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SHORT COMMUNICATION

# Low temperature bacterial expression of the neutral amino acid transporters SLC1A5 (ASCT2), and SLC6A19 (B0AT1)

**Michele Galluccio[1] · Marta Pantanella[1] · Deborah Giudice[1] · Stefania Brescia[1] · Cesare Indiveri[1,2]**

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## Abstract

It is well established that *Escherichia coli* represents a powerful tool for the over-expression of human proteins for structure/function studies. In many cases, such as for membrane transporters, the bacterial toxicity or the aggregation of the target protein hamper the expression limiting the application of this tool. The aim of this study was finding the appropriate conditions for the expression of reluctant proteins that is the human neutral amino acid transporters ASCT2 and B0AT1, that have great relevance to human health in cancer therapy and in COVID-19 research, respectively. The cDNAs coding for the proteins of interest were cloned in the pCOLD I vector and different *E. coli* strains (BL21 codon plus RIL, and RosettaGami2) were cultured in absence or in presence of glucose (0.5–1%), at low temperature (15 °C), and low inducer concentrations (10–100 μM). Cell growth and protein production were monitored by optical density measurements and western blotting assay, respectively. Even though in different conditions, the expression of both amino acid transporters was obtained.Reducing the growth rate of specific *E. coli* strains by lowering the temperature and the IPTG concentration, together with the addition of glucose, two reluctant human neutral amino acid transporters have been expressed in *E. coli*. The results have a potentially great interest in drug discovery since ASCT2 is an acknowledged target of anticancer therapy, and B0AT1 together with ACE2 is part of a receptor for the SARS-Cov-2 RBD proteins.

**Keywords** Amino acid · SLC · Protein expression · B0AT1 · ASCT2 · COVID-19

## Introduction

*Escherichia coli* represents the most used expression bacterial host, due to its easy handling, low costs, fast growth, and high expression yield. However, in many cases bacteria refuse to express human proteins for several reasons [1]. In these cases, the most frequently adopted strategy is that of switching to yeast or to mammalian cell lines. However, the yield in purified protein obtained with these approaches is often very poor and the cost, especially with mammalian cells, is much higher than the *E. coli* system [2]. Major difficulties in over-expressing human proteins have been encountered in the case of integral membrane proteins, such as transporters, which contain extensive hydrophobic regions and lead to bacterial cell toxicity [3]. This may be due to an imbalance between the amount of molecular chaperones, such as Dna K and GroEL, with respect to the recombinant protein that could trigger protein aggregation and inclusion bodies formation [4]. Another problem may arise from the saturation of the bacterial Sec translocon, leading to cell toxicity [5]. It is important to find some solutions to these problems since transporters are of great interest for the scientific community. They constitute a significant fraction of the human proteome (−10%) [6]. Among membrane transporters, SoLute Carriers (SLCs) are particularly interesting since these transporters reveal to be crucial in cell metabolism and count more than 400 members. These proteins control absorption and distribution of nutrients in the human body, ion transport and catabolite removal [7]. Since amino acids fall in the nutrient category with a huge diversity of cellular roles, the systematic study of the related transporters is one of the most attractive research topic, to date [8].

✉ Cesare Indiveri
cesare.indiveri@unical.it

[1] Department of DiBEST (Biologia, Ecologia, Scienze Della Terra) Unit of Biochemistry and Molecular Biotechnology, University of Calabria, Via Bucci 4C, 87036 Arcavacata Di Rende, CS, Italy

[2] CNR Institute of Biomembranes, Bioenergetics and Molecular Biotechnologies (IBIOM), Via Amendola 122/O, 70126 Bari, Italy

A further reason for this interest is the altered expression of several amino acid transporters in human pathologies. In particular, the up-regulation of amino acid transporters such as ASCT2 coded by SLC1A5 gene is reported in virtually all human cancers to fulfill the increased demand of glutamine [9]. Another transporter whose interest has increased a lot in the last months is SLC6A19 also known as B0AT1. Some mutations of this transporters are causative of the Hartnup disorder (OMIM 234500) [10]. Moreover, starting from the serendipity identification of a B0AT1 inhibitor [11], the pharmacological silencing of this transporter has been suggested as a treatment of Type 2 diabetes [12]. More importantly, B0AT1 has been described as a component of the SARS-Cov-2 cell receptor [13]. Both these transporters could not be expressed in *E. coli* to date [8, 14]. While its production has been previously unsuccessful in bacteria, the human ASCT2 protein has been successfully expressed in the eukaryote *Pichia pastoris* [15]. Its structure was then recently solved by Cryo-EM thanks to its successful expression in yeast [16]. B0AT1 has been expressed in HEK293 FreeStyle for solving the structure in complex with ACE2 [13]. In this scenario, approaches for achieving the low cost expression of this type of proteins are of great interest. Several approaches have been exploited in the case of other transporters to produce the protein of interest (POI), such as using different *E. coli* strains and promoters, codon biasing, implementing growth medium, changing inducer concentrations, introducing specific tags [1, 17–19]. Notwithstanding all the cited efforts, a "universal" expression strategy was never found. One of the tricks adopted sometimes for reducing protein aggregation is lowering the growth temperature. However, the available tools for this strategy only recently have been improved. In this work, a strategy based on very low growth temperature and inducer concentration, in presence of glucose, in combination with some specific *E. coli* strains, BL21 codon plus RIL, and RosettaGami2, is proposed as a solution for the expression of the human transporters B0AT1 and ASCT2. The described strategy can be then extended to the expression of other transporters.

## Materials and methods

Chemicals used for experiments, protease inhibitor cocktail (P8849), Human Dermal Fibroblasts (106-05A), Nickel Affinity Gel (HIS-Select®—P6611) and the Monoclonal Anti-polyHistidine-Peroxidase antibody (A7058) were from Sigma-Aldrich; Anti-ASCT2 (V501) was from Cell Signaling; pCOLD I plasmid from Takara; restriction endonucleases and specific reagents for cloning from Thermo scientific; *E. coli* RosettaGami2 strain from Novagen; BL21 codon plus RIL from Agilent technologies; codon optimized hB0AT1sequence was from Genscript.

### Cloning of hASCT2

To clone the cDNA encoding for human ASCT2 transporter (SLC1A5) (UniProtKB: Q15758; GenPept accession no. NP_005619.1), total RNA was isolated from primary human fibroblasts and reverse-transcribed. The 1626 bp cDNA corresponding to the hASCT2 encoding sequence was then amplified using the forward and reverse primers 5′-GGGAATTCCATATGGTGGCCGATCCTCCT CGAGACT-3′ and 5′-CCGGAATTCTTACATGACTGA TTCCTTCTCAGAG-3′, containing the *Nde* I and *Eco* RI sites, respectively. The amplified cDNA was cloned in the pCOLD I expression vector. The resulting recombinant plasmid, defined as pCOLD I-hASCT2, encodes a 6His-tagged fusion protein corresponding to the hASCT2 carrying the extra N-terminal sequence MNHKVHHHHHHIEGRH.

### Expression of hASCT2 transporter

To produce the 6His-hASCT2 recombinant protein, *Escherichia coli* RosettaGami2 cells, were transformed with the pCOLD I-hASCT2. Selection of transformed colonies was performed on LB-agar plates added with 100 µg/mL ampicillin and 34 µg/mL chloramphenicol. Different media (LB or TY 2X or M9 at pH 7.0) were prepared in absence or in presence of different glucose concentrations (0.5 or 1%). According to the number of conditions to be tested, colonies were inoculated in a different volume of a specific medium, and cultured overnight at 37 °C under rotary shaking (160 rpm). The day after, the culture was diluted 1:10 in fresh medium added with the specific antibiotics. When the optical density measured at 600 nm wavelength was 0.8–1, the cultures were shocked on ice for 10 min (under manual agitation every 1 min), then incubated for 40 min at 15 °C under rotary shaking (160 rpm). Different IPTG concentrations (from 0.1 to 1 mM) were tested to induce protein expression with the exception of one aliquot, grown in absence of inducer, (negative control). The cultures were continued for up to 24 h at 15 °C at 160 rpm. Every two hours, aliquots were collected and centrifuged at 3000×*g*, and at 4 °C for 10 min; the pellets were stored at −20 °C. A bacterial pellet aliquot, after thawing, was dissolved in a resuspension buffer (20 mM Hepes/Tris, 200 mM NaCl pH 7.5) added with protease inhibitor cocktail according to manufacturer instructions. The bacterial suspensions were sonified in an ice bath for 10 min (pulse of 1 s on, and 1 s off) at 40 W, using a Vibracell VCX-130 sonifier. The insoluble cell fractions were analyzed by SDS-PAGE and western blotting.

### Cloning of hB0AT1

The cDNA sequence coding for hB0AT1 transporter (SLC6A19) (UniprotKB: Q695T7; GenPept accession no. NM_001003841.3) was obtained by reverse transcription of the total RNA extracted as described in Cloning of hASCT2 section, followed by PCR amplification using the following forward and reverse primers: 5′-GGGAATTCCATATGG TGAGGCTCGTGCTGC-3′ and 5′-ACGCGTCGACTC AGTACTTCAGGTCCCCGTTCA-3′, containing the *Nde* I and *Sal* I restriction sites, respectively. Either wild type or codon optimized cDNA sequence were sub-cloned in the pCOLD I expression vector. The resulting recombinant plasmid, defined as pCOLD I-hB0AT1, encodes a 6His-tagged fusion protein corresponding to the hB0AT1 carrying the extra N-terminal sequence MNHKVHHHHHHIEGRH.

### Expression of hB0AT1 transporter

To produce the 6His-B0AT1 protein, *E. coli* BL21 codon plus RIL cells, were transformed with the pCOLD I-hB0AT1 construct. Selection of transformed colonies was performed on LB-agar plates added with 100 µg/mL ampicillin and 34 µg/mL chloramphenicol. Different media (LB or TY 2X or M9 at pH 7.0) prepared in absence or in presence of different glucose concentrations were tested. A colony was inoculated overnight at 37 °C under rotary shaking (160 rpm). The day after, the culture was diluted 1:20 in fresh medium added with the specific antibiotics. When the optical density measured at 600 nm wavelength was 0.8–1, the culture was treated before induction as described for hASCT2 expression. Different IPTG concentrations (from 0.01 to 1 mM) were tested to induce protein expression with the exception of one aliquot, grown in absence of inducer, (negative control). The cultures were continued for up to 24 h at 15 °C at 160 rpm. Every two hours, aliquots were collected, treated as described for hASCT2 expression, and analyzed by SDS-PAGE and western blotting.

### Purification of the hB0AT1 protein

In order to purify 6His-hB0AT1 protein, the pellet deriving from 12 mL of the bacterial suspension obtained as described above, was washed with 0.1 M Tris/HCl pH 8.0, and centrifuged at 12,000×*g* for 10 min. The new pellet was solubilized adding 400 µL of 8 M urea, 100 µL of 100 mM DTE, 54 µL of 10% sarkosyl, and 546 µL of a renaturing buffer (A) containing: 0.1% sarkosyl, 200 mM NaCl, 10% glycerol, and 20 mM Tris/HCl at pH 8.0. After centrifugation (12,000×g, 10 min, 4 °C), the supernatant was added to a His select $Ni^{2+}$ affinity gel column (0.5 × 7.5 cm; 1.5 mL = 1 column volume) equilibrated with 10 column volumes of a renaturing buffer A, and then mixed over night at 4 °C in a fixed angle rotator for tubes. The day after, the protein resin mix (about 15 mL) was transferred in a column and packed by gravity. 5 mL washing buffer (0.1% DDM, 200 mM NaCl, 10% glycerol, 5 mM DTE, 20 mM Tris/HCl at pH 8), 15 mL washing buffer added with 10 mM imidazole, 7 mL of washing buffer added with 200 mM imidazole were used for eluting proteins in 42 fractions (1 mL). Purified hB0AT1 protein was mainly present in the fractions 38–39.

### Western blot assay

hASCT2 and hB0AT1 were immuno-detected using the Monoclonal Anti-polyHistidine–Peroxidase antibody 1:10,000. In case of hASCT2, the immunostaining was also performed using a rabbit anti-ASCT2 diluted 1:1000. In this case peroxidase-linked anti-rabbit IgG was used as a secondary antibody (1:10,000).

## Results and discussion

### ASCT2 expression

*Escherichia coli* RosettaGami2 cells, were transformed with the pCOLDI-hASCT2 construct. This strain allows enhanced disulfide bond formation and harbors extra copies of unique types tRNAs that are typically rare in *E. coli* allowing expression of eukaryotic proteins. To prevent possible toxic effect following leakage expression of the POI, the effect of glucose addition during cell growth was tested [20]. Different colonies were separately inoculated in 10 mL of LB broth in presence of glucose 0.5% or 1% in comparison with the control without glucose. After 16 h, a 1:10 dilution in fresh LB containing the same glucose concentration was performed and the growth was monitored every 30 min. During the exponential phase of growth (OD ~ 0.8), the cultures were shocked on ice for 10 min, then incubated for 40 min at 15 °C to improve the transcription of the pCOLDI-ASCT2 mRNA and the stability of the 5′-UTR according to the feature of the cold shock protein A promoter [21]. Then, 0.4 mM IPTG was added to induce the synthesis of the POI. As expected, the addition of either 0.5% or 1% glucose (Fig. 1a) in the medium increased the rate of growth with respect to the absence of glucose (Fig. 1a). As a consequence of IPTG addition, an OD reduction was observed in any condition, until 6 h after induction, highlighting a putative toxic effect following the production of the POI, as previously observed [22]. The OD reduction was faster in the presence of glucose than in its absence, indicating that glucose induces a more toxic condition, probably related to a higher rate of heterologous protein production. At 22 h after IPTG addition, the toxic effect (OD

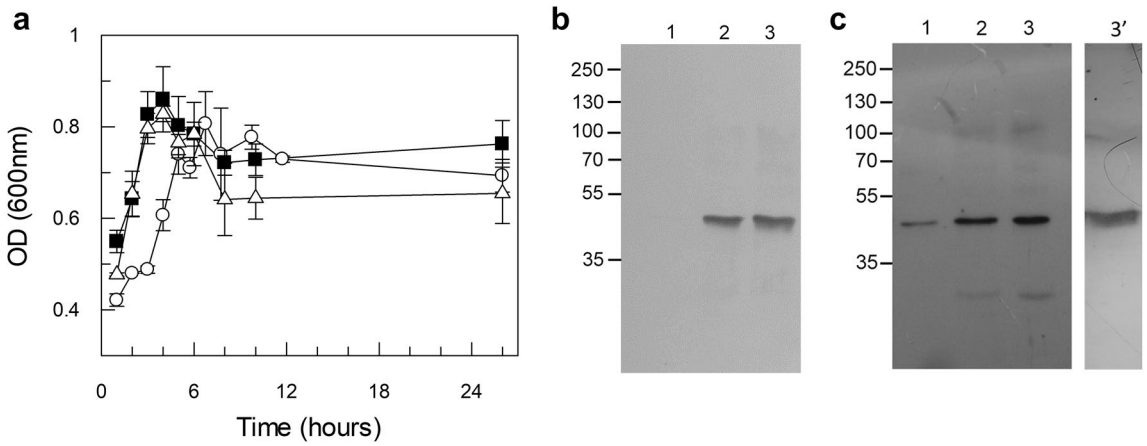


**Fig. 1** Effect of glucose and IPTG concentration on ASCT2 production. **a** RosettaGami2(DE3)pLysS cells transformed with hASCT2-pCOLDI construct were grown in absence (open circle) or in presence of 0.5% (filled square) or 1% (open triangle) glucose, respectively. The protein synthesis was induced with 0.4 mM IPTG at 15 °C for up to 22 h. For measuring OD values, bacterial suspensions were diluted 1:4 and the absorbance values were adjusted according to the dilution factor. The values are means ± S.D. from three cell growths. **b** Western blotting of the samples. Pellet of RosettaGami2(DE3)pLysS transformed with hASCT2-pCOLDI construct grown for 6 h in presence of 0.4 mM IPTG, and in absence (lane 1) or in presence of 0.5% (lane 2) or 1% (lane 3) glucose, respectively. **c** Western blotting of pellets deriving from RosettaGami2(DE3)pLysS transformed with hASCT2-pCOLDI construct grown for 6 h in presence of 1 mM IPTG (lane 1), 0.4 mM IPTG (lane 2), and 0.1 mM IPTG (lane 3), respectively; (lane 3′) western blotting of the same pellet as in lane 3 but immuno-detected by anti ASCT2 antibody

reduction) decreased. Indeed, in absence of glucose, a slow OD reduction was still observed, that was not present in the glucose containing cultures. These differences may be due to metabolic effects of glucose, which are not related to the POI, which is not more produced after 6 h (see below). No evident differences were observed on SDS-PAGE analysis of the samples deriving from cells grown in presence or in absence of the inducer (not shown). In order to detect the possible expression of the protein, a western blotting assay was performed analysing the insoluble fractions of induced cell lysates, that should contain the membrane proteins (Fig. 1b). While in absence of glucose small if any immuno-reactivity was observed (Fig. 1b, lane 1), in the presence of glucose a strong reactivity, higher at 1% glucose, indicated the presence of hASCT2 (Fig. 1b, lanes 2, 3). No further increase in protein production was observed after 6 h of induction (not shown). The effect of IPTG concentration on protein expression was tested. While increasing the IPTG concentration up to 1 mM (Fig. 1c, lane 1) caused a reduction of expression with respect to the control (0.4 mM IPTG, Fig. 1c, lane 2), reducing the IPTG concentration to 0.1 mM (Fig. 1c, lane 3), increased the expression of the POI. No improvements were obtained reducing further the IPTG concentration (not shown). The data indicated that slowing down the rate of expression by lowering the growth temperature and the IPTG concentration, but, together with the addition of glucose, the protein is tolerated and its production is improved probably because the saturation on Sec translocon is reduced thus preventing bacterial death [5]. The effect of glucose could be due to both energization of bacteria and action on the lac promoter. The immunostaining performed by an anti C-terminus antibody (Fig. 1c, lane 3′) confirmed that the ASCT2 produced by *E. coli* is a full length protein.

## B0AT1 expression and purification

Despite the 16 Cys residues, double of hASCT2, hB0AT1 protein expression was favoured in BL21 codon plus RIL cells, probably due to a lower toxicity of the target protein. Indeed, *E. coli* BL21 cells are known to have a better performance in high-cell-density fed-batch cultures compared to RosettaGami2 strain [23]. A colony of hB0AT1-pCOLDI transformed strain was inoculated overnight in LB broth in presence (0.5% or 1%) or in absence of glucose and after 16 h, diluted 1:20 in fresh LB medium containing the same glucose concentration. Cell growth was monitored every 30 min until log phase (OD ~ 0.8) when a cold shock was performed as described for hASCT2. 0.4 mM IPTG was then added to induce protein synthesis. As observed for RosettaGami2, the addition of glucose increased the growth rate with respect to control (Fig. 2a), but differently from hASCT2, the OD decrease continued until 28 h (Fig. 2a). No differences between cultures grown in absence or in presence of IPTG were observed by SDS-PAGE protein analysis (not shown). The production of hB0AT1 was detected by western blotting (Fig. 2b). The protein amount was higher after 22 h of induction in

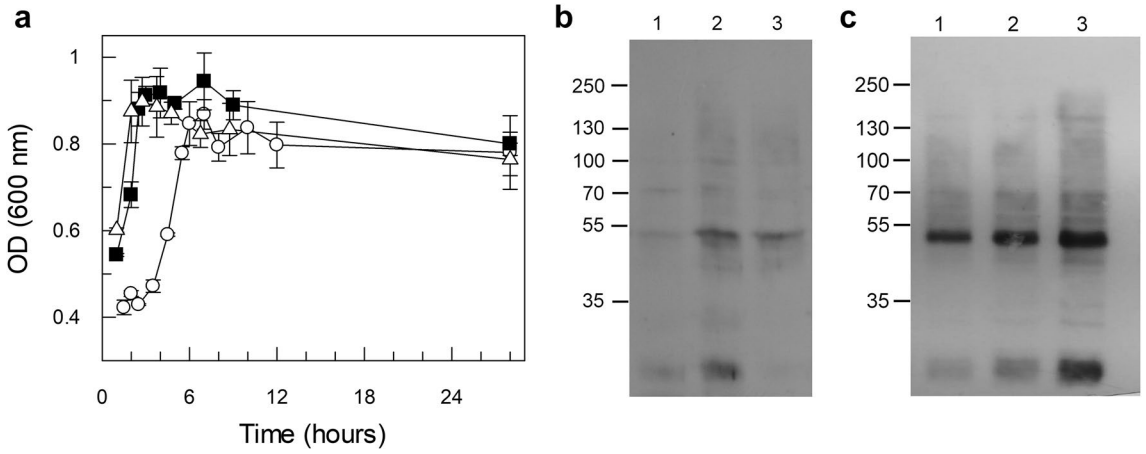


**Fig. 2** Effect of glucose and IPTG concentration on B0AT1 production. **a** BL21 codon plus RIL cells transformed with hB0AT1-pCOLD I construct were grown in absence (open circle) or in presence of 0.5% (filled square) or 1% (open triangle) glucose, respectively. The protein synthesis was induced with 0.4 mM IPTG at 15 °C for up to 22 h. For measuring OD values, bacterial suspensions were diluted 1:4 and the absorbance values were adjusted according to the dilution factor. The values are means ± S.D. from three cell growths. **b** Western blotting of the samples. Pellet of BL21 codon plus RIL transformed with hB0AT1-pCOLDI construct grown for 6 h in presence of 0.4 mM IPTG, and in absence (lane 1) or in presence of 0.5% (lane 2) or 1% (lane 3) glucose, respectively. **c** Western blotting of pellets deriving from BL21 codon plus RIL transformed with hB0AT1-pCOLD I construct grown for 22 h in presence of 0.4 mM IPTG (lane 1), 0.1 mM IPTG (lane 2), and 0.01 mM IPTG (lane 3), respectively

presence of 0.5% glucose (Fig. 2b, lanes 1, 2). No further improvements were obtained increasing glucose to 1% (Fig. 2b, lane 3). A critical parameter for increasing protein production was the IPTG concentration. The protein synthesis increased reducing the IPTG to 0.1 mM (Fig. 2c, lane 2) and was maximal at 0.01 mM (Fig. 2c, lane 3). The production of hB0AT1 was more efficient than hASCT2. The higher efficiency of production was probably related to the slower rate of synthesis of hB0AT1 with respect to hASCT2, and by the lower toxicity of the protein. No changes were observed using the codon optimized cDNA. Thus, starting from the insoluble fraction of 12 mL of cell lysate produced according to (Fig. 2c, lane 3), the purification of hB0AT1 protein was attempted. To this aim, the protein was subjected to a denaturation step during solubilization and subsequent on-column refolding during purification, as previously described for other SLC transporters [22, 24]. The first step was the addition of 8 M Urea, followed by disulphide bridge reduction with DTE and then the mild ionic detergent Sarkosyl. After centrifugation, the supernatant was mixed overnight with a Nickel chelating affinity resin. For refolding, the strong detergent sarkosyl was substituted by dodecyl-maltoside (DDM). The binding of the POI to the resin was really effective, as very poor reactivity, if any, against anti-His antibody was observed in the corresponding passthrough fraction (Fig. 3b, lane 3). After washing with 10 mM imidazole (Fig. 3a, lane 4), B0AT1 was eluted by 200 mM imidazole (Fig. 3a, lane 5). The protein was identified by western blotting (Fig. 3b, lanes 2, 5).

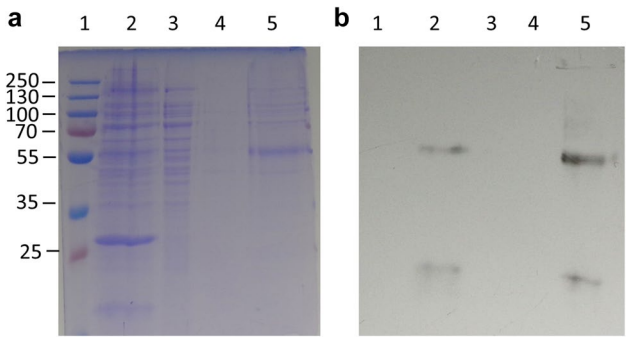


**Fig. 3** Purification of hB0AT1 protein. **a** SDS-PAGE of selected fractions. *Lane 1* Molecular weight marker, *lane 2* insoluble fraction of induced cell lysate, *lane 3* passthrough fraction 31, *lane 4* wash fraction 24, *lane 5* elution fraction 38. **b** Western blotting of the samples as in (**a**)

## Conclusions

The expression of ASCT2 and B0AT1 was only achieved after checking a huge number of combination of plasmids, *E. coli* strains and growth conditions improving a strategy used for organic cations transporters [18]. The temperature range was enlarged and additional parameters/tools were exploited. The results described, indicate that slowing down the growth rate of specific *E. coli* strains by decreasing the temperature and the IPTG concentration, together with the addition of glucose act synergistically on the capacity of bacteria to produce two heterologous

proteins that are not at all produced under usual growth conditions i.e., temperature ranging from 28 to 37 °C and IPTG ranging from 0.5 to 1.5 mM. The use of pCOLDI vector allowed us to substantially decrease the temperature of growth. It is, however, not possible to predict which factor is more relevant for reducing the toxic action of the heterologous proteins and, hence, to derive a general protocol. Very interestingly, the changes in bacteria metabolism revealed in a significant production of a hot membrane protein, that is the B0AT1 amino acid transporter. This protein is in complex with ACE2 constituting part of the receptor for the SARS-Cov-2 RBD proteins [13]. Thus, the possibility to produce B0AT1 at low cost and high yield can be useful for studying interaction with compounds that may have application as COVID-19 drugs.

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## Compliance with ethical standards

**Conflict of interest** The authors have no conflict of interest.

**Research involving human participants and/or animals** This article does not contain any studies with human participants or animals performed by any of the authors.

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