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**Chapter Title:** Overexpression of the ABC transporter BmrA within intracellular caveolæ in *Escherichia coli*

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**Abstract:**

We described here the overproduction and oriented membrane insertion of membrane protein inside intracellular vesicles named heterologous caveolæ within *E. coli*. The method is described with BmrA, a multidrug efflux pump from *Bacillus subtilis*. BmrA is produced in these vesicles, thanks to the co-expression with the canine caveolin-1 $\beta$ , one of the two isoforms of caveolin-1. Enriched by sucrose gradient, the caveolæ-containing fraction allows to probe the ATPase and Hoechst 33342 transport activities, the latter displaying a higher specific activity than the same without caveolin-1 $\beta$ .

**Key Words**

ABC transporter, membrane protein overexpression, membrane caveolæ, cytoplasmic vesicles.

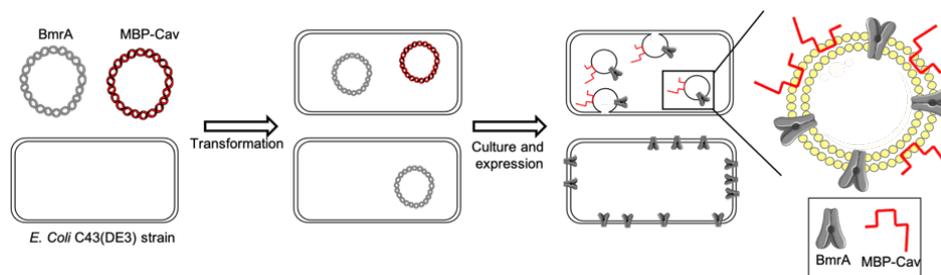
**1. Introduction**

Membrane proteins are key drug targets that drive many researches on these objects [1]. Overexpression is thus often used to obtain the large quantity of proteins required and achieve adequate purity and homogeneity levels. For membrane proteins, this overexpression has often been a major bottleneck. Indeed, overexpression can be toxic to cells, frequently requiring a mild overexpression rather than a massive production, and the quality of the produced protein requires meticulous and rigorous testing. Some documented examples show that a gentler expression of membrane proteins often results in a protein more active than the same protein produced in a larger quantity [2].

The orientation of the membrane protein can also be a critical point for their function, a problem especially concerning multidrug efflux pumps which translocate drugs from cytosol to the extracellular space. Membrane caveolæ are submicroscopic pits of the membrane found in higher eukaryotes [3]. These 60-80 nm wide pits are distinct from typical membrane invagination, being formed by the recruitment of a specific caveolin-1 protein and showing features distinct from clathrin-coated pits. Caveolæ are cell-type specific with cells being devoid of them (Kidney), or at the opposite can represent 50% of the plasma membrane surface for endothelial cells or adipocytes [3, 4]. Recently, heterologous caveolæ have been

successfully described in *E. coli* by heterologous expression of human caveolin-1 [5], opening the way to their use as membrane protein overexpression, being a reservoir for cell membrane [6]. Membrane caveolæ are made from the plasma membrane, which imposes the orientation of the expressed protein exposing the intracellular region of the protein to the cytosol once the caveolæ is generated.

The *Bacillus subtilis* ABC transporter BmrA is a multidrug transporter recognizing and transporting many structurally unrelated drugs [7-9]. Its overexpression confers resistance to *B. subtilis* against cervimycin C, an antibiotic secreted by a biotope competitor [10]. Here, we report the functional overexpression of BmrA within heterologous caveolæ in *E. coli* (Figure 1) allowing to probe the transport of drugs.



## 2. Materials

### 2.1. Plasmids

- The gene coding for BmrA WT and BmrA E504A are inserted in the pET-15b vector (Invitrogen) bearing ampicillin resistance.
  - The gene coding for the fusion protein Maltose-Binding Protein-caveolin-1 $\beta$  (MBP-Cav) is inserted into the pETM-40 vector (EMBL). The periplasm addressing sequence of MBP has been removed. The plasmid bears kanamycin resistance.
- Both proteins carry an N-terminal 6xHis tag.

### 2.2. Lab equipments

- Class-II Biological safety cabinet for sterile experiments
- Petri dishes
- 4 x 5L glass Erlenmeyer for bacterial culture
- New Brunswick™ Innova® 44 incubator, temperature regulated
- Low-speed centrifuge for 15/50 mL Falcon-type tubes
- Cell disrupter: Cell-D Constant Systems Ltd for bacterial lysis
- SDS-PAGE: Mini PROTEAN Tetra cell (Bio-Rad) and related devices, 0.75 mm spacer plate, 10 or 15-wells comb
- 96-wells plates (Greiner)
- Xenius SAFAS spectrophotometer for ATPase activity and substrate transport measure
- 1 mL Quartz cuvette

### 2.3. SDS-PAGE

- Acrylamide 4X/bis-acrylamide : 40%, 37.5:1 (Euromedex)
- Loading buffer Laemmli (4X): 8% (v/v) SDS, 100 mM Tris-HCl pH 8.0, 0.025% (w/v) Bromophenol blue, 2.8 M  $\beta$ -mercaptoethanol, 40% (v/v) glycerol
- Separating buffer stock solution: 1.5 M Tris-HCl pH 8.8

- Stacking buffer stock solution: 0.5 M Tris-HCl pH 6.8
- Aqueous SDS solution, 10% (w/v)
- Ammonium persulfate aqueous (APS) solution 10% (w/v), kept at -20°C.
- N, N, N', N'- tetramethylethylenediamine (TEMED, Sigma-Aldrich)
- Running buffer stock solution: Tris-Glycine-SDS (TG-SDS) 10X (Euromedex)
- Pre-stained molecular weight marker (10-170 kDa) (Euromedex)
- Staining solution: Coomassie buffer: 0.025% (w/v) Coomassie blue R250, 1% (v/v) ethanol, 25% (v/v) isopropanol, 10% (v/v) acetic acid
- Destaining solution: 10% (v/v) acetic acid

#### **2.4. Cells and media for cell culture**

- Bacteria : *E. coli* C43(DE3) strain
- SOC medium: 2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl<sub>2</sub>, 10 mM MgSO<sub>4</sub> and 20 mM glucose (NEB).
- Antibiotics stock solution: 40 mg/mL kanamycin (Sigma-Aldrich) and 50 mg/mL ampicillin resuspended in dH<sub>2</sub>O(Sigma-Aldrich). Kept at -20°C.
- LB media: 10 g/L tryptone, 5 g/L yeast extract and 10 g/L NaCl. The powder is dissolved in deionized water (making a total weight of 25 g/L) and sterilized by autoclaving 20 min at 121 °C. Right before use, the medium is supplemented with antibiotics: ampicillin 50 µg/mL for BmrA expression and kanamycin 40 µg/mL when caveolin is expressed.
- LB-agar media: LB media supplemented with 15 g/L agar and antibiotic(s)
- Isopropyl β-D-1-thiogalactopyranoside (IPTG) 0.5 M stock solution (Sigma-Aldrich)

#### **2.5. Membrane preparation and sucrose gradient**

- Bacterial lysis and membrane washing buffer: 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5 mM EDTA
- Glass homogenizer (50 ml Dounce)
- Sucrose: stock solution of 60% (w/v) sucrose (Sigma-Aldrich) used to prepare dilution to final concentrations of 20, 25, 30, 35, 40, 45, 50 and 55% (v/v) in 50 mM Tris-HCl pH 8.0, 150 mM NaCl
- BCA assay: 1 mg/mL bovin serum albumin (ThermoFisher) and Pierce™ BCA Protein Assay Kit
- Centrifuge with 15/50 mL tubes rotor and ultracentrifuge (Optima XPN-80, Beckman-coulter) with 50.2 Ti and SW41 Ti rotor

#### **2.6. ATPase activity**

- Inhibitors: 1 mM sodium orthovanadate (Na<sub>3</sub>VO<sub>4</sub>), 2 mM Na<sub>2</sub>S, 5 mM NaN<sub>3</sub>
- ATPase activity assay buffer: 30 mM Tris pH 8.0, 100 mM NaCl, 10 mM KCl, 2 mM MgCl<sub>2</sub>, 0.1 mg/mL pyruvate kinase (PK, Sigma-Aldrich), 0.1 mg/mL lactate dehydrogenase (LDH, Sigma-Aldrich), 1 mM phosphoenolpyruvate (PEP), 5 mM ATP-Mg (final concentration from a 100 mM mix made of 200 mM ATP + 200 mM MgCl<sub>2</sub> added in equal parts), 0.6 mM NADH

#### **2.7 Hoechst 33342 transport assays**

- Transport buffer: 50 mM HEPES-NaOH pH 8.0, 5 mM MgCl<sub>2</sub>, 8.5 mM NaCl
- ATP stock solution: 200 mM
- Hoechst 33342 stock solution: 200 µM

### 3. Methods

#### 3.1. *E. coli* co-transformation with genes coding for BmrA and MBP-Cav1 $\beta$

To generate heterologous caveolæ and to have BmrA inserted into them, MPB-Cav1 $\beta$  and BmrA are overexpressed simultaneously. This is achieved by having the two plasmids at the same time within *E. coli* cells by co-transformation and selection using two different antibiotics.

1. Fifty nanograms of each supercoiled circular plasmid *pET15b-bmra* [9] and *pETM40-mbpcav* [11] are added to 100  $\mu$ L C43(DE3) *E. coli* competent cells, for 30 minutes on ice.
2. Heat shock at 42 °C for 30 seconds, then place back on ice,
3. Add 300  $\mu$ L SOC medium under biological safety cabinet and incubate 1 h at 37 °C under agitation.
4. Spread 50  $\mu$ L of the cell mix onto LB-agar medium supplemented by ampicillin and kanamycin.
5. Incubate overnight at 37 °C, upside-down.
6. Controls for downstream experiments are achieved by co-transformation of mutant BmrA E504A (inactive) with *pETM40-mbpcav* to compare to the WT protein. Controls of BmrA transformed alone, without the plasmid coding for MBP-Cav, are also performed to compare expression and activity levels.

#### 3.2. Culture and overexpression

1. A freshly transformed colony is transferred into 20 mL LB-medium (in a 100 mL flask) supplemented with adequate antibiotics.
2. Incubate this pre-culture overnight at 37 °C under agitation (180 rpm)
3. Inoculate 4 x 500 mL of LB medium (with adequate antibiotics) in 5 L Erlenmeyer to a final OD<sub>600</sub> of 0.1 with the overnight pre-culture.
4. Incubate at 37°C under agitation until OD<sub>600</sub> reaches 0.6 and induce expression with 0.5 mM IPTG. Incubate overnight at 22°C under agitation.
5. Centrifuge cultures at 5,000 xg, 4 °C for 15 min with JLA 9.1000 rotor to collect bacteria
6. With spatula, transfer the pellet in 50 mL-tube, suspend them in 40 mL of 50 mM Tris-HCl pH 8.0, 150 mM NaCl to wash bacteria. Centrifuge again as previously and discard the supernatant.
7. If cell lysis is not performed immediately, store pellets at -80 °C.

#### 3.3. Membrane preparation

1. Resuspend bacteria in 50 mL per 2- Liter of culture with lysis buffer: 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5 mM EDTA and benzonase. From this point, keep everything at 4°C or on ice.
2. Lyse cells with a cell disrupter such as Cell-D Constant Systems Ltd with one pass at 1.4 kbar. If others cell disrupters are used, refer to the manufacturer's guidelines in order to keep vesicles intact (see **Note 1**).
3. Centrifuge cell lysate at 5,000 xg, 15 min, 4°C to pellet cell debris and intact cells. Collect supernatant and discard pellets.
4. Centrifuge supernatants at 100,000 xg, 1h, 4°C using 50.2 Ti rotor and suspend pellet with 1 mL of 50 mM Tris-HCl pH 8, 150 mM NaCl, 0.5 mM EDTA, anti-protease. Use a glass homogenizer and pestle to homogenize.

Take a 10  $\mu\text{L}$  aliquot to determine protein concentration in membranes using a BCA assay (following manufacturer's procedures, PIERCE) with an approximate 20-fold dilution of the membranes.

- Homogenized membranes can be stored in cryo-tubes and frozen in liquid nitrogen until further use.

### 3.4 Probe BmrA expression on SDS-PAGE, quantify expression using pre-purified protein

- Prepare a 10% separating gel by mixing 9.6 mL of water, 5 mL of 40% acrylamide/bis-acrylamide solution, 5 mL of 1.5 M Tris-HCl pH8.8, 200  $\mu\text{L}$  of 10% SDS, 200  $\mu\text{L}$  of 10% APS (see **Note 5**) and 20  $\mu\text{L}$  of TEMED. Pour it between 2 glass plates and add water at the surface of the gel to ensure a flat surface. Let polymerize and prepare a 4% stacking gel: 3.1 mL of water, 550  $\mu\text{L}$  of 40% acrylamide/bis-acrylamide solution, 1.3 mL of 0.5 M Tris-HCl pH6.8, 50  $\mu\text{L}$  of 10% SDS, 50  $\mu\text{L}$  of 10% APS and 5  $\mu\text{L}$  of TEMED. Remove water on separating gel and add stacking gel, place a 15-wells comb and let polymerize.
- Pipet 10  $\mu\text{g}$  of total protein, add water to a final volume of 10  $\mu\text{L}$  and add 3  $\mu\text{L}$  of 4x loading buffer.
- Load 12  $\mu\text{L}$  sample and run 10 min at 90 V then 1.5 h at 120 V
- Add known amount of purified BmrA [9] on the gel for comparison. For pure protein, incubate only 5 minutes with loading buffer to minimize aggregating artifacts on the gel.
- After electrophoresis, incubate gel in staining solution for 30 min and decolorating solution for 1 h.
- Quantify the intensity of bands corresponding to BmrA by densitometry, using for example ImageJ.

### 3.5. ATPase activity

For each type of membranes, 2 tubes are prepared, with  $\text{VO}_4^-$  and without  $\text{VO}_4^-$

Reaction buffer	Reaction mix	- $\text{VO}_4^-$ mix	+ $\text{VO}_4^-$ mix
30 mM Tris-HCl pH8.0 100 mM NaCl 10 mM KCl 2 mM $\text{MgCl}_2$	5 mM ATP-Mg 5 mM $\text{NaN}_3$ 2 mM $\text{Na}_2\text{S}$ 0.6 mM NADH 0.1 mg/mL PK 1 mg/mL PEP 0.1 mg/mL LDH Reaction buffer (final volume of 180 $\mu\text{L}$ per reaction)	5 mM ATP-Mg 5 mM $\text{NaN}_3$ 2 mM $\text{Na}_2\text{S}$ Reaction buffer (final volume of 46 $\mu\text{L}$ per reaction)	5 mM ATP-Mg 5 mM $\text{NaN}_3$ 2 mM $\text{Na}_2\text{S}$ 1 mM $\text{VO}_4^-$ Reaction buffer (final volume of 46 $\mu\text{L}$ per reaction)

- Calculate each necessary volume for "-  $\text{VO}_4^-$  mix", "+  $\text{VO}_4^-$  mix", "reaction mix" and each reagent
- Prepare  $\text{Na}_2\text{S}$  and  $\text{NaN}_3$  stock solution (they must be prepared just before the experiment and cannot be stored) (see **Note 2**).
- Incubate  $\text{VO}_4^-$  stock solution for 10 minutes at 95°C in dry bath before dilution (see **Note 3**).
- Prepare reaction buffer and ATP-Mg solution.
- Prepare "- $\text{VO}_4^-$  mix" and "+  $\text{VO}_4^-$  mix".

- Place 24  $\mu\text{L}$  of each type of membranes in 2 different tubes.
- Add 46  $\mu\text{L}$  of “-  $\text{VO}_4^-$  mix” or “+  $\text{VO}_4^-$  mix” to the corresponding tube and incubate for 10 min at 30 °C under shaking.
- Prepare reaction mix and incubate at 30 °C.
- For each tube with or without  $\text{VO}_4^-$ , dispatch in 3 x 20  $\mu\text{L}$  in a 96-wells UV plate.
- Add 180  $\mu\text{L}$  of reaction mix in each well and read immediately NADH absorbance at 340 nm for 20 min at 30 °C.

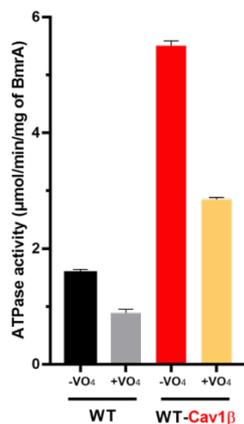
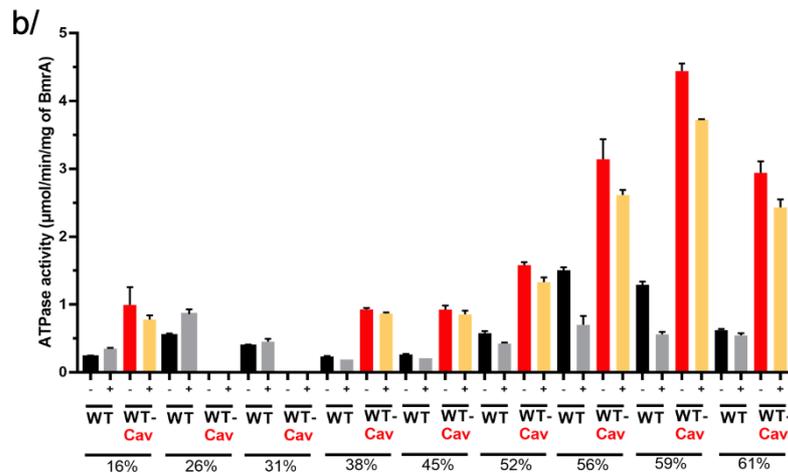
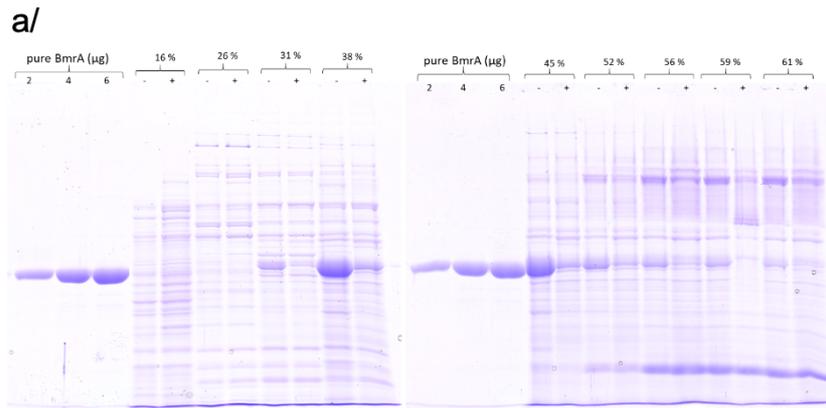


Figure 2 shows a typical example of BmrA ATPase activity on membranes. BmrA specific activity is the difference between the total activity, and the vanadate inhibited activity. Overexpression of BmrA in presence of caveolin-1 $\beta$  increases BmrA specific activity, being the result of fewer amount of BmrA produced but showing better activity. Note that the use of a BmrA inactive mutant is also a good control to differentiate specific activities.

### 3.6. Sucrose gradient and SDS-PAGE analysis

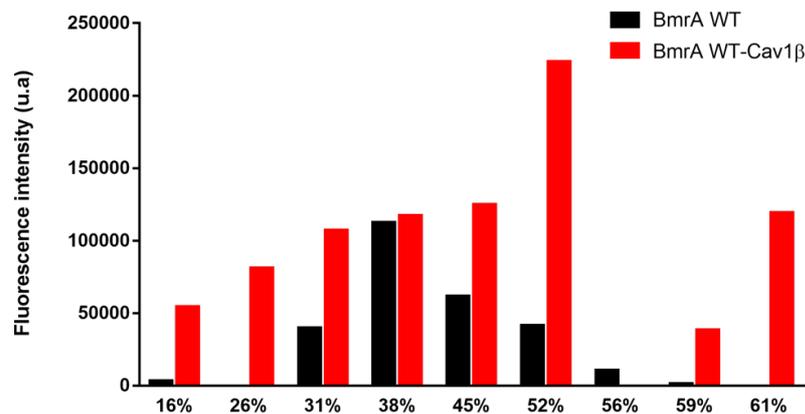
- Prepare 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55% and 60% (w/v) sucrose in 50 mM Tris-HCl pH 8.0, 150 mM NaCl.
- A sucrose gradient is prepared in SW41 rotor tube by pipetting first 1 mL of 60% sucrose solution and placing it at the bottom of the tube. Then, 1 mL of each sucrose concentration are added one after another in decreasing concentration order, by gentle pipetting to minimize disruption of the sucrose layers. At the top, add the volume of membranes corresponding to 10 mg of protein (see **Note 4**).  
When adding the sucrose solution, pour the solution slowly against the tube walls to not disturb the sucrose layers.
- Centrifuge overnight (about 15 h) at 150,000 xg, 4 °C.
- Collect 1 mL fraction one by one from top to bottom and keep them in Eppendorf tube. It is a good idea to store each fraction in aliquots of 0.5 mL in liquid nitrogen.  
Resuspend the pellet in 1 mL of 50 mM Tris-HCl pH 8.0, 150 mM NaCl.
- Analyze fractions of the gradient by SDS-PAGE by mixing 10  $\mu\text{L}$  of sample with loading buffer and incubate 30 min at room temperature before loading on acrylamide/bis-acrylamide gel, as described before.
- Each fraction can be analyzed for activity as described above.

Figure 3 shows a representative result of sucrose gradient separation of a membrane fraction. Noticeably, BmrA, overexpression in the presence of caveolin-1 $\beta$  results in a lower amount of BmrA produced, but the ATPase activity is remarkably higher. This is not surprising as it has been observed after changing BmrA overexpression on other *E. coli* cell lines [2].



### 3.7 Høchst 33342 transport assays

- The Høchst 33342 transport is recorded at 25°C with  $\lambda_{\text{excitation}} = 355 \text{ nm}$  and  $\lambda_{\text{emission}} = 457 \text{ nm}$  and bandwidth of 8 nm.
- In the quartz cuvette, put membranes corresponding to 50 µg of proteins and complete to 1 mL with transport buffer.
- One minute later add 10 µL of 200 µM Høchst 33342, final [Høchst 33342] = 2 µM.
- One minute later add 10 µL of 200 mM ATP, final [ATP] = 2 mM.
- Record fluorescence until equilibrium is reached.



#### 4. Notes

1. Do not disrupt the cells too much to keep vesicles intact.
2.  $\text{NaN}_3$  and  $\text{Na}_2\text{S}$  are toxic and must be prepared under chemical fume hood.
3.  $\text{VO}_4^-$  must be heated to break down aggregates. It can modify absorbance at 340 nm so control with  $\text{VO}_4$  alone is needed.
4. To prevent dilution of gradient and biasing reproducibility, do not add too much protein on the gradient
5. APS is stored at  $-20^\circ\text{C}$  and must be thaw just before the experiment.

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