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Promiscuous partnering and independent activity of MexB, the multidrug transporter protein from *Pseudomonas aeruginosa*.

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SYNOPSIS

The MexAB-OprM drug efflux pump is central to multidrug resistance of *Pseudomonas aeruginosa*. The ability of the tripartite protein to confer drug resistance to the pathogen is crucially dependent on the presence of all three proteins of the complex. However, the role of each protein in formation of the intact functional complex is not well understood. One of the key questions relates to the (in)ability of MexB to act independently of its cognitive partners, MexA and OprM. Here we demonstrate that in the absence of MexA and OprM, MexB can (i) recruit AcrA and TolC from *E. coli* to form a functional drug efflux complex, (ii) transport the toxic compound ethidium in a gram-positive organism where the periplasmic space and outer membrane are absent, and (iii) catalyse transmembrane chemical proton gradient (ΔpH)-dependent drug transport when purified and reconstituted into proteoliposomes. Our results represent the first evidence for drug transport by an isolated RND type multidrug transporter, and provide a basis for further studies into the energetics of RND-type transporters and their assembly into multiprotein complexes.

ABBREVIATIONS

RND, Resistance-Nodulation-cell Division / TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene p-toluenesulfonate / DDM, β -D-dodecyl-maltoside / IMP, inner-membrane protein / MFP, membrane fusion protein / OMP, outer membrane protein / IPTG, Isopropyl β -D-1-thiogalactopyranoside / DSP, Dithiobis[succinimidyl propionate]/ CCCP, Carbonyl cyanide 3-chlorophenylhydrazone

INTRODUCTION

P. aeruginosa is a pathogen that easily infects immuno-compromised, hospitalised patients. It is also the main cause of mortality of patients with Cystic Fibrosis and is characterised by an innate resistance to a wide spectrum of antibiotics [1]. Multidrug resistant strains of gram-negative organisms such as *P. aeruginosa* rely on tripartite protein assemblies that span both the inner and outer membranes to pump cytotoxic compounds from the cell. *P. aeruginosa*

expresses several such multidrug transporter complexes of which MexAB-OprM is the predominant system involved in intrinsic drug resistance. In the tripartite complex MexB is the inner membrane spanning protonmotive force dependent xenobiotic efflux protein (IMP), OprM is the outer membrane protein (OMP) and MexA is the periplasmic membrane fusion protein (MFP). AcrAB-TolC from *E. coli* is a homologous system with MexB and AcrB sharing 70% identity, MexA and AcrA sharing 57% identity and OprM and TolC sharing 19% identity.

Crystal structures have been reported for MexA [2-4], MexB [5] and OprM [6]. The crystal structure of MexA reveals that the protein is comprised of a membrane proximal domain, a β -barrel domain, a lipoyl domain and an α -helical hairpin domain. OprM is a trimeric channel with an outer membrane spanning, pore forming β -barrel domain and a periplasmic α -helical domain. MexB was found to be a homotrimer in mass spectrometry [7] and X-ray crystallography [5]. Each monomer of MexB consists of 12 transmembrane α -helices constituting the inner membrane domain. The connecting loops between TM1 and TM2 as well as TM7 and TM8 fold into two large periplasmic domains, which form the pore domain and OprM docking domain. The overall architecture of the proteins from the MexAB-OprM complex closely resembles that of the proteins in the AcrAB-TolC complex from *Escherichia coli* [4,8,9,10,11].

The structure of an assembled tripartite complex has not been determined yet and despite the available crystal structures of the individual components the details of assembly remain largely unknown. The interaction of a tight complex between all three pump components in vivo was confirmed as the tripartite MexAB-OprM complex could be purified from overproducing *E. coli* or *P. aeruginosa* cells without the need for chemical cross-linking [12]. Genetic and biochemical studies further suggested that the interaction between MexA and OprM occurs through the α -helical hairpin of MexA and α -helical barrel of OprM below the equatorial domain [13-16]. Also, MexB most probably interacts with the periplasmic tip of OprM via its top β -hairpin regions [14]. The details of the interaction of MexB with MexA are much less clear, but several studies implied the importance of the β -barrel domain in MexA for interaction with MexB [17,18]. Cross-linking data for AcrA with AcrB suggest extensive contact between the lipoyl, β -barrel and membrane proximal domains of AcrA and the periplasmic domain of AcrB [4].

Most studies indicate that the MFPs and IMPs form highly specific interactions that do not allow interchange between homologues pumps. For instance, MexA and MexB cannot be functionally interchanged with any of the other Mex systems in *P. aeruginosa* [12,19]. MexAB is also strict in its requirement for OprM. Although MexAB can form a partially functional complex with OprJ [19], it cannot form a functional complex with OprN from *P. aeruginosa* [20] or TolC from *E. coli* [16,21,22]. However, recently, functional interaction of MexB with AcrA and TolC has also been reported [17], raising questions about the exchangeability of tripartite components from different organisms.

Another question that also remains is whether or not RND multidrug transporters can function independently from their MFP and OMP. The RND multidrug transporters AcrB and AcrD from *E. coli* have been purified and reconstituted into lipid vesicles. For both proteins, the transport activity was dependent on the presence of the MFP AcrA [23,24].

In this study we have investigated the role of the MFP and OMP in drug efflux by inner membrane proteins by (i) expressing MexB in *E. coli* in the absence of MexA and OprM, (ii) expressing MexB in the gram-positive organism *L. lactis* where the periplasmic space and

outer membrane are absent and (iii) purifying and reconstituting MexB in proteoliposomes and measuring drug transport in the absence of the other protein partners from the tripartite complex.

EXPERIMENTAL

Cloning of MexB and MexA in *E. coli*

MexB was cloned in *E. coli* as described previously [7] to yield plasmid pMexBH. The *mexA* gene was amplified from genomic DNA of *P. aeruginosa* strain PAO1 by PCR by using Pfu Turbo polymerase (Stratagene) and primers 5' GGA ATT CCA TAT GTC CGG AAA AAG CGA GGC GCC GCC 3' (forward) and 5' CGC GGA TCC GCG TCA GCC CTT GCT GTC GGT TTT CG 3' (reverse). The primers were designed to amplify residues 24–383, thereby omitting the periplasmic signal sequence at the N terminus. In addition, Cys-24 was mutated to Ser, to prevent attachment of a fatty acid. This PCR produced a DNA fragment of 1,080 bp with unique restriction sites at the 5' end (*NdeI*) and the 3' end (*BamHI*). After restriction enzyme digestion, the fragment was ligated into *NdeI*-*BamHI* restricted pET28b(+) (Novagen). This protocol generated a construct containing a gene for the $\Delta(1-23)$, C24S mutant of MexA with an N-terminal hexahistidine tag attached via a thrombin cleavage site and was designated pHMexA_{AN}. The cloned PCR product was sequenced to ensure that only the intended changes were introduced.

Construction of the D407N MexB mutant

The D407N mutation was introduced in the *mexB* gene in the *E. coli* vector pMexBH by PCR using KODHotstart DNA polymerase (Novagen) and the forward primer 5' CTT GCT GGT GAA CGA CGC CAT CGT GGT G 3' and reverse primer 5' GAT GGC GTC GTT CAC CAG CAA GCC GAT GGC 3'. The mutant *mexB* gene was then subcloned into pET41a+ as an *NdeI*, *XhoI* fragment to yield pMexBH D407N. The mutated *mexB* gene was sequenced to ensure that only the intended changes were introduced.

Creation of the *E. coli* TolC knock-out mutant

The *tolC* gene on the chromosome of *E. coli* BW25113 was deleted using the method described by Datsenko and Wanner [25]. The primers used were 5'- TAA TTT TAC AGT TTG ATC GCG CTA AAT ACT GCT TCA CCA CGT GTA GGC TGG AGC TGC TTC -3' and 5'- GTA TCT TTA CGT TGC CTT ACG TTC AGA CGG GGC CGA AGC CCA TAT GAA TAT CCT CCT TAG -3' which contained priming sites 1 or 2 from pDK4 with 40 nucleotides before or after the *tolC* gene respectively. The deleted *tolC* locus was marked by genes conferring chloramphenicol resistance. The antibiotic resistance gene was removed by the pCP20-encoded recombinase [25]. Positive clones were verified by PCR and drug susceptibility tests. The BW25113 strain was converted to a (DE3)-lysogen, but all cytotoxicity and drug transport assays employed basal levels of expression without induction.

MexB cloning in *L. lactis*

MexB was cloned into *L. lactis* by digesting pMexBH and pMexBH D407N with *NdeI* and *XhoI* (NEB). The non histagged fragments of MexB and D407N MexB that were released were then cloned into the *NcoI*-*XhoI* restricted lactococcal pNZ8048 expression vector [26].

Cytotoxicity assays

E. coli BW25113 cells with deletions in AcrB, AcrAB or TolC were used to propagate the control (pET41a+) or the MexB (pMexBH) expressing plasmids. All experiments employed

basal levels of expression without induction. Cells were grown to an OD₆₆₀ of 0.2 in LB containing kanamycin (25 µg/ml). Cytotoxic drugs were added to the cell suspensions at increasing concentrations. For the determination of viable cell counts, cells were incubated at 37°C for 3 h. The bacterial cultures were then serially diluted up to 10⁹-fold and plated on LB agar plates containing kanamycin. Colonies were counted and the survival ratios were expressed as a percentage of the colony-forming units observed at a given concentration of antibiotic over that observed without added antibiotics. For determining the inhibitory effect of drugs on the growth rate of cells, the cell densities were monitored by measuring the OD₆₆₀ of the cultures every 10 min for 6 h in a VersaMax plate reader (Molecular Devices, CA). The relative growth rates were determined, and the IC₅₀ values were calculated. Half-maximal inhibitory concentration (IC₅₀) is the concentration of drug necessary to reduce the growth rate of cells by 50%. For oxacillin, the final OD₆₆₀ was used instead of the growth rate. Data represent the mean and standard error of three independent experiments, each carried out in duplicate.

Cytotoxicity assays on *L. lactis* NZ9000 Δ lmrA Δ lmrCD cells were carried out in the same way as for the *E. coli* cells with the following changes. Cells were grown in M17 medium (Difco) containing 0.5% glucose and chloramphenicol (5 µg/ml) to an OD₆₆₀ of 0.3 and protein expression was induced by the addition (1 : 2000) of the supernatant of the nisin-A producing *L. lactis* strain NZ9700 [26] (containing approximately 5 ng nisin-A/ml), before ethidium was added at increasing concentrations.

Substrate transport in *E. coli* cells

LB-Broth Miller (Formedium) containing 25 µg/ml kanamycin was inoculated (1 : 50 dilution) with an overnight culture of *E. coli* BW25113 cells with deletions in AcrB, AcrAB or TolC, propagating various constructs. Cells were incubated shaking at 37°C until an OD₆₆₀ of 0.5 was reached. The cells were harvested by centrifugation (3,000 g, 10 minutes, 4°C), then washed twice by resuspension in 50 mM KPi (pH 7.0) containing 5 mM MgSO₄ and sedimented by centrifugation (3,000 g, 10 minutes, 4°C). The cells were then resuspended in the same buffer to an OD₆₆₀ of 0.5 and incubated for 3 min at room temperature in the presence of 25 mM glucose to energise the cells. The fluorescence measurement was started and 30 seconds later 2 µM EtBr, 0.125 µM Hoechst 33342 or 0.25 µM TMA-DPH were added. The fluorescence was followed as a function of time in a Perkin-Elmer LS 55B fluorimeter. Excitation and emission wavelengths and excitation and emission slit widths were 500, 580, 5 and 10nm respectively for ethidium; 355, 457, 10 and 4nm respectively for Hoechst 33342; 350, 425, 5 and 5nm respectively for TMA-DPH.

Cross-linking in *E. coli* cells

E. coli BW25113 (DE3) cells with genomic deletions in either AcrB or TolC and propagating pMexBH were grown in LB medium containing kanamycin (25 µg/ml). When the optical density reached ~0.6 protein production was initiated by the addition of IPTG (1 mM) and cells were grown for another two hours. Cells were harvested and resuspended in 2 ml of cross-linking buffer (50 mM KPi pH 7.4 containing 150 mM NaCl) and energised by the addition of 0.5 % glucose or de-energised by incubation with the protonophore CCCP (10 µM). DSP (5 mM) was added to the cell suspensions and the cells were incubated for 30 min before the reaction was quenched by the addition of 50 mM Tris pH 7.5. After 15 min at room temperature ISO vesicles were prepared and the histidine tagged MexB was purified as described previously. Proteins were separated by SDS-PAGE and then transferred onto polyvinylidene difluoride. The blot was probed with anti-AcrA antibody and visualised by an antigoat antibody-HRP conjugate.

Ethidium transport in *L. lactis* cells

L. lactis strain NZ9000 Δ lmrA Δ lmrCD [27,28] was used as a host for pNZ8048 derived plasmids in this study. The cells were grown at 30°C in sterile M17 medium (Oxoid) supplemented with 0.5 % (w/v) glucose and containing chloramphenicol (5 µg/ml). Overnight cultures of *L. lactis* NZ9000 Δ lmrA Δ lmrCD harbouring the pNZMexB or control plasmids were inoculated into fresh medium by 50-fold dilution. When an OD₆₆₀ of about 0.5 was reached, protein expression was induced by the addition (1: 1000) of the supernatant of the nisin-A producing *L. lactis* strain NZ9700 [26] (containing approximately 10 ng nisin-A/ml). The cells were grown for another hour to allow protein expression and were then harvested, washed and assayed for transport as described for *Substrate transport in E. coli* cells.

Preparation of inside-out membrane vesicles

M9 medium (25 mM KH₂PO₄, 40 mM Na₂HPO₄, 10 mM NaCl, 20 mM NH₄Cl, 2 mM MgSO₄·7H₂O, 0.2 mM CaCl₂, 0.5% glucose) containing kanamycin (25 µg/ml) was inoculated with an overnight culture of BW25113 Δ AB propagating pMexBH or pMexBH D407N. Cells were grown at 30°C until an OD₆₆₀ of 0.7-0.8 was reached when gene transcription was induced by the addition of 1 mM IPTG. The cells were harvested another two hours later by centrifugation (13,000 g, 15 minutes, 4°C) and washed by resuspension in 100 mM KPi (pH 7.0) followed by centrifugation (13,000 g, 15 minutes, 4°C). The cell pellet was resuspended in 100 mM KPi (pH 7.0) and DNase (10 µg/ml) was added. The cells were lysed by three passages through a Basic Z 0.75 kW Benchtop cell disruptor (Constant Systems) at 20kpsi. The lysate was subjected to low-speed centrifugation (13,000 g, 10 minutes, 4°C) to remove cell debris, and the resulting supernatant was subjected to high-speed centrifugation (125,000 g, 40 minutes, 4°C) to collect the inside-out membrane vesicles. The pellet, containing the inside-out membrane vesicles was resuspended in 50 mM KPi (pH 7.0) containing 10 % (v/v) glycerol to a protein concentration of about 50 mg/ml and stored in liquid nitrogen. The protein concentration of the inside-out membrane vesicles was determined by the D_C Protein Assay (Bio-Rad) with BSA as a standard.

Protein purification

MexB was purified essentially as described in Mokhonov *et al.* [29]. Inside-out membrane vesicles containing MexB were solubilised by shaking at 4°C for one hour in a volume of solubilisation buffer (10 mM Bis-Tris pH 7.4, 20% glycerol, 500 mM NaCl, 2 % β -D-dodecyl-maltoside (DDM) and 10 mM imidazole pH 8.0) sufficient to produce a final protein concentration of 5 mg/ml. Unsolubilised protein was removed by high-speed centrifugation (150,000 g, 30 minutes, 4°C). Ni-NTA resin (Sigma) was equilibrated by washing three times with 5 resin volumes of deionised water, each time sedimenting the resin by centrifugation (1000 g, 3 minutes, 4°C), and then twice with 5 resin volumes of buffer A (10 mM Bis-Tris pH 7.4, 10 % glycerol, 500 mM NaCl, 0.1 % DDM and 10 mM imidazole), each time sedimenting by centrifugation as before. Solubilised protein was added to the equilibrated resin and allowed to bind at 4°C for at least an hour. Then the resin was transferred to a column (Bio-Rad) and the unbound protein was allowed to drain through. The resin in the column was washed with a total of 30 resin volumes buffer A and then with a total of 30 resin volumes of buffer B (10 mM Bis-Tris pH 6.0, 10 % glycerol, 500 mM NaCl, 0.1 % DDM and 10 mM imidazole). Histidine-tagged protein was eluted in 3 resin volumes of elution buffer (10 mM Bis-Tris pH 7.4, 10% glycerol, 200 mM NaCl, 0.1 % DDM and 300 mM imidazole), of which the first 0.5 resin volume were discarded. Total purified protein was assayed using the colorimetric D_C protein assay (Bio-Rad) with BSA as a standard.

For MexA purification, BL21 (DE3) cells propagating the MexA over-expressing pHMexA_{AN} plasmid were grown in LB medium at 37°C. When the cell density reached and OD₆₆₀ of ~0.7 protein production was initiated by the addition of IPTG (1 mM). Cells were harvested and prepared as described in *Preparation of inside-out membrane vesicles*. The supernatant after the high-speed spin was directly applied to Ni-NTA resin (Sigma) and MexA was purified as described for MexB, but without any DDM in the buffers.

Hoechst 33342 binding to purified MexB

Hoechst 33342 binding to purified protein was carried out in 2 ml reaction mixtures containing 25 µg of purified protein in 10 mM Bis-Tris (pH 7.4). Hoechst 33342 was added to the solution in a stepwise fashion to a final concentration of 1.5 µM, when no major changes in fluorescence were detected. Measurements were performed in an LS-55B luminescence spectrometer at excitation and emission wavelengths of 355 and 457 nm, respectively, and slit widths of 10 and 5 nm, respectively. The binding constants B_{\max} and K_d were determined using the relationship $B = B_{\max}[S]/(K_d + [S])$, in which drug binding is represented by B, the drug concentration by [S], the maximal binding by B_{\max} , and the drug concentration yielding $1/2B_{\max}$ by the dissociation constant K_d .

Reconstitution of purified MexB in proteoliposomes

For the preparation of liposomes, commercially available *E. coli* total lipid extract (Avanti Polar Lipids) were further purified by extraction with acetone-ether to yield *E. coli* polar lipids. *E. coli* total lipid extract (100 mg) was dissolved in 2 ml of chloroform and then slowly dripped into 10 ml of ice-cold nitrogen flushed acetone containing 2 µl of β-mercaptoethanol. The mixture was stirred for at 4 °C overnight and centrifuged in glass tubes for 15 min at 3000 g. The pellet was dried under nitrogen gas and dissolved in 10 ml of nitrogen-flushed diethyl ether containing 2 µl of β-mercaptoethanol for 10 min at room temperature. Following centrifugation for 10 min at 1600 g the supernatant was evaporated to dryness in a rotary evaporator. The residue was weighed and dissolved in chloroform, and egg phosphatidylcholine (PC) (Avanti Polar Lipids) was added to the polar lipids at a PC to *E. coli* lipid ratio of 1 : 3 (w/w). Once mixed, the lipids were dried as a thin film under nitrogen gas and stored at -20°C until required. For the reconstitution of purified MexB into Triton X-100-destabilized liposomes, the lipids were hydrated at a concentration of 20 mg/ml in 20 mM KPi (pH 7.0) containing 100mM potassium acetate and 1 mg/ml sonicated calf thymus DNA. The lipids were subjected to two freeze-thaw cycles and extruded 11 times through a 400 nm polycarbonate filter using a 1 ml LiposoFast-Basic extruder (Avestin). The resulting liposomes were diluted to a concentration of 4 mg/ml in 20 mM KPi (pH 7.0) containing 100 mM potassium acetate and destabilized by the addition of 2-3 mM Triton X-100 at 0.25 mM increments until the OD₅₄₀ of the liposome suspension reached a maximum [30]. Purified MexB in elution buffer was then added to a protein to lipid ratio of 1:100 (w/w). After incubation for 30 min at room temperature, 80 mg of hydrated polystyrene Bio-Beads (Bio-Rad) were added per millilitre of liposome suspension to remove the detergent (34, 35). Following incubation for 2 h at 4 °C, these beads were replaced twice by fresh Bio-Beads (80 mg/ml), which were incubated at 4 °C for 2 h and overnight, respectively. Before use, Bio-Beads were hydrated by one wash in methanol, followed by one wash in ethanol and five washes with ultrapure H₂O. The proteoliposomes were collected by centrifugation at 164000 g for 30 min and resuspended to 1 mg of membrane protein/ml in 20 mM potassium phosphate buffer (pH 7.0) containing 100 mM sodium acetate and 2 mM MgSO₄. Proteoliposomes were immediately used in transport assays.

Hoechst 33342 transport in proteoliposomes

To impose diffusion gradients [31], proteoliposomes in 20 mM potassium phosphate buffer pH 7 containing 2 mM MgSO₄, 100 mM potassium acetate and 10 nmol valinomycin (to prevent the formation of a reverse $\Delta\psi$) per mg protein were diluted 1:100 (to 10 μ g protein per ml) into the same buffer (no gradient) or into 20 mM potassium phosphate buffer pH 7 containing 2 mM MgSO₄ and 50 mM K₂SO₄ to generate a Δ pH (interior alkaline). After 30 sec, 0.125 μ M Hoechst 33342 was added and the fluorescence of the Hoechst–DNA complex formed inside the proteoliposomes was followed over time in an LS-55B luminescence spectrometer at excitation and emission wavelengths of 355 and 457 nm, respectively, and slit widths of 10 and 5 nm, respectively.

RESULTS

MexB can functionally complement AcrB in *E. coli* cells lacking AcrB.

In order to gain insight into the activity of MexB when not associated with its cognitive partners MexA and OprM, plasmid pMexBH encoding his-tagged MexB was expressed in *E. coli* strain BW25113 Δ AcrB, which lacked the MexB homologue, AcrB. Surprisingly, expression of MexB alone confers resistance on the cells to the toxic compound ethidium. The IC₅₀ of the survival ratio increased from 19 ± 3 μ M for the non-expressing control cells to 67 ± 11 μ M for MexB expressing cells (Figure 1A). The resistance conferred by MexB on its own was also compared to that of the tripartite MexAB-OprM pump. When all three components of the MexAB-OprM pump were expressed, the IC₅₀ of the survival ratio increased to 395 ± 35 μ M (Figure 1A). Similarly, when the effect of increasing the concentration of ethidium on the maximum specific growth rate was studied, MexB expressing cells exhibited a significant resistance to ethidium with IC₅₀ values of 38 ± 4 μ M, 110 ± 14 μ M and 335 ± 21 μ M for non-expressing, MexB expressing and MexAB-OprM expressing cells, respectively (Figure 1B and Table 1). The observed resistance could be explained if MexB was able to interact with the periplasmic binding protein AcrA and outer membrane pore TolC from *E. coli* and so complements the missing AcrB. The role of AcrA and TolC in MexB function was further investigated by expressing MexB in *E. coli* BW25113 Δ AcrAB which lacked both AcrA and AcrB and *E. coli* BW25113 Δ TolC, which lacked TolC. Deletion of TolC from the genomic DNA of strain BW25113 resulted in an increased drug sensitive phenotype; cytotoxicity assays had to be carried out at lower drug concentrations than that used for strains with the AcrB or AcrAB deletion. Cytotoxicity assays revealed that MexB in *E. coli* could confer resistance on cells to a range of compounds such as the antibiotics oxacillin and novobiocin, the toxic compounds ethidium and TPP and the detergent SDS (Table 1). The observed resistance was dependent on the presence of both AcrA and TolC, as no increase in resistance was observed when MexB was expressed in cells which lacked either AcrA or TolC. The expression of MexB was equal in all the BW25113 (DE3) *E. coli* cells used (results not shown). As a negative control, MexB with the inactivating D407N mutation [32] was also expressed in *E. coli* strain BW25113 Δ AcrB. No resistance was detected for the D407N mutant of MexB, which confirmed that the observed resistance was originating from MexB, as this mutant was expressed equally to wild type MexB (results not shown). The cells expressing only MexB displayed the same substrate specificity as the tripartite MexAB-OprM pump. However, the IC₅₀ values for MexB-expressing cells were several fold lower than that of the MexAB-OprM-expressing cells for all drugs tested (Table 1). The resistance to ethidium conferred by the tripartite MexAB-OprM in *E. coli* is similar to that of the native AcrAB-TolC complex. However differences were observed in the resistance conferred to other compounds, with the MexAB-OprM

complex being able to confer a higher level of resistance to novobiocin than the native AcrAB-TolC, but a lower level of resistance to SDS and TPP (Table 1).

MexB display AcrA and TolC dependent efflux of fluorescent drugs from *E. coli* cells.

To test whether drug extrusion from the cell is the underlying mechanism of drug resistance in *E. coli* Δ AcrB expressing MexB, we measured the extrusion of fluorescent substrates from the *E. coli* strains BW25113 Δ AcrB, BW25113 Δ AcrAB and BW25113 Δ TolC. Hoechst 33342 is a toxic compound that is virtually non-fluorescent in aqueous solution, but has a high fluorescent quantum yield when present in the hydrophobic environment of the lipid bilayer or when bound to DNA. Therefore, Hoechst 33342 efflux from cells can be measured as a reduction in fluorescence. When cells were pre-energised by the addition of glucose, the uptake of Hoechst 33342 in cells expressing MexB was significantly lower than that observed in the control cells without MexB and was only slightly higher than cells expressing the tripartite MexAB-OprM complex. Initial influx rates of 18.9 ± 0.2 , 5.7 ± 0.5 and 3.6 ± 0.3 a.u./min were obtained for the non-expressing control cells, the MexB-expressing cells and the MexAB-OprM-expressing cells respectively (Figure 2A). The most efficient reduction in Hoechst 33342 uptake was observed for the AcrAB-TolC complex with an initial influx rate of 0.9 ± 0.2 a.u./min (Figure 2A). No difference in Hoechst 33342 influx was observed between the control and MexB-expressing cells in strains where AcrA or TolC has been deleted (results not shown). In addition no Hoechst 33342 efflux was observed for the D407N mutant of MexB, again confirming that Hoechst 33342 efflux was indeed dependent on the presence of MexB (results not shown). These results demonstrate that MexB can partner with AcrA and TolC to form a functional efflux pump. The fluorescence of ethidium and TMA-DPH, which is enhanced in hydrophobic environments, was also used to assay their transport from the cells. Similar to the results obtained for Hoechst 33342 transport and in accordance with the cytotoxicity assays for ethidium, MexB was able to efflux both ethidium (Figure 2B) and TMA-DPH from cells (Figure 2C). In both cases, the passive influx was reduced (higher efflux) in the MexAB-OprM expressing cells compared to the cells which only express MexB (Figure 2B and Figure 2C). No significant difference was observed between the transport of ethidium and TMA-DPH by MexAB-OprM or the native AcrAB-TolC complex (Figures 2B and 2C).

To investigate if a physical interaction between MexA and MexB occurs, His-tagged MexB was purified using Ni-affinity chromatography from cells lacking AcrB or lacking TolC as indicated in Figure 2D. Purified fractions were subjected to SDS-PAGE and blotted onto a PVDF membrane. The blot was developed with anti-AcrA antibody. A high affinity complex forms between AcrA and MexB as AcrA co-purified with MexB even in the absence of cross-linker. In the presence of the cross-linker DSP, AcrA is observed as a higher oligomer complex. Formation of the AcrA-MexB complex is not dependent on the cells being energised as the complex was observed in the absence of glucose and presence of the protonophore CCCP. Formation of the AcrA-MexB complex was dependent on the presence of TolC as no complex was observed in the cells lacking TolC (Figure 2D). AcrA did not bind non-specifically to the Ni-affinity resin as no AcrA was observed when the purification was done with cells propagating the non-expressing control plasmid (Figure 2E). A homogenous preparation of MexB is obtained from these preparations as indicated by the Coomassie stained gel (Figure 2F).

Independent drug transport by MexB in a gram-positive bacterium

The surprising promiscuity of MexB that apparently allows it to form a functional complex with the *E. coli* proteins, AcrA and TolC, raises questions as to the exact role of the periplasmic binding protein and the outer membrane pore in MexB functionality. Are binding

of these proteins to MexB necessary for the formation of functional MexB *per se* or is complex-formation just a necessity to move drugs across the periplasm and outer membrane? We have addressed this question by expressing MexB in the gram-positive organism *Lactococcus lactis*. In this way there is no periplasmic space or outer membrane to traverse in order to transport drugs out of the cell. MexB and D407N MexB were expressed in an *L. lactis* strain in which genomic multidrug transporter genes *lmrA* and *lmrCD* were deleted [27]. Both MexB proteins express equally well in *L. lactis* cells (Figure 3A). Surprisingly, reduced ethidium uptake was observed for the MexB expressing cells in comparison to the D407N MexB and non-expressing control samples with initial influx rates of 19.8 ± 0.9 , 37.8 ± 0.9 and 40.1 ± 2.1 a.u./min for MexB-expressing cells, D407N MexB-expressing cells and control cells respectively (Figure 3B). Ethidium transport by MexB in *L. lactis* was also compared with that of the LmrP and LmrCD drug efflux proteins from *L. lactis*. LmrP transports ethidium very efficiently as hardly any ethidium is entering the cells (Figure 3B). The initial influx rate of ethidium into the cells is higher for the LmrCD expressing cells compared to the MexB expressing cells, but then ethidium accumulate at a lower steady state level than in the MexB expressing cells (Figure 3B). However the higher expression level of LmrCD compared to MexB could also be responsible for the lower level of ethidium accumulation in the LmrCD expressing cells (Figure 3A). The expression of MexB also confers resistance to the *L. lactis* cells to ethidium. In correlation with the transport assays, expression of LmrP conferred the highest level of resistance followed by LmrCD and then MexB (Table 2).

Independent drug transport by purified and reconstituted MexB

To further confirm the ability of MexB to interact with transported ligands independently of other tripartite components we studied the properties of purified MexB in detergent solution and when functionally reconstituted into proteoliposomes. First, MexB and D407N MexB were purified and the ability of the D407N MexB transport-negative mutant to interact with Hoechst 33342 was assessed in a drug binding assay that utilised the unique fluorescent properties of the dye. As Hoechst 33342 is not fluorescent in aqueous solution, the binding of Hoechst 33342 to the hydrophobic binding pocket can be observed as an increase in fluorescence. Equimolar amounts of MexB and D407N MexB were titrated with increasing concentrations of Hoechst 33342. The Hoechst 33342 fluorescence was corrected for non-specific binding and the data was plotted versus the Hoechst 33342 concentration. The data was fitted with a one binding site hyperbola and yielded R^2 values of 0.979 and 0.949 for MexB and D407N MexB respectively. No significant differences were obtained between the binding parameters of MexB and D407N MexB (Figure 4A and Table 3), indicating that D407N MexB retains the ability to bind Hoechst similar to the wild type protein, even though transport activity is abolished. As another control, drug binding to purified MexA was measured. No drug binding to MexA could be observed, as the Hoechst 33342 fluorescence was equal to that of the buffer alone (Figure 4A).

MexB and D407N MexB were reconstituted into proteoliposomes and the Hoechst 33342 transport activity was measured. The (proteo)liposomes were loaded with DNA and potassium acetate inside and the reconstitution method used should yield proteins that are orientated in a right side-out fashion [30]. Therefore, when the proteoliposomes are diluted into a buffer that does not contain potassium acetate, to allow the outward diffusion of acetic acid and the subsequent generation of a Δ pH (interior alkaline), an *in vitro* model for intact cells is created containing DNA and a basic internal pH. The generation of a Δ pH activates MexB which is a drug/ H^+ antiporter and hence would efflux Hoechst 33342 when a basic inside pH is generated. The D407N MexB mutant was purified and reconstituted into proteoliposomes to the same level as the wild type protein (Figure 4B). Imposition of the

Δ pH in the proteoliposomes reduced the passive diffusion of Hoechst 33342 in the MexB containing proteoliposomes, but not in the empty liposomes or the proteoliposomes containing the transport negative mutant D407N MexB. Initial influx rates for Hoechst 33342 of 6.64 ± 0.91 , 1.74 ± 0.32 and 7.24 ± 0.78 a.u./min were observed for the control liposomes and the proteoliposomes containing MexB or D407N MexB respectively (Figure 4C). Addition of purified MexA to the MexB-containing proteoliposomes had no effect on the Hoechst 33342 transport. In the absence of the Δ pH, no Hoechst 33342 transport was observed in the MexB-containing proteoliposomes (Figure 4D). These results indicate that Hoechst 33342 transport is dependent on MexB and that MexB can mediate Hoechst 33342 efflux by itself in a Δ pH-dependent manner.

DISCUSSION

Drug resistant gram-negative bacteria need to transport drugs over two membranes and the periplasmic space. This is normally affected by tripartite assemblies of an inner membrane protein an outer membrane factor and a membrane fusion protein, such as the MexAB-OprM protein complex from *P. aeruginosa*. Previous studies indicated that MexB could only form a functional complex with MexA and OprM [19]. However, we have found that MexB is promiscuous and can partner with AcrA and TolC from *E. coli* to form an active complex. MexB-AcrA-TolC displays the same substrate recognition profile as the MexAB-OprM complex but is less efficient in conferring resistance to the toxic substances (Figure 1 and Table 1). Our results correspond well with that of another study that also found that MexB expressed in *E. coli* cells can confer resistance to some drugs [17]. Our complementary study now demonstrates MexB-AcrA-TolC mediated efflux of the fluorescent compounds Hoechst 33342, ethidium and TMA-DPH. Consistent with the resistance data, MexB-AcrA-TolC was less efficient than the MexAB-OprM or native AcrAB-TolC complexes in transporting these compounds (Figure 2). Even though MexB-AcrA-TolC and the MexAB-OprM complex can efflux Hoechst 33342 from cells at $0.125\mu\text{M}$, these complexes do not confer resistance upon the *E. coli* cells to Hoechst 33342. This is probably due to the inability of MexB in *E. coli* to transport Hoechst 33342 at the concentrations at which it becomes toxic to the BW25113 Δ AcrB cells ($2\mu\text{M}$).

OprM and TolC share only a modest sequence homology (about 20% identity, compared to 70% for MexB and AcrB and 58% for MexA and AcrA). It is therefore not surprising that TolC cannot support MexB activity through functional complementation of OprM in the presence of MexA [16,22]. Models of the AcrAB-TolC complex predict a sparse interaction between the β hairpins in the AcrB crown and the periplasmic end of TolC [4,33]. Hence, a combination of MexB and TolC is tolerated as long as the third protein in the complex is AcrA. In this sense MexAB is different from MexXY and MexCD both of which can act together with TolC [21,34]. The question remains if an intact tripartite assembly is needed for function of the individual IMP proteins or if this is only a requisite for bridging the two membranes of the gram-negative organisms. To answer this question we have first expressed MexB in a gram-positive bacterium that lacks the outer membrane. Surprisingly, energy dependent drug efflux was still observed for the *L. lactis* Δ LmrA Δ LmrCD cells expressing MexB (Figure 3) and MexB conferred ethidium resistance on the cells (Table 2). As RND proteins and MFP analogues are found in gram-positive organisms [35-37], it cannot be ruled out that MexB activity in *L. lactis* is dependent on an endogenous MFP. In view of the absence of OMP proteins in *L. lactis*, the observations on MexB-dependent drug transport in this organism, provides evidence that MexB can work alone.

To avoid the possibility of MexB interactions with a hitherto unidentified MFP in *L. lactis*, we have taken advantage of the *E. coli* expression system to purify sufficient amounts of

protein at a very high purity to functionally reconstitute MexB in proteoliposomes. Transport of Hoechst 33342 was observed in the presence of a Δ pH (inside basic), but not in the absence of the imposed chemical proton gradient. Transport was also not observed for D407N MexB, in which the aspartate residue that is predicted to be involved in H^+ coupling was mutated to neutral asparagine. The addition of MexA had no effect on MexB mediated Hoechst 33342 transport in the proteoliposomes (Figure 4). This is the first time that drug transport is reported for a RND transporter reconstituted in proteoliposomes in the absence of MFS and OMP proteins. Previously, the MexB homologue, AcrB, had been reconstituted in lipid vesicles but drug transport was not measured directly. Instead, the increase in the fluorescence of NBD-lipids was measured as it was removed from donor vesicles to acceptor vesicles and this lipid transport was dependent on the presence of the MFP, AcrA [24]. Similarly, AcrD, another RND drug transport protein from *E. coli* has also been reconstituted and the proton flux upon addition of aminoglycosides was measured. This proton flux, as well as gentamycin uptake, was dependent on the presence of AcrA [23]. In neither of these cases was a transport-negative mutant included in the measurements. Our results showing independent, Δ pH-driven drug transport by an RND multidrug transporter in proteoliposomes make a useful contribution to our understanding of the transport mechanism of RND transporters. Moreover, it allows new opportunities to study the molecular mechanism of RND type drug transporters and their associated MFPs.

The ability of membrane fusion proteins to stimulate drug transport or ATPase activity of integral membrane transporter proteins has led to the speculation that the membrane fusion proteins are actively involved in the transport process [38] and drug binding sites have been identified in some MFPs [39,40]. However, we did not observe Hoechst 33342 binding to MexA, which would refute a direct involvement of this MFP in Hoechst 33342 transport at least. Similar to other MFPs [23,24,41], MexA might increase the drug binding affinity of MexB, which remains to be investigated. Interestingly, Hoechst 33342 binds the D407N mutant and the wild type protein with the same affinity. This observation agrees with the notion of Asp407 in MexB and AcrB being involved in proton coupling [32,42] rather than directly interacting with substrates. Yu and co-workers observed a drug-binding site formed by the loop between TM3 and TM4 in wild-type AcrB [43,44] that was collapsed in the D407N mutant, leading to the suggestion that the D407N mutant would be unable to bind drugs. The Hoechst 33342 binding we observed might have resulted solely from binding to a site in the periplasmic domain, where no large structural alterations were observed for the D407N mutant [43].

To our knowledge this is the first time that independent drug transport was observed for an RND transporter in the absence of the outer membrane pore or the membrane fusion protein. It is also the first time that drug transport in proteoliposomes has been verified by showing that no drug transport can be observed for a transport-negative mutant protein even though this mutant binds the substrate with equal affinity as the wild-type protein. The functional reconstitution and independent activity of MexB opens up exciting new possibilities for further mechanistic studies on RND-type transporters and their functional regulation by MFP and OMP partners in a tripartite complex. It will also allow investigations on the mechanism of coupling between drug transport and proton transport, and determination of drug/ H^+ stoichiometries.

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TABLES

Table 1: IC₅₀ values for BW25113 cells harbouring the control and MexB expressing plasmids in the presence of the tested substrate

The IC₅₀ value is the concentration of substrate required to reduce the maximum growth rate of the cells by 50%.

Genomic deletion	Proteins expressed	IC ₅₀				
		EtBr (μM)	NOV (μM)	OX (μM)	SDS (μg/ml)	TPP (μg/ml)
ΔAcrB	Control	38 ± 4	35 ± 7	47 ± 2	79 ± 1	19 ± 3
ΔAcrB	MexB	110 ± 14	165 ± 7	220 ± 40	327 ± 15	37 ± 4
ΔAcrAB	Control	36 ± 6	27 ± 5	50 ± 1	75 ± 7	21 ± 1
ΔAcrAB	MexB	40 ± 1	32 ± 5	49 ± 1	72 ± 12	20 ± 4
ΔTolC	Control	12 ± 3	6 ± 0	8 ± 1	12 ± 1	10 ± 1
ΔTolC	MexB	12 ± 2	7 ± 1	9 ± 1	11 ± 1	10 ± 4
ΔAcrB	D407N MexB	35 ± 4	48 ± 4	27 ± 7	83 ± 7	17 ± 7
ΔAcrAB	MexAB-OprM	335 ± 21	>400	nd*	1225 ± 30	132 ± 15
ΔAcrB	AcrB	378 ± 42	70 ± 10	447 ± 8	>2000	400 ± 18

*nd = not determined

Table 2: IC₅₀ values for *L. lactis* NZ9000 ΔLmrA ΔLmrCD cells harbouring the control, MexB, LmrP or LmrCD expressing plasmids in the presence of ethidium

The IC₅₀ value is the concentration of substrate required to reduce the maximum growth rate of the cells by 50%.

Protein expressed	μ _{max} (h ⁻¹)	IC ₅₀ (μM)
Control	0.249 ± 0.013	6 ± 0.29
MexB	0.109 ± 0.002	8.25 ± 0.35
LmrP	0.213 ± 0.004	13.25 ± 0.07
LmrCD	0.162 ± 0.025	11.75 ± 0.35

Table 3: Parameters for Hoechst 33342 binding to purified MexB and D407N MexB

	B_{\max}	K_D
Wild-type MexB	304.3 ± 10.9 a.u.	0.786 ± 0.074 μ M
D407N MexB	282.9 ± 20.9 a.u.	0.847 ± 0.157 μ M

FIGURE LEGENDS**Figure 1: MexB confers resistance upon *E. coli* cells that lack AcrB.**

(A) Cell survival in the presence of ethidium. MexB-expressing (■), MexAB-OprM expressing (▲) and non-expressing control (●) BW25113 Δ AcrB cells were incubated at 37°C in the presence of the indicated concentrations of ethidium. After 3h of incubation, the cell suspensions were serially diluted and plated. Colonies were counted and the survival ratios were calculated as the percentage of colony-forming units observed at a given concentration of ethidium to that observed without added ethidium. (B) Cell growth in the presence of ethidium. MexB-expressing (■), MexAB-OprM expressing (▲) and non-expressing control BW25113 Δ AcrB cells (●) were grown at 37°C in the presence of the indicated concentrations of ethidium and the A_{660} was measured every ten minutes. The growth rate was determined for each concentration of cytotoxic agent. The growth rate in the absence of cytotoxic agent was taken as 100% and did not differ between the different samples (0.514 ± 0.021 /h). All values represent the mean and standard error of five independent experiments.

Figure 2: MexB mediates drug transport from *E. coli* cells that lack AcrB by recruiting AcrA and TolC.

Non-expressing control (1), MexB-expressing (2), MexAB-OprM expressing (3) and AcrB expressing (4) BW25113 Δ AcrB cells were grown to an OD_{660} of 0.6. Cells were harvested, washed thrice in 50 mM KPi buffer pH 7.0 containing 5mM $MgSO_4$ and resuspended to a final OD_{660} of 0.5. Glucose (20mM) was added to generate metabolic energy. Uptake of fluorescent substrates was followed over time after the addition as indicated by the arrow of (A) 0.125 μ M Hoechst 33342, (B) 2 μ M ethidium or (C) 0.25 μ M TMA-DPH. The results show representative traces from three different experiments with different batches of cells. (D) AcrA co-purified with MexB. MexB expressing cells were grown and harvested as described for the fluorescent assays. The cross-linker DSP, glucose and the protonophore CCCP were added to the cells as indicated. ISO vesicles were prepared from the cells and the histagged MexB was purified using Ni-affinity chromatography from cells lacking AcrB (lanes 1-3) or lacking TolC (lane 4). Samples were analysed by SDS-PAGE, followed by immunoblotting with anti-AcrA antibody. (E) AcrA does not bind non-specifically to the Ni-NTA affinity resin but requires the interaction with His-tagged MexB. MexB was purified using Ni-affinity chromatography from cells lacking AcrB propagating the non-expressing control plasmid (lane 1) or the MexB expressing plasmid (lanes 2 and 3). Samples were treated as described in (D) and the blot was probed with anti-AcrA antibody. (F) Coomassie stained gel of MexB purified and treated as described in (D) showing the level of enrichment. The molecular weight markers are indicated in lane 1 and purified MexB in lane 2.

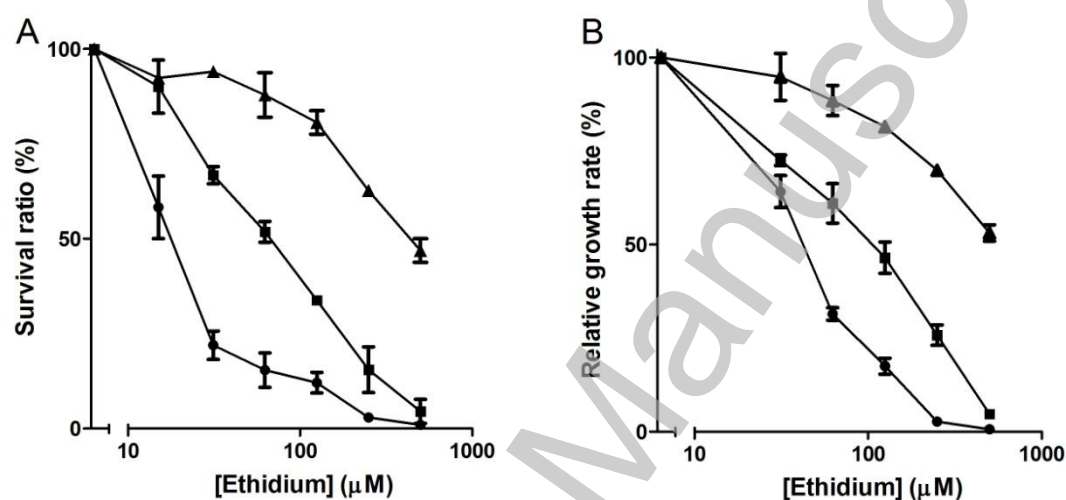
Figure 3: MexB is over-expressed and transport-active in *L. lactis*.

(A) ISO membrane vesicles were prepared from the control, MexB, D407N MexB, LmrP and LmrCD expressing cells. The total membrane proteins (30µg/lane) were separated with SDS-PAGE and visualised with Coomassie Brilliant Blue. The positions of MexB, LmrP, LmrC and LmrD are indicated with arrows. (B) *L. lactis* NZ9000 ΔLmrA ΔLmrCD cells harbouring various plasmids were grown at 30°C until an OD₆₆₀ of 0.4 was reached. Protein expression was initiated by the addition of nisin and allowed to continue for one more hour. Cells were harvested, washed thrice in 50mM KPi buffer pH 7.0 containing 5mM MgSO₄ and resuspended to a final OD₆₆₀ of 0.5. Glucose (20 mM) was added to generate metabolic energy. Ethidium was added where indicated by the arrow and the uptake of ethidium was followed over time. The results show representative traces from four different experiments with different batches of cells.

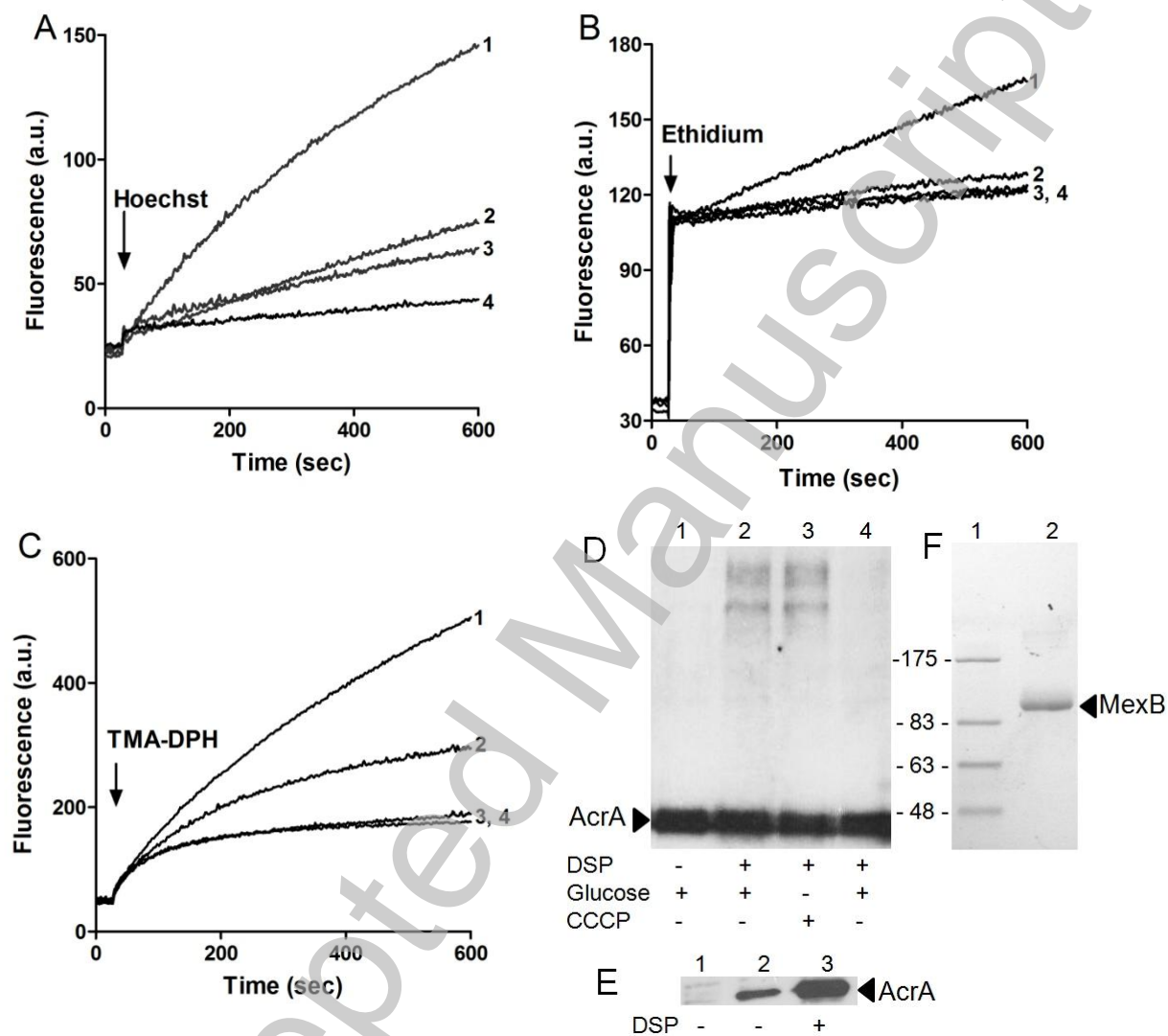
Figure 4: MexB in proteoliposomes can transport Hoechst 33342 in the absence of auxiliary proteins.

MexB and D407N MexB were purified from *E. coli* strain BW25113 ΔAcrAB. (A) Hoechst 33342 binding to purified MexB (●), D407N MexB (■), MexA (▲) and binding buffer containing DDM at the same concentration as in the protein samples (○) was measured by fluorimetry in the presence of increasing concentrations of the dye. The data from MexB and D407N MexB were corrected for non-specific binding of Hoechst 33342 and were fitted to a hyperbola. The data represent the means and standard error of four independent experiments with protein purified from different batches of vesicles. (B) Coomassie Blue stained SDS-PAGE (10%) of MexB and D407N MexB purified by Ni-NTA and reconstituted into proteoliposomes and of purified MexA. The positions of MexB and MexA are indicated. (C, D) Hoechst 33342 transport in empty control liposomes (1), proteoliposomes containing MexB (2), or D407N MexB (3) and proteoliposomes containing MexB pre-incubated with purified MexA (4). (Proteo)liposomes were diluted in 20mM KPi buffer (pH 7.0) containing 50mM K₂SO₄ and 2mM MgSO₄ to generate a pH gradient (basic inside; C) or in 20mM KPi buffer (pH 7.0) containing 100mM KAcetate and 2mM MgSO₄ for no pH gradient (D). Hoechst 33342 was added as indicated by the arrow and the Hoechst 33342 fluorescence was measured over time. The results show representative traces from three different experiments with proteins purified from different batches of vesicles.

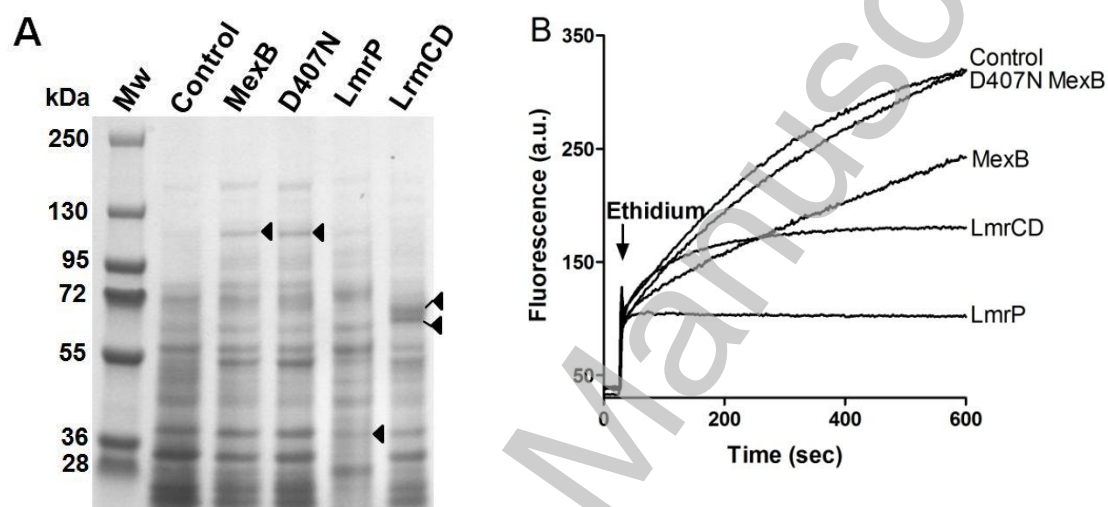
Welch *et al.*, Figure 1



Welch *et al.*, Figure 2



Welch *et al.*, Figure 3



Welch *et al.*, Figure 4

