

Membrane protein production in *Lactococcus lactis* for functional studies

Daphné Seigneurin-Berny, Martin S. King, Emiline Sautron, Lucas Moyet, Patrice Catty, François André, Norbert Rolland, Edmund R.S. Kunji, Annie Frelet-Barrand

▶ **To cite this version:**

Daphné Seigneurin-Berny, Martin S. King, Emiline Sautron, Lucas Moyet, Patrice Catty, et al.. Membrane protein production in *Lactococcus lactis* for functional studies. Mus-Veteau Isabelle. Heterologous Expression of Membrane Proteins. Methods in Molecular Biology, 1432, Humana Press, pp.79-101, 2016, Methods in Molecular Biology, 978-1-4939-3637-3. 10.1007/978-1-4939-3637-3_6 . hal-01352902

HAL Id: hal-01352902
https://hal.science/hal-01352902

Submitted on 13 Oct 2022

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Membrane Protein Production in *Lactococcus lactis* for Functional Studies

Daphne Seigneurin-Berny, Martin S. King, Emiline Sautron, Lucas Moyet, Patrice Catty, François André, Norbert Rolland, Edmund R.S. Kunji, and Annie Frelet-Barrand

Abstract

Due to their unique properties, expression and study of membrane proteins in heterologous systems remains difficult. Among the bacterial systems available, the Gram-positive lactic bacterium, *Lactococcus lactis*, traditionally used in food fermentations, is nowadays widely used for large-scale production and functional characterization of bacterial and eukaryotic membrane proteins. The aim of this chapter is to describe the different possibilities for the functional characterization of peripheral or intrinsic membrane proteins expressed in *Lactococcus lactis*.

Key words *Lactococcus lactis*, Membrane proteins, Expression, Transport assays

1 Introduction

In the past decades, *Lactococcus lactis*, a Gram-positive bacterium traditionally used in food fermentations, has emerged as a useful system for functional expression of prokaryotic and eukaryotic membrane proteins (MPs) [1]. *L. lactis* is an attractive alternative host for *Escherichia coli*, especially for eukaryotic MPs, because of (1) its moderate proteolytic activity, (2) the absence of inclusion body formation and endotoxin production [2, 3], (3) the efficient targeting of MPs into a single glycolipid cytoplasmic membrane [2, 4, 5], and (4) its ability to express MPs in their oligomeric state [2, 6]. This facultative anaerobe-aerobe lactic acid bacterium (LAB) grows at 30 °C with a doubling time of 35–60 min [7]; it is easy and inexpensive to grow and genetic methods and vector systems are well developed [8]. In addition to the classical cloning techniques, different strategies have been developed to obtain a larger number of recombinant clones [9, 10].

The expression of heterologous proteins in *L. lactis* has been facilitated both by advances in genetic methods and by new developments in molecular biology techniques. Using these tools, various vectors containing either constitutive or inducible promoters have been developed to obtain increased levels of proteins and to control their production. They currently constitute the basis of all expression systems in *L. lactis* [11]. The tightly regulated nisin-controlled gene expression (NICE) system is the most commonly used [12]. This promising and powerful expression system is based on genes involved in the biosynthesis and regulation of the antimicrobial peptide, nisin. When a gene of interest is placed upstream of the inducible promoter PnisA on a plasmid, its expression can be induced by the addition of subinhibitory amounts of nisin (0.1–5 ng/mL) to the culture medium [13]. The NICE system has proved to be highly versatile and is widely used in pharmaceutical, medical, and bio- and food-technology applications [14]. This well-characterized system is nowadays widely used for functional studies of homologously and heterologously expressed soluble and membrane proteins from diverse origins (prokaryotic or eukaryotic), topologies, and sizes (for reviews, see [1] and [14]). Moreover, in the last years, three structures have been obtained after expression of MPs using the NICE system [15–17] as well as several domain structures of human membrane proteins [18, 19].

In this chapter, we will give some examples of eukaryotic MPs for which functional analysis has been carried out after their expression in *L. lactis* using the NICE system. These functional characterizations can be performed on: (1) whole-cell bacteria, (2) membrane extracts, (3) fused membrane vesicles, (4) proteoliposomes after reconstitution of the MPs in phospholipids, using radioactive substrates, or (5) directly solubilized and purified membrane proteins [2, 3, 10, 12, 18, 19].

2 Materials

2.1 Growth of Recombinant Bacteria and Expression of the Proteins of Interest

1. *Lactococcus lactis* NZ9000 and nisin-producing NZ9700 strains (NIZO; *see* **Notes 1** and **2**).
2. M171GChl medium: M17 broth, 1% [w/v] glucose, 10 μg mL^{-1} chloramphenicol (*see* **Note 3**).
3. Laboratory glassware bottles (Schott bottles).
4. Incubator for cell growth.
5. Appropriate buffers for bacterial resuspension (*see* **Note 4**).

2.2 Isolation of Lactococcal Membranes with the Cell Disruptor

1. One Shot (Constant Cell Disruption Systems, Northants, UK) (*see* **Note 5**).
2. Appropriate buffers for protein resuspension (*see* **Note 4**).

2.3 SDS-PAGE and Detection of Recombinant Protein

1. Acrylamide-bis ready-to-use solution, 30% [w/v] (37.5:1).
2. 8× Laemmli resolving gel buffer: 3 M Tris–HCl pH 8.8 (60.6 g Tris–HCl resuspended in Milli-Q water; adjust to pH 8.8 at 25 °C with 12 N HCl. Store at room temperature).
3. 4× Laemmli stacking gel buffer: 0.5 M Tris–HCl pH 6.8 (363 g Tris–HCl resuspended in Milli-Q water; adjust to pH 6.8 at 25 °C with 12 N HCl. Store at room temperature).
4. Aqueous solution 20% [w/v] sodium dodecyl sulfate (SDS).
5. Ammonium persulfate: Prepare 10% [w/v] solution in water and immediately freeze in single-use (200 µL) aliquots at –20 °C.
6. Tetramethylethylenediamine (TEMED).
7. Resolving gels (10% acrylamide): 3.3 mL of 30% [w/v] acrylamide solution, 1.25 mL of 8× Laemmli resolving gel buffer, 50 µL of 20% [w/v] SDS, 5.3 mL of water, 10 µL of TEMED, and 100 µL of 10% [w/v] ammonium persulfate (*see* **Note 6**).
8. Stacking gels (5% [w/v] acrylamide): 2.8 mL of 30% [w/v] acrylamide solution, 1.25 mL of 4× Laemmli stacking gel buffer, 25 µL of SDS 20% [w/v], 2.8 mL of water, 5 µL of TEMED, and 50 µL of 10% [w/v] ammonium persulfate.
9. Laemmli running buffer (10×): For 1 L, 144.2 g of glycine (192 mM), 30.3 g of Tris–HCl (25 mM); add 50 mL of 20% [w/v] SDS (0.1% final concentration) and Milli-Q water. Store at room temperature.
10. Molecular weight marker.
11. Reducing sample buffer (4×): 0.08 M Tris–HCl, pH 6.8, 40% [v/v] glycerol, 1% [w/v] SDS, 0.1 mM bromophenol blue, 10 mM dithiothreitol. Store at –20 °C.
12. Sample buffer: 100 mL reducing buffer and 20 mL of 20% [w/v] SDS.
13. Control protein: Recombinant *Strep*-tag II fusion protein, MW about 28 kDa (0.1 mg mL^{-1}) (IBA, Goettingen, Germany).
14. System for protein transfer to nitrocellulose membranes (central core assembly, holder cassette, nitrocellulose filter paper, fibber pads, and cooling unit).
15. Protein transfer buffer: dilute running buffer 1× with ethanol to a final concentration of 20% [v/v]. Store at 4 °C.
16. Nitrocellulose or polyvinylidene difluoride (PVDF) membranes.
17. 3 MM paper from Whatman.
18. Protein-specific antibody or conjugate specific to the affinity tag (*see* **Note 7**).
19. Bio-Safe Coomassie (Biorad).
20. Electrochemiluminescence (ECL) detection kit.

2.4 Functional Characterization of Membrane Proteins Expressed in L. lactis

2.4.1 Dehydrogenase Assay on Purified Protein (ceQORH)

1. Solubilization buffer: 50 mM MOPS pH 7.8 containing 0.5 or 1 M NaCl.
2. 10 mM Tris–HCl pH 8.0.
3. Ni-NTA resin (Qiagen).
4. Binding buffer: 5 mM imidazole, 0.5 M NaCl, 20 mM Tris–HCl pH 7.9.
5. Wash buffer: 60 mM imidazole, 0.5 M NaCl, 20 mM Tris–HCl pH 7.9.
6. Elution buffer: 0.5 M imidazole, 0.25 M NaCl, 10 mM Tris–HCl pH 7.9.
7. Dehydrogenase reaction buffer: 100 μM NADPH, 100 μM nitroblue tetrazolium (NBT), 10 mM Tris–HCl pH 8.0.
8. PD10 column (GE Healthcare).
9. Eppendorf centrifuge.
10. Lipids (P3644, Sigma).
11. Spectrophotometer.

2.4.2 Phosphorylation Assays with AtHMA6 and AtHMA8 Using L. lactis Membranes

1. ATP phosphorylation buffer: 20 mM Hepes pH 7.0, 100 mM KCl, 5 mM $MgCl_2$, 300 mM sucrose (*see* **Note 8**).
2. Pi phosphorylation buffer: 20 mM Hepes pH 6.0, 10 mM $MgCl_2$, 20% [v/v] DMSO (*see* **Note 9**).
3. Metal solutions: prepare solutions at concentration ranging from 1 to 100 μM in phosphorylation buffer or water (*see* **Note 10**).
4. 1 mM ^{32}Pi (10–100 μCi $nmol^{-1}$, Perkin Elmer, 7 μL): Add 1 mL of 1 mM H_3PO_4 (prepared in 100 mM Hepes pH 5.6) directly in the tube containing the isotope ^{32}Pi. Filter the solution through a 0.2 μm membrane. To avoid loss of the solution in the filter, push the volume of the solution stayed in the filter using an empty syringe. Use 10 μL/reaction. Store at 4 °C.
5. 10 μM [γ-^{32}P]ATP (50–500 μCi $nmol^{-1}$, Perkin Elmer, 7 μL): for 10 reactions, add 1 μL of [γ-^{32}P]ATP to 110 μL of a solution of 10 μM ATP (*see* **Note 11**). Prepare a stock solution of 400 mM ATP in H_2O, aliquot in small volumes, and store at −20 °C. 10 μM ATP is prepared freshly from one stored aliquot of 400 mM ATP.
6. Stop buffer: 1 mM KH_2PO_4 in 7% [v/v] trichloroacetic acid (TCA). Store at 4 °C.
7. Denaturing buffer: 5 mM Tris-PO_4 pH 5.0, 6.7 mM urea, 400 mM DTT, 5% [w/v] SDS, 0.004% [w/v] orange G (*see* **Note 12**).
8. Chelator mix: 1 mM bicinchoninic acid (BCA) and 100 μM bathocuproine disulfonate (BCS) (*see* **Note 13**).

9. Resolving gel: 6 mL of Acrylamide/Bis 24/0.8% [w/v] solution (*see* **Note 14**), 4.5 mL of 4× resolving gel buffer (260 mM Tris-H_3PO_4 pH 6.5, 0.4% SDS), 7.4 mL of H_2O, 18 µL of TEMED, and 100 µL of 10% [w/v] ammonium persulfate.
10. Stacking gel: 1.2 mL of Acrylamide/Bis 24/0.8% [v/v] solution, 2 mL of 4× stacking gel buffer (260 mM Tris-H_3PO_4 pH 5.5, 0.4% SDS), 4.64 mL of H_2O, 7.5 µL of TEMED, and 160 µL of 10% [w/v] ammonium persulfate.
11. Running buffer: 0.17 mM MOPS (pH 6.0 adjusted with 2 M Tris), 0.1% [w/v] SDS. Store at 4 °C before use (do not store for more than 1 week) (*see* **Note 15**).
12. Acetic acid 15% [v/v].
13. Gel staining medium: acetic acid/isopropanol/water, 10/25/65 [v/v/v], supplemented with 2.5 $g.L^{-1}$ of Coomassie Brilliant Blue R250.
14. Gel destaining medium: 30% [v/v] ethanol.
15. Eppendorf centrifuge.
16. Gel electrophoresis apparatus (Bio-Rad Protean 3 or equivalent), with the various accessories needed for protein separation by electrophoresis (combs, plates, and casting apparatus).
17. Phosphorimaging device (*see* **Note 16**).

2.4.3 Transport Assays with Whole Cells Expressing AtAATP1/NTT1

1. 50 mM potassium phosphate buffer pH 7.0.
2. 3.33 nM [α-^{32}P]ATP (3000 mCi $mmol^{-1}$, Perkin Elmer). Add 1 µL to 50 mM potassium phosphate buffer pH 7.0 containing 1.5 mM cold ATP (A9062; Sigma) to obtain a 50 µM solution. Store on ice until use.
3. Filter membranes 0.45 µm (HAWP02500; Millipore).
4. Scintillation vials.
5. d.d. water.
6. Polymeric Vacuum Filter Holder (1225 Sampling Manifold; Millipore).
7. Multi-Purpose Scintillation Counter.

2.4.4 Transport Assays with Whole Cells and Fused Membrane Vesicles Expressing Mitochondrial Carriers

1. *E. coli* polar lipid extract (Avanti Polar Lipids).
2. Egg yolk phosphatidylcholine (Avanti Polar Lipids).
3. Nitrogen.
4. Diethyl ether.
5. Substrate/inhibitor, 10× stock (*see* **Note 17**).
   - ADP to give a final concentration of 5 mM
   - Carboxyatractyloside (CATR; Sigma) to give a final concentration of 2 µM

- Bongkrekic acid (BKA; Sigma) to give a final concentration of 2 μM

6. Extruder Set (Avanti Polar Lipids).
7. 1 μm polycarbonate filter (Whatman); filter supports (Whatman).
8. PD-10 column (GE Healthcare).
9. 2 mL Eppendorf tubes.
10. Hamilton robot (with vacuum manifold).
11. 96-well MultiScreenHTS-Hi Flow-FB opaque, Barex plastic plates (pore size = 1 μm; Millipore).
12. 96-well MultiScreenHTS-HA opaque, Barex plastic plates (pore size = 0.45 μm; Millipore).
13. 1.5 μM [^{14}C]-ADP (60 mCi mmol^{-1} = 2.22 GBq mmol^{-1}; Perkin Elmer) prepared in PIPES buffer to start the transport assays with mitochondrial carriers.
14. BackSeal black backing paper (Perkin Elmer).
15. MicroScint-20 (Perkin Elmer).
16. MultiScreen sealing tape, clear backing paper (Perkin Elmer).
17. TopCount (Perkin Elmer).

3 Methods

3.1 Growth of Recombinant Bacteria and Expression of the Proteins of Interest

The gene(s) of protein(s) of interest have to be cloned first into an expression vector containing nisin-inducible promoter either through classical cloning methods [2] or other strategies developed in the last years to facilitate cloning [9, 10]. First trials could be performed following protocols already described [2, 10, 20–22]. Here we present protocols that can be optimized for each protein of interest.

1. Inoculate M17G1Chl precultures with concentrated glycerol stocks or frozen cell stocks of recombinant bacteria carrying the gene of protein of interest and bacteria carrying the empty vector as negative control.
2. Incubate overnight at 30 °C without shaking (*see* **Notes 18** and **19**).
3. Inoculate M17G1Chl with 1/40^{e} precultures (*see* **Note 20**).
4. Incubate cultures at 30 °C; measure OD_{600nm} every 45 min to construct growth curve (doubling roughly every 45 min).
5. Induce protein expression by addition of homemade nisin (*see* **Note 21**) at OD_{600nm} from 0.5 to 0.8 depending on proteins of interest (*see* **Note 22**).

6. Swirl immediately to prevent cell lysis; return the flasks to the 30 °C incubator for further 2.5–4 h (*see* **Note 23**).
7. Depending on functional tests, bacteria are either centrifuged, resuspended with an appropriate buffer (*see* **Note 4**) and centrifuged again before storage at −20 °C (*see* Subheading 3.2), or directly used (*see* Subheading 3.4.1) or snap-frozen and stored in liquid nitrogen (*see* Subheading 3.4.4).

3.2 Isolation of Lactococcal Membranes with the Cell Disruptor

1. Resuspend the bacteria into the appropriate buffer (*see* **Note 4**).
2. Disrupt the bacteria by twofold passages through a One Shot at 35,000 p.s.i. (2.3 kbars).
3. Centrifuge 100,000 × *g*, 15 min, 4 °C and transfer the supernatant into ultracentrifuge tubes.
4. Centrifuge 100,000 × *g*, 1 h, 4 °C; resuspend the pellet and homogenize into the appropriate buffer (*see* **Note 4**).
5. Snap-freeze and store at −80 °C or in liquid nitrogen until use.

3.3 Detection of the Recombinant Protein Produced

1. Prior to the experiment, prepare acrylamide gels for protein electrophoresis, the gel apparatus according to the manufacturer's specifications, and the different gel solutions (stacking gel, acrylamide separating gel; *see* **Note 6**).
2. Heat the protein samples at 95 °C for 5 min to solubilize the proteins (*see* **Note 24**). Load protein samples, one molecular weight marker, and positive controls in defined quantities.
3. Run gels for 1 h at room temperature at 150 V with constant voltage (*see* **Note 25**).
4. After electrophoresis, perform the transfer for 1 h 30 min at 100 V in protein transfer medium prior to Western blotting analysis.
5. Recover the nitrocellulose membrane and rinse the membrane with water. The following incubation and washing steps require agitation on a rocking plate at room temperature.
6. Perform Western blotting analysis and/or Coomassie blue staining using protocols already established or given by manufacturers.
7. Perform ECL detection (Figs. 1 and 2)

3.4 Functional Characterization of Membrane Proteins

3.4.1 Dehydrogenase Assay on Purified Protein (ceQORH)

The chloroplast envelope Quinone OxidoReductase Homologue (ceQORH) protein from *Arabidopsis thaliana* is a peripheral protein associated with the chloroplast envelope through electrostatic interactions [24]. This nuclear-encoded protein is devoid of a classical and cleavable transit peptide and uses an alternative targeting pathway for its import into the chloroplast [25, 26]. The ceQORH protein is structurally related to bacterial, fungal, and animal proteins with known quinone oxidoreductase function. In an earlier

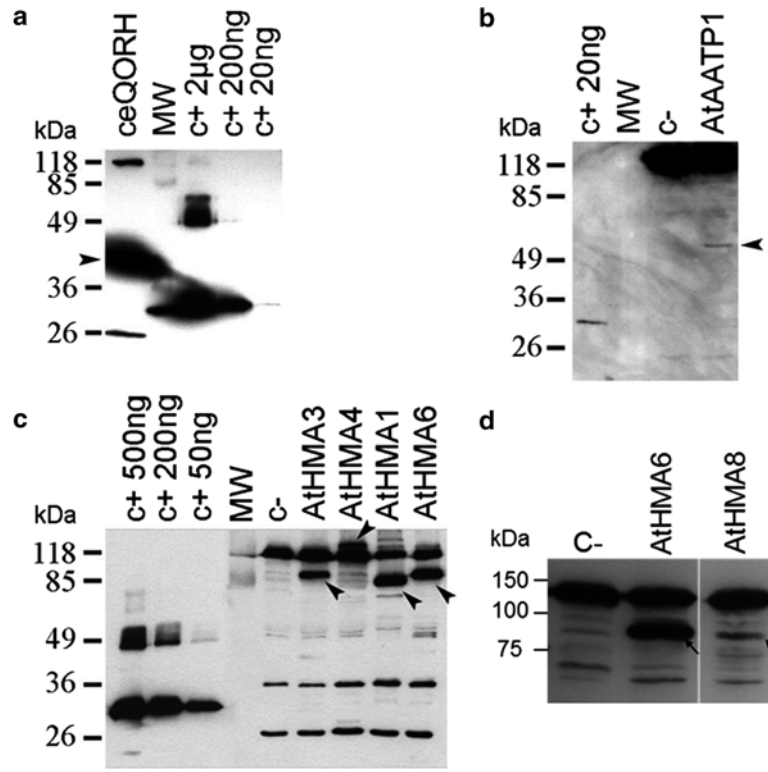


Fig. 1 Expression of plant membrane proteins in *L. lactis*. (**a**) Production of the peripheral protein ceQORH. (**b**–**d**) Production of Arabidopsis transmembrane proteins (the nucleotide transporter, AtAATP1 in panel **b** and the P_{1B}-ATPases in panels **c** and **d**). Total membrane proteins (10 µg, 20 µg, 15 µg, and 50 µg in panels **a**, **b**, **c**, and **d** respectively) were separated in a 10 % SDS-PAGE and analyzed by western blot performed using a horseradish peroxidase (HRP) conjugate specific to *Strep*-tag II. *Arrows* indicate the positions of the expressed proteins. In panels **a** and **b**, expressed proteins contain an additional N-terminal sequence resulting from the translation of the attB sites. In panels **b**, **c**, and **d**, c- meanscrude membrane proteins derived from bacteria containing the empty pNZ8148 vector. In panels **a**, **b**, **c**, defined amounts of a positive control protein (c+, *Strep*-tag II protein) were loaded to estimate the expression levels of the recombinant proteins. Adapted from [10] and [23]

work, an NADPH quinone oxidoreductase activity was detected in the chloroplast envelope [27], and this activity was assumed to be associated with the presence of the ceQORH protein. To investigate its enzymatic properties, we first expressed it in *Escherichia coli* but the protein was mostly recovered in inclusion bodies. By contrast, in *L. lactis*, the ceQORH protein was well produced and recovered in the membrane fraction, representing 20–30 % of total membrane proteins. This relatively high percentage is closed to levels of expression obtained for prokaryotic membrane proteins produced in *L. lactis* [1]. Performing dehydrogenase activity assays

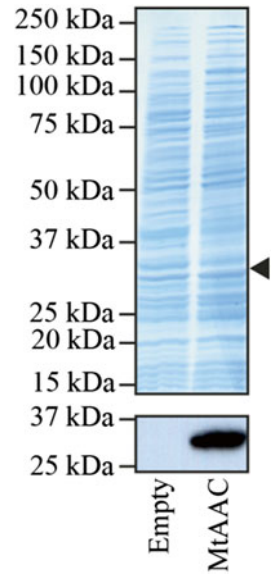
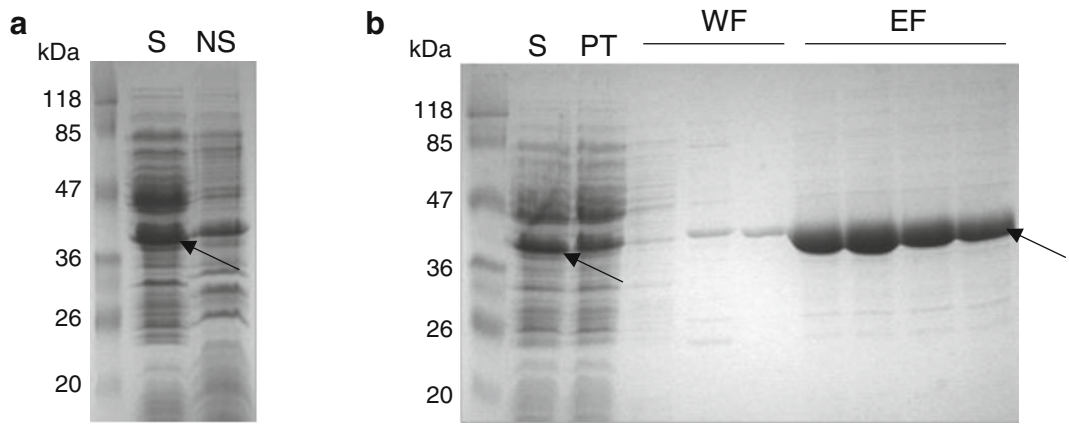


Fig. 2 Expression of the mitochondrial ADP/ATP carrier from the thermophilic fungus *Myceliophthora thermophile* (MtAAC). Instant blue-stained 12% SDS-PAGE gel (*top*) and western blot (*bottom*) of lactococcal membranes of the control strain (without protein) and strain expressing MtAAC. The *arrowhead* indicates the position of the expressed protein. Approximately 15 μg of total protein was loaded per lane. For Western blotting, the proteins were transferred to PVDF membranes and probed with a 1:20,000 dilution of a chicken IgY antibody for 1 h, followed by an anti-chicken-HRP conjugate at 1:20,000 dilution for another hour. The blotted membrane was developed using Amersham ECL Western blotting detection system for 30 min

on the purified protein using artificial substrates (NBT), we were able to establish that the ceQORH protein indeed exhibits NADPH-dependent dehydrogenase activity, and that this activity requires a lipid environment [10].

1. Incubate *L. lactis* membrane proteins (1 mg mL^{-1}) in 50 mM MOPS pH 7.8 containing 0.5 or 1 M NaCl for 45 min at 4 °C (*see* **Note 26**). Mix gently the sample every 15 min.
2. Following treatment, centrifuge membranes at 160,000 × *g*, for 1 h, at 4 °C to separate solubilized proteins in the supernatant from insoluble endogenous membrane proteins in the pellet. Keep the supernatant for further purification steps (Fig. 3a).
3. Desalt the supernatant on a PD-10 column against Binding buffer.
4. Pre-equilibrate the Ni-NTA resin in Binding buffer (*see* **Note 27**).
5. Incubate the solubilized proteins (*see* **Note 28**) with the pre-equilibrated Ni-NTA resin and mix gently by shaking (200 rpm on a rotary shaker) at 4 °C for 60 min.



c

Dehydrogenase activity of the ceQORH protein produced in L. lactis	
System	Specific activity of the ceQORH protein (nmol substrate/mg protein/min)
Complete	19.28 (100%)
Without ceQORH	0 (0%)
Without PC	1.97 (10,2%)
Without NADPH	0 (0%)
Without NBT	1.14 (5,9%)

Fig. 3 Functional characterization of the purified plant peripheral protein ceQORH. (**a**) *Lactococcus* membranes expressing the 6× His-tag ceQORH are solubilized in the presence of 1 M NaCl. After centrifugation, the solubilized (S) and nonsolubilized (NS) proteins are analyzed by SDS-PAGE and Coomassie blue staining of the gel. The 6× His-tag ceQORH protein is indicated by the arrow. (**b**) Purification of the 6× His-tag ceQORH protein using a Ni-NTA resin. Each fraction obtained during the purification process is analyzed by SDS-PAGE. PT: solubilized proteins not bound to the resin, WF: wash fractions, EF: elution fractions. (**c**). Dehydrogenase activity of the purified recombinant 6× His-tag ceQORH protein using the artificial substrate NBT. The impact of the lack of every reaction components (protein, phosphatidylcholine (PC), NAPDH, NBT) was tested. The specific activity determined for the 6× His-tag ceQORH protein (100 %) was 19.3 nmol of formazan/mg 6× His-tag ceQORH protein/min. Adapted from [10]

6. Load the solubilized proteins/Ni-NTA mixture onto a column.
7. Wash twice with Washing buffer.
8. Elute the His-tagged ceQORH protein with Elution buffer (*see* Fig. 3b for a typical purification).
9. Desalt the eluted His-tag ceQORH protein against 10 mM Tris–HCl pH 8.0.

For dehydrogenase activity assay (see **Note 29**):

10. Incubate 1 µg of purified ceQORH with 1 µg of lipids at 30 °C for 30 min (final volume of 20 µL).
11. Start the reaction by addition of 150 µL of 10 mM Tris–HCl containing 100 µM NADPH and 100 µM NBT.

12. Follow the formation of Formazan at 560 nm using a Spectrophotometer.
13. Deduce the enzymatic activities from the OD measurement using the molar absorption coefficient (*see* **Note 30**, Fig. 3c).

3.4.2 Phosphorylation Assays with AtHMA6 and AtHMA8 Using L. lactis Membranes

P_{IB}-ATPases (reviewed in [28]) belong to the large family of P-type ATPases that are transmembrane proteins responsible for the transport of ions and phospholipids across plasma and organelle membranes using the energy provided by ATP hydrolysis. Like all P-type ATPases, P_{IB}-ATPases (or HMA for Heavy Metal ATPase) are composed of a transmembrane domain M containing the transport site and determining ion selectivity, and of three cytosolic loops constituting the catalytic domain. P_{IB}-ATPases have six to eight predicted transmembrane helices and have been classified into several subgroups according to their ionic specificity [29]. Recently, the crystal structure of a prokaryotic P_{IB}-ATPase, LpCopA, has been solved providing new topological information on these enzymes [30]. In *Arabidopsis* species, AtHMA6 and AtHMA8 are two chloroplastic ATPases of the PIB-1 subgroup involved in Cu transport across the chloroplast envelope and the thylakoid respectively [31, 32]. The enzymatic properties of these two transmembrane proteins could be assessed using in vitro biochemical assays [23, 33] after their successful and efficient expression in *Lactococcus lactis*. Phosphorylation assays performed on lactococcal membranes expressing these exogenous P_{IB}-ATPases (*see* **Note 31**) could provide information about (1) the kinetic parameters of the enzyme using phosphorylation from ATP (*see* Fig. 4a–c), and (2) the apparent affinity for the translocated metal using phosphorylation from Pi (Fig. 4b). These phosphorylation assays can be performed on all P_{IB}-ATPases whatever their ionic specificity to assess their enzymatic properties.

Phosphorylation from ATP:

1. Prepare a mix containing 0.5 mg mL^{-1} of *L. lactis* membranes, with metals or chelators at the desired concentration (*see* **Note 32**) and complete with ATP phosphorylation buffer to a final volume of 90 µL.
2. Start the reaction by addition of 10 µL of 10 µM [γ-^{32}P]ATP (1 µM final). Vortex the suspension.
3. Stop the reaction 30 s later (*see* **Note 33**) by addition of 1 mL ice-cold Stop buffer. Vortex the suspension and incubate 30 min on ice.
4. Centrifuge for 15 min, at 15,000 × *g*, 4 °C and keep the pellet (*see* **Note 34**).
5. Wash the pellet with 1 mL of ice-cold Stop buffer and centrifuge for 15 min, at 10,000 × *g*, 4 °C.

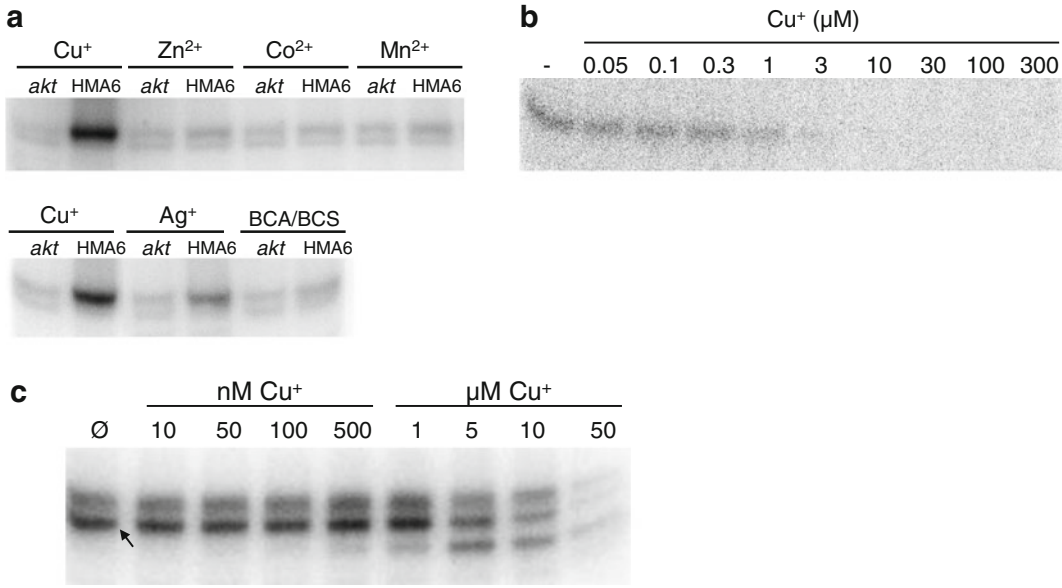


Fig. 4 Functional characterization of plant PIB-ATPases, AtHMA6 and AtHMA8 using phosphorylation assays. (**a**) Phosphorylation assays from ATP on lactococcal membranes expressing HMA6 and an inactive form of HMA6 ("akt") in the presence of 5 μM of various metals. Phosphorylation signal was observed only in the presence of Cu^+ and to a lesser extend with Ag^+ (*see* [33] for more information). (**b**) Phosphorylation assays from Pi on lactococcal membranes expressing HMA6 and in presence of various concentration of Cu^+. In that case, phosphorylation was inhibited by the transported metal, here Cu^+ [33]. (**c**) Phosphorylation assays from ATP on lactococcal membranes expressing HMA8. Phosphorylation signal of HMA8 is indicated by the *arrow*. One can notice that HMA8 was phosphorylated in the absence of added Cu^+ in the media (Ø) and that its phosphorylation was inhibited by high concentration of Cu^+ (*see* [23] for more details)

6. Repeat the wash step once.
7. Resuspend the pellet in 35 μL of Denaturing buffer. Vortex vigorously during 1–2 min.
8. Load the samples onto an acidic SDS-acrylamide gel (*see* **Note 35**).
9. After electrophoresis (about 1 h at 40 mA per gel), incubate the gel in the acetic acid solution for 10 min at room temperature.
10. Place the gel between "saran film" and expose it against a phosphorimager screen (*see* **Note 16**) in common film cassette overnight at room temperature.
11. Analyze the phosphorylation signal using a phosphorimaging device (*see* Fig. 4a–c).
12. Stain the gel (to check the amount of loaded proteins) by incubation for 30 min in the Gel staining medium and then in the Gel destaining medium.

Phosphorylation assays from Pi:

1. Prepare a mix containing 1 mg mL^{-1} of *L. lactis* membranes, metals, or chelators (*see* **Note 32**) in Pi phosphorylation buffer to a final volume of 90 µL.
2. Incubate the mix 5 min at 30 °C.
3. Start the reaction by addition of 10 µL of 1 mM ^{32}Pi (100 µM final). Vortex the suspension and incubate the reaction mix at 30 °C for 10 min.
4. Stop the reaction by addition of 1 mL ice-cold stop buffer. Vortex the suspension and incubate 30 min on ice.
5. Then proceed as described in **steps 4–12** for phosphorylation assays from ATP (Fig.4b).

3.4.3 Transport Assays with Whole Cells Expressing AtAATP1/NTT1

The nucleotide transporter 1, AtAATP1/NTT1, a highly hydrophobic membrane protein with 12 predicted transmembrane domains [34], is localized within the inner membrane of the chloroplast envelope [35]. This translocator imports ATP in exchange of ADP and Pi [36] in contrast to mitochondrial ATP/ADP translocators [37]. It supplies energy to chloroplasts used by storage plastids required for starch synthesis and to allow nocturnal anabolic reactions in chloroplasts [38].

1. Centrifuge 4350 × *g*, 10 min twice and resuspend recombinant bacteria into 30 mL to a final concentration of 100 mg mL^{-1} (3 mg/30 µL) in ice-cold 50 mM potassium phosphate buffer pH 7.0.
2. Add 50 µM [α-^{32}P]ATP (3000 mCi $mmol^{-1}$; Perkin Elmer) diluted in ice-cold 50 mM potassium phosphate buffer pH 7.0 to each sample (*see* **Note 36**).
3. Incubate at 25 °C for planned time periods and stop nucleotide uptake by addition of 1 mL of ice-cold potassium phosphate buffer.
4. Filtrate the cells through a 0.45 µm filter under vacuum.
5. Wash three times with 1 mL of ice-cold potassium phosphate buffer.
6. Transfer filter to a scintillation vial and add 3.5 mL of d.d. water
7. Measure the radioactivity retained on the filters in a scintillation counter.
8. Generate graphs using the KaleidaGraph version 4.02 (Synergy Software) and fit experimental data with the appropriate curve (i.e., single exponential) (Fig. 5).

3.4.4 High-Throughput Transport Assays of Mitochondrial Carriers

Mitochondrial carriers link the biochemical pathways of the cytosol and the mitochondrion matrix by transporting metabolites, nucleotides, inorganic ions, and cofactors across the mitochondrial inner

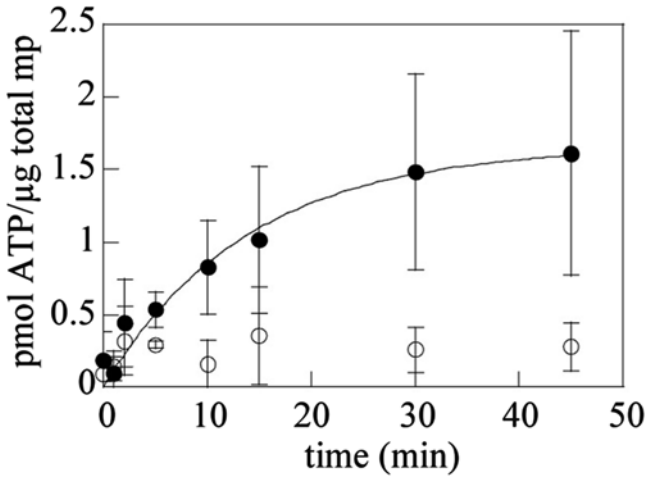


Fig. 5 Functional characterization of the nucleotide translocator AtAATP1/NTT1. *L. lactis* cells expressing AtAATP1/NTT1 (*closed circles*) or *L. lactis* cells containing the empty vector (as a negative control, *open circles*) were incubated with 50 mM [α-32P] ATP for the indicated time periods. Data are represented by the mean and standard deviation of four independent experiments. Adapted from [10]

membrane [39, 40]. The topology is pseudo-symmetric threefold, consisting of a barrel of six transmembrane α-helices with three short α-helices in the matrix loops [41]. Mitochondrial carriers function as monomers [42], but the aspartate/glutamate carrier was found to be dimeric in its resolved structure [18]. A single substrate-binding site has been identified in the central part of the cavity [43], which is located between two salt bridge networks on the matrix and cytoplasmic side of the carriers [44]. The opening and closing of the carrier could be coupled to the disruption and formation of the two salt bridge networks, changing the accessibility of the central substrate to either side of the membrane in an alternative way [44, 45]. Many mitochondrial carriers have not yet been identified and many aspects of their transport mechanism are unsolved. Therefore, it is important to develop transport assays that allow their characterization in high-throughput experiments.

Transport assays can be performed either with washed whole lactococcal cells when *L. lactis* has an endogenous pool of substrates, e.g., adenosine di- and tri-phosphate nucleotides or with fused membrane vesicles. For the latter, membranes are isolated, fused with liposomes by freeze thawing, and extruded in the presence of internal substrate to create single membrane vesicles, after which the external substrate is removed by gel filtration. In both cases transport assays are initiated by the addition of radiolabeled substrate. The automated procedures are illustrated with the characterization of the mitochondrial ADP/ATP carrier from *Myceliophthora thermophila*, a thermophilic fungus.

For transport assays on whole cells, snap-thaw the cell pellets (*see* Subheading 3.1) by gentle pipetting whilst the falcon tube is immersed in warm water (*see* **Note 37**) and adjust the OD_{600nm} to approximately 25.

For transport assay on ***fused membrane vesicles***, liposomes have to be prepared prior to the fusions with *L. lactis* membranes isolated (*see* Subheading 3.2) to form fused membrane vesicles.

1. Allow the frozen lipids to defrost at room temperature.
2. Prepare liposomes in a 3:1 weight ratio of *E. coli* polar lipid extract to egg yolk phosphatidylcholine; dry chloroform under a stream of nitrogen, ensuring all the chloroform to be evaporated.
3. Add 5 mL diethyl ether, vortex, and evaporate under a stream of nitrogen.
4. Vortex the lipids for 3 h in PIPES buffer to get a 20 mg mL^{-1} stock and homogenize.
5. Aliquot, flash-freeze, and store in liquid nitrogen.
6. Use 5 mg liposomes (250 μL of 20 mg mL^{-1} stock) and 1 mg of isolated *L. lactis* membranes (*see* Subheading 3.2); dilute to 1 mL with PIPES buffer.
7. Fuse the membranes by 6 cycles of snap-freezing in liquid nitrogen, and thawing to room temperature for 45 min; the fused membranes can be stored in liquid nitrogen.
8. Thaw the membranes, and add 50 μL of a 10× stock of substrate/inhibitor to 450 μL fused membranes for a final volume of 500 μL.
9. Equilibrate a 1 μm polycarbonate filter in PIPES.
10. Sandwich two supports (four needed in total) on either side of filter, assemble the extruders and push through some buffer to remove any air pockets and extrude each sample 11 times at room temperature (*see* **Note 38**); keep samples on ice before and after extrusion.
11. Prepare the PD-10 column; equilibrate with buffer as per instructions.
12. Gently add 400 μL post-extrusion vesicles onto each column; let the vesicles absorb into the column, add a further 2.1 mL buffer, and discard the flow-through.
13. Let the column run dry, add 1.6 mL of buffer, and collect the sample in a 2 mL Eppendorf tube.
14. Dilute each sample fourfold before use (1.5 mL into a final volume of 6 mL PIPES, which will give approximately 5 μg protein per well if using 100 μL vesicles, and enough protein for quadruple trials with 11 time points per trials); keep remaining undiluted sample for BCA protein determination.

High-throughput transport assays can be carried out using a Hamilton MicroLab Star robot. The first five steps are programmed to be carried out by the robot in 96-well format, allowing eight different uptake experiments to be performed simultaneously.

1. Pipet 100 μL bacteria ($OD_{600nm} = 25$) into the wells of a 96-well MultiScreen$_{HTS}$-Hi Flow-FB plate (pore size = 1 μm) or fused membrane vesicles (5 μg) into a 96-well MultiScreen$_{HTS}$-HA plate (pore size = 0.45 μm), while the plate is placed on a vacuum manifold.
2. Initiate transport with the addition of 100 μL PIPES buffer containing 1.5 μM of ^{14}C-labeled nucleotide.
3. Incubate at room temperature for planned time periods, the longer time points being added first.
4. Stop transport by filtration followed immediately by the addition of 200 μL of ice-cold PIPES buffer to all wells.
5. Wash wells two times with 200 μL ice-cold PIPES buffer.
6. Leave the plates to dry overnight.
7. Stick black backing paper on the underside of the filter plate.
8. Add 200 μL of MicroScint-20 to each well using the robot.
9. Stick clear backing paper on the topside of the filter plate; stand for at least 4 h to allow filter dissolution.
10. Load the plates into the TopCount Scintillation Counter. Initial rates are determined from the linear part of the uptake curves (first 60 s) (*see* Fig. 6).

4 Notes

1. Nisin can be either commercial or produced by the nisin-producing strain NZ9700. We have noticed that homemade nisin gave rise to higher amounts of proteins compared to commercial one [21].
2. Recombinant bacteria are generated through transformation with an expression vector containing the gene of interest; in our studies, we use pNZ8148.
3. The concentration of antibiotic (chloramphenicol) could vary from 5 to 10 μg mL^{-1}.
4. The buffers for bacterial and protein resuspension have to be adjusted to the protein of interest (50 mM Tris–HCl pH 8.0 for ceQORH [10] and 20 mM HEPES pH 6.0 for AtHMA6 and AtHMA8 [33] containing 6 or 20% glycerol for protein resuspension respectively; 50 mM potassium phosphate buffer pH 7.0 for AtNTT1 [10]; 10 mM PIPES pH 7.0, 50 mM NaCl for mitochondrial carriers [3]).

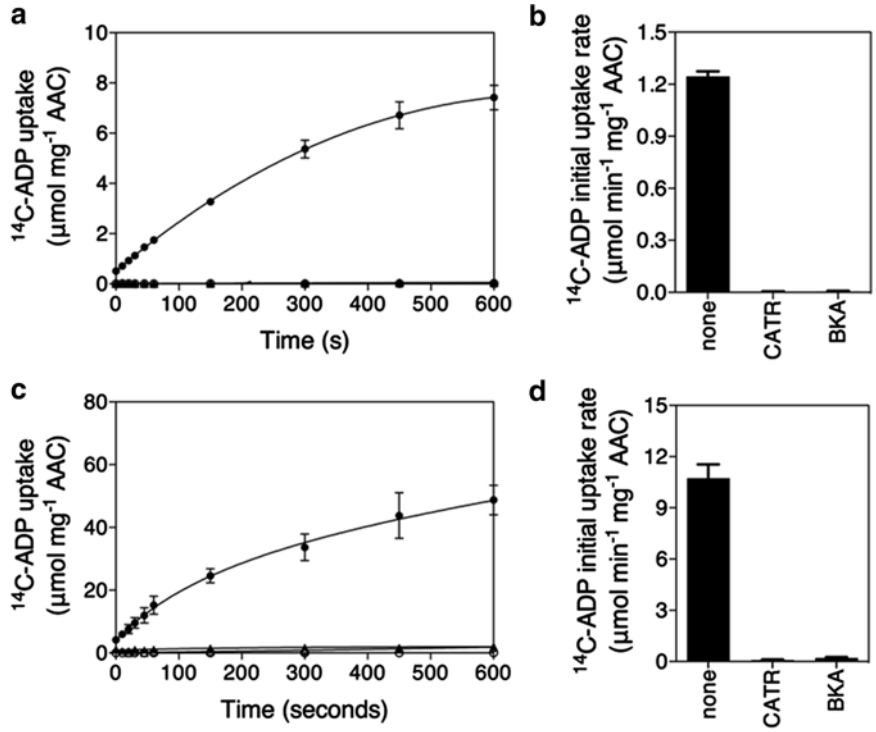


Fig. 6 Functional characterization of the mitochondrial ADP/ATP carrier from *Myceliophthora thermophile* (MtAAC) expressed in *L. lactis* by high-throughput transport assays. (**a**) Whole-cell uptake assays using *L. lactis* expressing MtAAC in the absence (*closed circles*) or presence of the specific inhibitors carboxyatractyloside (CATR; *closed triangles*) or bongkrekic acid (BKA; *open triangles*), both at 20 µM. The control shown is vector without the insert encoding MtAAC (*empty circles*). (**b**) Specific initial rates of ^{14}C-ADP uptake on whole cell over the first 60 s in the absence (none) or the presence of inhibitors (CATR or BKA), background subtracted. (**c**) Transport assays with fused membrane vesicles of *L. lactis* expressing MtAAC preloaded with 5 mM ADP in the absence (*open circles*) or presence of CATR (*closed triangles*) or BKA (*open triangles*). As control, transport was carried out with vesicles expressing MtAAC, but not loaded with internal substrate (*open circles*). (**d**) Specific initial of ^{14}C-ADP uptake on fused membrane vesicles over 60 s in the absence (none) or in the presence of inhibitors (CATR or BKA), background subtracted. Transport was initiated with the external addition of 1.5 µM ^{14}C-ADP. The *error bars* represent the standard deviation of four assays

5. One Shot and other disruption systems from Constant Cell Disruption System are the most suitable to disrupt the lactococcal cell wall with a yield in crude membranes improved by more than fivefold when compared to that obtained by lysozyme and French Press treatment [10].
6. The percentage of acrylamide in the resolving gel has to be adapted to the molecular weight of the protein of interest.
7. Western blot analysis can be performed using either protein-specific antibody or conjugate specific to the affinity tag.
8. HEPES buffer is preferentially used since it has low affinity for metals. $MgCl_2$ is required to favor Mg-ATP as substrate.

9. KCl is prohibited in the Pi phosphorylation buffer, since it accelerates the dephosphorylation rate.
10. According to the ionic specificity of the P_{IB}-ATPases, various metal solutions can be used (i.e., $CuSO_4$/$CuCl_2$, $AgNO_3$/AgCl, $ZnSO_4$, $MnSO_4$, or $NiCl_2$). Usually, different dilutions are prepared to perform the phosphorylation tests in the presence of metal concentrations ranging from 0.05 to 100 μM. To test Cu in its monovalent form Cu^+, a reducing agent has to be added in the reaction mix. Preferentially use Na_2SO_3 at 500 μM. Note that the pH of the solution can change the speciation of the metal (*see* Pourbaix diagrams) and thus its chemical properties (i.e., its solubility and then its interaction with the ATPase).
11. For phosphorylation assays on P_{IB}-ATPase well expressed in *Lactococcus* membranes, half amount of radiolabeled ATP can be used (i.e., 0.5 μL in 110 μL of unlabeled 10 μM ATP).
12. DTT is sensitive to oxidation and can then lose its reducing power within prolonged exposure to the air. This may result in an insufficient denaturation of the sample.
13. Metal chelators are used as controls. BCA/BCS are usually used as monovalent metal chelators and Cu chelators, whereas EGTA is used as a broad range divalent metal chelator. These chelators are therefore used to inhibit the catalytic cycle of ATPases. BCA and BCS are prepared in H_2O at a concentration of 1 mM and 100 μM respectively, stored in small aliquots at −20 °C.
14. The mix Acrylamide/Bis 24/0.8 % is prepared from two independent solutions: 60 mL of 40 % [w/v] Acrylamide and 40 mL of 2 % [w/v] Bis-acrylamide. Premixed solutions of acrylamide/Bis-acrylamide can also be used.
15. The pH value of the gel and the running buffer must be lower than 7 to avoid the hydrolysis of the aspartyl-phosphate bound.
16. Instead of using phosphorimaging device, it is possible to use autoradiography films, exposed in common film cassettes and then revealed using developer and fixer solutions.
17. The mitochondrial ADP/ATP carriers exchange ADP against ATP and they are inhibited by carboxyatractyloside (CATR) and bongkrekic acid (BKA) [46].
18. Shaking with a gentle rotation (90 rpm) avoids the sedimentation of bacteria at the bottom of the bottle.
19. The M17 medium could be prewarmed at 30 °C overnight. This step allows gaining time to reach the correct OD. Nevertheless, it might be better to add glucose and antibiotics on the day of culture because of the instability of antibiotic at 30 °C, and to check if the medium is not contaminated.

20. Cultures can be inoculated with a larger volume of precultures ($1/20^{e}$ to get an OD600 around 0.1). This can help to shorten the time to get the appropriate OD for induction.
21. The dilution of nisin to add for induction (0.0001–0.005) has to be optimized in order to get higher amounts of proteins produced and must be determined for every new preparation of nisin [47].
22. Several values for OD_{600nm} have been reported for induction of protein expression: from 0.5 for mitochondrial carriers [2] to 0.8 for plant membrane proteins [10].
23. The induction time needs to be optimized for each protein of interest. For the proteins analyzed here, an induction time of 2.5 and 4 h allowed production of sufficient amounts of protein for analyses of mitochondrial carriers and plant membranes proteins respectively.
24. Heating of the samples for 5 min at 95 °C is not necessary. Solubilization also works with an incubation of the samples in the sample buffer for 5 min at room temperature.
25. The migration parameters (voltage/time) have to be adapted to the protein of interest.
26. Membrane proteins either peripherally or intrinsically associated with membranes have to be solubilized to become soluble in aqueous solution before purification steps. The ceQORH protein is a peripheral protein which interacts with the inner envelope membrane of chloroplast through electrostatic interactions. This kind of protein can be solubilized with mild treatments using high salt-containing solution [24].
27. For more information concerning the purification of 6× Histidine-tag proteins, refer to the handbook of IBA (IBA, Goettingen, Germany).
28. Here we used a 6× Histidine-tag for the detection and purification of the recombinant proteins (this tag was fused to the *N*-terminal part of the protein). However, other affinity tags can be used like the *Strep*-tag II [10].
29. Dehydrogenase assays are performed with NBT chloride as an artificial substrate, and the reaction (NBT reduction to formazan) is monitored using a spectrophotometer. An absorption coefficient of 15,000 M^{-1} cm^{-1} for the formazan product at 560 nm is used to calculate the initial velocity [48]. Alternatively, NADPH oxidation can be recorded in the initial linear phase of decay of absorbance at 340 nm using a molar extinction coefficient of 6250 M^{-1} cm^{-1}.
30. The specific activity determined for the purified ceQORH protein was 19.3 nmoles of formazan per mg of ceQORH protein. min^{-1}. Note that the specific activity of the ceQORH protein

produced in *E. coli* was found 2.5 times lower than that of the ceQORH protein produced in *L. lactis* [10].

31. AtHMA6 and AtHMA8 belong to the family of P-type ATPases, that are multispanning membrane proteins that translocate ions across plasma or organelle membranes at the expense of ATP consumption [49]. The catalytic cycle of P-ATPases can be schematically reduced to a four-step process accounting for the coupling of ion motion to ATP hydrolysis, involving the formation of transient phosphorylated states initiated by ATP γ-phosphate transfer to the conserved aspartic residue of the DKTGT motif. In its free state (E), the P-ATPase binds cytoplasmic ions at its high affinity membrane site (**step 1**). ATP, bound to the large cytoplasmic domain of the transporter, is then hydrolyzed, leading to the formation of a phosphorylated enzyme (**step 2**). The ion bound phosphorylated form of the enzyme (Me.E ~ P) undergoes important conformation changes leading to metal release at the extracytoplasmic side of the membrane (**step 3**). In the metal-free phosphorylated enzyme E-P, the aspartyl-phosphate bound is then hydrolyzed to bring the enzyme back to its free state E (**step 4**). In many cases, ATPases can be phosphorylated from ATP in the forward direction (physiological condition; **steps 1–4** of the cycle) and from Pi in the backward direction (**step 4** only). Both reactions rely on the presence of the ion to be transported. Whereas phosphorylation from ATP only occurs on the ion(s) bound form of the transporter, transported ion(s) do competitively inhibit phosphorylation from Pi. Hence, the latter also gives access to the ionic specificity of the enzyme. In addition, involving simple equilibrium reaction (**step 4**), phosphorylation from Pi also allows to determine more precisely the apparent affinity of the transporter to the transported ion.
32. Usually it is better to test a wide range of metal concentration from 0.05 to 100 μM (concentration of the metal in the assay, i.e., in 100 μL). If needed in the assay, chelators are added to a final concentration of 1 mM (EGTA) and 1 mM/100 μM (BCA/BCS mix).
33. Depending on the activity of the studied P_{IB}-ATPase, the reaction time can vary. The incubation can also be performed at 4 °C to slow down the reaction.
34. The supernatant highly radioactive has to be removed carefully.
35. The use of acidic condition for the SDS-PAGE analysis is essential to avoid the hydrolysis of the aspartyl-phosphate bound and to detect the phosphorylated intermediates.
36. Each condition has to be tested in triplicate experiments at minimum.

37. Quickly thawing the pellets is vital for transport activity.
38. The syringes are not airtight at 4 °C for prolonged periods of time and will leak.

Acknowledgments

This study received financial support from the Commissariat à l'Energie Atomique et aux Energies Alternatives (CEA), the Centre National de la Recherche Scientifique (CNRS), the French National Institute for Agricultural Research (INRA), the University Joseph Fourier (Grenoble), and the Medical Research Council UK. E.S. was funded by a joint grant from the GRAL Labex (Grenoble Alliance for Integrated Structural Cell Biology: ANR-10-LABEX-04) and the CEA.

References

1. Bakari S, André F, Seigneurin-Berny D, Delaforge M, Rolland N, Frelet-Barrand A (2014) Lactococcus lactis, recent developments in functional expression of membrane proteins. In: Mus-Veteau I (ed) Membrane proteins production for structural analysis. Springer, New-York, USA, pp 107–132, Chapter 5
2. Kunji ERS, Slotboom DJ, Poolman B (2003) Lactococcus lactis as host for overproduction of functional membrane proteins. Biochim Biophys Acta 1610:97–108
3. Kunji ERS, Chan KW, Slotboom DJ, Floyd S, O'Connor R, Monné M (2005) Eukaryotic membrane protein overproduction in Lactococcus lactis. Curr Opin Biotechnol 16:546–551
4. Monné M, Chan KW, Slotboom DJ, Kunji ERS (2005) Functional expression of eukaryotic membrane proteins in Lactococcus lactis. Protein Sci 14:3048–3056
5. Bernaudat F, Frelet-Barrand A, Pochon N, Dementin S, Hivin P, Boutigny S, Rioux JB, Salvi D, Seigneurin-Berny D, Richaud P, Joyard J, Pignol D, Sabaty M, Desnos T, Pebay-Peyroula E, Darrouzet E, Vernet T, Rolland N (2011) Heterologous expression of membrane proteins: choosing the appropriate host. PLoS One 6, e29191
6. Herzig S, Raemy E, Montessuit S, Veuthey JL, Zamboni N, Westermann B, Kunji ERS, Martinou JC (2012) Identification and functional expression of the mitochondrial pyruvate carrier. Science 337:93–96
7. Gasson MJ, de Vos WM (eds) (1994) Genetics and biotechnology of lactic acid bacteria. Blackie Academic and Professional, London, United Kingdom
8. Morello E, Bermúdez-Humarán LG, Llull D, Solé V, Miraglio N, Langella P, Poquet I (2008) Lactococcus lactis, an efficient cell factory for recombinant protein production and secretion. J Mol Microbiol Biotechnol 14:48–58
9. Geertsma ER, Poolman B (2007) High-throughput cloning and expression in recalcitrant bacteria. Nat Methods 4:705–707
10. Frelet-Barrand A, Boutigny S, Moyet L, Deniaud A, Seigneurin-Berny D, Salvi D, Bernaudat F, Richaud P, Pebay-Peyroula E, Joyard J, Rolland N (2010) Lactococcus lactis, an alternative system for functional expression of peripheral and intrinsic Arabidopsis membrane proteins. PLoS One 5, e8746
11. Pontes DS, de Azevedo MS, Chatel JM, Langella P, Azevedo V, Miyoshi A (2011) Lactococcus lactis as a live vector: heterologous protein production and DNA delivery systems. Protein Expr Purif 79:165–175
12. Mierau I, Kleerebezem M (2005) 10 years of the nisin-controlled gene expression system (NICE) in Lactococcus lactis. Appl Microbiol Biotechnol 68:705–717
13. de Ruyter PG, Kuipers OP, de Vos WM (1996) Controlled gene expression systems for Lactococcus lactis with the food-grade inducer nisin. Appl Environ Microbiol 62: 3662–3667

14. Zhou XX, Li WF, Ma GX, Pan YJ (2006) The nisin-controlled gene expression system: construction, application and improvements. Biotechnol Adv 24:285–295
15. Erkens GB, Berntsson RP, Fulyani F, Majsnerowska M, Vujičić-Žagar A, Ter Beek J, Poolman B, Slotboom DJ (2011) The structural basis of modularity in ECF-type ABC transporters. Nat Struct Mol Biol 18:755–760
16. Berntsson RP, ter Beek J, Majsnerowska M, Duurkens RH, Puri P, Poolman B, Slotboom DJ (2012) Structural divergence of paralogous S components from ECF-type ABC transporters. Proc Natl Acad Sci U S A 109:13990–13995
17. Malinauskaite L, Quick M, Reinhard L, Lyons JA, Yano H, Javitch JA, Nissen P (2014) A mechanism for intracellular release of Na+ by neurotransmitter/sodium symporters. Nat Struct Mol Biol 21:1006–1012
18. Thangaratnarajah C, Ruprecht JJ, Kunji ERS (2014) Calcium-induced conformational changes of the regulatory domain of human mitochondrial aspartate/glutamate carriers. Nat Commun 5:5491
19. Harborne SP, Ruprecht JJ, Kunji ERS (2015) Calcium-induced conformational changes in the regulatory domain of the human mitochondrial ATP-Mg/Pi carrier. Biochim Biophys Acta 1847:1245–1253
20. Frelet-Barrand A, Boutigny S, Kunji ER, Rolland N (2010) Membrane protein expression in Lactococcus lactis. Methods Mol Biol 601:67–85
21. Boutigny S, Sautron E, Frelet-Barrand A, Moyet L, Salvi D, Rolland N, Seigneurin-Berny D (2015) Functional expression of plant membrane proteins in Lactococcus lactis. Methods Mol Biol 1258:147–165
22. King MS, Boes C, Kunji ERS (2015) Membrane protein expression in Lactococcus lactis. Methods Enzymol 556:77–97
23. Sautron E, Mayerhofer H, Giustini C, Pro D, Crouzy S, Ravaud S, Pebay-Peyroula E, Rolland N, Catty P, Seigneurin-Berny D (2015) HMA6 and HMA8 are two chloroplast Cu+–ATPases with different enzymatic properties. Biosci Rep 35(3): pii:e00201
24. Miras S, Salvi D, Ferro M, Grunwald D, Garin J, Joyard J, Rolland N (2002) Non-canonical transit peptide for import into the chloroplast. J Biol Chem 277:47770–47778
25. Miras S, Salvi D, Piette L, Seigneurin-Berny D, Grunwald D, Reinbothe C, Joyard J, Reinbothe S, Rolland N (2007) Toc159- and Toc75-independent import of a transit sequence-less precursor into the inner envelope of chloroplasts. J Biol Chem 282:29482–29492
26. Chang W, Soll J, Bolter B (2014) A new member of the psToc159 family contributes to distinct protein targeting pathways in pea chloroplasts. Front Plant Sci 5:239
27. Jager-Vottero P, Dorne AJ, Jordanov J, Douce R, Joyard J (1997) Redox chains in chloroplast envelope membranes: spectroscopic evidence for the presence of electron carriers, including iron-sulfur centers. Proc Natl Acad Sci U S A 94:1597–1602
28. Rosenzweig AC, Arguello JM (2012) Toward a molecular understanding of metal transport by P(1B)-type ATPases. Curr Top Membr 69: 113–136
29. Arguello JM (2003) Identification of ion-selectivity determinants in heavy-metal transport P1B-type ATPases. J Membr Biol 195: 93–108
30. Gourdon P, Liu XY, Skjorringe T, Morth JP, Moller LB, Pedersen BP, Nissen P (2011) Crystal structure of a copper-transporting PIB-type ATPase. Nature 475:59–64
31. Shikanai T, Muller-Moule P, Munekage Y, Niyogi KK, Pilon M (2003) PAA1, a P-type ATPase of Arabidopsis, functions in copper transport in chloroplasts. Plant Cell 15: 1333–1346
32. Abdel-Ghany SE, Muller-Moule P, Niyogi KK, Pilon M, Shikanai T (2005) Two P-type ATPases are required for copper delivery in Arabidopsis thaliana chloroplasts. Plant Cell 17:1233–1251
33. Catty P, Boutigny S, Miras R, Joyard J, Rolland N, Seigneurin-Berny D (2011) Biochemical characterization of AtHMA6/PAA1, a chloroplast envelope Cu(I)-ATPase. J Biol Chem 286:36188–36197
34. Kampfenkel K, Möhlmann T, Batz O, Van Montagu M, Inze D, Neuhaus HE (1995) Molecular characterization of an Arabidopsis thaliana cDNA encoding a novel putative adenylate translocator of higher plants. FEBS Lett 374:351–355
35. Neuhaus HE, Thom E, Möhlmann T, Steup M, Kampfenkel K (1997) Characterization of a novel eukaryotic ATP/ADP translocator located in the plastid envelope of Arabidopsis thaliana L. Plant J 11:73–82
36. Trentmann O, Jung B, Neuhaus HE, Haferkamp I (2008) Nonmitochondrial ATP/ADP transporters accept phosphate as third substrate. J Biol Chem 283:36486–36493
37. Haferkamp I, Fernie AR, Neuhaus HE (2011) Adenine nucleotide transport in plants: much more than a mitochondrial issue. Trends Plant Sci 16:507–515
38. Tjaden J, Mohlmann T, Kampfenkel K, Henrichs G, Neuhaus HE (1998) Altered plas-

tidic ATP/ADP-transporter activity influences potato (Solanum tuberosum L.) tuber morphology, yield and composition of tuber starch. Plant J 16:531–540

39. Kunji ERS (2012) Structural and mechanistic aspects of mitochondrial transport proteins. In: Ferguson S (eds), Comprehensive Biophysics, Elsevier. pp 174–205
40. Palmieri F (2014) Mitochondrial transporters of the SLC25 family and associated diseases: a review. J Inherit Metab Dis 37:565–575
41. Pebay-Peyroula E, Dahout-Gonzalez C, Kahn R, Trezeguet V, Lauquin GJ, Brandolin G (2003) Structure of mitochondrial ADP/ATP carrier in complex with carboxyatractyloside. Nature 426:39–44
42. Kunji ERS, Crichton PG (2010) Mitochondrial carriers function as monomers. Biochim Biophys Acta 1797:817–831
43. Robinson AJ, Kunji ERS (2006) Mitochondrial carriers in the cytoplasmic state have a common substrate binding site. Proc Natl Acad Sci U S A 103:2617–2622
44. Robinson AJ, Overy C, Kunji ERS (2008) The mechanism of transport by mitochondrial carriers based on analysis of symmetry. Proc Natl Acad Sci U S A 105:17766–17771
45. Ruprecht JJ, Hellawell AM, Harding M, Crichton PG, Mccoy AJ, Kunji ERS (2014) Structures of yeast mitochondrial ADP/ATP carriers support a domain-based alternating-access transport mechanism. Proc Natl Acad Sci U S A 111:E426–E434
46. Klingenberg M (2008) The ADP and ATP transport in mitochondria and its carrier. Biochim Biophys Acta 1778:1978–2021
47. Mierau I, Leij P, van Swam I, Blommestein B, Floris E, Mond J, Smid EJ (2005) Industrial-scale production and purification of a heterologous protein in Lactococcus lactis using the nisin controlled gene expression system NICE: the case of lysostaphin. Microb Cell Fact 4:15
48. Hillar A, Loewen PC (1995) Comparison of isoniazid oxidation catalyzed by bacterial catalase-peroxidases and horseradish peroxidase. Arch Biochem Biophys 323:438–446
49. Kuhlbrandt W (2004) Biology, structure and mechanism of P-type ATPases. Nat Rev Mol Cell Biol 5:282–295