Strategies for Over-Expression of Human Membrane Transport Systems

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Membrane proteins are crucial for life, because they allow the various body regions to communicate with each other. Ten percent of human genes encode for membrane transport systems, which are key components in maintaining cell homeostasis. They are involved in the transport of nutrients, catabolites, vitamins, and ions, allowing the absorption and distribution of these compounds to the various body regions. In addition, roughly 60% of FDA-approved drugs interact with membrane proteins, among which are transporters, often responsible for pharmacokinetics and side effects. Defects of membrane transport systems can cause diseases; however, knowledge of the structure/function relationships of transporters is still limited.

Keywords: membrane proteins ; over-expression ; channels

1. Membrane Transport Systems: A Brief Overview

1.1. Solute Carrier (SLC)

The SLC superfamily currently includes more than 450 transport proteins grouped into 65 families that transport a wide variety of substances across cell membranes [1][2]. Within each family, protein members share at least 20–25% sequence similarity with at least one other member of the family [2]. SLCs transport a wide variety of molecules, including sugars, amino acids, vitamins, nucleotides, inorganic and organic ions, oligopeptides, and xenobiotics/drugs [3]. Among the SLC transporter superfamily, some members exhibit broad substrate specificity, and others transport only one or a restricted group of biomolecules. Many members are still ‘orphans’, with no known substrate. The SLC superfamily includes either (i) facilitative transporters that act as a simple gatekeeper for a compound that passively moves down its concentration gradient, or (ii) secondary active transporters moving two substrates; one substrate moves down its electro/chemical gradient, providing the free energy to drive the transport of the other substrate against the concentration gradient. A hydropathy plot analysis performed on SLC proteins showed that they contain one to sixteen transmembrane (TM) domains, although most (~83%) are characterized by seven to twelve TM domains [4]. Two of the most common structural folds for SLC members are the major facilitator superfamily (MFS) and the leucine transporter (LeuT)-like folds [4]. Due to the difficulty in expressing and purifying these proteins, relatively few high-resolution structures for human SLCs have been solved [1]. To date, only 41 out of 458 (8.95%) are available in the PDB database. To unravel new molecular and functional aspects of known SLC transporters and to deorphanize the still unknown members, it is crucial to over-express the protein for performing in vitro functional assays. In the following sections, the strategy used for the bacterial expression of SLC transporters is described.

1.2. ABC Transporters

The ABC is one of the biggest protein transporter superfamilies present in all living organisms [5][6]. Human ABC transporters possess an ATP-binding cassette, also known as the ‘nucleotide-binding domain’ (NBD). The NBD presents several highly conserved motifs, including the Walker A and Walker B sequences, the ABC signature motif, the H loop, and the Q loop. Besides the NDB domain, ABC transporters also contain trans-membrane domains (TMDs) characterized by several hydrophobic α-helices. The ABC transporter core unit consists of two NBDs that bind and hydrolyze ATP, and two TMDs that are involved in substrate recognition and translocation across the lipid membrane [7]. In humans, there are 49 known ABC genes classified into seven different families (A–G) depending on their amino acid sequence and, eventually, on their protein domains [8]. Mutations in at least 11 of these genes are already known to cause severe inherited diseases such as cystic fibrosis and X-linked adrenoleukodystrophy (X-ALD) [7]. A variety of substances such as ions, carbohydrates, lipids, xenobiotics, and drugs are actively transported out of cells or into cellular organelles [9]. Most human ABC transporters are expressed as full-length proteins, whereas seven members of the B family, four of the D family, and five of the G family are expressed as “half-transporters” that must dimerize to be functional [7][8]. In the

following sections, researchers focus on human ABC transporter family members that are expressed in *E. coli* and functionally characterized.

1.3. Channels

Ion channels are integral membrane proteins that selectively conduct ions across the cellular membranes. Ion channels possess common structural characteristics that include a water-filled permeation pathway, also called a pore, which allows ions to flow across the cell membrane, and a selectivity filter, which is the narrowest part of the pore and is responsible for ion species selection [10]. Ion channels have a gating mechanism regulating the switch between the open and closed conformations. On the basis of the switch regulator, ion channels can be divided into voltage-gated ion channels, which are regulated by changes in membrane potential, and ligand-gated ion channels, modulated by the binding of a ligand such as a hormone or a neurotransmitter. Ion channels are ubiquitous and are involved in the regulation of many physiological events such as excitability, contraction, cell-cycle progression, and metabolism. Thus, defects in ion channel function can impair important biological processes, leading to a wide range of diseases [11].

2. Successful Over-Expression of Transport Systems

2.1. SLC1

The SLC1 family includes seven members involved in amino acid traffic in cells. The family members are divided in two groups according to substrate specificity and transport mode [12]. The first group (members A1, A2, A3, A6, and A7), known as EAATs (excitatory amino acid transporters), includes transporters with high affinity for the negatively charged amino acids glutamate and aspartate; the second group includes SLC1A4 and A5, known as ASCTs (alanine, serine, and cysteine transporters), involved in the traffic of several neutral amino acids in a broad set of tissues [13]. The members of the SLC1 family possess eight membrane-spanning domains, are glycosylated, and contain between 524 and 574 amino acids. Even though the 3D-structures of the last two members have recently been solved by Cryo-EM using *P. pastoris* as the expression host [14][15], the ASCT2 protein was also produced in *E. coli* [16]. The expression strategy employed the use of the Rosetta-gami2 strain, which joins the human tRNA supply and is involved in disulfide formation. For successful expression, a low temperature strategy had to be used to reduce protein toxicity. To achieve this, the pCOLD I vector—which carries the cold shock protein (csp), a promoter activated at low temperature—was used for cDNA cloning. Before the addition of 0.4 mM IPTG, a cold shock (10 min on ice and 40 min at 15 °C) was performed to improve the transcription of the ASCT2 mRNA and the stability of the 5′-UTR according to the feature of the cold-shock protein A promoter [17]. To antagonize the protein toxicity observed as cell culture OD reduction, glucose was added to prevent leakiness, and the growth temperature post-induction was kept at 15 °C to reduce the basal metabolism.

2.2. SLC2

The SLC2 family includes 14 GLUT members involved in the transport of monosaccharides, polyols, and other small carbon compounds across eukaryotic cell membranes [18]. The GLUT proteins contain about 500 amino acid residues, are glycosylated, and have 12 membrane-spanning domains. The human GLUT1 transporter, coded by the SLC2A1 gene, is the first example of a human transporter expressed in *E. coli* [19]. The cDNA coding for hGLUT1 was cloned into a pGTSD12 plasmid with an upstream prokaryote-type ribosome binding site in a T7 promoter/T7 polymerase expression system. The strategy consisted of exploiting the insertion of the recombinant GLUT1 protein in the cell membrane of the SR425 *E. coli* strain, which was rid of all the bacterial glucose transporter genes (*pts*G, *ptsM*, *gal*) and transformed with the gene coding for the T7 RNA polymerase under the control of the heat-inducible pL promoter. In this case, the transport function of hGLUT1 could be assayed directly in the bacterial host. ^{14}C-glucose transport inhibited by 2-deoxy-D-glucose and D-glucose, but not by L-glucose, was observed, confirming the expression of the target protein. In this system, the kinetics of transport were also studied, highlighting that—as observed in erythrocytes—glucose transport is inhibited by cytochalasin B and by mercuric chloride [19].

2.3. SLC3

The SLC3 family includes two members—SLC3A1 (also named rBAT) and SLC3A2 (also named 4F2hc or CD98hc)—that share about 20% of their amino acid sequence identity [20]. Both proteins are N-glycosylated: ~94 and ~85 kDa for the mature glycosylated forms of rBAT (685 aa) and 4F2hc (630 aa), respectively. The two proteins are type-II membrane N-glycoproteins with a single TMD and an intracellular N-terminus. They are characterized by a bulky extracellular C-terminus domain (50–60 kDa) that has been expressed in *E. coli*. Being highly water soluble, this domain has been crystallized and its structure solved by X-ray diffraction [21]. Even though these proteins are classified as members of the SLC superfamily, they are not directly involved in solute transport, but form heterodimers with some members of the SLC7

family, which are the subunits competent for transport [22]. cDNA coding for the entirety of SLC3A2 was cloned in a pGEX-4T1 vector that includes an N-terminal GST tag. The corresponding protein could then be over-expressed in Rosetta(DE3)pLysS, probably due to the higher solubility of the chimeric protein (SLC3A2-GST) with respect to the sole SLC3A2. The chimeric protein was purified on a glutathione Sepharose 4B affinity column, then cleaved using thrombin treatment [23].

2.4. SLC5

The SLC5 family includes 12 members that are sodium-dependent transporters involved in intestinal absorption (SLC5A1) or in the renal re-absorption (SLC5A2) of sugars [24]. The sole member of this family expressed in *E. coli* is SLC5A1 [25]. The use of a BL21 strain defective in the outer membrane protease (OmpT), together with low incubation temperatures (16 °C) and transcriptional regulation from the lac promoter/operator, have been crucial to reducing proteolytic degradation. Because bacterial cotransporters possess significantly shorter N-terminal hydrophilic extensions with respect to their eukaryote counterparts [26], amino acid residues 12–28 were removed, promoting insertion in the *E. coli* membrane. To recover the over-expressed protein and assay the transport function, SLC5A1 was solubilized with FosCholine-12 detergent, purified, and reconstituted in proteoliposomes in an active form [25].

2.5. SLC6

This family includes 20 secondary active co-transporters with 12 membrane-spanning domains that utilize a chemiosmotic Na^+ gradient and/or Cl^- to couple the transport of their substrates across a membrane [27]. The serotonin transporter (SLC6A4) was expressed and targeted to *E. coli* membranes by combining codon optimization and tRNA supply in different strains and media [28]. Another member of the SLC6 family, the SLC6A19 (also named B0AT1), was expressed in *E. coli* exploiting human tRNA supplementation (BL21 CodonPlus RIL strain), combining a cold shock strategy (csp promoter, see SLC1A5) with a very low inducer concentration (10 µM) in the presence of 0.5% glucose [16]. This protein is in complex with ACE2, constituting part of the receptor for the SARS-CoV-2 RBD proteins [29]. The production of B0AT1 in *E. coli* at a high yield can be useful for studying the interaction with compounds, which may have the potential for application as COVID-19 drugs [30][31].

2.6. SLC7

The SLC7 family includes 13 members divided in two subfamilies: the cationic amino acid transporters (CATs, SLC7A1–4, and SLC7A14), and the light or catalytic subunits (L-type amino acid transporters LATs, and SLC7A5-13) of the heteromeric amino acid transporters (HATs); these are mostly exchangers with a broad spectrum of substrates, ranging from neutral to negatively charged amino acids [20]. The members of this family differ in length—ranging from 470 amino acids for the SLC7A13 to 771 amino acids for the SLC7A14 members—and, consequently, in TM domains (12–14). SLC7A5 was over-expressed in the Rosetta(DE3)pLysS strain, (human tRNA supply) under standard conditions such as 4 h of 0.4 mM IPTG induction at 28 °C [23]. The addition of an N-terminal 6His tag was crucial for IMAC purification of a protein that was refolded on a column and reconstituted into proteoliposomes. Several structure/function relationships were also defined thanks to the production of several mutants, with relevance to physiology and pathology [32][33][34].

2.7. SLC17

The SLC17 family is a group of nine structurally related membrane proteins that mediate the transport of organic anions. SLC17A1–4s, also known as type-I phosphate transporters, are involved in the sodium-dependent transport of inorganic phosphate and other organic anions, such as urate and para-aminohippurate [35]. The member SLC17A5, also known as sialin, catalyzes the lysosomal transport of sialic acid and acidic sugar, including glucuronic acid. The SLC17A6-8s (vGLUTs) localize to synaptic vesicles, but each appears to have a different distribution among other cell membranes where they are involved in glutamate transport [36]. The SLC17A9 member (VNUT) is expressed in several tissues in mammals and is involved in vesicular nucleotide transport [37]. The SLC17 family members share a similar topology, since they are predicted to have 12 TM domains with intracellular N- and C-*termini*, as confirmed by the recently solved structure of the rat orthologous SLC17A6 [38]. Two out of nine members of the SLC17 family were expressed in *E. coli*, exploiting a strategy that combines low temperature expression and membrane targeting using N-terminal and/or C-terminal fusion peptides, constituting 120 amino acids of the YbeL bacterial protein (named β- domain) [39]. In particular, β-domains were added at both the N- and C-termini of the SLC17A5 and only at the C terminus of the SLC17A9 cDNAs. The fusion constructs were cloned in a pET-28a(+) vector. C43 cells were induced with 1 mM IPTG at 18 °C for 16 h in order to promote the insertion of the proteins in the membrane of the bacterial host. The proteins were purified using Ni-NTA chromatography and reconstituted in liposomes in a functionally active state [39].

2.8. SLC18

The four members of the SLC18 family mediate the transport of neurotransmitters (SLC18A1-A3) or polyamines (SLC18B1) [40][41]. Most computer-based predictions suggest 12 TMs with the N- and C-termini facing the intracellular milieu and a larger luminal loop between the first and second transmembrane domains. For the expression of the SLC18A3/VAChT, which mediates the transport of acetylcholine, the same strategy adopted for the members of the SLC17 family was used. The insertion in the C43(DE3) membrane was triggered by adding a YbeL tag at both the N- and C-termini of the protein after cloning in a pET-28a(+) vector. The induction of protein synthesis occurred at 18 °C for 16 h in the presence of 1 mM IPTG [42]. The use of a 6His tag allowed purification via Ni-NTA affinity chromatography, followed by MALDI mass spectrometry analysis. Which confirmed the production of the target protein [42].

2.9. SLC22

The SLC22 family is consists of at least 31 transporters expressed on both the apical and basolateral surfaces of epithelial cells, where they direct small-molecule transport between the body fluids and vital organs, such as the kidney, liver, heart, and brain [43][44]. The family includes organic cation transporters (OCTs), novel organic cation transporters (OCTNs), and organic anion transporters (OATs) with different modes of transport. They have been defined as “drug transporters” due to their role in the absorption and excretion of drugs. These proteins share 12 α-helical TM domains, a large extracellular domain between TM1 and TM2, and a large intracellular domain between TM6 and TM7 [45]. Two members of the OCTN subfamily (SLC22A4 and SLC22A5) were over-expressed in *E. coli* with different strategies [46][47]. The cDNA coding for SLC22A4/OCTN1 was cloned in the pH6EX3 expression vector, and the Rosetta(DE3)pLysS strain was used for human tRNA supply. Protein synthesis was induced with 0.4 mM IPTG at 28 °C for 6 h and a 6His-tagged protein was purified and functionally reconstituted in proteoliposomes [47][48][49]. After codon optimization, the use of the Lemo21 strain strongly increased the production of the target protein [50]. The over-expression of OCTN1 and the production of several mutants allowed researchers to reveal the structure/function relationships between this transporter and the molecular basis of human diseases [48][49][51]. Moreover, a specific antibody (anti-OCTN1) was produced using the over-expressed protein [52]. Despite the high sequence similarity (86.5%), the same approach was not effective for SLC22A5/OCTN2. For the production of the protein, the use of an N-terminal GST tag was exploited, but the amount of protein recovered after expression in Rosetta(DE3)pLysS and tag removal was quite low [46]. Interestingly, the conservative substitution of the second codon (R2K), introducing the statistically most present codon at the second position of *E. coli* genes [53], allowed the production of the target protein in a much larger amount with respect to the wild-type protein [46].

2.10. SLC25

With its 53 members, the SLC25 family is the largest among the solute carrier families. Based on sequence similarities, human MCs cluster into many different clades, suggesting a large variety of transported substrates, among which are nucleotides, carboxylates, amino acids [54]. Most MCs contain about 300 amino acids, with six TMs and the N- and C-termini protruding towards the cytosolic side of the inner membrane. For the expression of several members of the SLC25 family, neither human tRNA supply nor codon optimization was necessary (**Table 1**). The most-used strategy for the expression of several members of the SLC25 family was based on inclusion body formation, obtained by combining a BL21(DE3) derivative strain with a pET or T7 derivative plasmid (**Table 1**). In particular, the first protein over-expressed in a bacterial host, and then purified and assayed in an in vitro system, was the bovine oxoglutarate carrier, which was cloned in a pRUN vector [55]. Then, the oxodicarboxylate (ODC, SLC25A21), and glutamate carrier 1 and 2 (SLC25A22 and SLC25A18, respectively) were also cloned in the pRUN plasmid. The corresponding proteins were expressed as inclusion bodies after culturing C0214 cells for 4.5 h in the presence of 0.4 mM IPTG at 37 °C, and reconstituted in proteoliposomes in an active form [56][57]. The same method with a similar plasmid or strain was used for studying the carnitine/acylcarnitine translocase (CACT) [58]; the ornithine/citrulline carrier (ORNT1) [59]; the basic amino acid transporter (SLC25A29/ORNT3) [60]; the S-adenosylmethionine transporter (SLC25A26) [61]; the ATP-Mg/Pi transporters APC1 (SLC25A24) and APC3 (SLC25A25) [62]; and the peroxisomal transporter of coenzyme A, FAD and NAD^+ (SLC25A17/PMP34) [63]. This strategy was also used for the production of all the UCP isoforms (see **Table 1**). Indeed, Ivanova et al. cloned all five UCPs in pET-21a(+) and expressed the corresponding proteins in BL21(DE3) as a 6His-tagged protein in inclusion bodies, after 3h of 1 mM IPTG induction at 37 °C [64]. Following purification, the UCPs were reconstituted in stable, small, unilamellar vesicles, as confirmed by circular dichroism analysis [64]. The same expression strategy was adopted for the expression of UCP2 and UCP3, which were refolded from inclusion bodies using a dialysis method [65][66]. A completely different approach was used for the expression of the UCP1 protein, in which the insertion of the protein into the *E. coli* membrane was obtained by adding the small periplasmic leader sequence (PelB), provided by the pET-26b(+) vector and culturing BL21 CodonPlus(DE3)-RIPL with an auto-induction method [67]. The

protein was extracted from the *E. coli* membrane, purified, and reconstituted into phospholipid bilayers in an active form [68]. In addition to *E. coli*, *L. lactis* was also used for the expression of the regulatory domains of aralar1 (SLC25A12) and citrin (SLC25A13). In particular, the N- and C-terminal domain of both proteins were cloned in the pNZ8048 expression vector, produced as 8His-tagged proteins in *L. lactis* NZ9000, purified using a nickel-Sepharose high-performance column, and crystallized [69].

Table 1. Strategy adopted for membrane protein expression.

Protein/Alias	Plasmid	Tag	Strain	Strategy	Function	Reference
SLC1A5/ASCT2	pCOLD-I	N-Ter 6His	Rosetta-gami2	Low temperature/glucose	ND	[16]
SLC2A1/GLUT1	pGTSD12	None	SR425	P_L promoter, membrane insertion	M	[19]
SLC3A2/4F2hc	pGEX-4T1	N-Ter-GST	Rosetta(DE3)pLysS	28 °C	ND	[23]
SLC5A1/SGLT1	pTMH-6FH	FLAG	BL-21	0.3 mM IPTG, 16 °C, 5 h	P	[25]
SLC6A4/SERT	TAGZyme pQE-2	N-Ter 8His	BL21 CodonPlus (DE3) RP	Recovery from membrane	S	[70]
SLC6A19/B0AT1	pCOLD-I	N-Ter 6His	BL21 codonPlus RIL	Low temperature/low IPTG/glucose	ND	[16]
SLC7A5/LAT1	pH6EX3	N-Ter 6His	Rosetta(DE3)pLysS	0.4 mM IPTG, 28 °C, 4 h	P	[23]
SLC17A5/Sialin	pET-28a(+)	N-Ter and C-Ter β domain	C43(DE3)	Membrane insertion, 1 mM IPTG, 18 °C, 16 h	M	[39]
SLC17A9/VNUT	pET-28a(+)	N-Ter β domain	C43(DE3)	Membrane insertion, 1 mM IPTG, 18 °C, 16 h	M	[39]
SLC18A3/VAChT	pET-28a(+)	N- and C-ter YbeL	C43(DE3)	Membrane insertion, 1 mM IPTG, 18 °C, 16 h	M	[42]
SLC22A4/OCTN1	pH6EX3	N-Ter 6His	Rosetta(DE3)pLysS	0.4 mM IPTG 28 °C, 6 h	P	[47]
SLC22A4/OCTN1	pH6EX3	N-Ter 6His	Lemo21(DE3)	Codon optimization, 0.4 mM IPTG 28 °C, 6 h	P	[50]
SLC22A5/OCTN2	pET-21a(+)	C-Ter 6His	Rosetta(DE3)pLysS	R2K mutation	N	[46]
SLC22A5/OCTN2	pET-41a(+)	GST	Rosetta(DE3)pLysS	28 °C, 6 h	N	[46]
SLC25A7/UCP1	pET-26b(+)	PelB/6His/TEV	BL21 CodonPlus (DE3)-RIPL	Auto-induction method	S,P	[67]
SLC25A7/UCP1	pET-21a(+)	N-Ter 6His	BL21(DE3)	1 mM IPTG, 37 °C, 3 h	S,P	[64]
SLC25A8/UCP2	pET-21a(+)	N-Ter 6His	BL21(DE3)	1 mM IPTG, 37 °C, 3 h	S,P	[64]
SLC25A8/UCP2	pET-21a(+)	None	BL21(DE3)	1 mM IPTG, 30 °C, 6 h	P	[66]
SLC25A8/UCP2	pMW172	None	C41	2 h, 1 mM IPTG at 37 °C	ND	[65]
SLC25A9/UCP3	pET-21d(+)	N-Ter 6His	BL21(DE3)	1 mM IPTG, 37 °C, 3 h	S,P	[64]
SLC25A9/UCP3	pET-21a(+)	None	BL21(DE3)	1 mM IPTG, 30 °C, 6 h	P	[66]
SLC25A9/UCP3	pET-24a(+)	None	BL21(DE3)	1 mM IPTG, 37 °C, 2 h	P	[71]
SLC25A12/Aralar1	pNZ8048	N-Ter 8His	*L. lactis* NZ9000	Codon optimization	C,P	[69]
SLC25A13/Citrin	pNZ8048	N-Ter 8His	*L. lactis* NZ9000	Codon optimization	C,P	[69]
SLC25A14/UCP5	pET-21a(+)	N-Ter 6His	BL21(DE3)	1 mM IPTG, 37 °C, 3 h	S,P	[64]
SLC25A15/ORNT1	pET-21a(+)	C-Ter 6His	C0214	0.4 mM IPTG, 28 °C 4 h	P	[59]
SLC25A17/ PMP34	pET-21b	T7	Rosetta-gami B	30 °C inclusion bodies	P	[63]

Protein/Alias	Plasmid	Tag	Strain	Strategy	Function	Reference
SLC25A18/GC2 SLC25A22/GC1	pRUN	None	C0214(DE3)	0.4 mM IPTG, 37 °C 4.5 h	P	[57]
SLC25A20/CACT	pMW7	None	C0214(DE3)	0.4 mM IPTG, 37 °C 4 h	P	[58]
SLC25A21/ODC	pRUN	None	C0214(DE3)	0.4 mM IPTG, 37 °C 4.5 h	P	[56]
SLC25A24/APC1 SLC25A25/APC3	pQE30	N-Ter 6His	M15(pREP4)	Manufacturer's instructions	P	[62]
SLC25A26/SAMC	pRUN	None	BL-21 CodonPlus(DE3)-RIL	0.4 mM IPTG, 37 °C 4.5 h	P	[61]
SLC25A27/UCP4	pET-21a(+)	N-Ter 6His	BL21CodonPlus (DE3)-RIPL	1 mM IPTG, 37 °C, 3 h	S,P	[64]
SLC25A29/ORNT3	pRUN	None	Rosetta-gami B(DE3)	37 °C	P	[60]
SLC29A1/ENT1	pHAT20	3xFLAG	BL21(DE3)	<25 °C		[72]
SLC30A8/ZnT8	pTrxFus	N-Ter Thioredoxin	GI724	Inclusion bodies	ND	[73]
SLC30A8/ZnT8	pTrxFus	N-Ter Thioredoxin	GI698	Intracellular soluble fraction	ND	[73]
SLC35C1/FUCT1	pJOE2702	ompA/FLAG	BW25113(DE3)	Codon optimization, membrane insertion	M	[74]
SLC35F3	pZE12luc	None	MW25113	Membrane insertion	M	[75]
SLC38A9	pH6EX3	N-Ter 6His	Lemo21(DE3)	Codon optimization, 39 °C, 2 h	P	[76]
SLC52A2/RFVT2	pH6EX3	N-Ter 6His	Rosetta(DE3)	Codon optimization	P	[77][78]
ABCB10	pET19b	N-Ter 6His	Rosetta2, BL21-CodonPlus (DE3)-RIPL	Codon optimization	L,S	[79]
MDR1	pPOW-B2	None	UT5600	Membrane insertion	M	[80]
hTRPV3	pWaldo–GFPe	GFP	BL21(DE3)-R3-pRARE 2	1 mM IPTG, TB medium, at 18 °C,	B	[81]
EAG1	pRSET-A	N-Ter 6His	BL21(DE3)	1 mM IPTG, 37 °C, 4 h	ND	[82]
MiRP1	pET-21b	C-Ter 6His	BL21(DE3) CodonPlus RP	1 mM IPTG, 37 °C, 4 h	S	[28]
ROMK1	pET-28a(+)	Several tags	BL21(DE3)-pLysS	Codon optimization, membrane insertion	L	[83]
CLIC4	pET-22b(+)	C-Ter 6His	BL21(DE3)	1 mM IPTG, 37 °C, 6 h	C	[84]
MICU1	pGEX-6p-1	N-Ter GST	BL21 DE3	0.5 mM IPTG, 16 °C, 20 h	S	[85]
MICU2-NΔ84-CΔ28	pET-28a(+)	N-Ter 6His	BL21 DE3	0.5 mM IPTG, 16 °C, 20	S	[85]
hVDAC1	pET-21a(+)	C-Ter 6His	BL21(DE3)	1 mM IPTG, 37 °C, 3 h	N,PP	[86][87]
hVDAC1 hVDAC2	PDS56/RBII	C-Ter 6His	M15(pREP4)	1 mM IPTG, 37 °C, 5–6 h	PP,S	[88]
hVDAC3	pET-21a(+)	C-Ter 6His	BL21(DE3)	1 mM IPTG, 37 °C, 3 h	PP	[89]

B: black lipid membranes; C: Crystallization; L: lipid nanodiscs; M: membrane-targeting; N: NMR; ND: not determined; P: proteoliposomes; PP: planar phospholipid membranes; S: spectroscopic analyses.

2.11. SLC29

The SLC29 family includes four members present in most cell types and tissue, designated equilibrative nucleoside transporters (ENTs) because of the properties of the first-characterised member of the family, hENT1. ENT1 and ENT2, have a similar broad substrate specificity for purine and pyrimidine nucleosides, but ENT2 is also competent in nucleobases transport [90]. ENT1 and ENT2 are localized in the basolateral membrane of polarized cells. ENT3 is widely distributed and is present in lysosomal and mitochondrial membranes [91]. ENT4 is present on plasma membranes and transports adenosine and monoamines to the brain and heart [92]. The bacterial expression of SLC29A1/ENT1 tagged with an N-HAT-3X-FLAG was obtained in BL21(DE3) under the control of a lac promoter. The use of this promoter was crucial, since it allowed a slow constitutive transcription rate that was well tolerated by the translational machinery of the bacterial host when cultured below 25 °C [72].

2.12. SLC30

The SLC30 family includes 10 members involved in Zn^{2+} homeostasis in the body. Based on sequence similarities, the SLC30 family members can be divided into four subfamilies. Despite the difference in amino acid length—ranging from 323 amino acids for SLC30A2 to 765 amino acids for SLC30A5—all the members of the family are predicted to have six TM domains and a histidine/serine-rich loop between TM4 and TM5. The N- and C-terminal ends are localized intracellularly, facing the cytosol [93]. A member of this family, ZnT8 (SLC30A8)—and in particular, the C-terminal domain of the transporter (amino acids 268–369)—was identified as a target of autoantibodies in type-I Diabetes Mellitus (DM) [94]. These auto-antibodies were detected in more than 60% of patients with type 1 DM [94]. Thus, the over-expression of the C-terminal domain can help in the diagnosis of type-I DM. The codon-optimized sequence coding for the C-terminal domain of the ZnT8 transporter was cloned in a specific plasmid and the corresponding peptide was produced as thioredoxin (Trx), tagged either in inclusion bodies using the GI724 strain or in the soluble fraction of cell lysate culturing GI698 cells (**Table 1**). The peptide was successfully purified in a properly folded state that could be useful for developing new low-cost tests for the detection of ZnT8 autoantibodies [73].

2.13. SLC35

The SLC35 family includes thirty-one members that are divided into seven subfamilies, from SLC35A to SLC35G, also named nucleotide sugar transporters (NST); they connect the synthesis of activated sugars in the nucleus or cytosol to glycosyltransferases that reside in endoplasmic reticulum (ER) and/or Golgi apparatus [95]. This connection is crucial for regulating sugar- and organelle-specific protein glycosylation [96], and there are further indications that some members of this family may also form multiprotein complexes with glycosyltransferases [97]. For the majority of the SLC35 family members, a topology characterized by 10 TM α-helices connected by short loops, with N- and C-termini located on the cytoplasmic side, is predicted [96]. The expression of the GDP-L-fucose antiporter SLC35C1 in an active form was obtained in the BW25113(DE3) *E. coli* strain specifically deleted (Δ*fucAK)* [74]. The expression strategy combined the codon optimization of the gene and the use of a N-terminal OmpA signal sequence, promoting the insertion of the target protein in the bacterial membrane. The cultures were induced for five hours with 0.01% L-rhamnose. To study the GDP-L-fucose transport *in vitro*, inside-out vesicles of the OmpA-FucT1 expressing *E. coli* strains were produced and kinetic parameters were also measured, confirming the production of the target protein in a functional state [74]. Another member of the SLC35 family, (the putative thiamine transporter, member F3), was successfully produced in *E. coli* [75]. The plasmid pZE12luc with a strong and tightly regulated $P_L$lacO1 promoter was used for cloning the target gene [98]. The MW25113 strain with either thiamine transport [ThiBPQ = sfuABC] or with its biosynthetic [*ThiH*] genes inactivated was used for producing the transporter in the bacterial membrane. The uptake of [^{3}H]-thiamine confirmed the expression of the protein in a folded conformation and allowed researchers to perform functional studies [75].

2.14. SLC38

The SLC38 family includes eleven members and is part of a phylogenetic cluster of amino acid transporters comprising the SLC32 and SLC36 families [99]. They mediate the Na^+-dependent net uptake and efflux of small neutral amino acids. They are particularly expressed in cells that grow actively, or in cells with active amino acid metabolism, such as the liver, kidney, and brain [100]. The overall structure most likely consists of 11 (TM) α-helices with the N-terminal domain protruding towards the cytosol. One of the most interesting members of this family is A9, which is a lysosomal amino acid transporter involved in sensing amino acid sufficiency for the activation of the mechanistic target of rapamycin complex 1 (mTORC1), which regulates cell growth and proliferation [76][101]. The heterologous expression of the A9 member of the SLC38 family in *E. coli* caused cell death, probably as a consequence of Sec-translocon saturation. To overcome this problem, a codon optimization strategy with modulation of RNA polymerase T7 activity was attempted [76]. Culturing a Lemo21(DE3) strain in the presence of 0.1 mM rhamnose to induce lysozyme T7 production (see Section 3.2), but for

only 2 h at 39 °C, allowed the synthesis of the target protein, which was reconstituted in proteoliposomes in an active form [76][102].

2.15. SLC52

The SLC52 family includes three members (SLC52A1-3) involved in regulating riboflavin homeostasis. SLC52A1 is highly expressed in the placenta and small intestine, SLC52A2 is ubiquitous, and SLC52A3 is predominantly expressed in the testes and small intestine [103]. Riboflavin is the precursor of the flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), which are essential cofactors for numerous enzymes (i.e., oxido-reductases, monooxygenases, oxidases); thus, the alteration of the flavoproteome can cause severe diseases [104]. Hydropathy profile analysis predicts 11 TM domains for all members of the family. To express member 2 of this family (SLC52A2), its codon-optimized cDNA was cloned under the control of the strong tac promoter, and the production of the corresponding 6His-tagged protein was obtained by culturing the Rosetta(DE3) strain at 28 °C in the presence of 0.4 mM IPTG [78]. The protein was purified by nickel-chelating affinity chromatography and reconstituted in proteoliposomes in a folded state; this allowed the discovery of novel aspects of the transport mechanism, such as inhibition by FMN and the interaction with Ca^{2+} ions [78]. Moreover, structure/function relationships were characterized thanks to the bacterially expressed proteins [77][78].

2.16. ABCB10

A small number of ATP-binding cassette (ABC) transporters are found in mitochondria. Most are half-transporters of the B-group-forming homodimers, and their topology suggests that they function as exporters [105]. The proteins contain an N-terminal transmembrane domain (TMD) and a C-terminal nucleotide-binding domain (NBD), which form homodimers for full functionality. To obtain the bacterial expression of the ABCB10 protein, the key factors were the use of a codon-optimized sequence and an N-terminal 6His tag obtained after cloning in the pET-19b vector. The cultivation of Rosetta 2, BL21-CodonPlus(DE3)-RIPL or Lemo21(DE3) gave similar positive results, allowing the production of the protein of interest after 3 h of induction at 30 °C in presence of 0.5 mM IPTG. The 6His-ABCB10 protein was solubilized from *E. coli* membranes and purified using Ni-NTA affinity chromatography. The folded state of the purified protein was assessed by circular dichroism spectra and reconstituted into a small lipid-bilayer system known as nanodiscs [79].

2.17. MDR1/P-gp

MDR1, also known as P-glycoprotein (P-gp), is a member of the ABC superfamily, present in both prokaryotes and eukaryotes, which regulate the traffic of various molecules and the extrusion of xenobiotics from cells. This 170-kDa membrane protein confers cross-resistance to many natural drugs such as anthracyclines, actinomycin D, Vinca alkaloids, and peptide antibiotics [106]. The strategy adopted for the bacterial expression of MDR1 was the use of the heat-inducible pL promoter. The full-length cDNA coding for MDR1 was cloned into a pPOW-B2 vector. UT5600 cells were cultured at 30 °C and the expression of the target protein was induced, shifting the temperature to 42 °C for 30 min. The production of the MDR1 protein in the *E. coli* membrane was confirmed by transport assays in which the uptake of ^{3}H TPP^{+} (tetraphenylphosphonium) was measured [80]. Some years later, Linton and Higgins produced a lot of chimeric proteins of truncated P-gp fused to β-lactamase, to solve the enigma of the different topology when expressed in bacterial or mammalian cells. They concluded that P-gp could misfold during *E. coli* expression due to the misrecognition of multiple P-gp sequences as topogenic signals [107]. Altogether, the described works on ABC transporters indicate that, in the case of ABCB10, *E. coli* is suitable, likely due to its nature as a mitochondrial protein whose properties resemble those in bacteria. On the other hand, in other cases, researchers can expect that new technology would make *E. coli* a more favorable host.

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