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Evaluation of cell damage caused by cold sampling and quenching for metabolome analysis

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Abstract Cell damage during sampling and quenching for metabolome analysis have been investigated at whole sample level using an OD-based method and ATP loss investigation, and at single cell level by means of flow cytometry. *Escherichia coli* was cultivated in shake flasks and sampled into several cold quenching solutions during exponential growth phase varying quenching solution composition and sampling temperature. For single cell analysis, the samples were incubated with selective propidium iodide dye and analysed via flow cytometry to differentiate between intact and damaged cells. It was found that every combination of quenching solution, temperature, or cooling rate tested influenced the *E. coli* cell membrane integrity indicating rupture which will not only let the dye in, but also intracellular ATP out of the cells, which is not desired in in vivo metabolome analysis.

Keywords Cell leakage · Quenching · Flow cytometry · Propidium iodide · Membrane integrity

Introduction

Cell leakage during cold-shock quenching has been reported over the last years by numerous research groups seeking for an ideal solution to stop cell activity of pro- and eukaryotic cells in metabolome studies (Bolten et al. 2007; Link et al. 2008; Spura et al. 2009; Villas-Bôas et al. 2007). This phenomenon has been reported long before the boom of the "omics"-era as an answer to cold and osmotic stress, reporting the release of intracellular components like cations and small non-electrolyte molecules (Haest et al. 1972), phosphorylated glucose analogs (Delobbe et al. 1971), thiomethylgalactosidase, valine and galactose (Leder 1972), amino acids (Wargel et al. 1970), and even whole membrane proteins (Neu and Heppel 1965; Nossal and Heppel 1966; Piperno and Oxender 1966). Nevertheless, up to date, no cold quenching agent or technique has been found that enables an accurate estimation of intracellular concentrations of intermediate metabolites with high turnover rates since cell leakage prevents its unambiguous quantification (Villas-Boas and Bruheim 2007).

Besides the development of reproducible sampling procedures for quantitative analysis of the low intracellular metabolite concentrations, the sampling procedure should ensure that the present rapid changing state of the culture is correctly reflected in the measurements (Schaedel and Franco-Lara 2009). Therefore, a sampling procedure for accurate metabolome analysis is only guaranteed when cell integrity and minimal cell damage of the sampled microbial population are also guaranteed. Until now, cell wall and membrane damage has been assumed as the main reason for preventing detailed analysis of in vivo metabolite concentration and distribution (Spura et al. 2009; Villas-Bôas et al. 2007; Villas-Boas and Bruheim 2007), since the contact to any quenching solution might cause the unspecific loss of a variety of metabolites in an unknown amount (Bolten et al. 2007). However, validation of this presumption through whole or single cell analysis during or after the quenching process is actually the exception than the rule for metabolome studies.

To picture the stresses that microbial cells must withstand during rapid cold and quenching sampling, several analogies to works on cell membranes applied to cryopres-

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ervation or to complement-mediated bacterial killing (bactericidal chilling), e.g., for food product preservation, can be named, including (Fay and Farías 1976; Kato and Bito 1978; Beney and Gervais 2001 and references therein; Cao-Hoang et al. 2008; Saragusty et al. 2009 and references therein): (1) increase in the surface to volume ratio through volumetric changes and membrane shrinkage and stretching in response to cold hyperosmotic solutions, (2) toxicity effects of the quenching solution, (3) thermotropic phase transition of microbial membrane phospholipids in response to decreasing temperature, (4) freeze-induced dehydration, (5) osmotic-induced dehydration, (6) loss of cell turgor pressure, (7) plasmolysis, (8) possible mechanical stresses induced by extracellular ice formation as well as cell–cell and cell–sampler walls interactions, (9) ionic and electrical effects resulting from preferential incorporation of some ionic species into the ice, (10) the effects of elevated solute concentration and intracellular ice formation (IIF), which are cooling-rate dependent, (11) cold-induced lipid peroxidation and loss of superoxide dismutase activity, (12) recrystallization during sample preparation for analytics, which is warming-rate dependent, (13) decrease of cell membrane fluidity and increase of its viscosity due to mechanical compression and temperature drop, (14) non-specific membrane lipid extraction through organic solvents in the quenching solution, (15) influence of hydrodynamic energy peaks due to the hydromechanical shear stress through the rapid liquid withdraw generated in the sampling device channels at high sampling rates.

Still, the numerous stresses at which the microbial cells are subject, different aqueous quenching solutions, e.g., buffered methanol (Bolten et al. 2007) or glycerol/saline (Villas-Boas and Bruheim 2007), have been tested in the last years comparing metabolome loss during the quenching and extraction process searching for the ideal composition which enables the minimal influences on cell leakage caused by either osmotic or cold shock (Villas-Bôas et al. 2007). Britten and McClure (1962) determined an osmotic equilibrium of intra- and extracellular metabolites in growing *Escherichia coli* cells. They observed that changes of the osmolarity of the surrounding media removed all the free intracellular amino acids out of the cells (Villas-Bôas et al. 2007). Based on this osmotic principle, the use of a solution which does not alter the cell physiology should therefore reduce osmolaric leakage (Villas-Boas and Bruheim 2007).

The use of methanol as the basic component of quenching solutions has the advantage that very low temperatures can be applied during the cold sampling and quenching process to stop the cell metabolism. The choice of the starting temperature of the quenching solution has to be made carefully because it adds to the amount of leakage during the sampling process (Canelas et al. 2008). A downside of aqueous solutions containing organic solvents is the removal of several intracellular metabolites from bacterial cells (Villas-Bôas et al. 2007) or even cell lysis. Nonetheless, the cold methanol method, originally developed for quenching yeast cells (de Koning and van Darn 1992), still represents the only alternative where the biomass can be separated from the extracellular medium with good efficiency. Extracting cells without removing the media first often leads to severe misjudgement of intracellular concentrations due to extracellular metabolite fractions (Bolten et al. 2007; Canelas et al. 2008). In addition, media components like salts may also interfere with many analytical techniques (Canelas et al. 2008).

Investigation of the possible causes for cell leakage due to sampling and quenching for metabolome analysis can be accomplished through determination of cell membrane integrity by means of flow cytometry. As indicated by Nebe-von-Caron and Badley (1995), the evaluation of membrane integrity is the most definite proof of cell viability since this is an indicator that a cell can potentially generate gradients and is therefore able to carry on metabolic activity. Cells with damaged or compromised membranes are considered as dead (Díaz et al. 2010; Shapiro 2003), since larger membrane defects may deprive the cell of materials it would normally accumulate, may expose it to toxins it would normally exclude, and therefore the cell would not be able to maintain or generate the electrochemical gradient and hence the membrane potential (Shapiro 2003; Díaz et al. 2010).

Flow cytometry is a high-throughput quantitative analysis technology which enables the characterization of cell populations at single cell level. Based on the scattering and/or fluorescence signals (either through auto-fluorescence or using fluorescent dyes) generated after single cell illumination with a laser beam or UV, the individual response intensity is correlated to structural and/or functional cell parameters. The ability of common cytometers to analyze a very large number of particles (around 5,000 cells per second) while simultaneously measuring multiple cellular parameters per cell enables the classification of cells into populations (Díaz et al. 2010 and references therein).

To label damaged cells, several non-vital dyes like propidium bromide, 7-ADD, or propidium iodide (PI) can be applied. These dyes, known as impermeant, are incapable to pass intact cell membranes or walls therefore staining only damaged or dead cells (Shapiro 2003). The advantage of PI is an unnecessary use of a second dye to exclude living cells (Zamai et al. 1996). PI intercalates with DNA and RNA inside the cell, forming a red fluorescent complex (Jones and Senft 1985). The fluorescence signal can then be detected to determine the dye distribution within the cell population (Diaz et al. 2009; Jepras et al. 1995).

In the present work, the effect of several quenching solutions and initial temperatures on cell damage during sampling and quenching for metabolome analysis was studied for *E. coli* by means of flow cytometry. To quantify the degree of cell damage, a PI-staining detection accounting for cell membrane integrity was used. Still, the applicability of the PI-staining method itself was studied too, since several groups have reported PI accumulation in some intact bacteria or cells at exponential growth phase (Díaz et al. 2010; Nebe-von-Caron et al. 2000; Shi et al. 2007; Quirós et al. 2009). Because completely ruptured cells, which also cause metabolite loss, cannot be detected with flow cytometry, total cell rupture was instead quantified by monitoring the reduction of optical density (OD) of treated samples (Delobbe et al. 1971; Svarachorn et al. 1991; Tsuchido et al. 1995) referred to that of untreated cells. A simultaneous analysis of ATP concentrations in quenching solution supernatant and cell extract was performed to account for key metabolite leakage. Throughout the manuscript, the concept of "whole sample level" refers to measurements performed which delivered integral values of the sample taken, like the estimation of the optical density (OD). Contrastingly, flow cytometry delivers differentiated information per events/cells present in the sample, i.e., splitting the integral information into quantitative single cell characteristics and therefore this kind of analysis is referred to as "single cell level".

Material and methods

Strain and culture medium

For this study, *E. coli* strain MG1655 was used. Cultivation was done in minimal medium (Hiller et al. 2007) with only 0.255 g/L $MgSO_4$*7H_2O. Agar plates for cell viability tests consisted of 20 g/L agar, 20 g/L yeast extract, 10 g/L peptone, and 11 g/L glucose monohydrate. Chemicals were purchased from Sigma-Aldrich.

Cultivation

E. coli preculture was inoculated with 200 μL of a glycerol stock stored at −86°C and grown at 30°C over night at 120 rpm in 100 mL of culture medium in 500 mL Erlenmeyer flasks with four baffles in an incubator (Inkubations-Schüttelschrank BS4, Braun, Melsungen, Germany). For the main culture, the preculture was centrifuged and re-suspended in the minimal medium so that 1 mL of the solution was sufficient to inoculate the main culture. Cultures were grown at 37°C starting with an OD_{660} of 0.4 under the same conditions as the preculture.

Quenching

For quenching tests, 4 mL samples from exponential growing culture were taken with a 10 mL electrical glass pipette (Hirschmann, Herrenberg, Germany). The samples were put directly into Falcon tubes containing 16 mL of precooled quenching solutions. Tests were done in triplicates. The quenching solutions themselves were cooled down to −40°C with a cryostat (Lauda, Lauda-Königshofen, Germany). Lower temperatures of the quenching solution were achieved by a −86°C freezer. Afterwards, the falcon tubes were placed in the cryostat at −40°C, and samples were added when the solution reached the target initial temperature. Falcon tubes were weighted for exact sample size determination and centrifuged (5 min, −19°C, 10,000×*g*, Biofuge stratos, Heraeus). Samples from quenching solutions supernatant were stored at −86°C until ATP determination. Additives in cultivation broth were ectoine and hydroxyectoine. Additives in 60% MeOH quenching solution included 70 mM HEPES (Hiller et al. 2007), 40% glycerine/NaCL (5.6% *m*/*v*, saline solution) (Link et al. 2008), glutaraldehyde (GA) (Tsuchido et al. 1995), trehalose (5% *m*/*v*), and mannitol (0.5% *m*/*v*). In addition, 60% glycerine/NaCL (5.6% *m*/*v*, saline solution) (Villas-Boas and Bruheim 2007) was tested.

Monitoring of cell damage

The quenched cell pellet was re-suspended in 4 mL 1% PBS buffer (with 8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4, and 0.24 g/L KH_2PO_4) and placed on ice. OD_{660} was determined, and samples were diluted to 0.1 with PBS buffer. This was done as well to untreated samples from the main culture for comparison. Cell lysis was monitored by reduction of OD_{660} after re-suspension in 4 mL PBS buffer. As parameter, the OD recovery ratio, φ was used:

$$\varphi \equiv \frac{OD_{660,Recovered}}{OD_{660,Original}} \times 100\% \quad (1)$$

where $OD_{660,Recovered}$ is the OD_{660} value of the re-suspended sample and $OD_{660,Original}$ the value of the culture itself. Membrane integrity was determined by incubation with PI (10 μg/mL) for 4 min and measurement of fluorescence with a flow cytometer (Cell Lab Quanta™ Flow Cytometer, Beckman Coulter, Brea, USA).

The Sideward Scatter (SS) signal was used as a trigger signal, and red fluorescence (FL3) was detected through a 670 nm long pass filter. Sample rate never exceeded 1,000 events/s. Signals were logarithmically amplified, and PMT settings were adjusted to particular PI-staining method. Untreated cells from cultivation caused a single peak during FL3 measurement at low fluorescence intensities. These measurements were taken in all cases as reference.

Normalization of FL3 data was done via particular single cell electronic volume measurements to determine the "fluorescence concentration". Cells from quenching solution stained with PI led to two separate peaks, where one was located in the same area as the one from untreated cells and was therefore associated with living, undamaged cells. The area of both peaks was determined, and the area ratios were related to undamaged and damaged cell pool fractions.

ATP in quenching solution supernatant and cell extract was determined using the ATP determination kits of Biaffin (supernatant) and Invitrogen (extract). Extraction was performed using 1 mL of buffered water (30 mM TEA, pH 7.5) at 95°C for 15 min in water bath (Hiller et al. 2007).

Results

Cell lysis through cold sampling

Complete cell rupture/lysis was monitored following the method reported by Delobbe et al. (1971) and Tsuchido et al. (1995) employing the OD recovery ratio, φ. The influence of the main aqueous component methanol on cell damage was first tested by varying its concentration in the quenching solution using a starting temperature for sampling of −20°C. Despite its concentration, methanol had, in essence, a negative effect on the OD recovery ratio of the re-suspended cell pellet, and none of the solutions permitted the complete recovery of the culture OD (Fig. 1). The OD recovery ratio φ ranged from 75% to 85% indicating that about 15% to 25% of the *E. coli* cells in the sample underwent a volume loss and/or lysis process with basically direct metabolite loss into the quenching solution. Using methanol as sole component of a quenching solution therefore provokes cell rupture not only through the shock caused by the high osmotic difference suffered by the cells entering the pure aqueous methanol solution from the culture medium but also through methanol as cell-permeating substance itself (Villas-Boas and Bruheim 2007).

In a second step, the influence of the starting temperature of the quenching solution on cell damage was tested for *E. coli* taking a broader temperature range than that described in Canelas et al. (2008) for yeast. In this case, 100% methanol with a starting temperature below −40°C and a maximum mixing temperature below −20°C was identified as optimal conditions for yeast quenching using a quenching solution-sample volume ratio of 1:5.

In the presented study, temperatures between 0°C and −76°C with a sample quenching solution ratio of 1:5 (Canelas et al. 2008) were used. Results showed that with higher temperatures, the OD recovery ratio declined more and more sinking below 60% at 0°C. The highest OD recovery ratio value of about 88% was reached with samples taken with the sharpest temperature shift using a starting temperature of −76°C (Fig. 2). Using −76°C as sampling temperature was also the only method appropriate to keep the resulting mixture sample/quenching solution temperature below −20°C using this method, which has been traditionally considered to be sufficient to stop the metabolism (Weuster-Botz 1999).

In a third study, the OD recovery ratio test was further applied to other quenching solutions reported in literature which made use of several additives to the methanol-based formulation (Fig. 3).

Mixing 60% methanol with different additives resulted in about 30% loss for HEPES buffered solution (−50°C) (Hiller et al. 2007) which was even more than in un-buffered 60%

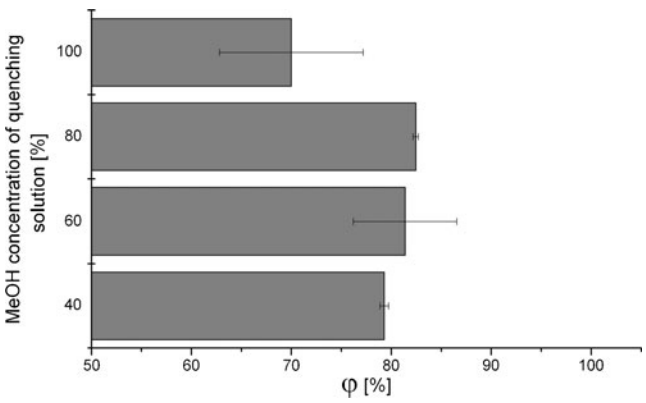

Fig. 1 OD recovery ratio φ vs. methanol concentration. After the cells were quenched at −20°C, centrifuged, and re-suspended in PBS buffer, no culture recovered the original optical density indicating the existence of volume loss and/or lysis process

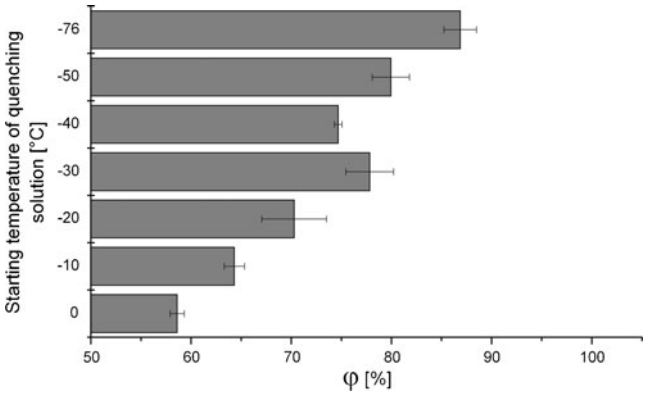

Fig. 2 OD recovery ratios dependent on initial sampling temperature of the quenching solution. Lowering the starting temperature reduced the fraction of lysed cells during the quenching process noticeably. Quenching was performed with a 100% MeOH quenching solution to avoid viscosity problems at low temperatures

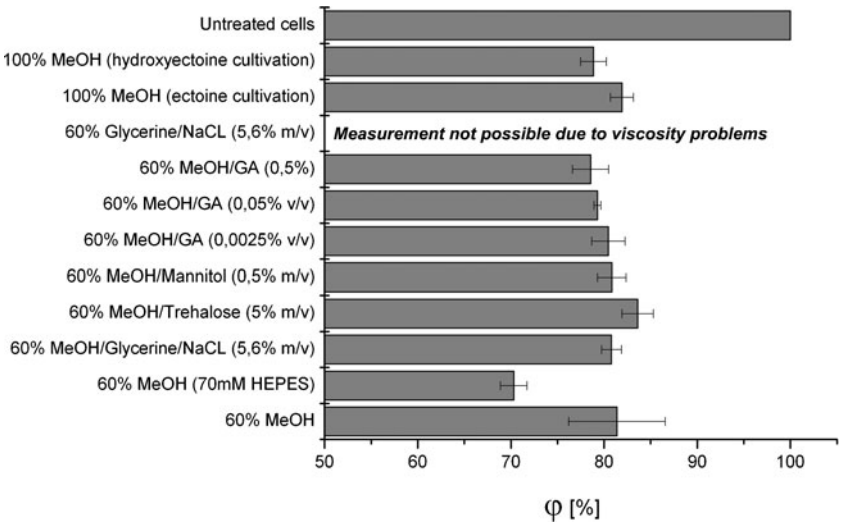

Fig. 3 Effect of quenching solution composition. All solutions containing 60% MeOH led to equal φ values, except for MeOH buffered with HEPES. The saline glycerine solution could not be separated from the cells due to high viscosity of the resulting mixture. Cultivation with ectoine and hydroxyectoine as additives and subsequent quenching in 100% MeOH did not change φ markedly (starting temperature of sampling: GA, trehalose, and mannitol −40°C, Glycerine −20°C, rest −50°C). The use of NaCl in the solution's legends describes the saline solutions used

methanol (20%). The 60% methanol/glycerine (saline) solution reported by (Link et al. 2008) had nearly the same effect as 40% methanol (19%) at a sampling temperature of −50°C. However, the saline solution of Villas-Boas and Bruheim (2007) caused severe problems during sampling and sample preparation. In general, bad mixing was reached due to its extremely high viscosity at −20°C generating a kind of two-phase fluid with discernible cell broth/quenching solution boundaries using pipettes. Cell re-suspension for OD measurements was therefore not possible to perform adequately, because cells could also not be homogeneously separated from the quenching solution with the available equipment, and it would have been necessary to transfer the quenching solution mixture to several smaller tubes for better, but time-consuming centrifugation. Given that samples could not be kept on low temperatures throughout the process, cells quenched in this solution were not further used for extraction. Trehalose (5% *m*/*v*) and mannitol (0.5% *m*/*v*), which have been used traditionally as cryoprotectants (Rudolph et al. 1986; Tibbett et al. 2002; Sztein et al. 2001) led to 16% and 19% loss, respectively. Glutaraldehyde (GA), which is a cell wall stabilizing agent and was already applied by Tsuchido et al. (1995) to stabilize membrane lipids in *Bacillus subtilis* cells (Granboulan and Leduc 1967), was tested in different concentrations as well and led to 19–21% loss of OD (−40°C).

Certainly, a fraction of these differences is also due to the quick re-suspension process in 4 mL of PBS buffer rather than in the loss of the original OD value, i.e., few cells could also be removed from the quenching supernatant after centrifugation of the sample to obtain the cell pellet. The removal of some cells cannot be completely avoided during the procedure. The PBS buffer itself appeared to have no effect on untreated cells but did not completely prevent re-suspended quenched cells to undergo further lysis. Still, the decrease on the OD recovery ratio is a good and direct indicator for the effect of the cold sampling and quenching process on whole cell damage.

Single cell damage through cold sampling

After cold sampling, quenched cells were re-suspended in PBS buffer, incubated with PI, and analyzed in a flow cytometer. The measured fluorescence was automatically normalized to the single cell electronic volume estimated via the Coulter-counter principle. Including the analysis of non-treated not quenched *E. coli* cells, two distinguishable peaks were detected, one related to intact and one to damaged cells emitting fluorescence due to PI labeling. The technique was validated with heat-treated cells in combination with agar cultivation of the same samples.

Hiller et al. (2007) used SYTO 9 and PI in microtiter plates to validate the cell viability during the quenching process. Cells (99.1±0.8%) were detected as viable with an intact cell wall. Opposite findings at the single cell scale in this study suggest that cell membrane is disrupted during the quenching process. Essentially, every single quenching solution tested resulted in a distinct pool of damaged cells (Fig. 4). The variation of the methanol concentration at a starting temperature of −20°C showed that 40% or 60% of methanol led to around 50% to 60% of PI-labeled cells. Using 80% or even 100% of methanol led to about 100% labeling, and therefore to 100% membrane damaged cells. Culturability of the quenched cells, i.e., the existence of colony-forming units, was confirmed using agar plates. Barely any cells were detected in samples quenched with 80% MeOH, none in 100%, and a closed biological film with 40% and 60% MeOH (data not shown), while temperature variation did not change the influence of 100% MeOH on PI labeling.

The addition of HEPES (Hiller et al. 2007) as a buffering agent and GA (Tsuchido et al. 1995) in concentrations of

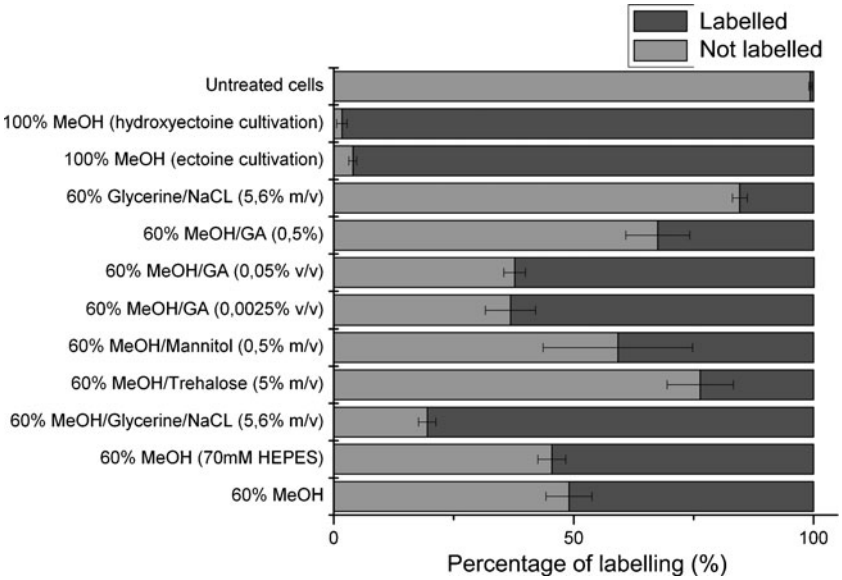

Fig. 4 Relative fraction of PI-labeled cells using different methanol concentrations and supplements in the quenching solution. Buffered methanol (70 mM HEPES), 0.0025% and 0.05% GA did not change the pattern. Trehalose, mannitol, 0.5% GA, and glycerine increased the amount of not labeled cells. The minimal damage was caused by the glycerine/saline solution

0.0025% and 0.05% did not change the resulting percentage of labeled cells in 60% methanol. The use of a combination of 60% methanol and glycerine with the same amount of NaCL (saline solution, Villas-Boas and Bruheim 2007) resulted in an even higher percentage. Patterns were shifted to unlabeled cells only by using 0.5% GA (33%) and the cryoprotectants mannitol (41%) as well as trehalose (24%), and also by the solution based on glycerine.

As for the saline solution of Villas-Boas and Bruheim (2007), although it was not possible to estimate an accurate OD recovery ratio for this sample, the isolation and further dilution in PBS buffer for cytometric analysis was possible. The supernatant itself was filtered through a 0.2-μm filter unit and was analyzed for ATP. In relation to single cell damage, it could be confirmed that the saline solution can be considered as a gentile quenching solution, since it resulted in the least percentage of labeled cells.

ATP leakage through cold sampling

To search for a functional relationship between the damage of cells to the loss of intracellular metabolites, ATP concentration in supernatant and cell extract was analyzed varying the starting temperature of the 100% methanol quenching solution. The ATP concentration in the quenching solution supernatant and in the cell extract were examined, since it has been shown in the past that ATP can be used as an appropriate indicator for metabolite leakage, as it is usually not excreted from the cell interior under normal cultivation conditions and only present in low concentrations in the culture media (Link et al. 2008; Nasution et al. 2006).

Variation in methanol concentration in the quenching solution at a starting temperature of −20°C led to a high loss of ATP during the whole quenching process (Fig. 5). In general, high extraction values were observed in solutions with high MeOH concentrations.

Contrastingly, lower starting temperatures led to higher ATP concentration in the cell extract (Fig. 6). That is, less extracellular ATP was detected in the quenching solution supernatant with temperatures below −40°C, which can be a result of diminished whole cell damage. As for the intracellular ATP extracted from the quenched cells, two groups were distinguishable with a sampling temperature of −30°C as threshold value. While in the first group, fairly constant ATP concentrations around 1,600 $\mu g/g_{DCW}$ were measured for cell extracts sampled with temperatures equal to or below −30°C, lower ATP concentrations of approx. 1,200 $\mu g/g_{DCW}$ were detected for sampling temperatures above −30°C.

Of all additives and cryoprotectants tested in combination with 60% methanol, like HEPES, trehalose, mannitol, glycerine, or GA, only glycerine led to a higher ATP concentration in the cell extract (Fig. 7). However, although the glycerine-based solution led to a quite high ATP concentration in the supernatant, the high concentration could be due to slower chilling and, therefore, to subtle cell treatment after the quenching process.

The compatible solutes ectoine and hydroxyectoine in the cultivation media did not affect the ATP concentration in the supernatant of 100% MeOH quenching solution or in the cell extract noticeably, which probably simply means that, due to a lack of stress during cultivation, none of the stress reducing agents was assimilated and in the quenching process its protecting effects were negligible. An extra indication should be given to the low ATP concentration obtained with solutions containing GA 0.05% and 0.5%. It

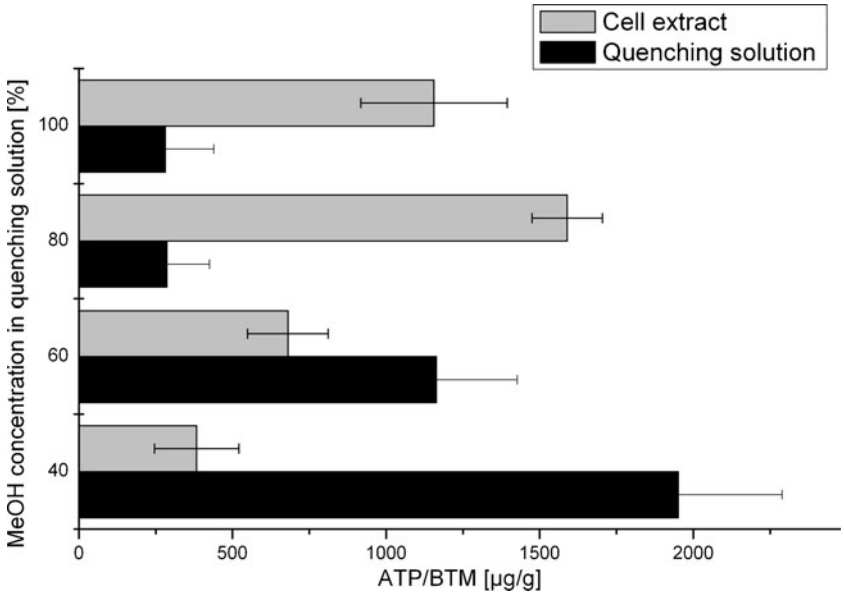

Fig. 5 Methanol quenching solutions with starting temperature −20°C. Using lower methanol concentrations (40%, 60%) led to a high ATP concentration detected in the quenching solution. Methanol (80%) still led to a loss of ATP, but a high concentration was detected in the cell extract which was higher than the one using 100% methanol

is probable that GA interfered with luciferase in the ATP determination kit fixing the lysine residues which are essential for enzyme activity (Kumita et al. 2000; Sung and Kang 1998).

The results of ATP determination for high methanol concentration and low starting temperature matched the findings of Canelas et al. (2008) for *Saccharomyces cerevisiae* but not those of Wittmann et al. (2004), where lower methanol concentrations led to a significant decrease of metabolite loss from the cells. Unfortunately, the ATP leakage results neither correlated with the corresponding OD recovery ratios for whole cell damage (e.g., Figs. 1 and 5) nor with the flow cytometer analysis for single cell damage (see Figs. 4 and 5).

Discussions

Leakage due to cold shock or osmotic stress is considered the major source of experimental inaccuracies for metabolomic studies in microorganisms due the non-specific intracellular metabolite loss during sampling using cold quenching solutions. The results presented in this study indicate that the membrane integrity is indeed negatively influenced by every

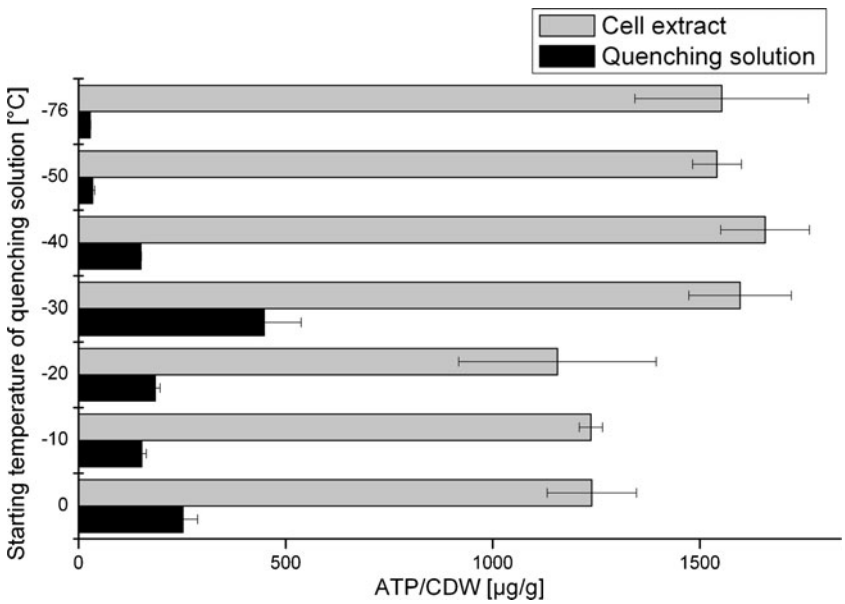

Fig. 6 Dependence of ATP recovery as function of the starting temperature in quenching solutions. Starting temperatures below −30°C led to higher ATP concentrations in the quenched biomass and lower extracellular ATP concentrations in the quenching solution supernatant

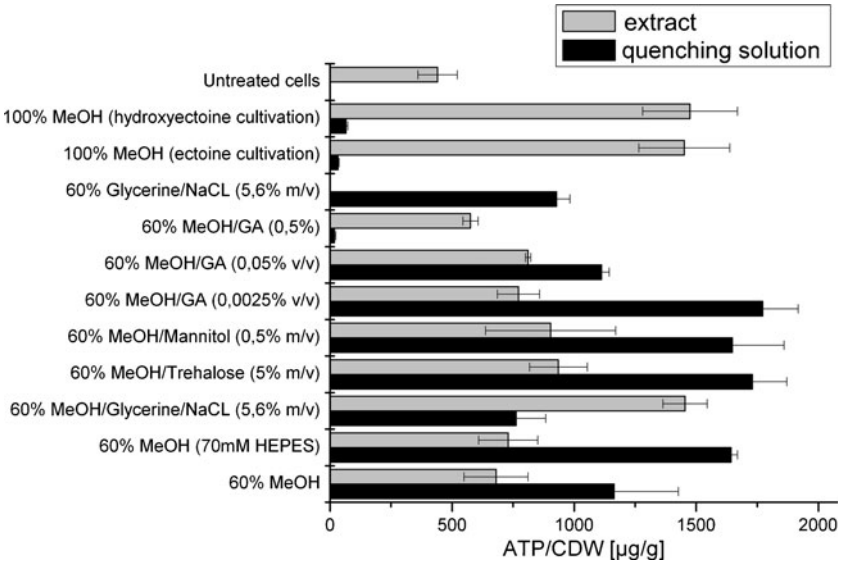

Fig. 7 Any of the tested quenching solutions led to ATP leakage, shown by ATP in the quenching solution supernatant after sampling. The addition of ectoine or hydroxyectoine to the cultivation media did not improve the performance of 100% MeOH solutions (see Fig. 6). Cells could not be separated from the glycerine solution, therefore no extraction took place. Starting temperatures for quenching solutions from *top* to *bottom*: −40°C, −40°C, −50°C, −40°C, −40°C, −40°C, −50°C, −50°C, −20°C, −50°C, −50°C

one of the quenching solutions reported in literature, independent of their composition, but with different damage levels. Cell damage and resulting metabolite leakage can occur mainly due to two different phenomena: total lysis with resulting cell fragmentation or single cell damage, e.g., reflected in the loss of the membrane integrity, with consequent cell permeabilization. While the OD recovery ratio of the re-suspended cells after quenching gives a first quantitative indication to complete cell damage, flow cytometry enables the quantification of cell damage at the single cell level based on the membrane integrity.

In general, methanol concentrations lower than 100% in the quenching solution led to a less damaged *E. coli* cell pool, whereas variation of the temperature did not change the impact of methanol significantly, both indicated by PI labeling. Using different supplements as cryoprotectants or osmolytes also changed the influence of both methanol concentration and starting temperature on the ATP concentration spectrum. For example, raising the salt concentration in the quenching solution to lower the osmotic shock in combination with the use of the cryoprotectant glycerol can improve the output of ATP in the cell extract but still led to a high ATP concentration in the quenching solution supernatant.

The use of different starting temperatures provokes fairly constant single cell damage during the cold sampling process. With an almost constant value of approximately 99% PI-labeled single cells, almost no differences between pure methanol quenched samples were observed. These results are confirmed for the whole cell damage too. As discussed before, it was observed that with starting temperatures equal to or below −30°C, OD recovery ratios lower than 75% were estimated, while for sampling temperatures above −30°C, higher OD recovery ratios were obtained with a consequent diminished contribution of whole cell disruption to ATP leakage. Matching to these findings, the concentrations of intracellular ATP estimated from the cell pellet extract were lower for temperatures equal to or below −30°C and higher and almost constant for sampling temperatures above −30°C (see Fig. 5), implying a more adequate sampling procedure for intracellular metabolome analysis under the late conditions. Since only ATP was investigated as a model metabolite in the presented study, different results might be obtained for other intracellular metabolites. Considering the ATP findings, the OD recovery ratio method and the flow cytometer analysis might confirm the assumption of Wittmann et al. (2004), who reported high losses of intracellular metabolites in methanol-based quenching solution: metabolite leakage takes place because of whole cell damage and permeated single cells. Lower methanol concentration led to only partially labeled samples, indicating a still intact cell fraction in the biomass pellet, which has been erroneously reported before as the intact metabolite pool using the cold sampling method without consideration of leakage (Lange et al. 2001; Theobald et al. 1993).

However, the fundamental question still remaining is why *E. coli* cells are leaky when using any methanol-based solutions for rapid cold sampling and quenching? From several studies, it is clear that at least three different kinds of events can cause important variations in the membrane structure and may induce cell damage: thermal stress, osmotic stress, and the use of organic solvents. At physiological temperatures and levels of hydration, the most relevant functional state of the phospholipids bilayer of biological membranes is in a fluid, lamellar, liquid crystalline phase (Mille et al. 2002), which act as a molecular sieve allowing small hydrophilic molecules to

enter into the periplasm of the cell. Maintenance of a specific bulk lipid state would therefore correlate with a functional pore-like structure required for nutrient transport (Janoff et al. 1979).

In bacteria, phospholipids can adopt various fluid and solid phases characterized by different spatial arrangements (van Meer et al. 2008). It is known that bacterial phospholipids may suffer from gel (L_β) to liquid crystalline (L_α) phase transitions undergoing a thermotropic, cooperative and, usually, reversible process in response to environmental stresses but especially to temperature changes (Haest et al. 1972; Cronan and Vagelos 1972; Morrisett et al. 1975; Eze 1993; Beney and Gervais 2001). The temperature at which the transition occurs is called the phase transition temperature, and it depends on various growth and environmental factors. The classical fluid mosaic model of the membrane (Singer and Nicolson 1972) considers a leaflet (bilayer) of phospholipids with considerable motional freedom in which the membrane integral proteins and other molecules float. In this context, the term membrane fluidity represents the dynamic properties of lipids within the bilayer. In general, during phase transition, the long range order of the bilayer is preserved. Above the transition temperature, the phospholipids bilayer is in the liquid crystalline state (L_α) with short range disorder of the acyl chains, which are melted and mobile. Below the transition temperature, the phospholipids are in the gel state (L_β) with short range order being the acyl chains rigid and immobile (Eze 1993). For example, increasing the degree of saturation of the fatty acyl chains results in a lowering of the transition temperature (Fay and Farías 1976; Kato and Bito 1978; Jones et al. 1987; Parola et al. 1990; Morein et al. 1996; Thieringer et al. 1998; Yamanaka 1999).

It has been shown before that below certain temperature and depending on the fatty acid composition of the lipid bilayer, the membrane of *E. coli* cells become fragile causing the rupture of the permeability barrier of the cells (Haest et al. 1972). Thereby, the amount of released intracellular cations and non-electrolyte molecules increases with decreasing temperature of the sampling medium. For example, a phase transition between 18°C and 30°C has been observed with the ultimate loss of contact between cell membrane and cell wall after rapid cold shock or mechanical stress, due to a sudden decrease in the cytoplasmic volume enclosed by the cell membrane. The phase transitions exist not only in the cells membranes of *E. coli*, but also in the lipids extracted from them as demonstrated by Morrisett et al. (1975) for cells grown on media supplemented with elaidic acid. Simple bilayer lipid mixtures, which have been extensively used as biophysical approaches emulating cell membranes, have revealed rich-phase behavior with the coexistence of even two-fluid phases, e.g., liquid-ordered and -disordered domains, with different physical properties within a single membrane plane (Albon and Sturtevant 1978; Feigenson 2006, 2007; van Meer et al. 2008). In the case of the *E. coli* membranes, the observation of multiple phase transitions was suggested as to be caused by a heterogeneous distribution of the membrane lipids into multiple domains and its preferential association with particular proteins within the bilayer and, therefore, be the reason for the absence of some transitions in the membranes, but not in the extracted lipids (Morrisett et al. 1975). In any case, no matter if the chilling commonly used for deactivation with cold sampling and quenching is performed slow or rapid: the change in temperature implies the passage through at least one lipid membrane phase transition of cells cultivated at physiologic temperatures.

Still, *E. coli* cells grown at low temperatures are less sensitive to membrane transitions caused by cold shock by increasing the levels of unsaturated fatty acids (Kato and Bito 1978; Parola et al. 1990; Yamanaka 1999). These changes in membrane lipid composition causes lower phase transition temperatures and thus compensate order and rigidity of membrane lipids under cold shock. Similar experimental evidence has also been obtained for the Gram-positive bacteria *B. subtilis* (Delobbe et al. 1971; Svarachorn et al. 1991; Tsuchido et al. 1995; Beranová et al. 2008). However, even when it has been reported that the reorganization of the membrane components like proteins and phospholipids induced by temperature and osmotic pressure variation could prevent cell mortality (Mille et al. 2002), cell adaptation would be not possible under the extreme and abruptly change in temperature and osmotic stress occurring during rapid cold sampling and quenching. For example, since induction of cold shock proteins in *E. coli* is preceded by a 4-h lag after cold shift from 37°C to 10°C (Jones et al. 1987), and the characteristic times for rapid cold sampling and quenching are far below this time, the bacteria would not be able during this process to compensate for the membrane transition from a fluid state to a non-fluid state by means of changes in the membrane lipid composition (Morein et al. 1996; Thieringer et al. 1998; Yamanaka 1999). This fact might consistently explain why many samples used for metabolome experiments using either Gram-negative or Gram-positive bacteria that grow rapidly, e.g., at the exponential growth phase, have leaky membranes. An enhanced resistance has been found in *E. coli* and *B. subtilis* cells that have grown in minimal medium, which are reported to recover better than cells grown on rich medium since their lipid membrane order is smaller due to enlarged cell diameters (Parola et al. 1990; Beranová et al. 2008). These experimental observations would imply that *E. coli* cells sampled from the exponential growing phase and incubated on rich medium are more sensitive to a cold shock and, therefore, present more metabolome leakage than cells sampled either from the

stationary phase or from steady-state cultivations grown on minimal medium, as confirmed experimentally with membrane fluidity measurements performed by Cao-Hoang et al. (2008) for *E. coli* and *B. subtilis*.

The late hypothesis is reinforced by the work of Janoff et al. (1979) who found out that the reason of the differences in sensitivity to membrane transitions caused by cold shock lies in the lipid order to disorder transition suffered by the outer membrane, but not by the cytoplasmic membrane of *E. coli* cells, which was not dramatically affected by the temperature of growth. Their experimental evidence suggests that the cytoplasmic membrane presumably exists in the gel+liquid crystalline state during cellular growth at 12°C and 20°C, but in a liquid crystalline state when *E. coli* cells were grown at 37°C and 43°C, while the outer membrane apparently existed in a gel+liquid crystalline state at all incubation temperatures (Janoff et al. 1979). This fact can be the key event to understand the occurrence of metabolite leakage in *E. coli* during rapid cold sampling and quenching, and also give a hint to its location: while the outer membrane undergoes only one phase transition for all incubation temperatures, the cytoplasmic membrane experiences one phase transition for growth at 12°C and 20°C, but suffers from two-phase transitions for growth at 37°C and 43°C. The existence of more phase transitions would not only explain the lower sensitivity of cells grown at low temperatures, but also indicate a more extensive damage of the cytoplasmic membrane than of the outer membrane due to the twofold phase transition if they are irreversible. Since Gram-positive bacteria only have a cytoplasmic membrane but no outer membrane, similar damages due to phase transitions may be expected; however, in contrast to *E. coli* studies, extremely scarce experimental evidence on thermotropic membrane characterization has been reported for them.

Besides the total temperature change, the cooling rate itself has been reported to cause extended cell damage in *E. coli* and *B. subtilis* cells too (Svarachorn et al. 1991; Cao-Hoang et al. 2008). For example, studies on the permeability of *E. coli* membrane to permease accumulated substrates showed that the bacterial membrane is exquisitely sensitive to isoosmotic cold shock and the substrate pools formed may suffer almost total loss if the cells are rapidly chilled from 25°C to 0°C during sampling (Leder 1972). As suggested by the authors, the isoosmotic shock causes crystallization of the liquid-like lipids within the membrane and therefore a rapid efflux of permease-accumulated substrates would be facilitated through the hydrophilic channels created in the phase change process.

As showed by Cao-Hoang et al. (2008) with *E. coli* and *B. subtilis* cells, sensitivity to rapid cold shock is a function of the growth phase: exponentially growing cells were more sensitive to the rapid cold shock than stationary growing cells. The measurements indicated that exponentially growing cells have a more fluid membrane than stationary growing cells at the physiological temperature before the cold shock occurred and concluded that the instantaneous decrease in membrane fluidity induced by the rapid cold shock of exponentially growing cells was higher than that of stationary growing cells. Moreover, they made anisotropy measurements comparing the effects of low against rapid cooling rates, clearly demonstrating that bacterial cells growing exponentially experienced a permanent loss of the membrane integrity (irreversible membrane rigidification) caused only by the rapid cold shock, but not by slow chilling. As the authors argue, this loss in membrane integrity would be unfavorable to the maintenance of homeostasis, giving rise to discontinuities in the gelled membrane and creating hydrophilic channels which facilitate the outflow of cell components, i.e., one more cause of metabolome loss. Additionally, metabolic compound lost is also a function of solubility which itself is dependent on the particular temperature. Therefore, cells may lose their integrity due to transitions to low temperatures of, e.g., −76°C (Fig. 2), but the loss of metabolites is avoided by reduced solubilities in methanol as a solvent as for the case of peptides (Scanu and Edelstein 1971).

Although Cao-Hoang et al. (2008) claim to have carried out PI measurements and found a far greater PI uptake in bacterial cells from the exponential growth phase upon the rapid chilling than during slow chilling, these measurements unfortunately are not available for comparison with the actual results achieved in our study. In any case, both studies point out that the membrane of exponentially growing *E. coli* cells was highly permeabilized after a rapid cold treatment inducing irreversible alteration of the membrane integrity and that this chilling-induced damage is exclusively due to a physic effect, which is the rapidity of the temperature downshift. Still, metabolome leakage during quenching is intriguing and may be caused not only by cold shock. For example, using 40% of methanol at −20°C led to a partially PI-labeled sample. This indicates that cells must still be intact otherwise PI would go through the cell membrane. Quantification of ATP in contrast proved a high leakage, which can only be due to loss of ATP passing through the cell wall. Methanol (100%), on the other hand, led to an almost 100%-labeled cell pool. Still, less ATP was detected in the quenching supernatant. Increasing the temperature difference of growth conditions and quenching solution, and therefore the cold shock, even led to a decreasing amount of detectable ATP in the supernatant and high ATP concentrations in the cell extract but still to an almost completely PI-labeled cell pool.

In addition to cold shock, osmotic shock has been named a possible cause for metabolome leakage in different works and corresponding quenching solutions adapted to undermine detrimental effects. Thus, the addition of salts, to adapt the ionic strength of the quenching solution, and

cryoprotectants like trehalose or mannitol has been used traditionally in quenching solutions for rapid sampling to simultaneously reduce the damage from cold shock (Bolten et al. 2007; Villas-Boas and Bruheim 2007). As has been proposed by Nossal and Heppel (1966), the use of salts like $MgCl_2$ in solutions as medium to avoid osmotic shock maintains high viability ratios, being hypothesized that the cells have in this solution a tendency to stick together and might not mix perfectly on dilution. In the case of *E. coli*, it is well known that it suffers a drastic decrease in cell volume when confronted to increased osmotic pressure. This volume change can induce a mechanical effect on the membrane as primary site of injury, deforming it by cell shrinkage that can lead to final membrane disruption, which explains cell mortality due to osmotic stress (Mille et al. 2002 and references therein). The increased area-to-volume ratio of the cytoplasmic membrane leads to irreversible surface changes, such as membrane vesiculation, which induces surface depletion and, when subjected to fast rehydration, cannot even recover their initial internal volume, and rupture (Mille et al. 2002). Similar to increased osmotic pressure, it has been demonstrated that the mechanical effect of hydrostatic pressure causes a closer packing of the hydrocarbon chains of phospholipids in *E. coli*, which is analogous to the alteration caused by cooling (Casadei et al. 2002). At least for eukaryotic cells, dramatic changes in area by ca. 50% are also possible when a phase transition occurs, as for example by cooling, affecting even the bounded proteins and, therefore, altering protein–protein contacts and behavior (Feigenson 2006).

Finally, as for the interaction of the hydrophobic core of the lipid bilayer with alcohols and organic solvents, as those used in standard quenching solutions, it has been reported that they mainly influence the fluidity of *E. coli* cell membranes destabilizing them due to increased mobility of the acyl groups, increased permeability to hydrophilic solutes of cells and liposome membranes, even enabling a transition from a lamellar to hexagonal state of the inner membrane (Beney and Gervais 2001 and references therein).

Considering that besides *E. coli* and *B. subtilis*, several other Gram-positive and Gram-negative bacteria like *Gluconobacter oxydans*, *Pseudomonas putida*, *Zymononas mobilis* (Bolten et al. 2007), and *Corynebacterium glutamicum* (Bolten et al. 2007; Wellerdiek et al. 2009) have also presented cell leakage of intracellular metabolites, it is consequently valid to ask if only damage to the cytoplasmic membrane make these bacteria especially sensitive to cold shock, but also ask why other microorganisms like the yeast *Saccharomyces cerevisiae* presents minimal cell leakage after cold shock (Mashego et al. 2003, 2007; Canelas et al. 2008, 2009; Spura et al. 2009).

In contrast to *E. coli* and *B. subtilis*, no irreversible rigidification of the membrane, i.e., reversible phase transition of exponentially growing cells was observed for *S. cerevisiae* (Cao-Hoang et al. 2008). The same research group have especially focused on studying the mortality of *S. cerevisiae* cells exposed to a combination of thermal and osmotic treatments and the leakage of cellular components through the unstable membrane when lipid phase transition occurs (Laroche et al. 2001; Simonin et al. 2008; Gervais and Martinez de Marañon 1995). First evidence to the cause of minimal leakage may be found in the reversible phase transition, which indicates a more robust membrane of *S. cerevisiae* than those of bacteria. As proposed by Cao-Hoang et al. (2008), the answer may lie in the composition of the membrane. *E. coli* has three main membrane phospholipids, which occur frequently in prokaryotic and eukaryotic cells: phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and diphosphatidylglycerol (DPG) (Cronan and Vagelos 1971; Morein et al. 1996). In contrast, yeast cells contain sterols, which are particularly involved in the resistance of the cell to different types of stress. Sterols, such as cholesterol and ergosterol, decrease the fluidity of the L_α phase of membranes and above a certain concentration even suppress the transition of pure phospholipid bilayers from the L_α to the L_β phase, conferring on the membrane a stable fluidity over the transition temperature range (Bottema et al. 1983; Beney and Gervais 2001). That leakage of solutes across membranes associated with either pressure or thermotropic phase transitions in membrane lipids containing high portions of cholesterol also occurs, has been early demonstrated in human (Drobnis et al. 1993) and bovine (Saragusty et al. 2009) sperm cells, a phenomenon which reinforces the hypothesis of cell damage of the membranes exclusively due to physic effects. Resuming the findings of the membrane characteristic of *E. coli* cells: when growing at temperatures around 37°C and inoculated in complex media and sampled at the exponential growing phase cells are far more sensitive to damage arising from rapid cold sampling and quenching than cells cultivated at lower temperatures with minimal media and sampled from the stationary growth phase, no matter what kind of quenching solution is used and if the final sample temperature is around or below 0°C.

In this work, it was demonstrated that the use of methanol as sole component of an aqueous quenching solution causes ca. 15% to 25% cell rupture and lysis of *E. coli*. Most probable causes for this cell damage, which could be monitored using the OD recovery ratio as parameter, are the organic solution as solvent and possible osmotic shock suffered by cells entering the methanol quenching solution from the culture medium. Contrastingly to the negative effect of methanol concentrations, the starting temperature of the quenching solution plays an important role in diminishing *E. coli* cell lysis. In this sense, the use of the term “cold shock” as possible explanation for

cell damage in intracellular metabolome analysis literature may be misleading as was discussed elsewhere (Cao-Hoang et al. 2008). According to the present results, actually a positive effect is reached, i.e., whole cell damage diminishment was found for decreasing quenching temperatures. Therefore, it can be concluded that *E. coli* cells are more sensitive to lysis through thermal shock with OD recovery ratios varying from ca. 58% to 88% than to variations in the composition of the quenching solution, where OD recovery ratios ranged from 75% to 85%. From the single cell analysis, the main conclusion of the present study is that rapid cold sampling and quenching neither with methanol nor with the recently proposed ethanol-based solution (Spura et al. 2009) would be well-suited to avoid unspecific metabolome leakage in Gram-negative and Gram-positive bacteria, because both methods would inevitably induce irreversible alteration of the membrane integrity through chilling, cell fragmentation, and permeabilization damage of the membranes exclusively due to physic effects, like the rapidity of the temperature downshift, increase of osmotic stress, or unspecific solvent extraction. It is clear that due to its facility for application and broad spread use, the cold sampling and quenching method is still an alternative for rapid inactivation of enzymatic cell activity but very inconvenient and even burdensome when reaching the final analytical stage: a differential method, like the one presented by Canelas et al. (2008) and Taymaz-Nikerel et al. (2009), is unavoidable for reliable metabolome analysis requiring independent measurements in the cell extract, supernatant, and culture broth.

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