# Final report OTKA NNF 78930

## Methods and results

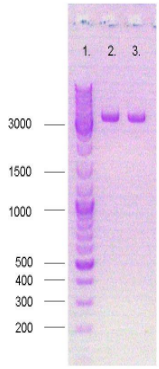
**Introduction of the gene of interest AcrB honologue protein into the plasmid pTTQ18**

The genes encoding the multidrug transporters were amplified from the corresponding samples of genomic DNA using appropriate oligonucleotides. These oligonucleotides were designed to introduce an *BamH*I site at the 5' end and a *Xho*I site at the 3' end, in order to promote the subsequent ligation with the 4.56 kb pTTQ18/RGS(His)$_6$ fragment. The genes were amplified by polimerase chain reaction and the PCR products were isolated from an agarose gel and then digested with *BamH*I and *Xho*I.

In order to clone each of the genes into the pTTQ18 plasmid vector, the „empty" plasmid was isolated from *E. coli* strain BLR and digested with the restriction endonucleases *BamH*I and *Xho*I to yield a linear DNA fragment. This fragment [pTTQ18 with the RGS(His)$_6$ coding DNA sequence] was isolated from an agarose gel.

Ligation reactions were performed using T4 ligase at 16°C overnight. The ligated product was subsequently transformed into *E. coli* DH5α [*fhuA2* Δ*(argF-lacZ)U169 phoA glnV44 Φ80* Δ*(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17* ] cells and recombinant clones were selected on LB plates containing ampicillin. Automated DNA sequencing was used to confirm the presence of each gene and the absence of any adventitious base changes. The plasmid was then transformed into *E. coli* strain BL21 (DE3) Novagen™ [$F^-$ *ompT hsdS*$_B$($r_B^-$ $m_B^-$) *gal dcm (DE3*)] for expression studies.

The following genes were successfully cloned into pTTQ18 plasmid without any difficulty: BMEI 1645, BMEI 0895, HI 0895, BCE 0788, BC0714 and AcrB.




**PCR reaction of *Helicobacter pylori* (HP0607)**

1. DNA ladder. 2, 3. DNA fragment of HP0607 separated on 1% agarose gel. The PCR product is around 3000 base pair as expected.

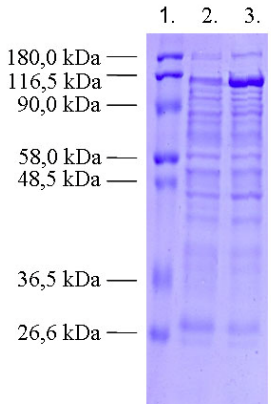
The PCR reaction was not successful with *Acinetobacter baumannii* (ACIAD0783), *Pseudomonas aeruginosa* (PA0158), *Pectobacterium carotovorum* (ECA1169) and *Klebsiella pneumoniae*-ban (KPN00443). We either didn't get any PCR products or several non-specific fragments appeared when we analyzed the reactions on agarose gel. We tried different newly developed enzymes and even gradient PCR to solve the problem.

**Expression of the target protein and isolation of the membrane**

For small-scale investigation of protein expression, 50 mL cultures in 250 mL flasks were used. Maintenance and growth of these *E. coli* BL21 strains was achieved by culturing the bacteria in Luria Broth (LB) liquid medium or on plates of the before mentioned medium containing 1.5% agar. Ampicillin (at least 100 μg/ml) was used throughout all stages of growth in order to maintain plasmid integrity. Total membranes were prepared from sphaeroplasts by the water lysis method from small scale expression (up to 50 ml).

Total membrane vesicles were prepared from 1000 ml cultures in 2 l flasks. The cells were disrupted by French press. The total membrane was separated by differential centrifugation from other cell debris and washed to remove the EDTA before affinity chromatography (Ni-NTA).

For both small-scale tests and larger scale production of inner membranes, the growth of the *E. coli* strains was allowed to continue until the cell density had reached an $A_{600}$ of approximately 0.5. At this point the expression of the cloned gene was induced by the addition of 0.2 mM isopropyl-ß-D-thiogalactopyranoside (IPTG). Growth continued for 3 h after the induction of the *tac* promoter at 37°C.




**Expression test of AcrB protein.**

1. molecular weight marker, 2. uninduced sample, 15 μg of total membrane after water lysis, 3. induced sample 15 μg of total membrane after water lysis.

Similar result was observed when BMEI 1645 was studied.

**Detection of expressed histidine-tagged transport proteins**

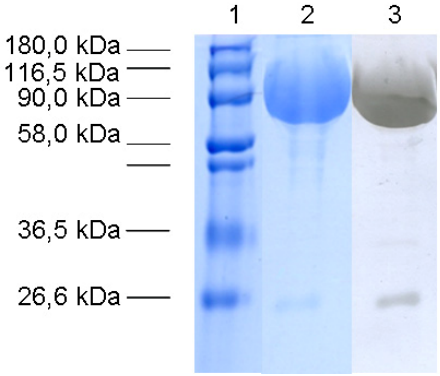
*E. coli* BL21(DE3) cells harbouring each plasmid were cultured in 2YT medium and expression trials performed with different concentrations of IPTG (0.0-1.0 mM). IPTG at a concentration of 0.2 mM was sufficient for the maximal expression of the AcrB$(His)_6$ and BMEI1645$(His)_6$ proteins.

Membrane samples were prepared for analysis of the overexpressed protein by SDS-PAGE. An IPTG-inducible protein was observed to migrate at a molecular mass of approximately 100 kDa for both AcrB$(His)_6$ and BMEI1645$(His)_6$. The identity of the overexpressed protein in the membranes was confirmed by Western blotting with anti-His antibody.

**Solubilization and purification of histidine-tagged transport proteins**

Initial purification of AcrB$(His)_6$ and BMEI1645$(His)_6$ was achieved by Immobilised Metal Affinity Chromatography (IMAC). Some minor contaminants are visible like bands of lower (in case of AcrB) and higher (in case of BMEI 1645) molecular weight.

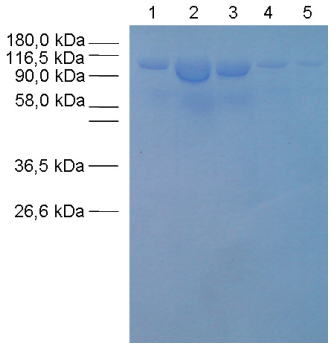
The conditions for solubilisation and purification, i.e. dodecyl-β-D-maltoside (DDM) concentration for solubilisation and imidazole concentration for IMAC vary depending on the characteristics of each individual transport protein. However, the generic conditions described here have proved generally useful. The efflux transporters were initially solubilised in 20 mM Tris pH 8.0, 5 mM imidazole pH 8.0, 300 mM NaCl 20% glycerol and 1% DDM. After washing out the unspecifically bound proteins, AcrB$(His)_6$ and BMEI1645$(His)_6$ were eluted by the following buffer: 20 mM Tris pH 8.0, 200 mM imidazole pH 8.0, 150 mM NaCl, 5% glycerol and 0.05% DDM. Before size exclusion chromatography buffer exchange was performed by using Econo-Pac 10DG column (Bio-Rad).




**Purification of AcrB protein.**

1. molecular weight marker, 2. Commassie stained purified concentrated protein, 3. Silver stained purified concentrated protein.

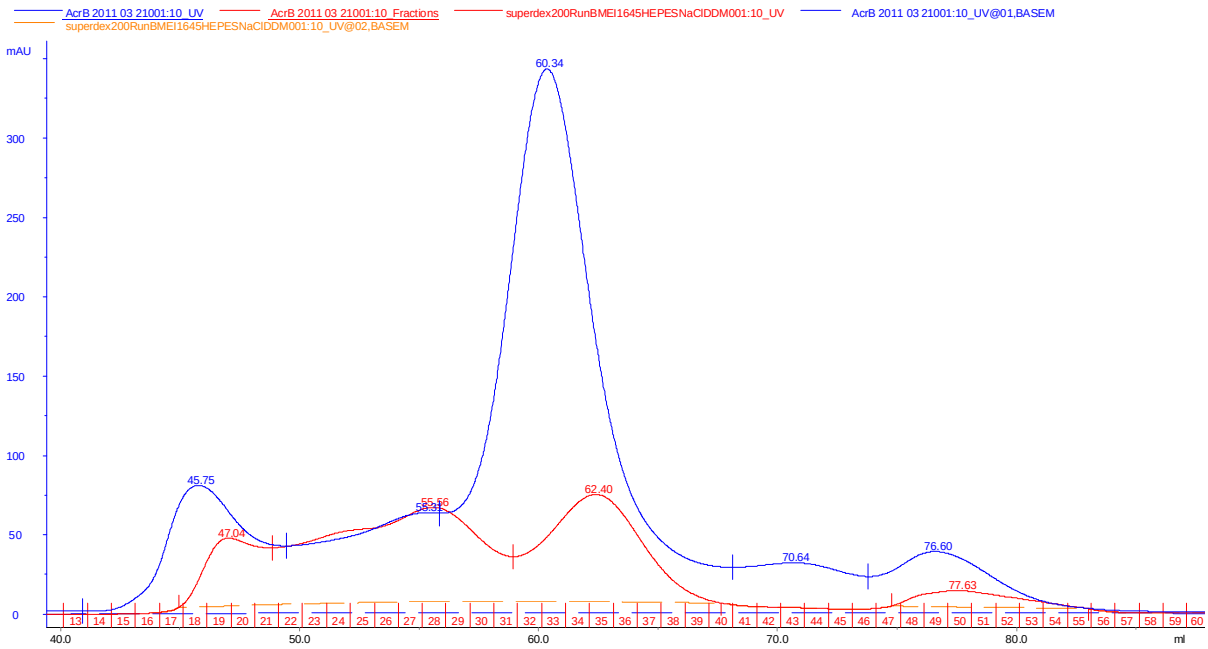
A lower molecular weight contaminant was detected however it did not influence the crystallization.




**Purification of BMEI 1645 protein.**

1-5. different fractions during the elution of the protein by imidazole off NiNTA resin.

It is also important to test for its monodispersity, because aggregated material usually inhibits crystallisation. Size exclusion chromatography is an established technique for the purification of proteins on the basis of their size. In the case of membrane proteins isolated in detergents, the apparent molecular size is increased by the presence of the detergent micelle. Even so, this method can be used as both a purification step, in addition to IMAC, and a way to assess monodispersity.

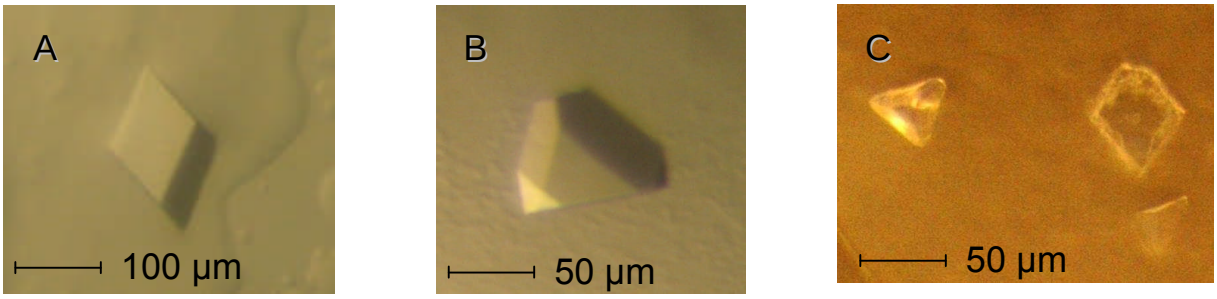



**Size exclusion chromatography (S200 16/60) of AcrB and BMEI 1645.** Blue line represents the purification profile of AcrB with a smaller void volume peak aound 45.75 ml and a nice symmetrical peak around 60.34 ml. The purified and concentrated protein showed a minor contamination at lower molecular weight. Red line represents the purification profile of BMEI 1645 protein. Besides the void volume peak two additional peaks are visible. The peak at 55.56 ml contained a higher molecular weight contamination that didn't give a positive immunoblot, so it cannot be a higher oligomeric state of our target protein. The peak at 62.40 also contained BMEI 1645 transporter.

Although BMEI 1645 showed nicely purified protein on SDS poliacrylamide gels when the sample was passed through size exclusion chromatograpy two major peaks were observed (red line). Both peaks contained the target protein so before we move on with the crystallization we have to clarify this result. AcrB showed only a single symmetrical peak. The contamination didn't influence either the shape of the curve or the crystallization of the protein.

**Crystallisation trials on the purified proteins**

After purification, crystallisation trials were set up using a previously developed comercially not available crystal screen at 18°C. At present, we prefer to retain glycerol in the crystallisation drop as this may provide a ready made cryo-protectant depends on its concentration. AcrB native protein crystals were appeared in two different crystallization conditions 2-3 weeks after the crystallization started. Two different shapes of crystals were observed (see figure below „A" and „B"). One of the condition with lower pH mainly gave form „A" crystals while „B" type crystals were observed in case of higher pH (condition2). As type „A" crystals grew bigger we started to optimize that crystallization condition with a matrix screen and checked the appearance and the size of the crystals. When we found the best condition for the crystallization of AcrB we started to test the effect of a β-peptid foldamer in different concentrations. According to our observation the addition of β-peptid foldamer decreased drastically the time that was necessary for the first crystals to appear. Two-three days needed for the crystallization of AcrB in this case. However the crystals, grew from the solution with β-peptid foldamer, were smaller around half size like the „native" crystals therefore the crystallization condition needs to be further optimized.




**Crystallization of AcrB protein.** „A" Crystals with contition1. „B" crystals with condition2. „C" crystals with ß-peptid foldamer.

## Summary:

The OTKA NNF 78930 grant helped to set up a protein crystallization laboratory in Szeged in the Institute of Pharmaceutical Analysis. Our major goal is to be able to start from cloning and finish the project with a new protein structure. The collegues were mostly unexperienced with the different techniques and methods applied during the last two years. This grant gave them a very good opportunity to study and pratice molecular biology methods, protein expression, purification and crystallization. Éva Kalmár who was employed by the project is working on her PhD now with a solid background provided by this support. Henriett Diána Szűcs is planning to apply for a PhD scholarship of the graduate school of USZ Faculty of Pharmacy. Moreover Lívia Marton had nice progress with the malaria transporters collaboration with SOLVO Zrt. Recently a new project student joined the group Enikő Ludányi who is working on the cloning and expression of AcrA to study the interactionof AcrA and AcrB.

During the last 24 months we have achieved most of our aims. Unfortunately we had sometimes logistic problems not having the right equipment. We are grateful for the help of HAS BRC Department of Biochemistry.

## Publications:

**Book chapters:**

- Saidijam M, Bettaney KE, Leng D, Ma P, Xu Z, Keen JG, Rutherford NG, Ward A, Henderson PFJ, Szakonyi G, Ren Q, Paulsen IT, Nes I, Kroeger JK, Kolsto A (2011) The MFS efflux proteins of Gram-positive and Gram-negative bacteria. In: Advences in Enzymology and Related Areas of Molecular Biology, ed. Toone EJ. John Wiley & Sons Inc. pp. 147-166.

**Presentations:**

- Szakonyi G: Investigation of AcrB homologue transporters. ATENS meeting, Dublin, Ireland, 2009. December 10-11.
- Marton L, Róna G, Mészáros Á: Investigation of transporter interactions of antimalarials. SZTE TDK Conference, Szeged, 2010. March 24-27. (II. award).
- Szűcs HD: Beta-foldamers, as crystallization adjuvants? SZTE TDK Conference, Szeged, 2011. February 8-12. (I. award).
- Szűcs HD: Beta-foldamers, as crystallization adjuvants? XXX. OTDK, Debrecen, 2011. April 7-9.

**Posters:**

- Szűcs HD, Dombi G, Szakonyi G: Cloning expression and crystallization of AcrB membrane protein. 40. Membrane-Transport Conference, Sümeg, 2010. May 18-21.
- Kalmár E, Szűcs HD, Dombi G, Szakonyi G: Expression of AcrB homologue membrane proteins in Escerichia coli. 40. Membrane-Transport Conference, Sümeg, 2010. May 18-21.
- Marton L, Róna G, Mészáros Á, Szakonyi G, Márki-Zay J: Investigation of transporter interactions of antimalarials. 40. Membrane-Transport Conference, Sümeg, 2010. May 18-21.
- Márki-Zay J, Gedey Sz, Szakonyi G, Mészáros Á, Róna G, Marton L, Jakab K. Investigation of transporter interactions of antimalarials. Magyar Laboratóriumi Diagnosztikai Társaság 55. Nagygyűlése, Pécs, 2010.08.26-28.
- Kalmár E, Csordás-Tóth E, Dombi G, Szakonyi G: Problems occur during the expression of AcrB homologue membrane proteins. 41. Membrane-Transport Conference, Sümeg, 2011. May 17-20.
- Szűcs HD, Dombi G, Szakonyi G: Beta-foldamers, as crystallization adjuvants? 41. Membrane-Transport Conference, Sümeg, 2011. May 17-20.

**Diploma work:**

- Szűcs HD: Beta-foldamers, as crystallization adjuvants?