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Dear Dr. DSousa:
Thank you for your patience and your efforts to make this protocol as useful as possible to your readers. We have significantly expanded the section on the operation of the French Press. There are videos available and we have used those as guidelines to create a story line for this manuscript that is also unique.
Sincerely

Paul

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TITLE:

Characterization of Membrane Transporters by Heterologous Expression in *E. Coli* and Production of Membrane Vesicles

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KEYWORDS:

Membrane transporter, antiporter, polyamine transport, polyamine exchanger, membrane vesicles, French press, kinetic analysis, radioisotope transport assays, GABA

SUMMARY:

We describe a method for the characterization of proton-driven membrane transporters in membrane vesicle preparations produced by heterologous expression in *E. coli* and lysis of cells using a French press.

ABSTRACT:

Several methods have been developed to functionally characterize novel membrane transporters. Polyamines are ubiquitous in all organisms, but polyamine exchangers in plants have not been identified. Here, we outline a method to characterize polyamine antiporters using membrane vesicles generated from the lysis of *Escherichia coli* cells heterologously expressing a plant antiporter. First, we heterologously expressed *AtBAT1* in an *E. coli* strain deficient in polyamine and arginine exchange transporters. Vesicles were produced using a French press, purified by ultracentrifugation and utilized in a membrane filtration assay of labeled substrates to demonstrate the substrate specificity of the transporter. These assays demonstrated that *AtBAT1* is a proton-mediated transporter of arginine, γ-aminobutyric acid (GABA), putrescine and spermidine. The mutant strain that was developed for the assay of *AtBAT1* may be useful for the functional analysis of other families of plant and animal polyamine exchangers. We also hypothesize that this approach can be used to characterize many other types of antiporters, as long as these proteins can be expressed in the bacterial cell membrane. *E. coli* is a good system for the characterization of novel transporters, since there are multiple methods that can be employed to mutagenize native transporters.

INTRODUCTION:

Proteins involved in the trafficking of metabolites constitute an essential level of physiological regulation, but the vast majority of plant membrane transporters have not yet been functionally characterized. Several strategies have been implemented to characterize novel transport proteins. Heterologous expression in model organisms such as *E. coli* and eukaryotic cells such as yeast, *Xenopus* oocytes, mammalian cells, insect cells and plant cells have all been used to determine their transport activity[1]. Eukaryotic cells are favored for the expression of eukaryotic proteins, because the basic cellular composition, signal transducing pathways, transcription and translation machineries are compatible with the native conditions.

Yeast has been an important model organism for the characterization of novel transport proteins in plants. The first plant transport protein that was successfully expressed in yeast (*Saccharomyces pombe*) was the hexose transporter HUP1 from Chlorella[2]. Since then, many plant transport proteins have been functionally characterized using a yeast expression system. These include, plant sugar transporters (SUC1 and SUC2[3], VfSUT1 and VfSTP1[4]) and the auxin transporters (AUX1 and PIN[5]). Disadvantages of utilizing yeast to express plant proteins can include impaired activity of plastid-localized proteins because yeast lacks this organelle, mistargeting[6], and formation of misfolded aggregates and activation of stress responses in yeast due to overexpression of membrane proteins[7-9].

Heterologous expression of transport proteins in *Xenopus* oocytes have been widely used for the electrophysiological characterization of transporters[10]. The first plant transport proteins characterized using heterologous expression in *Xenopus* oocytes were the Arabidopsis potassium channel KAT1[10] and the Arabidopsis hexose transporter STP1[11]. Since then, *Xenopus* oocytes have been employed to characterize many plant transport proteins such as plasma membrane transporters[12], vacuolar sucrose transporter SUT4[13] and vacuolar malate transporter ALMT9[14]. An important limitation of *Xenopus* oocytes for transport assays is that the concentration of intracellular metabolites cannot be manipulated[1]. Moreover, professional knowledge is required to prepare *Xenopus* oocytes and the variability of the oocyte batches is difficult to control.

Heterologous expression in the model organism *E. coli* is an ideal system in terms of characterization of novel plant transport proteins. With a fully sequenced genome[15], the molecular and physiological characteristics of *E. coli* are well known. Molecular tools and techniques are well established[16]. In addition, different expression vectors, non-pathogenic strains and mutants are available[17-19]. Furthermore, *E. coli* has a high growth rate and can be easily grown under laboratory conditions. Many proteins can be easily expressed and purified at high amounts in *E. coli*[9]. When proteins cannot be assayed directly in cellular systems, reconstitution of proteins into liposomes has also been a successful, albeit challenging innovation for the characterization of purified membrane proteins. Functional characterization of the plant mitochondrial transport proteins including solute transporters such as phosphate transporters in soybean, maize, rice and Arabidopsis, dicarboxylate-tricarboxylate carrier in Arabidopsis have been accomplished by using this model system[20,21]. However, recombinant proteins of the tomato protein SICAT9 were found to be nonfunctional in reconstitution experiments, and other members of the CAT transporter family were found to be nonfunctional in *Xenopus* oocyte

assays[22]. Thus, additional molecular tools are needed for the characterization of membrane transporters.

Five polyamine transport systems are found in *E. coli*[23]. They include two ABC transporters mediating the uptake of spermidine and putrescine, a putrescine/ornithine exchanger, a cadaverine/lysine exchanger, a spermidine exporter and a putrescine importer. The putrescine exchanger PotE was originally characterized using a vesicle assay, where inside out vesicles were prepared by lysing cells with a French press and measuring the uptake of radiolabeled putrescine into the vesicles in exchange for ornithine[24]. Vesicle assays were also used to characterize a calcium transporter, which mediated the transport of calcium in response to a proton gradient[25]. These experiments prompted us to develop a strategy for the characterization of other polyamine exchangers. We first created a strain of *E. coli* deficient in *PotE* and *CadB* exchangers. Here, we demonstrate the functional characterization of a plant polyamine antiporter by heterlogous expression in the modified *E. coli* strain, generation of membrane vesicles using a French press, and radiolabeled assays.

PROTOCOL:

1. Generation of the *E. coli* double knock out mutant with P1 transduction

1.1. Obtain the *E. coli* single-knockout mutant strains *ΔPotE* and *ΔCadB* from the *E. coli* Genetic Stock Center (http://cgsc.biology.yale.edu).

NOTE: The *ΔPotE* strain is kanamycin resistant[26] and the *ΔCadB* strain is tetracycline resistant[27].

1.2. Construct the *PotE/CadB* double knockout (DKO) strain using the P1 transduction protocol[28].

1.2.1. Lysate preparation

1.2.1.1. Dilute an overnight culture of *ΔPotE* in fresh LB (1:100) supplemented with 10–25 mM $MgCl_2$, 5 mM $CaCl_2$, and 0.1-0.2% glucose.

1.2.1.2. Grow at 37 ˚C for 1-2 h.

1.2.1.3. Add 40 µL of P1 phage lysate to the culture, and continue growing at 37 ˚C. Monitor for 1–3 h until the culture has lysed

NOTE: When the culture is lysed, cellular debris will be visible in the tube and the culture will have a significant loss of turbidity.

1.2.1.4. Add several drops of chloroform (50-100 µL) to the lysate and vortex. Centrifuge at 12,000 x *g* for 2 min to remove cellular debris and transfer the supernatant to a fresh tube.

1.2.2. Transduction

1.2.2.1. Grow *ΔCadB* strain overnight in LB medium.

1.2.2.2. Harvest the cells by centrifugation at 4,000 x *g* for 2 min and resuspend in 1/5-1/3 the harvested culture volume in fresh LB supplemented with 100 mM $MgSO_4$ and 5 mM $CaCl_2$.

1.2.2.3. Add the *ΔCadB* cell suspension to the tube with phage from step 1.2.1.4 and mix rapidly.

1.2.2.4. Add 200 µL of 1 M Na-Citrate (pH5.5), then add 1 mL of LB. Incubate at 37 °C for 1 h to allow the expression of the antibiotic resistant marker.

1.2.2.5. Spin cells at 4000 x *g* for 5 min.

1.2.2.6. Resuspend in 100 µL LB supplemeted with 100 mM Na-Citrate (pH 5.5) and vortex well to disperse cells.

1.2.2.7. Select the recombinant strains on LB agar plates with 50 µg/mL kanamycin and 10 µg/mL tetracycline and then confirm by Polymerase Chain Reaction (PCR) with appropriate primers[26,27].

1.2.2.8. Verify the PCR fragments by sequencing.

2. Expression of the target gene (AtBAT1) in *E. coli* mutant

2.1. Amplify the full-length sequence of the target gene by PCR and insert into the Gateway entry vector pENTR/D-TOPO[29].

NOTE: In this case ATBAT1.1 was amplified using forward primer 5'CGGCGATCAATCCTTTGTT and reverse primer 5'GCTAAGAATGTTGGAGATGG, an initial denaturation at 98 °C for 2 min and then 30 cycles of denaturation at 98 °C for 0.1 min, annealing at 59 °C for 0.3 min, extension at 72 °C for 0.40 min and final extension at 72 °C for 10 min. The PCR product was purified using a PCR cleanup kit, quantified using a spectrometer. Insertion of the PCR product into the Gateway vector was done following the manufactrurers directions.

2.1.1. Transform the entry vector into competent Top10 *E. coli* cells by heatshock and select for successful recombinants on LB media with 50 µg/mL kanamycin following standard molecular cloning protocols[30].

2.1.2. Extract plasmid DNA using a plasmid extraction kit, following the manufacturer's directions, and sequence to confirm the correct insertion.

2.2. Transfer the target gene into the *E. coli* destination vector, pBAD-DEST49 by Gateway LR cloning to create an expression vector[29].

2.2.1. Transform the expression vector into competent Top10 *E. coli* cells by heatshock for 30 seconds and select successful recombinants on LB media with 100 µg/mL ampicillin[30].

2.2.2. Extract plasmid DNA[30] and sequence to confirm the correct insertion.

2.3. Transform expression vector into *E. coli* ΔpotE740(del)::kan, ΔcadB2231::Tn10 and select on LB plates with ampicillin, kanamycin, and tetracycline[30].

2.4. In order to determine the optimal concentration of arabinose for induction, express the target gene under the control of the *araBAD promoter* in LB media with gradient concentrations of arabinose as described[29].

2.5. Collect the cell pellets and assess protein expression by SDS-PAGE and visualization of protein bands as described in the product manual[29].

NOTE: The *E. coli* ΔpotE740(del)::kan, ΔcadB2231::Tn10 was used as the control sample without BAT1 expression. *E.coli* double knock out mutant cells with the empty pBAD-DEST49 vector was not used as a control due to the presence of the ccdB gene in the vector.

3. Generation of inside-out membrane vesicles

3.1. Culture the *E. coli* mutant cells expressing the target protein by growing a single colony overnight in 5 mL of polyamine-free media (2% glycerol, 6 g of K_2HPO_4, 3 g of KH_2PO_4, 0.5 g of NaCl, NH_4Cl, 50 mg of thiamine, 0.79 g yeast complete synthetic media adenine hemisulfate (10 mg/L), L-Arginine (50 mg/L), L-Aspartic acid (80 mg/L), L-Histidine hydrochloride monohydrate (20 mg/L), L-Leucine (100 mg/L), L-Lysine hydrochloride (50 mg/L), L-Methionine (20 mg/L), L-Phenylalanine (50 mg/L), L-Threonine (100 mg/L), L-Tryptophan (50 mg/L), L-Tyrosine (50 mg/L), L-Valine (140 mg/L), Uracil (20 mg/L)). Transfer this culture to 500 mL of polyamine-free media supplemented with 0.0002% arabinose and grow until the cell culture reaches an OD_{600} of 0.6-0.8 (usually ~5 h).

3.2. Collect the cell pellet by centrifugation at 2,000 x *g* for 15 min at 4 °C. Resuspend the pellet and wash three times with 0.1 M potassium phosphate buffer, pH 6.6, containing 10 mM EDTA (Buffer 1). The final volume of cells in Buffer 1 after the third spin should be 10 mL.

3.3. Generate inside-out membrane vesicles by French Press treatment (10,000 p.s.i.) of the intact cells using a 35 mL French pressure Cell.

3.4. Prior to loading the sample into the standard French pressure cell, lubricate the piston and O-rings to make it easier to insert into the cell.

NOTE: These instructions are specific for the use of the standard pressure cell[31].

3.4.1. Carefully screw the closure plug into the lower end of the cell. Ensure that the cell is right-

side up based on the lettering on the side of the cell. Insert the piston into the cell and move it into the cell, until the max fill line on the piston reach the top of the piston. Flip the unit over and place on the provided stand between the three posts.

3.4.2. Pour the cell suspension into the body of the cell.

NOTE: We have been using only 10 mL, so there will be plenty of room left in the French pressure cell chamber, which has a capacity of 35 mL.

3.4.3. Mount the standard pressure cell on the French pressure cell unit.

3.4.3.1. First check that the power is off. On the left side of the panel is the **Ratio Selector** switch. It has three positions: **Down** at the end of the run, **Low** for using a smaller pressure cell, and **High** for use with the standard French pressure cell. If the French press was previously used with the smaller cell the piston may not be fully retracted. Set this switch to **Down.**

3.4.4. Turn the machine on with the switch on the RHS at the back of the unit and turn the **Pause-Run** switch to the **Run** position. This will result in the piston being fully retracted. Move the switch back to **Pause,** and shut the machine off.

3.4.5. Loosen the thumb screws and move the safety clamp that spans to two stainless steel columns to the side. It will swing out to the right. With one hand holding the base of the closure plug in place, pick the French pressure cell up with both hands, and rotate it 180°, so the piston is now in the upright position. Set it onto the platform, and move the safety clamp back in place so that this clamp holds the French pressure cell in position.

3.4.6. Note the **Flow Valve assembly** on the **closure plug** needs to be accessible to the operater and positioned so that the valve can be turned freely. Open the the flow valve assembly with a few counter clockwise turns. Position the the arms of the piston so that they are oriented in a two o'clock and eight o'clock position. Tighten the thumbs screws so that the standard pressure cell is held in place.

3.4.7. Insert the **Sample outlet tube** into the closure plug, and connect a short piece of flexible tubing so that the broken cell debris can be collected in a falcon tube. We usually use an 300 mL ice-filled beaker to hold the falcon tube upright, and to keep the cell debris chilled.

3.4.8. Make sure the **Pause-Run** switch is set to to **Pause**, and the **valve assembly** is in the **open position**. Turn the machine back to on, adust the **Ratio Selector** switch to **High**, and turn the **Pressure Increase Valve** on the front panel to the right about a half turn.

3.4.9. The piston will begin to rise from the base to displace the air in chamber. Direct the air coming out of the Tygon tubing attached to the sample outlet tube to the tube in the beaker.

3.4.10. When the first drops of liquid come out, close the the **flow valve assembly** by turning it

clockwise. This creates a metal to metal seal, with the pressure cell, so do not overtighten.

3.4.11. The front of the French Press has a chart on the front panel to generate the proper pressure on the biological cells inside the pressure cell. In this case, increase the pressure by turning the **Pressure Increase Valve** clockwise, until the **Gauge** reaches 640 psi. This will create an internal pressure of 10,000 psi.

3.4.12. Once the cell has reached target pressure open the flow valve assembly slightly by turning it counter clockwise . Adjust the the opening of the valve to allow a flow rate of only about 10 drops per min.

3.4.13. When the stop line on the piston body reaches the top of the flow cell. Switch the **Pause-Run Switch** to **Pause**. This is critical because having the piston inserted farther into the cell will damage the unit. Open the **flow valve assembly** and collect the remaining drops.

3.4.14. Turn **Ratio Selector** switch to **Down**, and then set the **Pause Run** switch to **Run**. When the bottom plate is fully retracted set the **Run** switch to **Pause**, and turn the machine off.

3.4.15. Remove the French pressure cell from the French press, and disassemble for cleaning. Store all parts dry with a light covering of glycerol. Remove and replace any gaskets or seals with signs of wear.

3.5. Remove unbroken cells and cell debris of the French press eluent by centrifugation at 10,000 x *g* for 15 min at 4 °C. Discard the pellet, and transfer supernatant to ultracentrifuge tubes.

3.6. Pellet membrane vesicles from the resulting supernatant by ultracentrifugation at 150,000 g for 1 h at 4 °C.

3.7. Wash the membrane vesicles once, without resuspension, in 1 mM Tris-maleate, pH 5.2 containing 0.14 M KCl, 2 mM 2-mercaptoethanol and 10% glycerol and resuspend in the same buffer (Buffer 2)[23] using a Dounce tissue grinder. Typically the membrane fraction from 500 mL of culture would be suspended in 5 mL of buffer, and a concentration of 5-10 mg of membrane protein/mL using the Biochinic Acid assay[32].

3.8. Store 100 µL aliquots of membrane preparations at -80 °C in 1.5 mL microcentrifuge tubes.

4. Western blot and orientation of transporter assay

4.1. Wash and resuspend vesicles from step 3.7 in 30 mM Tris pH 7.8 + 0.1 mM $CoCl_2$.

4.2. To 100 µL samples, add 1 µL of 2 mg/mL carboxypeptidase A in 0.1 M NaCl, 30 mM Tris, 0.2 mM $CoCl_2$ pH 7.8.

4.3. Inculate for 20 min at 20 °C. Stop digestion by adding 5 µL of 0.5 M NaEDTA, 0.5 M 2-mercaptoethanol pH 7.5.

4.4. To fully inactivate the enzyme, incubate the solution for 1 h at room temperature.

NOTE: Vesicles without the catboxypeptidase A treatment were used as a control.

4.5. Analyze samples by electrophoresis in the presence of lithium dodecyl sulfate. Add 5-10 µL of 150 mg/mL lithium dodecyl sulfate, 450 mg/mL glycerol, 0.1 mg/mL bromophenol blue, 0.4 M Tris pH 7.5 to 100 µL of sample.

4.6. Perform immunoblotting by laying a nitrocellulose filter moistened with 25 mM sodium-hydrogen phosphate pH 7.5 upon the polyacrylamide gel. Place these between two moistened cellulose filters and finally between two moistened plastic scouring pads. Place this assemblage between the electrodes of a chamber containing 25 mM sodium-hydrogen phosphate pH 7.5 at 2-4 °C.

4.7. Allow electrotransfer of proteins to proceed for 3 h at 20 V and 2-3 A.

4.8. Block the nitrocellulose membrane overnight at 2 °C in a blocking buffer of 0.15 M NaCl, 10 mM Tris, pH 7.5 and 0.5 mg/mL Tween 20[33].

4.9. Incubate the nitrocellulose filter for 2 h with a 1:5000 dilution of Anti-His (C terminal)-HRP antibody in the blocking buffer (20 mL) and wash twice with 50 mL of buffer for a total of 60 min.

4.10. To visualize the immunoblot, dissolve 6 mg of 4-chloro-1-naphthol in 20 mL of denatured alcohol and add 80 mL of 15 mM Tris pH 7.5 and 50 µL of 30% H_2O_2. Bathe the filter paper in the substrate solution for 20 min. The reagent will react with the antibody to form a blue precipitate on the nitrocellulose membrane. When sufficient color has developed, rinse the membrane with water, and allow to dry.

5. Transport assay

5.1. Incubate 100 µL aliquots of membrane vesicles at 12 °C for 5 min.

5.2. Initiate transport by adding radiolabeled polyamines to the membrane vesicles at a final concentration of 50 µM (unless otherwise stated). Make ^{3}H-substrate (spermidine or putrescine) solutions in an assay buffer consisting of 10 mM Tris-HCl, 10 mM potassium phosphate, pH 8.0 and 0.14 M KCl[23] (Buffer 3) with modifications depending on the assay.

5.3. Conduct transport assays for 1 min at 12 °C in 1.5 mL microfuge tubes.

5.4. After 1 min, transfer the reaction mixtures to filtration manifold and filter through a 0.45 µm nitrocellulose membrane filter.

5.4.1. Add 3 mL of ice-cold assay buffer containing a 10-fold higher concentration of unlabeled polyamines followed by 3 mL of assay buffer without the polyamines to reduce nonspecific binding.

5.4.2. Transfer the washed filters to 20 mL disposable scintillation vials containing 10 mL of scintillation liquid and determine radioactivity by using a liquid scintillation counter. The scintillation counter measures the radioactivity in the sample and reports it as disintegrations per min (dpm).

5.5. Calculate the net polyamine uptake as the difference between the uptake at one minute by vesicles incubated at 12 °C and uptake at 0 min by vesicles incubated on ice.

NOTE: The mass of substrate imported into the vesicles is calculated as follows. If the sample volume in the microfuge tube is 0.2 µL and the starting concentration of substrate is 100 µM, then the total mass of substrate in the microfuge tube is 20 x 10^{-12} M. Because of the very high specific activity of commercially labelled substrates (often 2.22 x10^{9} dpm/mM) the mass of the isotope added can be ignored in the calculation. So if the total amount of isotope that was added to the microfuge tube was 100 x 10^{6} dpm and the vesicles had a net uptake of 1000 dpm, then the total mass of substrate taken up by the vesicles was 1% of the total number of moles of substrate or 2 x 10^{-13} M.

5.5.1. Determine K_m for the substrate by measuring the uptake of 10, 25, 50, 250 and 500 µM radiolabeled substrate into vesicles expressing the target protein. Calculate Michaelis-Menten kinetics using a nonlinear regression method using the Michaelis Menton model of the statistical software package[34].

5.6. For the competition experiments, add 100 µM, 500 µM, 1 mM, 1.5 mM or 2 mM nonlabelled competitive substrate made in the assay buffer to the 1.5 mL microcentrifuge tube containing 100 µL of vesicles at 12 °C.

5.6.1. Add radiolabeled polyamine (50 µM) to the microcentrifuge tube simultaneously.

5.6.2. Measure radioactivity trapped inside vesicles as mentioned above by repeating steps 5 through 5.4.2.

5.6.3. Determine apparent K_m ($K_{m,app}$) for the competitive substrates by measuring the uptake of 10, 25, 50, 250 and 500 µM radiolabeled polyamines in the presence of 100 µM or higher nonlabelled substrate and using a nonlinear regression method to plot the curve.

REPRESENTATIVE RESULTS:

The major steps in this protocol are summarized pictorially in **Figure 1**. Briefly, *E. coli* cells deficient in all polyamine exchangers and expressing *AtBAT1* are cultured, centrifuged, washed with a buffer and subjected to cell lysis using a French press. Lysis tends to produce vesicles that

are mostly inside-out and trap the buffer outside the cells. Cell debris is removed by centrifugation, and a second ultracentifugation step is used to collect a membrane pellet. The membrane pellet is resuspended in Tris-Maleate buffer pH 5.2 and stored at -80 °C. Transport assays are done at 12 °C, which was found to be optimal for maintaining membrane stability. Assays are initiated by the addition of radiolabeled substrate and a shift in the pH of the buffer suspension of vesicles to pH 8.0. After 1 min, ice-cold assay buffer with unlabeled substrates is added to stop the uptake of the radiolabel into the vesicles. Radiolabelled vesicles are trapped by filtration through nitrocellulose membranes. Membranes are transferred to scintillation vials and radiolabel on the membranes is determined by liquid scintillation counting.

A western blot is used to verify that AtBAT1 is translocated to vesicles (**Figure 2**). Probing the blot with an Anti-His C-terminal antibody revealed a fusion construct protein of approximately 72.3 kDa (**Figure 2**, Lane 2). Digestion of the vesicles prior to SDS-PAGE resulted in a dimunition, but not a complete loss of the probe signal (**Figure 2**, lane 3) . The decrease in the probe signal as a consequence of carboxypeptidase A suggests that most of the C-terminal residues are on the outside of the vesicles.

In this assay system, vesicles are suspended in a buffer at pH 5.2 so that the pH inside the vesicles equilibrates with the buffer. Transport of the radiolabeled substrate into the vesicles at pH 5.2 is initiated by suspending the vesicles in a pH 8.0 buffer, thus creating a pH gradient of pH 2.8 across the membrane. At 12 °C, uptake of radiolabeled spermidine by the vesicles was highest at 1 min, and remained linear over 3 min (**Figure 3A**). Therefore, the incubation time for the transport assay was fixed at 1 min. To account for non-specific binding of radiolabel, the vesicles were incubated at 0 °C in the presence of radiolabeled substrate for one minute, and these counts were subtracted from uptake of ladiolabel at higher temperatures.

Figure 3B shows the uptake of radiolabeled spermidine into the vesicles after one minute. There was no net uptake of isotope by membrane vesicles that were prepared and stored at pH 8.0, as there was no proton gradient across the vesicle membrane. To demonstrate the effect of dissipation of the artificial proton gradient, the membrane vesicles were incubated in pH 8.0 buffer for 10 min prior to the addition of labelled substrate[25]. Under these conditions, a minimal uptake of radiolabeled substrate was shown. Uptake of radiolabeled spermidine was also minimal in vesicles prepared with *E. coli* cells deficient in the polyamine exchangers C*adB* and *PotE*. Taken together, these results indicate that the proton driven uptake of spermidine was due to the BAT1 protein (**Figure 3A,B**).

To determine the substrate specificity of the protein, K_m values were calculated by measuring the uptake of radiolabeled substrate at 10, 25, 50, 100, 250 and 500 μM concentrations. The K_m for spermidine, putrescine and arginine were 55 ± 12 μM, 85 ± 20 μM and 1.4 ± 0.5 mM, respectively, indicating that this protein is a high affinity polyamine and arginine exchanger (**Figure 4**).

Affinity of the transporter for a particular substrate can also be determined indirectly by using competition assays. Here, we have utilized two methods to evaluate the competition between two substrates. In the first method, the uptake of 50 μM radiolabeled spermidine was measured

in the presence of increasing concentrations of the nonlabelled competing substrate (**Figure 5A**). In the second method, the apparent K_m for spermidine was calculated by measuring the uptake of increasing concentrations of radiolabeled spermidine in the presence of 100 μM nonlabelled competing substrate (**Figure 5B**). Competition assays revealed that GABA is a competitive inhibitor of spermidine with a $K_{m,app}$ of 164 ± 15 μM (**Figure 5A**,**B**). Furthermore, measuring the uptake of 50 μM radiolabeled spermidine in the presence of varying concentrations of different amino acids revealed that AtBAT1 is also capable of transporting glutamate and alanine at mM concentrations (**Figure 6**).

FIGURE LEGENDS:

Figure 1. Schematic representation of the method. (**A**) Schematic representation outlining key steps in the preparation and purification of membrane vesicles from *E. coli*. (**B**) Schematic representation outlining key steps in transport assay of membrane vesicle preparations using radiolabeled substrates.

Figure 2. Western blot showing expression of AtBAt1 in purified vesicles. Bands were visualized using horseradish peroxidase conjugated anti-His (C-term)-HRP antibody. **Lane 1**, Prestained protein ladder. **Lane 2**, Purified vesicles expressing AtBAT1.1 showing a band of the expected size of the fusion protein. **Lane 3**, purified vesicles expressing AtBAT1.1 were pretreated with carboxypeptidase A prior to SDS electrophoresis and western blotting. Equivalent amounts of vesicles (protein) were added to each lane. Decreased staining indicates that the C-terminal of the protein in most vesicles is degraded by by protease digestion.

Figure 3. Transport activity of vesicles showing the effect of BAT1 protein expression and the importance of a pH gradient. (**A**) Time dependent uptake of ^{3}H labeled spermidine in vesicles expressing BAT1 with an internal pH of 5.2 and introduced to a buffer at pH 8.0. In the control assay, the vesicles were added to the assay buffer at pH 8.0, 10 min prior to the addition of ^{3}H labeled spermidine to enable dissipation of the proton gradient. Then uptake of radiolabel into the vesicles was assessed over a 1 min interval. (**B**) Uptake of ^{3}H labeled spermidine in the presence of a proton gradient (internal pH of 5.2), in the absence of a proton gradient (internal pH of 8), in vesicles added to the assay solution 10 min prior to the addition of radiolabeled spermidine and in vesicles made from *E. coli* mutant cells not expressing BAT1. Uptake into vesicles was monitored for 1 min. All values are presented as mean ± SE of five replicates. Data analysis was performed using a student's t-test and * indicates a significant difference from the control (p value < 0.05).

Figure 4. *In vitro* assays of polyamine and arginine transport activity of BAT1. (**A**) The K_m values for spermidine and putrescine uptake are 55 ± 12 μM and 85 ± 32 μM respectively. (**B**)The K_m for arginine uptake is 1.4 ± 0.5 mM. All values are presented as mean ± SE of five replicates.

Figure 5. GABA is a competitive inhibitor of Spermidine transport by BAT1. (**A**) Uptake of ^{3}H labeled spermidine by vesicles expressing AtBAT1.1 was significantly reduced in the presence of 100 μM or 500 μM GABA. (**B**) Apparent K_m for spermidine uptake by BAT1.1 was increased to 164

± 20 μM in the presence of 100 μM GABA. All values are presented as mean ± SE of five replicates. Data analysis was performed using a student's t-test and * indicates a significant difference from the control (*p* value < 0.05).

Figure 6. Glutamate and alanine are competitive inhibitors of spermidine transportby BAT1. Spermidine uptake was significantly reduced in the presence of 1 mM non-labeled glutamate and 1.5 mM non-labeled alanine. All values are presented as mean ± SE of five replicates. Data analysis was performed using a student's t-test and * indicates a significant difference from the control (*p* value < 0.05).

DISCUSSION:

In the present study, we outline a method for the characterization of an antiporter by first expressing the protein in *E. coli* and then generating membrane vesicles, so that the heterologously-expressed protein can be assayed in a cell-free system. In addition to equipment found in most molecular biology labs, this strategy requires the use of a French press, an ultracentrifuge, and access to a facility to conduct radioisotope assays.

A basic requirement of this technique is that the heterologous protein is correctly targeted to the plasma membrane of *E. coli*. This strategy may also be useful for functional analysis of organellar transporters since the plastid ADP glucose transporter was successfully localized to the *E. coli* cell membrane and functionally characterized[35]. The vector (pBAD-DEST49) used in these experiments contains an N-terminal thioredoxin protein to increase the solubility of the translated product. N-terminal fusions of a small *B. subtilus* protein mystic, have been found to enable more efficient targeting of membrane transporters to the cytoplasmic membrane[36]. However, misfolding events, and the failure of the proteins to be properly integrated into the cytoplasmic membrane are potential problems that preclude the use of bacterial expression systems for many types of transporters[1].

Membrane vesicles have also been used to characterize plant transporters[37,38]. As the vesicles lack the essential energy sources such as ATP and enzymes, the interference from active transporters and other metabolic activity is minimal. Thus, this system is ideal for the analysis of passive translocations such as metabolite exchangers. The everted membrane vesicles, in particular, can be applied to the characterization of exporters and antiporters since the composition of the internal solution can be manipulated by changing the composition of buffer 1. Furthermore, using French press or ultrasound sonication is fairly efficient in generating inside-out membrane vesicles from intact *E. coli* cells. 95% of the vesicles generated by ultrasound sonication or French press have everted membranes[39,40]. PotE, the *E. coli* antiporter of putrescine and ornithine, was the first polyamine antiporter that was characterized using inside-out membrane vesicles[23]. We used P1 transduction to create a specific mutant strain for the characterization of a polyamine antiporter, and this strain may be useful for the characterization of other animal, fungal or plant polyamine exchangers. We also envision that other *E. coli* strains with two or more gene deletions might be useful for the characterization of other plant and animal exchange transporters using membrane vesicles.

The most critical step in this protocol is the expression of the protein in the *E. coli* mutant system. An *E. coli* expression vector with an inducible promoter is utilized to promote tight, dose dependent regulation of the heterologous gene expression. The presence of N terminal and C terminal tags such as His-patch Thioredoxin, V5 epitope or 6xHis in the vector is useful for detection and purification of the protein. In addition, the presence of a thioredoxin fusion protein which is a component of the pBAD49 vector, can increase translation efficiency and, in some cases, solubility of eukaryotic proteins expressed in *E. coli*[41]. The different codon choices in Arabidopsis and *E. coli* could challenge protein expression in *E. coli.* It is known that codon optimization can impressively increase heterozygous proteins expression in *E. coli*[42]. In the vesicle assay, codon optimized AtBAT1.2 showed a higher exchange activity than non-codon optimized AtBAT1.1 in *E. coli* cells (data not shown), demonstrating that codon optimization was helpful to enhance the expression and function of heterologously expressed proteins in bacterial cells. The production of membrane vesicles by careful adjustment of the valve to maintain a slow even drip of lysed cells is also a key step in the procedure. After ultracentrifugation, we have found that resuspension of membrane vesicles in a Dounce homogenizer minimizes sample to sample variation between aliquots of membrane vesicles that are prepared and subsequently stored at -80 °C.

A limitation of *E. coli* expression systems is that they are incapable of post-translational modifications such as N-glycosylation or acetylation. Absence of these protein modifications might impact protein activity[1]. However, mutants capable of performing these modifications have been identified and can be used as a tool to express proteins that require such modifications[43]. The generation of sufficient amounts of the expressed protein could be a challenge due to unfolding and aggregation as inclusion bodies, failure of the protein to be properly integrated in to the cytoplasmic membrane, mistargeting and mis-regulation due to lack of post translational modifications

A minor limitation of this technique is that it does not provide evidence for the natural orientation of the transporter. This can be accomplished by taking advantage of the N or C terminal tags and immunological methods. The accessibility of a particular terminus of the protein in vesicles can be achieved by the digestion of all accessible, and therefore, presumably external termini of the carrier, electrophoresis of the protein in the presence of sodium dodecyl sulfate, transfer to nitrocellulose filters and detection of the remaining, internal termini with antibodies[40].

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DISCLOSURES:
The authors have nothing to disclose.

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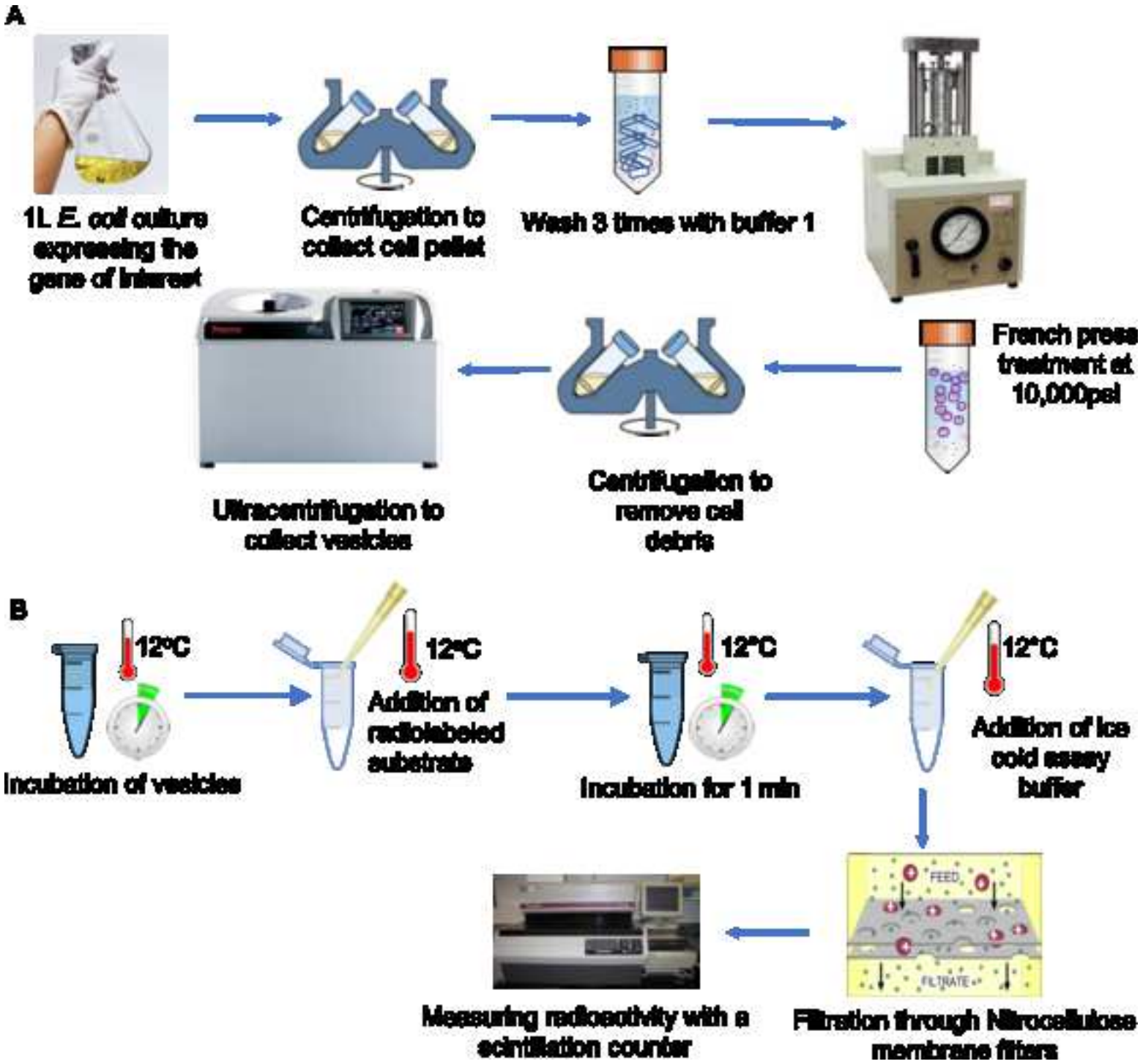

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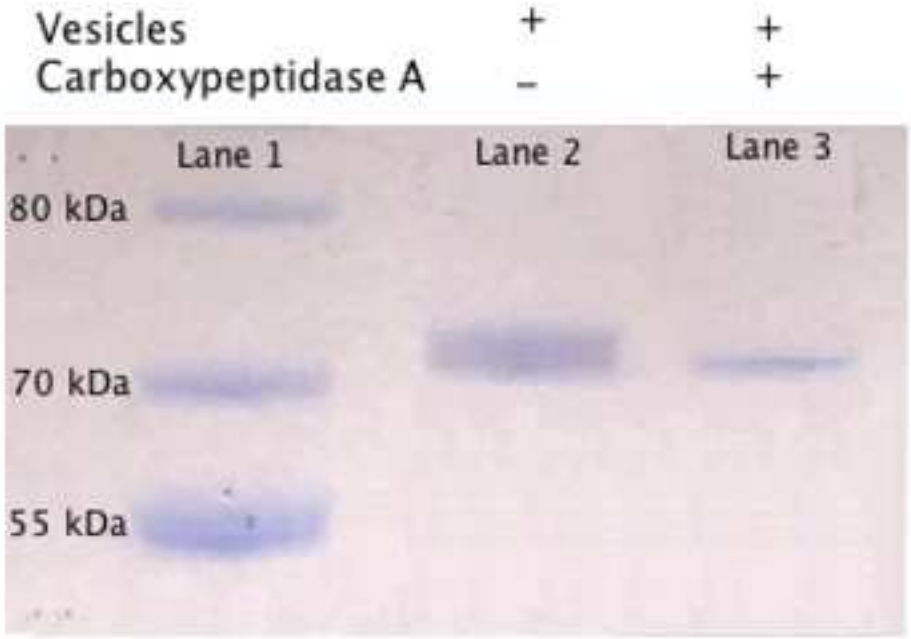

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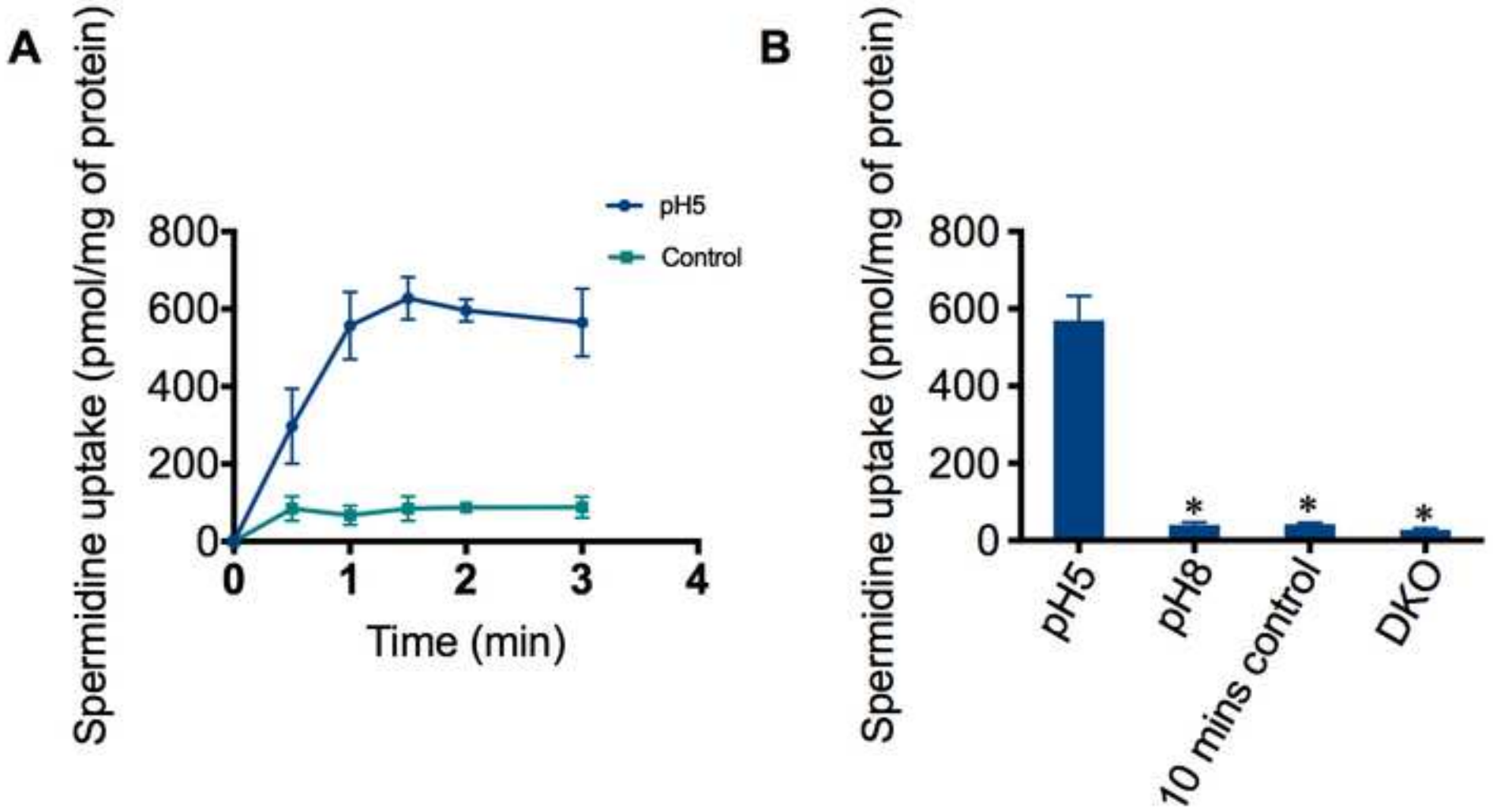

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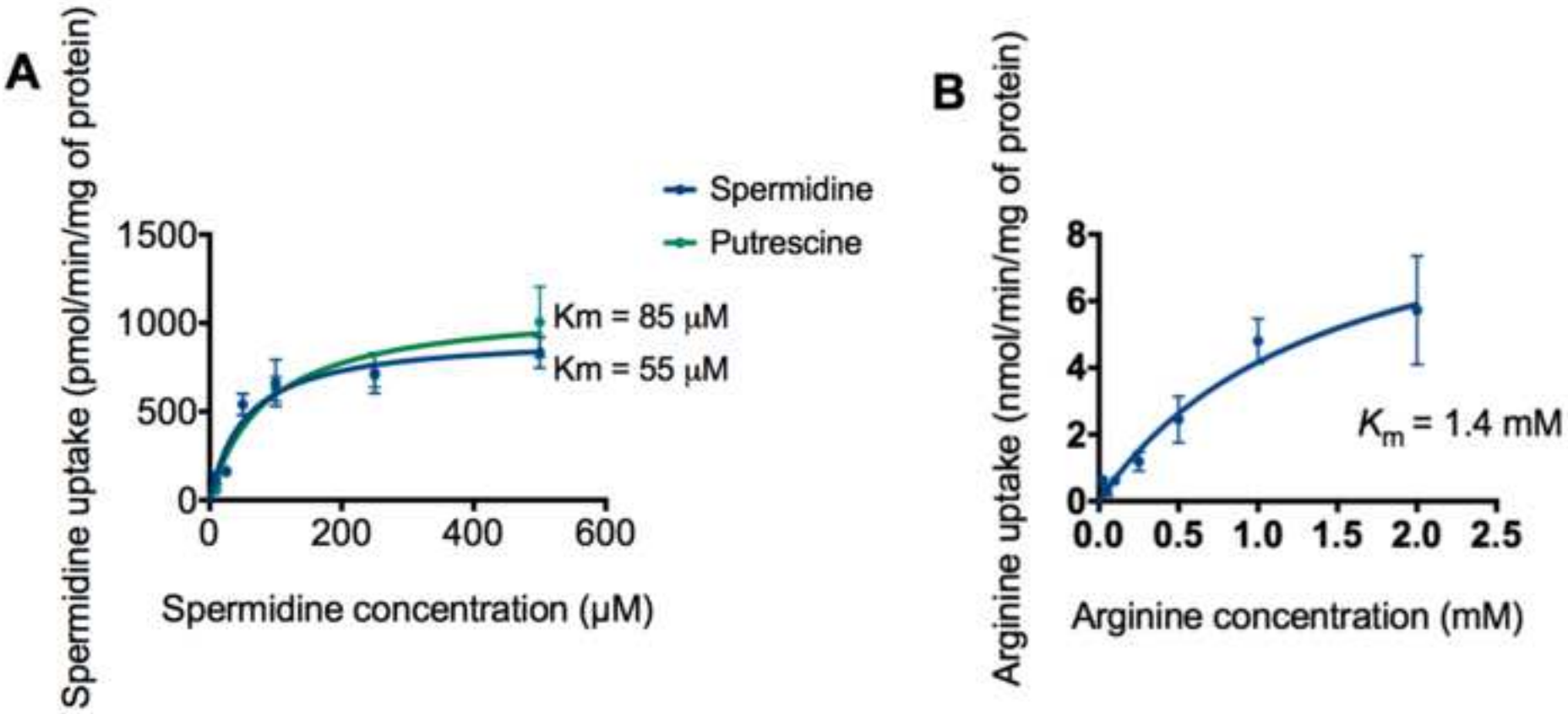

Figure 5

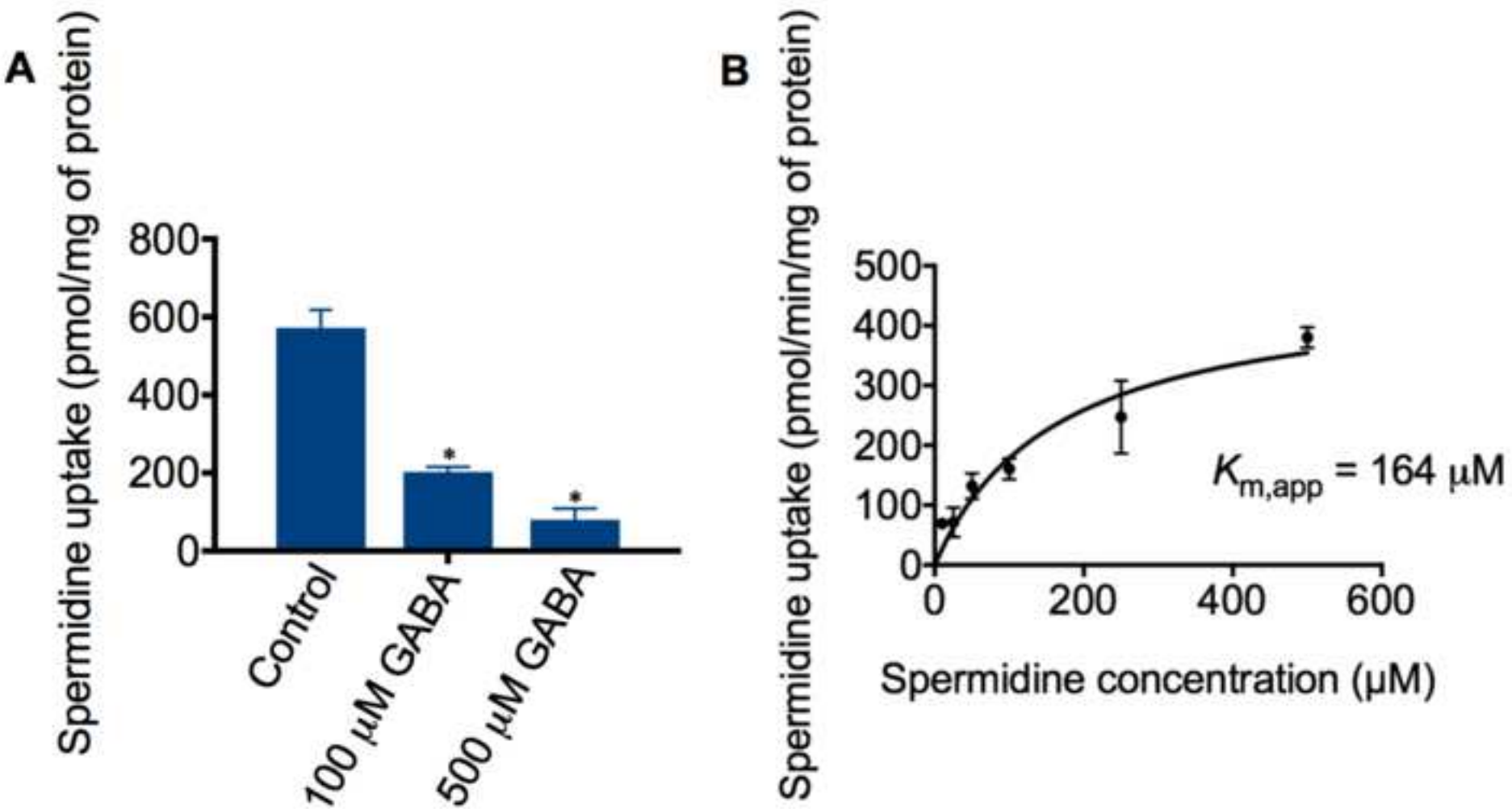

Figure 6

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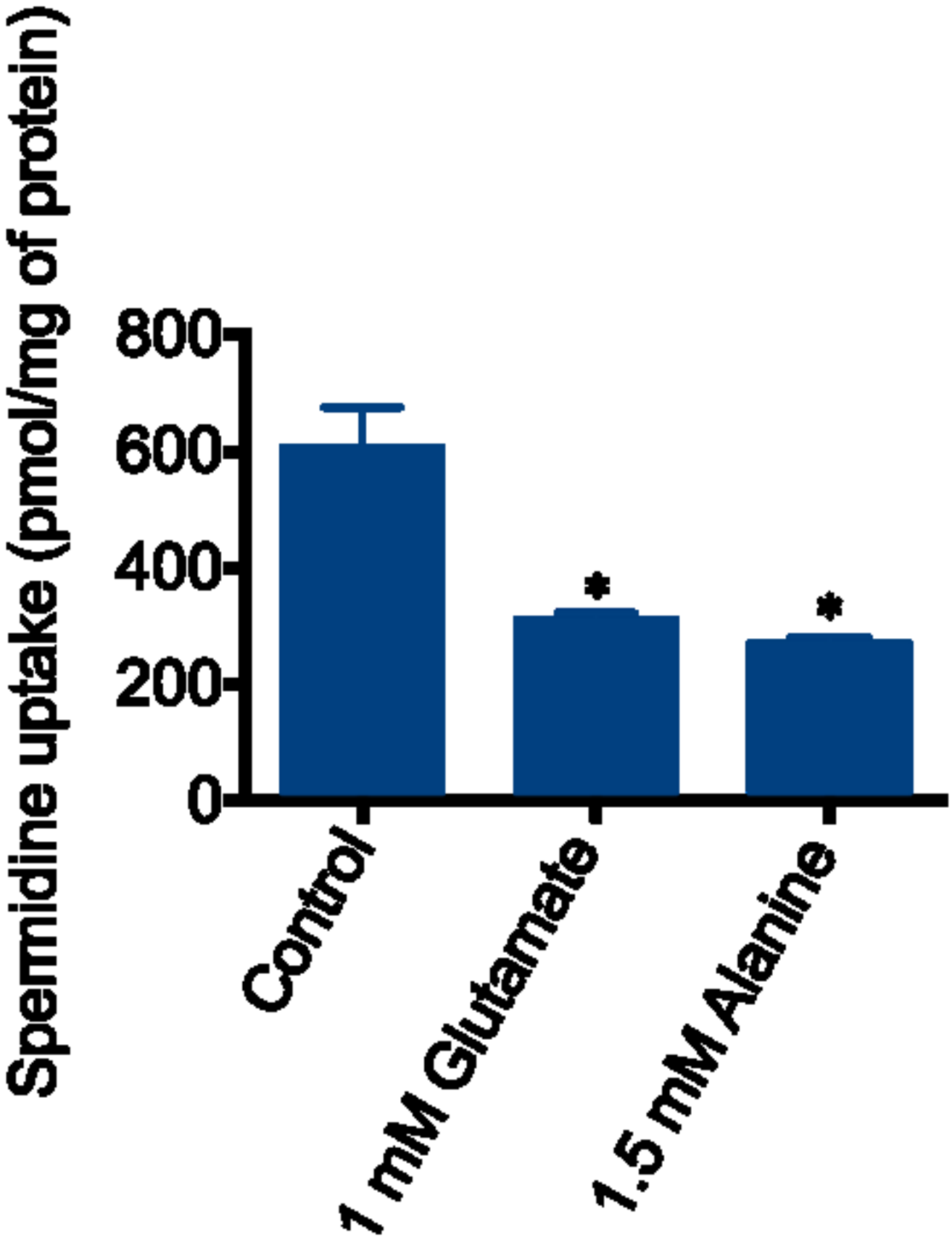

Name of Material/Equipment	Company	Catalog Number
2-mercaptoethanol	Sigma-Aldrich	M6250
^{3}H-putrescine	PerkinElmer	NET185001MC
^{3}H-spermidine	PerkinElmer	NET522001MC
4-chloro-1-naphthol	Sigma-Aldrich	C8890
^{14}C arginine	Moravek Inc.	MC137
Arginine	Sigma-Aldrich	A-5006
Anti-His (C-term)-HRP antibody	Thermofisher	R931-25
Arabinose	Sigma-Aldrich	A3256
BCA protein assay kit	ThermoFisher	23227
Bromophenol blue	Bio-Rad	161-0404
Carboxypeptidase B	Sigma-Aldrich	C9584-1mg
Centrifuge	Sorvall	
Dounce tissue grinder	LabGenome	7777-7
Ecoscint-H	National Diagnostics	LS275
EDTA	Sigma-Aldrich	
Filtration manifold	Hoefer	FH225V
French Pressure Cell	Glen Mills	FA-080A120
GABA	Sigma-Aldrich	A2129
Glutamate	Sigma-Aldrich	G6904
Glycerol		
GraphPad Prism software		
Hydrogen peroxide	KROGER	
Potassium Chloride	J.T. Baker	3040-01
Liquid scintillation counter	Beckman	LS-6500
Maleate	Sigma-Aldrich	M0375
Nanodrop	ThermoFisher	
Nitrocellulose membrane filters	Merck Millipore	hawp02500

PCR clean up kit	Genscript	
Potassium Phosphate dibasic	ThermoFisher	P290-500
putrescine	fluka	32810
Potassium Phosphate monobasic	J.T.Baker	4008
Spermidine	Sigma-aldrich	S2501
Strains :E. coli ΔpotE740(del)::kan, ΔcadB2231::Tn10	This manuscript	Available upon request.
Tris-base	Research Products	T60040-1000
Ultracentrifuge	Sorvall MTX 150	46960
Ultracentrifuge tubes	ThermoFisher	45237
Vector: pBAD-DEST49	ThermoFisher	

Lithium dodecyl sulphate

Sodium-hydrogen phosphate
Nirotrocellulose membranes
SDS Page precast gel
Sodium Chloride

Tween 20

	Comments/Description
Detects the C-terminal polyhistidine (6xHis) tag, requires the free carboxyl group for detection	
	Pierce BCA protein asay kit.
SS-34 fixed angle rotor and GA-6 fixed angle rotor	
Corning 7777-7 pyrex homogenizer with pour spout.	
scintillation cocktail	
http://www.graphpad.com/prism/Prism.htm	
0.45 µM	

QuickClean II

Strain is deficient in the *PotE* and *CadB* polyamine exchangers.

Thermo Fisher S150-AT fixed angle rotor
Centrifuge tubes for S150-AT rotor
Gateway expression vector for *E. coli*

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Bowling Green State University

Department of Biological Sciences

August 19, 2019

Alisha D'Souza, Ph.D.
Senior Review Editor
JoVE
617.674.1888

Dear Dr. DSousa:

My apologies for the delay in getting this returned to you. When I got back from holidays, there were issues with my computer, that needed to be resolved. We have made revisions to Figures 1&2, and minor adjustment to the text as requested. And, I needed the time to read the manuscript over again. Specific issues raised by reviewers are addressed below.

RE comments by Reviewer1:
One remaining question I have despite the modifications to the paper is: do antiporters really work in the transport of a single substrate and if so, is determining kinetic constants for the transport of a single substrate really valid? This question probably just results from my ignorance on the subject, but it seems counterintuitive that a putrescine/arginine antiporter would work in the absence of the other exchange substrate,

I think Reviewer1 has been confused by the term putrescine/arginine antiporter. This moniker is intended to indicate some of the substrates that this transporter can mediate transfer across a membrane using a pH gradient. Many transporters, and we believe BAT1 falls in this "group", are in fact polyspecific. They are capable of transporting a group of structurally related compounds. Describing their ability to transport compounds using Michaelis-Menton kinetics is a standardized way of showing their relative specificity for different substrates. The primary substrates that are transferred, will also be affected by availability of substrates, and protein modifications that alter transporter activity. Because most, but NOT all of the vesicles produced by this method are inside out, (Figure 2) this method can be used to identify specific substrates used by the transporter. It is not intended to be used to describe a structural model for how the transporters function *in vivo*.

Reviewer 4
Major Concerns:
A detailed description of how the E.coli mutant strain PotE CadB should be included as this is the main biological material required for the experiments.

This has been done.

Reviewer 5
Please improve the quality of Fig. 1 and Fig. 2.
Done.

Reviewer 6
Lines 382-383: A claim is made that BAT1 also transports glutamate and alanine. However, this is not demonstrated in the figure. It is only shown that less spermidine is taken up in the presence of glutamate and alanine. Thus, glutamate and alanine could also be transport inhibitors. Also, this experiment is shown in Figure 6 (not Figure 5)

WE had tried to minimize the discussion of BAT1 specificially, because our primary focus in this paper is get people to use this method to characterize other proteins. That has not been the focus of the reviewers. To address their concerns we have rephrased the text to indicate that glutamate and alanine inhibit polyamine transport. It should be noted that in prior work, heterologous expression of BAT1 in yeast or plant cells has identified these compounds as substrates for this enzyme.
Those references have been added to the introduction.

Sincerely

Paul

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