Structural and functional studies of an in vitro fully assembled *Pseudomonas aeruginosa* efflux pump

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Efflux pumps from Gram negative bacteria are on the front line of bacterial resistance.
Tackling the mechanism of efflux outside the complexity of the bacterial cell
a « bottom-up » approach

*Pseudomonas aeruginosa*

**Efflux pumps**

**in vitro assay**

**tools for the rational manipulation of membrane proteins**

- bacterial strains for the heterologous expression of « difficult » membrane proteins
- new detergents (amphipathic polymers)
- biomimetic systems: lipid nanodiscs, liposomes, lipid cubic phases ...
Reconstitution of the proteins in nanodiscs and electron microscopy analysis of the tripartite complex

discussion:

« direct contact » model

versus

« tip-to-tip » model


**controversy:**

« direct contact » model

versus

« tip-to-tip » model


We show that the pump adopts assembles along the tip-to-tip model.
Membrane transport

Design of an *in vitro* assay for membrane protein transport

**Pre-requisites**
- Compartimentation
- Relevant index of transport
- Energization of the system
- Compartimentation: reconstitution into liposomes

- Relevant index of transport

Fluorescence de la pyranine (UA)

\[ \Delta F = 40\% \]

\[ \Delta pH = 0.5 \]

Pyranine
Design of an assay for transport across a tripartite efflux pump

pyranine fluorescence decreases
EthB fluorescence increases
concomitant!
pH inside of the liposomes as a function of time, in the presence of EthB

EthB concentration is 5 μM. Error bars represent the standard deviation (n = 3).

The pH inside of the liposomes as a function of time, in the presence of EthB.

*EthB concentration is 5 μM. Error bars represent the standard deviation (n = 3).*

Acidification of the liposomes is biphasic

- **fast phase**
  - $H^+$ spontaneous diffusion
  - $[H^+]$ gradient

- **slow phase**
  - Charge gradient
  - $[H^+]$ gradient
  - Equilibrium
Ethidium bromide fluorescence as a function of time

The ethidium bromide concentration is 2 μM. Error bars represent the standard deviation (n = 3).

SATISFACTORY AND USEFUL BUT ... FRUSTRATING!

MAKING THIS ASSAY QUANTITATIVE ...

- control the orientation
- obtain homogenous population of proteoliposomes
- quantitate the number of protein per liposome
- measure time resolved fluorescence variations at the msec time scale
- measure the catalytic parameters of the pump
Reconstituting the activity of an efflux pump in vitro: a quantitative approach

D. Puvanendran
PhD Student

ESCMID eLibrary
Determination of the protein and lipid quantity in the proteoliposomes

Estimation of the quantity of protein per liposome

Quality control by DLS and SEC MALS

mean diameter = 103.9 nm
polydispersity = 0.127

\[ N_{\text{lipids/liposome}} = \frac{4\pi R^2 + 4\pi (R-h)^2}{a} \]

\[ N_{\text{lipids/liposome}} = 86780.61 \]

\[ N_{\text{proteins/liposome}} = \frac{n_{\text{proteins/liposome}} \times Na}{m_{\text{proteins/liposome}} / MW_{\text{OprM trimer}} \times Na} = 3.62 \text{ proteins/liposome.} \]
Activity measurements

Stopped-flow fluorimetry

Driving syringes

Fixed spectrometer

Mixing chamber

Hard-stop
Activity measurements

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Acidification buffer

Liposomes/proteoliposomes
valinomycin = proton / potassium ionophore

=> collapse of the charge gradient

the slow phase is indeed rate-limited by an opposite charge gradient
Transport = acceleration of acidification
Liposomes without proteins

MexABwt proteoliposomes

MexABmut proteoliposomes
SF measurements: we indeed reach the msec time-scale and confirm our initial conclusions.
SF measurements: again, we observe that the presence of OprM has a dramatic effect
Strep-mediated pulldown of proteoliposomes allows for an identification of the formation tripartite complexes.

100 µL MexAB proteoliposome
100 µL OprM proteoliposome
800 µL buffer
incubate at room temperature
add magnetic bead => pellet the complex
wash the pellet and SDS PAGE
Strep-mediated pulldown of proteoliposomes allows for an identification of the formation tripartite complexes.
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Incubate at room temperature

Add magnetic bead => pellet the complex

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Strep-mediated pulldown of proteoliposomes allows for an identification of the formation tripartite complexes.

100 µL MexAB proteoliposome

100 µL OprM proteoliposome

800 µL buffer => generation of pH gradient in the presence of substrate ★

incubate at room temperature

add magnetic bead => pellet the complex

wash the pellet and SDS PAGE
Strep-mediated pulldown of proteoliposomes allows for an identification of the formation tripartite complexes.

The proton motive force indeed promotes disassembly of the complex.

CONCLUSIONS

- The pump assembles along the « tip-to-tip » model.
- Transport is very rapid (msec time scale).
- We confirm that OprM has a significant effect on transport.
- There is disassembly of the pump upon transport.
take-home message: a mutual interplay

Disassembly → Transport → Opening → Assembly

substance-induced coupled activity

OprM → MexA → MexB