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Simple purification and characterization of soluble and homogenous ABC-F translation factors from *Enterococcus faecium*

Fanny DEMAY¹, Marc HALLIER¹, Sylvie GEORGEAULT¹, Emmanuelle COM²⁻³, Vincent CATTOIR⁴⁻⁵, Renan GOUDE¹, and Reynald GILLET^{1*}

¹Univ. Rennes, CNRS, Institut de Génétique et Développement de Rennes (IGDR) UMR 6290, Rennes, France

²Univ Rennes, Inserm, EHESP, Irset (Institut de Recherche en Santé, Environnement et Travail) - UMR_S 1085, F-35000 Rennes, France

³Univ Rennes, CNRS, Inserm, Biosit UAR 3480 US_S 018, Protim core facility, F-35000 Rennes, France

⁴Department of Clinical Microbiology, Rennes University Hospital, Rennes, France

⁵Univ. Rennes, Inserm, UMR 1230 BRM, Rennes, France

*Corresponding author

Abstract

The family of ATP-binding cassette F proteins (ABC-F) is mainly made up of cytosolic proteins involved in regulating protein synthesis, and they are often part of a mechanism that confers resistance to ribosome-targeting antibiotics. The existing literature has emphasized the difficulty of purifying these recombinant proteins because of their very low solubility and stability. Here, we describe a rapid and efficient three-step purification procedure that allows for the production of untagged ABC-F proteins from *Enterococcus faecium* in the heterologous host *Escherichia coli*. After four purified ABC-F proteins were produced using this protocol, their biological activities were validated by *in vitro* experiment. In conclusion, our study provides an invaluable tool for obtaining large amounts of untagged and soluble ABC-F proteins that can then be used for *in vitro* experiments.

Keywords

ABC-F protein; antibiotic resistance; SUMO; Ni-NTA chromatography; heparin chromatography

Introduction

The ATP-binding cassette F (ABC-F) family of proteins is present in most genomes, and is mainly involved in the regulation of protein synthesis [1]. Whereas the majority of ABC proteins are involved in transport processes through their transmembrane domains, ABC-Fs are cytoplasmic proteins that contain two ATP-binding cassette domains linked together by a double alpha-helix linker (Figure 1A,B). Several of these proteins interact directly with the ribosome and function as ribosomal protection proteins (RPPs) thus conferring resistance to ribosome-targeting antibiotics [2], [3], [4]. The purification of ABC-F proteins is **essential for understanding** the molecular mechanisms involved in these ribosomal interactions, as well as their link to resistance. To accomplish this, recombinant proteins are required for *in vitro* studies, but while certain ABC-F proteins have been easily overexpressed and purified, several studies have shown that the proteins in this family are very rarely soluble in aqueous media, and are usually quite difficult to purify [5], [6].

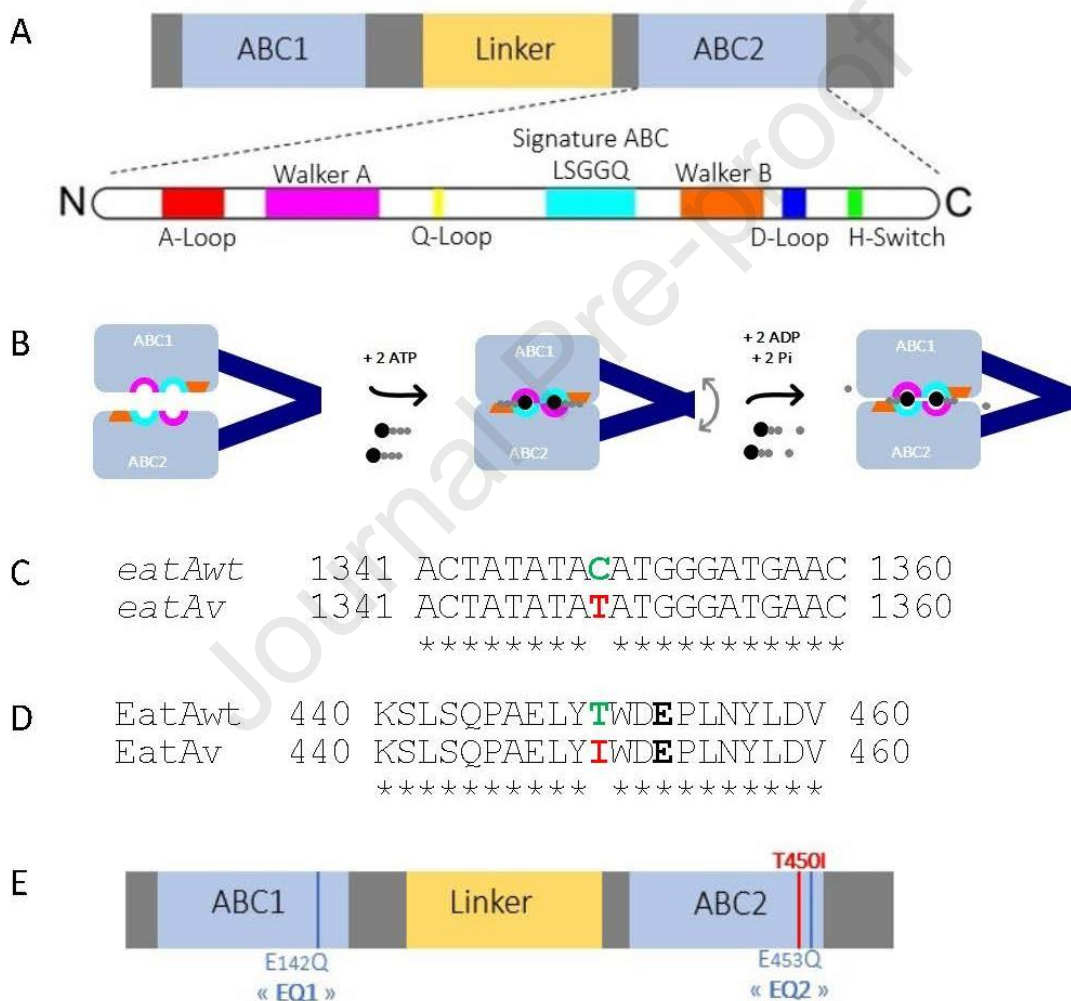


Figure 1. Representation of the primary and secondary structure of an ABC-F protein and properties of EatA proteins. A) Domain architecture of an ABC-F protein. Two ATP-binding cassettes (ABC1 and ABC2) are associated with a linker. The zoom shows the conserved motifs of an ABC domain, with Walker A and the ABC motif LSGGQ involved in ATP binding, and Walker B involved in ATP hydrolysis. **B)** Schematic view of an ABC-F protein folded into a head-to-tail sandwich in order to bind and hydrolyze two ATP molecules. **C)** Nucleotide sequence alignments of the *eatAwt* and *eatAv* genes showing the C1349T point mutation conferring resistance to lincosamide, streptogramin A, and pleuromutilin antibiotics (LSAP phenotype). **D)** Peptide sequence alignments of the resulting *EatAwt* and *EatAv* proteins in the ATP-binding cassette F (ABC-F) family. Threonine (T) is green, isoleucine (I) (T450I for Thr450Ile substitution) is red, and the conserved E453 catalytic site is in bold. **E)** Locations of the Thr450Ile substitution found in the variant *Eat(A)* protein and specific mutations (EQ1 and EQ2) made to inactivate the ABC domains.

To work around this problem of expression of recombinant proteins in the *E. coli* heterologous host, a small ubiquitin-like modifier (SUMO) protein can be used as a tag to improve cytosolic solubility [7]. This small (11 kDa) eukaryotic protein is involved in varied cellular processes [8], and the SUMO moiety actually facilitates the proper folding of many recombinant proteins. When fused to a target protein, the SUMO tag acts as a chaperone, enhancing protein folding and expression by promoting solubility in the *E. coli* cytosol [7]. Because protein folding is promoted, purification becomes easier, especially because SUMO can be combined with an upstream 6-histidine (6His) sequence for the pre-purification of the SUMOylated protein using immobilized metal ion affinity chromatography (IMAC). The 6His-SUMO tag can then be removed by the ubiquitin-like protease 1 (Ulp1). Note that the 6His-Ulp1 protease can be easily expressed and purified in the laboratory, making this step much cheaper than commercial proteases. The last purification step consists of separating the free SUMO moiety and Ulp1 protease from the recombinant protein. The pET-SUMO cloning kit handbook recommends performing a "reverse His-tag" purification by putting the cleavage mix into a nickel column. The untagged protein can thus be purified directly into the flow-through, while the 6His-SUMO tag, 6His-Ulp1 protease, and uncleaved tagged proteins all bind to the column. This approach has often been described in the literature, but an additional gel-filtration or anion-exchange step is often then necessary to finish removing any residual SUMO proteins and Ulp1 proteases [9], [10].

The wild-type enterococcal ABC transporter gene *eat(A)* is a 1,503-bp gene coding for a protein in the ABC-F family, and it was first described in 2013 [11]. A point mutation (C1349T, Figure 1C) was also described within that gene that leads to an amino acid substitution (Thr450Ile, Figure 1D) in the Walker B motif of the resulting protein's second ABC domain (Figure 1E). This single mutation confers resistance to lincosamide, streptogramin A, and pleuromutilin antibiotics (LS_AP phenotype). These three antibiotics are members of unrelated families of chemical compounds but they all target the peptidyl transfer centre of the bacterial ribosome. The mutated allelic variant was named *eat(A)v*.

In order to explore how a single substitution located 3 residues upstream from the catalytic site of ATP hydrolysis leads to antibiotic resistance, we expressed and purified large amounts of recombinant Eat(A) and Eat(A)v proteins. Two specific mutations were done in each ABC domain to inactivate the two Walker B motifs (E142Q in Walker B1 and E453Q in Walker B2, Figure 1E). To characterize the ATPase activity of these proteins, we did set up an alternative three-step purification protocol based on a combination of nickel and heparin columns. This process for the sequential purification of SUMO-tagged ABC-F and then untagged proteins is fast, inexpensive, and easily performed in the laboratory using standard columns and equipment. Using it, we were able to purify four untagged ABC-F proteins from *Enterococcus faecium*, and were also able to characterise their ATPase activity. This easy procedure should improve the *in vitro* study of this type of ABC-F antibiotic-resistant proteins.

Materials and Methods

1. Materials

DH5 α chemically competent *Escherichia coli* cells were used for amplifying plasmid constructs. Protein expression was done using BL21(DE3) *E. coli* cells. Isopropyl- β -D-thiogalactopyranoside (IPTG) and antibiotics were purchased from Sigma-Aldrich. The Champion pET SUMO protein expression system (K300-01, Invitrogen) contained the pET SUMO vector, PCR and transformation reagents, Mach1-T1 competent *E. coli* cells, One Shot BL21(DE3) chemically competent *E. coli* cells, and SUMO protease. Q5 polymerase was purchased from New England Biolabs (M0491S). The AKTA start system, HisTrap FF column (1 mL, 17-5319-01), HiTrap Desalting column (5 mL, 17-1408-01), and HiTrap Heparin HP column (1 mL, 17-0406-01) were obtained from GE Healthcare. PCR production and agarose extract cleaning were done using a Macherey-Nagel NucleoSpin Gel and PCR Clean-up kit (740609-50), and their NucleoSpin Plasmid kit (7400588-50) was used for plasmid extractions. Protein quantification was done using a ThermoScientific Pierce BCA Protein

Assay Kit (23250), while a Sigma-Aldrich Malachite Green Phosphate Assay Kit (MAK307) was used for inorganic phosphate quantification. ATP (R1441) and GTP (R1461) were purchased from Fisher Scientific. SIGMAFAST EDTA-free Protease Inhibitor Cocktail Tablets were purchased from SIGMA (S8830-20TAB). Plasmid pFGET19-ulp1 was purchased from Addgene (64697) for the expression and purification of 6His-ulp1.

2. Plasmid DNA constructs

Unless otherwise noted below, cloning and transformation protocols were based on the manual provided with the Champion pET-SUMO Protein Expression System (Invitrogen). The open reading frames of wild-type and variant forms of *eat(A)* were amplified from *Enterococcus faecium* strains HM1070 and TC5 [11] using the primers Feat(A)SUMO (**atgt**ctaaaaatcgaaataaaaaatctg) and Reat(A) (ttagattctctcaagagaaat**aatc**). Amplifications were done using Q5 polymerase as follows: initial denaturation (98 °C, 30 sec); 30 cycles of denaturation (98 °C, 10 sec), annealing (55 °C, 30 sec), and elongation (72 °C, 60 sec); and a final extension (72 °C, 10 min). The PCR products were analysed by electrophoresis on a 1% agarose gel stained with ethidium bromide and cleaned using a NucleoSpin kit. PCR products were then incubated with Taq polymerase in the presence of dATP (72 °C, 20 min) in order to add the 3'-deoxyadenosine (dA) residues necessary for TA cloning. The resultant PCR product was ligated by T4 DNA ligase into linearized pET-SUMO vector containing deoxythymidine (dT) overhangs. The reaction (10 µL) was incubated overnight at 15 °C. Ligation products were transformed into One Shot Mach1-T chemically competent *E. coli* cells for plasmid propagation by heat shock at 42 °C for 30 sec, and transformants were incubated overnight at 37 °C on LB agar plates with kanamycin (50 µg/mL). Recombinant plasmids were identified using colony PCR with the T7 forward primer (taatacgactcactataggg) and the *eat(A)* medR reverse primer (gcatcagcaccactgctcc). Clones containing the plasmids that were correctly oriented were selected and grown overnight in 10 mL of LB media, then plasmid DNA was extracted using a NucleoSpin Plasmid kit. Each recombinant plasmid was sequenced.

3. Site-directed mutagenesis (SDM)

SDM experiments were performed as per the instructions provided with the Stratagene QuikChange Site-Directed Mutagenesis kit. The substitution E142Q inactivates the first ABC domain of the *eat(A)* gene, and the corresponding mutant was named "EQ1". The primers used for this first SDM were cccgtaatcgat**ca**ccaacggatcatttgg (EQ1) and ccaaatgattcgttggtgatcgattaacggg (EQ1R), with the codon modified during this mutagenesis (GAA replaced by CAA) shown here in bold. The second ABC domain was inactivated by modifying E453Q using the EQ1 mutated plasmid as a template. The plasmid construct harbouring the mutations in both ABC domains was named "2EQ" (for EQ1 + EQ2). For the second SDM, the primers used to obtain the wt double mutant *eat(A)*wt2EQ were ctatata**cat**gggat**ca**accattgaattttgg (EQ2wtF) and ccaaataattcaatggttgatccatgtatatag (EQ2wtR), while to obtain the double mutant *eat(A)*v.2EQ, the primers were ctatata**cat**gggat**ca**accattgaattttgg 3' (EQ2vF) and ccaaataattcaatggttgatccatgtatatag (EQ2vR). As before, the codon modified during this mutagenesis (GAA replaced by CAA) is bold, while the "ACA" (threonine) and "ATA" (isoleucine) codons are underlined.

The 50 µL PCR reaction contains 50 ng of template DNA, 5 µL of 10X buffer, 1 µM each of the two forward and reverse primers, 1 mM of dNTP mix, and 1 µL of PfuTurbo polymerase. The reaction conditions were as follows: initial denaturation (95 °C, 1 min); then 16 cycles of denaturation (95 °C, 30 sec), annealing (55 °C, 1 min), and elongation (68 °C, 7 min); and a final extension (68 °C, 10 min). Afterwards, 1 µL of DpnI enzyme was gently mixed into the amplification product, then incubated at 37 °C overnight. Finally, 1 µL of the mix was transformed into XL10-Gold ultracompetent cells by heat shock at 42 °C for 30 sec. In order to validate the SDM, all recombinant plasmids were sequenced along the entire ORF.

4. SUMO-protease expression and purification

The pGGT19_Ulp1 (Addgene) inducible plasmid carrying 6His-Ulp1 was transformed into competent *E. coli* BL21(DE3) cells and plated on LB agar plates containing 50 µg/mL kanamycin. A single colony of transformants was inoculated into 50 mL of LB medium supplemented with 50 µg/mL kanamycin, and this culture was grown overnight at 37 °C under agitation of 180 rpm. The following morning, 500 mL of LB culture medium supplemented with 50 µg/mL of kanamycin was inoculated with the overnight culture to an initial OD₆₀₀ of 0.05 and grown at 30 °C at an agitation of 180 rpm. When the OD₆₀₀ reached 0.8, IPTG (1 mM final) was added to the culture. Growth continued for 5 h at 30 °C and 180 rpm. The cells were harvested by centrifugation (7,000 rpm for 10 min at 4 °C). The cell pellet was washed with 5 mL of phosphate-buffered saline (PBS). After another centrifugation, the pellet was frozen at -80 °C to enhance cell lysis. The cell pellet was resuspended in 10 mL of lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.01 mM DTT, and 0.02 mM EDTA) with protease inhibitor tablets (Sigma-Aldrich, S8830-20TAB), then lysed using an SLM Instruments French press (3 cycles at 16,000 psi). The crude extract was clarified by centrifugation (13,000 rpm for 45 min at 4 °C) and filtered (0.45 µM) before being applied to a HisTrap FF nickel Sepharose column (GE Healthcare) previously equilibrated with lysis buffer containing 10 mM imidazole (flow rate 0.5 mL/min). To remove non-specific proteins, a washing buffer was used (50 mM Tris-HCl pH 8.0, 500 mM NaCl, 0.01 mM DTT, 0.02 mM EDTA, 20 mM imidazole, and 0.1% Triton X100). Finally, an elution gradient buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.01 mM DTT, 0.02 mM EDTA, 250 mM imidazole, and 1% glycerol) was used to elute the 6His-Ulp1. To avoid precipitation of the protein, DTT (5 mM final) was immediately added to the eluted fractions. These chromatography column fractions were then analysed by 15% SDS-PAGE and stained with Coomassie blue dye to identify the 28-kDa protein of interest. To allow binding to the heparin column, the purest fractions were pooled and diluted to reduce NaCl concentrations to just 150 mM. The diluted fractions were then loaded onto the HiTrap Heparin HP column (GE Healthcare) pre-equilibrated with buffer A (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 5 mM DTT, 0.02 mM EDTA, and 1% glycerol). The protein of interest was then eluted with a NaCl gradient in buffer B (50 mM Tris-HCl pH 8.0, 1 M NaCl, 5 mM DTT, 0.02 mM EDTA, and 1% glycerol). Finally, to reduce salt concentrations and to remove imidazole, the eluted fractions were pooled and applied to a Sephadex G-25 HiTrap Desalting column. The fractions containing the 6His-Ulp1 protein were pooled, glycerol was added to a final concentration of 20%, and the aliquots were flash-frozen for storage at -80 °C. The protein concentration was determined using a Pierce BCA protein assay kit.

5. Expression and purification of ABC-F proteins

The protein expression and purification processes were exactly the same for each of the four Eat(A) proteins. The inducible pET-SUMO plasmid carrying *eat(A)* were transformed into *E. coli* BL21(DE3) competent cells and plated into LB agar containing kanamycin 50 µg/mL. A single colony of transformants was inoculated into 50 mL of LB culture medium supplemented with 50 µg/mL kanamycin and grown overnight at 37 °C with agitation of 180 rpm. The next morning, 1 L of LB media supplemented with 50 µg/mL of kanamycin was inoculated with the overnight culture to an initial OD₆₀₀ of 0.05 and grown at 37 °C with agitation of 180 rpm. When the OD₆₀₀ reached 0.5-0.6, IPTG (0.1 mM final) was added. Growth continued at 16 °C under agitation of 180 rpm until the next morning. After around 20 h, cells were harvested by centrifugation (5,000 G for 10 min at 4 °C), and the pellet was washed with 10 mL of PBS. After another centrifugation, the pellet was frozen at -80 °C to improve cell lysis. The cell pellet was resuspended in 20 mL of lysis buffer (pH 8.0: 50 mM HEPES-KOH pH 7.5, 700 mM KCl, 2 mM MgCl₂, 20 mM imidazole, 0.1% Tween-20, and 10% glycerol) with EDTA-free protease inhibitor tablets. It was lysed using the same French press (3 cycles at 16,000 psi) then sonified in a Branson Sonifier 450 (2 min, 50% duty cycle, output 3). The crude extract was then clarified by centrifugation (15,000 G for 15 min at 4 °C). The soluble fraction was filtered (0.45 µm) and applied on a HisTrap FF column previously equilibrated with the lysis buffer (flow rate 0.5 mL/min). After a wash step performed with 40 mM imidazole and 2 M KCl, 6His-SUMO-Eat(A) protein was eluted with a gradient of buffer C (pH 8.0: 50 mM HEPES-KOH pH 7.5, 300 mM KCl, 2 mM MgCl₂, 500 mM imidazole, 0.1% Tween-20, and 10% glycerol). The fractions eluted

from chromatography columns were analysed using 10% SDS-PAGE and stained with Coomassie blue. The purest fractions were pooled and loaded onto Sephadex G-25 HiTrap Desalting columns pre-equilibrated with desalting buffer (pH 8.0: 50 mM HEPES-KOH pH 7.5, 300 mM KCL, 2 mM MgCl₂, 0.1% Tween-20, and 10% glycerol). The desalted proteins were mixed with a molar ratio of two 6His-Ulp1 proteases for one 6His-SUMO-Eat(A) protein in the presence of 1 mM of DTT. Cleavage was performed over night at 18°C under stirring. After around 16 h, the cleavage mix was diluted by half with unsalted buffer (50 mM HEPES-KOH pH 7.5, 2 mM MgCl₂, 0.1% Tween-20, and 10% glycerol) to reduce salt before being loading onto a HiTrap heparin HP column pre-equilibrated with buffer D (50 mM HEPES-KOH pH 7.5, 75 mM KCL, 2 mM MgCl₂, 0.1% Tween-20, and 10% glycerol). Untagged proteins were eluted with an NaCl gradient in buffer E (50 mM HEPES-KOH pH 7.5, 1 M NaCl, 2 mM MgCl₂, 0.1% Tween-20, and 10% glycerol). Fractions containing the ABC-F protein were pooled and concentrated on an Amicon 3MWCO 3-kDa filter at 20 °C, and glycerol was added to a final concentration of 20%. Aliquots were flash-frozen in liquid nitrogen and stored at -80 °C. Protein concentrations were determined using a BCA assay kit.

6. SDS-PAGE and Western-Blot Analysis

Proteins were separated by SDS-PAGE using 10% acrylamide gels and visualized by Coomassie blue staining. For immunoblot analysis, proteins were transferred to hybond-P PolyVinylidene Fluoride (PVDF) membrane (Amersham). The membrane was probed using a rabbit anti-peptide (Genscript, Piscataway, NJ, USA) directed against the CEWSRSREGKTKKQ sequence of the EatA protein. A horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin (Sigma-Aldrich) was used as the secondary antibody. The molecular weight standards used were from Thermo Scientific.

7. Protein identification by mass spectrometry

Gel piece was in gel digested following steps previously described [12] with some modifications. Briefly after several washes in acetonitrile and 100 mM ammonium bicarbonate, proteins in gel piece were reduced with 65mM DTT in 100mM ammonium bicarbonate (15min at 37°C) then alkylated with 135mM iodoacetamide in 100mM ammonium bicarbonate (15min at room temperature in the dark). After several washes in acetonitrile and 100 mM ammonium bicarbonate, proteins were digested with 10 ng/μL of a Trypsin/LysC mix (Promega) in 25 mM ammonium bicarbonate /ProteaseMAX™ 0.01% (Promega) overnight at 37°C. Finally, peptides were collected after recovering steps in first 70% acetonitrile/0.1% formic acid and after 100% acetonitrile. Peptides were then evaporated in a vacuum centrifuge to 40 μL and purified using Phoenix spin cartridge (Preomics) according to manufacturer's instructions. Approximately 200 ng of the resulting peptides were analyzed by nanoLC-MS/MS using a nanoElute (Bruker Daltonik GmbH, Bremen, Germany) coupled with a TimsTOF Pro mass spectrometer (Bruker) using PASEF acquisition mode as previously described (Girard et al., 2022, DOI:10.1021/acs.jproteome.2c00496). Peptide and protein identification were performed using the Mascot (Mascot server v2.6.2; <http://www.matrixscience.com>) database search engine and its automatic decoy database search to calculate a false discovery rate (FDR). MS/MS spectra were queried against the sequence of the Eat(A)_v_LSAP-resistance protein, the *E. coli* SwissProt proteins (23,230 sequences, 2023_02 release) and a common proteomic contaminant database from the Max Planck Institute of Biochemistry, Martinsried (247 sequences). Mass tolerance for MS and MS/MS was set at 15 ppm and 0.05 Da. The enzyme selectivity was set to full trypsin with one miscleavage allowed. Protein modifications were fixed carbamidomethylation of cysteines, variable oxidation of methionine. Identification results from Mascot (.dat files) were imported into the Proline Studio software (v2.2.0) [13] that was then used for validation as previously described [14]. Identified peptides were validated with a peptide rank of 1 and filtered based on Mascot score values to obtain a false discovery rate (FDR) of 1% at the PSM level.

8. ATPase characterization

Determination of the ATPase activity of the purified ABC-F proteins was performed using the Malachite Green assay kit. The reaction mixture contained 2 μ M protein, 500 μ M ATP in 40 μ L. Reactions were started when ATP was added, and hydrolysis reactions were incubated at 37 °C for 30 min. After this period, the samples were diluted 1:1 in water, and the reactions were stopped by adding 20 μ L of the working reagent (supplied with the kit). The generated amounts of inorganic phosphate were determined by comparing the absorbance at 620 nm with a standard phosphate curve. Bovine serum albumin (BSA) was used as a negative control. The data reported are averages of triplicate samples analysed with GraphPad Prism software. Statistical analysis was performed with unpaired t-tests, with a P-value \leq 0.05 considered significant.

Results and discussion

1. Construction & expression of recombinant Eat(A) proteins

In the first experiments, we expressed each form of the Eat(A) protein (wild type EatAwt, variant EatAv, and the wild type 2EQ and variant 2EQ mutants EatAwt2EQ, and EatAv2EQ). This was done via conventional cloning of their genes into a pET-22b(+) vector that allowed the addition of a 6-histidine tag to the C-terminal part of the Eat(A) proteins. The purifications of the four proteins were then all optimized in the same manner (described below). After many attempts to improve the expression parameters, including varying the IPTG concentrations, culture temperatures, and durations, we consistently found that the proteins accumulated in inclusion bodies (data not shown), thereby impeding their purification in a native form. Such a failure to express these ABC-F proteins due to complete insolubility is in accordance with what has already been described in the literature for other members of the family [4], [5]. We therefore decided to use the 6His-SUMO tag approach, used for many years to improve the expression and purification of insoluble proteins. The open reading frames that code for the four versions of *eatA* were cloned into the pET-SUMO vector, a T7-*lac* promoter vector carrying an N-terminal 6His-SUMO tag with a SUMO protease cleavage site. We used DNA sequencing to ensure the correct insertion of the ORF into the cloning vector. The resulting final construct was called “6His-SUMO-Eat(A)” (Figure 2).

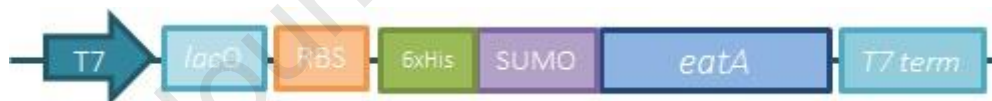


Figure 2. Schematic diagram of recombinant Eat(A) expression system using the pET-SUMO vector. T7-*lacO* is for T7-*lac* promoter; RBS for Ribosome Binding Site; 6xHis for 6-Histidine tag; SUMO for small ubiquitin-like modifier tag; *eatA* for enterococcal ABC transporter gene; T7term for T7 terminator.

The Eat(A)wt purification procedure described here is exactly the same as that used for the other three forms of the protein. To begin, we transformed the 6his-SUMO-Eat(A) vector into BL21(DE3) competent *E. coli* cells, and we expressed the recombinant protein at 16 °C for 20 h. Under these growth and induction conditions, we found the Eat(A) protein fused to the SUMO moiety in the soluble fraction. We then purified the 6His-SUMO-Eat(A) protein through a nickel affinity column (Figure 3A). The purest fractions were pooled (Figure 3B, fractions 16 to 27) and desalted on a G-25 Sepharose column to discard imidazole, as the protease is only active if there is less than 150 mM of this reagent (see Methods). At this step, a protein of approximately 72 kDa was mainly detected on a 10% SDS-PAGE gel (Figure 3B), showing that the SUMO moiety allows a large fraction of the protein to be recovered in the soluble phase instead of inclusion bodies.

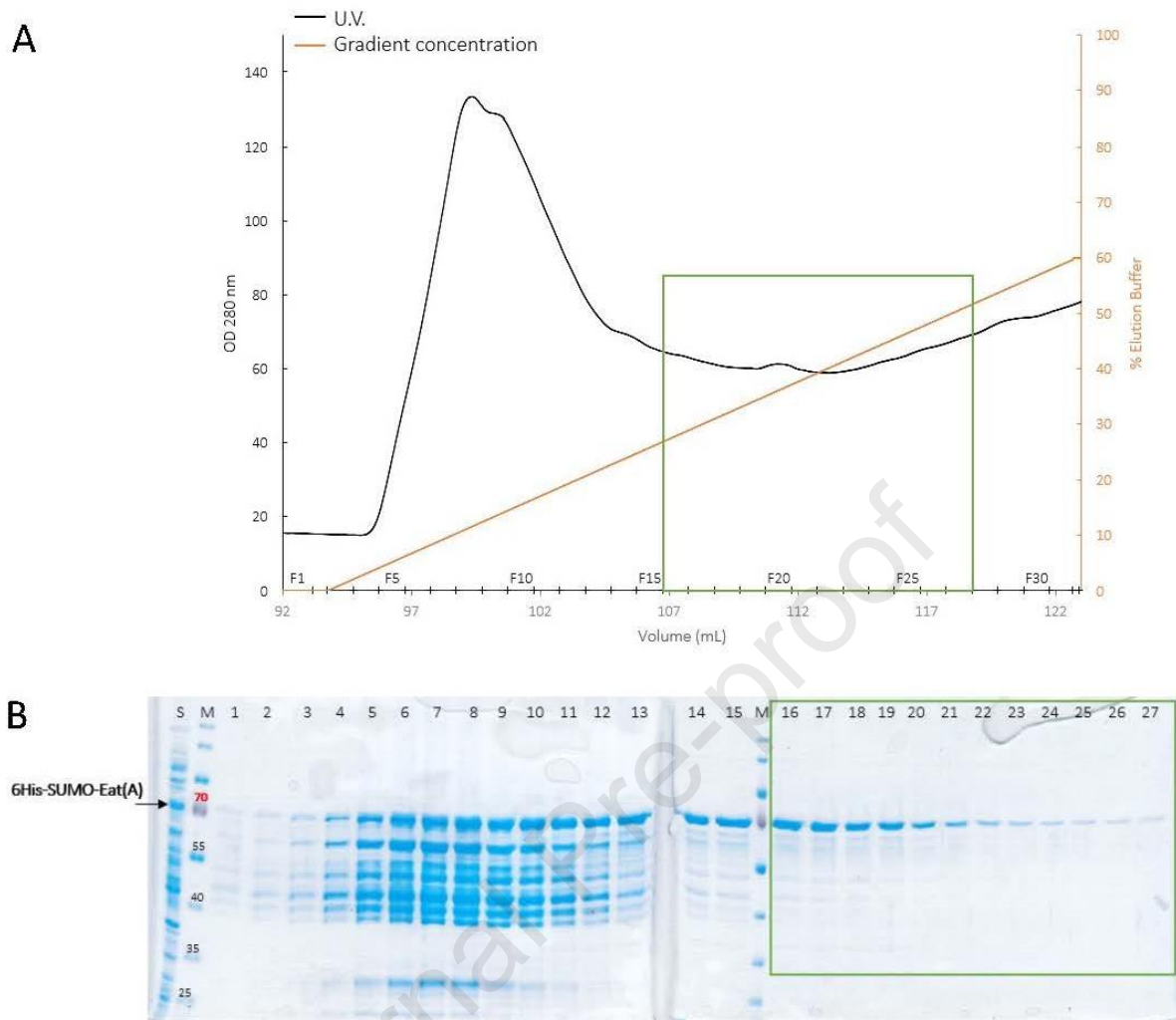


Figure 3. Purification of an ABC-F protein using a simpler protocol based on 6his-SUMO-Eat(A) on an Ni-NTA column. A) Elution profile of Ni-NTA chromatography. UV curve and elution buffer gradient are in black and orange, respectively. The purest fractions of 6His-SUMO-Eat(A) (F16 to F27) are indicated by a green rectangle. **B)** Coomassie blue stained 10% SDS-PAGE gel from different sample fractions. Lane M: pre-stained protein marker. Lane S: induced supernatant after lysis and centrifugation. Lanes 1 to 27: Fractions from elution gradient. The arrow indicates the 6His-SUMO-Eat(A) protein (~72 kDa). Green rectangle represents fractions containing purest tagged Eat(A) proteins

2. Purification of the Ulp1 protease and removal of the 6his-SUMO tag

The 6His-SUMO tag can be easily removed using the highly specific Ulp1 protease, a member of the cysteine protease superfamily that precisely recognises the SUMO group's C-terminal Gly-Gly motif [15]. Although the use of cleavable purification tag sites is commonly used for producing recombinant proteins such as TEV or thrombin cleavages, the advantage of using Ulp1 instead is that no additional amino acids are left at the N-terminals of the proteins of interest. We therefore used plasmid pFGET19-ulp1 to express Ulp1 fused to an N-terminal 6His tag. The protein was expressed in the BL21(DE3) strain, and the soluble 6His-tagged Ulp1 was bound on an Ni-NTA affinity column then eluted using an imidazole gradient (50-500 mM). SDS-PAGE analysis of the elution fractions revealed that Ulp1 was co-purified with several contaminants. To solve this problem, we performed another round of purification on a heparin column (see Materials and Methods). As a result of these two successive purifications, we obtained a final concentration of 8 mg/mL of pure Ulp1 proteins (Supplementary Figures 1 and 2). To examine the cleavage activity of the Ulp1 protein with the recombinant 6His-SUMO-Eat(A) protein, we tested various protein/protease ratios, incubation times, and temperatures.

We found that the optimal conditions for digestion are overnight at room temperature (~18 to 20°C) using two molecules of Ulp1 for one molecule of 6His-SUMO-Eat(A) protein (Figure 4B, lane C)

3. Heparin purification of untagged ABC-F proteins

After cleavage, the protein of interest must be isolated from the rest of the mix. As described in the introduction, the usual “reverse His-tag” protocol may not be sufficient for separating the untagged protein of interest from the rest of the cleavage mix, and will thus require additional purification steps. This could have been done through a size exclusion chromatography. However, this method requires a highly concentrated sample to be loaded on the exclusion column. Unfortunately, during the concentration step the protein precipitated rapidly, avoiding the use of this purification method. Therefore, we tested a different purification approach based on a heparin column. Heparin is a glycosaminoglycan polysaccharide with numerous sulphate groups. From a biological point of view, heparin plays an anticoagulant role, maintaining normal blood flow by reducing the risk of clot formation. Heparin is used for protein purification because some proteins have an intrinsic affinity to it, but also because of its activity as a cation exchanger. At physiological pH, heparin’s negatively charged sulphate groups interact with the basic groups of proteins, particularly amino acids such as arginine and lysine. Heparin therefore allows protein purification by combining affinity and ion exchange on the same column [16]. Heparin’s numerous anionic sulphate groups are expected to mimic the polyanionic structure of the nucleic acid, thus favouring interactions with DNA and RNA binding proteins [17].

Because ABC-F proteins interact with the ribosome, especially with ribosomal RNA [3], [10], we explored this method for isolating the cleaved protein from the rest of the mix. After the overnight cleavage step, we diluted the mix to reduce the salt concentration (from 300 to 150 mM KCl) to allow the binding of the protein to the heparin column. We then eluted the various components of the cleavage mix using an NaCl salt gradient. The untagged ABC-F (58 kDa) was eluted at a salt concentration of 350 mM, followed by 6His-SUMO tag (17 kDa) and finally 6His-Ulp1 (28 kDa) (Figure 4). We pooled the fractions containing the untagged EatA proteins (Figure 4B, fractions 12-16). Because the protein aggregates when concentrated at 4 °C we performed the centrifugation at 20 °C on a 3MWCO centrifugal filter. We used this shortened procedure to purify four ABC-F proteins: Eat(A)wt, Eat(A)v, and the inactivated mutants Eat(A)wt.2EQ and Eat(A)v.2EQ. From 1 L of culture, we obtained a final concentration of approximately 1.2 to 2 mg/mL of proteins (23-35 µM).

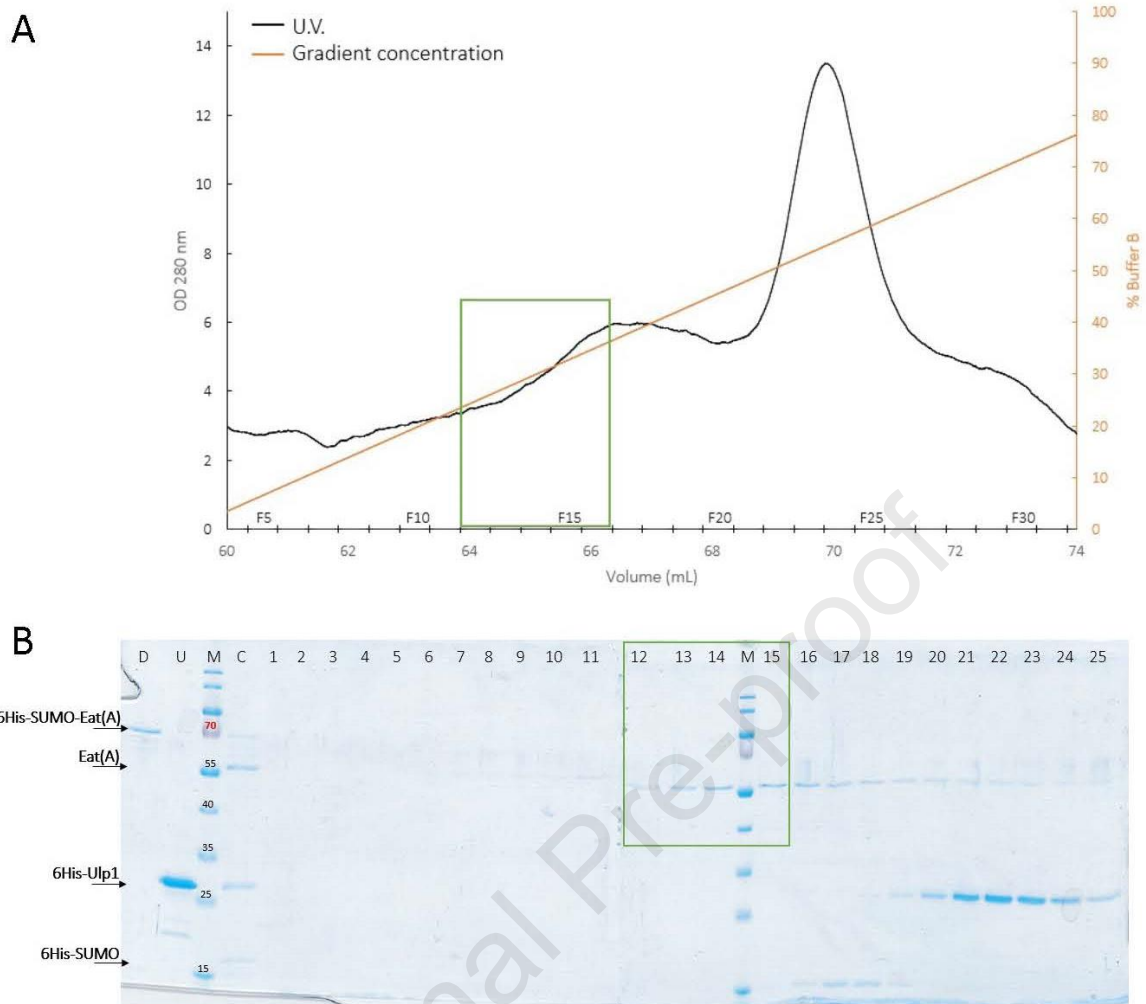


Figure 4. Untagged Eat(A) purification on a heparin column after Ulp1 cleavage. A) Elution profile of heparin chromatography. UV curve and elution buffer gradient are in black and orange, respectively. The fractions containing untagged Eat(A) proteins (F12 to F15) are indicated by a green rectangle. **B)** Coomassie blue stained 12% SDS-PAGE gel from different sample fractions. Lane D: 6His-SUMO-EatA protein; Lane M: pre-stained protein marker; Lane U: 6His-Ulp1 protein; Lane C: 6His-SUMO-EatA after Ulp1 (5,4 ug) cleavage. Lanes 1 to 25: Fractions from elution gradient. Green rectangle represents fractions containing untagged Eat(A) proteins.

4. Confirmation of the identity of the purified Eat(a)v protein

In order to confirm the identity of the protein we did perform Western-Blot as well as mass spectrometry analyses. Purified Eat(a)v was first separated on an SDS-PAGE gel and then transferred to a PVDF membrane. Despite a slight degradation that occurred during the purification process the experiment confirms the recognition of Eat(a)v by a specific antibody (Figure 5A)

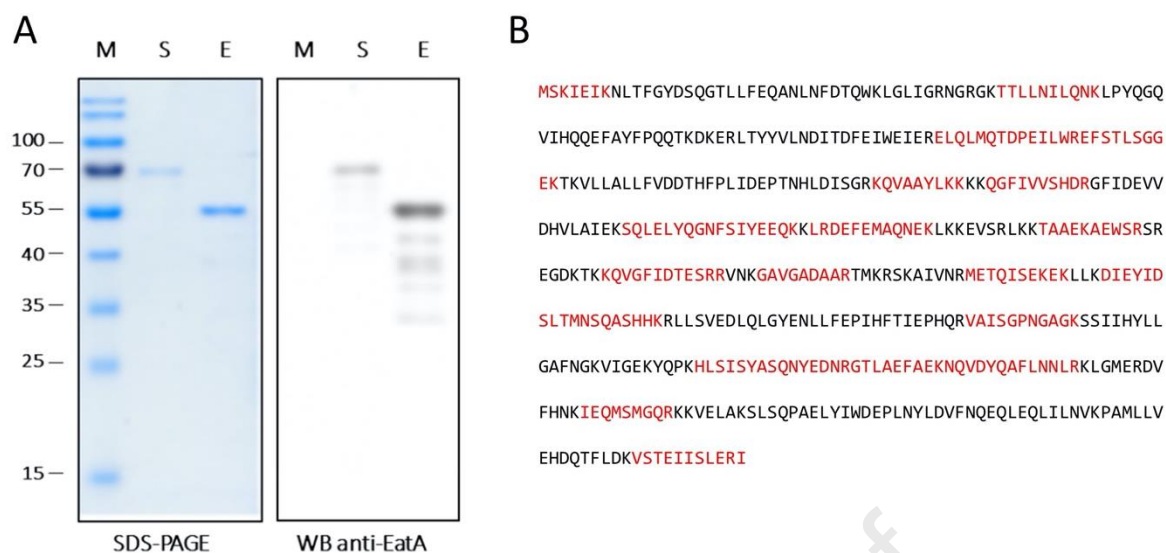


Figure 5. Analyses of the purity of the Eat(A) protein by SDS-PAGE, Western blot and Mass spectrometry. A) Purified 6His-SUMO-EatA (2ug, lane S) and Eat(A) (8ug, lane E) proteins were separated by SDS-PAGE and either stained with Coomassie Brilliant blue or transferred to a PVDF membrane. The membrane was probed with an anti-peptide specific for the EatA protein and revealed by chemiluminescence using an HRP-conjugated goat anti-rabbit immunoglobulin. Lane M: pre-stained protein marker. B) A total of 43,2% of the sequence were covered by the identified peptides

To ensure definitively the protein identity we then characterized it by mass spectrometry. The purified Eat(A)v was separated on a 10% SDS-PAGE and the major band, at 58 kDa was excised and subjected to in-gel digestion with trypsin. NanoLC MS/MS mass spectrometry analysis identified the Eat(a)v protein with the most specific spectra (376), confirming that the protein visualized in the extracted band is the protein of interest. Twenty-six peptides derived from Eat(a)v sequence were identified (Supplementary Table 1) and a total of 43,2% of the sequence were covered by the identified peptides (Figure 5B).

5. Analysis of the ATPase activity of the purified Eat(A) proteins

The Eat(A)v variant has a T450I substitution located 3 amino acids upstream from the E453 catalytic site of the second ABC domain. We therefore decided to test whether this mutation has an impact on the protein's ATP hydrolysis activity. We used the Malachite Green colorimetric assay to measure the amounts of inorganic phosphate (Pi) released by the purified proteins after ATP hydrolysis. This assay is based on the formation of a complex between the dye and phosphomolybdate under acidic conditions that produce a species with a maximum absorbance of around 620 nm, directly proportional to the Pi released during hydrolysis (see Materials and Methods). In a reaction volume of 40 μ L, 50 pmol of proteins were incubated with 500 μ M ATP for 30 min at 37 $^{\circ}$ C. We determined the Pi levels by comparing the optical density values read at 620nm with a range of Phosphate standards. As shown in Figure 6, BSA, a negative control, shows no signal as compared to ATP by itself. Although Eat(A) has two ABC domains that can bind and hydrolyse ATP [10], [11], we observed that neither the Eat(A)wt wild-type form of the protein nor its wt2EQ inactivated form showed significant hydrolysis activity, as their signals are comparable to the controls (both ATP alone and BSA). In contrast, the variant protein has statistically significant ATPase activity as compared to the same negative controls. When both Walker B motifs are inactivated, this activity was lost (Figure 6, V and V2EQ mutants). This reduction of ATPase activity is comparable to what has already been described in the literature when both Walker B motifs are inactivated [18]. Our results show that only the Eat(A) variant form has ATP hydrolysis activity when by itself, which suggests that mutation Thr450Ile of the Eat(A) protein leads to the activation of ATP hydrolysis.

The set of experiments discussed here confirms that our protocol allows simple purification of functional ABC-F proteins.

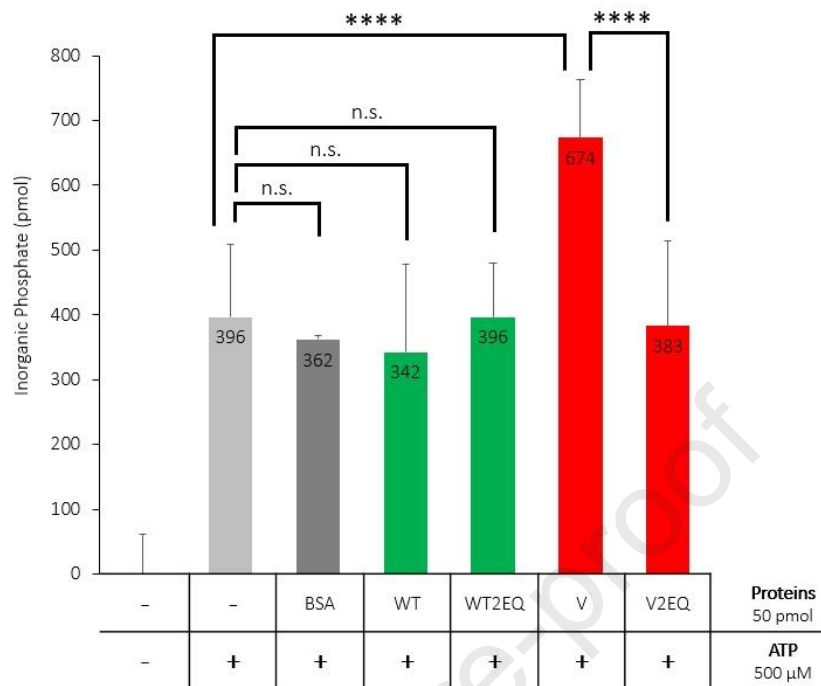


Figure 6. Measure of the ATPase activity of purified Eat(A) proteins. Eat(A) wild type (WT) and Eat(A) variant (V) proteins, and their double mutants (WT2EQ and V2EQ) were incubated with ATP for 30 min at 37 °C. Inorganic phosphate was quantified and compared to the reaction mixture without ATP. BSA was used as negative control. The only statistically significant differences ($p \leq 0.0001$ after unpaired t-tests) were seen between the ATP alone and the first variant V, as well as between the mutants themselves (V and V2EQ). Data are the means of three independent assays. Error bars represent Standard deviation. n.s., not statistically significant; ****, $p \leq 0.0001$ from unpaired t-test.

Conclusion

ABC-F proteins have recently been described as regulatory factors of protein synthesis, and in particular as determinants of resistance to ribosome-targeted antibiotics [2]. Structural biology studies have also highlighted their interactions with the ribosome. However, the literature also highlights difficulties in purifying these proteins, limiting *in vitro* investigations into the underlying molecular mechanisms affecting ribosomal translation. Here, we have presented an easy three-step purification procedure for obtaining pure and soluble ABC-F protein fractions. This begins with affinity purification of soluble 6His-SUMO-Eat(A) proteins through IMAC. The native protein is then cleaved of its SUMO tag by the specific protease Ulp1, and finally the untagged ABC-F protein is purified through a heparin column. This improved protocol is an invaluable tool for obtaining large amounts of untagged and soluble ABC-F proteins that can then be used for *in vitro* experiments, as shown by the characterisation of ATPase activity we have presented here.

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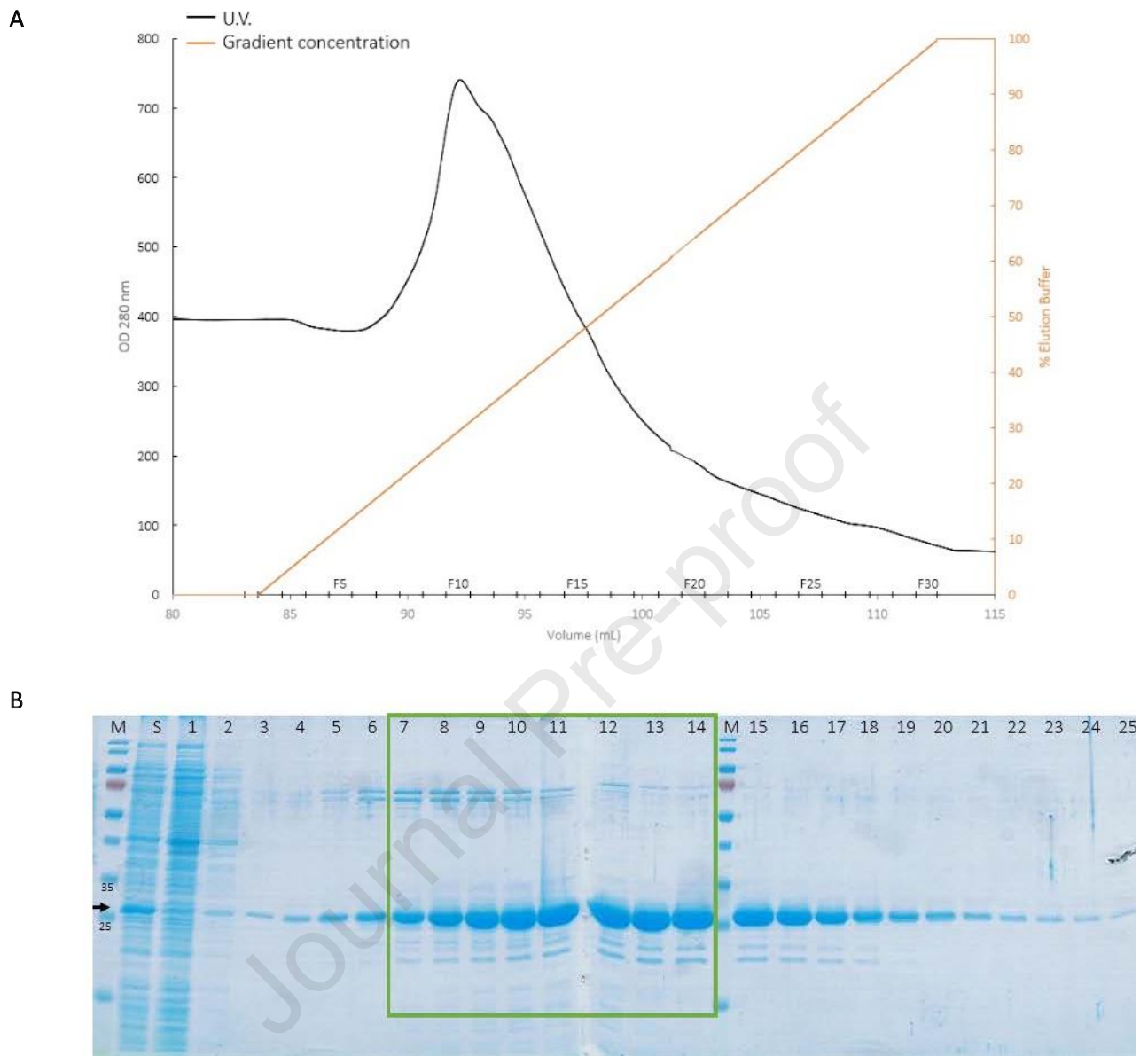
Funding F.D. was funded by a doctoral grant from the French Ministère de l'Enseignement supérieur, de la Recherche et de l'Innovation. This work was supported by the Fondation pour la Recherche Médicale (Equipe

FRM EQU202203014663). Protim Core Facility was supported by structural grants from Biogenouest, Infrastructures en Biologie Santé et Agronomie (IBISA), and the Conseil Régional de Bretagne.

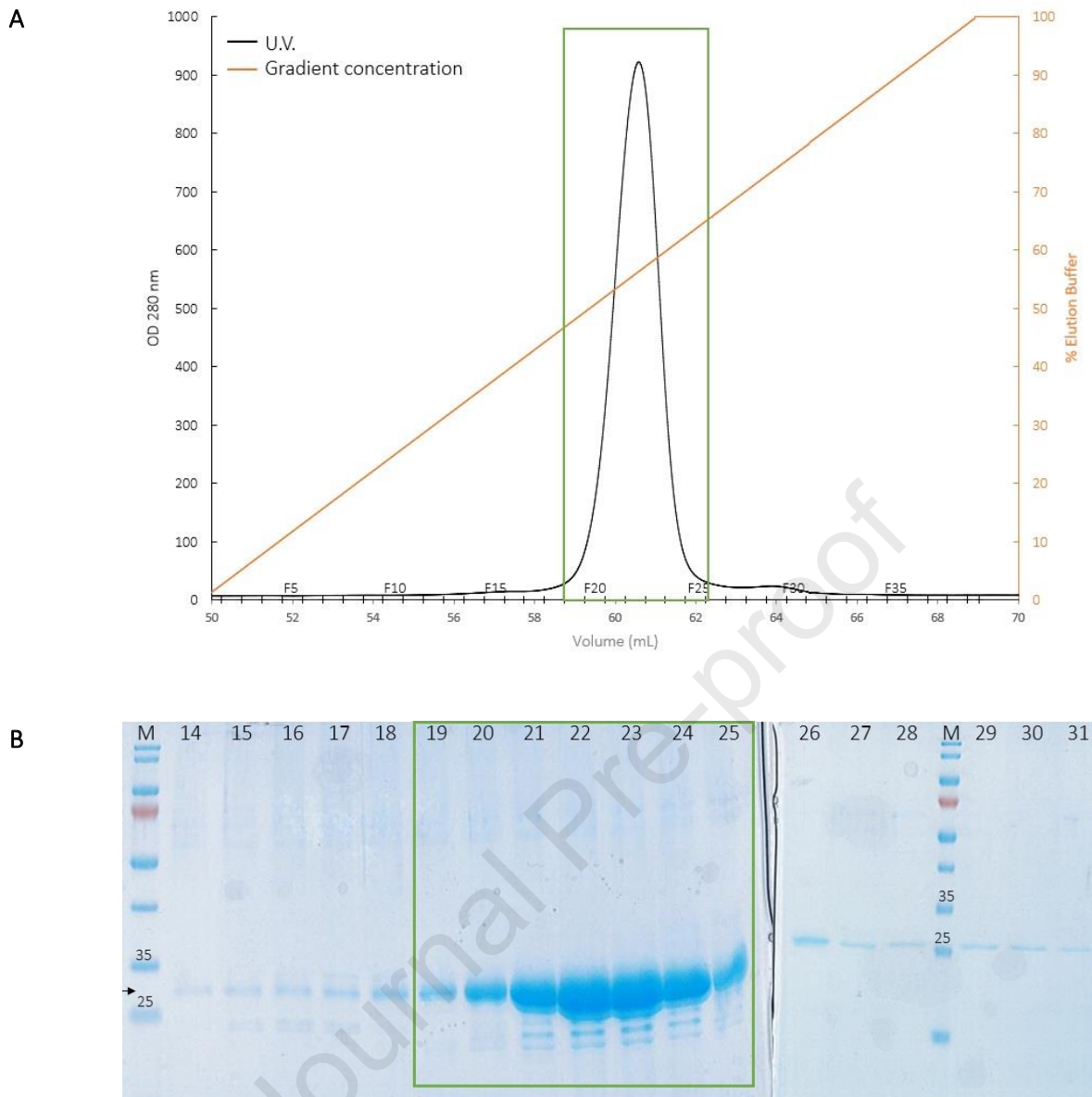
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Supplementary Data



Supplementary Figure 1. 6His-Ulp1 purification on Ni-NTA column. A) Elution profile of Ni-NTA chromatography. UV curve and elution buffer gradient are in black and orange, respectively. **B)** Coomassie blue stained 12% SDS-PAGE gel from different sample fractions. Lane M: pre-stained protein marker. Lane S: Induced supernatant after lysis and centrifugation. Lanes 1 to 24: Fractions from elution gradient. The arrow indicates the 6His-Ulp1 protein (~28 kDa).



Supplementary Figure 2. 6His-Ulp1 purification on Heparin column. A) Elution profile of heparin chromatography. UV curve and elution buffer gradient are in black and orange, respectively. **B)** Coomassie blue stained 12% SDS-PAGE gel from different sample fractions. Lane M: pre-stained protein marker; Lanes 14 to 31: Fractions from elution gradient. The fractions containing 6His-Ulp1 proteins (F19 to F25) are indicated by a green rectangle.

Table 1. Detection by nanoLC-MS/MS of the peptides derived from Eat(A)v expressed in E. coli. Are indicated in columns: Prev. AA: Previous Amino-Acid in the protein sequence ; Peptide: sequence of the identified peptides; Next AA: Next Amino-Acid in the protein sequence ; Length: length of the identified peptides; PTMs: Post Translational Modifications detected; PSM Score : Peptide Spectrum Match Score; Start: start position of the identified peptides in the protein sequence; Stop: stop position of the identified peptides in the protein sequence; Calc. Mass: Calculated Mass of the theoretical peptide ; Exp. MoZ: Experimental Mass to Charge Ratio; Ppm: Difference between experimental and theoretical masses; Charge : charge of the peptide ions detected; Rank: Rank of the identified sequence; RT: Retention Time of the peptides; Protein Set Count: number of Protein Sets in which the peptides are assigned; ProteinSets : Name of the Protein Sets in which the peptides are assigned.

Prev. AA	Peptide	Next AA	Length	PTMs	PSM Score	Start	Stop	Calc. Mass	Exp. MoZ	Ppm	Charge	Missed Cl.	Rank	RT	Protein Set Count	Protein Sets
-	MSKIEK	N	7	Oxidation (M1)	25,92	1	7	863,478607	432,748	3,2825215	2	1	1	9,997784	1	1 (Eat(A)v_LSAP-resistance)
K	ITLLNLIQNK	L	10		55,02	45	54	1156,681549	579,34906	1,7424759	2	0	1	35,091667	1	1 (Eat(A)v_LSAP-resistance)
R	ELQLMQTDPEILWR	E	14	Oxidation (M5)	83,97	99	112	1786,892349	894,45331	-0,15707916	2	0	1	35,46317	1	1 (Eat(A)v_LSAP-resistance)
R	EFSTLSGGEK	T	10		66,19	113	122	1053,497849	527,75823	3,8455255	2	0	1	14,832667	1	1 (Eat(A)v_LSAP-resistance)
R	KQVAAYLK	K	8		44,65	153	160	919,549072	460,78411	4,987176	2	1	1	11,8336	1	1 (Eat(A)v_LSAP-resistance)
K	QVAAYLK	K	8		43,56	154	161	919,549072	460,78352	3,7067435	2	1	1	10,975467	1	1 (Eat(A)v_LSAP-resistance)
K	QVAAYLK	K	7		40,83	154	160	791,454117	396,73607	4,3744636	2	0	1	17,7425	1	1 (Eat(A)v_LSAP-resistance)
K	QSFVAVSHDR	G	10		70,64	164	173	1156,598923	579,30737	1,0918223	2	0	1	30,222834	1	1 (Eat(A)v_LSAP-resistance)
K	SQELVYQGNFVVEEQK	K	17		54,37	189	205	2074,984711	1038,49915	-0,46364966	2	0	1	33,2435	1	1 (Eat(A)v_LSAP-resistance)
K	LRDFFVIAQNEK	L	12	Oxidation (M7)	67,32	207	218	1524,68782	763,35489	4,8522882	2	1	1	12,500893	1	1 (Eat(A)v_LSAP-resistance)
R	DEFFVIAQNEK	L	10	Oxidation (M5)	51,14	209	218	1255,502655	628,76372	8,137464	2	0	1	10,779217	1	1 (Eat(A)v_LSAP-resistance)
K	TAAEKAWEISR	S	10		26,12	229	238	1147,562149	574,78877	0,72983384	2	1	1	9,587466	1	1 (Eat(A)v_LSAP-resistance)
K	KVGFIDTESR	R	11		88,76	247	257	1278,656815	640,33924	5,554118	2	1	1	17,094831	1	1 (Eat(A)v_LSAP-resistance)
K	QVGFIDTESR	R	10		78,09	248	257	1150,561859	576,29038	3,7732854	2	0	1	22,3645	1	1 (Eat(A)v_LSAP-resistance)
K	QVGFIDTESR	V	11		42,14	248	258	1306,662964	436,56386	5,182926	3	1	1	17,897667	1	1 (Eat(A)v_LSAP-resistance)
K	GAVGADAAR	T	9		57,42	262	270	786,398407	394,20877	5,810407	2	0	1	6,2764163	1	1 (Eat(A)v_LSAP-resistance)
R	METQISEK	E	8	Oxidation (M1)	53,52	282	289	980,448441	491,22905	-4,98034	2	0	1	8,217183	1	1 (Eat(A)v_LSAP-resistance)
R	METQISEK	L	10	Oxidation (M1)	31,36	282	291	1237,585983	619,80233	3,3276849	2	1	1	7,3143835	1	1 (Eat(A)v_LSAP-resistance)
K	DIEYDLSLTWNSQASHHK	R	18	Oxidation (M10)	60,17	295	312	2103,95311	702,32849	4,998636	3	0	1	27,984667	1	1 (Eat(A)v_LSAP-resistance)
R	VAISGPNAGK	S	11		72,65	342	352	969,524338	485,77078	2,7482173	2	0	1	11,0539665	1	1 (Eat(A)v_LSAP-resistance)
K	HLSISYASQNYEDNR	G	15		58,55	376	390	1795,8125	599,6138	3,931435	3	0	1	20,195333	1	1 (Eat(A)v_LSAP-resistance)
R	GTLAFAEK	N	9		58,02	391	399	964,486542	483,25212	3,2550402	2	0	1	22,049665	1	1 (Eat(A)v_LSAP-resistance)
K	NQVDYQAFNLNLR	K	13		48,28	400	412	1593,789932	797,90736	6,4143195	2	0	1	35,205	1	1 (Eat(A)v_LSAP-resistance)
K	IEQVSVGGR	K	9	Oxidation (M4); Oxidation (M6)	54,91	425	433	1110,479767	556,24898	3,2728257	2	0	1	3,3258002	1	1 (Eat(A)v_LSAP-resistance)
K	VSTEISLER	I	10		71,44	490	499	1145,629181	573,82363	3,0732534	2	0	1	28,824	1	1 (Eat(A)v_LSAP-resistance)
K	VSTEISLERI	-	11		36,78	490	500	1258,713242	630,36233	-2,4858658	2	1	1	34,663166	1	1 (Eat(A)v_LSAP-resistance)

Prev. AA	Peptide	Next AA	Length	PTMs	PSM Score	Start	Stop	Calc. Mass	Exp. MoZ	Ppm	Charge	Missed Cl.	Rank	RT	Protein Set Co	Protein Sets
-	MSKIEIK	N		7 Oxidation (M1)	25.92	1	7	863.478607	432.748	3.2825215		2	1	9.997784	1 1	(Eat(A)v_LSAP-resistance)
K	TLLNILQNK	L	10		55.02	45	54	1156.681549	579.34906	1.7424759		2	0	35.091667	1 1	(Eat(A)v_LSAP-resistance)
R	ELQLMQTDPE	E	14	Oxidation (M5)	83.97	99	112	1786.892349	894.45331	-0.15707916		2	0	35.46317	1 1	(Eat(A)v_LSAP-resistance)
R	EFSTLSGGEK	T	10		66.19	113	122	1053.497849	527.75823	3.8455255		2	0	14.832667	1 1	(Eat(A)v_LSAP-resistance)
R	KQVAAYLK	K	8		44.65	153	160	919.549072	460.78411	4.987176		2	1	11.8336	1 1	(Eat(A)v_LSAP-resistance)
K	QVAAYLKK	K	8		43.56	154	161	919.549072	460.78352	3.7067435		2	1	10.975467	1 1	(Eat(A)v_LSAP-resistance)
K	QVAAYLK	K	7		40.83	154	160	791.454117	396.73607	4.3744636		2	0	17.7425	1 1	(Eat(A)v_LSAP-resistance)
K	QGFIVVSHDR	G	10		70.64	164	173	1156.598923	579.30737	1.0918223		2	0	30.222834	1 1	(Eat(A)v_LSAP-resistance)
K	SQLELYQGNFSI	K	17		54.37	189	205	2074.984711	1038.49915	-0.46364966		2	0	33.2435	1 1	(Eat(A)v_LSAP-resistance)
K	LRDEFEMAQN	L	12	Oxidation (M7)	67.32	207	218	1524.68782	763.35489	4.8522882		2	1	12.500883	1 1	(Eat(A)v_LSAP-resistance)
R	DEFEMAQNEK	L	10	Oxidation (M5)	51.14	209	218	1255.502655	628.76372	8.137464		2	0	10.779217	1 1	(Eat(A)v_LSAP-resistance)
K	TAAEKAWSR	S	10		26.12	229	238	1147.562149	574.78877	0.72983384		2	1	9.587466	1 1	(Eat(A)v_LSAP-resistance)
K	KQVGFIDTESR	R	11		88.76	247	257	1278.656815	640.33924	5.554118		2	1	17.094831	1 1	(Eat(A)v_LSAP-resistance)
K	QVGFIDTESR	R	10		78.09	248	257	1150.561859	576.29038	3.7732854		2	0	22.3645	1 1	(Eat(A)v_LSAP-resistance)
K	QVGFIDTESRR	V	11		42.14	248	258	1306.662964	436.56386	5.182926		3	1	17.897667	1 1	(Eat(A)v_LSAP-resistance)
K	GAVGADAAR	T	9		57.42	262	270	786.398407	394.20877	5.810407		2	0	6.2764163	1 1	(Eat(A)v_LSAP-resistance)
R	METQISEK	E	8	Oxidation (M1)	53.52	282	289	980.448441	491.22905	-4.98034		2	0	8.217183	1 1	(Eat(A)v_LSAP-resistance)
R	METQISEKEK	L	10	Oxidation (M1)	31.36	282	291	1237.585983	619.80233	3.3276849		2	1	7.3143835	1 1	(Eat(A)v_LSAP-resistance)
K	DIEYIDSLTMNSR	R	18	Oxidation (M10)	60.17	295	312	2103.95311	702.32849	4.998636		3	0	27.984667	1 1	(Eat(A)v_LSAP-resistance)
R	VAISGPNAGK	S	11		72.65	342	352	969.524338	485.77078	2.7482173		2	0	11.0539665	1 1	(Eat(A)v_LSAP-resistance)
K	HLSISYASQNYE	G	15		58.55	376	390	1795.8125	599.6138	3.931435		3	0	20.195333	1 1	(Eat(A)v_LSAP-resistance)
R	GTLAFAEK	N	9		58.02	391	399	964.486542	483.25212	3.2550402		2	0	22.049665	1 1	(Eat(A)v_LSAP-resistance)
K	NQVDYQAFLNK		13		48.28	400	412	1593.789932	797.90736	6.4143195		2	0	35.205	1 1	(Eat(A)v_LSAP-resistance)
K	IEQMSMGQR	K	9	Oxidation (M4); O	54.91	425	433	1110.479767	556.24898	3.2728257		2	0	3.3258002	1 1	(Eat(A)v_LSAP-resistance)
K	VSTEIISLER	I	10		71.44	490	499	1145.629181	573.82363	3.0732534		2	0	28.824	1 1	(Eat(A)v_LSAP-resistance)
K	VSTEIISLERI	-	11		36.78	490	500	1258.713242	630.36233	-2.4858658		2	1	34.663166	1 1	(Eat(A)v_LSAP-resistance)

Highlights

- The proteins from ATP-binding cassette F proteins family (ABC-F) are difficult to purify because of their very low solubility and stability.
- We describe a rapid and efficient three-step purification procedure for obtaining large amounts of untagged and soluble ABC-F proteins
- This invaluable tool that can then be used for *in vitro* experiments.

Journal Pre-proof

Author statement: FD and MH performed biochemical and purification experiments; SG conducted microbiology experiments; EC performed the proteomic analyses. RGo, VC and RGi conceived the project. MH and RGo designed the experiments. FD, MH and RGi wrote the manuscript.

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