



Changes in efflux pump activity of *Clostridium beijerinckii* throughout ABE fermentation

Barbora Branska¹ · Maryna Vasylykivska¹ · Hana Raschmanova¹ · Katerina Jureckova² · Karel Sedlar² · Ivo Provaznik² · Petra Patakova¹

Received: 8 April 2020 / Revised: 5 December 2020 / Accepted: 17 December 2020 / Published online: 6 January 2021
© The Author(s), under exclusive licence to Springer-Verlag GmbH, DE part of Springer Nature 2021

Abstract

Pumping toxic substances through a cytoplasmic membrane by protein transporters known as efflux pumps represents one bacterial mechanism involved in the stress response to the presence of toxic compounds. The active efflux might also take part in exporting low-molecular-weight alcohols produced by intrinsic cell metabolism; in the case of solventogenic clostridia, predominantly acetone, butanol and ethanol (ABE). However, little is known about this active efflux, even though some evidence exists that membrane pumps might be involved in solvent tolerance. In this study, we investigated changes in overall active efflux during ABE fermentation, employing a flow cytometric protocol adjusted for Clostridia and using ethidium bromide (EB) as a fluorescence marker for quantification of direct efflux. A fluctuation in efflux during the course of standard ABE fermentation was observed, with a maximum reached during late acidogenesis, a high efflux rate during early and mid-solventogenesis and an apparent decrease in EB efflux rate in late solventogenesis. The fluctuation in efflux activity was in accordance with transcriptomic data obtained for various membrane exporters in a former study. Surprisingly, under altered cultivation conditions, when solvent production was attenuated, and extended acidogenesis was promoted, stable low efflux activity was reached after an initial peak that appeared in the stage comparable to standard ABE fermentation. This study confirmed that efflux pump activity is not constant during ABE fermentation and suggests that undisturbed solvent production might be a trigger for activation of pumps involved in solvent efflux.

Key points

- Flow cytometric assay for efflux quantification in Clostridia was established.
- Efflux rate peaked in late acidogenesis and in early solventogenesis.
- Impaired solventogenesis led to an overall decrease in efflux.

Keywords Flow cytometry · Efflux pump · Ethidium bromide · ABE fermentation · Clostridium

Introduction

Biological production of biofuel is a promising future technology for sustainable energy conservation in a post-petroleum

era. Biobutanol is one of the potential chemicals that can be produced through a microbiological pathway, acetone-butanol-ethanol (ABE) fermentation, by bacterial strains of solventogenic clostridia. Clostridia have a great potential in the field mainly due to their ability to utilize an exceptionally wide range of substrates (Tracy et al. 2012), which predisposes them to be potential producers of bioenergy from various renewable and waste streams (Zhao et al. 2019; Branska et al. 2020). The biggest bottleneck that hinders economically viable production of biobutanol is its low final titre, which is dictated by solvent tolerance (Patakova et al. 2018). Clostridia possess a wide range of mechanisms allowing them to survive in increased concentrations of solvents (Patakova et al. 2018; Cheng et al. 2019) and one of such defence mechanism is

✉ Barbora Branska
barbora.branska@vscht.cz

¹ Department of Biotechnology, University of Chemistry and Technology Prague, Technická 5, 166 28 Prague, Czech Republic

² Department of Biomedical Engineering, Faculty of Electrical Engineering and Communication, Brno University of Technology, Technická 12, 616 00 Brno, Czech Republic

enhanced expression of efflux pumps that can pump toxic compounds out of the cell into the extracellular environment so that it improves biotechnological process performance through strengthening the flux of product out of the cell. Although transport of most uncharged low-molecular-weight substances was previously assumed only to be mediated by passive diffusion, there is evidence now that this may not be the case and many substances are transported by more or less promiscuous transmembrane transporters (Fernandes et al. 2003; Kell et al. 2015; Boyarskiy and Tullman-Ercek 2015). Up to now, seven families of prokaryotic efflux pumps have been described, including five well-known groups: small multidrug resistance (SMR), major facilitator superfamily (MFS), multidrug and toxic compound extrusion (MATE), resistance nodulation division (RND) and ATP binding cassette (ABC) transporters, and two newly described groups, proteobacterial antimicrobial compound efflux (PACE) (Hassan et al. 2015; Spengler et al. 2017) and p-amino benzoyl-glutamate transporter (AbtG) (Delmar and Yu 2015). These transporters have been thoroughly studied, in particular in connection with multidrug resistance (MDR) of pathogenic microorganisms; however, similar mechanisms are probably natural to most bacteria for their defence against a wide range of antimicrobial agents including solvents. Indeed, some studies point to the possibility of transport of various drugs across the phospholipid moiety being minor (Kell et al. 2014). The role of efflux pumps in the transport of short-chain alcohols like n-butanol, isobutanol, ethanol, etc. has been intensively researched, but so far with rather contradictory conclusions. Transcriptomic data revealed increased expression of particular efflux pumps in strains that are more tolerant to butanol (Reyes et al. 2011, 2013) and it has been experimentally confirmed that overexpression of selected pumps (Basler et al. 2018) or modification of existing ones leads to increased tolerance to butanol (Bui et al. 2015; Zhang et al. 2018; Basler et al. 2018; He et al. 2019). However, it is likely that not only one particular transporter is involved in the export of butanol from the cell, but multiple different pumps could operate in synergistic mode (Turner and Dunlop 2015). The space within a cell membrane is rather limited when compared to intracellular space, and increased expression of transmembrane pumps can lead to changes in natural membrane proteome (Wagner et al. 2007) and thus affect cell fitness or viability. Some balance should exist between the negative effect of overexpression of transporters and the actual concentration and toxicity of antimicrobial substances (Boyarskiy et al. 2016). Hence, the expression of efflux pumps is tightly regulated and changes throughout the life cycle of bacteria in response to actual needs. Mostly a basal level of expression can be found under standard conditions while overexpression is induced by particular stimuli (Sun et al. 2014).

While the sporulation-inclusive life cycle and biphasic production schedule of solventogenic clostridia barely allow cells

to remain in the same physiological state (Branska et al. 2018) for even a short period of time, dynamic changes in efflux activity throughout ABE fermentation are expected. Considering that efflux activity may play a fundamental role in defence against toxic substances, we wanted to know how efflux activity develops during the production of intrinsic stressors such as organic acids and solvents during ABE fermentation. Nowadays, efflux is analysed mostly in clinically important isolates of pathogenic bacteria with enhanced efflux using an indirect minimal inhibition concentration (MIC) assay, but effective protocols for direct monitoring of efflux that depends on changing physiology are scarce. Therefore, we focused on the development of a reliable methodology for the quantification of efflux in the culture of solventogenic clostridia. An optimized methodology based on ethidium bromide (EB) efflux was then applied to monitor changes of efflux during standard ABE fermentation and ABE fermentation with impaired solventogenesis (the so-called “acid crash” phenomenon) for *Clostridium beijerinckii* strain NRRL B-598. The data obtained were compared with the expression profile of genes annotated as transporters, which were acquired in an independent but similar experiment (Sedlar et al. 2018; Patakova et al. 2019).

Materials and methods

Cultivation

Bacterial strain *C. beijerinckii* NRRL B-598 from the ARS/NRRL culture collection was cultivated either in an anaerobic chamber Concept 400 (Ruskin) or a bioreactor Multiforce (Infors HT) according to protocols described previously (Kolek et al. 2016a; Patakova et al. 2019). For assay optimization experiments, cultures were inoculated directly from spores; 0.5 ml of a spore suspension was heat-treated (80 °C, 2 min) and transferred into an Erlenmeyer flask containing 50 ml of TYA medium (50 g/l glucose, 6 g/l tryptone (Sigma-Aldrich), 2 g/l yeast extract (Merck), 3 g/l ammonium acetate, 0.5 g/l KH_2PO_4 , 0.3 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.01 $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$). An exponentially growing overnight culture was then used for tuning the efflux assay. Experiments in bioreactors were conducted under similar conditions for both phenotypes, i.e. standard ABE and impaired solventogenesis, the only differences being in the initial glucose concentration (40 and 50 g/l, respectively) and in an establishment of an anaerobic atmosphere in the bioreactor. For standard ABE fermentation, reactors were bubbled with CO_2 for 15 min, which led to a decrease in pH that was adjusted afterwards with 1 M NaOH to a pH value of 6.3 prior to inoculation. For induction of the acid crash-like phenotype, reactors were bubbled with N_2 and therefore no pH adjustment was necessary.

Efflux assay

For accumulation experiments, 1 ml of the sample was transferred from an Erlenmeyer flask into an Eppendorf tube and centrifuged for 1 min at 8000 g. After centrifugation, the supernatant was discarded and the pellet was resuspended in either a saline solution or TYA medium containing EB at a concentration of 2 µg/ml. Fluorescence was measured immediately and the mean fluorescence in the FL3 channel (> 670 nm) was recorded together with the precise time interval after EB addition. For each point, 50 µl of cell suspension were transferred into 1 ml of deionized water and placed onto the SIP of a BD flow cytometer (Accuri C6). At least 5000 particles were analysed per sample. An accumulation assay with increasing concentrations of EB was carried out similarly, the only change being an increasing concentration of EB in the solutions added and incubation time of 5 min. CCCP (carbonyl cyanide m-chlorophenyl hydrazone) was diluted in DMSO (dimethyl sulfoxide) and added into TYA and saline solution with EB to reach a final concentration of 5 µg/ml. Beside this experiment, a similar concentration of CCCP was added to the growing culture; however, it resulted in sharp declination in culture viability and thus no more data were generated by this design. Control experiments were carried out to show that DMSO had no impact on final fluorescence (data not shown). For the direct efflux assay, the calculated amount of cell suspension corresponding to 1 ml of culture with $OD\ 0.5 \pm 0.1$ was transferred into an Eppendorf tube and centrifuged and placed into the anaerobic chamber where the supernatant was discarded and the pellet was resuspended in 1 ml of saline solution containing 2 µg/ml EB and incubated for 5 min. After EB loading into cells, the suspension was centrifuged (8000 g, 1 min) and the supernatant was discarded. Then, the pellet was resuspended in 1 ml of saline solution or TYA medium with 20 g/l of glucose, and fluorescence was measured immediately by flow cytometry as described above. From the time course of fluorescence, the efflux activity was quantified as an absolute value of slope calculated using MS Excel function “slope”. The slope was calculated from at least three values of mean fluorescence (arbitrary units) obtained after EB removal at regular intervals within the first 15 min. All mean fluorescence values were calculated automatically using FC software CFlowPlus. The methodology did not allow us to measure the samples in technical replicates; therefore error bars within charts with cytometric data indicate variance between biological replicates and not analytical variations.

Analytics

Glucose and metabolite concentrations were analysed by HPLC with ion exchange-based separation using as a stationary phase IEX H form, 250 × 8 mm (Watrex). The mobile

phase was 0.5 mM H₂SO₄ in demineralized water at a flow rate of 1.0 ml/min. Detection was refractometric for all analytes and quantification was based on calibration curves of pure substances. Cell concentration was measured as absorbance at 600 nm expressed as optical density (OD) using a Varian Cary 50 UV-Vis spectrophotometer. Viability was measured as the number of solely propidium iodide (PI)-positive cells after staining with a combination of PI and carboxyfluorescein diacetate as described elsewhere (Branska et al. 2018) and analysed employing a BD Accuri C6 flow cytometer.

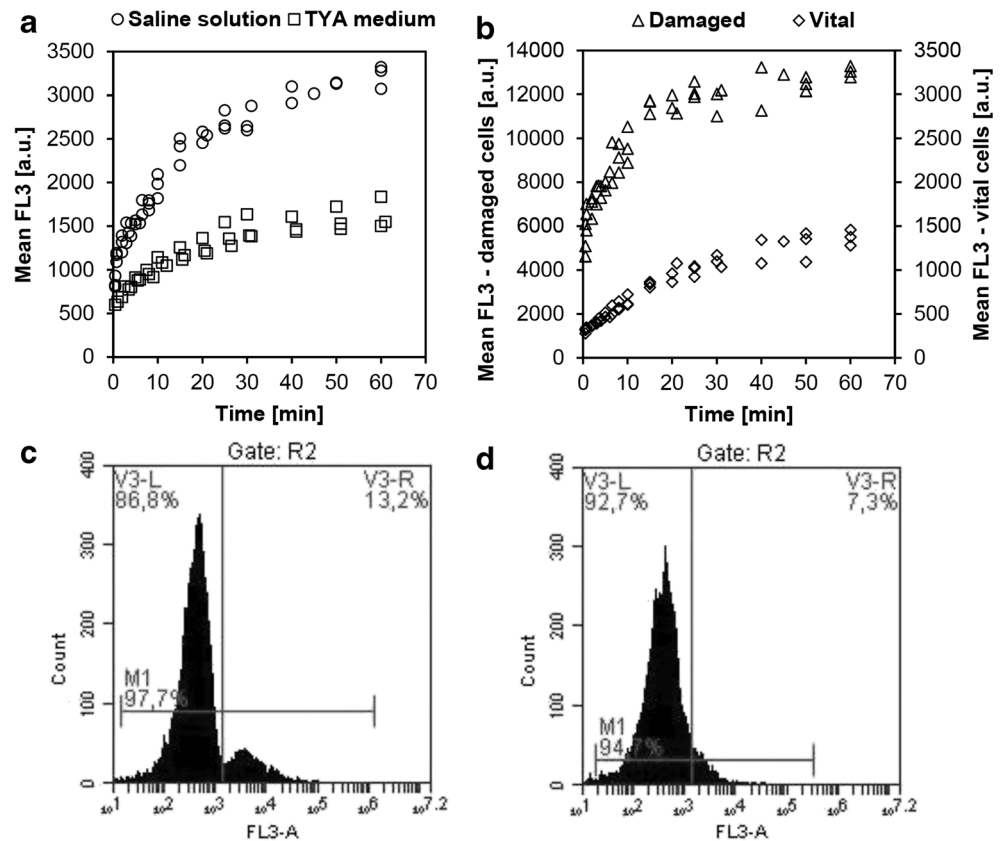
Transcriptomic analysis was performed as a part of another study (Sedlar et al. 2018). Putative transporters were selected based on the publication of Jureckova et al. (2018) and the annotation available in the GenBank database under accession number CP011966.3. Count tables generated during previous studies (Sedlar et al. 2018; Patakova et al. 2019) were normalized using the R/Bioconductor DESeq2 package (Love et al. 2014) using a built-in DESeq2 function. A heatmap representing transcription of selected genes using Z-score was generated with the R package ggplot while similarities in expression profiles were analysed using Ward’s hierarchical agglomerative clustering considering the Manhattan distance (Murtagh and Legendre 2014).

Results

Ethidium bromide accumulation assay

All experiments were performed using ethidium bromide (EB), a fluorescent probe that is one of the most commonly used (Viveiros et al. 2008; Paixão et al. 2009; Patel et al. 2010; Rodrigues et al. 2011; Pal et al. 2020) in efflux assays as it serves as a universal substrate for a wide range of efflux pumps. Actually, two different experimental designs can be used in an accumulation assay. The first is a direct measurement of the increase in fluorescence of EB after its addition to a cell suspension; for this design, lower efflux activity leads to a more rapid increase in fluorescence. The second method is the addition of an increasing EB concentration to cells, in which case cells are able to actively efflux EB up to some particular concentration and EB exceeding this concentration can penetrate the cells, leading to an increase in fluorescence. Both methods were applied to an exponentially growing (motile, rod, non-sporulating) culture of *C. beijerinckii* NRRL-B598. Fluorescence was measured at the individual cell level using flow cytometry in two environments, a saline solution to mimic an environment without a source of energy and TYA medium, containing both a source of energy and all required nutrients. Results are shown in Fig. 1a from which it is obvious that without an energy source, accumulation takes place much faster than with an energy source, confirming that

Fig. 1 Increase in mean fluorescence of cells as a function of incubation time with ethidium bromide (EB; 2 $\mu\text{g}/\text{mL}$). **(a)** Accumulation of EB in time in two different solutions, saline solution (circles) and TYA medium (squares). **(b)** Dynamic fluorescence accumulation within damaged (triangles) cells and vital cells (diamonds) in saline solution, and FC histograms of cells stained in **(c)** saline solution showing the division of cells into two subpopulations (left, active vital cells; right, cells with impaired ability to pump out EB) and **(d)** TYA medium where no apparent division was observed



EB can be used to measure energy-dependent active efflux from *C. beijerinckii*. The accumulation assay in saline solution also identified two distinct populations (Fig. 1b and c), one of apparently higher fluorescence (probably less active or injured cells) and a second with low fluorescence (viable cells). Focusing on these two subpopulations, we could see a different dynamics of EB uptake. Fluorescence of intact cells with lower fluorescence increased steadily for approximately 20 min and then the rate of increase declined, although the cells continued to take up the EB probe. Less active, injured or dead cells with higher fluorescence took up EB very rapidly, reaching nearly steady fluorescence in approximately 15 min. When the accumulation assay was performed in TYA medium, only one population of lower fluorescence intensity was observed (Fig. 1d).

The second method, i.e. cell exposure to increasing EB concentration, was also performed in both solutions with an energy source (TYA medium) and without (saline solution); see Fig. 2. We expected that fluorescence of cells challenged with a low concentration of dye would be comparable to the level of autofluorescence and that by exceeding a certain EB concentration accumulation would occur, resulting in a step-wise increase in fluorescence. However, no such course was observed; instead, fluorescence increased linearly in the range from 0.25 up to 2 $\mu\text{g}/\text{mL}$ EB, and at 5 $\mu\text{g}/\text{mL}$, fluorescence was probably influenced by saturation of nucleic acid binding

sites with EB. Even though the fluorescence of cells loaded with EB in TYA medium was lower, the course of fluorescence change with increasing EB concentration was similar. To understand this phenomenon better, we added carbonyl cyanide m-chlorophenyl hydrazone (CCCP), a substance that is used in efflux assays for its ability to uncouple a proton gradient across the membrane. CCCP had a negligible effect on the time course of mean fluorescence with increasing EB concentration (Fig. 2). It should be noted that data are presented only for the experiment where CCCP was added to accumulation reaction only; protocols with the addition of CCCP

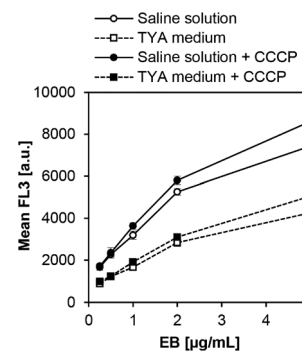


Fig. 2 Mean fluorescence of cell culture loaded for 5 min with increasing concentration of EB in the presence of saline solution, TYA medium and with and without addition of carbonyl cyanide m-chlorophenyl hydrazone (CCCP; 5 $\mu\text{g}/\text{mL}$)

to the growing culture led to a significant decline in viability with almost 100% of inactivated cells after 2 h.

Direct efflux measurement

Direct measurement of efflux was performed as follows: cells were first pelleted by centrifugation to remove all possible energy sources in the cultivation medium and then loaded with EB in saline solution to enable EB to enter the cells under conditions minimizing the effect of active efflux during dye loading. After loading of the dye, the cells were centrifuged and saline solution with EB was replaced by TYA medium or saline solution without EB. Fluorescence was measured immediately for 60 min. The resulting curves (Fig. 3) showed a clear difference between changes in fluorescence over time in the culture without an energy source (saline solution) versus the culture supplemented with an energy source (TYA medium). In saline solution, fluorescence remained constant or slightly increased, which was probably the result of a slightly increased amount of EB intercalated into nucleic acids (EB increases its fluorescence upon intercalation). In contrast, fluorescence of cells in TYA medium declined quickly due to active efflux of the EB. Thus, the direct efflux assay clearly distinguished between energy-dependent efflux and passive diffusion. Direct efflux measurement in an energy-supplemented environment was therefore chosen for direct monitoring of efflux. Data shown in Fig. 3 indicate that fluorescence declined quickly after EB depletion, and at later stages, the decline slowed down. Therefore, the interval of the first 15 min after EB depletion was chosen for the evaluation of efflux rate and linear regression was fitted to the experimentally obtained time course of mean fluorescence of cells. The absolute value of the slope for at least three points

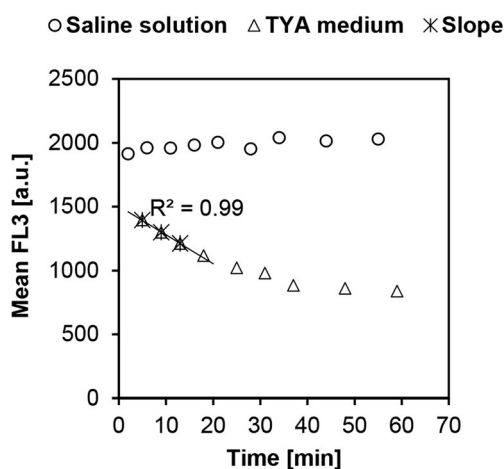


Fig. 3 Fluorescence of cell culture after removal of ethidium bromide (EB) and suspension of cells in TYA medium and saline solution. The linear slope depicted in the chart shows a linear regression of data obtained within the selected time interval for evaluation of efflux

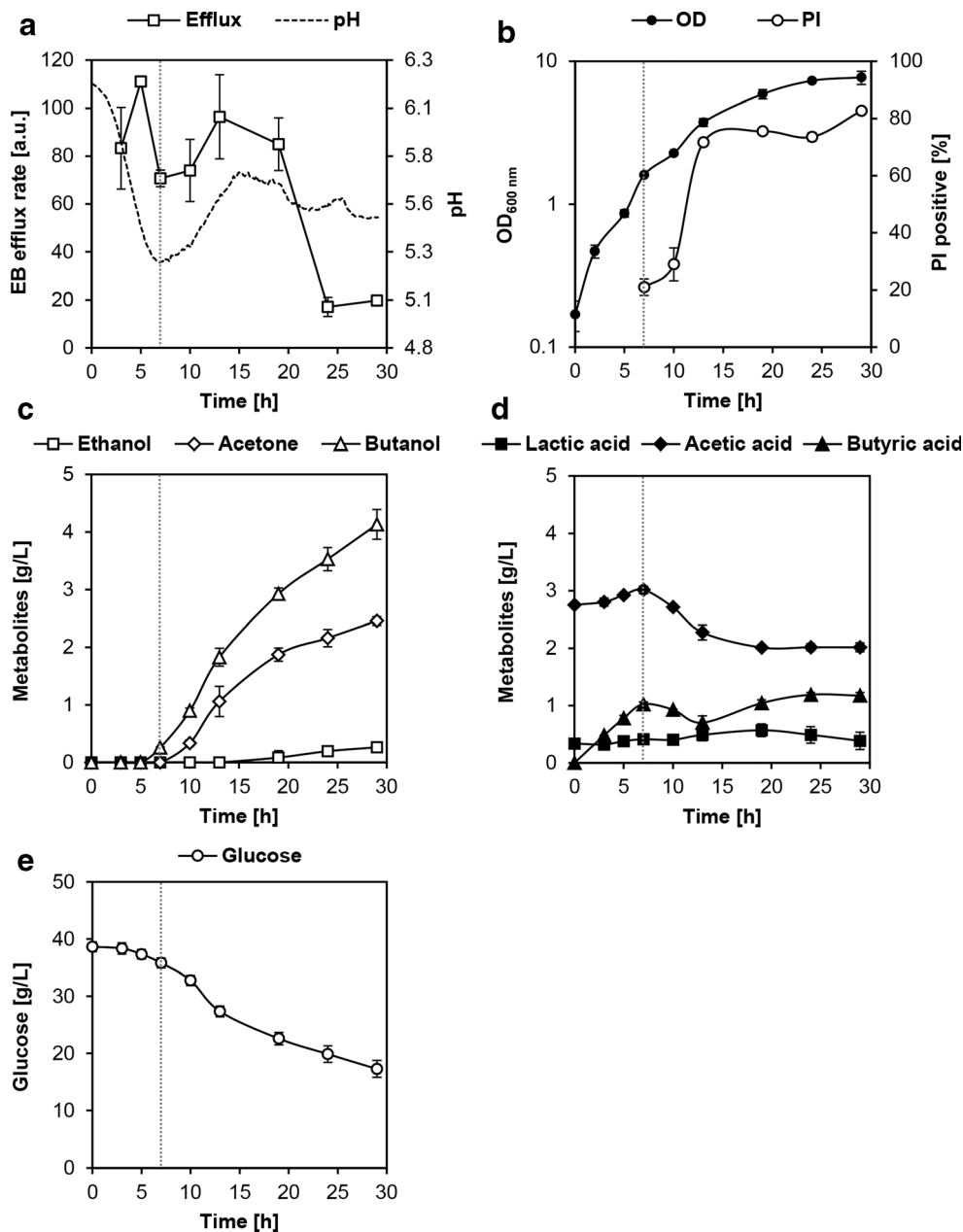
within the interval of 0–15 min after EB removal was used for quantification of efflux in clostridia subjected to two types of cultivation experiment (Figs. 4 and 5).

Efflux changes during standard ABE fermentation

Changes in efflux activity throughout the time course of standard ABE fermentation are shown in Fig. 4, together with the main growth and metabolic parameters and basic analysis of culture fitness using cytometric detection of physiological changes after staining with carboxyfluorescein diacetate and propidium iodide (PI). The efflux rate during a typical ABE process was high throughout the majority of the fermentation. The highest value was reached in late acidogenesis, an apparent drop was recorded in the transient switch stage between acidogenesis and solventogenesis, but it was soon restored and remained high up to the 24th hour when efflux declined strongly to low values, where it remained until the end of the cultivation. We have considered the possible effect of inactive cells in the population; therefore, a viability assay was performed on all samples. While the viability analysis revealed a significant increase in % of PI-positive cells after the shift to solventogenesis, there was no other apparent change up to the 24th hour and metabolic profiles suggested continuing fermentation. The authors would like to note here that, under some conditions, PI positivity does not necessarily mean cell death (Shi et al. 2007) and might reflect some physiological changes including, among others, initiation of sporulation (Jones et al. 2008; Tracy et al. 2008; González-Peñas et al. 2015; Branska et al. 2018). The first mature spores were observed in the culture ($0.8\% \pm 0.2$) shortly after the 24th hour, indicating successful accomplishment of the sporulation cycle.

As there are published data of transcriptomic analysis of the strain tested under similar conditions in the NCBI Sequence Read Archive (SRA) under accession number SRS2757230, we analysed the expression of putative transporters (Jureckova et al. 2018) and grouped them according to their expression profiles (Fig. 5). Ward's hierarchical clustering considering Manhattan distance among particular genes according to their expression profiles revealed two major groups, each of them further dividing into two well-distinguished sub-groups reflecting different physiological patterns. This gene clustering into two main groups was not limited only to transport-related genes but was probably a common phenomenon observed in independent transcriptomic studies of *C. beijerinckii* (Wang et al. 2012; Sedlar et al. 2018). The main groups represented a distinction between genes active predominantly in acidogenic plus early solventogenic stages and genes actively transcribed in later stages or during the whole period analysed. The subdivision of the first group reflects differences between acidogenesis (Fig. 5, red depicted cluster) and the switch into the

Fig. 4 Parameters of standard ABE fermentation showing (a) efflux activity and pH, (b) growth and proportion of inactive cells in culture, (c) concentration of solvents, (d) acids and (e) glucose. The vertical line represents the transition between the acidogenic phase (left) and the solventogenic (right) determined by the change in pH



solventogenic stage (Fig. 5, yellow depicted cluster) while the second group is formed either by genes that are more involved in metabolism during the transition into a stationary or sporulating culture or by genes that cannot be unambiguously assigned to any of the above groups.

Seemingly there are various genes switched on and off during particular stages of ABE fermentation, which may explain the phase-dependent fluctuation in efflux activity. Those genes active predominantly in the acidogenic phase have apparently higher RPKM (Reads Per Kilobase Million) values when compared to the group of genes more transcribed in later stages (see Supplemental_material_1). Much deeper and more

thorough studies should be performed to reveal interconnection between nonspecific efflux activity assayed with EB and specific gene expression of particular pumps. Nevertheless, some general phenomena can be observed. Within the transmembrane proteins and their subunits depicted in Fig. 5, a clear abundance of ABC transporters can be found within the first main group with a higher prevalence in the acidogenic phase (Fig. 5, red cluster). On the other hand, transmembrane subunits of RND family transporters are more frequent in mid to late solventogenic stage (the second major group), together with MATE and MFS families.

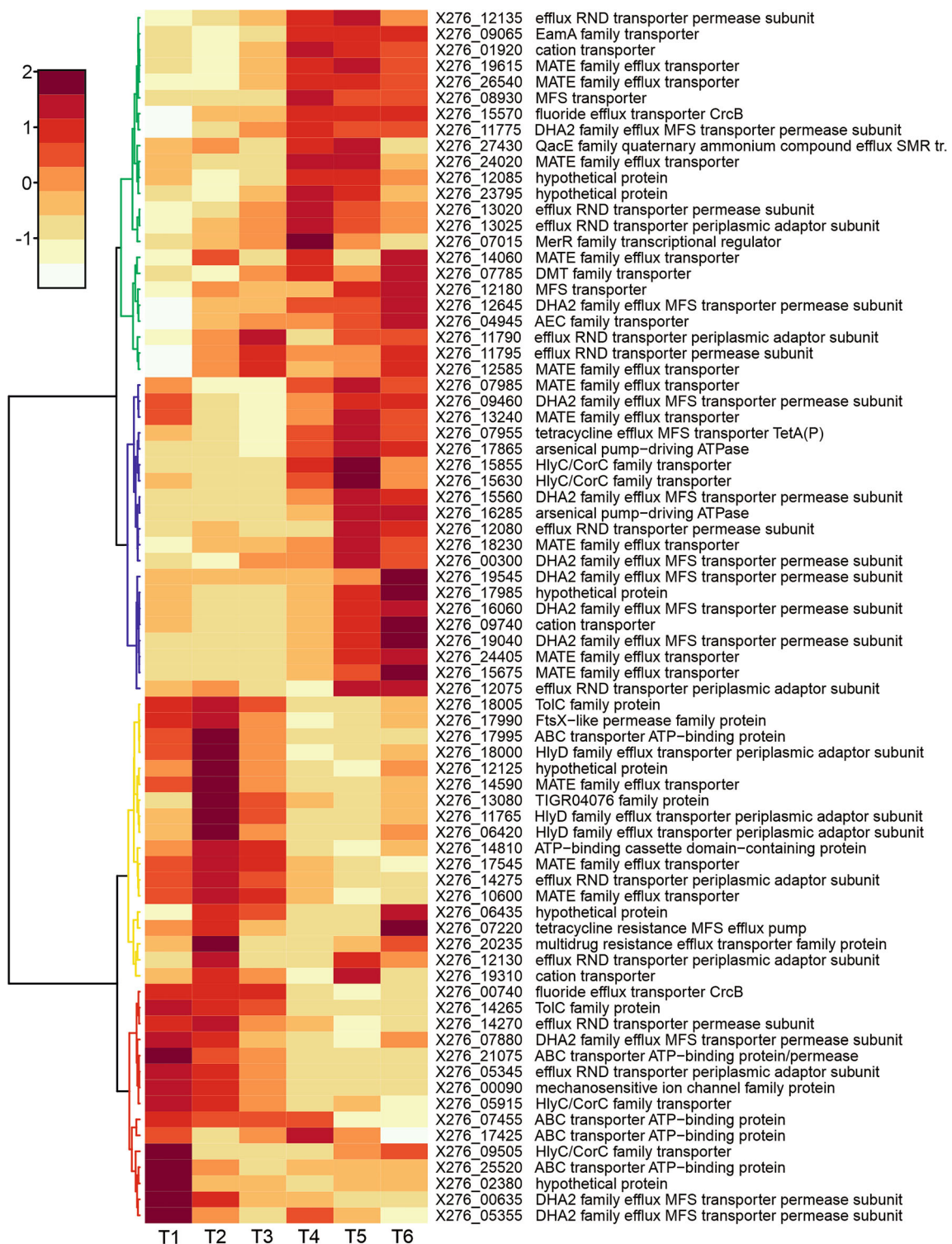


Fig. 5 Transcriptional profiles of genes coding proteins putatively involved in efflux activity of *C. beijerinckii* NRRL-B598 visualized on the heatmap using a Z-score related to an average expression of each gene. Genes are clustered using Ward's hierarchical clustering considering Manhattan distance among transcriptional profiles. T1–T6 are six sampling time points (for more information see Sedlar et al. (2018)),

namely, T1 represents an exponentially growing, acid-producing culture, 3.5 h after initiation of ABE fermentation; T2, the switch from acidogenesis to solventogenesis indicated by a pH turning point at 6 h; T3–T6 early to late solventogenesis and sporulation initiation, sampling times 8.5, 13, 18 and 23 h, respectively

Efflux changes during ABE fermentation with impaired solventogenesis

Since the highest efflux activity was observed for the acidogenic culture in the standard ABE fermentation, we wanted to explore whether this applies generally for acid-producing cultures. The sole acid production is typical for so-called “acid crash”, a phenomenon that sometimes can be observed in a non-pH-controlled cultivation of solventogenic clostridia (Maddox et al. 2000; Schuster et al. 2001; Seo et al. 2017). It is characterized by an accumulation of organic acids while a switch to the solventogenic stage is omitted and cells continue to grow at a decreased rate with acid production leading finally to detrimental acidification of the extracellular environment and cell death. *C. beijerinckii* NRRL B-598 tends to display a phenotype similar to “acid crash” accidentally but can be induced by particular changes in medium preparation as well, although factual triggers for its induction are poorly understood.

We measured efflux activity during cultivation when increased acid production was reached by slightly changed protocol for preparation of the culture medium (see Materials and methods section). Even though the pH curve (Fig. 6) implied “acid crash” behaviour, subsequent HPLC analysis proved suppressed solventogenesis, and after all, some production of solvents apparently started, even though with significantly lower titres. It seemed more like disrupted acid re-utilization than total inhibition of solvent production. Nevertheless, increased toxicity of the acidic environment (decreased pH) together with increasing acid concentration was reached as originally intended. Efflux rate, surprisingly, decreased strongly at the stage when, in standard ABE fermentation, the switch to solventogenesis would occur (which is indicated by cessation of the sharp pH fall). Very low values of efflux rate were observed up to the 24th hour of cultivation when the culture viability dropped significantly and cells started to die from the accumulation of negative factors in the surrounding environment. A decrease in efflux activity was not the only apparently changed physiological feature observed at the switch. The growth rate was also significantly slowed, possibly reflecting attenuated cell activity related to decreased ability of cells to actively pump EB out of the cells. Moreover, no sporulation events were observed throughout the whole fermentation.

Discussion

Even though efflux pumps are highly discussed especially in connection with increased antibiotic resistance in bacteria (Sun et al. 2014; Spengler et al. 2017; Du et al. 2018), their field of action can be much more extensive; e.g. in the case of solventogenic clostridia, active efflux might play a role in cell defence mechanisms against toxicity of solvents produced

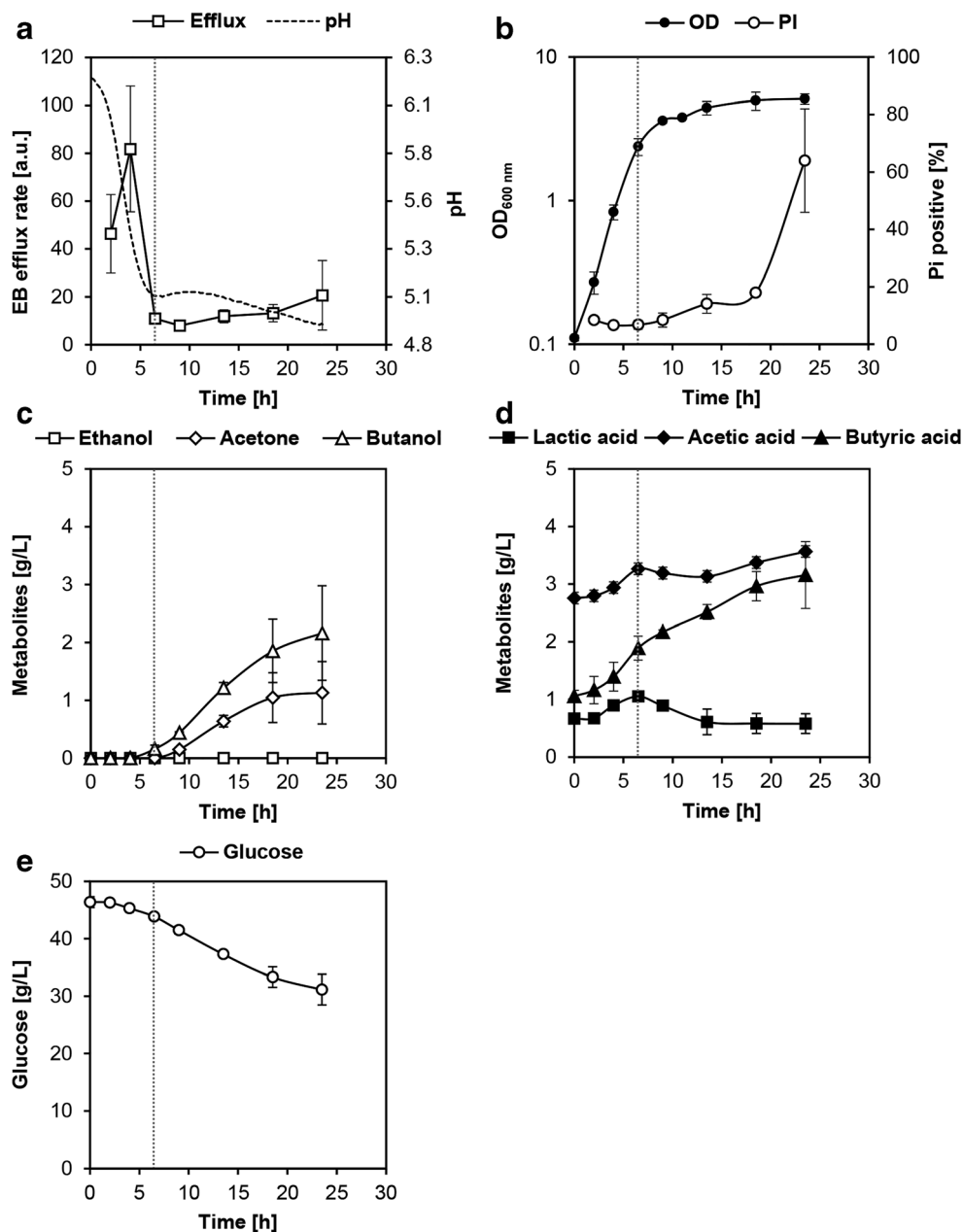
(Patakova et al. 2018; Kusumawardhani et al. 2018). Nowadays, an effort is being made towards the development of high-producing solvent-tolerant strains, e.g. with targeted overexpression of genes encoding heat shock proteins (HSPs) (Tomas et al. 2003; Liao et al. 2017). Increasing the efflux rate of a toxic product might represent another approach for developing hyper-producing mutants. However, knowledge on this issue is still very limited, mainly due to limited tools for studying this phenomenon.

In this work, it was shown that flow cytometry-based methodology could serve for at-line monitoring of active efflux during various cultivation experiments, reflecting the simultaneous activity of a whole range of various transporters. Our original intention was to determine whether the production of solvents accompanied by increasing adverse environmental stress may lead to an increased efflux rate. The results indicated the highest efflux in the acidogenic stage of ABE fermentation, independent of the current butanol concentration. Actually, high efflux pump activity during acidogenesis is not surprising. Increasing acid concentration puts increasing demands on transporting acids and H⁺ ions (Bowles and Ellefson 1985; Guan and Liu 2020) from the cells in order to maintain a stable intracellular environment. Efflux of H⁺ ions occurs at the expense of ATP hydrolysis (Senger and Papoutsakis 2008); because the production of acids at this stage of fermentation is rising, an intensive efflux leads to depletion of cellular energy. In accordance with the expected high activity of ABC transporters during acidogenesis, transcription profile of genes related to ABC pumps clustered within the group of genes exhibiting higher expression during acidogenesis and early solventogenesis (Fig. 5). The switch to solventogenesis can be considered a rescue mechanism from the negative impact of decreasing pH caused by the accumulation of butyric and acetic acids. Results similar to ours were obtained for *C. acetobutylicum* ATCC 824, where a high rate of H⁺ ion efflux was reported at the beginning of the acidogenic phase, with a decrease towards the end, coinciding with the maximum production of butyric and acetic acids (Senger and Papoutsakis 2008).

Re-utilization of acids was observed after the 7th hour of cultivation (Fig. 4), which could lead to a lower rate of efflux. Simultaneously, low concentrations of solvents were already present in the medium; therefore, activation of other pumps capable of solvent excretion could be possible. Increased expression of genes encoding efflux pumps such as RND transporter subunits belonging to the RND family (the most probable family involved in solvent efflux (Kusumawardhani et al. 2018)) was observed in all four major clusters but with higher prevalence in solventogenic phase, supporting the putative function of this type of pumps in the transportation of solvent across cytoplasmic membrane.

The group of genes encoding putative efflux pumps activated in mid to late solventogenesis (Fig. 5) included various

Fig. 6 Parameters of cultivation with impaired solventogenesis: (a) efflux activity and pH, (b) growth and proportion of inactive cells in culture, (c) concentration of solvents, (d) acids and (e) glucose. The vertical line represents the transition between the acidogenic phase (left) and expected solventogenic phase (right) determined by the change in pH



transporters of RND, MATE and MFS efflux pump families and decreased incidence of ABC pumps was observed. At this stage, increased expression of the MerR family of transcriptional regulators was also observed. Regulators belonging to this family are characterized by their response to environmental stimuli, including oxidative stress or antibiotics (Brown et al. 2003), and some members of the family are reported to act as activators of efflux transporters in *Bacillus* (Ahmed et al. 1994).

Contrary to our expectations, efflux did not increase with rising solvent concentration, but rather the opposite. During late solventogenesis, cells ceased active pumping of EB. At this stage, the first mature spores were released from mother

cells and a decrease in viability suggested cells were approaching the end of ABE fermentation. This type of predictive repression of physiological functions and loss of viability prior to attaining an inhibitory concentration of solvent has been reported previously in a culture of *C. beijerinckii* NRRL-B598 (Branska et al. 2018).

An unambiguous difference between efflux rates during standard ABE and acid crash-like fermentation indicates the possible involvement of efflux pumps in solvent transport. The role of particular efflux pumps in short-chain organic solvent transport has been studied through overexpression of genes for various types of efflux pumps that might be involved. Outcomes of this research mostly admit the active

efflux of solvents as one of the possible transport mechanisms; however, the issue is not fully understood yet. Particularly for butanol, the extended screening of non-native RND efflux pumps in *E. coli* (Dunlop et al. 2011) has disproved the active efflux of butanol. Other reports describe a positive impact of particular pumps expression on the survival of cells in the presence of butanol, or on production, particularly TtgABC (Basler et al. 2018) and SrpABC (Kieboom et al. 1998; Bui et al. 2015), both from *Pseudomonas putida* and MdtJ (Zhang et al. 2018) and AcrB (Reyes et al. 2011; Fisher et al. 2013; He et al. 2019) from *E. coli*. Most studies consider the RND-type pumps as the best candidates for solvents extrusion, but another pump families might also be involved. For example, upregulation of ABC-type pumps was observed upon butanol challenge of acidogenic *C. acetobutylicum* chemostat cells (Schwarz et al. 2012) and improvement of isopentenol production was recorded after expression of certain genes encoding ABC transporter in *E. coli* (Foo et al. 2014). Many published studies concerning solvent efflux used non-native pumps from aerobic production systems and were tested in Gram-negative bacteria with mainly externally added solvents; therefore, conclusions from them should be taken cautiously if compared to the results obtained for solventogenic clostridia. Unfortunately, there is a limited number of studies available directly for solventogenic clostridia or their native pumps, probably because the genetic transformation of clostridia is an experimental challenge in itself (Kolek et al. 2016b). However, some reports are beginning to emerge, e.g. the very recent study successfully utilized RND-type pump from *Pseudomonas putida* for the enhancement of tolerance of *Clostridium saccharoperbutylacetonicum* to lignocellulose-derived inhibitors and butanol (Jiménez-Bonilla et al. 2020). Another example is the overexpression of a putative ABC-type transporter and related regulator in *C. acetobutylicum* (Yang et al. 2020) which led to faster growth and solvent formation. In general, the role of efflux pumps in solvent tolerance seems to have a solid base with an increasing number of studies supporting this presumption (see recent review (Vasylykivska and Patakova 2020)). Another major issue is the harmful effect of an abundant presence of efflux pumps in the cell membrane. Generally, the ratio of membrane proteins to cytoplasmic ones is low due to limited space in the membrane compared to the cytoplasmic compartment (Kell et al. 2015). An increased expression of membrane-bound subunits of pumps might lead to disruption of membrane homeostasis (Wagner et al. 2007). Therefore expression of efflux pumps must be precisely tuned (Harrison and Dunlop 2012; Turner and Dunlop 2015; Boyarskiy et al. 2016; Basler et al. 2018). Moreover, cooperation between pumps might be needed for increased tolerance (Rojas et al. 2001; Turner and Dunlop 2015). Even though these studies used non-native pumps and artificial regulation systems, we believe that assessment of overall pump activity

might be very helpful in searching for interconnections between increased tolerance and pump activity; cooperation and sensitive control might take place in natural systems too.

Screening of pump activities has become a common approach for the detection of antibiotic resistance caused by increased activity of multidrug efflux pumps (Patel et al. 2010). However, in multidrug resistance studies, cultivation-based determination of the MIC of EB serves as an indicator of stably increased efflux activity. With regard to the natural purpose of efflux pumps and the need for tight regulation, it seems obvious that an efflux rate must change according to actual cell requirements. Hence a method that would monitor efflux rate throughout the time course of cultivation might be very useful. Two quantitative assays for bacterial cultures or individual cells that can be used for such monitoring are described in the literature: an indirect accumulation assay (Rodrigues et al. 2011) and direct measurement of efflux of a suitable indicator. In order to identify a suitable method for quantification of efflux in *C. beijerinckii*, we tested both approaches in various experimental arrangements. An accumulation assay might be influenced by different factors. We think that the most critical is altered levels of EB penetration into cells due to changed permeability of the membrane, caused by an alteration in the composition of the cytoplasmic membrane (Blair and Piddock 2016) at various stages of ABE fermentation. This is a well-known phenomenon in solventogenic clostridia as an adaptive mechanism to increase in the solvent concentration (Kolek et al. 2015; Kusumawardhani et al. 2018). While the efflux of solvents is an energy-demanding process (Matsumoto et al. 2002), we searched for methodologies capable of distinguishing between active and passive fluxes of EB by modification of methodologies described previously (Viveiros et al. 2008; Paixão et al. 2009; Costa et al. 2018). During the experimental examination, we recognized different patterns of accumulation and efflux of EB depending on whether the energy source was present or not. Nevertheless, we chose the direct efflux measurement method, because EB accumulated in the absence of an energy source and afterwards pumped out in a glucose-containing growth medium provided a simpler and clearer quantification of efflux activity. The method based on quantification of the rate of active nonspecific efflux of EB from cells revealed that overall efflux activity changed significantly throughout the cultivation probably as a consequence of induction and suppression of a new set of transporters. Given the original plan to correlate efflux results with butanol production, we would like to mention that performing the analysis with EB we were able to reflect the total efflux rather than the specific efflux of solvents. Even so, we believe that knowledge of efflux changes introduced in this work helps understanding its fluctuation during the ABE fermentation as well as a possible contribution to the tolerance of solventogenic clostridia. At the same time, flow cytometry using different fluorescent probes can be

an important tool for studying this area, as shown, for example, in a recent study where extensive screening of both efflux and influx was successfully performed using other fluorescent probes for mutants with a knockout of genes for various transporters (Jindal et al. 2019).

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00253-020-11072-2>.

Authors' contributions BB wrote the manuscript and optimized the efflux assay; BB together with MV and HR performed the experiments; KS and IP designed the study. KS and KJ analysed the data. PP supervised the lab work and all authors contributed to the manuscript preparation. All authors discussed the results and read and approved the final manuscript.

Funding This work has been supported by grant project GACR 17-00551S.

Data availability Data generated or analysed during this study are included in this published article; transcriptomic data for analysed genes are available as the Supplementary file 1. Bacterial strain is available in the ARS/NRRL culture collection.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval This article does not contain any studies performed by any of the authors using human participants or animals.

References

- Ahmed M, Borsch CM, Taylor SS, Vázquez-Laslop N, Neyfakh AA (1994) A protein that activates expression of a multidrug efflux transporter upon binding the transporter substrates. *J Biol Chem* 269:28506–28513
- Basler G, Thompson M, Tullman-Ercek D, Keasling J (2018) A *Pseudomonas putida* efflux pump acts on short-chain alcohols. *Biotechnol Biofuels* 11:136. <https://doi.org/10.1186/s13068-018-1133-9>
- Blair JMA, Piddock LJV (2016) How to measure export via bacterial multidrug resistance efflux pumps. *MBio* 7. <https://doi.org/10.1128/mBio.00840-16>
- Bowles LK, Ellefson WL (1985) Effects of butanol on *Clostridium acetobutylicum*. *Appl Environ Microbiol* 50:1165–1170
- Boyarskiy S, Tullman-Ercek D (2015) Getting pumped: membrane efflux transporters for enhanced biomolecule production. *Curr Opin Chem Biol* 28:15–19. <https://doi.org/10.1016/j.cbpa.2015.05.019>
- Boyarskiy S, Davis López S, Kong N, Tullman-Ercek D (2016) Transcriptional feedback regulation of efflux protein expression for increased tolerance to and production of n-butanol. *Metab Eng* 33:130–137. <https://doi.org/10.1016/j.ymben.2015.11.005>
- Branska B, Pechacova Z, Kolek J, Vasylykivska M, Patakova P (2018) Flow cytometry analysis of *Clostridium beijerinckii* NRRL B-598 populations exhibiting different phenotypes induced by changes in cultivation conditions. *Biotechnol Biofuels* 11:99. <https://doi.org/10.1186/s13068-018-1096-x>
- Branska B, Fortova L, Dvorakova M, Liu H, Patakova P, Zhang J, Melzoch M (2020) Chicken feather and wheat straw hydrolysate for direct utilization in biobutanol production. *Renew Energy* 145: 1941–1948. <https://doi.org/10.1016/j.renene.2019.07.094>
- Brown NL, Stoyanov JV, Kidd SP, Hobman JL (2003) The MerR family of transcriptional regulators. *FEMS Microbiol Rev* 27:145–163. [https://doi.org/10.1016/S0168-6445\(03\)00051-2](https://doi.org/10.1016/S0168-6445(03)00051-2)
- Bui LM, Lee JY, Geraldi A, Rahman Z, Lee JH, Kim SC (2015) Improved n-butanol tolerance in *Escherichia coli* by controlling membrane related functions. *J Biotechnol* 204:33–44. <https://doi.org/10.1016/j.jbiotec.2015.03.025>
- Cheng C, Bao T, Yang ST (2019) Engineering *Clostridium* for improved solvent production: recent progress and perspective. *Appl Microbiol Biotechnol* 103:5549–5566
- Costa SS, Viveiros M, Pomba C, Couto I (2018) Active antimicrobial efflux in *Staphylococcus epidermidis*: building up of resistance to fluoroquinolones and biocides in a major opportunistic pathogen. *J Antimicrob Chemother* 73:320–324. <https://doi.org/10.1093/jac/dkx400>
- Delmar JA, Yu EW (2015) The AbgT family: a novel class of antimetabolite transporters. <https://doi.org/10.1002/pro.2820>
- Du D, Wang-Kan X, Neuberger A, van Veen HW, Pos KM, Piddock LJV, Luisi BF (2018) Multidrug efflux pumps: structure, function and regulation. *Nat Rev Microbiol* 16:523–539. <https://doi.org/10.1038/s41579-018-0048-6>
- Dunlop MJ, Dossani ZY, Szmidi HL, Chu HC, Lee TS, Keasling JD, Hadi MZ, Mukhopadhyay A (2011) Engineering microbial biofuel tolerance and export using efflux pumps. *Mol Syst Biol* 7:487. <https://doi.org/10.1038/msb.2011.21>
- Fernandes P, Ferreira BS, Cabral JMS (2003) Solvent tolerance in bacteria: role of efflux pumps and cross-resistance with antibiotics. *Int J Antimicrob Agents* 22:211–216. [https://doi.org/10.1016/S0924-8579\(03\)00209-7](https://doi.org/10.1016/S0924-8579(03)00209-7)
- Fisher MA, Boyarskiy S, Yamada MR, Kong N, Bauer S, Tullman-Ercek D (2013) Enhancing tolerance to short-chain alcohols by engineering the *Escherichia coli* AcrB efflux pump to secrete the non-native substrate n-Butanol. <https://doi.org/10.1021/sb400065q>
- Foo JL, Jensen HM, Dahl RH, George K, Keasling JD, Lee TS, Leong S, Mukhopadhyay A (2014) Improving microbial biogasoline production in *Escherichia coli* using tolerance engineering. *MBio* 5:1932–1946. <https://doi.org/10.1128/mbio.01932-14>
- González-Peñas H, Lu-Chau TA, Moreira MT, Lema JM (2015) Assessment of morphological changes of *Clostridium acetobutylicum* by flow cytometry during acetone/butanol/ethanol extractive fermentation. *Biotechnol Lett* 37:577–584. <https://doi.org/10.1007/s10529-014-1702-3>
- Guan N, Liu L (2020) Microbial response to acid stress: mechanisms and applications. *Appl Microbiol Biotechnol* 104:51–65
- Harrison ME, Dunlop MJ (2012) Synthetic feedback loop model for increasing microbial biofuel production using a biosensor. *Front Microbiol* 3. <https://doi.org/10.3389/fmicb.2012.00360>
- Hassan KA, Liu Q, Henderson PJF, Paulsen IT (2015) Homologs of the *Acinetobacter baumannii* Acel transporter represent a new family of bacterial multidrug efflux systems. *MBio* 6:e01982–e01914. <https://doi.org/10.1128/mBio.01982-14>
- He X, Xue T, Ma Y, Zhang J, Wang Z, Hong J, Hui L, Qiao J, Song H, Zhang M (2019) Identification of functional butanol-tolerant genes from *Escherichia coli* mutants derived from error-prone PCR-based whole-genome shuffling. *Biotechnol Biofuels* 12:73. <https://doi.org/10.1186/s13068-019-1405-z>
- Jiménez-Bonilla P, Zhang J, Wang Y, Bliersch D, de-Bashan LE, Guo L, Wang Y (2020) Enhancing the tolerance of *Clostridium saccharoperbutylacetonicum* to lignocellulosic-biomass-derived inhibitors for efficient biobutanol production by overexpressing efflux pumps genes from *Pseudomonas putida*. *Bioresour Technol* 312: 123532. <https://doi.org/10.1016/j.biortech.2020.123532>
- Jindal S, Yang L, Day PJ, Kell DB (2019) Involvement of multiple influx and efflux transporters in the accumulation of cationic fluorescent

- dyes by *Escherichia coli*. BMC Microbiol 19:1–16. <https://doi.org/10.1186/s12866-019-1561-0>
- Jones SW, Paredes CJ, Tracy B, Cheng N, Sillers R, Senger RS, Papoutsakis ET (2008) The transcriptional program underlying the physiology of clostridial sporulation. Genome Biol 9:R114. <https://doi.org/10.1186/gb-2008-9-7-r114>
- Jureckova K, Koscova P, Sedlar K, Kolek J, Patakova P, Provaznik I (2018) *In silico* prediction of genes coding efflux pumps in *Clostridium beijerinckii* NRRL B-598. In: Vesely M, Hrdlicka Z, Hanika J, Lubojacky J (eds) Proceedings of the 6th International Conference on Chemical Technology. Czech Society of Industrial Chemistry, Czech Republic, pp 86–90. <https://www.icct.cz/AngiologyKlon-ICCT/media/system/2018/ICCT-2018-Proceedings.pdf>
- Kell DB, Oliver SG, De Koning HP (2014) How drugs get into cells: tested and testable predictions to help discriminate between transporter-mediated uptake and lipoidal bilayer diffusion. <https://doi.org/10.3389/fphar.2014.00231>
- Kell DB, Swainston N, Pir P, Oliver SG (2015) Membrane transporter engineering in industrial biotechnology and whole cell biocatalysis. Trends Biotechnol 33:237–246. <https://doi.org/10.1016/j.TIBTECH.2015.02.001>
- Kieboom J, Dennis JJ, Zylstra GJ, De Bont JAM (1998) Active efflux of organic solvents by *Pseudomonas putida* S12 is induced by solvents. J Bacteriol 180:6769–6772
- Kolek J, Patakova P, Melzoch K, Sigler K, Rezanka T (2015) Changes in membrane plasmalogens of *Clostridium pasteurianum* during butanol fermentation as determined by lipidomic analysis. PLoS One 10:e0122058. <https://doi.org/10.1371/journal.pone.0122058>
- Kolek J, Branska B, Drahoukoupil M, Patakova P, Melzoch K (2016a) Evaluation of viability, metabolic activity and spore quantity in clostridial cultures during ABE fermentation. FEMS Microbiol Lett 363:fnw031. <https://doi.org/10.1093/femsle/fnw031>
- Kolek J, Sedlar K, Provaznik I, Patakova P (2016b) Dam and Dcm methylations prevent gene transfer into *Clostridium pasteurianum* NRRL B-598: development of methods for electrotransformation, conjugation, and sonoporation. Biotechnol Biofuels 9:14. <https://doi.org/10.1186/s13068-016-0436-y>
- Kusumawardhani H, Hosseini R, de Winde JH (2018) Solvent tolerance in bacteria: fulfilling the promise of the Biotech Era? Trends Biotechnol 36:1025–1039
- Liao Z, Zhang Y, Luo S, Suo Y, Zhang S, Wang J (2017) Improving cellular robustness and butanol titers of *Clostridium acetobutylicum* ATCC824 by introducing heat shock proteins from an extremophilic bacterium. J Biotechnol 252:1–10. <https://doi.org/10.1016/j.jbiotec.2017.04.031>
- Love MI, Huber W, Anders S (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 15:550. <https://doi.org/10.1186/s13059-014-0550-8>
- Maddox I, Steiner E, Hirsch S, Wessner S, Gutierrez N, Gapes J, Schuster K (2000) The cause of “acid-crash” and “acidogenic fermentations” during the batch acetone-butanol-ethanol (ABE-) fermentation process. J Mol Microbiol Biotechnol 2:95–100
- Matsumoto M, de Bont JAM, Isken S (2002) Isolation and characterization of the solvent-tolerant *Bacillus cereus* strain R1. J Biosci Bioeng 94:45–51. [https://doi.org/10.1016/S1389-1723\(02\)80115-3](https://doi.org/10.1016/S1389-1723(02)80115-3)
- Murtagg F, Legendre P (2014) Ward’s hierarchical agglomerative clustering method: which algorithms implement Ward’s criterion? J Classif 31:274–295. <https://doi.org/10.1007/s00357-014-9161-z>
- Paixão L, Rodrigues L, Couto I, Martins M, Fernandes P, de Carvalho CCCR, Monteiro GA, Sansonetty F, Amaral L, Viveiros M (2009) Fluorometric determination of ethidium bromide efflux kinetics in *Escherichia coli*. J Biol Eng 3:18. <https://doi.org/10.1186/1754-1611-3-18>
- Pal S, Misra A, Banerjee S, Dam B (2020) Adaptation of ethidium bromide fluorescence assay to monitor activity of efflux pumps in bacterial pure cultures or mixed population from environmental samples. J King Saud Univ - Sci 32:939–945. <https://doi.org/10.1016/j.jksus.2019.06.002>
- Patakova P, Kolek J, Sedlar K, Koscova P, Branska B, Kupkova K, Paulova L, Provaznik I (2018) Comparative analysis of high butanol tolerance and production in clostridia. Biotechnol Adv 36:721–738
- Patakova P, Branska B, Sedlar K, Vasylykivska M, Jureckova K, Kolek J, Koscova P, Provaznik I (2019) Acidogenesis, solventogenesis, metabolic stress response and life cycle changes in *Clostridium beijerinckii* NRRL B-598 at the transcriptomic level. Sci Rep 9:1371. <https://doi.org/10.1038/s41598-018-37679-0>
- Patel D, Kosmidis C, Seo SM, Kaatz GW (2010) Ethidium bromide MIC screening for enhanced efflux pump gene expression or efflux activity in *Staphylococcus aureus*. Antimicrob Agents Chemother 54:5070–5073. <https://doi.org/10.1128/AAC.01058-10>
- Reyes LH, Almario MP, Kao KC (2011) Genomic library screens for genes involved in n-butanol tolerance in *Escherichia coli*. PLoS One 6:e17678. <https://doi.org/10.1371/journal.pone.0017678>
- Reyes LH, Abdelaal AS, Kao KC (2013) Genetic determinants for n-butanol tolerance in evolved *Escherichia coli* mutants: cross adaptation and antagonistic pleiotropy between n-butanol and other stressors. Appl Environ Microbiol 79:5313–5320. <https://doi.org/10.1128/AEM.01703-13>
- Rodrigues L, Ramos J, Couto I, Amaral L, Viveiros M (2011) Ethidium bromide transport across *Mycobacterium smegmatis* cell-wall: correlation with antibiotic resistance. BMC Microbiol 11:35. <https://doi.org/10.1186/1471-2180-11-35>
- Rojas A, Duque E, Mosqueda G, Golden G, Hurtado A, Ramos JL, Segura A (2001) Three efflux pumps are required to provide efficient tolerance to toluene in *Pseudomonas putida* DOT-T1E. J Bacteriol 183:3967–3973. <https://doi.org/10.1128/JB.183.13.3967-3973.2001>
- Schuster KC, Goodacre R, Gapes JR, Young M (2001) Degeneration of solventogenic *Clostridium* strains monitored by Fourier transform infrared spectroscopy of bacterial cells. J Ind Microbiol Biotechnol 27:314–321. <https://doi.org/10.1038/sj.jim.7000146>
- Schwarz KM, Kuit W, Grimmmler C, Ehrenreich A, Kengen SWM (2012) A transcriptional study of acidogenic chemostat cells of *Clostridium acetobutylicum* - cellular behavior in adaptation to n-butanol. J Biotechnol 161:366–377. <https://doi.org/10.1016/j.jbiotec.2012.03.018>
- Sedlar K, Koscova P, Vasylykivska M, Branska B, Kolek J, Kupkova K, Patakova P, Provaznik I (2018) Transcription profiling of butanol producer *Clostridium beijerinckii* NRRL B-598 using RNA-Seq. BMC Genomics 19:415. <https://doi.org/10.1186/s12864-018-4805-8>
- Senger RS, Papoutsakis ET (2008) Genome-scale model for *Clostridium acetobutylicum*: part II. Development of specific proton flux states and numerically determined sub-systems. Biotechnol Bioeng 101:1053–1071. <https://doi.org/10.1002/bit.22009>
- Seo S-O, Janssen H, Magis A, Wang Y, Lu T, Price ND, Jin Y-S, Blaschek HP (2017) Genomic, transcriptional, and phenotypic analysis of the glucose derepressed *Clostridium beijerinckii* mutant exhibiting acid crash phenotype. Biotechnol J 12:1700182. <https://doi.org/10.1002/biot.201700182>
- Shi L, Gunther S, Hubschmann T, Wick LY, Harms H, Muller S (2007) Limits of propidium iodide as a cell viability indicator for environmental bacteria. Cytometry A 71A:592–598. <https://doi.org/10.1002/cyto.a.20402>
- Spengler G, Kincses A, Gajdacs M, Amaral L (2017) New roads leading to old destinations: efflux pumps as targets to reverse multidrug resistance in bacteria. Molecules 22:468. <https://doi.org/10.3390/molecules22030468>
- Sun J, Deng Z, Yan A (2014) Bacterial multidrug efflux pumps: mechanisms, physiology and pharmacological exploitations. Biochem

- Biophys Res Commun 453:254–267. <https://doi.org/10.1016/J.BBRC.2014.05.090>
- Tomas CA, Welker NE, Papoutsakis ET (2003) Overexpression of groESL in *Clostridium acetobutylicum* results in increased solvent production and tolerance, prolonged metabolism, and changes in the cell's transcriptional program. Appl Environ Microbiol 69:4951–4965. <https://doi.org/10.1128/AEM.69.8.4951-4965.2003>
- Tracy BP, Gaida SM, Papoutsakis ET (2008) Development and application of flow-cytometric techniques for analyzing and sorting endospore-forming Clostridia. Appl Environ Microbiol 74:7497–7506. <https://doi.org/10.1128/aem.01626-08>
- Tracy BP, Jones SW, Fast AG, Indurthi DC, Papoutsakis ET (2012) Clostridia: the importance of their exceptional substrate and metabolite diversity for biofuel and biorefinery applications. Curr Opin Biotechnol 23:364–381. <https://doi.org/10.1016/J.COPBIO.2011.10.008>
- Turner WJ, Dunlop MJ (2015) Trade-offs in improving biofuel tolerance using combinations of efflux pumps. ACS Synth Biol 4:1056–1063. <https://doi.org/10.1021/sb500307w>
- Vasylykivska M, Patakova P (2020) Role of efflux in enhancing butanol tolerance of bacteria. J Biotechnol 320:17–27
- Viveiros M, Martins A, Paixão L, Rodrigues L, Martins M, Couto I, Fährnich E, Kern WV, Amaral L (2008) Demonstration of intrinsic efflux activity of *Escherichia coli* K-12 AG100 by an automated ethidium bromide method. Int J Antimicrob Agents 31:458–462. <https://doi.org/10.1016/j.ijantimicag.2007.12.015>
- Wagner S, Baars L, Ytterberg AJ, Klussmeier A, Wagner CS, Nord O, Nygren, Van Wijk KJ, De Gier J-W (2007) Consequences of membrane protein overexpression in *Escherichia coli*. Mol Cell Proteomics 6(9):1527–1550. <https://doi.org/10.1074/mcp.M600431-MCP200>
- Wang Y, Li X, Mao Y, Blaschek HP (2012) Genome-wide dynamic transcriptional profiling in clostridium beijerinckii NCIMB 8052 using single-nucleotide resolution RNA-Seq. BMC Genomics 13:102. <https://doi.org/10.1186/1471-2164-13-102>
- Yang Y, Lang N, Zhang L, Wu H, Jiang W, Gu Y (2020) A novel regulatory pathway consisting of a two-component system and an ABC-type transporter contributes to butanol tolerance in *Clostridium acetobutylicum*. Appl Microbiol Biotechnol 104:5011–5023. <https://doi.org/10.1007/s00253-020-10555-6>
- Zhang Y, Dong R, Zhang M, Gao H (2018) Native efflux pumps of *Escherichia coli* responsible for short and medium chain alcohol. Biochem Eng J 133:149–156. <https://doi.org/10.1016/J.BEJ.2018.02.009>
- Zhao T, Tashiro Y, Sonomoto K (2019) Smart fermentation engineering for butanol production: designed biomass and consolidated bioprocessing systems. Appl Microbiol Biotechnol 103:9359–9371

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.