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Comparison of preservation methods for bacterial cells in cytomics and proteomics

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Abstract

Cell sampling during long-term experiments usually requires reliable storage of cells for later analysis. In this study, we evaluated three different preservation strategies (sodium azide fixation, deep freezing and vacuum drying) with regard to their effects on bacterial cells. *Pseudomonas putida* was used as a model organism and stored for shorter (2 d) and longer periods (28 d). The impact of the treatments (preservation method, duration) was evaluated on the level of single cells using flow cytometry and on the population level using protein mass spectrometry. On the single cell level, the effect of sodium azide fixation was found to be small ($1.01 \leq sd \leq 1.76$) for short term and larger for long term storage ($1.59 \leq sd \leq 2.33$), as determined by FlowFP fingerprinting. In contrast, deep frozen and vacuum dried cells showed properties highly similar to fresh reference cells, even after extended storage ($0.5 \leq sd \leq 1.2$). On the population level, the mass spectrometric analysis revealed about 800 proteins for each sample and storage condition. The proteome profiles evaluated by label-free quantification showed that variation within functional groups was least for deep frozen and vacuum dried cell samples after 2 d (sd log2 relative protein quantity < 1) and marginally increased after 28 d. In contrast, sodium azide fixation caused higher variations between functional groups although the number of detected proteins and the respective peptide coverage excluded protein degradation. In conclusion, deep freezing was found to be the method of choice, but simple vacuum drying of cells with storage at 4°C can be a convenient alternative.

Keywords: Fixation; Cryopreservation; Vacuum drying; Bacteria; Flow cytometry; Mass spectrometry.

Abbreviations:

F, fresh**; VD,** vacuum drying**; DF,** deep freezing**; SAF,** sodium azide fixation**; FCM,** flow cytometry**; MS,** mass spectrometry

1. Introduction

The number of samples that can be analyzed by flow cytometry is steadily increasing and recently reached 1536-well plate format as a platform for parallel analysis [1]. The advent of high-throughput analyses raises the question of efficient sample preservation, both for plain flow cytometry and for cell sorting in combination with other *Omics* techniques in microbiology [2]. With such applications in mind, the recovery of whole intact cells after storage is essential for flow cytometric analysis and the reliability of this recovery is particularly important for long-term experiments. The process of sample preservation consists of two parts, sample preparation and sample storage. Here, we define sample preparation as a measure to stop cellular activity and prevent cells from alteration or decay. Usually relatively harsh methods derived from histology were used for preparation, commonly known as fixation. For instance, alcohols and alde-

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hydes have the advantage of inactivating biohazardous specimens [3]. In general, different fixation and preparation techniques have different benefits and drawbacks, as they change the sample in characteristic ways. The risk of further changes caused by subsequent storage until sample analysis is mainly determined by the storage time and temperature [4]. The diversity of preparation techniques and storage conditions is large, and the choice depends on the kind of analysis samples are subjected to.

The most widely-used fixation methods for microbial cells are alcohol- or aldehyde-based methods, which mainly work via protein cross-linking (aldehydes), cell permeabilization and water removal (alcohol) [3, 5]. Although aldehydes permit easy fixation and storage even at room temperature (RT), major drawbacks include alterations of cell morphology, cell aggregation, loss of biomolecules and increased autofluorescence [3, 6, 7]. Another chemical preservation method, which can be also regarded as fixation, is the use of (sodium-) azide, which was proven useful for microorganisms [8, 9]. Azide inhibits the terminal enzyme of the respiratory chain and thereby prevents energy production of aerobic cells [10-12]. Metal salts like barium, nickel and molybdenum in combination with 10% sodium azide proved especially useful for anaerobic bacteria and provided highly reproducible cytometric patterns for at least nine days [13, 14]. These chemical preservation techniques are usually sufficient for flow cytometry, but destructive effects like protein cross-linking in case of aldehydes disqualify a method for protein mass spectrometry. A method widely used in biobanking is cryopreservation by deep freezing (DF) at temperatures below -60°C with addition of a cryoprotective agent, such as glycerol or sugars [15-17]. Deep freezing was suitable for preservation of methane oxidizing bacteria as tested by flow cytometry [18], but performed poorly for natural microbial communities [13]. In the food and pharmaceutical industry, drying of bacterial cells by different techniques is used for product conservation [19, 20]. Freeze drying, spray drying or low temperature (~0°C) vacuum drying were shown to preserve different bacterial strains [21-23], but have not been applied in single cell analysis.

In this study, we compared three different preparation and storage procedures suitable for single cell analysis and simple enough for standard laboratory application: Sodium azide fixation (SAF) followed by storage at 4°C, vacuum drying (VD) followed by storage at 4°C, and deep freezing (DF) with liquid nitrogen and subsequent storage at -80°C. The bacterial strain *Pseudomonas putida* was used as model organism for analysis by flow cytometry (FCM) and by protein mass spectrometry (MS). The combination of both techniques is promising, as cells with different characteristics can be identified and sorted by FCM, and the subpopulation's proteome can be analyzed by MS. Subpopulation proteomics was already performed for an artificial mixture of bacteria [24] and for pure cultures of *P. putida* [2]. Besides MS, FCM can also be coupled with other downstream applications like RNA isolation [25, 26]. In the presented study, FCM and MS were used to evaluate the effect of the selected preservation methods (VD, DF, SAF) on cell characteristics (DNA content, light scatter) and protein profile, respectively, in comparison to fresh samples (F). A gate-free similarity fingerprinting method was applied for analyzing cytometry data [27] and a novel functional clustering algorithm was used for comparison of proteome profiles.

2. Materials and Methods

2.1. *Bacterial strains and cultivation conditions*

Pseudomonas putida KT2440 (source: DSMZ – German Collection of Microorganisms and Cell Cultures) was cultivated overnight at 30°C on a rotary shaker (180 rpm) in minimal medium (Na_2HPO_4 6 g/L, KH_2PO_4 3 g/L, NaCl 0.5 g/L, NH_4Cl 1 g/L, $MgSO_4$ 0.5 g/L, $CaCl_2$ 15 mg/L, $ZnSO_4$ x $7H_2O$ 3.6 mg/L, $CuSO_4$ x $5H_2O$ 0.625 mg/L, H_3BO_3 0.15 mg/L, $FeSO_4$ x $7H_2O$ 6 mg/L, $CaCO_3$ 5 mg/L, $MnSO_4$ x $7H_2O$ 3 mg/L, $CoSO_4$ x $7H_2O$ 0.7 mg/L) with 2 g/L glucose as sole carbon and energy source. A 500-ml shaking flask with 100 ml of minimal medium was inoculated with a volume of overnight culture corresponding to an initial optical density of 0.05 at 600 nm (OD_{600nm}, $d_{cuvette}$ = 0.5 cm). For comparison, *Escherichia coli* DH5α (source: DSMZ) was cultivated overnight at 37°C on a rotary shaker (180 rpm) in LB medium. A 500 ml shaking flask with 100 ml of LB medium was inoculated to an initial OD_{600nm} of 0.05. The growth of the cells was monitored by measurement of OD_{600nm} up to a maximum incubation time of 12 h.

2.2. *Cell preparation and storage*

Cells of *P. putida* were collected every two hours for a total of 12 hours after inoculation covering all cell cycle stages (Figure 1B). For *E. coli,* cells from the lag and exponential growth phases at 0, 1 and 2 hours were used. Samples were taken at various time points by centrifugation of up to 2 ml cell suspension in a microcentrifuge (Heraeus Fresco 21) for 5 min at room temperature and 5,000 x g, and the supernatant was discarded. For DF, the cells were resuspended in 1 ml phosphate buffered saline (PBS; 6 mM Na_2HPO_4, 1.8 mM NaH_2PO_4, 145 mM NaCl, pH 7.2) containing 15 % (v/v) glycerol as cryoprotective agent, incubated for 10 min on ice and shock frozen in liquid nitrogen for subsequent storage at -80°C. For VD, any residual medium was removed and the cell pellet was vacuum dried for 30 min at 30°C using a vacuum concentrator (N-Biotek Micro-Cenvac). The dried cell pellet was stored at 4°C. For SAF, the cells were resuspended in 2 ml 10 % (v/v) sodium azide (NaN_3) and stored at 4°C.

2.3. Cell staining

Fresh (only *P. putida*), deep frozen and sodium azide fixed cells were centrifuged for 5 min at RT and 5,000 x g and the supernatant was discarded. The resulting cell pellets as well

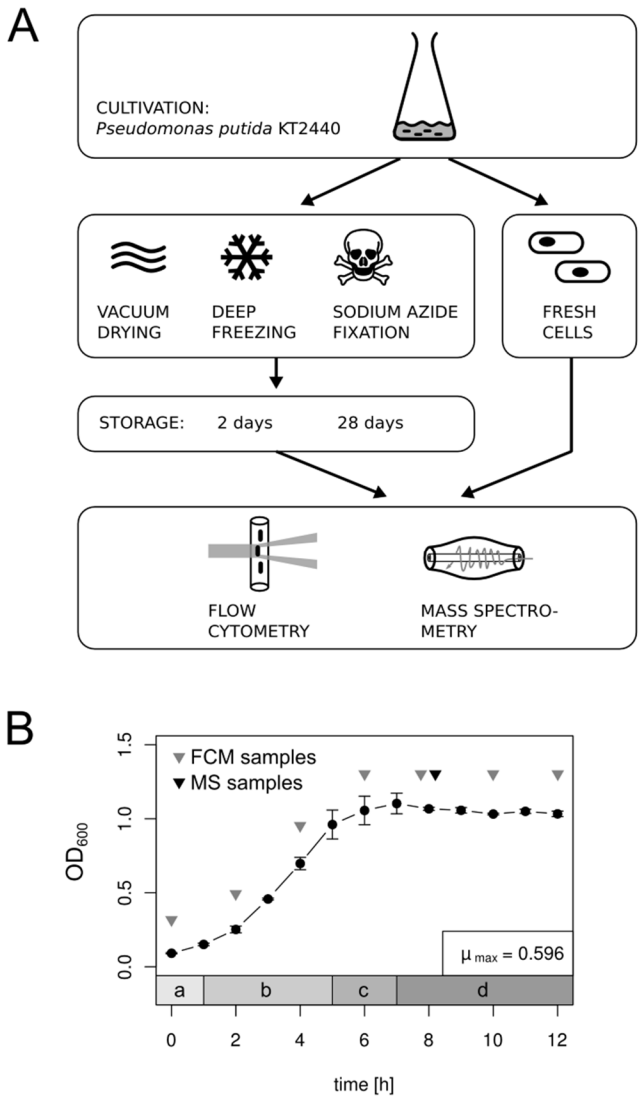

Figure 1. Experimental design of the study. (**A**) Bacterial cultures were grown in shaking flasks, samples were taken at different time points and prepared for storage by vacuum drying (VD), deep freezing (DF) and sodium azide fixation (SAF). The samples were stored for 2 d or 28 d and then analyzed by flow cytometry and mass spectrometry. Fresh samples (F) were directly analyzed and served as reference. (**B**) Growth of *P. putida* KT2440 was monitored by optical density measurement ($OD_{600nm,}$ $d_{cuvette}$ = 0.5 cm) and covered lag phase (a), exponential (b), early stationary (c) and stationary (d) growth phase. Samples for flow cytometry were taken every 2 h (▼) and for mass spectrometry at 8 h of growth (▼).

as VD pellets were resuspended in ice cold PBS by repeated pipetting, and the OD_{600nm} was adjusted to 0.05. For DNA staining, 1 ml of the cells was harvested by centrifugation, and the pellet was resuspended in 1 ml of permeabilization buffer (0.3 M citric acid, 5 g/L Tween 20) and incubated for 10 min on ice. After centrifugation the supernatant was removed and the cells were resuspended in 1 ml DNA staining solution (0.68 µM 4',6-diamidino-2'-phenylindole (DAPI), 0.4 M Na_2HPO_4) [28] and incubated for at least 15 min at RT. Prior to flow cytometry, cell clusters were removed by filtration using a membrane of 50 µm pore diameter (CellTrics, Partec) to prevent clogging of the cytometer nozzle. A pumping of DAPI by fresh cells, as reported for several nucleic acid stains [29], was not observed.

2.4. *Flow cytometry and data analysis*

Flow cytometry was performed using a MoFlo cell sorter (Beckman-Coulter, USA) as described in [24]. The DAPI fluorescence was determined using a multi-line UV laser (333–365 nm, 100 mW) for excitation, and emission was detected in the FL4 channel (450±30 nm). Prior to all measurements, the instrument was adjusted using fluorescent beads and a biological standard. Data were recorded with the Summit v4.3 software (Beckman-Coulter) and further analyzed using the Bioconductor framework for R [30]. The electronic noise was removed (forward scatter, threshold of 25) to prevent bias of similarity analyses. For better comparability, the dominant peaks of the forward scatter (FSC) and DAPI (FL4) channels were normalized (warpSet function, variable peak number, grouping on parameter time) using the Bioconductor *flowStats* package [31]. Similarity fingerprinting with the FSC and FL4 channels (standard deviation method, five recursions) was performed using the *FlowFP* package [27].

2.5. *Identification of proteins by LC-MS-MS*

For proteomics, cells of *P. putida* KT2440 (three technical replicates) were harvested after 8 h of growth and either stored as described above or directly prepared for MS by resuspension in 2 ml PBS. The cell number was determined using flow cytometry and a sample volume corresponding to 1 x 10^8 cells was harvested by centrifugation and resuspended in 25 µl 25 mM ammonium bicarbonate buffer (NH_4HCO_3, pH 7.8) with 1 µl acetonitrile and 5 µl of trypsin (0.25 µg/µl, Promega, Madison, USA) for proteolytic digestion. The samples were incubated over night at 37°C with continuous shaking (180 rpm) and the digestion was stopped by addition of formic acid (FA, 0.1 % (v/v) final concentration). Cell debris was removed by centrifugation for 10 min at 13,000 x g and RT and the supernatant was transferred to a fresh 0.5 ml-tube. Samples were stored at -20°C until analysis. The peptide solution was then purified using the ZipTip protocol (Millipore, Bedford, USA), dried using a vacuum concentrator and the remaining peptides resuspended in FA. The solution was sonicated in a water bath for 5 min prior to injection. Peptides were separated and measured by a high-pressure liquid chromatography (nano-UPLC) system (nano -Acquity, Waters) coupled to an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) as described in [32]. Continuous scanning of eluted peptide ions was carried out between 300-1600 m/z, automatically switching to MS/MS CID mode on ions exceeding an intensity of 3000.

The retrieved raw data were analyzed by MaxQuant (version 1.2.2.5) [33] with the genome sequence of *P. putida*

KT2440 as the database. The MaxQuant settings can be found in more detail in supplementary Table S1. The label-free quantification (LFQ) values were used for protein quantification and can be found in supplementary Table S3. For peptide mapping, the original mass spectra were further analyzed by Thermo Discoverer (v.1.2.0.208), Mascot (v 2.3) and the NCBInr database (as of February 2013) with a restriction to sequence entries of *P. putida* KT2440 (available in supplementary Table S4).

2.6. *Proteome mapping*

The mean and standard deviation of the obtained LFQ values were calculated for each triplicate. The relative protein quantity was calculated as ratio of the protein quantity of the respective stored sample to the fresh sample (F). To add biological information to the detected proteins, their subcellular localization was predicted *in silico* by PSORTb v3.0 [34]. Furthermore, proteins were annotated using the KEGG BRITE functional hierarchy for *P. putida* KT2440 (http://www.genome.jp/kegg-bin/get_htext?ppu00001.keg, [35]), adding four hierarchical levels (here called 'system', 'process', 'pathway' and 'protein'). Sunburst treemaps were created using a custom recursive function in R (supplementary information S2).

3. Results

3.1. *Cell preparation and storage*

The influence of preservation method and storage time on the stability of bacterial cells was investigated. To this end, three different cell parameters (FSC, SSC and DNA content) were selected as indicators and analyzed by FCM. Likewise, the protein profile of the same samples was analyzed by MS. Samples of *P. putida* KT2440 were taken at various time points during batch cultivation. Hence, the cells display a different morphology, DNA content and possibly also sensitivity to preparation methods due to various growth stages. At every time point, cells were either directly analyzed by flow cytometry (fresh, F) or after preservation by VD, DF or SAF as depicted in Figure 1A. Since VD has not been used for cell preservation in cytometry before, the method was optimized regarding drying temperature and duration. Out of three different drying durations (10, 30, 60 min) and two temperatures (30°C, 60°C), the most distinct distribution (FSC, DNA) was obtained at 30°C for 30 min (supplementary Figure S1). Likewise, DF was tested with 15 and 50 % (v/v) glycerol in PBS as a cryoprotective agent, but no difference was observed (supplementary Figure S2).

3.2. *Analysis of flow cytometric pattern similarity*

Samples of *P. putida* KT2440 from seven time points were prepared by four different methods (F, VD, DF, SAF) and either directly analyzed (F) or stored for 2 d and 28 d (VD, DF, SAF). The light scattering and DNA content of the cells were analyzed by flow cytometry (Figure 2A). The side scatter signal showed no remarkable differences between samples and was disregarded for similarity analysis. Predominantly, the FSC signal showed a unimodal distribution, which shifted according to the growth of the cells. The cellular DNA content changed during cultivation, ranging from a single chromosome equivalent (C1n) to two or more copies

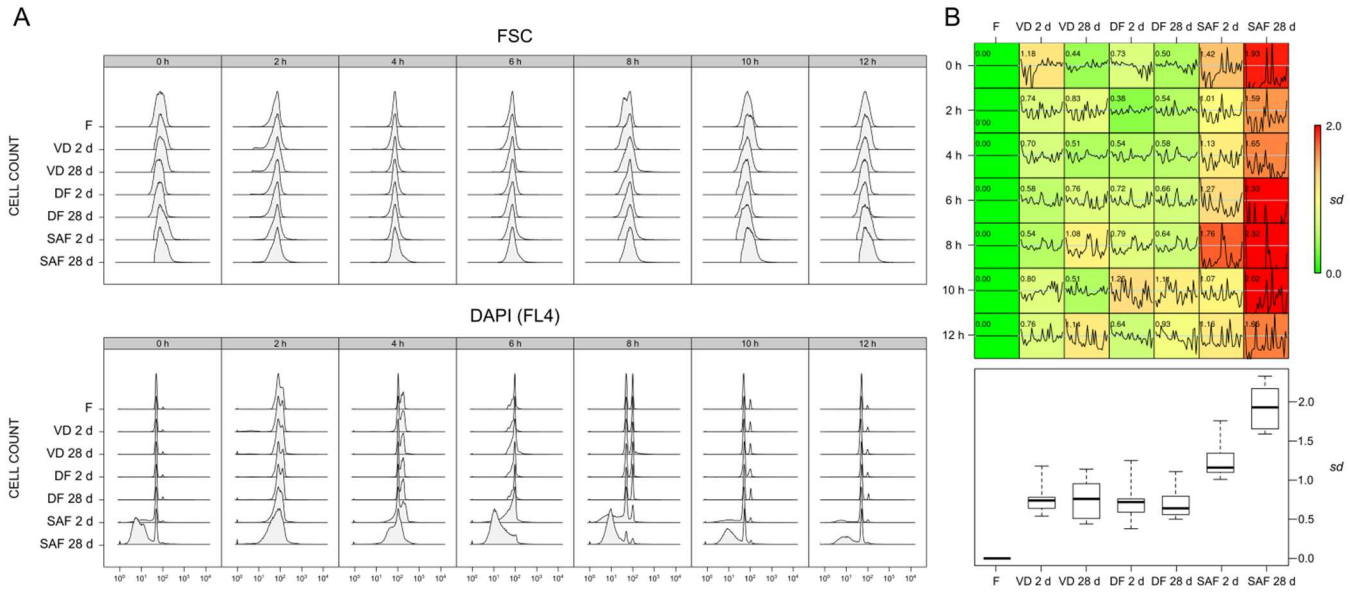

Figure 2. Similarity analysis of stored *P. putida* KT2440 cells using flow cytometry. The cells were either directly analyzed (F) or prepared by vacuum drying (VD), deep freezing (DF) and sodium azide fixation (SAF) and stored for 2 d or 28 d. (**A**) Histograms of forward scatter (FSC, upper panel) and DAPI fluorescence (FL4, lower panel) after 0-12 h of incubation. (**B**) Similarity fingerprint of stored samples in comparison to the fresh sample (F). Given is the standard deviation *sd* as index for bin differences, which increases with increasing dissimilarity. The retrieved *sd* values for each method are summarized in a box plot.

(≥ C2n). The DNA pattern of the stored samples was highly similar to the fresh samples for VD and DF, but SAF treated cells showed a certain proportion of cells below the C1n peak after 2 d, which further increased after 28 d of storage. To quantify similarity of flow cytometric patterns a fingerprinting method (*FlowFP*) was employed, involving FSC and DNA (Figure 2B). It is based on a probability binning algorithm, which constructs a model from a reference sample based on the distribution of events in it. Here, the respective fresh sample (F) of each time point served as the reference sample. This is then compared to the stored samples yielding an index for similarity, the standard deviation of bin differences (*sd*). It is 0 for identical samples and increases with increasing dissimilarity. The sensitivity limit of the method was determined by comparison of two virtual halves of the same sample F, yielding an $sd \leq 0.25$ (supplementary Figure S3). An equal similarity to the fresh reference sample was found for VD and DF ($0.5 \leq sd \leq 1.2$, Figure 2B) independent of the storage duration. Samples stored by SAF were less similar after 2 d ($1.01 \leq sd \leq 1.76$) and particularly after prolonged storage of 28 d ($1.59 \leq sd \leq 2.33$). The growth phase of *P. putida* had only a small influence on the similarity to the reference sample. For comparison, a similar experiment was conducted using another bacterial strain, *E. coli* DH5α. In contrast to *P. putida*, cells of the exponential growth phase were more amenable to alteration than lag phase cells (supplementary Figure S4). Furthermore, the deleterious effect of SAF was much less pronounced for *E. coli* than for *P. putida*.

3.3. *Identification of proteins by MS*

Flow cytometry is a powerful application for analysis and sorting of cells, and can be readily combined with other downstream applications such as proteomics. However, the storage of cells until sorting may influence their protein profile. Therefore, we tested the ability of the selected methods (DF, VD, SAF) to preserve the protein composition of *P. putida* cells for two time periods (2 d, 28 d). Cell samples were acquired at the early stationary growth phase (8 h) and either stored, or instantly analyzed serving as the reference (F). The cellular proteins were analyzed by shotgun mass spectrometry with label-free quantification. A total number of 971 different proteins was detected across all samples, with a range of 793 to 858 different proteins present in at least one replicate per single sample (Figure 3A). The number of proteins common to all samples was 751 (intersection, IS), indicating comparably good protein recovery for all preservation techniques, although the number of recovered proteins across replicates varied by each method (supplementary Figure S5). Particularly, samples stored for 28 d with VD and SAF showed a reduced number of detected proteins per replicate as low as 611 and 713, respectively. Overall, the obtained coverage was in the range of other MS based studies for *P. putida*, which found 604 to 2383 different proteins [36 -39]. Here, the 971 identified proteins showed a different distribution in theoretical subcellular localization (as determined *in silico* by PSORTb) compared to the 5350 proteins annotated at www.pseudomonas.com (Figure 3B). For instance, the proportion of cytoplasmic proteins was higher for

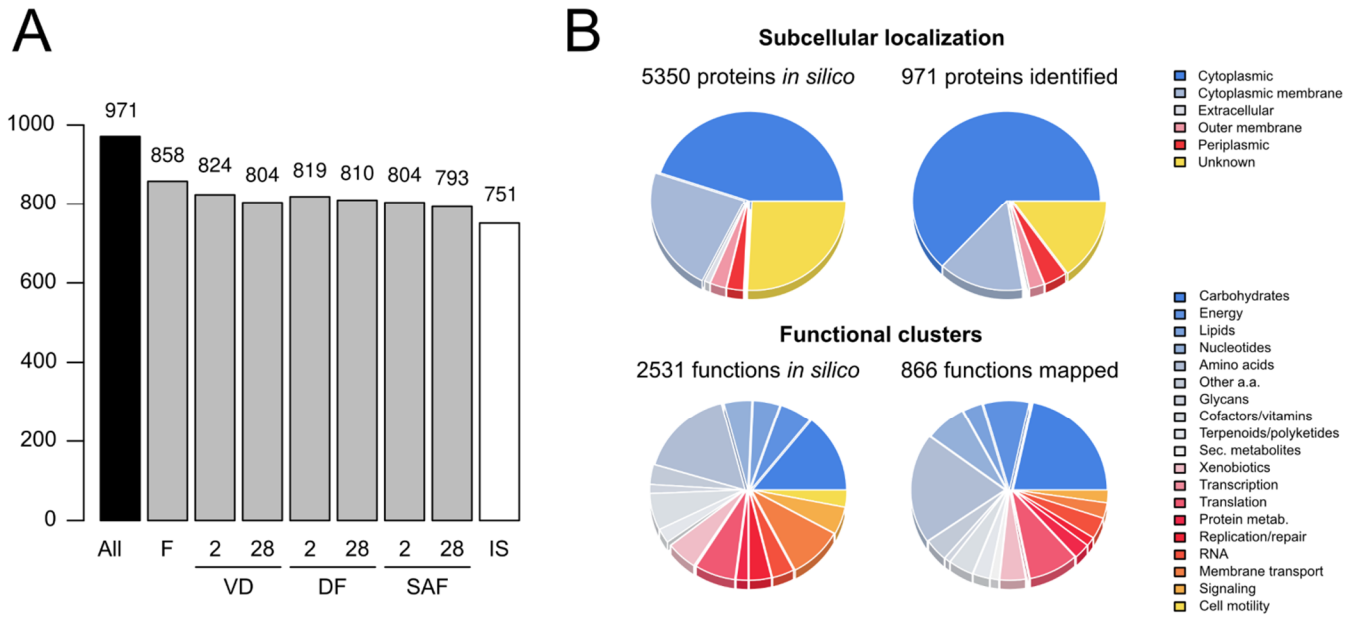

Figure 3. Proteins detected by mass spectrometry after storage of *P. putida* KT2440 cells. (**A**) Number of unique proteins present in at least one replicate per sample for fresh (F), vacuum dried (VD), deep frozen (DF) and sodium azide fixed cells (SAF). The intersection (IS) denotes the number of proteins common in all samples. (**B**) Subcellular localization of all 971 detected proteins in comparison to 5350 protein encoding sequences in the *P. putida* database (www.pseudomonas.com). A subset of the identified proteins was mapped to 866 functions by KEGG BRITE, compared to 2531 functions of all annotated proteins.

the experimental data (63 % instead of 45 %), whereas the proportion of inner membrane localized proteins was smaller (15 % instead of 23 %). To gain a deeper insight into the nature of the identified proteins, 463 out of 971 unique proteins were assigned to 866 functional annotations using the KEGG BRITE database (Figure 3B), which comprised a total of 1494 unique proteins assigned to 2531 functional annotations for *P. putida* KT2440. The clustering of proteins according to the second highest hierarchy level (here called 'process') with 19 subgroups illustrates the over-representation of detected proteins in carbohydrate, energy and amino acid metabolism. An under-representation was observed for proteins involved in membrane transport, signal transduction and cell motility.

3.4. *Protein profile similarity of stored samples*

Besides the number of proteins identified per sample, the relative quantity of the detected proteins was taken into account to test the impact of the three preservation methods on cell protein composition. Therefore, we investigated if certain functional clusters of proteins were more prone to alteration than others. The hierarchical annotation by KEGG BRITE was used to arrange proteins according to the top three out of four hierarchical levels ('system', 'process' and 'pathway'). Protein clusters were drawn as a sunburst treemap (Figure 4), where each layer of the treemap represents one hierarchical level, starting with the broadest level as innermost layer ('system') and ending with the most detailed layer at the surface ('pathway'). The width of a sector corresponds to the number of proteins within the functional group, and the color represents the standard deviation of the $\log_2$ relative protein quantity (*sd*), with the fresh sample (F) as reference. For example, a yellow or red color indicates a high *sd* and therefore a higher or lower quantity of (some) proteins in a group, compared to the proteins of the fresh sample. Thus, storage-induced variations affecting only certain functional groups can be easily spotted. The variation was small within the most groups for VD and DF samples ($sd < 1$), although the pathways for methane, glycine, pyruvate and glyoxylate metabolism as well as xenobiotics biodegradation showed increased variations ($1.2 \leq sd \leq 2.0$). The storage time of 2 or 28 d had no significant influence on the protein profile. However, a stronger overall variation was displayed by SAF treated samples already after 2 d of storage. After 28 d the variation was further increased for proteins involved in oxidative phosphorylation, protein export, ABC transporter and sulfur and lysine metabolism ($1.8 \leq sd \leq 2.2$). The variation in protein quantity within functional groups is an indicator for the impact of the preservation method. To further elucidate the cause for this variation, we analyzed the peptide coverage obtained by MS for 60 selected proteins of different chain lengths across samples (supplementary Figure S6). For these 20 largest, 20 smallest and 20 proteins of medium polypeptide chain length, the peptide coverage was very similar for all samples and no decay at the termini was observed. This finding suggests, that degradation of proteins was not the cause for the deviating protein profile of SAF cells in comparison to DF and VD cells. Furthermore, even a slightly increased number of peptides was detected for the SAF treated cells after 28 d compared to the 2 d stored cells. For VD, the effect was inverse with fewer peptides detected after 28 d compared to 2 d.

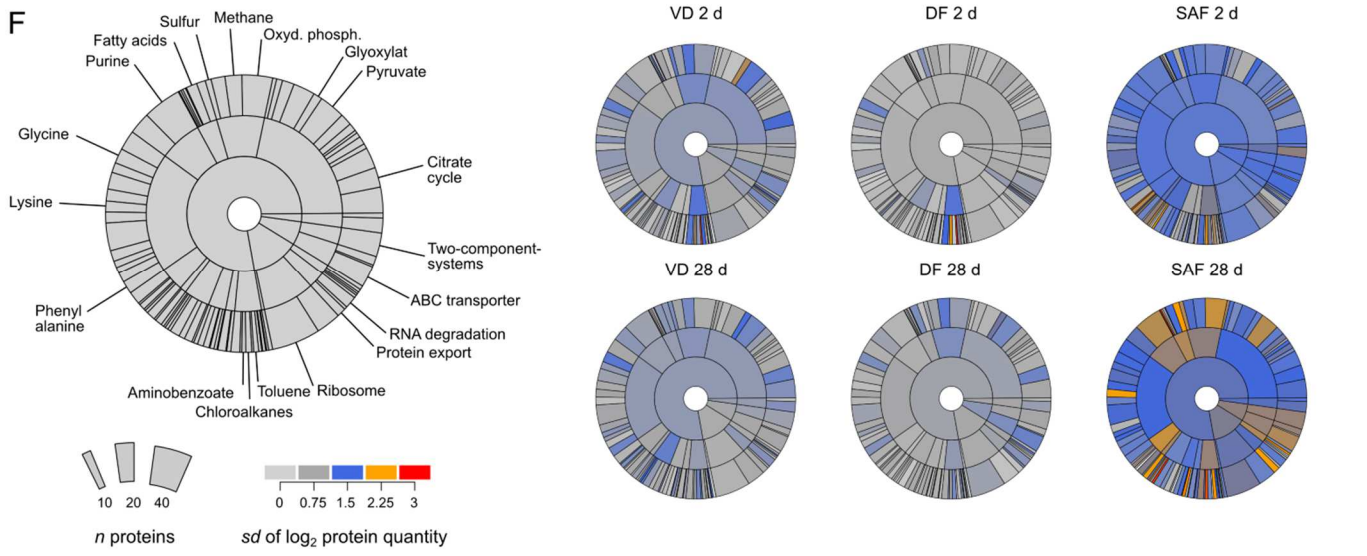

Figure 4. Functional clustering of proteins derived from cell samples prepared by VD, DF and SAF and stored for 2 d and 28 d. The sunburst treemaps represent 463 proteins mapped to functional groups using KEGG BRITE for visualization of sample variation. Treemaps consist of three hierarchically ordered layers ('system', 'process', 'pathway') with the most general ('system') being in the center. Each sector represents a group, with sector width encoding the number of proteins *n* and color encoding standard deviation of the $\log_2$ relative protein quantity (*sd*), compared to the reference (fresh cells, F). Thus, a gray color represents low variation in protein quantity and a yellow or red color a high variation.

4. Discussion

In this study, we tested three different preservation techniques (VD, DF, SAF) for bacterial cells with respect to their influence on sample integrity. These methods were selected for speed, simplicity, and omission of organic solvents. The effect of short-term and long-term storage was compared using *P. putida* as model organism. The two techniques used here –FCM and MS– are state-of-the-art for the analysis of microbes by addressing one specific *Omics* level each, namely cytomics and proteomics. The combination of both techniques can be used to obtain a detailed picture of the cellular protein interior for different subpopulations, as was recently shown for microorganisms [2, 24]. However, this kind of experiments requires the reliable preservation of cell samples before FCM and MS are applied. Using cytometry, we found VD and DF to preserve scatter characteristics and DNA content equally well, with high similarity to fresh cells even after long term storage (28 d). Based on the results for *P. putida*, a threshold of $sd = 1.5$ can be considered appropriate for indicating high sample similarity, as measured by *FlowFP* fingerprinting (Figure 2). The similarity of SAF samples was generally lower, mainly due to differing DNA patterns of the cells.

Likewise, the protein profile of VD and DF samples for *P. putida* showed higher similarity to the fresh sample than that of SAF samples as determined by MS. Interestingly, the peptide coverage of 60 selected proteins showed no decreased numbers of detected peptides for SAF, but even a slight increase after prolonged storage (28 d). A possible cause for this may be that the three dimensional structure of proteins is disturbed or unfolded over time due to the high concentration of sodium azide salt. Depending on the nature of the used salt, cellular proteins may be more amenable for whole cell tryptic digestion under such conditions [40]. Nevertheless, the increase of peptide recovery over time is not a desirable effect, since it might influence the comparability between samples. Regarding VD and DF, the higher similarity to fresh cells in proteome profiles was also reflected on the level of peptide coverage. Most likely, the absence of an aqueous environment (VD) and the very low temperature (DF) preserves protein structure more effectively.

However, functional clustering revealed that proteins of specific pathways are more prone to alteration than others, and these pathways coincided for VD and DF (e. g. glycine and pyruvate metabolism). Similar results were found in a proteomic study with human cells, where protein degradation during cold storage affected not all proteins in a sample and not all sample types equally [41]. Furthermore, the number of detected proteins in two of three replicates of VD and SAF was considerably lower after 28 days of storage, pointing towards the superiority of low storage temperatures (-80° C) over moderate ones (4°C). The loss of culturability of stored bacteria in relation to elevated storage temperature or time is a known phenomenon, both for freeze-dried and deep-frozen cells [15, 17, 20]. But little is known about the integrity of biomolecules in whole cells, when stored at different temperatures. What was shown at least, is the beneficial effect of deep storage temperatures (≤ -80°C) on protein stability for already isolated protein extracts [41, 42].

Most storage procedures for whole cells are optimized for biobanking, aiming at resuscitation of cells after storage. For this purpose, cryopreservation procedures like deep freezing and freeze drying may be the most important preservation methods [4]. But not every specimen is equally cryotolerant. Some microbial genera like *Helicobacter* or *Neisseria* are notoriously difficult to freeze or to recover [4] and complex microbial communities may require a completely different treatment (anaerobic sampling, metal ion treatment) for stabilization [13]. Moreover, the preservation of cells for single-cell analysis or for resuscitation are two different objectives, and the chosen technique is not necessarily suitable for both. However, cryopreservation is a preferred option, as the majority of cells is cryotolerant and the required equipment is affordable even for small laboratories [4]. The storage temperature for cryopreservation should preferably be lower than -20°C, which was reported to result in degradation of serum proteins compared to -80°C [42]. If storage capacities at -80°C are limited, alternatives like freeze drying and low temperature vacuum drying may be considered [22, 23], The vacuum drying procedure applied here preserved the cellular DNA content, light scattering properties, and protein profile with similar efficiency as deep freezing. It requires neither chemical treatment nor other technical effort than a generic vacuum concentrator, and storage of dried cells can take place at 4°C.

Whichever technique is chosen, it is necessary to test the applicability of the desired work flow to the target organism. The bacterial species covered here are not representative for all bacterial genera, but are commonly used in biotechnology. The presented preservation methods are intended for the use in sub-population proteomics, a combination of flow cytometric cell sorting and protein mass spectrometry recently applied for microbes [2, 24, 43]. As the process of cell sorting may impose further stress on recovered cells and change protein abundances independent of storage, it was omitted here. However, the results of this study may be of interest for other analytical disciplines as well. Regarding flow cytometry for instance, other markers such as fluorescent proteins are often used and their function should be conserved by DF [2]. Proteins, however, belong to the more stable biomolecules. And although more 'delicate' biomolecules such as RNA could be stronger affected by unfavorable storage conditions, the findings of this study may very well apply to these biomolecules as well.

5. Conclusions

Three different methods for the preservation of bacterial cells –vacuum drying (VD), deep freezing (DF), sodium azide fixation (SAF)– were evaluated using flow cytometry and protein mass spectrometry. Cells of *P. putida* were stored for 2 d and 28 d and the similarity to a fresh reference sample

quantified by cytometry fingerprinting and proteome profiling. Both DF and VD ensured high agreement between stored and fresh samples as analyzed by flow cytometry, whereas SAF samples showed reduced similarity. Furthermore, 971 different proteins were identified across all samples, and 463 of these proteins were functionally clustered and revealed susceptibility of certain protein groups to alteration, depending on the preservation method. Overall, most of the functional groups displayed low variation in protein quantity for VD and DF samples, but high variation in case of SAF, particularly after 28 d. Interestingly, no peptide decay — for example at the protein termini — was found for SAF but rather an increase in peptide coverage. We assume, that the protein structure is made more amenable for trypsin digestion by the action of sodium azide. Nevertheless, DF and VD are recommended for use in flow cytometry and further downstream applications like protein mass spectrometry, whereas SAF should be avoided for *P. putida*.

6. Supplementary material

Supplementary data and information is available at: http://www.jiomics.com/index.php/jio/rt/suppFiles/115/0.
Supplementary Material includes Figures S1 (Evaluation of vacuum drying (VD) conditions for *P. putida* KT2440 using flow cytometry), S2 (Evaluation of deep freezing (DF) conditions for *P. putida* KT2440 cells using flow cytometry), S3 (Internal variation of identical samples when using FlowFP fingerprinting [27]), S4 (Test of different storage methods for *E. coli* DH5α using flow cytometry), S5 (Variability of protein number and quantity across replicates as identified by mass spectrometry) and S6 (Peptide coverage of selected proteins). Supplementary material further includes S1 for MaxQuant settings, S2 for the R treemap function, and tables S3 and S4 for the list of detected proteins by MaxQuant and by Thermo Discoverer/Mascot, respectively.

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