

The targeted expression of nucleotide sugar transporters to the *E. coli* inner membrane

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Abstract

The heterologous expression of functional mammalian integral membrane proteins represents a significant hurdle towards investigating their structure/function relationship. We have therefore utilised the OmpA signal sequence to deliberately target the expression of a mammalian nucleotide sugar transporter, the murine CMP-sialic acid transporter, to the *E. coli* inner membrane. The recombinant CMP-sialic acid transporter activity was subsequently evaluated using either *E. coli* spheroplasts or mixed phosphatidylcholine-*E. coli* inner membrane proteoliposomes.

Key Words: membrane proteins, nucleotide sugar transporter, OmpA signal sequence, protein expression

1. Introduction

Membrane proteins represent approximately 30% of the total prokaryotic and eukaryotic proteome. However, integral membrane proteins represent only about 0.3% of all the protein structures elucidated; with an even smaller percentage if only the structures of eukaryotic membrane protein are considered (1, 2). Despite the vast array of expression systems currently available, the main bottleneck in eukaryotic membrane protein crystallization and elucidation of structure-function relationships remains the quantitative expression and

purification of functional protein **(1, 2)**. The expression of eukaryotic integral membrane proteins in prokaryotic expression systems (e.g. *E. coli*) typically leads to the sequestering of these recombinant proteins as inclusion bodies, from which functional membrane protein is often difficult to recover **((3)** and references therein).

The CMP-sialic acid transporter (CST) is a Golgi resident hydrophobic protein with 10 putative transmembrane domains that catalyses the transport of CMP-sialic acid (the universal donor substrate for sialyltransferases) into the Golgi apparatus of eukaryotic cells **(4)**. The CST is a member of a highly conserved family of multiple membrane spanning proteins collectively referred to as nucleotide sugar transporters **(5)**. Therefore, our interest **(6, 7)** in not only probing the relationship between CST structure and function, but also in generating protein suitable for structural elucidation, led us to develop an efficient system for the heterologous expression of integral membrane proteins in *E. coli*. This system utilizes the OmpA signal sequence to target integral membrane proteins to the *E. coli* inner membrane.

2. Materials

2.1 Protein expression and *E. coli* inner membrane isolation

1. pFLAG-mCST **(8)** (see **Note 1** and **Fig. 1**).
2. Electrocompetent *E. coli* BL21 (Merck Biosciences, Darmstadt, Germany) (see **Note 2**).
3. 50 mg/mL Ampicillin: Dissolve 1 g Ampicillin sodium salt in 20 mL Milli Q H₂O (see **Note 3**) and sterilize by passing through a 0.2 µm filter. Aliquot and freeze at -20°C.

4. LB (Luria-Bertani) medium: Dissolve 10 g Tryptone (Oxoid, Cambridge, UK) 5 g Yeast Extract (Oxoid) and 10 g NaCl in 1 L Milli Q H₂O and sterilise by autoclaving at 121°C for 20 min. Store at room temperature.
5. LB Agar-Ampicillin plates: Add 15 g Agar Bacteriological to LB medium described above prior to autoclaving. Allow medium to cool to approximately 55°C and add Ampicillin (50 mg/mL) to a final concentration of 100 µg/mL. Immediately pour (approximately 25-30 mL) into 10 cm diameter Petri dishes and allow to set at room temperature. Store at 4°C.
6. 1 M IPTG: Dissolve 2.83 g Isopropyl-β-D-thio-galactopyranoside (IPTG) in 8 mL Milli Q H₂O. Bring to 10 mL with additional Milli Q H₂O and sterilize by passing through a 0.2 µm filter. Freeze at -20°C.
7. Phosphate Buffer saline (PBS): Dissolve 8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄, 0.24 g KH₂PO₄ in approximately 800 mL of Milli Q H₂O and adjust pH to 7.4. Bring to 1 L and store at room temperature.
8. PBS-Protease inhibitor cocktail: Dissolve 1 Complete Protease Inhibitors Cocktail Tablet (Roche Applied Science, Castle Hill, NSW, Australia) in 50 mL PBS and supplement with 0.5 M EDTA (pH 8) to a final concentration of 1 mM.
9. Sucrose solutions (w/w): For all sucrose concentrations the indicated amounts are dissolved in 120-150 mL Milli Q H₂O. Ten mL of 0.1 M EDTA (pH 7.5) is then added and the solution is adjusted to 200 mL by the addition of Milli Q H₂O.

55% sucrose, 138.3 g; 50% sucrose, 123.0 g; 45% sucrose, 108.2; 40% sucrose, 94.1 g; 35% sucrose, 80.6 g; 30% sucrose, 67.6 g.

2.2 Evaluation of recombinant membrane protein functionality

2.2.1 Spheroplasting of *E. coli* cells

1. 200 mM Tris-HCl (pH 8): Dissolve 24.23 g Tris base in 900 ml of Milli Q water and adjust pH to 8 with HCl. Bring to 1 L and store at room temperature.
2. 200 mM Tris-HCl (pH 8) containing 1 M sucrose: Dissolve 24.23 g Tris base and 342.3 g sucrose in 500 ml of Milli Q water, bring volume to 900 mL and adjust pH to 8 with HCl. Bring to 1 L and store at room temperature.
3. Egg white lysozyme (*see Note 4*).
4. 1 M MgCl₂ solution: Dissolve 10.2 g Magnesium Chloride Hexahydrate in 20 ml of Milli Q water and sterilize by passing through a 0.2 µm filter. Aliquot and freeze at -20°C.
5. 100 mM EDTA (pH 7.6) solution: Dissolve 3.73 g of EDTA-Na₂ dihydrate in 70 mL Milli Q water and while stirring adjust to pH 7.6 with NaOH. Bring to 100 mL and store at room temperature.

2.2.2 Evaluation of protein functionality: Generation of mixed phosphatidylcholine-inner membrane proteoliposomes

1. Phosphatidylcholine (Type XI-E, 100 mg/ml in chloroform).
2. 10 mM Tris-HCl (pH 7) containing 2 mM MgCl₂: Prepare 1 M Tris-HCl (pH 7) by dissolving 12.11 g Tris base in 70 ml of Milli Q water and adjust pH to 7 with HCl. Bring to 100 mL and store at room temperature. Add 1 mL 1 M Tris-HCl (pH 7) and 0.2 mL 1 M MgCl₂ solution to 98.8 mL of H₂O, mix and store at 4°C.

3. Methods

3.1 Protein expression and inner membrane isolation

The protocol provided is for the targeted expression of the mouse CMP-sialic acid transporter (mCST) to the *E. coli* inner membrane using the OmpA leader sequence under optimal conditions (15°C, 0.1 mM IPTG, 3 h). These conditions were determined by evaluating various temperatures (15°C, 20°C, 25°C and 37°C), IPTG concentrations (0.1 - 1 mM) and induction times (up to 4 h). Therefore, the same would need to be determined for each protein expressed using our system; however, the basic methodology described is in essence the same regardless of temperature, IPTG concentration and induction times used.

The OmpA signal sequence targets recombinant proteins to the Sec translocase (the *E. coli* translocation machinery in the inner membrane) (3). The isolation of *E. coli* inner membrane following the targeted expression of membrane proteins using the OmpA leader sequence serves two purposes. Firstly, it is used to verify the localisation of the recombinant CST to the inner membrane. Secondly, the isolated inner membrane fraction can subsequently be used to generate mixed phosphatidylcholine-inner membrane proteoliposomes (described in 3.2.2) for the evaluation of recombinant CST functionality.

1. Transform electrocompetent *E. coli* BL21 cells with pFLAG-mCST (see **Note 2**) and select for positive transformants by plating on LB Agar-Ampicillin plates (see **Note 5**).
2. An overnight culture of *E. coli* BL21 transformed with pFLAG-mCST is prepared by inoculating 3 mL LB medium containing 100 µg/mL ampicillin with a single transformant (colony) and incubating at 37°C for 16 h with shaking (225 rpm).

3. Two mL of the resulting overnight culture is used to inoculate 1 L LB medium containing 100 µg/mL ampicillin and the culture is incubated at 37°C with shaking until an OD₆₀₀ (*see Note 6*) of 0.4-0.5 is reached. At this stage the culture is then shifted to an incubator set at 15°C (*see Note 7*) and incubated with shaking until an OD₆₀₀ of 0.6-0.7 is reached (generally takes 45-60 min).
4. At an OD₆₀₀ of 0.6-0.7 protein expression is induced by the addition of IPTG to a final concentration of 0.1 mM (induced cells) (*see Note 8*).
5. After 3 h incubation at 15°C cells are harvested by centrifugation (4000 x g, 15 min, 4°C) and suspended in PBS-Protease inhibitor cocktail supplemented with 1 mM EDTA.
6. Induced *E. coli* cells whilst on ice are lysed by sonication using an ultrasonic processor fitted with a tapered microtip probe set at 40% maximum output for a 30 sec pulse followed by a 30 sec pause on ice. This is repeated for 4 cycles. The resulting total cell lysate is pre-cleared by centrifugation (20,000 x g, 30 min, 4°C), this removes un-lysed cells, cell debris and inclusion bodies (*see Note 9*).
7. A total membrane fraction (*see Note 10*) is subsequently obtained by further centrifugation of the supernatant following pre-clearing by ultra-centrifugation at 100,000 x g, 4°C, 1 h.
8. The total membrane fraction is layered on a 30-55% sucrose gradient (Ultra Clear Tubes, Beckman, Part number 344058) essentially as described by Osborn and Munson (**9**) and shown in **Fig. 2**, and centrifuged at 100,000 x g for 18 h at 4°C using a swing-out bucket rotor (eg. SW32 Ti, Beckman Coulter, Fullerton, CA, USA).
9. Following centrifugation 1 mL fractions are carefully removed beginning at the top of the tube and set on ice.

10. The protein concentration of each fraction is determined using the BCA protein assay (Pierce, Rockford, IL, USA) or similar protein assay kit.
11. Protein is separated by SDS-PAGE (*see Note 11*) and detected by Western blot analysis (*see Note 12*). The result from a typical isolation using a sucrose density gradient of the *E. coli* inner membrane expressing recombinant CST is shown in **Fig. 3**.
12. Fractions identified containing recombinant CST are pooled and used immediately.

3.2 Evaluation of recombinant membrane protein functionality

The following protocols describe two alternative methods that permit the evaluation of recombinant CST functionality after targeted expression to the *E. coli* inner membrane. The first utilises spheroplasted *E. coli* cells (described in 3.2.1), where the peptidoglycan network connecting the inner and outer membrane is destabilised using lysozyme leading to the almost complete removal of the outer membrane without cell lysis. This treatment results in the *E. coli* inner membrane becoming exposed, allowing the functionality of the recombinant protein localised to the membrane to be evaluated. The use of spheroplasts to evaluate recombinant membrane protein functionality has a number of advantages: (i) they are relatively quick to prepare, thus minimising sample handling; (ii) they are suitable for small-scale pilot experiments; (iii) they can be easily collected by centrifugation or filtration because of their size; and (iv) based on the positive-inside rule (**10**), the orientation of the recombinant protein within the membrane should be uniform. Therefore, the use of spheroplasted cells represents an ideal starting point for determining recombinant protein functionality. However, the integration of a recombinant membrane protein can, if expression is not tightly regulated, destabilise the inner membrane making spheroplasting difficult due to cell lysis. If

spheroplasting cannot be achieved we recommend isolation of the inner membrane as described in 3.1, and the generation of mixed phosphatidylcholine-inner membrane proteoliposomes (described in 3.2.2).

The generation of mixed proteoliposomes is particularly necessary if the transport activity of a recombinant solute transporter, such as the CST, is to be assessed. A significant advantage of using mixed proteoliposomes, particularly when evaluating recombinant solute transporter functionality, is the ability to pre-load the proteoliposomes with substrates or inhibitors. That is, many solute transporters function *via* an antiporter mechanism where the influx of one molecule is driven by the efflux of a counter-molecule, such a mechanism is utilised by the CST (7). Therefore, the ability to pre-load proteoliposomes with substrates or inhibitors allows detailed evaluation of recombinant transporter functionality.

3.2.1 Spheroplasting of *E. coli* cells

Spheroplasting was performed essentially according to Witholt *et al.*, (1976) (11, 12).

1. Following induction with 0.1 mM IPTG (3.1 step 5) collect the *E. coli* cells by centrifugation (4000 x g, 15 min, 4°C). Resuspend the cells at 40 mg/ml in 200 mM Tris-HCl buffer (pH 8.0) (*see Note 13*).
2. Dilute the cell suspension with an equal volume of 200 mM Tris-HCl (pH 8.0) containing 1 M sucrose (final concentration: 200 mM Tris-HCl (pH 8.0) containing 0.5 M sucrose).
3. Add EDTA (pH 7.6) to a final volume equivalent to 0.5% of the cell suspension obtained at step 2.
4. Add lysozyme to a final concentration of 60 µg/ml

5. Induce osmotic shock by diluting the cell suspension with an equal volume of Milli Q water, under constant and gentle stirring.
6. Monitor spheroplasting progression by light microscopy (*see Note 14*).
7. When spheroplasting efficiency reaches 80 to 85%, add MgCl_2 to a final concentration of 20 mM to stabilize the spheroplasts.
8. *E. coli* spheroplasted in this manner can be now collected by centrifugation, resuspended in an appropriate buffer systems and used for evaluation of functionality (eg. binding, enzyme activity or transport assays).

3.2.2 Generation of mixed phosphatidylcholine-inner membrane proteoliposomes

1. An appropriate amount (*see Note 15*) of the 100 mg/mL phosphatidylcholine solution is transferred to a glass round bottom flask and the bulk of the chloroform removed quickly by rotary evaporation. The lipid cake is then transferred to a high vacuum pump and left to dry overnight.
2. The resulting lipid cake is rehydrated with 10 mM Tris-HCl (pH 7) containing 2 mM MgCl_2 to give a final phosphatidylcholine concentration of 30 mg/ml.
3. The lipid suspension is extruded 11 times with the Avanti Lipid Mini-Extruder using a polycarbonate filter with 200 nm diameter (*see Note 16*).
4. The phosphatidylcholine unilamellar vesicle suspension is then diluted in 10 mM Tris-HCl (pH 7) containing 2 mM MgCl_2 to a final concentration of 3 mg/ml and stored on ice until required.
5. The inner membrane fraction purified on sucrose gradient as described above (3.1) is mixed with the phosphatidylcholine vesicles at a ratio of 1:10 (w/w).

6. Fusion of purified inner membranes with unilamellar phosphatidylcholine vesicle is induced by snap-freezing in liquid nitrogen followed by thawing at room temperature. This freeze-thaw cycling is repeated 5 times (*see Note 17*).
7. After the last freeze-thaw cycle, the mixed phosphatidylcholine-inner membrane proteoliposomes are once again extruded through a 200 nm polycarbonate filter 11 times and immediately applied to the determination of membrane protein functionality (eg. binding, enzyme activity or transport assays).

4. Notes

1. pFLAG-mCST was constructed as described in (**8**). The coding sequence of the full-length mCST incorporating *XhoI* and *BglIII* sites was ligated into the corresponding sites of pFLAG-ATS (summarised in **Fig. 1**). Protein expression is controlled by the IPTG inducible *tac* promoter, a hybrid of the *E. coli trp* and *lac* promoters. All genetic manipulations are performed in the *recA E. coli* strain DH5 α .
2. Due to the use of a *tac* promoter, *E. coli* DE3 lysogenic strains (required when using the T7 promoter) are not required for protein expression. We use *E. coli* BL21 (*F*⁻ *ompT hsdS_B (r_B⁻ m_B⁻) gal dcm*) because, as is the case with all other *E. coli* B strains, *E. coli* BL21 is deficient in the *Ion* protease and lacks the *ompT* outer membrane protease. There are a number of methods available for preparing electrocompetent *E. coli* cells (see Sambrook and Russell (**13**)), or electrocompetent *E. coli* BL21 can be purchased from Merck Biosciences.
3. All solutions should be prepared in water that has a resistivity of 18.2 M Ω -cm and is of the highest possible purity. This is referred to as Milli Q H₂O in this text.

4. Be aware that different lysozyme preparations might have very different specific activities. It is therefore necessary to empirically identify optimal lysozyme concentrations depending on the preparation used.
5. It is recommended not to use *E. coli* BL21 or similar expression strains for the preparation of long-term glycerol stocks of the plasmid since they are not *recA* strains. In order to identify plasmid and/or protein toxicity, upon transformation it is recommended that a toxicity test be performed. This is performed by plating the same dilution of transformed cells onto LB agar, LB agar supplemented with 100 µg/mL ampicillin (plasmid toxicity), LB agar supplemented with both 100 µg/mL ampicillin and 1 mM IPTG (protein toxicity) and comparing the number of cells obtained on each plate (*14, 15*).
6. OD₆₀₀ refers to the optical density of a bacterial suspension read at a wavelength of 600 nm using a standard spectrophotometer. That is, light passing through a bacterial suspension is scattered, and the amount of scatter is an indication of the biomass present in the suspension.
7. Maintaining an incubator temperature of 15°C is nearly impossible to achieve in standard laboratories; however, an easy solution is to place an incubator in a 4°C cold-room and use the incubator's temperature control to set the temperature to 15°C. To avoid shock responses, whenever possible it is preferable to reduce the temperature stepwise rather than shifting abruptly from 37°C to 15°C, thus allowing the cell culture to equilibrate and reach the desired OD₆₀₀ at 15°C before inducing protein expression by the addition of IPTG.
8. *E. coli* cells (approximately 50 mL) can be removed prior to IPTG induction (referred to as un-induced cells) and analysed by Western Blotting. This analysis allows the

investigator to assess the stringency of the induction process; that is, if any “leaky” protein expression occurred in the absence of IPTG.

9. It is critical that the *E. coli* suspension is kept cold during sonication in order to avoid heat denaturation of proteins, and that foaming of the sample be minimised. This can be achieved by keeping the tube containing the *E. coli* suspension in a beaker of ice during the entire sonication process, and by placing the microtip probe greater than 10 mm below the surface of the sample. The sonication settings described have been optimised for an ultrasonic processor with a net power output of 750 Watts (eg. Sonics Vibra Cell VC 750). Therefore, sonication settings will need to be adjusted depending on energy output of a given sonicator. To monitor cell lysis, a small aliquot of the total cell lysate is removed following each sonication cycle and centrifuged (13, 000 x g, 30 sec, 4°C). The protein content in the resulting supernatant is determined using a standard protein assay (eg. Bradford Assay). The corresponding sonication cycles required to give maximum protein content can then be determined.
10. This fraction contains both the *E. coli* inner and outer membrane.
11. There are numerous standard protocols available for performing SDS-PAGE. We perform SDS-PAGE essentially as described by Laemmli, 1970 on a 10% acrylamide gel using the Mini-PROTEAN 3 Cell Electrophoresis System (Bio-Rad). However, samples are prepared for SDS-PAGE by dilution 1:1 with a modified 2 x SDS-PAGE sample buffer (0.125 M Tris-HCl (pH 6.8), 4% SDS, 20% Glycerol, 0.2 M dithiotheritol (DTT), 0.02% Bromophenol Blue, 7 M urea and 2 M thiourea) and incubated at 30°C for 15 min. To avoid carbamylation of proteins, it is recommended not to heat samples containing urea at temperatures in excess of 37°C (**16**). Moreover, at higher temperature hydrophobic proteins, such as the mCST, tend to aggregate in

the presence of SDS, resulting in the appearance of high molecular weight smears rather than discrete protein bands (17).

12. There are numerous standard methodologies available for performing Western blotting. We transfer protein following separation by SDS-PAGE to a PVDF membrane using the Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad) for 1 hr instead of the 15 min described by the manufacturer. Blocking of PVDF membrane is performed using 2% (w/v) skim milk powder in Tris Buffered Saline (TBS, pH 7.4) containing 0.1% Tween-20. The primary antibodies that can be utilised for Western blot detection are anti-FLAG M1 (recognises N-terminal DYKDDDDK; dilution 1:10,000) or anti-FLAG M2 (recognises DYKDDDDK dilution; 1:10,000). We commonly use horseradish peroxidase-conjugated anti-mouse IgG (Bio-Rad, Hercules, CA, USA; diluted 1:10,000) and the SuperSignal[®] West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA) as the substrate for horseradish peroxidase. All antibody solutions are made up in 2% skim milk powder in TBS containing 0.1% Tween-20. Immunoreactive bands are visualised by exposing X-ray film (Kodak, Australia) to the PVDF membrane in a X-ray film cassette (Kodak) for up to 30 min (the exact exposure time will depend on expression levels).
13. It is recommended that freshly induced *E. coli* cells be used to prepare spheroplasts and not cells that have been frozen.
14. To follow spheroplasting efficiency by light microscopy, transfer a small aliquot (5 μ L) of the *E. coli* suspension onto a glass slide and cover with a cover slip. Over time, as spheroplasting progresses, the shape of the *E. coli* cells will change from a rod-like to a round morphology. When 80-85% of the cells appear round spheroplasting can be terminated.

15. The amount of phosphatidylcholine and therefore the volume of rehydration buffer to be used are dependent on the number of samples to be prepared. When pre-loaded proteoliposomes are required, substrates or inhibitors can be added to the rehydration buffer at this stage. Pre-loaded proteoliposomes can subsequently be retrieved either by gel filtration chromatography (eg. using PD-10 column) or by ultracentrifugation.
16. Several extrusion apparatus can be purchased from different suppliers depending on the specific applications, from hand driven small-scale analytical extruders for semi-preparative applications to fully automated devices suitable for handling large sample volumes. We use a small manual extrusion apparatus from Avanti (The Mini-Extruder). When using this device make sure to extrude the lipid suspension an odd number of times. By doing so it is possible to ensure that contaminants present in the donating syringe are not passed into the receiving syringe and therefore carried through to subsequent phases of the experimental procedure. When critical, proteoliposomes size distribution can be monitored either by gel filtration chromatography or dynamic light scattering **(18-20)**.
17. This freeze-thawing method for the generation of proteoliposomes takes advantage of the spontaneous fusing of lipid vesicles when the temperature is lowered to below their phase transition temperature **(21)**.

References

1. Grisshammer, R., and Tate, C.G. (2003) Overexpression of integral membrane proteins. *Biochim. Biophys. Acta* **1610**, 1.
2. Link, A.J., and Georgiou, G. (2007) Advances and challenges in membrane protein expression. *AIChE Journal* **53**, 752-756.

3. Drew, D., Froderberg, L., Baars, L., and de Gier, J.W. (2003) Assembly and overexpression of membrane proteins in *Escherichia coli*. *Biochim. Biophys. Acta* **1610**, 3-10.
4. Eckhardt, M., Muhlenhoff, M., Bethe, A., and Gerardy-Schahn, R. (1996) Expression cloning of the Golgi CMP-sialic acid transporter. *Proc. Natl. Acad. Sci. USA* **93**, 7572-7576.
5. Caffaro, C.E., and Hirschberg, C.B. (2006) Nucleotide sugar transporters of the Golgi apparatus: from basic science to diseases. *Acc. Chem. Res.* **39**, 805-12.
6. Tiralongo, J., Abo, S., Danylec, B., Gerardy-Schahn, R., and von Itzstein, M. (2000) A high-throughput assay for rat liver Golgi and *Saccharomyces cerevisiae*-expressed murine CMP-*N*-acetylneuraminic acid transport proteins. *Anal. Biochem.* **285**, 21-32.
7. Tiralongo, J., Ashikov, A., Routier, F., Eckhardt, M., Bakker, H., Gerardy-Schahn, R., and von Itzstein, M. (2006) Functional expression of the CMP-sialic Acid transporter in *Escherichia coli* and its identification as a simple mobile carrier. *Glycobiology* **16**, 73-81.
8. Maggioni, A., von Itzstein, M., Gerardy-Schahn, R., and Tiralongo, J. (2007) Targeting the expression of functional murine CMP-sialic acid transporter to the *E. coli* inner membrane. *Biochem. Biophys. Res. Commun.* **362**, 779-84.
9. Osborn, M.J., and Munson, R. (1974) Separation of the inner (cytoplasmic) and outer membranes of Gram-negative bacteria. *Methods Enzymol* **31**, 642-53.
10. White, S.H., and von Heijne, G. (2004) The machinery of membrane protein assembly. *Curr. Opin. Struct. Biol.* **14**, 397-404.
11. Witholt, B., Boekhout, M., Brock, M., Kingma, J., Heerikhuizen, H.V., and Leij, L.D. (1976) An efficient and reproducible procedure for the formation of spheroplasts from variously grown *Escherichia coli*. *Anal Biochem* **74**, 160-70.

12. Witholt, B., Heerikhuizen, H.V., and De Leij, L. (1976) How does lysozyme penetrate through the bacterial outer membrane? *Biochim. Biophys. Acta* **443**, 534-44.
13. Sambrook, J., and Russell, D.W. (2001) Molecular Cloning: A Laboratory Manual. Vol. 1. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press
14. Dumon-Seignovert, L., Cariot, G., and Vuillard, L. (2004) The toxicity of recombinant proteins in *Escherichia coli*: a comparison of overexpression in BL21(DE3), C41(DE3), and C43(DE3). *Protein Expr. Purif.* **37**, 203-6.
15. Miroux, B., and Walker, J.E. (1996) Over-production of proteins in *Escherichia coli*: mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels. *J. Mol. Biol.* **260**, 289-98.
16. Stark, G.R., Stein, W.H., and MAoore, S. (1960) Reactions of the Cyanate Present in Aqueous Urea with Amino Acids and Proteins. *J Biol. Chem.* **235**, 3177-3181.
17. Ragan, C.I. (1986) Analysis of membrane protein composition by gel electrophoresis. In: *Techniques for the Analysis of Membrane Proteins* (Ragan, C.I., Cherry, R.J., eds). London: Chapman and Hall, 1-25.
18. Mui, B., Chow, L., and Hope, M.J. (2003) Extrusion technique to generate liposomes of defined size. *Methods Enzymol.* **367**, 3-14.
19. Vojta, A., Scheuring, J., Neumaier, N., Mirus, O., Weinkauf, S., and Schleiff, E. (2005) Determination of liposome size: a tool for protein reconstitution. *Anal. Biochem.* **347**, 24-33.
20. Olson, F., Hunt, C.A., Szoka, F.C., Vail, W.J., and Papahadjopoulos, D. (1979) Preparation of liposomes of defined size distribution by extrusion through polycarbonate membranes. *Biochim. Biophys. Acta* **557**, 9-23.

21. Kasahara, M., and Hinkle, P.C. (1976) Reconstitution of D-glucose transport catalyzed by a protein fraction from human erythrocytes in sonicated liposomes. *Proc. Natl. Acad. Sci. USA* **73**, 396-400.

Figure Captions

Fig. 1. pFLAG-mCST was constructed as described in (8). The coding sequence of the full-length mCST was amplified by PCR using pTRc-ME8HA (7) as the template and a forward primer that introduced an *Xho*I site and a reverse primer that introduced a *Bgl*II site. The resultant PCR product was digested with *Xho*I and *Bgl*II and ligated into the corresponding sites of pFLAG-ATS to generate pFLAG-mCST. Using this plasmid the mCST can be expressed in *E. coli* BL21 fused to an N-terminal OmpA signal sequence, shown in bold, and the 8 amino acid residue FLAG-tag under the control of the IPTG inducible *tac* promoter (a hybrid of the *E. coli trp* and *lac* promoters).

Fig. 2. The *E. coli* inner and outer membrane can be separated by layering a total membrane fraction onto a 30-55% sucrose gradient as shown. The volumes required to generate this gradient in a 38 mL Ultra Clear centrifuge tube are indicated and centrifugation is performed at 100,000 x g for 18 h at 4°C using a swing-out bucket rotor. The outer membrane accumulates at the 30%-35% sucrose interface and the inner membrane accumulates at the 45%-50% sucrose interface. The membrane fractions are represented as the dotted bands.

Fig. 3. A typical separation of the *E. coli* inner membrane incorporating the recombinant mCST from the outer membrane using a sucrose density gradient. (A) One mL fractions were carefully removed beginning at the top of the centrifuge tube and analysed for protein content.

(B) Recombinant mCST content in the fractions was varified by Western blotting using anti-FLAG mAb M2 as the primary antibody. The region of the blot corresponding to 30 kDa, the expected molecular mass of mCST, is shown. Twenty microgram of protein was loaded per lane.

Figure 1

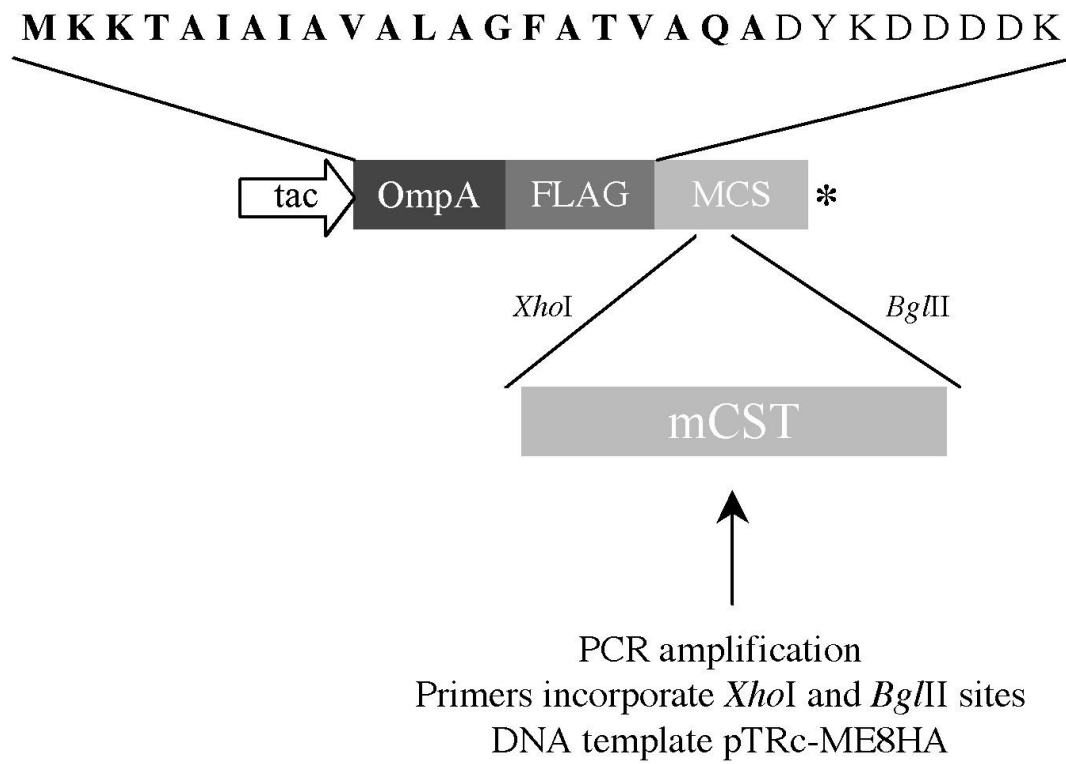


Figure 2

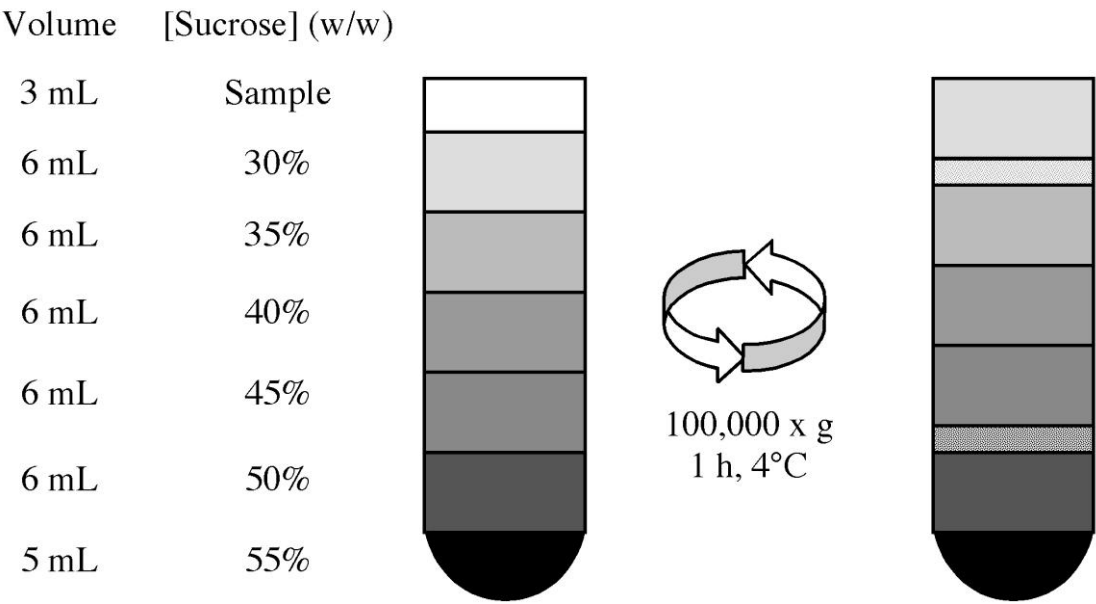


Figure 3

