vesicles

Add substrate at 5 μ M at the same time as liposomes (« time of incubation is the key », *see Note 23*).

2. Initiate the transport reaction by adding between 5 to 20 μ l HCl 0.15 M (Fig. 3).
3. Monitor fluorescence of probes at 25 °C with continuous stirring using a SAFAS Xenius spectrofluorometer. Measurements are made using the “dual wavelengths” mode of the spectrofluorometer. Pyranine fluorescence ($\lambda_{ex} = 455$ nm and $\lambda_{em} = 509$ nm) and ethidium bromide fluorescence ($\lambda_{ex} = 300$ nm and $\lambda_{em} = 600$ nm) are monitored as a function of time (Fig. 3b and c, respectively).
4. To evaluate the effect of accessory protein, MexA, on the transport of substrates by reconstituted MexB, add palmitoylated MexA in the same time as MexB during the reconstitution procedure (*see Subheading 3.5, step 3*).
5. To confirm that the observed transport reaction depends on the presence of MexB in vesicles, perform the same procedure with an inactive mutant of MexB.

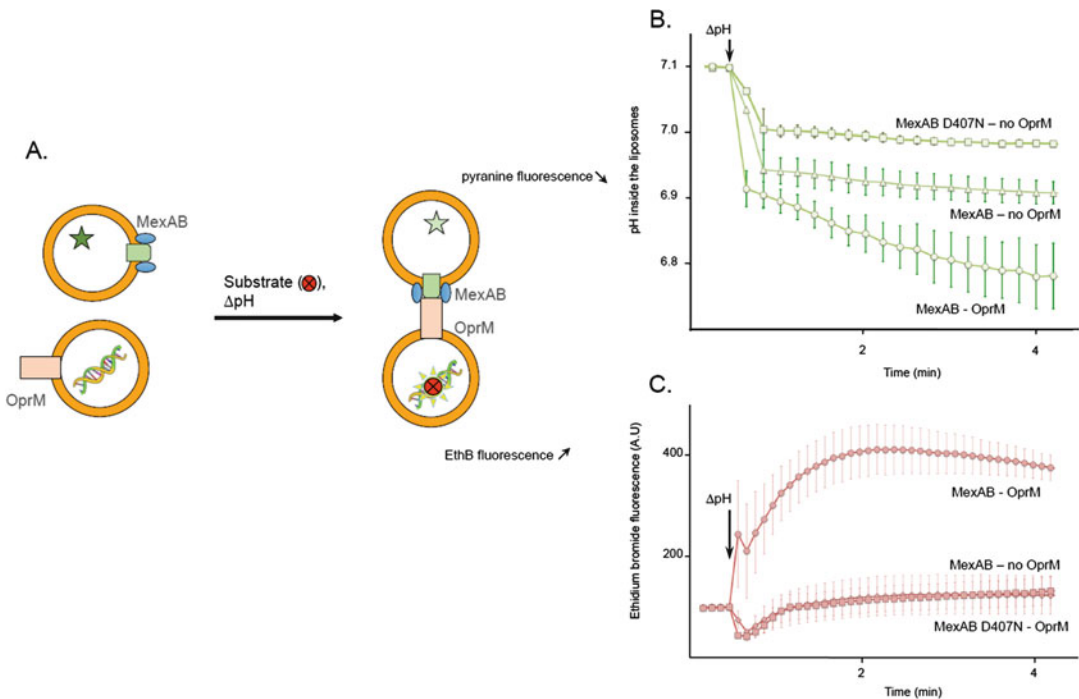


Fig. 3 Assay schematics and reconstitution of the tri-partite MexAB-OprM efflux pump. **(a)** Proteins are reconstituted to mimic the arrangement of the pump within the two-membrane envelope of *P. aeruginosa*. For this purpose, MexB alone or in combination with MexA is reconstituted into one population of proteoliposomes that contain pyranine, whereas OprM is reconstituted into a different population of proteoliposomes containing RNA. Both types of liposomes are mixed in the presence of Mg^{2+} ions and ethidium bromide. The transport reaction is initiated by imposing a proton gradient. **(b)** Time-dependent changes in pyranine fluorescence intensity of proteoliposomes containing a wild-type (*triangle*) or an inactive version (*squares*) of MexB. Effect of OprM on the MexAB-induced acceleration of acidification (*circle*). **(c)** Time-dependent changes in ethidium bromide fluorescence intensity of proteoliposomes containing OprM (*circle* and *square*), or of empty liposomes (*diamond*), mixed with liposomes containing MexAB. As a negative control, an inactive version of MexAB is also used (*square*)

4 Notes

1. Proteins encoded on pUC151A plasmid are constitutively expressed and do not require any induction for the overexpression of AcrB. Typical purification profiles can be found, e.g., in Fig. 1, Ref. [20], for MacAB and in Fig. 1, Ref. [40], as for MexAB.
2. 5% TX was used only to extract AcrB, MacA, MacB and TolC proteins from the *E. coli* membranes. During purification steps, storage and biochemical assays, concentration of detergent can be decreased according critical micelles concentration (CMC) value of the detergent. 0.2% TX was used to maintain purified

membrane proteins in soluble form. MexB was not stable enough in TX so we had to use DDM.

3. The choice of TX as a detergent for MacA and MacB proteins solubilization was determined by the value of the ATP-ase activity of purified and/or reconstituted MacB. We have found that the ATP-ase activity of MacB was irreversibly inhibited by DDM or OG, but not by TX. After exposure MacB to DDM the specific activity was as low as 0.035 mol ATP/min/mol MacB. In comparison, MacB purified using TX maintained two orders higher activity. Furthermore stimulating effect of MacA on the ATP-ase activity of MacB purified using DDM was no longer detected even after detergent removal. High concentration of imidazole inhibited the ATP-ase activity of MacB therefore only 100 mM imidazole elution fractions containing MacB were taken for further assays.
4. We found that MacA is not structurally stable in *E. coli* containing chromosomal OmpT protease. The protein is cleaved by OmpT protease during the detergent solubilization step. To purify a full-length stable MacA, we deleted *ompT* gene from the chromosome of *E. coli* BW25113 cells by method described in [38].
5. C8POE is a detergent that is known to specifically solubilize membrane proteins from the inner membrane. Upon solubilization and ultracentrifugation, proteins from the outer membrane are found in the pellet. The latter must be washed extensively before being subjected to solubilization with the proper detergent. An alternative solution consists in separating the outer membrane from the inner membrane by subjecting them to a sucrose gradient: membranes are loaded on top of a two-layer sucrose cushion (1.5 M/0.5 M sucrose), ultracentrifuged for 1 h at $100,000 \times g$ and the outer membranes are found in the 1.5 M sucrose phase.
6. After detergent exchange AcrB is stable only for a few hours and should be used for reconstitution into proteoliposomes immediately. Do not exceed the AcrB concentration at this step higher than 0.3 mg/ml.
7. Although not physiologically relevant, the use of cholesterol turned out to dramatically improve our reconstitution protocols, most probably thanks to the tightening effect of cholesterol on membranes.
8. Preparation of liposome samples as monodisperse and pure as possible is mandatory. A good quality control of the preparation helps very much the reproducibility of the procedures. We routinely use dynamic light scattering (DLS) to check the quality of the liposomes at every step of the reconstitution protocol. Sucrose gradients and cryo-electron microscopy are

also used to assess the dispersity of the final proteoliposome suspension. Following similar lines, the buffer used to prepare the liposomes and that of the buffer used to solubilize them should be isotonic. We systematically measure the osmolarity of our buffers.

9. Liposome solubilization upon addition of increasing concentrations of detergent can be described as a three-phase process. Phase I corresponds to detergent partitioning into the liposome membranes up to a threshold corresponding to the point where membranes are saturated with detergent, the R_{sat} value. During phase II, detergent is no longer incorporated into the liposomes but is instead found as free monomers in solution. The concentration of free detergent increases until formation of detergent micelles and lipid/detergent mixed micelles. Phase III corresponds to the situation where liposomes are completely solubilized, the R_{sol} value. R_{sat} and R_{sol} , which are specific for the type of lipid and detergent used, should be measured before any membrane protein reconstitution assay.
10. Mixing MacA and MacB proteins prior reconstitution into proteoliposomes resulted in reconstitution of highly active ATP-ase complex (Fig. 1b). To study the effect of MacA on the ATP-ase activity of MacB using single-protein vesicles requires intermixing of bilayers by additional sonication round [20]. The resulting activity will be significantly higher than for MacB alone or when MacA and MacB proteoliposomes are combined in the same reaction without intermixing of bilayers (Fig. 1d). We have concluded that ATP-ase activity of MacAB in membrane vesicles requires a physical interaction between MacA and MacB within a bilayer and that MacA activates MacB in proteoliposomes.
11. Molar ratio between lipids and detergent used during reconstitution reaction is very important for achieving of functionally active MacB ATP-ase (Fig. 1c). We prepared MacAB vesicles with constant concentration of TX (0.45%) and different concentration of *E. coli* phospholipids. Detergent was not removed in this experiment. The molar ratio of detergent:lipid varied from 5.7 to 2.8, 1.4 and 0.9. Based on previous observations [39] and our results, we concluded that at the detergent:lipid ratio 5.7 lipids were completely solubilized in TX and interfered with the activity of MacAB complex. At the detergent:lipid ratio of 2.8 and below, there is mixed population of lipids micelles and vesicles, which resulted in significant ATP-ase activity of MacAB vesicles. At the molar ratio 0.9, reconstituted lipid vesicles dominate, which coincides with the highest level of MacAB ATP-ase activity.

12. Detergent removal can be also achieved by extensive dialysis against the reconstitution buffer without detergent or simply diluting the concentration of detergent in sample below CMC level. We found that using SM-2 Adsorbent Bio-Beads simplifies this step.
13. To determine the optimal concentration of KCl we titrated the effect of KCl on the ATP-ase activity of reconstituted proteoliposomes. 50 mM KCl was the minimal concentration required for full activity of MacAB proteoliposomes.
14. We have tried to incorporate various types of nucleic acids (sonicated calf thymus DNA, PCR products, genomic DNA) but the resulting suspension turned out to be unsatisfactory (*see Note 8*). By opposite, RNA scaffolds are very stable and soluble. Their use improved very much the reconstitution protocol.
15. The described protocol of MacB reconstitution into proteoliposomes does not allow a control of the MacB orientation in prepared vesicles. Analysis of the ATP-ase activity of MacAB proteoliposomes loaded with ATP showed that MacB is predominantly inserted with NBD domains inside of vesicles. The protocol described for MexB has been optimized in order to favor a 100% incorporation inside-out. To do so, we take extreme care to solubilize the liposomes with a detergent concentration precisely equal to that needed to saturate the membranes. In such conditions, the protein is supposed to insert unidirectionally with its hydrophobic part first.
16. Purified MacB^{His} has basal ATP-ase activity in detergent solution. To measure MacB activity in detergent micelles follow the procedure described in Subheading 3.7 with one modification. Include 0.2% TX in all buffers used in reaction. Substrates and accessory proteins should be also prepared in reaction buffer.
17. To characterize the kinetics of ATP hydrolysis by reconstituted MacAB complex, in reaction we used increasing concentrations of ATP up to 3 mM. We found that at these reaction conditions, ATP hydrolysis by MacAB complex reaches saturation at about 0.3–1 mM ATP depending on protein and liposomes preparations (Fig. 1b).
18. To verify the specificity of MacA stimulation of the MacB ATP-ase activity, we perform the preparation of MacAB proteoliposomes using different ratios between MacB and MacA during reconstitution. We evaluated the final concentration of MacA and MacB in proteoliposomes preparations by SDS-PAGE (*see Subheading 3.5, step 3*) and measured the ATP-ase activity. We found that MacA stimulation was concentration dependent with the half-maximum being achieved when MacB:MacA ratio was about 2:3. Furthermore, AcrA

protein, the accessory protein of another drug efflux pump AcrB, did not show any stimulation activity when used in the same experimental set up. Thus, we proved that the effect of MacA is specific to MacB protein. Addition of TolC-proteoliposomes into the reaction mixture did not further stimulate the activity of the pump.

19. The pH-sensitive fluorescent probe pyranine is water soluble and does not readily cross a lipid bilayer. Since the pyranine fluorescence changes with the pH value of environment in a linear fashion, this probe can be used to calibrate changes in ΔpH caused by active drug transport in AcrB-proteoliposomes. Dilution of vesicles loaded with pyranine into a buffer with pH 6.0 will create ΔpH across the membrane with alkaline inside and acidic outside. Only slow decrease in pyranine fluorescence will be observed due to spontaneous leakage of H^+ . Addition of nigericin will make the membrane permeable to proton and cause immediate collapse of ΔpH .
20. Valinomycin is a lipid-soluble ionophore with high selectivity against potassium ions (K^+). It binds K^+ and facilitates their transport across lipid bilayers. Addition of valinomycin to K^+ -lipid vesicles into an isosmotic NaCl-containing buffer leads to generation of an electrical potential across the membrane. Then transmembrane potential is converted to an interior-acid ΔpH in the presence of 0.1 M Cl^- . In the presence of AcrB and its substrates ΔpH dissipates rapidly due to the proton efflux coupled with inward transport of drugs.
21. AcrB expels multiple substrates from cells providing multidrug resistance to bacteria. Some of the substrates include lipophilic molecules capable of diffusion through the lipid barrier. Using the transport assays with fluorescent lipids as substrates for AcrAB pump (*see* Subheading 3.9), Zgurskaya and Nikaido [10] experimentally showed that AcrA stimulated the lipid transport by AcrB. Fluorescent derivatives of phospholipids, N-NBD-PE and N-Rh-PE, were included for AcrB vesicles preparation (Fig. 2a). When these two lipids are present at high concentrations in the same bilayer, the fluorescence of NBD is quenched due to the fluorescence energy transfer to the rhodamine-containing lipid (N-Rh-PE). With the AcrB-dependent efflux of fluorescent lipids, their concentration in AcrAB-proteoliposomes decreases by transfer into acceptor vesicles and de-quenching of NBD fluorescence can be observed and quantified. AcrA triggers faster de-quenching thus showing the stimulating role of the accessory protein during the active transport (Fig. 2b).
22. MexB is a *P. aeruginosa* homolog to AcrB. Using the transport assays described in Subheading 3.10, Verchère et al. reported a

system where the functional assembly of the tripartite efflux pump could be mimicked (Fig. 3). By using specific reporters incorporated into proteoliposomes containing either transporter or the outer membrane channel, the observed transport occurs through the pump and not by a simple passive diffusion [17]. On the one side, pyranine loaded into MexAB proteoliposomes allows the monitoring of pH variations upon transport through MexB (transport is coupled to the counter transport of protons, hence by the acidification of the liposome). On the other side, RNA is loaded into the OprM-proteoliposomes. The substrate, ethidium bromide, a RNA intercalating agent, is added in the outer medium, corresponding to the periplasmic space where the drug is supposed to reach its binding sites. Upon generation of a proton gradient, ethidium bromide is transported by MexB, at the expense of proton counter transport, through OprM, making it possible for its intercalation into RNA. The concomitance of the two signals (decrease of the fluorescence of pyranine/increase of the fluorescence of ethidium bromide) is the evidence for transport through the pump.

23. Incubation is a key parameter. Indeed, we had noticed that the system had to equilibrate for a significant period of time for the liposome-to-liposome transport to occur. This was later confirmed in an assay specifically dedicated to the in vitro monitoring of the pump assembly [40].

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