Roskilde
University

Physiological response in E. coli to YdgR overexpression depends on whether the protein has an intact function

Sajid, Salvia; Hernandez-Salas, Lilia Patricia; Rafiq, Maria; Lund, Torben; Girke Jørgensen, Mikkel; Honore, Bent ; Poskjær Christensen, Lars; Hansen, Poul Robert; Franzyk, Henrik; Mirza, Osman; Krishna Prabhala, Bala

Published in:
Biochemical and Biophysical Research Communications

DOI:
10.1016/j.bbrc.2023.04.032

Publication date:
2023

Document Version
Publisher's PDF, also known as Version of record

Citation for published version (APA):
Sajid, S., Hernandez-Salas, L. P., Rafiq, M., Lund, T., Girke Jørgensen, M., Honore, B., Poskjær Christensen, L., Hansen, P. R., Franzyk, H., Mirza, O., & Krishna Prabhala, B. (2023). Physiological response in E. coli to YdgR overexpression depends on whether the protein has an intact function. *Biochemical and Biophysical Research Communications*, *661*, 42-49. https://doi.org/10.1016/j.bbrc.2023.04.032

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain.
- You may freely distribute the URL identifying the publication in the public portal.

Take down policy
If you believe that this document breaches copyright please contact rucforsk@kb.dk providing details, and we will remove access to the work immediately and investigate your claim.

Download date: 08. Feb. 2025

Biochemical and Biophysical Research Communications 661 (2023) 42–49

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Physiological response in *E. coli* to YdgR overexpression depends on whether the protein has an intact function

Salvia Sajid [a, c], Lilia Hernandez Salas [b], Maria Rafiq [c], Torben Lund [b], Mikkel Girke Jørgensen [d], Bent Honoré [e], Lars Porskjær Christensen [a], Paul Robert Hansen [c], Henrik Franzyk [c], Osman Mirza [c], Bala Krishna Prabhala [a, *]

[a] *Department of Physics, Chemistry and Pharmacy, University of Southern Denmark, Campusvej 55, 5230, Odense M, Denmark*
[b] *Department of Science and Environment, Roskilde University, Universitetsvej 1, 10.1, DK-4000, Roskilde, Denmark*
[c] *Department of Drug Design and Pharmacology, University of Copenhagen, Universitetsparken 2, 2100, København Ø, Denmark*
[d] *Department of Biochemistry and Molecular Biology, University of Southern Denmark, Campusvej 55, 5230, Odense M, Denmark*
[e] *Department of Biomedicine, Aarhus University, Nordre Ringgade 1, 8000, Aarhus C, Denmark*

A R T I C L E I N F O

Article history:
Received 30 March 2023
Received in revised form
13 April 2023
Accepted 14 April 2023
Available online 14 April 2023

Keywords:
YdgR
Transport protein overexpression
POT/PTR family
E. coli BL21(DE3)pLysS
Metabolomics/proteomics

A B S T R A C T

Membrane transport proteins are essential for the transport of a wide variety of molecules across the cell membrane to maintain cellular homeostasis. Generally, these transport proteins can be overexpressed in a suitable host (bacteria, yeast, or mammalian cells), and it is well documented that overexpression of membrane proteins alters the global metabolomic and proteomic profiles of the host cells. In the present study, we investigated the physiological consequences of overexpression of a membrane transport protein YdgR that belongs to the POT/PTR family from *E. coli* by using the lab strain BL21 (DE3)pLysS in its functional and attenuated mutant YdgR-E33Q. We found significant differences between the omics (metabolomics and proteomics) profiles of the cells expressing functional YdgR as compared to cells expressing attenuated YdgR, e.g., upregulation of several uncharacterized y-proteins and enzymes involved in the metabolism of peptides and amino acids. Furthermore, molecular network analysis suggested a relatively higher presence of proline-containing tripeptides in cells expressing functional YdgR. We envisage that an in-depth investigation of physiological alterations due to protein overexpression may be used for the deorphanization of the y-gene transportome.

© 2023 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

1. Introduction

E. coli has been a primary choice for the recombinant production of proteins due to its comprehensive physiological and genetic characterization as well as its high potential to produce foreign proteins [1]. The optimization of recombinant protein expression in *E. coli* has mostly been accomplished through trial-and-error adjustments of basic parameters like expression vectors, medium composition, host strains, and growth temperature [2]. Poor growth or absolute growth inhibition of the host cells has been observed as a result of overexpression of recombinant proteins, for instance, it was reported that both overproduction and under-expression of membrane proteins led to severe growth alterations in L. lactis [3]. Walker et al. first discovered *E. coli* BL21 (DE3) mutant strains that carry naturally occurring suppressor mutations, which reduced the toxicity caused by the synthesis of cytotoxic proteins under the control of the potent T7 promoter [4]. These strains are commonly utilized to express more membrane proteins, largely because they enable increased biomass production. Interestingly, it was later discovered that the mutations in these strains lowered the translational efficiency of T7 RNA polymerase. Wagner et al. reported that overexpression of membrane proteins in *E. coli* BL21 (DE3)pLysS resulted in an accumulation of cytoplasmic aggregates, chaperones, soluble proteases, as well as numerous precursors of periplasmic and outer-membrane proteins [5]. Nevertheless, BL21 (DE3)pLysS has been used to express several membrane proteins such as YiiP (zinc transporter) and AdiC (arginine, agmatine antiporter) as well as outer-membrane proteins like porins (OmP) [6]. Previously, it has been reported that overexpression of membrane proteins in *E. coli* not only alters the expression of other proteins (both soluble and membrane-

* Corresponding author.
E-mail address: bapra@sdu.dk (B.K. Prabhala).

https://doi.org/10.1016/j.bbrc.2023.04.032
0006-291X/© 2023 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

associated) [7], but also triggers responses similar to antimicrobial resistance [8] as well as changes in membrane lipid composition [9]. However, only a few reports describe the consequences of the overexpression of a membrane protein with attenuated activity. Therefore to address this gap we used a prototypical peptide transporter, YdgR (mainly transporting di- and tripeptides) [10] from *E. coli*, as a model and overexpressed both functional YdgR and its attenuated variant YdgR-E33Q to investigate the changes and/or differences in their metabolomic and proteomic profiles.

2. Materials and methods

2.1. Expression

Overexpression of YdgR was performed as described previously [11–16].

2.2. Fluorescence-based assays

Competition assays were performed on *E. coli* BL21 (DE3)pLysS cells overexpressing functional YdgR, attenuated YdgR, and empty vector as described previously, where H-[β-Ala]-Lys (AMCA)-OH (β-alanyl-lysyl-N^{ε}-7-amino-4-methyl coumarin-3-acetic acid) was used as a fluorescent reporter substrate and assay procedures as previously described [11–16]. Harvested cells were resuspended in modified Hank's Balanced Salt Solution (HBSS) consisting of $CaCl_2$, 1.26 mM; $MgCl_2$, 0.49 mM; $MgSO_4$, 0.41 mM; KCl, 5.33 mM; KH_2PO_4, 0.44 mM; NaCl, 138 mM; Na_2HPO_4, 0.34 mM; D-glucose, 5.56 mM; $NaHCO_3$, 4.17 mM and MES, 10 mM at pH 6.5. The resuspended cells were transferred to a 96-well fluorescence microplate, and the fluorescence was measured at 340 nm/460 nm as excitation and emission wavelengths.

Efflux assays were performed as previously described [17]. Briefly, the cells were incubated with 10 μM ethidium bromide, 100 μM catechin and 100 μM reserpine at 37 °C and shaking at 500 rpm for 10 min, after which the assay was terminated by the addition of 500 μL ice-cold buffer. The cells were then centrifuged at 14,000 rpm at 4 °C for 1 min. The pellet was washed thrice with ice-cold buffer (pH 6.5). The cell suspension was measured for fluorescence at 526 nm and 605 nm as excitation and emission wavelengths. BMG CLARIOstar plate-reader (BMG Labtech, Ortenburg, Germany) was used to record fluorescence data. Data were analyzed by using GraphPad Prism version 9.2.

2.3. Proteomics analysis

An amount of 200 μL cell suspension at $OD_{600} = 10$ was dissolved in lysis buffer (5% sodium dodecyl sulfate, 50 mM triethylammonium bicarbonate (TEAB), pH 8.5) and stored at −20 °C. Cells dissolved in the lysis buffer were sonicated on ice, incubated with agitation at 99 °C for 5 min and centrifuged at 16,000×g for 10 min. The protein concentration in the supernatant was measured by using infrared spectrometry, and 100 μg protein was alkylated, and tryptic digested with the suspension trapping method [18], using S-Trap microcolumns (Protifi, Farmingdale, NY, USA), essentially as previously described [19]. The peptide concentration was estimated by tryptophan fluorescence, and 1 μg peptide was dissolved in 0.1% formic acid was analyzed by label-free quantification (LFQ) nano liquid chromatography–tandem mass spectrometry in triplicate. Each sample was injected two times (technical duplicates).

The mass spectrometry platform consisted of an Ultimate 3000 connected to an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific Instruments, Waltham, MA, USA) and was operated with the universal method settings as described [19]. Raw files were used for LFQ analysis using MaxQuant version 1.5.7.4 [20]. The UniProt (https://www.uniprot.org) *E. coli* K12 database used for identification was downloaded on April 4, 2022. Settings were as described [19]. The created protein groups file was entered into Perseus version 1.6.14.0 [21]. Data were filtered for peptides only identified by site in the reverse database and peptides considered as contaminants. At least two unique peptides were required for identification. In total 1794 proteins were identified. For pairwise proteome comparison, a two-sided *t*-test was performed with a significance level (p value) of <0.05 and a fold-change of >1.5.

2.4. Liquid chromatography-mass spectrometry (LC-MS)-based metabolomics analysis

Cells and media were separated and lyophilized after harvesting (Zirbus Technology). The dry residual content was reconstituted in 100 μL of 50% acetonitrile in water containing 0.1% formic acid. The samples were centrifuged at 14,000 rpm for 20 min. The clear filtered cell lysate/medium suspension (150 μL) was transferred to HPLC glass vials with inserts. Untargeted metabolomics investigations were carried out on an Agilent 6460 quadrupole time-of-flight mass spectrometer (Q-TOF-MS), coupled to an Agilent 1290 HPLC system (Agilent, Santa Clara, California). In each run 5 μL cell lysate/medium suspension were injected and analyzed for peptides. Peptides were separated on a Waters HPLC XBridge C18 analytical column (150 mm × 4.6 mm; particle size: 3 μm; pore size: 100 Å, Milford, Massachusetts) at 25 °C. The mobile phases consisted of Milli-Q water containing 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B). The HPLC gradient started with 5% of solvent B at a flow rate of 0.3 mL/min and both flow rate and gradient were linearly increased to 100% B and 0.5 mL/min in 20 min, respectively, and maintained for 3 min, before being restored to starting conditions after 7 min. The total run time was 30 min.

Sample ionization was achieved in the electrospray ionization (ESI) positive mode. The capillary voltage was set to 4000 V, the drying gas was heated to 225 °C, and the flow rate was 13 L/min. The scan range was set in the range *m*/*z* 50–1000 for both MS1 and MS2. The top 8 ions in each scan were selected for fragmentation. The scans were run at collision energy 0 and 25. The sheath gas flow was set at 12 L/min at a temperature of 400 °C, while the fragmentor was set at 250 V. The acquisition rate was set at 1 spectra/s and 1000 milliseconds/spectrum for both MS1 (5997 spectrums in total) and MS2 spectra (5953 spectrums in total). Ammonium formate clusters were used as mass calibrant, and injected at regular intervals by the solvent reservoir such that the mass spectra were calibrated at least three times in a single run. The metabolomics data analysis was performed by using the XCMS [41] server with parameter ID1. The results table from the XCMS was downloaded, and the PCA plots were generated by using SPSS (28.0.1.0). The output tables were plotted by using GraphPad Prism 9.2.

2.5. Gas chromatography-mass spectrometry (GC-MS) based metabolomics analysis

A GC-MS analysis was performed to observe low-molecular weight extracellular compounds (50–600 Da). An aliquot of 50 μL of cell suspension was processed according to the procedure described previously [22], using N-Methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA, Merck, Germany) as a derivatization agent. Untargeted metabolomics was performed by using a Tracer 1300 gas chromatograph (Thermo Fisher Scientific, Milan, Italy), coupled to an ISQ 7000 Single Quadrupole mass spectrometer system (Thermo Fisher Scientific, Milan, Italy). The GC column used was an Agilent® DB-5MS column (30 m × 0.25 mm i. d.,

$d_f = 0.25$ μm liquid phase, Santa Clara, California). Hydrogen (H_2) was applied as carrier gas with a flow rate of 1.2 mL/min and 110 kPa head pressure. Once derivatized, 2 μL of the sample was injected into the system in splitless mode. The oven was programmed to an initial 50 °C with a 1 min hold time, followed by an intermediate step of 260 °C with a rate of 5 °C/min and an immediate change to 325 °C with 10 °C/min and a hold time of 10 min. The MS transfer line was set at 280 °C and the ion source temperature was set at 250 °C.

For data analysis, the files were processed in the XCalibur® Qual Browser, where background noise was subtracted, and a pre-identification was performed by using the NIST Library. The metabolites were fully identified by using MS-Dial (RIKEN) based on the Fatty Acids Methyl Esters Retention Index [22] identification, using the MS/MS Positive Fiehn Public database from RIKEN Metabolomics [23]. All the information from a total of 42 identified metabolites was exported from MS-Dial to be used for the statistical analysis.

2.6. Microscopy

Resuspended cells after the uptake assay were centrifuged at 14,000 rpm and 25 °C for 1 min. The supernatant was removed, and the cell pellet was resuspended and incubated for 5 min in a buffer containing 5 mM glucose and 0.5 mM potassium thiocyanate. The cells were centrifuged again at the same speed, and temperature for 1 min, and then they were resuspended in 86% glycerol to prepare the slides for differential interference contrast (DIC) microscopy. Images were taken by Zeiss Axio Observer 7 with a Plan-Apochromat 63 × /1.4, oil objective by using Zeiss software. Images were processed in Image J for presentation.

2.7. Classical molecular networking workflow description

A molecular network was created by using the online workflow from the Global Natural Product Social Molecular Networking (GNPS) (https://ccms-ucsd.github.io/GNPSDocumentation/) on the GNPS website (http://gnps.ucsd.edu). The data were filtered by removing all MS/MS fragment ions within ± 17 Da of the precursor ion. MS/MS spectra were window-filtered by choosing only the top 6 fragment ions in the ± 50 Da window throughout the spectrum. The precursor ion mass tolerance was set to 0.2 Da with an MS/MS fragment ion tolerance of 0.5 Da. A network was then created, where edges were filtered to have a cosine score above 0.7 and more than 6 matched peaks. Furthermore, edges between two nodes were kept in the network only if each of the nodes appeared in the other's respective top 10 most similar nodes. Finally, the maximum size of a molecular family was set to 100, and the lowest-scoring edges were removed from molecular families until the molecular family size was below this threshold. The spectra in the network were then searched against GNPS' spectral libraries. The library spectra were filtered in the same manner as the input data. All matches kept between network spectra and library spectra were required to have a score above 0.7 and at least 6 matched peaks [24].

3. Results and discussion

3.1. The cellular effects of YdgR overexpression depend on whether it is functional

In the present study, we overexpressed YdgR and its attenuated variant, YdgR-E33Q, and the activity of these overexpressed transport proteins was tested by using the reporter substrate H-[β-Ala]-Lys (AMCA)-OH (Fig. 1A). As previously observed [12], the uptake of H-[β-Ala]-Lys (AMCA)-OH was inhibited 5-fold in the presence of trialanine (AAA) as a competitor, indicating the presence of functional YdgR. The attenuated YdgR was also tested by using H-[β-Ala]-Lys (AMCA)-OH, and in this case no transporter-mediated uptake was observed, thereby confirming the attenuated activity of YdgR. Furthermore, no inhibition of the fluorescence of H-[β-Ala]-Lys (AMCA)-OH was observed in the presence of AAA as a competitor (Fig. 1A). A similar trend was observed for cells harboring empty vector (Fig. 1A). To understand the effects of overexpression of membrane proteins on cell physiology, the cells expressing empty vector, and functional YdgR were subjected to microscopy. We observed elongation of *E. coli* cells when functional ydgR was overexpressed compared to cells harboring empty vector (Fig. 1B and C). This is in contrast with the previous study, when physiological alterations in the cell morphology were reported due to the depletion of an outer membrane cell division protein, ygbQ [25].

To further obtain an overview of cellular physiology as a function of overexpression of membrane protein, the proteomic profile of cells harboring empty vector was compared with cells expressing attenuated YdgR. Secondly, cells expressing attenuated YdgR were compared to cells expressing functional YdgR to get an overview of how cellular physiology depends upon protein function. The global proteomics profiles showed a significant difference between cell lines expressing empty vector, attenuated YdgR, or functional YdgR (Fig. 1D). The cells expressing attenuated YdgR and functional YdgR were more similar as compared to the cells expressing empty vector, which most likely arise from the fact that both cell lines share the feature of overexpression of a membrane protein (Fig. 1D).

Furthermore, to compare the differential expression of proteins as a function of transporter overexpression, all differentially regulated proteins between attenuated YdgR and the empty vector were plotted (Fig. 1E). Significantly upregulated proteins are highlighted in green, while significantly downregulated proteins are highlighted in red. As expected, YdgR is significantly upregulated in cells expressing attenuated YdgR (Fig. 1E). Previously, it was shown that expression levels of protein do not change due to impaired activity [16]. As a result of overexpression, we observed that FhuA, an outer membrane ferric siderophore transporter (for ferrichrome, a cyclic hexapeptide) [26] was downregulated (Fig. 1E). It shows that the overexpression of ydgR was accompanied by the downregulation of another family of peptide transporters. Downregulation of ferrichrome as a function of overexpression of membrane proteins has also been reported in *L. lactis* [3,27]. Furthermore, it also observed that several sugar transporters were also downregulated, such as XyiF (xylose), MglA (galactose), and MglC (galactoside) as a function of overexpression (Fig. 1E). Downregulation in sugar importers has often been associated with the recycling of carbon sources and improved energy yields. This mechanism has been used for the development of cell factories and increases the yields of lycopene and GABA [28]. Moreover, Sec translocase (SecY) was observed to be upregulated as a function of overexpression. When a membrane protein is overexpressed, it appears to be accompanied by the upregulation of proteins carrying and inserting the synthesized proteins into the membrane [7].

On the other hand, to compare the differential expression of proteins as a consequence of the expression of functional YdgR, a volcano plot was prepared for the differentially regulated proteins between attenuated YdgR and functional YdgR (Fig. 1F). Several proteins were upregulated as a function of overexpression of attenuated YdgR, which also includes many uncharacterized proteins such as yegD, ycgM, yecA. It might be possible that any of these y-proteins could be efflux transporters. For instance, it has been reported that a previously uncharacterized protein, yjiO, later

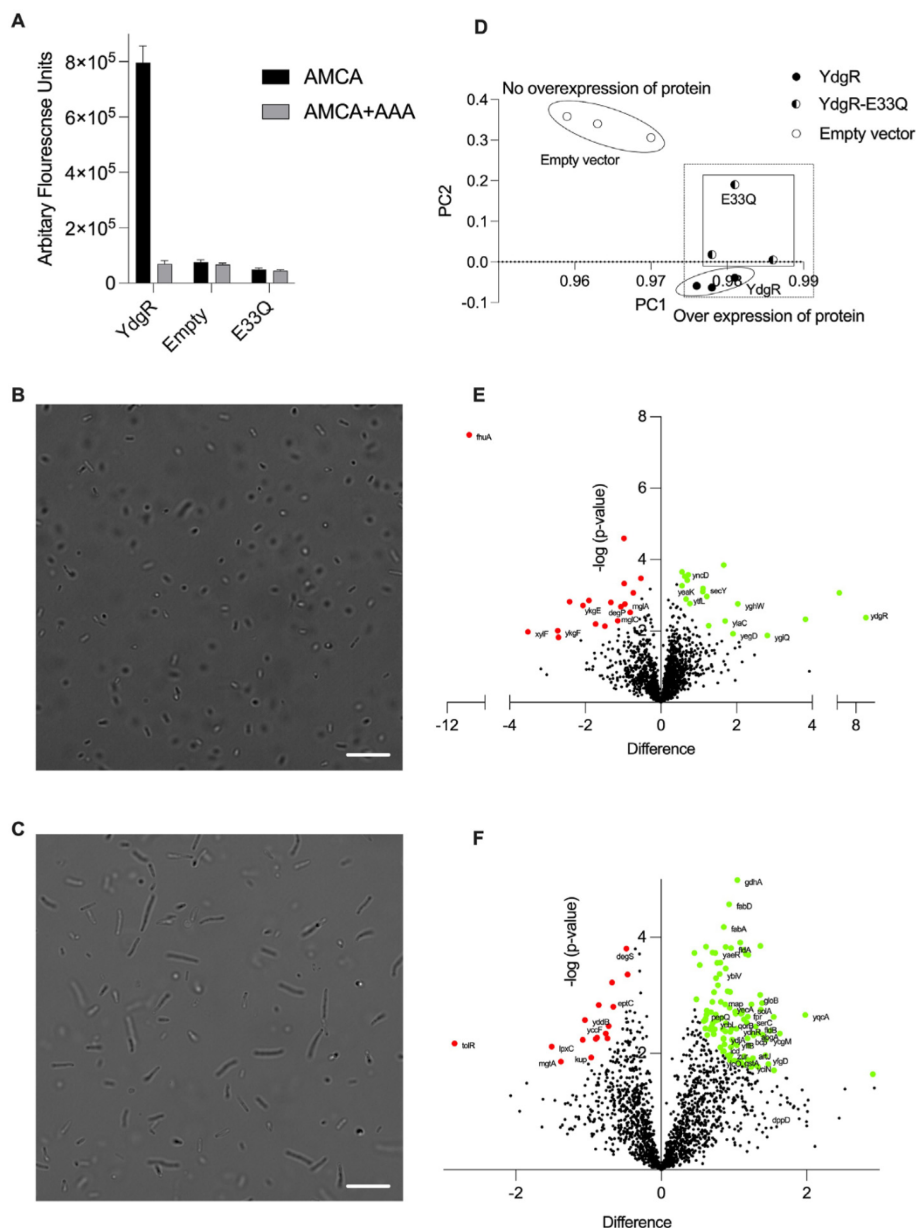

Fig. 1. (A) Uptake of H-[β-Ala]-Lys (AMCA)-OH (abbreviated as AMCA above) in the absence and presence of AAA as a competitor in cells overexpressing YdgR, attenuated YdgR-E33Q, or empty vector. (B) Microscopy images showing the phenotype of *E. coli* BL21 (DE3)pLysS cells harboring empty vector. (C) Microscopy images showing the phenotype of *E. coli* BL21 (DE3)pLysS cells overexpressing YdgR. (Scale bar = 10 μm) (D) PCA plot depicting differentially regulated proteins in terms of protein overexpression. (E) Volcanic plot depicting differentially regulated proteins due to overexpression of attenuated YdgR-E33Q as compared to empty vector. (F) Volcanic plot depicting differentially regulated proteins due to impaired functionality of transporter in attenuated YdgR-E33Q as compared to functional YdgR.

was found to be a protein associated with multidrug resistance (i.e., MdtM), which works as a gene cluster with corresponding genes such as yjiL to enhance multidrug resistance [29–31]. A number of peptides (especially proline- and serine-containing peptides) and amino acid-metabolizing enzymes were observed to be upregulated when functional YdgR was overexpressed (Fig. 1F). Similarly, several peptide uptake systems (and the corresponding degradation systems) were found to be upregulated when OpuA (glycine-betaine transporter) was upregulated [3]. Additionally, magnesium transporters (MgTs) were downregulated, which has also been observed when overexpressing other membrane proteins both in *E. coli* [32] and in *L. lactis* [3].

3.2. Both influx and efflux transporters are overexpressed as a function of YdgR overexpression

Previously, Wagner et al. reported that overexpression of a particular membrane protein alters the expression of other membrane proteins as well [7]. To confirm this finding in the case of YdgR, efflux assays were conducted by detection of ethidium bromide uptake. A significantly higher uptake of ethidium bromide was observed in cells overexpressing YdgR as compared to cells overexpressing attenuated YdgR or empty vector (Fig. 2A). Furthermore, in the presence of trialanine (AAA) as a competitor, ethidium bromide uptake was not inhibited, indicating a YdgR-independent uptake (Fig. 2A). This demonstrates that there is an upregulation of other influx transporters. To further probe the

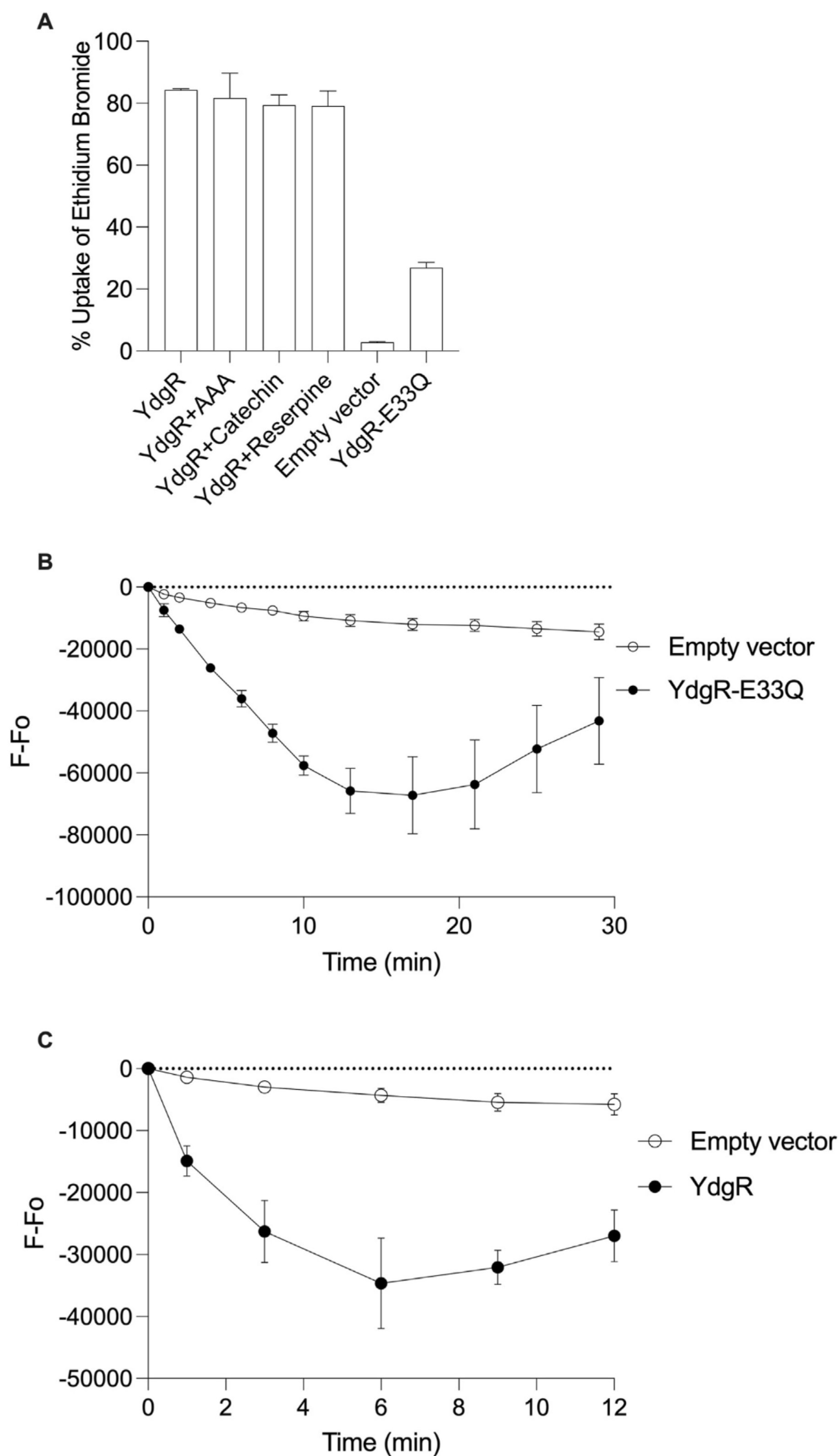

Fig. 2. (A) % Uptake of ethidium bromide in cells overexpressing YdgR in absence and presence of AAA, catechin and reserpine as competitors, and in empty vector and YdgR-attenuated mutant (i.e., YdgR-E33Q). (B) Ethidium bromide efflux kinetics in empty vector-harboring cells as compared to cells overexpressing mutant (YdgR-E33Q). (C) Ethidium bromide efflux kinetics in empty vector-harboring cells as compared to cells overexpressing functional YdgR.

Fig. 3. PCA plots depicting metabolomics analysis of extra- and intracellular fractions. (A) GC-MS analysis of extracellular metabolites. (B) LC-MS analysis on intracellular metabolites. (C) A representative molecular network of intracellular fractions of cells overexpressing functional YdgR (Red), attenuated YdgR-E33Q (green) and empty vector (blue). The labeled peptides are Ile-Pro-Ile (IPI), Leu-Pro-Lys (LPK) and His-Pro (HP). The edges are labeled by relative mass error. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

uptake of ethidium bromide, catechin and reserpine (known inhibitors of efflux pumps) were added as competitors. No inhibition in the uptake of the ethidium bromide was observed even in 10-fold higher concentrations of catechin or reserpine [33] (Fig. 2A).

To further confirm the upregulation of efflux transporters, we monitored the efflux in cells overexpressing attenuated YdgR (Fig. 2B) or functional YdgR (Fig. 2C) as compared to that seen for cells expressing an empty vector. A faster efflux was observed in cells overexpressing functional YdgR (Fig. 2C) as compared to cells overexpressing attenuated YdgR (Fig. 2B), indicating a concomitant

upregulation of several efflux transporters in response to overexpression of functional YdgR. Thus, it is evident from efflux assays that cell overexpressing functional YdgR are more differentially regulated with respect to transport proteins as compared to cells overexpressing attenuated YdgR. Furthermore, no changes in efflux of ethidium bromide in the presence of reserpine and catechin were observed, indicating both uptake and efflux are independent of existing transport mechanisms. It is anticipated that higher uptake of ethidium bromide in cells expressing YdgR might be due to either differential regulation of its transporter as a function of YdgR overexpression, or arise from the presence of several genes referring to the y-ome [34], which were also differentially regulated in attenuated YdgR-E33Q as compared to functional YdgR (Fig. 1F), indicating the involvement of transporters yet to be identified.

3.3. Metabolomics profiles are altered due to changes in proteomics

Differences in the proteome are often associated with concomitant changes in the metabolome [35]. To investigate the alterations caused by differential regulation of proteins, the cells were subjected to metabolomics with respect to both intracellular content by LC-MS and extracellular content by GC-MS. Based on the global metabolomics profile, it was observed that there were significant differences between the cells expressing empty vector and the cells expressing membrane proteins (attenuated YdgR or functional YdgR) with respect to the extracellular fractions (Fig. 3A). These observations are in line with the previously reported results [36], where the volatomics profiles for functional and dysfunctional overexpressed transporter proteins were significantly different. Moreover, cells expressing membrane proteins were found to possess elevated levels of amino acids such as leucine, glycine, and lysine. Furthermore, succinic acid and malic acid were found to be abundant in cells expressing functional YdgR. This has also been reported for expression of other membrane proteins in BL21DE3 cells [7].

Malic acid was reported to induce protective effects in *E. coli* against acidic stress at low pH [37]. Previously, we have observed that in cells expressing functional YdgR, the pH of the external compartment decreases significantly as compared to what is seen for cells expressing empty vector [12]. Therefore, higher levels of malic acid, as observed in the present study, might be a way to counteract a decreasing pH within *E. coli* cells. Also, for intracellular fractions marked differences were observed between cells expressing empty vector, attenuated, or functional YdgR (Fig. 3B). To further understand the metabolomics differences between these cell lines the data were subjected to GNPS classical molecular networking analysis of cells expressing functional YdgR (G1, red), attenuated YdgR (G2, blue) and empty vector (G3, green) (Fig. 3C). This resulted in a network, where only a few nodes could be annotated from the GNPS spectral libraries. Three different proline-containing peptides (Ile-Pro-Ile, Leu-Pro-Lys, and His-Pro) (Fig. 3C) were annotated. It is completely in agreement with the proteomics data, since X*aa*-Pro metabolizing enzymes were upregulated in cells expressing functional YdgR. It was also evident that a higher level of tripeptides was present in cells expressing functional YdgR as compared to that seen for cells expressing attenuated YdgR or empty vector (Fig. 3C). Furthermore, equal amounts of His-Pro were observed in cells expressing functional or attenuated YdgR, indicating a YdgR-independent entry into the cells.

Additional targeted peptidomics-based strategies must be applied in order to identify potential natural substrates of YdgR. Furthermore, this approach may also be applied as a tool to deorphanize members of the y-gene transportome. At present, it is known that *E. coli* contains four POTs (i.e., YdgR, YhiP, YjdL and YbgH) as well as other peptide permeases belonging to the ABC transporter family [38,39]. Marreddy et al. reported that DppA, a dipeptide permease (an ABC transporter), was upregulated in response to overexpression of functional YdgR, and similar results have also been observed in the case of *L. lactis* [3]. Overexpression of a membrane protein induces a number of physiological changes within the cell, and this has been well investigated in the literature [3,7,40]. Generally, following a simple growth curve of cells overexpressing membrane proteins can provide useful information on the nature of membrane protein overexpression. For instance, in *E. coli* (MG1655) expressing membrane transport protein GlpT (glycerol 3-phosphate transporter), no growth of cells was observed post-IPTG induction. This was speculated to be due to the transport of glycerol 3-phosphate and other several sugars, which exert toxic effects in the cell [40]. Similarly, a tendency to suppress cell growth was also observed when YdgR was overexpressed because of the transport of chloramphenicol (present during cultivation of cells) into the cells upon YdgR overexpression [16]. These observations further support the consensus that toxicity induced by membrane protein expression mainly can be attributed to the increased level of the protein itself. These observations have two important implications, of which the primary one is that a number of transport proteins are engineered and incorporated into microbial cell factories to improve the yields, however, concomitant changes in cellular physiology are often neglected. Secondly, transport assays to biochemically characterize membrane transport proteins are typically performed via point mutations, and the cellular response to this mutation is assumed to be negligible. Nevertheless, in the present study, we found a 3-fold higher number of significantly altered expression of different genes (cytoplasmic, membrane and orphan) in cells expressing functional YdgR as compared to cells expressing attenuated YdgR, thereby providing a basis for more in-depth studies of the cellular responses to alterations in the expression and/or function of transport proteins.

4. Conclusion

The results obtained in the present study emphasizes that cellular responses to mutations introduced to alter the function of membrane transport proteins are surprisingly extensive, and thus cannot be neglected. Significant differences were observed in both cellular morphology and omic profiles of functional versus attenuated YdgR as compared to empty vector harboring cells, indicating significant consequences of protein overexpression on whole cell-based assays involving this transporter.

Overall, the physiological implications of overexpression of the functional membrane transport protein YdgR and its attenuated variant YdgR-E33Q in the *E. coli* lab strain BL21 (DE3)pLysS resulted in substantial variations in the proteomics and metabolomics profiles for cells expressing functional YdgR as compared to those expressing attenuated YdgR. However, a more general conclusion regarding the effects of overexpression of transporter proteins cannot be drawn. Nevertheless, we believe that further use of this methodology may prove beneficial for the deorphanization of members of the y-gene transportome.

Declaration of competing interest

All authors declare no conflict of interest.

Acknowledgment

The Higher Education Commission, Pakistan, is greatly acknowledged for funding overseas Ph.D. scholarship for SS. We wish to acknowledge Carsten Uhd Nielsen for fruitful discussions,

Ahmed Basim Abduljabar for the technical assistance with proteomics, and Christoffel Dinant for microscopy at the Danish Cancer Society Research Center, Denmark. The mass spectrometer used for proteomics was funded by A.P. Møller og Hustru Chastine Mc-Kinney Møllers Fond til almene Formaal and the BMG Labtech plate reader used for fluorescence measurement was funded by the Carlsberg Foundation.

References

[1] F.R. Schmidt, Recombinant expression systems in the pharmaceutical industry, Appl. Microbiol. Biotechnol. 65 (4) (2004) 363–372.
[2] Tomohiro Makino, Georgios Skretas, George Georgiou, Strain engineering for improved expression of recombinant proteins in bacteria, Microb. Cell Factories 10 (May) (2011) 32.
[3] Ravi K.R. Marreddy, P. C. Pinto Joao, Justina C. Wolters, Eric R. Geertsma, Fabrizia Fusetti, Hjalmar P. Permentier, Oscar P. Kuipers, Jan Kok, Bert Poolman, The response of lactococcus lactis to membrane protein production, PLoS One 6 (8) (2011), e24060.
[4] B. Miroux, J.E. Walker, 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 (3) (1996) 289–298.
[5] Samuel Wagner, Mirjam M. Klepsch, Susan Schlegel, Ansgar Appel, Roger Draheim, Michael Tarry, Högbom Martin, et al., Tuning Escherichia coli for membrane protein overexpression, Proc. Natl. Acad. Sci. U. S. A 105 (38) (2008) 14371–14376.
[6] Marvin V. Dilworth, S. Piel Mathilde, Kim E. Bettaney, Ma Pikyee, Ji Luo, David Sharples, David R. Poyner, et al., Microbial expression systems for membrane proteins, Methods 147 (September) (2018) 3–39.
[7] Samuel Wagner, Baars Louise, A. Jimmy Ytterberg, Anja Klussmeier, Claudia S. Wagner, Olof Nord, Per-Åke Nygren, Klaas J. van Wijk, Jan-Willem de Gier, Consequences of membrane protein overexpression in Escherichia coli, Mol. Cell. Proteomics: MCP 6 (9) (2007) 1527–1550.
[8] Jack James, Benjamin Yarnall, Andy Koranteng, Jane Gibson, Tahmina Rahman, A. Declan, Doyle, Protein over-expression in Escherichia coli triggers adaptation analogous to antimicrobial resistance, Microb. Cell Factories 20 (1) (2021) 13.
[9] Kerstin Kanonenberg, Jorge Royes, Alexej Kedrov, Gereon Poschmann, Federica Angius, Audrey Solgadi, Olivia Spitz, et al., Shaping the lipid composition of bacterial membranes for membrane protein production, Microb. Cell Factories 18 (1) (2019) 131.
[10] Bala K. Prabhala, Rahman Moazur, Hussam H. Nour-Eldin, Flemming Steen Jørgensen, Osman Mirza, PTR2/POT/NPF transporters: what makes them tick? Advances in Protein Chemistry and Structural Biology 123 (2021) 219–240.
[11] Johanne Mørch Jensen, Fouzia Ismat, Gerda Szakonyi, Moazur Rahman, Osman Mirza, Probing the putative active site of YjdL: an unusual proton-coupled oligopeptide transporter from E. Coli, PLoS One 7 (10) (2012), e47780.
[12] Bala K. Prabhala, Nanda G. Aduri, M. Jensen Johanne, Heidi A. Ernst, Nida Iram, Moazur Rahman, Osman Mirza, New insights into the substrate specificities of proton-coupled oligopeptide transporters from E. Coli by a pH sensitive assay, FEBS (Fed. Eur. Biochem. Soc.) Lett. 588 (4) (2014) 560–565.
[13] Bala K. Prabhala, Nanda G. Aduri, Helle Hald, Osman Mirza, Investigation of the substrate specificity of the proton coupled peptide transporter PepTSo from shewanella oneidensis, Int. J. Pept. Res. Therapeut. 21 (1) (2015) 1–6.
[14] Nanda G. Aduri, K. Prabhala Bala, Heidi A. Ernst, Flemming S. Jørgensen, Lars Olsen, Osman Mirza, Salt bridge swapping in the EXXERFXYY motif of proton-coupled oligopeptide transporters, J. Biol. Chem. 290 (50) (2015) 29931–29940.
[15] Neha Sharma, Nanda G. Aduri, Anna Iqbal, Bala K. Prabhala, Mirza Osman, Peptide selectivity of the proton-coupled oligopeptide transporter from Neisseria meningitidis, J. Mol. Microbiol. Biotechnol. 26 (5) (2016) 312–319.
[16] Bala K. Prabhala, Nanda G. Aduri, Neha Sharma, Aqsa Shaheen, Arpan Sharma, Mazhar Iqbal, Paul R. Hansen, et al., The prototypical proton-coupled oligopeptide transporter YdgR from Escherichia coli facilitates chloramphenicol uptake into bacterial cells, J. Biol. Chem. 293 (3) (2018) 1007–1017.
[17] Laura Paixão, Liliana Rodrigues, Isabel Couto, Marta Martins, Pedro Fernandes, C. Carla, C.R. de Carvalho, Gabriel A. Monteiro, Filipe Sansonetty, Leonard Amaral, Miguel Viveiros, Fluorometric determination of ethidium bromide efflux kinetics in Escherichia coli, J. Biol. Eng. 3 (October) (2009) 18.
[18] Alexandre Zougman, Peter J. Selby, Rosamonde E. Banks, Suspension trapping (STrap) sample preparation method for bottom-up proteomics analysis, Proteomics 14 (9) (2014), 1006–1000.
[19] Maja Ludvigsen, Louise Thorlacius-Ussing, Henrik Vorum, Mary Pat Moyer, Mogens Tornby Stender, Ole Thorlacius-Ussing, Bent Honoré, Proteomic characterization of colorectal cancer cells versus normal-derived colon mucosa cells: approaching identification of novel diagnostic protein biomarkers in colorectal cancer, Int. J. Mol. Sci. (2020), https://doi.org/10.3390/ijms21103466.
[20] Stefka Tyanova, Tikira Temu, Juergen Cox, The MaxQuant computational platform for mass spectrometry-based shotgun proteomics, Nat. Protoc. 11 (12) (2016) 2301–2319.
[21] Stefka Tyanova, Tikira Temu, Pavel Sinitcyn, Arthur Carlson, Marco Y. Hein, Tamar Geiger, Matthias Mann, Jürgen Cox, The Perseus computational platform for comprehensive analysis of (prote)omics data, Nat. Methods (2016), https://doi.org/10.1038/nmeth.3901.
[22] Oliver Fiehn, Metabolomics by gas chromatography-mass spectrometry: combined targeted and untargeted profiling, in: Frederick M. Ausubel, et al. (Eds.), Current Protocols in Molecular Biology, vol. 114, 2016 (April): 30.4.1–30.4.32.
[23] Hiroshi Tsugawa, Aya Satoh, Haruki Uchino, Cajka Tomas, Makoto Arita, Masanori Arita, Mass spectrometry data repository enhances novel metabolite discoveries with advances in computational metabolomics, Metabolites 9 (6) (2019), https://doi.org/10.3390/metabo9060119.
[24] Mingxun Wang, Jeremy J. Carver, Vanessa V. Phelan, Laura M. Sanchez, Neha Garg, Peng Yao, Don Duy Nguyen, et al., Sharing and community curation of mass spectrometry data with global natural products social molecular networking, Nat. Biotechnol. 34 (8) (2016) 828–837.
[25] Nienke Buddelmeijer, Nicholas Judson, Dana Boyd, John J. Mekalanos, Jonathan Beckwith, YgbQ, a cell division protein in Escherichia coli and Vibrio cholerae, localizes in codependent fashion with FtsL to the division site, Proc. Natl. Acad. Sci. U. S. A 99 (9) (2002) 6316–6321.
[26] M. Bonhivers, L. Plançon, A. Ghazi, A. Boulanger, M. le Maire, O. Lambert, J.L. Rigaud, L. Letellier, FhuA, an Escherichia coli outer membrane protein with a dual function of transporter and channel which mediates the transport of phage DNA, Biochimie 80 (5) (1998) 363–369.
[27] Joao P.C. Pinto, Oscar P. Kuipers, Ravi K.R. Marreddy, Bert Poolman, Jan Kok, Efficient overproduction of membrane proteins in lactococcus lactis requires the cell envelope stress sensor/regulator couple CesSR, PLoS One 6 (7) (2011), e21873.
[28] Hwi-Min Jung, Im Dae-Kyun, Jae Hyung Lim, Gyoo Yeol Jung, Min-Kyu Oh, Metabolic perturbations in mutants of glucose transporters and their applications in metabolite production in Escherichia coli, Microb. Cell Factories 18 (1) (2019) 170.
[29] Andor Krizsan, Daniel Knappe, Ralf Hoffmann, Influence of the yjiL-mdtM gene cluster on the antibacterial activity of proline-rich antimicrobial peptides overcoming Escherichia coli resistance induced by the missing SbmA transporter system, Antimicrob. Agents Chemother. 59 (10) (2015) 5992–5998.
[30] Scarlett R. Holdsworth, Christopher J. Law, Multidrug resistance protein MdtM adds to the repertoire of antiporters involved in alkaline pH homeostasis in Escherichia coli, BMC Microbiol. 13 (May) (2013) 113.
[31] K. Nishino, A. Yamaguchi, Analysis of a complete library of putative drug transporter genes in Escherichia coli, J. Bacteriol. 183 (20) (2001) 5803–5812.
[32] Lada E. Petrovskaya, Rustam H. Ziganshin, Elena A. Kryukova, Alexander V. Zlobinov, Sultan Sh Gapizov, Lyudmila N. Shingarova, Vasiliy A. Mironov, Galina Yu Lomakina, Dmitriy A. Dolgikh, Mikhail P. Kirpichnikov, Increased synthesis of a magnesium transporter MgtA during recombinant autotransporter expression in Escherichia coli, Appl. Biochem. Biotechnol. 193 (11) (2021) 3672–3703.
[33] Isabel Couto, Sofia Santos Costa, Miguel Viveiros, Marta Martins, Leonard Amaral, Efflux-mediated response of Staphylococcus aureus exposed to ethidium bromide, J. Antimicrob. Chemother. 62 (3) (2008) 504–513.
[34] Sankha Ghatak, Zachary A. King, Anand Sastry, Bernhard O. Palsson, The Y-ome defines the 35% of Escherichia coli genes that lack experimental evidence of function, Nucleic Acids Res. 47 (5) (2019) 2446–2454.
[35] Warwick B. Dunn, Nigel J.C. Bailey, Helen E. Johnson, Measuring the metabolome: current analytical technologies, Analyst 130 (5) (2005) 606–625.
[36] Salvia Sajid, Ishika Aryal, Suleman Farooq Chaudhri, Frants Roager Lauritsen, Mikkel Girke Jørgensen, Håvard Jenssen, Bala Krishna Prabhala, MIMS as a low-impact tool to identify pathogens in water, Water 15 (1) (2023) 184.
[37] K. Bjornsdottir, F. Breidt Jr., R.F. McFeeters, Protective effects of organic acids on survival of Escherichia coli O157:H7 in acidic environments, Appl. Environ. Microbiol. 72 (1) (2006) 660–664.
[38] J.W. Payne, Transport and hydrolysis of peptides by microorganisms, Ciba Foundation Symposium 50 (1977) 305–334.
[39] D.R. Tyreman, M.W. Smith, G.M. Payne, J.W. Payne, Exploitation of peptide transport systems in the design of antimicrobial agents, in: Molecular Aspects of Chemotherapy, 127–42, Springer Berlin Heidelberg, Berlin, Heidelberg, 1992.
[40] Francesca Gubellini, Grégory Verdon, Nathan K. Karpowich, Jon D. Luff, Grégory Boël, Nils Gauthier, Samuel K. Handelman, Sarah E. Ades, John F. Hunt, Physiological response to membrane protein overexpression in E. Coli, Mol. Cell. Proteomics: MCP 10 (10) (2011). M111.007930.
[41] CA Smith, EJ Want, G O'Maille, R Abagyan, G Siuzdak, XCMS: processing mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching, and identification, Anal. Chem. 78 (2006) 779–787. https://doi.org/10.1021/ac051437y.