Differential proteomic analysis using isotope-coded protein-labeling strategies: Comparison, improvements and application to simulated microgravity effect on Cupriavidus metallidurans CH34

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Among differential proteomic methods based on stable isotopic labeling, isotope-coded protein labeling (ICPL) is a recent non-isobaric technique devised to label primary amines found in proteins. ICPL overcomes some of the disadvantages found in other chemical-labeling techniques, such as iTRAQ or ICAT. However, previous analyses revealed that more than 30% of the proteins identified in regular ICPL generally remain unquantified. In this study, we describe a modified version of ICPL, named Post-digest ICPL, that makes it possible to label and thus to quantify all the peptides in a sample (bottom–up approach). Optimization and validation of this Post-digest ICPL approach were performed using a standard protein mixture and complex protein samples. Using this strategy, the number of proteins that were identified and quantified was greatly increased in comparison with regular ICPL and cICAT approaches. The pros and cons of this improvement are discussed. This complementary approach to traditional ICPL was applied to the analysis of modification of protein abundances in the model bacterium Cupriavidus metallidurans CH34 after cultivation under simulated microgravity. In this context, two different systems – a 2-D clinorotation and 3-D random positioning device – were used and the results were compared and discussed.

Keywords:
Cupriavidus metallidurans CH34 / Differential proteomics / Isotope-coded protein labeling / Microbiology / Simulated microgravity

1 Introduction

Long-term manned space missions will require development of artificial ecosystems (called life support systems) able to recycle crew-produced wastes. Different artificial ecosystems already exist, at least theoretically, and rely on transformation by different bacteria of crew wastes into elements capable of sustaining photosynthetic growth of cyanobacteria and higher plants [1]. After developing and closing the ecological loop, one of the most challenging tasks of such a project is to evaluate the effect of space flight on bacteria communities present in the ecosystem. Our knowledge of microbial response to stimuli, such as microgravity, radiation and the combination of both, is very limited. However, previous studies have, for example, already highlighted enhancement of virulence of Salmonella enterica in space [2] or simulated microgravity [3] increased resistance to salt stress and antibiotics in Escherichia coli [4] and increased extracellular accumulation of various products [5–7]. Comparison of these results suggests that different organisms may differ substantially in their ecological interactions.
responses to low-shear and space environments. A better characterization of microbial response to the space environment is essential for the safe use of artificial ecosystems in future long-term manned space missions.

Because of the great number of constraints associated with space flight experiments (limited availability of power, crew time, flight opportunities, etc.), ground simulation of space conditions and in particular of microgravity has been widely used in the last 10 years. Different devices exist to simulate microgravity, the main one being the rotating wall vessel (RWV) [8]. The RWV is a bioreactor that allows a constant rotation of the gravitational field, resulting in a randomization of the gravity vector and thus creating weightlessness [9]. This device, or variants thereof, has made it possible to highlight the effect of low-shear-modeled microgravity (LSMMG) on metabolite production, stress resistance, growth kinetics and virulence (reviewed by Nickerson et al. [9]). In addition, Nickerson et al. [3] observed modification due to LSMMG at the proteome level using 2-D gels but without identifying the proteins involved. The same team has also used RWV to analyze the effect of simulated microgravity in a transcriptional analysis of S. enterica [10]. In this study, 163 genes were regulated in LSMMG in comparison with normal gravity. These genes belonged to diverse functional groups, such as iron uptake, lipopolysaccharide biosynthesis or virulence factor. In response to space flight cultivation, the expression of a number of genes of S. enterica was also shown to be modified [2]. Moreover, a conserved RNA-binding protein, named Hfq, was identified as a potential global regulator involved in the response to this environment. These findings were confirmed using the RWV, suggesting LSMMG was sufficient to reproduce part of the space conditions. Finally, Wilson et al. [11] have demonstrated a direct correlation between inorganic phosphate concentration and the phenotypic response of S. enterica. Once again RWV was used in this study to create ground-based, spaceflight-analogue conditions.

Analyzing the effect of LSMMG on E. coli, Tucker et al. [12] concluded that no specific response was associated with changes in gravity. They assume that changes observed in gene expression were more likely due to indirect effects created by the loss of the gravity vector, e.g. low shear, than to gravity itself.

In addition, using clinorotation to modulate microgravity, Vukanti et al. [13] have highlighted the activation of starvation-inducible genes as well as multiple stress-resistance-related genes in E. coli. This activation was supposed to be a side effect of modeled microgravity that creates nutrient depletion zones around the bacteria.

Moreover, it has been shown that the device used to simulate weightlessness could also influence the proteome response to this stimulus. Data from Barjaktarovic et al. [14] on Arabidopsis thaliana suggested that 2-D clinorotation devices (such as RWV) do not produce the same effect as 3-D random positioning (random positioning machine, RPM) devices. In this latter type of device, weightlessness is obtained by continuous and randomized repositioning of the gravity vector in three dimensions. If the RPM was extensively used to study cytoskeleton structure, motility of human cells [15] and plant gravitropism [16], only few studies related to the analysis of bacterial behavior have been reported so far [17, 18].

In this study, we used RWV and RPM to cultivate Cupriavidus metallidurans CH34 in modeled microgravity. This bacterium is not supposed to be part of a life support system, but was chosen as a model organism for several reasons. First, C. metallidurans CH34 has been isolated from polluted soils and its capacity to adapt to various environments has been studied extensively [19, 20]. Furthermore, numerous Cupriavidus and Ralstonia strains have been isolated from different compartments of numerous spacecraft-related sites (cooling system and drinking water of spacecraft, floor, air and surfaces of spacecraft assembly rooms, for review see [21]). Moreover, its full genome sequence was recently obtained and its 5945 genes were all submitted to expert annotation, making it possible to perform high-throughput proteomic analysis on this bacterium. Finally, it has already been cultivated in a space environment and some effects were highlighted at both the phenotypic and the molecular level [21]. Here, we used a quantitative shotgun proteomic analysis, based on isotope-coded protein labeling (ICPL) [18, 22, 23], to elucidate the global response of this model bacterium to simulated microgravity.

In the ICPL procedure, only lysine-containing peptides can be quantified, and consequently, only 70% of the identified proteins were quantified in this study, but also in the previously reported analyses [18, 23]. This drawback led our laboratory to modify ICPL protocol to label all peptides of the sample, so as to obtain quantitative data about every identified protein. This homemade procedure, called “Post-digest ICPL”, was optimized and validated using standard protein mixture as well as complex samples and its performance compared with “regular” ICPL and cICAT. The Post-digest ICPL was finally used to evaluate the impact of RPM-simulated microgravity on C. metallidurans CH34.

2 Materials and methods

2.1 Bacterial strain and growth conditions

Studies were performed using C. metallidurans CH34. All cultures were made at 21°C in 284 minimal liquid medium until an OD600nm of 0.4 (exponential phase) was reached [24]. The RWV container (Cellon, Luxembourg) was used not only for RWV-simulated microgravity, but also for RPM simulation and 1 × g control to avoid bias due to differences in culture aeration. The biocontainers used in RWV and RPM experiments were filled completely with ca. 58 mL of culture medium inoculated at an OD600nm of 0.1. Air bubbles were carefully removed through the sampling ports,
using syringes (without the needle), to avoid undesired shear stress. Gas exchange in the RWV bioreactors during growth was ensured by the gas-permeable silicone membrane present at the back of each RWV culture vessel. Bacterial growth in RWV conditions was allowed at a rotational speed of 25 rpm. The RPM was operated as a random walk 3-D-clinostat (basic mode) with an angular rotation velocity of 60°/s. The RWV vessels in the horizontal position were used as the control for both the RWV in the vertical position and the RPM cultivation. Control, RPM and RWV experiments were performed simultaneously in the laboratory of Prof. P. Pippia at the University of Sassari. All cultures were made in triplicate.

### 2.2 Protein extraction and labeling

Bacterial pellets, obtained by centrifugation, were washed twice in PBS. Protein extraction was performed using 6 M guanidinium chloride (Lysis buffer of ICPL kit, SERVA, Germany). The bacterial solution was then ultrasonicated for 3 × 15 s (80% amplitude, U50 IKAtechnik) and incubated for 20 min at room temperature. Supernatant was recovered by centrifugation (18,000 × g, 15 min, room temperature) and proteins assayed according to the Bradford method.

For the ICPL procedure, 100 μg of proteins were labeled using an ICPL kit (SERVA) and following manufacturer’s instruction. Briefly, after reduction and alkylation using iodoacetamide, proteins were labeled at protein N-termini and lysine by incubation for 2 h at room temperature with a light or heavy form of the ICPL reactant (N-nicotinoyloxy-succinimide). The labeling was stopped by quenching excess reagent with hydroxylamine. Finally, different samples were pooled (RWV or RPM with control). The proteins were recovered through acetone precipitation and dissolved in 60 μL of 10 mM phosphate buffer, pH 8.5, urea 1 M. After enzymatic digestion, 33 μg of tryptic peptides were submitted for labeling using 3 μL of ICPI-labeling reagent for 90 min at room temperature. This first labeling step was followed by the addition of 1.5 μL supplemental reagent and reaction was allowed for 90 additional minutes.

For the cICAT procedure, 100 μg of proteins were recovered through acetone precipitation and redissolved in cICAT lysis buffer, as recommended by the manufacturer. The labeling procedure and tagged peptide enrichment were made following the manufacturer’s instructions. The proteins were digested with trypsin and tryptic peptides were recovered from SCX column in a single fraction. Labeled peptides were then affinity captured and the tag was acid cleaved before analysis in MuDPIT.

### 2.3 MALDI-TOF analysis

For MALDI-TOF analysis, 1 μL of sample was mixed with 1 μL of matrix (5 mg/mL CHCA and 0.5 pmol/μL porcine rennin (Sigma) as internal standard, in 25% (v/v) ethanol, 25% (v/v) ACN, 0.05% (v/v) TFA), then spotted onto a MALDI sample plate and allowed to air dry. MALDI-TOF was performed using a M@LDITM mass spectrometer (Micromass, UK) equipped with a 337-nm nitrogen laser. The instrument was operated in the positive reflectron mode with 15 kV of source voltage, 2.5 kV of pulse voltage and 2 kV of reflecting voltage.

### 2.4 2-D nanoLC-MS/MS analysis

Twenty-five microgram (75 μg for cICAT) of peptides dissolved in loading solvent (ACN 5% (v/v); HCOOH 0.1% (v/v) in LC-MS-grade water) was loaded onto the first separation column (SCX, POROS10S, 10 cm, Dionex, The Netherlands), using an Ultimate 3000 system (Dionex), delivering a flow rate of 20 μL/min of loading solvent. Flow through was collected onto a guard column (C18 Trap, 300 μm id × 5 mm, Dionex). After desalting for 10 min, the guard column was switched online with the analytical column (75 μm id × 15 cm PepMap C18, Dionex) equilibrated in 96% solvent A (formic acid 0.1% in HPLC-grade water) and 4% solvent B (ACN 80%, formic acid 0.1% in HPLC-grade water). The peptides were eluted with an ACN gradient from 4 to 37% of solvent B in 100 min, 37 to 57% of B in 10 min and 57 to 90% of B in 10 min. The peptides adsorbed onto the SCX column were sequentially eluted using five salt plugs of 1, 5, 10, 100 and 1000 mM NaCl in the case of cICAT and nine plugs (1, 2.5, 5, 10, 25, 50, 100, 200, 1000 mM NaCl) in the case of ICPL-based methods. Each of these fractions was analyzed in RP chromatography as described for the SCX flow through.

For all the three labeling procedures, online MS analysis was performed using the ‘peptide scan’ option of an HCT ultra ion Trap (Bruker, Germany), consisting of a full-scan
MS and MS/MS scan spectrum acquisitions in ultrascan mode (26,000 m/z/s). Each analysis was performed in duplicate (technical replicate). Peptide fragment mass spectra were acquired in data-dependent AutoMS(2) mode with a scan range of 100–2800 m/z, five averages, and four precursor ions selected from the MS scan range of 300–1500 m/z. Precursors were actively excluded within a 0.5-min window after one spectrum, and all singly charged ions were excluded. The stable-isotope-labeling experiment (SILE) option of the mass spectrometer was activated. In this configuration, the precursor selection was based on detection of differentially expressed isotopic pairs. In this context, peptides selected for MS/MS analysis were required to have H/L > 1.33 or < 0.75. The SILE selection options authorized 1–4 labels/peptide and a charge state of +2 and +3. Peptide peaks were detected and deconvoluted automatically using Data Analysis 3.4 software (Bruker). Mass lists in the form of MASCOT Generic Files were created automatically and used as the input for MASCOT MS/MS Ions searches of the NCBI database release 20080704 using an in-house MASCOT 2.2 server (Matrix Science, UK). The search parameters used were: taxonomy = C. metallidurans CH34; enzyme = endo-Arg and endo-Glu; Max. Missed cleavages = 2; fixed modifications = carbamidomethyl (C); variable modifications = oxidation (M); peptide tolerance ± 1.5 Da; MS/MS tolerance ± 0.5 Da; peptide charge = 2+ and 3+; instrument = ESI-TRAP. Variable modifications also contain ICPL or cICAT tag with the corresponding specificity. Only proteins identified with a protein score above the MASCOT calculated ion score, defined as the 95% confidence level, were considered. In addition, proteins identified with a single peptide were submitted to manual validation, using criteria defined by Sarigolu et al. [25]: (i) fragmentation spectra with a long, nearly complete y and/or b-series; (ii) all lysines modified; (iii) number of lysines predicted from the mass difference of the labeled pair matching the number of lysines in the peptide sequence; (iv) detection of at least one modified lysine (if any in the sequence) in the fragment series. The false-positive discovery rate was estimated at the peptide level using the decoy option of the MASCOT search engine.

Warp-LC 1.2 was used for protein quantification. First, extracted ion chromatograms (EICs) were calculated by using the m/z values of identified peptides and summing the intensities of the mass peaks within a mass tolerance of 0.5 Da around the identified peptides. A retention time window of 0.4 min was considered around the elution time of the identified peptides. +2, +3 and +4 charge states were taken into account in the EIC generation by Data Analysis. The chromatographic peak finder in Data Analysis was used to detect peaks in the EIC traces and areas under the curve were calculated from the sum of the signal intensities times the distance between two successive data points (MS spectra). Only peptides with an ion score above 20 were considered for quantification. Quantitative data were systematically inspected manually and outlier ratios were manually recalculated. Protein ratios for which coefficients of variation were greater than 25% or quantified based on less than three peptides were also manually recalculated. For manual calculation, MS spectra were averaged along the elution window of the corresponding isotopic pair using Data Analysis software. A mass list of the averaged spectrum was created in Data Analysis. The areas under the curve, calculated thanks to the Data Analysis chromatographic peak finder, of the first three peaks of the isotopic distribution were summed for both the heavy and light components of an isotopic pair. The regulation ratio was calculated using the summed intensities.

For statistical analysis, all data were converted in the log space to maintain symmetry around zero. The global mean and SD of protein ratios were calculated for each couple of the sample. The cut-off point for protein differential abundance was determined, following Wang et al. [26], as the mean ± 2SD (95% confidence interval). At the individual level, protein differential abundance was assessed using the Student’s t-test, as already mentioned by Shi et al. [27] and Stevens et al. [28].

3 Results and discussion

3.1 Analytical method validation

To confirm that our analytical platform (combining data acquisition on HCT ultra and processing with Bruker’s software) can perform ICPL-based shotgun proteomic analysis, two standard protein mixtures from the ICPL kit (SERVA) were used. These mixtures contained the same three proteins but at different ratios. Abundance ratios were BSA 1:1, ovalbumin 4:1, carbonic anhydrase 1:2.

The ICPL label targets unmodified amino groups of lysine and the N-terminus. One weakness of lysine labeling is that trypsin does not cleave ICPL-modified lysine sites. Trypsin digestion of ICPL-labeled protein results in rather long peptides that are not easily detected by mass spectrometry, especially after gel electrophoresis. The benefit of a secondary enzymatic digestion was therefore evaluated. In this context, MALDI-TOF analysis was used to compare identified peptides whether the labeled protein mixture was treated with trypsin or with trypsin and endoproteinase Glu-C. Table 1 presents labeled peptides identified in both conditions. Clearly, a combination of trypsin and endoproteinase Glu-C digestion made it possible to identify more labeled peptides and this procedure was thus adopted for all our ICPL analyses.

The labeled protein mixture was subsequently analyzed using the 1DLC MS/MS procedure. As summarized in Table 1, the expected abundance ratios were observed for BSA, ovalbumin and carbonic anhydrase, thus providing evidence that our analytical platform is amenable to ICPL-based shotgun proteomic analyses.
3.2 Quantitative methods comparison

In a first set of experiments, the same couple of samples (RWV versus control) was analyzed using three different approaches to compare their efficiency: the frequently used and well-validated cICAT method, the more recently developed ICPL method and a homemade Post-digest ICPL. The Post-digest ICPL was developed in our laboratory because previous analyses revealed that more than 30% of the proteins identified in regular ICPL generally remain unquantified [18, 23]. In our study, many proteins were identified with no lysine-containing peptides. These unlabelled peptides cannot be used for quantification. In this context, our laboratory undertook the modification of the ICPL protocol to label samples after tryptic digestion. Labeling at the peptide level should allow tagging of all the peptides, thanks to their N-terminal primary amine, and thus make it possible to quantify all identified proteins. The general principle of the three approaches is depicted in Fig. 1.

The results of the three methods were compared in terms of number of proteins identified and quantified in a single run using the SILE option of the mass spectrometer, i.e. MS/MS precursor selection was based on detection of differentially expressed isotopic pairs. The three methods were also compared in terms of number of peptides/proteins useful for quantification.

As shown in Fig. 2A, this analysis revealed that ICPL used in Post-digest configuration made it possible to increase performance as more than 350 proteins could be identified. Moreover, only a few of them (2%) were not quantified due to ambiguous MS spectra. With the regular ICPL approach, nearly 300 different proteins were identified using the same SILE-based precursor selection procedure, but quantitative data were obtained for only 200 of them. In contrast, from the cICAT approach only 132 and 124 proteins were identified and quantified, respectively. In cICAT, the affinity capture step allowed for sample complexity reduction and thus higher amounts of starting material could be used. However, even while engaging three times more proteins, cICAT obtained the worst results in this study. Low yield in the labeled peptide affinity capture procedure and/or low recovery rate after the in-glass tag cleavage step could explain such results.

Moreover, about 50% of the proteins were quantified with at least three peptides in the case of ICPL and Post-digest ICPL and the latter method gets more than 10% of proteins quantified with at least eight peptides (Fig. 2B). On the contrary, using the cICAT procedure, 45% of the proteins were quantified using only one peptide. As the statistical power of the measured ratio increased with the number of analyzed peptides, ICPL and Post-digest ICPL could clearly be considered as more powerful methods.

3.3 Post-digest ICPL optimization

After a Post-digest ICPL labeling using the classical experimental procedure, we observed some non-labeled peptides. This incomplete labeling dramatically affected the standard deviation of quantitative data for the corresponding proteins. Moreover, incomplete derivatization of N-terminal peptides was also observed, if the Post-digest strategy was applied to a standard protein mixture (data not shown). In this context, optimization of the Post-digest-labeling procedure was undertaken using a standard protein mixture containing albumin, carbonic anhydrase type II, ovalbumin. Different optimization strategies were tested. Quantitative labeling was obtained by increasing the buffer capacity of the labeling solution (phosphate buffer 100 mM versus 10 mM), the reactant/substrate ratio as well as the reaction time allowed. After 90 min of incubation in the presence of reactant, an additional quantity of reactant was added (1/2 of initial reactant quantity) and the sample was then incubated for 90 min. Using this modified protocol, no residual singlet, i.e. unlabeled peptide, was found in MALDI-TOF analysis of the labeled protein mixture (Fig. 3). Moreover, from 25 μg of protein mix, no unlabeled peptides were identified in LC-MS/MS analysis (data not shown).

Finally, the expected ratio between light and heavy form was obtained for all the three proteins from the standard mixture (Table 2). Moreover, the number of quantified peptides per protein strongly increased. The optimized Post-digest ICPL was thus applied to analyze a complex protein sample. In this study, we used this approach to analyze the effect of simulated microgravity on the model bacterium C. metallidurans.

3.4 Simulated microgravity

In a preliminary analysis, three biological replicates for RPM, RWV and control conditions were quantitatively
Post-digest proteins were found to show a different relative abundance in biological replicates, showed that simulated microgravity induced a slight effect on RPM cultivation. In this context, we focused on RPM effect evaluation and thus submitted only the RPM samples to analysis using regular ICPL. In the cICAT protocol, proteins were labeled (1), mixed (2) and submitted to enzymatic digestion (3). Labeled peptides were then affinity purified (4) to reduce sample complexity before MuDPIT analysis (5). In regular ICPL, experimental steps were similar, but no sample complexity reduction was applied. In Post-digest ICPL, labeling occurred after tryptic digestion, so as all the peptides could be tagged at their N-terminal by the amine reactive tag.

Using this optimized Post-digest ICPL approach, 674 and 649 proteins were identified in two biological replicates (false-positive discovery rate: 0.39 and 0.52%, respectively). While in cICAT most of the proteins were only quantified using a single peptide (B), the majority of the proteins was quantified with more than two peptides in ICPL and Post-digest ICPL, thus allowing a more accurate relative quantification.

Figure 2. Comparison of performance obtained by cICAT, ICPL and Post-digest ICPL in terms of number of identified and quantified proteins (A) as well as in terms of number of quantified peptides/proteins (B). ICPL and Post-digest ICPL allowed identification of more proteins (A), the latter being able to quantify 98% of them. False-positive discovery rate was estimated at the peptide level using the decoy option of the MASCOT search engine. In cICAT, the affinity capture step allowed for sample complexity reduction and thus higher amounts of starting material can be used. While in cICAT most of the proteins were only quantified using a single peptide (B), the majority of the proteins was quantified with more than two peptides in ICPL and Post-digest ICPL, thus allowing a more accurate relative quantification.

pooled and submitted to analysis using regular ICPL. In this condition, 608 and 440 proteins were identified in RPM versus control and RWV versus control samples, respectively (false-positive discovery rate: 0.53 and 0.11%, respectively). Quantitative data were obtained for 423 and 313 of them, respectively (Supporting Information Tables S1 and S2). This preliminary analysis, performed on a pool of three biological replicates, showed that simulated microgravity induces a slight effect on C. metallidurans CH34. Only eight proteins were found to show a different relative abundance between RWV and control samples. Nevertheless, 36 proteins seem to be quantitatively affected by RPM cultivation. In this context, we focused on RPM effect evaluation and thus submitted only the RPM samples to quantitative proteomic analysis using the new optimized Post-digest ICPL.

Using this optimized Post-digest ICPL approach, 674 and 649 proteins were identified in two biological replicates (false-positive discovery rate: 0.39 and 0.52%, respectively). No incomplete ICPL labeling was observed among the 4372 detected proteins.
and 4173 peptides identified, demonstrating quantitative labeling using the optimized Post-digest ICPL procedure (Supporting Information Tables S3 and S4). While only 70% of the identified proteins could be quantified in regular ICPL, Post-digest ICPL allowed quantification of 95% of them. Only a few proteins were not quantified due to ambiguous MS spectra. For statistical analysis, only proteins quantified with at least two peptides were taken into account. The statistical thresholds for protein abundance ratio were determined to be greater than 1.8 or less than 0.55 and greater than 1.85 or less than 0.53 for the two biological replicates, respectively. Only proteins showing equivalent trends in both biological replicates and in the pooled sample were taken into account and are presented in Table 3. At the individual level, fold changes were assessed using the Student’s t-test.

Among the 36 proteins for which a significantly different relative abundance was detected in the preliminary analysis using a pooled sample, 27 were quantified in both separated biological replicates. Among them, 14 proteins showed a similar modification of abundance in both biological replicates and in the pooled sample. However, the relative abundance of nine proteins was only modified in one of the two biological replicates. These data clearly show significant inter sample variability and thus it is preferable to perform the analysis of separated biological replicates rather than pooled samples. It also justified that a differential relative abundance was only taken into account if observed in both biological replicates.

Despite the relatively low amplitude of protein abundance variations, we observed a complex response to RPM cultivation as it involves proteins associated with various cellular functions (Fig. 4). Among all proteins showing a change in abundance, we observed a significant increase in different stress proteins,
### Table 3. Proteins for which modification of abundance was observed in two biological replicates after RPM cultivation and Post-digest ICPL analysis

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Locus tag</th>
<th>NCBI ID</th>
<th>Sample 1</th>
<th>Sample 2</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Number of peptides used for quantification</td>
<td>Number of peptides used for quantification</td>
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<td>gi</td>
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<tr>
<td>Hypothetical protein, Rmet_5074</td>
<td>Rmet_5074</td>
<td>gi</td>
<td>94313995</td>
<td>2</td>
</tr>
<tr>
<td>Cold-shock DNA-binding protein family protein</td>
<td>Rmet_5816</td>
<td>gi</td>
<td>94314735</td>
<td>4</td>
</tr>
</tbody>
</table>

The threshold for the protein abundance ratio was determined to be greater than 1.8 or less than 0.55 and greater than 1.85 or less than 0.53 for the two biological replicates, respectively. At the individual level, fold changes were assessed using the Student’s t-test. SD: standard deviation; ND: No data; *Student’s t-test assessed statistical significance (t < 0.05).

a) Proteins for which decreased relative abundance was observed.
especially Universal stress proteins (USP). Three different Universal stress proteins (UspA1 (Rmet_0458), UspA3 (Rmet_1387) and UspA9 (Rmet_4395)) were significantly more abundant in simulated microgravity. UspA3 was also found to have increased in *C. metallidurans* after space flight [21]. Cold shock protein A (CspA, Rmet_5816) was also more abundant after RPM cultivation. Interestingly, anaerobic survival of *Pseudomonas aeruginosa* using pyruvate fermentation requires Usp-type stress proteins [29]. In contrast, a significant decrease in DnaK protein (Rmet_{3172}) was observed. Moreover, our results highlighted an increased quantity of cytochrome d1, heme region (Rmet_{3172}) not only in both RPM samples, but also in preliminary analysis conducted with an RWV device. This protein is a nitrite reductase and its high concentration in simulated microgravity could reflect a switch from oxygen to nitrite as the electron acceptor in respiratory metabolism. This switch could result from anoxic or micro-oxic conditions during growth in simulated microgravity due to decreased homogenization of the medium [30].

Generally, simulated microgravity could mean a reduction in homogenization, which could cause a change in interaction between bacteria and their environment. As observed for *E. coli* [12], the majority of the proteins whose abundance was reduced were involved in metal (CupA, CupC) or molecule transport (OmpA/MotB (Rmet_{0712}, Rmet_{2674}, Rmet_{2768}); Phosphonate transport (Rmet_{2994}, Rmet_{2994}); ABC-type transporter (Rmet_{0794}); Porin (Rmet_{3144}), which tends to show the fine tuning of the transport activity in the response of *C. metallidurans* to RPM cultivation.

Relative abundance of YaeT (Rmet_{1443}) was decreased in simulated microgravity. Proteins of this family are responsible for assembling proteins into the outer membrane of Gram-negative bacteria. The observed reduction in the amount of YaeT could explain the decreased abundance of a large number of membrane proteins after RPM-generated-simulated microgravity.

4 Concluding remarks

In contrast to other isotope-labeling approaches, the ICPL-labeling step is not limited to the peptide level but was developed to be applied on the protein level. ICPL allows reduction of complexity on the protein level by different fractionation steps such as chromatographic separation without sacrificing quantitation accuracy. This protein-labeling procedure has the major advantage that different species of a labeled protein (protein isoforms and post-translational-modified species) can be separated before MS analysis.

In this study, we have highlighted the great efficiency of ICPL in terms of number of proteins identified and quantified. The ICPL label is an isotope-coded nicotinoyl group coupled to an amino-reactive N-hydroxysuccinimide that targets unmodified primary amino groups. The majority of MS analysis requires prior processing of proteins into peptides with a specific protease. The most commonly used protease is trypsin, which is not able to cleave peptide bonds involving ICPL-modified lysine. Clearly, a combination of
trypsin and endoproteinase Glu-C for the digestion makes it possible to significantly increase the number of labeled peptides identified by MS analysis. This procedure is suitable for the complex mixtures of proteins that have been fractionated or not with SDS-PAGE.

Despite this improvement, about 30% of the identified proteins remained unquantified in all analyses we carried out. This same analytical weakness was also noticed in other recent studies [18, 23]. ICPL was thus adapted to a Post-digest protocol in order to increase the number of identified and quantified proteins. The Post-digest ICPL was optimized and validated using a standard protein mixture and complex protein samples. When used to analyze bacteria samples cultivated under RPM-simulated microgravity, the Post-digest ICPL approach made it possible to significantly increase the number of identified (674 versus 608) and quantified (640 versus 440) proteins. Post-digest ICPL can be compared with the iTRAQ method, in which labeling also occurs after tryptic digestion and that makes it possible to obtain quantitative data for all the identified peptides. One of the most important drawbacks of iTRAQ is that, as quantification is performed in the low mass range of MS/MS spectra, its use in ion trap remains challenging [23]. Moreover, using the SILE-based precursor selection option of the mass spectrometer, it is possible to focus sequencing effort on isotopic pairs, presenting a defined heavy/light ratio. As iTRAQ is an isobaric method, the discrimination of differentially quantified peptides can only be performed after MS/MS analysis. Finally, one of the strengths of ICPL labeling is that it increases the MALDI ionization efficacy and thus makes it possible to increase the sensitivity of the quantitative MALDI approach [22]. The Post-digest ICPL approach seems to be a complementary method to traditional ICPL labeling especially to obtain quantitative information about the 30% of proteins that were not quantified with this last approach. Nevertheless, in contrast with labeling at the protein level, labeling at the peptide level does not make it possible to separate previously and efficiently the different species of a labeled protein (protein isoforms and post-translational-modified species). Actually, for a functional study, bottom-up and top-down approaches will be necessary to acquire the most complete view of the proteome modification of a specific system. Nevertheless, the variation of protein abundance can reflect some critical and relevant cellular modifications, such as certain metabolism modifications. In this context, a bottom-up approach, such as Post-digest ICPL labeling, can allow researchers to detect the modification in abundance of some key proteins. Clearly, particular attention must be taken to analyze data obtained with the Post-digest ICPL approach due to the acquisition of incomplete structural information on forms of a protein serving as the source of peptides.

Post-digest ICPL has been applied to the preliminary analysis of RPM-generated-simulated microgravity effect on a model bacterium, C. metallidurans CH34. Microgravity in itself seems not to be involved in dramatic changes in the bacterial proteome. Nevertheless, in contrast with the RWV samples, we observed significant modifications in the abundance of some proteins in a sample submitted to RPM treatment. This observation agreed with those of other studies, for which these two different ways of simulating microgravity did not produce equivalent effects [14]. Our data suggest a complex response to C. metallidurans RPM cultivation involving proteins, especially membrane proteins, implicated in various cellular functions. This preliminary analysis suggests that it is essential to continue our efforts to analyze not only the effects of simulated microgravity and other spatial stress on model organisms, like in this study, but also on bacteria that would be used in artificial ecosystems.

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5 References


