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**Photobacterium profundum** under Pressure: A MS-Based Label-Free Quantitative Proteomics Study

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**Abstract**

*Photobacterium profundum* SS9 is a Gram-negative bacterium, originally collected from the Sulu Sea. Its genome consists of two chromosomes and an 80 kb plasmid. Although it can grow under a wide range of pressures, *P. profundum* grows optimally at 28 MPa and 15°C. Its ability to grow at atmospheric pressure allows for both easy genetic manipulation and culture, making it a model organism to study piezophily. Here, we report a shotgun proteomic analysis of *P. profundum* grown at atmospheric pressure compared to high pressure using label-free quantitation and mass spectrometry analysis. We have identified differentially expressed proteins involved in high pressure adaptation, which have been previously reported using other methods. Proteins involved in key metabolic pathways were also identified as being differentially expressed. Proteins involved in the glycolysis/glucogenesis pathway were up-regulated at high pressure. Conversely, several proteins involved in the oxidative phosphorylation pathway were up-regulated at atmospheric pressure. Some of the proteins that were differentially identified are regulated directly in response to the physical impact of pressure. The expression of some proteins involved in nutrient transport or assimilation, are likely to be directly regulated by pressure. In a natural environment, different hydrostatic pressures represent distinct ecosystems with their own particular nutrient limitations and abundances. However, the only variable considered in this study was atmospheric pressure.

**Introduction**

The deep sea comprises approximately 70% of the Earth’s biosphere. However, piezophiles (i.e., organisms that thrive at high pressure) have been less studied compared to other extremophiles. This is due to the difficulty of isolating and culturing them in a high pressure environment [1]. Understanding the biochemical mechanisms governing how they have adapted to live under high pressure may yield significant biotechnological and industrial applications [2].

An increase in hydrostatic pressure induces a reduction in cell volume, which affects biological reactions and cellular processes by altering macromolecular packing and hydration [3]. Therefore, any biological reaction or response for positive or negative changes in cell volume will be affected by pressure. This may include: protein-protein interactions, ribosome assembly, protein folding, DNA conformation and interactions as well as protein-small molecule interactions [3].

*Photobacterium profundum* SS9 is a deep sea Gram negative bacterium that was originally isolated from an amphipod homogenate collected from a depth of 2.5 km in the Sulu Sea from the Philippines [1]. *Photobacterium profundum* is in the *Photobacterium* subgroup of the family *Vibrionaceae* and is, therefore, closely related to other studied *Vibrio* species [4] such as *Vibrio cholerae* (the etiological agent of cholera) and *Vibrio vulnificus* (responsible for some types of seafood poisoning and infection through open wounds) [5]. The genome sequence for *P. profundum* SS9 has been recently published and consists of two chromosomes and an 80 kb plasmid [6]. *P. profundum* is well adapted to high pressure and grows optimally at 28 MPa and 15°C, which defines it as being both a piezophile (i.e., thrives under high pressure conditions) and as a psychrophile, (i.e. thrives under cold conditions). Interestingly, *P. profundum* SS9 can grow over a large range of pressures from atmospheric pressure (0.1 MPa) up to 90 MPa [1]. *P. profundum* SS9’s ability to grow at atmospheric pressure allows for the ease of genetic manipulation, culturing and the development of genetic tools, which are difficult to implement with many other piezophiles. For this reason, it has been adopted by the community as a model organism to study piezophily [1,7,8].

Several studies on *P. profundum* have shown drastic changes in both its gene expression and cellular morphology when pressure is shifted from 0.1 MPa (atmospheric pressure) to 28 MPa [4,6,9]. This is yet another reason for which *P. profundum* serves as a valuable piezo-tolerant model organism.

To date, two comparative transcriptomic studies have been performed on *P. profundum* at different pressures [4,6]. It is common practice to study global changes in an organism in...
response to a given perturbation by using a transcriptomic approach (i.e. quantifying mRNA expression) as an estimation of the protein expression level. Although a transcriptomic approach is an essential tool to decipher mechanisms in response to a perturbation, several studies have shown poor correlation between the level of mRNA and proteins with the exception of the few most abundant proteins [10–12]. This observation highlights the complex relationship between mRNA and protein levels in a cell or organism due to either the importance of protein turnover or the presence of miRNA.

Until recently, proteome-wide analysis of organisms has been a challenge due to proteins not being easily amplified (as there is currently no PCR equivalent for proteins). Additionally, proteomics provides a direct measure of the global protein expression level within cells and, therefore, suffers from a strong bias toward the detection of highly abundant proteins. Fortunately, the development of more sensitive mass spectrometers with faster acquisition rates, combined with various fractionation strategies, now allows for the detection of low abundant proteins.

While several quantitative proteomic approaches exist, each has its own inherent limitations. For example, 2DE suffers from a small dynamic range and a bias toward specific classes of proteins [10]. SILAC, although currently a gold standard in the field of quantitative proteomics, is currently still limited to well-characterised in vivo models and can suffer from being long and tedious to establish in new model organisms. In a similar way, $^{15}$N metabolic labelling suffers from a similar problem, since the medium composition has to be controlled and data analysis is still challenging due to heterogeneous $^{15}$N incorporation [13]. In vitro labelling strategies such as dimethylation introduce more complexity in the LC trace (reducing the number of proteins identified), isobaric labelling strategies can be rather expensive and some issues with iTRAQ accuracy and its precision have been documented by the work of Lilley's group [14].

Label-free quantitative proteomic approaches were established several years ago in the industrial proteomic field [15–17] and quite recently have emerged as credible quantitative tools by several academic research groups ([10,19], to name a few). Recently, several relatively straightforward commercial software programs have been developed (for review see [20]). There are several advantages to a label-free quantitation. For example, there is no need to grow the organism with an expensive stable isotope, the method doesn’t introduce more complexity by adding a heavy and a light component to each peptide, and theoretically there is no limitation from an experimental design point of view. This has made label-free quantitation an attractive strategy, even for small academic proteomic facilities, for the quantitation of changes in the cellular proteome resulting from a given perturbation.

Here, we report the first quantitative LC-MS label-free study to investigate Photobacterium profundum’s response to hydrostatic pressure changes for atmospheric pressure (0.1 MPa) and high pressure (28 MPa) conditions. We have identified a number of differentially expressed proteins involved in high pressure adaptation which have been previously reported, including dnaK (PBPRA1484) and GroEL (PBPRA3387) [3,4].

Several proteins involved in key metabolic pathways were differentially expressed; 11 proteins involved in the glycolysis/gluconeogenesis pathway were up-regulated at high pressure.
Figure 2. Scatter plot of the non-transformed protein intensity plotted against their median intensity group. In Fig. 2A, B, C, the biological triplicates grown at 0.1 MPa (replicates 1 to 3) is plotted against the 0.1 MPa median intensity group. In Fig. 2D, E and F, the biological triplicates grown at 28 MPa (replicate 1 to 3) is plotted against the 28 MPa median group. In Fig. 2G, the median intensity at 28 MPa is plotted against the median intensity at 0.1 MPa. Slope and regression coefficients are also highlighted in each plot. The non-transformed protein intensity was extracted using Progenesis (see material and methods section for details).

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Conversely, at atmospheric pressure (0.1 MPa) several proteins involved in the oxidative phosphorylation pathway were up-regulated. These observations suggest that *P. profundum* may use either fermentation or respiration metabolism depending on its environment (poor oxygen content is typical of the deep sea, while higher oxygen levels characterize the surface zone).

Finally, several of the identified proteins are regulated directly in response to the physical impact of pressure. It is plausible that proteins involved in nutrient transport or assimilation, for example, have their level of expression directly regulated by pressure. The various ocean layers, from the Epipelagic zone (0 to 200 m deep) to the Mesopelagic and Bathypelagic zone (200 to 4000 m deep) represent completely distinct ecosystems with their own particular nutrient limitations and abundances. This is not the case in our study, where the only variable considered was pressure. Therefore, we hypothesize that atmospheric pressure serves as a sensing mechanism by which *P. profundum* can detect its position (depth) in the ocean. Increased pressure induces dramatic changes in the proteomic composition of this organism. Combined, these changes may result in both increased membrane fluidity and adaptation to altered nutrient availability.

| Description                                | Protein Id   | Orthologs | Peptides used | Intensity ratio 28MPa/0.1MP | Reference             |
|--------------------------------------------|--------------|-----------|---------------|----------------------------|-----------------------|
| transcription activator ToxR               | PBPR1022     | ToxR      | 1             | 0.25                       | Welch et al 1998      |
| ompL_phop or porin-like protein L precursor| PBPR0600     | OmpL      | 6             | 0.18                       | Chi et al 1993        |
| chaperone protein DnaJ                     | PBPR0968     | DnaJ      | 7             | 0.53                       | Campanaro et al 2005  |
| DNA repair protein RecN                    | PBPR0964     | RecN      | 6             | 0.21                       | Vezzi et al 2005      |
| uvrD; DNA-dependent helicase II            | PBPR3513     | UvrD      | 8             | 0.38                       | Vezzi et al 2005      |
| pyruvate kinase (EC:2.7.1.40); K00873      | PBPR0428     |           | 17            | 0.59                       | Vezzi et al 2005      |
| phosphoglycerate kinase                    | PBPR3131     | Pgk       | 21            | 0.40                       | Vezzi et al 2005      |
| glucose-6-phosphate isomerase              | PBPR3328     | Pgk       | 20            | 0.62                       | Vezzi et al 2005      |

Table 1. Several proteins identified in this study and been previously reported in the literature to be piezo-sensitive.

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Materials and Methods

1. Materials
All chemicals were purchased from Sigma-Aldrich (UK) unless otherwise stated. Acetonitrile and water for LC-MS/MS and sample preparation were HPLC quality (Fisher, UK). Formic acid was Suprapure 98–100% (Merck, Darmstadt, Germany) and trifluoroacetic acid was 99% purity sequencing grade. Trypsin (modified, sequencing grade) was purchased from Roche Diagnostics (West Sussex, UK). All HPLC-MS connector fittings were from Upchurch Scientific or Valco (Hichrom and RESTEK, UK).

2. Photobacterium profundum culture and cell lysis
All Photobacterium profundum SS9 culture was performed anaerobically at 17°C in marine broth (28 g/liter 2216 medium; Difco Laboratories) supplemented with 20 mM glucose and 100 mM HEPES buffer (pH 7.5). To produce stock cultures, –80°C freezer stock of P. profundum SS9 was inoculated into 15 mL of marine broth at 17°C in sterile plastic tubes and allowed to grow to an OD of 1.5 at 600 nm. For the cultures to be used in the comparative proteomic study, 50 mL of marine broth was inoculated with100 μL of the stock cultures. The inoculum was then aliquoted into sterile plastic Pasteur pipettes [21–23] containing 6 mL of culture each, excluding air to avoid uneven hydrostatic pressure distribution and to ensure anaerobic conditions. Pasteur pipettes were then sealed with a Bunsen burner and a bag sealer. For growth at 0.1 MPa, pipettes were wrapped in aluminium foil and then incubated in a temperature-controlled room at 17°C. For high pressure growth, Pasteur pipettes were incubated at 28 MPa in a water-cooled pressure vessel 0.1–40 MPa at 17°C. Sets of P. profundum SS9 Pasteur pipette cultures from the same batch were grown simultaneously to stationary phase under two different pressure conditions in triplicate: 1) at high pressure (28 MPa) and 2) low pressure (0.1 MPa) for 5 days. The pipette cultures were then removed from their respective conditions and the cultures were harvested by centrifugation at 800 x g for 10 min. Cell pellets were then snap-frozen and stored at –80°C.

Prior to analysis, cell pellets were defrosted on ice and 200 μL of 8 M urea was added to each pellet. Cells were disrupted with 100 mg acid-washed beads (425–600um, Sigma UK) using a TissueLyser (Qiagen, Retsch, Germany) for 3 min at 30 Hz. Insoluble debris was removed by centrifugation (20 k x g 10 min at 4°C) and total protein was assayed using a Bradford kit (BioRad, UK).

3. Protein digestion and clean-up
Protein sample digestion was performed as described previously [24]. Peptide extracts were cleaned using a SupelClean C18 cartridge (Sigma-Aldrich, UK) and dried under low pressure. Peptide samples were stored at –20°C.

4. HPLC-MS analysis
Capillary-HPLC-MS/MS analysis was performed using an online system consisting of a micro-pump (1200 binary HPLC system, Agilent, UK) coupled to a hybrid LTQ-Orbitrap XL instrument (Thermo-Fisher, UK). The LTQ was controlled through Xcalibur 2.0.7. Samples were reconstituted in 10 μL of loading buffer before injection (8μL), and analyzed on a 2 hour gradient for data dependent analysis in a similar way as described previously [22].

5. Data Analysis
MS/MS data was searched using MASCOT Versions 2.4 (Matrix Science Ltd, UK) against the Photobacterium profundum subset of the NCBI protein database (January 2011 for a total of 5489 sequences) using a maximum missed-cut value of 2. Variable methionine oxidation and fixed cysteine carbamidomethylation were used in all searches; precursor mass tolerance was set to 7 ppm and MS/MS tolerance to 0.4 amu. The significance threshold (p) was set below 0.05 (MudPIT scoring). A peptide Mascot score of 20 was used in the final analysis, which corresponds to a global false discovery rate of 1.4% using a decoy database search. LC-MS label-free quantitation was performed using Progenesis (Nonlinear Dynamics, UK) as described elsewhere [25]. Protein conflict (peptides shared between different proteins) was confined as follows: conflict resulting from multiple sequence assignment to the same peak; we only used the sequence having the highest score. Conflict resulting from same peptide sequences assigned to different proteins, the assignment was singly attributed to the protein that had the highest number of peptides. Regarding the label-free quantitation, the total number of Features (i.e. intensity signal at a given retention time and m/z) was reduced to MS/MS peaks with charge of 2, 3, or 4+ and we only kept the five most intense MS/MS spectra per “Feature”. The subset of multi-charged ions (2+, 3+, 4+) was extracted from each LC-MS run and the ion intensities summed for normalization. Protein quantitation was performed as follows; for a specific protein, the associated unique peptide ions were summed to generate an abundance value. The measured protein abundances were transformed using an ArcSinH function (as the method of detection can generate a significant amount of near zero measurements for which a log transform is not ideal). The within group means were calculated to determine the fold change and the transformed data was then used to calculate the p-values using one way ANOVA. ArcSinH transformation was used only for the calculation of the p-value. Differentially expressed proteins were considered meaningful under the following conditions: Only proteins detected by two or more peptides, with an absolute ratio of at least 1.5 (i.e. 1.5 fold up-regulated or 0.667 down-regulated) and p<0.05 associated with the protein change.

Different bioinformatic analyses were performed in this study. Protein subcellular localization was determined using a combination of PSORTb v.3.0.2 [http://www.psort.org/psortb/index.html] [26] and CELLO [http://cello.life.nctu.edu.tw/] [27] in order to predict subcellular localisation of the proteins in a similar manner as presented by [28]. The protein GI number was then searched using NCBI BLAST to identify protein orthologs in better-characterized species, namely Vibrio and E. coli. The Kegg database [http://www.genome.jp/kegg/] was then used to identify pathway information and pathway enrichment was performed using Kobas v2.0 [http://kobas.cbi.pku.edu.cn/home.do] [29]. Data were converted using the latest PRIDE converter available v2.4.2 [30]. Data are available on the public data repository PRIDE [http://www.ebi.ac.uk/pride/]. All data are also available in Supplementary information S1 and proteins identified with a single peptide reported in the text are detailed (MS/MS spectra and assignment) in Supplementary information S2 the Table and Spectra.

Results and Discussion
All experiments were performed in biological triplicates [21–23] at 2 different pressures: 28 MPa and 0.1 MPa. After the cultures had been grown under their respective pressures (28 MPa and 0.1 MPa), they were all found to have a similar O.D. at 600 nm (ca. 1.5), suggesting they were all at the stationary phase of cell growth [22]. A total of 966 proteins (proteins with at least one unique peptide) were identified in this study. Of these proteins,
213 were differentially expressed between 28 MPa and 0.1 MPa, having a protein intensity ratio higher than 1.5, a p-value less than 0.05 and were identified with at least 2 unique peptides. The number of proteins being significantly down-regulated (i.e., ratio 28 MPa/0.1 MPa) with a p-value less than 0.05 and identified with at least 2 unique peptides was 168 proteins. All proteins identified in this study are reported in Supplementary information S1. Approximately 15% of the proteome was identified in this study with a likely bias toward the most highly abundant proteins.

Figure 1 illustrates a volcano plot of all p-values in function of the protein intensity ratio 28 MPa/0.1 M Pa. All values were extracted using Progenesis software. The significant number of changes detected by LC-MS are highlighted and clearly show that a global shotgun proteomic approach without intensive fractionation is sufficient to capture major changes associated with differences in growth at the 2 different pressures. In Figure 2, comparison of each individual protein’s intensities is reported in function of the median intensity for each protein within a group (2A, 2B, 2C for the 0.1 MPa, 2D, 2E, 2F for the 28 MPa). Comparison of the 2 median intensity groups is shown in 2G. Normalisation was performed by Progenesis on the different LC-MS runs and shows little difference between the runs and their corresponding median, with slope varying between 0.874 to 1.086 for each sample against their respective group median.

The sets of differentially expressed proteins were analyzed by pSORT and CELLO in order to establish their putative cellular localization (Figure 3). The dominant fraction of proteins identified was found to be in the cytoplasm, at 80.5% of the total of all differentially expressed proteins identified. Proteins from the cytoplasmic membrane were estimated at 6.7% and a similar observation was made for periplasmic proteins (5.2%). Proteins having an inner-membrane localization were slightly more represented (3.9%) than the those from the outer-membrane (1.9%) and extracellular localization were estimated at 1.3% of the total proteins. A similar pattern of localization was also found for the subgroup of proteins which were reported to be significantly differentially expressed under the different pressure regimes (data not shown).

Not surprisingly, the cellular distribution reveals that the outer-membrane proteins reported to have a crucial role in pressure sensing, are poorly represented in this study. A more focused study on how Photobacterium perceives pressure changes would benefit from a membrane enrichment strategy.

Several proteins identified in this study have been previously reported as being important for piezophilic growth (shown in Table 1). The transmembrane proteins ToxR and ToxS, for example, interact with each other and are thought to be both pressure sensing proteins as well as being involved in regulating the cellular response to pressure [31]. ToxR (PBPR1A022) was found to be down-regulated at 28 MPa, with a measured ratio of 0.25 (28 MPa/0.1 MPa) and having a p-value of 0.009, but was identified with only one unique peptide (see Supplementary information S1). ToxS (PBPR1A021P) was also one of the 966 proteins identified. However, we were unable to significantly evaluate its level of expression in relation to pressure (p-value 0.779, see Supplementary information S1). OmpL (PBPR1A1142), an outer membrane porin protein under the control of the ‘ToxR/S complex was one of the first pressure regulated genes to be found in Photobacterium profundum’ [32]. Our data correlates well with previous studies showing that OmpL is down-regulated at 28 MPa and was identified in this study with a protein intensity ratio of 0.10 (ratio intensity 20 MPa/0.1 MPa) a p-value of 0.006 and was identified with 6 peptides.

Other proteins with a predicted localization at the outer-membrane were also found significantly differentially regulated in function of pressure. AsmA (PBPR1A172), OmpA (PBPR1B0642) as well as a lipoprotein B (PBPR1A2806) were found up-regulated at 28 MPa and an outer membrane channel protein (PBPR1A0450) was found down-regulated at 28 MPa. In this study, however, DnaJ (PBPR1A0695) was found down-regulated at 28 MPa. Other proteins involved in piezo-sensitive mechanisms are also reported in Table 1 and compared with the literature.

Differentially expressed proteins were grouped into their respective pathways using KOBASE 2.0 (KEGG Orthology Based Annotation System). This classification was used to generate Supplementary information S3, where only those pathways showing a significant enrichment compared to Photobacterium profundum global genome (p-value 0.05 and less) were kept for up- and down-regulation.

Surface water and deep-sea water represent completely different physical and biochemical environments, having varying fundamental parameters. Compared to surface water, deep-sea conditions are characterized by higher pressure and the absence of light. Temperature gradients also exist, since deep-sea water is usually colder than surface water, with the exception of proximity to hot vents, where temperatures are much higher than 100°C. Other parameters that play crucial roles in biological processes include differences in oxygen, nitrate and nitrite concentrations, as well as dissolved inorganic phosphate content. Regarding oxygen levels, intermediate water (500 m–2500 m) contains less oxygen than surface and deep water. The compositional difference is partly attributed to the organic debris from the surface being decomposed while passing through this intermediate zone. Deeper sea water receives significantly less of this organic “rain” ([33] data interpreted by Copin-Montegut, 1993); Surface depletion in phosphate is attributed to a pronounced competition for scarce resources compared to the deeper zone [34]. On the other hand, the C:N ratio for particulate organic matter increases with depth and is associated with a preferential re-mineralisation of nitrogen compared to carbon during decomposition [35].

Different respiration modes driven by pressure have been previously suggested [36,37]. In our study, putative trimethyl-amine-N-oxide reductase (PBPR4216) and the anaerobic dimethyl sulfoxide reductase, subunit A (PBPR1B0330) were found to be up-regulated at high pressure, which suggests a form of anaerobic respiration at 28 MPa. One consequence of trimethylamine reduction is an increase in intracellular pH. The Protein tmA, cytochrome (PBPR1A2532) (identified with 1 peptide) is also up-regulated at high pressure, which could suggest a role in counter-balancing the putative alkalisation due to trimethylamine reduction [4]. In further regard to the up-regulation of the anaerobic respiration pathway, Periplasmic nitrate reductase (PBPR1A0854) (identified with one unique peptide) and nrfA, cytochrome c552 (PBPR1A1258) (identified with one unique peptide) were all found to be up-regulated at high pressure [35]. Interestingly, Cytochrome c oxidase, cbb3-type (PBPR1A834), involved in the oxidative phosphorylation pathway, was also up-regulated at high pressure. Cytochrome c oxidase cbb3-type, has a reduced proton pumping ability, but higher catalytic activity at low oxygen concentration which supports an enhanced requirement for this protein in low oxygen environments [38]. Cytochrome c oxidase bb3 type or quinol oxidase has been shown to be up-regulated under high pressure regimes [35]. In contrast, a set of 6 proteins involved in the oxidative phosphorylation pathway which is typical of aerobic respiration were found up-regulated at low pressure (see Supplementary information S3 and refer to NADH dehydrogenase, PBPR1A2956;
cytochrome d ubiquinol oxidase subunit I (PBPR2538), FO1 ATP synthase subunit gamma, delta and 2 subunit alpha (PBPR3605, PBPR3607, PBPR3606 and PBPR0134, respectively). These results suggest that pressure may regulate two different modes of respiration in *Photobacterium profundum* as highlighted in the work of Kato [34].

The transport of small molecules and membrane transporters are affected by changes in hydrostatic pressure [2]. We have identified a number of significantly differentially expressed ABC transporters, which were involved in ion, sugar and amino acid transporters across the cell membrane. Specifically, we have identified different subunits of the phosphate transport ATP-binding cassette-type (ABC-type) system, such as the phosphate ABC transporter ATP-binding protein (PBPR1391); phosphate ABC transporter, periplasmic phosphate-binding protein (PBPR1394) as well as PhoR, phosphate regulon sensor protein (PBPR0722) and the putative DNA-binding response regulator PhoB (PBPR0721), which are part of a two-component system responsible for responding to phosphate limitation [39], which were also down-regulated at 28 MPa (compared to 0.1 MPa). This may both be due to changes in requirements and availability of phosphate at different sea levels and pressures, or to the transport system having evolved to function at high pressure. Phosphate transport functions less effectively at 0.1 MPa and is, therefore, required in a greater abundance by the cells. This is particularly interesting since phosphorus is a key element in marine ecosystems [40]. A similar observation has been made in this study regarding an extracellular tungstate binding protein (PBPR1889), which was found to be up-regulated at 0.1 MPa. While we do not know the exact reason for this up-regulation, tungsten has a crucial role in the function of some oxidoreductases. Tungsten is a rare element in marine ecosystems, with the exception of hydrothermal systems [41].

We also identified a number of regulatory proteins that were significantly differentially expressed between pressure conditions and could, therefore, be new candidates for pressure-regulated gene expression. In *E. coli* the MarR (multiple antibiotic resistance regulator) family of transcriptional regulators are involved in the response to antibiotics and oxidative stresses. A MarR family regulator was also found to be present in our results showing a 7.8 fold increase from 28 MPa to 0.1 MPa (down-regulated at 28 MPa vs. 0.1 MPa) being quantified using 3 unique peptides.

A number of ribosomal proteins were differentially expressed between 28 and 0.1 MPa. Mesophilic ribosomes are one of the most pressure-sensitive structures in bacterial cells due to the particular large volume change associated with the assembly of the ribosome. An increase in pressure results in the dissociation of ribosomal subunits and the inability to form new ones [42–45]. A higher level of ribosomal protein subunits present at 28 MPa could allow for the existence of a constant number of assembled units independently of the pressure if the assembled structure is not favoured by high pressure. Analysis of the *P. profundum* genome identified 15 rRNAs, the largest reported for in any bacterium [6]. This, combined with the high level of variation within these rRNA operons, is thought to reflect *P. profundum* S99’s ability to rapidly respond to changes in pressure and the requirement to alter ribosomal structure in function of atmospheric pressure [46]. There were a total of 25 significantly up-regulated ribosomal proteins present in our data (see Supplementary information S3) and they represent an enrichment having a p-value of $3 \times 10^{-5}$. This is one of the highest enrichment factors obtained for any group of proteins identified in this study.

Transcriptome analysis at 0.1 MPa versus 28 MPa, showed an up-regulation of DnaK, DnaJ and GroEL [6,47]. It has been previously speculated that this could be a piezophilic response to survive shallow-water conditions when *P. profundum* is located far from the deep-sea [47]. In our study, we see that GroEL (PBPR3387) and DnaK (PBPR0697) are instead up-regulated at 28 MPa, while DnaJ is down-regulated. An anti-correlation between the proteomic and transcriptomic data has been previously highlighted with regards to proteins associated with the cellular stress responses in the work of Hack in 2004 [10]. This may well explain the observations made in this study. Our differing results for DnaK and DnaJ may also be due to their involvement in the very early phases of the cellular stress response. While all care was taken to harvest and freeze cells as quickly as possible, it may be that some stress response signals were activated as soon as the cell cultures were de-pressurized. Of course, this problem is intrinsic in all of the studies performed on *P. profundum* to date, and only limited to the most rapid changes in protein expression.

Specific and unique enzymes involved in the glycolysis/glucogenesis were identified as being differentially regulated in both of the pressure conditions being tested (a Kegg pathway diagram is presented in Supplementary information S4). Surprisingly, the enzyme involved in the phosphotransferase (PTS) system, glucose-specific IIBC component (EC:2.7.1.69) has 2 isoforms differentially expressed at each pressure (shown in yellow in Supplementary information S4). The isoform PBPR0861 with 169aa (PTS system glucose-specific transporter subunit) is up-regulated at high pressure while the other isoform (PTS system glucose-specific transporter subunits IIBC) (PBPR1203) with 477 aa is up-regulated at atmospheric pressure. The genes encoding both isoforms of this enzyme (EC:2.7.1.69) are located in different regions of the chromosome and support different putative functions for the isoforms in relation to the effect of pressure. A similar observation has been made with isoforms of glycerol-3-phosphate dehydrogenase (EC:1.2.1.12) where one isoform (PBPR2208) with 339 aa is up-regulated at 28 MPa and another isoform (PBPR2602) with 330 aa is up-regulated at 0.1 MPa.

Under high pressure, the enzyme alcohol dehydrogenase (PBPR2519), which converts acetaldehyde into alcohol, was found to be up-regulated. This suggests that the biochemical pathway responsible for the conversion of pyruvate into 2-Hydroxy-ethyl-ThPP is being activated. Interestingly, this observation implies that *P. profundum* may assume a fermentative metabolic phenotype under high pressure. How this shift in metabolism allows for cell survival under high pressures should be further investigated.

Two comparative transcriptomic studies have been performed on *Photobacterium profundum* under different pressure regimes [4,6]. In Figure 4, we compared the output from our current proteomic data with the published transcriptomic data. The overlap between the studies is only 82 proteins since the method of protein quantitation employed by each study is quite different. Empty circles represent the proteins identified in this study but with a low confidence quantitation level (p-value associated to the quantitation above 0.05). The filled circles are associated to those proteins, which were identified in this study and quantified with a p-value<0.05. In both cases, a trend between the proteomic and the transcriptomic study is observed (quadrant I and III contain 48 proteins). A possible mechanism for explaining anticorrelation between transcriptome (high ratio) and proteome (low ratio) is the presence of anti-sense RNA which could inhibit translation [48].

The presented dataset is too small to highlight meaningful trends in terms of protein function as highlighted by Hack [10]. That explains the observation of stress proteins having reciprocal trends in expression levels of mRNA (transcriptomic studies) versus
protein (proteomic studies). The overall observations made in this study are consistent with the observations reported by other studies of *P. profundum* [10–12].

Conclusions

We have analysed the proteome of *Photobacterium profundum* under different pressure regimes using a label-free quantitative proteomic analysis. An important fraction of this proteome is under tight regulation, with relatively highly abundant proteins being up- or down-regulated in function of the pressure. The data acquired in this study suggests that drastically altered modes of protein function exist under the different pressure regimes. As mentioned in other studies [4,6], the difference in marine environments is not only characterized by a fundamental physical differences (i.e., pressure, light and temperature) which can play an important role in protein assembly and transport, but they represent completely unique ecological niches. By using the same growth medium in both pressure conditions, we highlighted that nutrient intake by *P. profundum* is potentially modulated by pressure.

Several of the differentially expressed proteins have been previously identified as playing important roles in cellular adaptation to altered atmospheric pressure. However, some of the differentially expressed proteins either have not previously been identified in high-pressure adaptation mechanisms or were not regulated as expected.

The increase in the number of new organisms being sequenced provides the opportunity for new proteomics studies to be generated. To our knowledge, we are reporting one of the first proteomic studies on *P. profundum*, a key model organism for understanding pressure adaptation and may have a valuable role in industrial and biotechnology applications.

References

1. El-Hajj ZW, Alcock D, Tryfona T, Lauro FM, Sawyer L, et al. (2010) Insights into proteome dynamics from genomics studies on the deep-sea bacterium, *Photobacterium profundum* SS9. Ann N Y Acad Sci 1189: 143–148.
2. Bartlett DH (2010) Introduction to high-pressure bioscience and biotechnology. Ann N Y Acad Sci 1189: 1–5.
3. Bartlett DH, Kato C, Horikoshi K (1995) High pressure influences on gene and protein expression. Res Microbiol 146: 697–706.
4. Campanaro S, Vezzi A, Vitulo N, Lauro FM, D’Angelo M, et al. (2005) Laterally transferred elements and high pressure adaptation in *Photobacterium profundum* strains. BMC Genomics 6: 122.
5. Valerio E, Chaves S, Tenreiro R (2010) Diversity and impact of prokaryotic toxins on aquatic environments: a review. Toxins (Basel) 2: 2359–2410.
6. Vezzi A, Campanaro S, D’Angelo M, Simonato F, Vitulo N, et al. (2005) Life at depth: *Photobacterium profundum* genome sequence and expression analysis. Science 307: 1459–1461.
7. Bartlett DH, Welch TJ (1995) ompH gene expression is regulated by multiple environmental cues in addition to high pressure in the deep-sea bacterium *Photobacterium profundum* species SS9. J Bacteriol 177: 1008–1016.
8. Bartlett D, Wright M, Yayanos AA, Silverman M (1989) Isolation of a gene regulated by hydrostatic pressure in a deep-sea bacterium. Nature 339: 572–574.
9. Eloe EA, Lauro FM, Vogel RF, Bartlett DH (2008) The deep-sea bacterium *Photobacterium profundum* SS9 utilizes separate flagellar systems for swimming and swarming under high-pressure conditions. Appl Environ Microbiol 74: 6298–6305.
10. Hack CJ (2004) Integrated transcriptome and proteome data: the challenges ahead. Brief Funct Genomic Proteomic 3: 212–219.
11. Gyggy SP, Rochon Y, Franzu BR, Aebersold R (1999) Correlation between protein and mRNA abundance in yeast. Mol Cell 19: 1720–1730.
12. Futterer B, Latter GI, Monardos P, McLaughlin CS, Garrels JI (1999) A sampling of the yeast proteome. Mol Cell 19: 7357–7368.
13. Martin SF, Munagapati VS, Salvo-Chirnside E, Kerr LE, Le Bihan T (2011) Proteome turnover in the green alga *Ostreococcus tauri* by time course 15N metabolic labeling mass spectrometry. J Proteome Res.
14. Karp NA, Huber W, Sadowski PG, Charles PD, Hester SV, et al. (2010) Addressing accuracy and precision issues in iTRAQ quantitation. Mol Cell Proteomics 9: 1085–1097.
15. Stewart, II, Zhao L, Le Bihan T, Larsen B, Scocuzza S, et al. (2004) The reproducible acquisition of comparative liquid chromatography/tandem mass spectrometry data from complex biological samples. Rapid Commun Mass Spectrom 18: 1697–1710.
16. Wang W, Zhou H, Lin H, Roy S, Shafer TA, et al. (2003) Quantification of proteins and metabolites by mass spectrometry without isotopic labeling or spiked standards. Anal Chem 75: 4018–4026.
17. Le Bihan T, Robinson MD, Stewart II, Figuers D (2004) Definition and characterization of a “tryptosome” from specific peptide characteristics by nano-HPLC/MS/MS and in silico analysis of complex protein mixtures. J Proteome Res 3: 1130–1140.
18. Cox J, Mann M (2008) MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein identification. Nat Biotechnol 26: 1367–1372.
19. Mueller LN, Rimner O, Schmidt A, Letarte S, Bodenmüller B, et al. (2007) SuperHirn – a novel tool for high resolution LC-MS-based peptide/protein profiling. Proteomics 7: 3470–3480.
20. Nelson KA, Ali NA, Murdihardjo S, Murai E, Mariani M, et al. (2011) Less label, more free: approaches in label-free quantitative mass spectrometry. Proteomics 11: 535–553.
21. Chi E, Bartlett DH (1993) Use of a reporter gene to follow high-pressure signal transduction in the deep-sea bacterium *Photobacterium profundum* sp. strain SS9. J Bacteriol 175: 7533–7540.
22. Allen EE, Facciotti D, Bartlett DH (1999) Monounsaturated but not polyunsaturated fatty acids are required for growth of the deep-sea bacterium *Photobacterium profundum* SS9 at high pressure and low temperature. Appl Environ Microbiol 65: 1710–1720.
23. El-Hajj ZW, Tryfona T, Alcock DJ, Hasan F, Lauro FM, et al. (2009) Importance of proteins controlling initiation of DNA replication in the growth of the high-pressure-loving bacterium *Photobacterium profundum* strain SS9. J Bacteriol 191: 6303–6305.
24. Le Bihan T, Grima R, Martin S, Forster T, Le Bihan Y (2010) Quantitative analysis of low-abundance peptides in HeLa cell cytoplasm by targeted liquid chromatography/mass spectrometry and stable isotope dilution: emphasizing the distinction between peptide detection and peptide identification. Rapid Commun Mass Spectrom 24: 1093–1104.
25. Le Bihan T, Martin SF, Chirnside ES, van Ooijen G, Barrios-Llereña ME, et al. (2011) Shotgun proteomic analysis of the unicellular alga *Ostreococcus tauri*. J Proteomics 74: 2060–2070.
26. Yu NY, Wagner JR, Laird MR, Melli G, Rey S, et al. (2010) PSORTb 3.0: improved protein subcellular localization prediction with refined localization subcategories and predictive capabilities for all prokaryotes. Bioinformatics 26: 1608–1615.

27. Yu CS, Chen YC, Lu CH, Hwang JK (2006) Prediction of protein subcellular localization. Proteins 64: 643–651.

28. Lin X, Zhang H, Cui Y, Lin S (2012) High sequence variability, diverse subcellular localizations, and ecological implications of alkaline phosphatase in dinoflagellates and other eukaryotic phytoplankton. Front Microbiol 3: 233.

29. Xie C, Mao X, Huang J, Ding Y, Wu J, et al. (2011) KOBAS 2.0: a web server for annotation and identification of enriched pathways and diseases. Nucleic Acids Res 39: W316–322.

30. Barnes H, Vizcaino JA, Eidhammer I, Martens L (2009) PRIDE Converter: making proteomics data-sharing easy. Nat Biotechnol 27: 598–599.

31. Bartlett DH (2002) Pressure effects on in vivo microbial processes. Biochim Biophys Acta 1595: 367–381.

32. Welch TJ, Bartlett DH (1998) Identification of a regulatory protein required for pressure-responsive gene expression in the deep-sea bacterium Photobacterium species strain SS9. Mol Microbiol 27: 977–985.

33. Takahashi T, Broecker W, AE B (1981) Carbon cycle modelling, Bolin B, editor. New York: Wiley.

34. Worden AZ, Cuvelier ML, Bartlett DH (2006) In-depth analyses of marine microbial community genomics. Trends Microbiol 14: 331–336.

35. Aono E, Baba T, Ara T, Nishida T, Nakamichi T, et al. (2010) Complete genome sequence and comparative analysis of Shewanella violacea, a psychrophilic and piezophilic bacterium from deep sea floor sediments. Mol Syst Biol 6: 1216–1226.

36. Kato C, Qureshi MH (1999) Pressure response in deep-sea piezophilic bacteria. J Mol Microbiol Biotechnol 1: 87–92.

37. Takegai H, Ota Y, Haga M, Fujimoto H, Kato C, et al. (2011) Piezotolerance of the respiratory terminal oxidase activity of the piezophilic Shewanella violacea DS812 as compared with non-piezophilic Shewanella species. Biosci Biotechnol Biochem 75: 919–924.

38. Buchmann S, Warkentin E, Xie H, Langer JD, Ermler U, et al. (2010) The structure of cbh3 cytochrome oxidase provides insights into proton pumping. Science 329: 327–330.

39. Lamarche MG, Wanner BL, Crepin S, Harel J (2008) The phosphate regulon and bacterial virulence: a regulatory network connecting phosphate homeostasis and pathogenesis. FEMS Microbiol Rev 32: 461–473.

40. Paytan A, McLaughlin K (2007) The oceanic phosphorus cycle. Chem Rev 107: 563–576.

41. Adams MW (1998) The biochemical diversity of life near and above 100 degrees C in marine environments. J Appl Microbiol 85 Suppl 1: 108S–117S.

42. Gross M, Jannick K (1990) Pressure-induced dissociation of tight couple ribosomes. FEBS Lett 267: 239–241.

43. Landau JV (1967) Induction, transcription and translation in Escherichia coli: a hydrostatic pressure study. Biochim Biophys Acta 149: 506–512.

44. Alpas H, Lee J, Bezugli F, Kaledine G (2003) Evaluation of high hydrostatic pressure sensitivity of Staphylococcus aureus and Escherichia coli O157:H7 by differential scanning calorimetry. Int J Food Microbiol 87: 229–237.

45. Pavlovic M, Hornemann S, Vogel RF, Ehrmann MA (2005) Transcriptional response reveals translation machinery as target for high pressure in Lactobacillus sanfranciscensis. Arch Microbiol 184: 11–17.

46. Lauro FM, Tran K, Vezzi A, Vinulo N, Valde G, et al. (2008) Large-scale transposon mutagenesis of Photobacterium profundum S99 reveals new genetic loci important for growth at low temperature and high pressure. J Bacteriol 190: 1699–1709.

47. Simonato F, Campanaro S, Lauro FM, Vezzi A, D’Angelo M, et al. (2006) Piezophilic adaptation: a genomic point of view. J Biotechnol 126: 11–25.

48. Waldhauser JR, Rodriguez S, Coleman ML, Chisholm SW (2012) Transcriptome and proteome dynamics of a light-dark synchronized bacterial cell cycle. PLoS One 7: e43432.