Elsevier Editorial System(tm) for Journal of Proteomics
Manuscript Draft

Manuscript Number: JPROT-D-09-00257R1

Title: Response of Pseudomonas putida KT2440 to phenol at the level of membrane proteome

Article Type: SI: Analytical Proteomics

Section/Category: Original Article

Keywords: Membrane proteome; Phenol; Pseudomonas; Stress response; quantitative proteomics

Corresponding Author: Prof Isabel Sá-Correia,

Corresponding Author's Institution: Instituto Superior Técnico

First Author: Catarina Roma-Rodrigues

Order of Authors: Catarina Roma-Rodrigues; Pedro M Santos; Dirk Benndorf; Erdmann Rapp; Isabel Sá-Correia

Abstract: This study led to the extension and refinement of our current model for the global response of Pseudomonas putida KT2440 to phenol by getting insights into the adaptive response mechanisms involving the membrane proteome. A two-dimensional gel electrophoresis based protocol was optimized to allow the quantitative comparison of membrane proteins, by combining inner and outer membrane fractionation with membrane protein solubilization using the detergent dodecylmaltoside. Following phenol exposure, a coordinate increased content of protein subunits of known or putative solvent efflux pump systems (e.g. TtgA, TtgC, Ttg2A, Ttg2C, PP_1516-7) and a decreased content of porins OprB, OprF, OprG and OprQ was registered, consistent with an adaptive response to reduce phenol intracellular concentration. This adaptive response may in part be mediated by post-translational modifications, as suggested by the relative content of the multiple forms identified for a few porins and efflux pump subunits. Results also suggest the important role of protein chaperones, of cell envelope and cell surface and of a more active respiratory chain in the response to phenol. All these mechanistic insights may be extended to Pseudomonas adaptation to solvents, of possible impact in biodegradation, bioremediation and biocatalysis.

Response of *Pseudomonas putida* KT2440 to phenol at the level of membrane proteome

Catarina Roma-Rodrigues[a)], Pedro M. Santos[a) §], Dirk Benndorf[b)], Erdmann Rapp[c)], Isabel Sá-Correia[a)*]

a) IBB – Institute for Biotechnology and Bioengineering/Centre for Biological and Chemical Engineering, Instituto Superior Técnico, Av. Rovisco Pais 1049-001 Lisboa, Portugal.

b) Bioprocess Engineering, Otto von Guericke University, Universitätsplatz 2, 39106 Magdeburg, Germany

c) Bioprocess Engineering, Max Planck Institute for Dynamics of Complex Technical Systems, Sandtor Straße 1, 39106 Magdeburg, Germany

[*]Corresponding author

Professor Isabel Sá-Correia

IBB, Institute for Biotechnology and Bioengineering, Centre for Biological and Chemical Engineering, Instituto Superior Técnico, Av. Rovisco Pais, 1049-001 Lisboa, Portugal

Email: isacorreia@ist.utl.pt

Fax: +351 218 419 199

Phone: +351 218 417 682

[§] Present address: CBMA-Centre of Molecular and Environmental Biology, Department of Biology, University of Minho, Campus de Gualtar, 4715-057 Braga, Portugal

Running title: Phenol-dependent membrane proteome alterations

Keywords: Membrane proteome; Phenol; *Pseudomonas*; Stress response; quantitative proteomics

Abbreviations: TMF – Total Membrane Fraction; OMF – Outer Membrane Fraction; IMF – Inner Membrane Fraction; RND – Resistance-Nodulation-Division; LPS – Lipopolysaccharides; ABC – ATP-binding cassette

Abstract

This study led to the extension and refinement of our current model for the global response of *Pseudomonas putida* KT2440 to phenol by getting insights into the adaptive response mechanisms involving the membrane proteome. A two-dimensional gel electrophoresis based protocol was optimized to allow the quantitative comparison of membrane proteins, by combining inner and outer membrane fractionation with membrane protein solubilization using the detergent dodecylmaltoside. Following phenol exposure, a coordinate increased content of protein subunits of known or putative solvent efflux pump systems (e.g. TtgA, TtgC, Ttg2A, Ttg2C, PP_1516-7) and a decreased content of porins OprB, OprF, OprG and OprQ was registered, consistent with an adaptive response to reduce phenol intracellular concentration. This adaptive response may in part be mediated by post-translational modifications, as suggested by the relative content of the multiple forms identified for a few porins and efflux pump subunits. Results also suggest the important role of protein chaperones, of cell envelope and cell surface and of a more active respiratory chain in the response to phenol. All these mechanistic insights may be extended to *Pseudomonas* adaptation to solvents, of possible impact in biodegradation, bioremediation and biocatalysis.

1. Introduction

Strains belonging to the *Pseudomonas* genus have been widely used to elucidate the adaptive mechanisms underlying increased tolerance against toxic concentrations of organic solvents in Gram-negative bacteria [1-2] and, whether genetically prepared, to use them as carbon source [3-5]. Genome-wide expression analyses are being applied to get an integrative perspective of the physiology of microorganisms challenged with toxic compounds, including toxins, drugs and environmental pollutants. For instance, the alterations occurring in the proteome of *Pseudomonas* strains following sudden exposure to phenol were examined by quantitative proteomics based on two-dimensional gel electrophoresis (2-DE) and on the identification of proteins whose content was altered in response to this solvent by mass spectrometry (MS) [5-6]. However, these studies were focused on the soluble component of the proteome, including cytoplasmic and a few peripheral and outer membrane proteins. Results revealed that *Pseudomonas* exposure to toxic concentrations of phenol may lead to: i) the induction of the general and oxidative stress response; ii) the induction of proteins involved in energetic metabolism, suggesting a higher energy demand in phenol stressed cells; iii) the induction of fatty acid biosynthesis, as a recovery mechanism for damaged membrane phospholipids resulting from phenol-induced oxidative stress, and iv) the inhibition of cell division and motility [5-6]. Moreover, the increased content of homologous proteins with the same predicted or demonstrated biological function in both the experimental model strain *P. putida* KT2440 and *Pseudomonas* sp. M1 following their sudden exposure to phenol, indicated that both strains share adaptive mechanisms. *Pseudomonas* sp. M1 exhibits exceptional biodegrading ability and was isolated from an enriched culture of Rhine river sediments [7]. Remarkably, even though the genome sequence of strain M1 is not available, 60% of the total number of protein spots excised from the gels were positively identified by mass spectrometry, 70% of them matching *P. aeruginosa* proteins present in databases [5]. Based on the comparison of 16S rRNA gene sequences [5], the closest species to M1 strain is *P. citronellolis* but the

closest species with the genome sequenced is, indeed, *P. aeruginosa*. It is therefore likely that results emerging from studies on the solvent adaptive mechanisms using the model strain KT2440 may contribute to predict cellular responses towards toxic solvent exposure in environmental *Pseudomonas* strains, more suited to be explored in biodegradation and bioremediation.

Currently, the best studied mechanisms of adaptation to toxic solvents involve the modifications occurring at the level of cell membrane and cell wall, to counteract the effects of solvents on membrane stability and cell permeability and the reduction of the toxic compound concentration inside the cells through the activity of efflux pumps [1-2]. However, little is known about the global effects of solvent stress on the composition and eventual modification of the membrane proteome. This is mainly due to experimental limitations of the most commonly used proteomic analysis technique – 2-DE coupled to MS. Indeed, it is very challenging to establish a suitable protocol to keep hydrophobic integral membrane proteins in solution during the isoelectric focusing (IEF) procedure. Furthermore, many membrane proteins are in low abundance in the proteome and the use of proteases such as trypsin, for protein digestion, is often not suitable for protein identification by peptide mass fingerprinting due to a lack of cleavage sites in the membrane spanning domains [8]. Comprehensively, there are very few reports on studies carried out using 2-DE for the analysis of membrane proteins of Gram-negative bacteria and most of these studies are mainly focused on the analysis of outer membrane proteins [9-13]. However, once the drawbacks are circumvented or limited, 2-DE prevails as a very attractive high throughput methodology to study global protein expression. Indeed, it has the capacity to separate complex mixtures and to reveal the occurrence of post-translational modifications. Moreover, once a proteome reference map is produced, this can be used to readily identify protein spots in every 2-DE gel produced using the same protocol and organism, thus facilitating downstream data analysis.

The main objective of the present study was to elucidate, using 2-DE-based expression proteomics, the response of *P. putida* KT2440 membrane proteome to sudden exposure to a toxic concentration of phenol, leading to cell growth arrest before adapted growth resumption [6]. To

accomplish this goal, a 2-DE protocol was optimized to allow the separation and quantification of membrane proteins. The anticipated difficulties in handling hydrophobic proteins were partially surpassed by combining proteome fractionation and protein solubilization with the detergent dodecylmaltoside in the isoelectric focusing buffer for 2-DE. The successful application of this protocol led to the extension and refinement of our current model for the global response of *P. putida* KT2440 to phenol by getting insights into the adaptive mechanisms at the membrane proteome level.

2. Materials and Methods

2.1. Bacterial strain and cultivation conditions in presence or absence of phenol

The growth conditions used in this work reproduced those used in a previous study [6]. Briefly, *Pseudomonas putida* KT2440 cells were grown in basal mineral medium described by Hartmans et al. [14] supplemented with succinate 20 mM as carbon source. Cultures were grown in 250 mL Erlenmeyer flasks with 100 mL medium with orbital shaking (250 rpm), at 30 ºC, until the cultures reached a standardized optical density at 600 nm (OD_{600}) in the range of 0.7 - 0.8. These cultures were used to inoculate three identical 1000 mL Erlenmeyer flasks with 400 mL of fresh medium grown under the same conditions. When the cultures reached an OD_{600} of 0.2, phenol was added to two of the triplicate cultures in order to obtain a final concentration of 800 mg/L (phenol cultures). The third culture was left unsupplemented (control). Cells from the control culture and from one of the phenol cultures were harvested 1h after phenol addition: Ctrl and P1 samples, respectively. Cells from the other phenol supplemented culture were harvested after 5h of incubation following phenol supplementation: P5 sample. The three cultures (400 mL) were centrifuged for 5 minutes at 11 300 *g*, at 4 ºC, cells were washed with ice cold Tris-HCl buffer (10 mM Tris-HCl pH 8.5) and the pellet was stored at – 80 ºC until use.

2.2. Membrane proteome fractionation

Preparation of total membrane fraction proteins

To prepare the total membrane fraction, three independent cell samples were obtained for Control, Phenol 1h, and Phenol 5h conditions. For each cell sample, 5 stored cell pellets were resuspended in ice cold working buffer (10 mM Tris-HCl pH 8.5, 1x proteases inhibitors from Halt™ protease inhibitors cocktail kit (*Pierce*, Rockford, USA)) to a final volume of 12 mL. Cells were lysed by sonication on ice using 30 treatments of 20 s with 20 s intervals between treatments. Cell debris was removed with two centrifugations (3000 *g* for 30 min at 4 ºC) and the supernatant was transferred to an ultracentrifuge tube. The crude extract was fractionated by ultracentrifugation (99 000 *g* for 90 minutes at 4 ºC) giving rise to soluble and membrane protein fractions. The supernatant, corresponding to the soluble protein fraction, was discarded and the pellet, corresponding to the total membrane fraction, was washed three times with ice cold working buffer followed by ultracentrifugation (99 000 *g* for 60 min at 4 ºC) to reduce soluble protein contamination. For solubilization of total membrane fraction proteins, pelleted membranes were resuspended in 500 µL of SDS buffer (125 mM Tris-HCl pH 6.8, 22% (v/v) Glycerol, 4% (w/v) SDS, 0.05% (w/v) bromophenol blue and 1% (w/v) DTT), followed by sonication (5 treatments of 5 s with 20 s intervals between treatments), incubation for 2 h at room temperature and centrifugation (12 000 *g* for15 min at room temperature). The resulting supernatant, containing solubilized total membrane fraction proteins, was transferred to a fresh tube.

Preparation of inner and outer membrane fractions proteins

The procedure used to prepare the inner and outer membrane fractions was adapted from [15-16], using two independent cell samples obtained for Control, Phenol 1h and Phenol 5h conditions. For each cell sample, 12 stored cell pellets were resuspended in ice cold working buffer and cells were lysed by sonication on ice as described for total membrane fraction preparation. Cell debris was removed with two centrifugations (3000 *g* for 30 min at 4 ºC) and the supernatant was transferred to an ultracentrifuge tube. The crude extract was ultracentrifuged (99 000 *g* for 90 minutes at 4 ºC) and total membrane fraction was washed two times with ice cold working buffer followed by ultracentrifugation (99 000 *g* during 60 min at 4 ºC). The pelleted membranes were resuspended in 2 mL of ice cold

working buffer and placed on top of a isopycnic sucrose gradient (2.5 mL sucrose 80% (w/v), 3.5 mL sucrose 50% (w/v) and 3.0 mL sucrose 20% (w/v)) and ultracentrifuged (116 000 *g* for 20 h at 4 ºC). The differences in the density of the inner and outer membranes allowed their separation in the two interfaces formed between solutions with the three sucrose concentrations used. The band found between the 20% and 50% sucrose solutions was the fraction enriched with inner membrane proteins (Inner Membrane Fraction –IMF), while the band found between the 50% and 80% sucrose solutions corresponded to the fraction enriched with outer membrane proteins (Outer Membrane Fraction – OMF) [15]. The inner and outer membrane fractions were removed with a micropipette, and a final volume of 200 µL for each membrane fraction was obtained. For solubilization of proteins present in these membrane fractions, 50 µL of SDS buffer 4x (500 mM Tris-HCl pH 6.8, 88% (v/v) Glycerol, 16% (w/v) SDS, 0.2% (w/v) bromophenol blue and 4% (w/v) DTT) were added to the outer and inner membrane fractions. After 2h incubation at room temperature, the inner and outer membrane protein extracts were centrifuged (12 000 *g* for 15 min at room temperature) and the corresponding supernatants were transferred to a clean tube.

2.4. Two-dimensional gel electrophoresis

Protein concentrations in the total membrane fraction, outer membrane fraction and inner membrane fraction obtained from cells either or not cultivated with phenol were quantified using Plus One 2D-Quant kit (GE Healthcare). To eliminate contaminants, such as lipids, nucleic acids, SDS, and salts that may interfere with isoelectrical focusing, 100 µg of total membrane fraction proteins, 100 µg of outer membrane fraction proteins and 150 µg of inner membrane fraction proteins were precipitated using 2D-CleanUp kit (GE Healthcare), according to the manufacturer's instructions. Proteins from the three membrane fractions were gently resuspended in 100 µL of IEF buffer (7 M urea, 2 M thiourea, 1% (w/v) dodecylmaltoside, 2% (v/v) Pharmalytes 3-10 (GE Healhtcare), 10 mM DTT and traces of bromophenol blue), incubated at room temperature for 1 h followed by centrifugation (12 000 *g* during 15 min at room temperature) and the supernatant was transferred to a clean microtube. Immobiline

DryStrip (IPG strips, GE Healthcare) with 24 cm and a 3-10 non-linear pH range were rehydrated overnight with 450 µL of rehydration buffer (7 M urea, 2 M thiourea, 1% (w/v) dodecylmaltoside, 0.5% (v/v) Pharmalytes 3-10, 1.2% (v/v) Destreak reagent (GE Healthcare) and traces of bromophenol blue). Proteins were loaded by anodic cup-loading on IPG strips. A total of 21 gels were run, corresponding to 3 gels for total membrane fraction and 2 gels for inner and outer membrane fractions prepared for each one of the three different experimental conditions (Control, Phenol 1h and Phenol 5h). Isoelectric focusing was carried out on an Ettan IPGphor instrument (GE Healthcare) at a maximum voltage of 8 000 V. After IEF, strips were incubated for 15 min in equilibration buffer (2% SDS, 50 mM Tris-HCl pH 8.8, 6 M urea, 30% (v/v) Glycerol and traces of bromophenol blue) containing 1% (w/v) DTT, followed by 15 min incubation in the same buffer with 3% (w/v) of iodoacetamide. For the second dimension separation, strips were applied on top of 1.5 mm 12 % SDS-polyacrylamide gels. Electrophoresis was carried out in Ettan DALTsix instrument in two steps: the first step at 5 W / gel for 1 h and the second step at 17 W / gel for 3 h. After SDS-PAGE of membrane proteins, gels were carefully removed from the cassettes and incubated in a fixation solution (30% (v/v) ethanol and 10% (v/v) acetic acid) for at least 2 h. The fixation solution was gently poured off and gels were immersed in 340 mL of Flamingo staining solution (BioRad). Gel images were obtained by scanning in a Thyphoon Trio scanner (GE Healthcare) using a green laser (532 nm) with 555 nm emission filter.

2.5. Analysis of protein expression levels

Gel images were analyzed using Progenesis SameSpots (Nonlinear dynamics, NewCastle, UK). Protein spots were identified by using the automatic spot detection algorithm. Individual spot volumes were normalized against total spot volumes for a given gel. Statistical analysis was also performed by Progenesis SameSpots software package. Averages of protein abundance for each membrane fraction of each growth condition were also compared by their normalized volume using one-way ANOVA between test groups. This test returned a *p*-value that takes into account the mean

difference and the variance of a matched spot between sample conditions and also the sample size. Only statistically significant spots ($p < 0.05$) were selected for analysis. In a few cases (indicated in Table 2) spots with p between 0.05 and 0.1 were also analyzed. Differential expression between applied experimental conditions was quantified and a threshold of at least 2-fold increase or 0.5-fold decrease between averaged gels was considered. Spots that showed evidence of saturation were not included for further analysis.

Progenesis Samespots software was also used in Principal Component Analysis of 2-DE gel protein patterns and in Correlation analysis of abundance variation of proteins among control and phenol conditions.

2.6. Identification of proteins by peptide mass fingerprinting

After the analysis of protein expression levels, one gel of each prepared membrane fraction obtained from cells suddenly exposed to 800 mg/L phenol for 1h, was stained with silver nitrate. Spots of interest were excised manually from silver stained polyacrylamide gels, digested overnight with trypsin and the resulting peptides were eluted as described elsewhere [17] and concentrated by vacuum centrifugation. Fully automated online pre-concentration and separation of the tryptic-digested samples was performed using a set of capillary- and nanoHPLC instruments of the 1100 Series (Agilent, Waldbronn, Germany) operated in series. Mass spectrometric detection was carried out by online coupling nanoHPLC with a QSTAR XLTM (QqTOF) mass spectrometer (Applied Biosystems/MDS/Sciex, Darmstadt, Germany) operated in MS and MS/MS mode, as described before [18]. The instrument was equipped with an online nano-electrospray ion source (NanoSpray® II Source) and upgraded with a heated interface. A database search was conducted using the MS/MS ion search (MASCOT, http:www.matrixscience.com; [19]) using a MASCOT in house server. Search parameters were set as follows: all species; tryptic digest with a maximum of one missed cleavage; no fixed modification; variable modifications, oxidation of methionine (M) and carbamidomethylation (C) of cysteine; peptide masses were assumed to be monoisotopic; mass tolerance of 0.1 Da for the

precursor ion and 0.07 Da for product ions. For final confirmation, at least two product-ion spectra of different peptides of each identified protein were verified.

The proteins identified are shown in Tables A, B and C of Supplementary material. Protein names were attributed according to *P. putida* KT2440 genome annotation (http://cmr.jcvi.org/cgi-bin/CMR/GenomePage.cgi?org=gpp) or based on the highest homologous protein found in public databases. Whenever there was no protein symbol attributed, the protein is referred in the text by the respective locus tag number (e.g. protein PP_0001). Identified proteins were grouped by their functional category as described in *P. putida* KT2440 genome annotation. Protein localization was attributed according to the information provided in *Pseudomonas* Genome database (http://www.pseudomonas.com) or from known localizations reported in scientific literature. The number of transmembrane helices of the identified proteins was inferred using the TMHMM server (http://www.cbs.dtu.dk/services/TMHMM-2.0/) (Table C of Supplementary material).

3. Results and Discussion

3.1. Effects of phenol in *P. putida* KT2440 membrane proteome expression

In order to get clues into phenol-induced alterations occurring in *P. putida* KT2440 membrane proteome, cell samples for proteomic analysis were collected after 1h (P1) and 5h (P5) of cultivation following phenol addition to an exponentially growing bacterial culture (Figure 1). These conditions were selected aiming to obtain relevant information on the global response to phenol during the period of growth arrest (early response) and when adapted cells resumed growth, respectively, compared to the control sample (Ctrl) which was collected from an identical unsupplemented culture (Figure 1). Cell samples from each experimental condition were lysed and crude extracts were fractionated in order to obtain the total membrane fraction (TMF) and this fraction was fractionated in the outer membrane fraction (OMF) and the inner membrane fraction (IMF), as described in Materials and Methods section. Figure 2 shows representative Flamingo stained gels of TMF, OMF and IMF obtained from *P. putida* KT2440 cells harvested after 1 hour of cultivation following sudden exposure

to phenol (P1). Around 1400, 1000 and 850 protein spots with a pI range from pH 3 to pH 10 were separated and detected in TMF, OMF and IMF gels, respectively. In total, 247 protein spots were positively identified by mass spectrometry, being 64 collected from TMF, 85 from OMF and 98 from IMF (Tables A, B and C of Supplementary material). The selection of the collected spots was carried out based on a preliminary expression analysis which was indicative of the protein spots whose abundance was altered in response to phenol. A limited number of spots were also collected, not based on their differential content but to verify predicted identities, using previously reported reference maps [10-11, 20-21]. Interestingly, among the identified protein spots, a small group of spots were detected in the three proteome fractions (Table D of Supplementary material). All the identified protein spots were categorized according to their predicted localization (Tables A, B and C of Supplementary material). In Table 1, the overall distribution of the identified spots according to their subcellular localization is shown, highlighting that TM, OM and IM fractions were not solely composed of proteins with the predicted subcellular localization based on the fraction designation. This apparent discrepancy is not surprising since, in previous reports dealing with subproteome fractions, proteins not matching the anticipated subcellular localization were also detected [9, 11-13, 22]. The percentage of proteins with unexpected locations in the different fractions was found to be identical in the various independent experiments carried out and cannot be considered a fortuitous artifact of sample preparation. Proteomics has revealed that many proteins are present in unexpected cellular locations and the interpretation of proteomics data with an open mind is recommended [23]. Several factors may contribute to the apparent discrepancies registered, i) protein aggregation events occurring before or during sample processing, and ii) protein-protein interactions that were not fully disrupted before sample fractionation. For instance, it is shown in Table 1 that a very significant percentage of cytosolic proteins was detected in the IMF. It is known that a number of cytosolic proteins interact with inner membrane proteins, being therefore plausible the presence of cytosolic proteins in the membrane proteome, mainly in the IMF. In this context, proteins involved in membrane protein synthesis,

translocation and targeting are also expected to be present in the membrane proteome fractions, where they were actually detected (Tables A, B and C of Supplementary material).

A Principal Component Analysis (PCA) of the 2-DE gels obtained, clearly demonstrates that TMF and OMF gels are clustered in the same vicinity (Figure 3), suggesting that TMF 2-DE gel protein pattern is more related to OMF 2-DE gel protein pattern than to IMF 2-DE gel protein pattern. This observation is consistent with the considerable number of proteins that were identified in both TMF and OMF gels. From the analysis of Table 1, it is also possible to conclude that an effective enrichment in outer membrane or inner membrane proteins in the corresponding fraction was registered. From the 98 proteins identified in the IMF only thirteen proteins have predicted transmembrane helices (ten with 1 transmembrane helix, one with 2 transmembrane helices, and two with 3 transmembrane helices). Even if it is known that membrane proteins with multiple helices are poorly resolved in 2-DE, with our experimental approach there was a large number of protein spots corresponding to proteins that were separated and detected in the inner membrane fraction gels that were not identified since their abundance was not altered in response to phenol. In addition, some of the collected protein spots whose content changed following cell exposure to phenol could not be positively identified by mass spectrometry, probably due to their low abundance or due to their poor digestion. Therefore, at this phase, we cannot rank the experimental protocol used in this work with respect to its effectiveness for the separation of integral membrane proteins with several transmembrane helices.

Despite all the referred limitations, the current protocol used allowed a relatively high coverage of membrane proteins and consequently an increased understanding of the adaptive mechanisms to phenol occurring at the level of membrane proteome. The relative abundance of proteins in the different membrane fractions prepared from cells challenged with phenol (P1 and P5) was compared with control samples. The more pronounced phenol-induced alterations in the protein expression patterns are listed in Table 2. Several proteins were identified in multiple spots (Table E from Supplementary material) that in general, exhibited a similar content variation among phenol and

control conditions. Only the protein forms that exhibited a significant variation in their contents in phenol stressed and unstressed cells are listed in Table 2 (e.g. proteins OprF, OprH, TtgA and TtgC).

A correlation analysis concerning the variation in response to phenol of the whole set of proteins detected in each fraction stress (Figure 4), indicates that the abundance of the majority of the membrane proteins was affected 1h after phenol exposure. For example, in the case of inner membrane fraction proteins, the abundance of 70% of phenol-responsive proteins varied after 1h of cells exposure to phenol and maintained this modified abundance after 5h of phenol-induced stress. This observation suggests that major modifications occurring in phenol challenged cells are already registered during the early response. However, the modification of the abundance of some proteins was essentially registered in phenol adapted cells (Figure 4, 1st and 2nd patterns), suggesting that these proteins may play an important role in phenol-adapted growth (e.g. LolD, GacA or MltA/B).

3.2. Phenol-induced alterations in the content of solvent efflux systems

Different protein spots corresponding to the outer membrane subunit TtgC and to the membrane fusion protein subunit TtgA of the efflux pump TtgABC were identified in the three membrane fractions examined (Tables A, B and C from Supplementary material). The TtgABC is a Resistance-Nodulation-Division (RND) efflux transporter, associated to *P. putida* tolerance to toluene, styrene, xylene, ethlylbenzene and propylbenzene [1]. The content of both the periplasmic and outer membrane subunits was increased (up to 35-fold) after phenol exposure (Table 2), indicating that this efflux pump is required for KT2440 adaptation to phenol stress, presumably by decreasing the intracellular concentration of the toxicant. Although the alteration of the relative abundance of each TtgA and TtgC form was, in general, more pronounced after 5h of phenol exposure, some of the detected forms did not follow this pattern, suggesting that the biological activity of this transport system may be modulated via post-translational modifications.

Similarly, the abundance in the inner membrane proteome of the ATP binding protein Ttg2A and of the membrane fusion protein Ttg2C of an ABC-type transport system known to be involved in

resistance to organic solvents in *P. putida* [24], also increased after 1h and 5h of phenol addition. The increased abundance of a putative membrane fusion protein subunit of a RND efflux system encoded by PP_1516 was also registered (Table 2). The gene PP_1516 is clustered with PP_1517 gene, encoding an RND efflux pump inner membrane protein, forming a putative operon PP_1516-PP_1517 (Figure 5). Genes encoding this uncharacterized transport system exhibit homology with the AcrAB components of the AcrAB-TolC RND efflux pump of *E. coli*, suggesting that the *P. putida* proteins encoded by PP_1516 and PP_1517 genes may function in association with an outer membrane channel like the TolC protein, for phenol extrusion. In agreement with this hypothesis, the abundance of the two protein forms of TolC increased in response to phenol in *P. putida* KT2440 (Table 2). These results are consistent with the importance and functioning complexity of the efflux pumps in the adaptive response to organic solvent stress in *Pseudomonas* [1]. Both the ATP binding protein Ttg2A and the genes PP_1516 and PP_1517 were already described as being involved in toluene response of *P. putida* KT2440 [25], being strong candidates as determinants of phenol resistance through the active efflux of this and other aromatic solvents.

3.3. Phenol-induced alterations in the content of cell envelope associated proteins

The exposure of *P. putida* KT2440 cells to 800 mg/L phenol resulted in an altered content of several outer membrane proteins, in particular in the decreased abundance of porins OprB, OprG and OprQ (Table 2). Remarkably, the glucose porin OprB was previously related to phenol influx in *P. putida* KT2440 cells [26]. Therefore, these results suggest that cell exposure to phenol may trigger an adaptive decrease of outer membrane permeability by decreasing the abundance of these channel proteins with porin activity. This cellular response was previously hypothesized [27] and may act in coordination with the activation of efflux pump systems to reduce the intracellular concentration of phenol. Three protein spots (numbered 51, 52, 53, respectively) whose abundance varied in response to phenol were identified as the highly conserved porin OprF (Table 2), which is the major porin in several *Pseudomonas* species [28]. The abundance of spot 51, corresponding to OprF porin form with

a higher molecular weight, was found to increase following phenol exposure, whereas the abundance of the lower molecular weights forms corresponding to spots 52 and 53 was drastically reduced (Figure 3a and Table 2). In *Pseudomonas aeruginosa*, OprF adopts two different conformations, the closed channel conformer, constituted a N-terminal β-barrel domain and a C-terminal periplasmic α-helix domain; and 2) the open channel conformer, constituted by a single β-barrel domain that possess a larger number of β-strands with respect to the close channel conformer [29]. This closed channel conformer (95% of the OprF population), plays an important role in stabilization of cell envelope structure [29-30] while the open channel conformer (approximately 5 % of OprF population), that are responsible for the protein activity [29]. It is known that the β-barrel structures of OprF are recalcitrant to denaturation, leading to multiple forms with different motility in SDS-PAGE [31-32]. Therefore, it is likely that the spots 52 and 53 may correspond to partially denatured forms, whereas spot 51 may correspond to the fully denaturated OprF form. If the registered alterations of the relative abundance of the spots 51, 52 and 53 are the result of cell adaptation to phenol mediated by changes in the folding ratio between OprF closed and open channels is a question that certainly deserves further studies.

The abundance of several other proteins present in the external surface of the outer membrane was also modified in response to phenol. This is the case of protein OprH, which was found in nine forms (the content of eight forms increased more than 2-fold in response phenol, while the abundance of one form decreased 0.4-fold). As hypothesized for TtgABC efflux pump, this result suggests a possible post-translational modification of the protein following phenol exposure, presumably with impact on its biological function. Interestingly, the overproduction of OprH in *P. aeruginosa* was related with resistance to polycationic antibiotics and EDTA [33]. Our data also suggest an alteration at the level of cell lipopolysaccharides (LPS) in response to phenol stress, biased on the 4-fold increased abundance of the Lipid A 3-O-deacylase, PagL protein. In *P. aeruginosa*, PagL is an outer membrane protein involved in the alteration of the number of acyl chains in the LPS lipid A [34].

The abundance of the lipoprotein VacJ and of a GDSL (for the amino acid consensus sequence motif Gly, Asp, Ser, and Leu) lipase family protein, whose location is attributed to the surface of the

cell membrane, also increased in response to phenol. However, the increased abundance of the GDSL family lipase was only registered after 5h of phenol exposure corresponding to cell growth resumption under phenol stress. This protein is orthologous to *P. aeruginosa* EstA protein, an esterase related to the production of the biosurfactants rhamnolipids [35]. The production of the lipopeptide biosurfactant putisolvin in *P. putida* PCL1445 is dependent on the two-component system GacA-GacS [36] whose DNA response regulator was found to increase in abundance in phenol adapted cells (Table 2). Both rhamnolipids and putisolvin are low-molecular weight biosurfactants [35-36] which are involved in the alteration of cell surface and swarming motility [35]. These results together with former results from our laboratory (decreased content of FliC, one of the key components of flagella in response to phenol [6]) are suggestive of an alteration of the motility of phenol challenged cells. Further studies are required to confirm such hypothesis.

Results also showed an increased content of proteins having a role in physical stability and shape maintenance of Gram-negative bacteria [37-38]. The abundance of three different forms of the peptidoglycan associated lipoprotein OprL was found to increase 4-fold (Table 2). The gene that codes for OprL (also designated Pal) is located in a cluster of genes encoding the inner membrane proteins TolQ, TolR and TolA (Figure 5), and the periplasmic translocation protein TolB [39]. Together, these proteins form the Tol-Pal system [39], that, in *P. putida* KT2440, appears to be involved in the maintenance of outer membrane integrity [37] and to be necessary for the appropriate function of transport systems involved in the uptake of organic molecules such as succinate, proline and sucrose [40]. The 17 KDa surface antigen identified in this study as having an increased content after phenol-induced stress, is orthologous to the small lipoprotein SlyB which is conserved in different Gram-negative bacteria contributing to the integrity of the cell envelope [41].

An increased abundance (of about 3-fold) of the inner membrane Penicilin binding protein 5 (PBP5) was registered in response to phenol. The D-alanine carboxypeptidase PBP5, is involved in the removal of a terminal D-alanine residue from the pentapeptide chain in peptidoglycan biosynthesis [42]. The abundance of MurA (UDP-N-acetylglucosamine enolpyruvyl deacetylase), involved in the

first step of the bacterial cell wall biosynthesis [43], also increased in response to phenol. Altogether, these results suggest that KT2440 exposure to phenol triggers a global adaptive response involving de novo biosynthesis of peptidoglycan, in coordination with the alteration of outer membrane and cell surface components.

3.4. Phenol leads to the alteration of the content of proteins involved in protein synthesis and stabilization

Proteins involved in protein biosynthesis, specifically the 30S and 50S ribosomal proteins, the translation elongation factor Efp and four amino acid – tRNA synthetases, LysS, ArgS, TyrS and GlyQ, were found in all the membrane fractions examined (Table 2). This result is consistent with the known transfer of nascent membrane proteins in the membrane by translocase systems, such as SecABYEG system [44].

The abundance of the ATP-dependent protease Lon-1 and of the inner membrane protein HflK was also increased in response to phenol (Table 2). In *E.coli*, the Lon protein is involved in the proteolysis of heat shock-induced protein aggregates [45] while HflK is one subunit of the inner membrane protein complex HflKC that negatively modulates the proteolytic activity of the membrane bound ATPase FtsH [46], involved in the quality control of membrane proteins, being responsible for the degradation of integral membrane proteins and cytosolic proteins [46]. Remarkably, proteases FtsH and Lon act in concert with the DnaKJ-GrpE chaperone machinery to control stability and activity of the alternative sigma factor σ^{32} at the post-translational level [47]. In *P. aeruginosa*, σ^{32} associated RNA polymerase is involved in the activation of genes encoding heat shock protective factors in response to elevated temperatures [48]. Consistent with the induction of protein aggregation by phenol suggested before, and the consequent induction of proteins involved in protein aggregation recovery or recycling [6].

3.5. Increased content of proteins involved in electron transport in response to phenol

Proteins with an increased content in phenol challenged cells also include five proteins involved in the function of the respiratory chain, specifically: the iron-sulfur subunit of Ubiquinol-cytochrome c reductase (QcrA), the electron transfer flavoprotein-ubiquinone oxidoreductase, the subunit NuoCD of NADH dehydrogenase I, and the flavoprotein subunit SdhA of Succinate dehydrogenase complex (Table 2). Moreover, the abundance of the subunit AtpF of ATP synthase is higher in phenol stressed cells, 3-fold and 9-fold after 1h and 5h of phenol exposure, respectively (Table 2). The increased content under phenol stress registered for these peripheral proteins of the inner membrane suggest that the expression of the electron transport and ATP synthase proteins that are integral to the inner membrane may also be higher in phenol stressed cells, although they could not be identified using 2-DE based approach. The increased content of respiratory chain proteins is consistent with the extra-energetic requirements of cells that trigger energetically expensive adaptation mechanisms in order to survive, adapt and thrive under phenol challenge, as previously hypothesized [5-6]. Although the TCA cycle enzymes exhibit a decreased content in the membrane fractions obtained from phenol stressed cells, the level of the TCA enzymes was found to increase when the soluble fraction was examined in a previous study [6]. We believe that the presence of these enzymes in the membrane fraction is whether an artifact or due to their loose association with proteins of the membrane fraction.

3.6. Phenol-induced alterations in the content of proteins involved in iron metabolism and other proteins

Our results also suggest a decreased biosynthesis and uptake of the *Pseudomonas* characteristic siderophore pyoverdine [49] in response to phenol. Indeed, the abundance of L-ornithine N5 Oxygenase (PvdA), involved in the synthesis of pyoverdine, and of the pyoverdine receptor FpvA decreased in phenol stressed cells (Table 2). Moreover, two proteins recently related with pyoverdine synthesis [50], the SyrP protein and the protein encoded by the gene in PP_4222 locus also decreased in response to phenol (Table 2). Additionally, the abundance of the content of the majority of the

siderophore receptors identified in this study was found to decrease in response to phenol (Table 2). Consistent with the decreased abundance of siderophore receptors registered in the present study, the abundance of the central regulator Fur, that negatively controls the uptake of iron, was reported before in response to phenol in *P. putida* KT2440 [6].

The abundance of several other proteins of unknown function was also found to be altered under phenol stress (Table 2). Three proteins encoded by PP_0564, PP_5353 and PP_0258 genes increased 28-fold, 43-fold and 58-fold, respectively, after 5h of phenol stress, suggesting that they may have an important role in the adaptive response to phenol. Several other proteins whose abundance varied in response to phenol were previously found to be involved in resistance to other stresses that are known to destabilize the cell envelope. Specifically, the abundance of the ATP-binding protein subunit of the glycine betaine/carnitine/choline ABC transporter was found to duplicate. This transporter is an orthologue of the OpuC ABC transporter of *P. syringae* pv. Tomato str. DC3000 and glycine, betaine and carnitine are osmolytes that are transported by OpuC in response to osmotic and cold stress [51]. The abundance of the OsmE lipoprotein was also found to increase in response to phenol stress as reported to occurin *E. coli* under osmotic stress and during the decelerating phase of growth [52]. The outer membrane lipoprotein Blc is a lipocalin, which probably contributes to cell adaptation to both starvation and high growth medium osmolarity [53]. Fluorescence quenching experiments indicated that Blc binds fatty acids and phospholipids, suggesting a possible role in storage or transport of lipids necessary for membrane maintenance [54].

4. Conclusions

In this study, we have explored 2-DE-based expression proteomics focused on membrane proteins to refine and extend our current model for the global response of *Pseudomonas putida* KT2440 to phenol. The mechanistic model proposed is likely to be extensive to the adaptive response of Gram-negative bacteria to other organic solvents. The registered alterations clearly show that the majority of the phenol-responsive proteins present in the different membrane fractions are already

induced after 1h of cell exposure to phenol, during the early response, and reveal a very intricate combination of cell requisites to successfully adapt to phenol toxicity. A number of proteins detected in the membrane fractions examined have a predicted cytosolic location, according to *Pseudomonas* Genome database annotation. However, their percentage associated to the membrane fractions was found to be identical in the different independent experiments carried out and cannot be considered a fortuitous artifact resulting from sample preparation. Results indicate that the response to phenol involves an increased content of different solvent efflux pump systems together with a decreased content of outer membrane proteins, presumably leading to decreased cell permeability. These coordinated responses may also involve post-translational modifications, contributing to the decrease of the solvent concentration inside cells. Results are also suggestive of the induction of protective mechanisms to assure cell surface and cell structure integrity and of a more active function of the respiratory chain. In a previous work, we have focused our attention on the phenol-induced alterations occurring essentially at the level of the soluble proteome of KT2440 (more hydrophilic fraction of the proteome) [6]. The comparison of our former results with those reported in this work strongly suggests that, in general, the data are complementary and provide valuable insights into the understanding of the puzzling complexity of the adaptive mechanisms involved in solvent stress response in *Pseudomonas putida* KT2440*,* a relevant bacterial model for environmental studies. However, some discrepancies were registered. A number of proteins whose relative abundance changed following phenol exposure were identified in both cytosolic and membrane fractions. In the case of a number of proteins whose localization was predicted to be cytosolic, (e.g. the trigger factor (Tig), serine hydroxymethyltransferase (GlyA), NH(3)-dependent NAD(+) synthethase (NadE), heat shock protein (HtpG), and aconitate hydratase 2 (AcnB)), their relative abundance was found to increase in gels prepared from soluble fraction following phenol stress [6] whereas in the present work their content was found to decrease in the membrane fractions. In the case of proteins predicted to be associated with the membrane (e.g. the outer membrane proteins TolC, OprF and OprQ and the periplasmic subunits of different transporters), their phenol-induced alteration was found to occur mainly in the

gels prepared from membrane fractions. These discrepancies registered may be the result of specific protein-protein interactions (dependent on the abundance of the interacting partners) and the contaminations of the fractions with proteins whose localization is different. Therefore, expression proteomic analyses should be considered as a useful experimental approach to get clues to guide more detailed research to validate and deepen proteomic-based hypotheses.

As a whole, results emerging from this study provided additional mechanistic insights into solvent adaptation of the very versatile *Pseudomonas* genus, of possible impact in biodegradation, bioremediation and biocatalysis. Indeed, it is expected that the understanding of the adaptive strategies employed by *P. putida* KT2440 under phenol challenge can be useful to guide the design of more robust strains to be used in whole-cell biotransformations using two-phase solvent systems and in the bioremediation of solvent contaminated soils.

Acknowledgments

This work was supported by Fundação para a Ciência e Tecnologia (PhD scholarship SFRH/BD/38805/2007 to CRR).

Authors declare no financial and commercial conflicts of interest.

5. References

[1] J.L. Ramos, E. Duque, M.T. Gallegos, P. Godoy, M.I. Ramos-Gonzalez, A. Rojas, W. Teran and A. Segura, Mechanisms of solvent tolerance in Gram-negative bacteria. *Annu Rev Microbiol* **56** (2002), pp. 743-768.

[2] Y. Sardessai and S. Bhosle, Tolerance of bacteria to organic solvents. *Res Microbiol* **153** (2002), pp. 263-268.

[3] Y.N. Sardessai and S. Bhosle, Industrial potential of organic solvent tolerant bacteria. *Biotechnol Prog* **20** (2004), pp. 655-660.

[4] H.J. Heipieper, G. Neumann, S. Cornelissen and F. Meinhardt, Solvent-tolerant bacteria for biotransformations in two-phase fermentation systems. *Appl Microbiol Biotechnol* **74** (2007), pp. 961-973.

[5] P.M. Santos, V. Roma, D. Benndorf, M. von Bergen, H. Harms and I. Sá-Correia, Mechanistic insights into the global response to phenol in the phenol-biodegrading strain *Pseudomonas sp.* M1 revealed by quantitative proteomics. *OMICS* **11** (2007), pp. 233-251.

[6] P.M. Santos, D. Benndorf and I. Sá-Correia, Insights into *Pseudomonas putida* KT2440 response to phenol-induced stress by quantitative proteomics. *Proteomics* **4** (2004), pp. 2640-2652.

[7] S. Iurescia, A.M. Marconi, D. Tofani, A. Gambacorta, A. Paterno, C. Devirgiliis, M.J. van der Werf and E. Zennaro, Identification and sequencing of beta-myrcene catabolism genes from *Pseudomonas* sp. strain M1. *Appl Env Microbiol* **65** (1999), pp. 2871-2876.

[8] B.A. van Montfort, B. Canas, R. Duurkens, J. Godovac-Zimmermann and G.T. Robillard, Improved in-gel approaches to generate peptide maps of integral membrane proteins with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *J Mass Spectrom* **37** (2002), pp. 322-330.

[9] J.D. Boyce, P.A. Cullen, V. Nguyen, I. Wilkie and B. Adler, Analysis of the *Pasteurella multocida* outer membrane sub-proteome and its response to the *in vivo* environment of the natural host. *Proteomics* **6** (2006), pp. 870-880.

[10] X.X. Xia, M.J. Han, S.Y. Lee and J.S. Yoo, Comparison of the extracellular proteomes of *Escherichia coli* B and K-12 strains during high cell density cultivation. *Proteomics* **8** (2008), pp. 2089-2103.

[11] C. Xu, X. Lin, H. Ren, Y. Zhang, S. Wang and X. Peng, Analysis of outer membrane proteome of *Escherichia coli* related to resistance to ampicillin and tetracycline. *Proteomics* **6** (2006), pp. 462-473.

[12] M.P. Molloy, N.D. Phadke, J.R. Maddock and P.C. Andrews, Two-dimensional electrophoresis and peptide mass fingerprinting of bacterial outer membrane proteins. *Electrophoresis* **22** (2001), pp. 1686-1696.

[13] S. Marti, J. Sanchez-Cespedes, E. Oliveira, D. Bellido, E. Giralt and J. Vila, Proteomic analysis of a fraction enriched in cell envelope proteins of *Acinetobacter baumannii*. *Proteomics* **6** (2006), pp. S82-87.

[14] S. Hartmans, J.P. Smits, M.J. van der Werf, F. Volkering and J.A.M. de Bont, Metabolism of Styrene Oxide and 2-Phenylethanol in the Styrene-Degrading *Xanthobacter strain* 124X. *Appl Environ Microbiol* **55** (1989), pp. 2850-2855.

[15] K. Ishidate, E.S. Creeger, J. Zrike, S. Deb, B. Glauner, T.J. MacAlister and L.I. Rothfield, Isolation of differentiated membrane domains from *Escherichia coli* and *Salmonella typhimurium*, including a fraction containing attachment sites between the inner and outer membranes and the murein skeleton of the cell envelope. *J Biol Chem* **261** (1986), pp. 428-443.

[16] A.L. Horstman and M.J. Kuehn, Enterotoxigenic *Escherichia coli* Secretes Active Heat-labile Enterotoxin via Outer Membrane Vesicles. *J Biol Chem* **275** (2000), pp. 12489-12496.

[17] A. Shevchenko, M. Wilm, O. Vorm, O.N. Jensen, A.V. Podtelejnikov, G. Neubauer, P. Mortensen and M. Mann, A strategy for identifying gel-separated proteins in sequence databases by MS alone. *Biochem Soc Trans* **24** (1996), pp. 893-896.

[18] D. Vester, E. Rapp, D. Gade, Y. Genzel and U. Reichl, Quantitative analysis of cellular proteome alterations in human influenza A virus-infected mammalian cell lines. *Proteomics* **9** (2009), pp. 3316-3327.

[19] D.N. Perkins, D.J. Pappin, D.M. Creasy and J.S. Cottrell, Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* **20** (1999), pp. 3551-3567.

[20] T. Guina, M. Wu, S.I. Miller, S.O. Purvine, E.C. Yi, J. Eng, D.R. Goodlett, R. Aebersold, R.K. Ernst and K.A. Lee, Proteomic analysis of *Pseudomonas aeruginosa* grown under magnesium limitation. *J Am Soc Mass Spectrom* **14** (2003), pp. 742-751.

[21] D.D. Sriramulu, M. Nimtz and U. Romling, Proteome analysis reveals adaptation of *Pseudomonas aeruginosa* to the cystic fibrosis lung environment. *Proteomics* **5** (2005), pp. 3712-3721.

[22] D.G. Papasotiriou, S. Markoutsa, B. Meyer, A. Papadioti, M. Karas and G. Tsiotis, Comparison of the membrane subproteomes during growth of a new pseudomonas strain on lysogeny broth medium, glucose, and phenol. *J Proteome Res* **7** (2008), pp. 4278-4288.

[23] G.S. Butler and C.M. Overall, Proteomic identification of multitasking proteins in unexpected locations complicates drug targeting. *Nat Rev Drug Discov* **8** (2009), pp. 935-948.

[24] K. Kim, L. Sungjin, L. Kyunghee and L. Dongbin, Isolation and Characterization of Toluene-Sensitive Mutants from the Toluene-Resistant Bacterium *Pseudomonas putida* GM73. *J Bacteriol* **180** (1998), pp. 3692-3696.

[25] P. Dominguez-Cuevas, J.E. Gonzalez-Pastor, S. Marques, J.L. Ramos and V. de Lorenzo, Transcriptional Tradeoff between Metabolic and Stress-response Programs in *Pseudomonas putida* KT2440 Cells Exposed to Toluene. *J Biol Chem* **281** (2006), pp. 11981-11991.

[26] P.A. Kivistik, M. Putrins, K. Puvi, H. Ilves, M. Kivisaar and R. Horak, The ColRS Two-Component System Regulates Membrane Functions and Protects *Pseudomonas putida* against Phenol. *J Bacteriol* **188** (2006), pp. 8109-8117.

[27] S. Isken and J.A.M. de Bont, Bacteria tolerant to organic solvents. *Extremophiles* **2** (1998), pp. 229-238.

[28] T. Jaouen, E. De, S. Chevalier and N. Orange, Pore Size Dependence on Growth Temperature Is a Common Characteristic of the Major Outer Membrane Protein OprF in Psychrotrophic and Mesophilic Pseudomonas Species. *Appl Environ Microbiol* **70** (2004), pp. 6665-6669.

[29] E.M. Nestorovich, E. Sugawara, H. Nikaido and S.M. Bezrukov, *Pseudomonas aeruginosa* porin OprF: properties of the channel. *J Biol Chem* **281** (2006), pp. 16230-16237.

[30] E.G. Rawling, F.S. Brinkman and R.E. Hancock, Roles of the carboxy-terminal half of *Pseudomonas aeruginosa* major outer membrane protein OprF in cell shape, growth in low-osmolarity medium, and peptidoglycan association. *J Bacteriol* **180** (1998), pp. 3556-3562.

[31] W. Garten, I. Hindennach and U. Henning, The major proteins of the *Escherichia coli* outer cell envelope membrane. Characterization of proteins II* and III, comparison of all proteins. *Eur J Biochem* **59** (1975), pp. 215-221.

[32] E. Sugawara, E.M. Nestorovich, S.M. Bezrukov and H. Nikaido, *Pseudomonas aeruginosa* porin OprF exists in two different conformations. *J Biol Chem* **281** (2006), pp. 16220-16229.

[33] A. Bell, M. Bains and R.E.W. Hancock, *Pseudomonas aeruginosa* Outer Membrane Protein OprH: Expression from the cloned gene and function in EDTA and Gentamicin resistance. *J Bacteriol* **173** (1991), pp. 6657-6664.

[34] C.R. Raetz, C.M. Reynolds, M.S. Trent and R.E. Bishop, Lipid A modification systems in gram-negative bacteria. *Annu Rev Biochem* **76** (2007), pp. 295-329.

[35] S. Wilhelm, A. Gdynia, P. Tielen, F. Rosenau and K.E. Jaeger, The Autotransporter Esterase EstA of *Pseudomonas aeruginosa* Is Required for Rhamnolipid Production, Cell Motility, and Biofilm Formation. *J Bacteriol* **189** (2007), pp. 6695-6703.

[36] J.F. Dubern and G.V. Bloemberg, Influence of environmental conditions on putisolvins I and II production in *Pseudomonas putida* strain PCL1445. *FEMS Microbiol Lett* **263** (2006), pp. 169-175.

[37] J.J. Rodriguez-Herva, M.I. Ramos-Gonzalez and J.L. Ramos, The *Pseudomonas putida* peptidoglycan-associated outer membrane lipoprotein is involved in maintenance of the integrity of the cell cell envelope. *J Bacteriol* **178** (1996), pp. 1699-1706.

[38] I. Sonntag, H. Schwarz, Y. Hirota and U. Henning, Cell envelope and shape of *Escherichia coli*: multiple mutants missing the outer membrane lipoprotein and other major outer membrane proteins. *J Bacteriol* **136** (1978), pp. 280-285.

[39] M.A. Llamas, J. L.Ramos and J.J. Rodriguez-Herva, Transcriptional Organization of the *Pseudomonas putida tol-oprL* Genes. *J Bacteriol* **185** (2003), pp. 184-195.

[40] M.A. Llamas, J.J. Rodríguez-Herva, R.E.W. Hancock, W. Bitter, J. Tommassen and J.L. Ramos, Role of *Pseudomonas putida tol-oprL* Gene Products in Uptake of Solutes through the Cytoplasmic Membrane. *J Bacteriol* **185** (2003), pp. 4707–4716.

[41] M. Plesa, J.P. Hernalsteens, G. Vandenbussche, J.M. Ruysschaert and P. Cornelis, The SlyB outer membrane lipoprotein of *Burkholderia multivorans* contributes to membrane integrity. *Res Microbiol* **157** (2006), pp. 582-592.

[42] D. Hesek, M. Suvorov, K.I. Morio, M. Lee, S. Brown, S.B. Vakulenko and S. Mobashery, Synthetic Peptidoglycan Substrates for Penicillin-Binding Protein 5 of Gram-Negative Bacteria. *J Org Chem* **69** (2004), pp. 778-784.

[43] E.Z. Baum, D.A. Montenegro, L. Licata, I. Turchi, G.C. Webb, B.D. Foleno and K. Bush, Identification and Characterization of New Inhibitors of the *Escherichia coli* MurA Enzyme. *Antimicrob Agents Chemother* **45** (2001), pp. 3182-3188.

[44] A.J.M. Driessen and N. Nouwen, Protein Translocation Across the Bacterial Cytoplasmic Membrane. *Annu Rev Biochem* **77** (2008), pp. 643-667.

[45] R. Rosen, D. Biran, E. Gur, D. Becher, M. Hecker and E.Z. Ron, Protein aggregation in *Escherichia coli*: role of proteases. *FEMS Microbiol Lett* **207** (2002), pp. 9-12.

[46] A. Kihara, Y. Akiyama and K. Ito, A protease complex in the *Escherichia coli* plasma membrane: HflKC (HflA) forms a complex with FtsH (HflB), regulating its proteolytic activity against SecY. *EMBO J* **15** (1996), pp. 6122-6131.

[47] U. Jenal and R. Hengge-Aronis, Regulation by proteolysis in bacterial cells. *Curr Opin Microbiol* **6** (2003), pp. 163-172.

[48] E. Potvin, F. Sanschagrin and R.C. Levesque, Sigma factors in *Pseudomonas aeruginosa*. *FEMS Microbiol Rev* **32** (2008), pp. 38-55.

[49] I.J. Schalk, Metal trafficking via siderophores in Gram-negative bacteria: specificities and characteristics of the pyoverdine pathway. *J Inorg Biochem* **102** (2008), pp. 1159-1169.

[50] S. Matthijs, G. Laus, J.M. Meyer, K. Abbaspour-Tehrani, M. Schafer, H. Budzikiewicz and P. Cornelis, Siderophore-mediated iron acquisition in the entomopathogenic bacterium *Pseudomonas entomophila* L48 and its close relative *Pseudomonas putida* KT2440. *Biometals* **22** (2009), pp. 951-964.

[51] R.M. Kappes, B. Kempf, S. Kneip, J. Boch, J. Gade, J. Meier-Wagner and E. Bremer, Two evolutionarily closely related ABC transporters mediate the uptake of choline for synthesis of the osmoprotectant glycine betaine in *Bacillus subtilis*. *Mol Microbiol* **32** (1999), pp. 203-216.

[52] C. Gutierrez, S. Gordia and S. Bonnassie, Characterization of the osmotically inducible gene *osmE* of *Escherichia coli* K-12. *Mol Microbiol* **16** (1995), pp. 553-563.

[53] R.E. Bishop, The bacterial lipocalins. *Biochim Biophys Acta* **1482** (2000), pp. 73-83.

[54] V. Campanacci, R.E. Bishop, S. Blangy, M. Tegoni and C. Cambillau, The membrane bound bacterial lipocalin Blc is a functional dimer with binding preference for lysophospholipids. *FEBS Lett* **580** (2006), pp. 4877-4883.

Figure captions:

Figure 1: Growth curves of *Pseudomonas putida* KT2440. Cells were grown at 30 ºC, in basal mineral medium with 20 mM succinate as the carbon source. Growth was followed based on culture optical density at 600 nm (OD600). Three cultures were prepared. When the cell cultures reached an OD600 of 0.2, phenol was added to two of the cultures up to a final concentration of 800 mg/L (close squares). The third culture was left unsupplemented (open squares). Cells from the control culture (Ctrl) and from one of the phenol cultures (P1) were harvested 1h after phenol addition. Cells from the other phenol culture were harvested after 5h of incubation following phenol supplementation (P5).

Figure 2: Reference maps. Representative proteome maps of *Pseudomonas putida* KT2440 obtained using the total membrane fraction (TMF) (a), the outer membrane fraction (OMF) (b) and the inner membrane fraction (IMF) (c) obtained from cells suddenly exposed to 800 mg/L phenol for 1h. Proteins were separated by 2-DE (IEF/SDS-PAGE) and visualized by staining with Flamingo stain. The identified protein spots are numbered as in Tables A, B and C from supplementary material.

Figure 3: Principal Component Analysis (PCA) of protein patterns in 2-DE gels. The PCA used first principal component for analysis of total membrane fraction (TMF), outer membrane fraction (OMF) and inner membrane fraction (IMF) 2-DE gels prepared from cell samples of *P. putida* KT2440 cells challenged with phenol for 1h. Spots inside boxes represent each gel prepared for the indicated condition. The grey line represents the clustering of the proteins detected in the corresponding 2-DE gels.

Figure 4: Pattern profiles of the alteration of protein abundance after 1h (P1) or 5h (P5) of *P. putida* KT2440 exposure to 800 mg/L phenol compared with control condition (Ctrl). The number of proteins

exhibiting a specific expression pattern profile and examples of the identified proteins for each expression pattern profile are also shown.

Figure 5: Organization of genes encoding proteins responsive to phenol, as shown in Table 2. The genes encoding these proteins are referred in the manuscript as being organized in operons or in a cluster of genes that may form an operonic structure. If given, gene names are shown beneath the gene schemes and the gene locus numbers are shown above the gene schemes.

Table

Table 1: Percentage of *Pseudomonas putida* KT2440 genome encoded proteins and percentage of proteins identified in total membrane fraction, outer membrane fraction and inner membrane fraction associated to their putative or demonstrated subcellular localization. The percentage of proteins identified in the three membrane fractions, relative to the total number of proteins encoded by *P. putida* KT2440 genome, with different subcellular localizations, is also shown into brackets.

Theoretical subcellular localization of proteins	Genome encoded proteins	Total membrane fraction	Outer membrane fraction	Inner membrane fraction
Outer membrane	2.7 %	42 % (18.4 %)	35 % (19.7 %)	3 % (1.4 %)
Inner membrane	17.8 %	6 % (0.4 %)	-	36 % (3.0 %)
Periplasm	1.9 %	2 % (1.0 %)	1 % (1.0%)	9 % (6.9 %)
Cytoplasm	37.5 %	23 % (0.7 %)	27 % (1.1 %)	40 % (1.6 %)
Unknown	39.8 %	27 % (0.5 %)	37 % (1.4 %)	12 % (0.5 %)

Table

Table 2. Relative alteration (above 2-fold or below 0.5-fold) of protein content in *Pseudomonas putida* KT2440 membrane fractions after 1h and 5h of sudden exposure of cells to 800 mg/L phenol.

Spot (nr.) [a]	Protein description	NCBI RefSeq	Location [b]	Fold Change [c]		ANOVA (*p*) [d]
				P1/C	P5/C	
Cell envelope						
Biosynthesis and degradation of murein sacculus and peptidoglycan						
98	Membrane-Bound Lytic Murein Transglycosylase (MltB)	NP_746903	IM	1.2	2.0	2.7E-02
206	Outer membrane-bound lytic murein transglycolase A (MltA)	NP_747074	OM	1.5	2.1	6.4E-03
203	D-alanyl-D-alanine carboxypeptidase (PBP5)	NP_746908	IM	2.5	3.0	2.5E-02
Other						
2	Peptidoglycan-associated lipoprotein (OprL)	NP_743383	OM	3.8	4.3	2.1E-04
6	17 kDa surface antigen	NP_743292	OM	2.9	3.4	2.4E-03
12	Outer membrane lipoprotein, lipocalin (Blc)	NP_746160	OM	3.7	2.7	1.1E-03
61	VacJ family lipoprotein	NP_744312	OM	2.3	3.4	4.6E-04
210	Lipid A 3-O-deacylase (PagL)	NP_742898	Unknown	4.4	4.1	1.5E-03
211	Lipoprotein PP_1238 (NlpB)	NP_743398	IM	3.5	3.5	7.4E-04
Transport and binding proteins						
Amino acid, peptides and amines						
97	Spermidine/Putrescine ABC Transporter , ATP-Binding Protein (PotA)	NP_742577	IM	0.5	0.6	1.8E-02
101	Glycine Betaine/Carnitine/Choline ABC Transporter, ATP-Binding Protein, Putative (OpuC)	NP_743029	IM	2.2	0.6	9.6E-03
175	Amino acid ABC transporter, ATP-binding protein (AatP)	NP_743229	IM	2.7	3.3	6.1E-03
177	ABC efflux transporter, ATP-binding protein, putative	NP_742671	IM	2.2	2.4	2.2E-02
178	Branched-chain amino acid ABC transporter, ATP-binding protein (BraG)	NP_743298	IM	2.1	2.5	2.9E-03
184	Amino acid ABC transporter, ATP-binding protein	NP_742450	IM	3.1	1.9	2.4E-02
185	Basic amino acid ABC transporter, ATP-binding protein (AotP)	NP_746594	IM	2.6	2.3	2.9E-02
Porins						
51	OmpF family protein (OprF)	NP_744239	OM	2.9	2.8	1.1E-02
52	OmpF family protein (OprF)	NP_744239	OM	0.2	2.7	8.7E-02
53	OmpF family protein (OprF)	NP_744239	OM	0.04	2.1	1.0E-01
65	OmpA/MotB Domain Protein	NP_743659	OM	5.1	5.1	1.1E-02
76	OmpW Family Protein (OprG)	NP_742668	OM	0.3	0.5	4.4E-02

77	OmpA/MotB Domain Protein	NP_743282	OM	0.7	6.2	1.9E-02
84	OmpA/MotB Domain Protein	NP_746315	OM	1.8	2.2	4.7E-02
105	Porin B (OprB)	NP_743180	OM	0.4	1.1	7.1E-03
129	Outer Membrane Porin (OprQ)	NP_742435	OM	0.9	0.5	1.7E-02
Cations and iron carrying compounds						
35	Outer membrane ferripyoverdine receptor (FpvA)	NP_746334	OM	0.5	0.5	2.7E-03
37	TonB-dependent siderophore receptor	NP_745748	OM	0.7	0.3	3.1E-05
38	Outer membrane ferric siderophore receptor, putative	NP_745299	OM	0.9	0.5	1.3E-03
47	Outer membrane ferric siderophore receptor, putative	NP_745468	OM	2.2	0.9	1.1E-03
113	TonB-Dependent Siderophore Receptor	NP_742434	OM	0.5	0.5	1.3E-02
114	B12 Family TonB-Dependent Receptor (BtuB)	NP_742688	OM	1.4	2.6	4.3E-02
140	TonB-Dependent Siderophore Receptor	NP_742698	OM	0.5	0.4	3.3E-02
149	TonB-Dependent Hemoglobin/Transferrin/Lactoferrin Family Receptor (CirA)	NP_743167	OM	0.4	0.4	1.1E-02
Unknown substrate						
31	TolC family type I secretion outer membrane protein (TolC)	NP_746629	OM	2.3	2.7	7.1E-04
43	TolC family type I secretion outer membrane protein (TolC)	NP_747026	OM	2.9	4.9	1.9E-05
Cellular processes						
Toxin resistance						
4	Outer membrane protein H1 (OprH)	NP_743345	OM	4.0	3.5	5.7E-04
5	Outer membrane protein H1 (OprH)	NP_743345	OM	4.6	3.2	1.3E-04
8	Outer membrane protein H1 (OprH)	NP_743345	OM	4.8	2.5	1.1E-03
9	Outer membrane protein H1 (OprH)	NP_743345	OM	3.0	2.9	1.6E-03
10	Outer membrane protein H1 (OprH)	NP_743345	OM	0.4	0.4	7.8E-04
66	Outer membrane protein H1 (OprH)	NP_743345	OM	2.7	2.2	3.1E-02
69	Outer membrane protein H1 (OprH)	NP_743345	OM	1.6	6.8	4.0E-03
72	Outer membrane protein H1 (OprH)	NP_743345	OM	2.8	1.8	2.1E-02
123	Outer membrane protein H1 (OprH)	NP_743345	OM	2.8	2.4	7.8E-03
18	Multidrug/solvent RND efflux system, MFP Subunit, PP_1516 protein	NP_743673	P	4.9	6.0	1.6E-06
26	Multidrug/solvent RND efflux system, MFP subunit (TtgA)	NP_743545	P	2.2	1.8	1.4E-03
168	Multidrug/solvent RND efflux system, MFP subunit (TtgA)	NP_743545	P	18.0	35.0	1.3E-04
205	Multidrug/solvent RND efflux system, MFP subunit (TtgA)	NP_743545	P	1.5	2.0	1.5E-03

159	Multidrug/solvent RND efflux system, MFP subunit (TtgA)	NP_743545	P	11.0	15.0	5.9E-05
172	Multidrug/solvent RND efflux system, MFP subunit (TtgA)	NP_743545	P	0.6	0.4	1.1E-02
44	Multidrug/solvent RND efflux system, outer membrane lipoprotein (TtgC)	NP_743543	OM	4.5	7.5	1.5E-06
45	Multidrug/solvent RND efflux system, outer membrane lipoprotein (TtgC)	NP_743543	OM	2.8	4.5	1.1E-04
150	Toluene tolerance ABC transporter, periplasmic substrate-binding protein (Ttg2C)	NP_743121	P	3.4	3.1	5.2E-04
200	Toluene tolerance ABC efflux transporter, ATP-binding protein (Ttg2A)	NP_743119	IM	3.0	2.4	1.4E-02
Cell Division						
204	Cell division protein (ZipA)	NP_746391	IM	3.8	5.6	1.2E-02
Energy metabolism						
Electron transport						
15	Ubiquinol-cytochrome c reductase, iron-sulfur subunit (QcrA)	NP_743477	IM	1.9	2.9	3.6E-04
127	Cytochrome C Oxidase, Cbb3-Type, Subunit II (FixO)	NP_746367	Unknown	0.4	0.7	3.9E-02
192	Electron transfer flavoprotein-ubiquinone oxidoreductase, putative	NP_746320	IM	4.2	3.2	1.1E-02
196	NADH dehydrogenase I, C,D subunit (NuoCD)	NP_746240	IM	3.4	4.0	1,3E-02
ATP-proton motive force interconversion						
155	ATP synthase subunit B (AtpF)	NP_747517	IM	3.1	9.2	1.6E-03
TCA cycle						
27	Succinyl-CoA synthetase, beta subunit (SucC)	NP_746303	C	0.4	1.5	1.6E-03
82	Succinyl-Coa synthetase, alpha subunit (SucD)	NP_746302	Unknown	0.4	0.4	1.3E-03
29	Dihydrolipoamide acetyltransferase (KgdB)	NP_746305	C	0.7	0.5	4.2E-04
148	Isocitrate Dehydrogenase, NADP-Dependent (IDH)	NP_746142	Unknown	0.5	0.3	1.4E-02
193	Succinate dehydrogenase, flavoprotein subunit (SdhA)	NP_746308	IM	2.6	2.1	4.2E-02
215	Aconitate hydratase 2 (AcnB)	NP_744488	C	0.3	0.4	4.2E-02
234	Fumarate hydratase, class II (FumC)	NP_743105	C	0.3	0.2	3.1E-03
Amino Acids And Amines						
17	L-asparaginase, type II (AnsB)	NP_744601	P	1.9	3.6	4.7E-06
Glycolysis/Gluconeogenesis						
230	Glucose-6-phosphate isomerase (Pgi-1)	NP_743963	C	0.4	0.4	1.5E-02
245	Fructose-1,6-bisphosphatase (Fbp)	NP_747141	C	0.3	0.1	2.4E-07
Fatty acid and phospholipid metabolism						
Biosynthesis						

128	Acetyl-CoA Carboxylase Subunit Beta (AccD)	NP_744146	Unknown	2.0	1.8	4.1E-02
191	3-ketoacyl-(acyl-carrier-protein) reductase (FabG)	NP_742744	C	2.3	4.4	8.9E-04
Degradation						
50	GDSL family lipase (EstA)	NP_742584	OM	1.3	3.3	5.5E-04
116	Multifunctional Fatty Acid Oxidation Complex Subunit Alpha (FadB)	NP_744285	C	2.1	2.7	3.3E-02
Biosynthesis of cofactors, prosthetic groups, and carriers						
Isoprenoids						
99	4-hydroxy-3-Methylbut-2-En-1-Yl Diphosphate Synthase (IspG)	NP_743014	C	1.1	3.1	9.0E-03
Heme, porphyrin and cobalamin						
22	Oxygen-independent coproporphyrinogen III oxidase family protein; (HemN_C)	NP_745911	C	0.8	0.4	7.4E-04
24	Phytanoyl-CoA dioxygenase (PhyH)	NP_745913	Unknown	0.5	0.3	5.7E-06
174	Uroporphyrin-III C-methyltransferase, putative (HemX)	NP_742357	IM	3.6	4.6	7.6E-03
235	Oxygen-independent coproporphyrinogen III oxidase (HemN_C)	NP_746380	C	0.2	0.2	3.1E-03
Pyridine nucleotides						
42	Quinolinate synthetase (NadA)	NP_743391	C	0.2	0.2	7.5E-06
244	NH(3)-dependent NAD(+) synthetase (NadE)	NP_746974	C	0.2	0.3	1.6E-03
Pantothenate and coenzyme A						
247	Pantoate-beta-alanine ligase (PanC)	NP_746809	C	0.3	0.3	1.3E-02
Purines, pyrimidines, nucleosides, and nucleotides: purine ribonucleotide biosynthesis						
23	Adenylosuccinate lyase (PurB)	NP_746146	Unknown	2.3	1.7	1.2E-03
164	Cihydroorotate dehydrogenase (PyrD)	NP_744245	Unknown	3.2	3.10	1.7E-02
231	Inosine-5-monophosphate dehydrogenase (GuaB)	NP_743192	C	0.3	0.4	1.0E-03
Aminoacid metabolism						
90	3-deoxy-7-Phosphoheptulonate Synthase (AroF-2)	NP_745224	C	2.1	1.3	3.9E-02
110	2-isopropylmalate Synthase (LeuA)	NP_743186	C	2.6	2.1	2.6E-03
112	Serine Hydroxymethyltransferase (GlyA)	NP_742832	C	0.7	0.5	1.3E-02
214	Argininosuccinate synthase (ArgG)	NP_743249	Unknown	0.4	0.3	2.2E-02
242	Tetrhydrodipicolinate succinylase (DapD)	NP_743687	C	0.3	0.1	5.8E-04
246	Branched-chain amino acid aminotransferase (IlvE)	NP_745648	C	0.1	0.2	2.9E-03
Protein synthesis						
Translation factors						

238	Translation elongation factor P (Efp)	NP_744013	C	0.2	0.2	1.6E-03
Ribosomal proteins - synthesis and modification						
75	50S Ribosomal Protein L7/L12 (RplL)	NP_742612	C	0.4	0.2	1.5E-03
216	30S ribosomal protein S1 (RpsA)	NP_743928	C	0.2	0.2	2.4E-02
tRNA aminoacylation						
41	Lysyl-tRNA synthetase (LysS)	NP_743653	C	0.2	0.4	2.0E-05
221	Arginyl-tRNA synthetase (ArgS)	NP_747190	C	0.3	0.2	2.6E-02
233	Tyrosyl-tRNA synthetase (TyrS)	NP_742602	C	0.3	0.2	1.5E-03
240	Glycyl-tRNA synthetase, tetrameric type, alpha subunit (GlyQ)	NP_742231	C	0.3	0.2	1.1E-03
Protein fate						
Degradation of proteins, peptides, and glycopeptides						
118	ATP-Dependent Protease (Lon-1)	NP_743601	C	3.0	2.1	3.8E-02
212	HflK protein	NP_746995	IM	3.4	3.7	5.2E-04
Protein folding and stabilization						
7	Heat shock protein (Hsp20)	NP_744133	Unknown	8.0	1.8	2.4E-07
11	Peptidyl-prolyl cis-trans isomerase B (PpiB)	NP_745047	C	0.3	0.7	3.3E-05
49	Trigger factor (Tig)	NP_744448	C	0.6	0.4	1.6E-05
121	Methionine Sulfoxide Reductase A (MsrA)	NP_742503	Unknown	0.5	0.6	1.3E-02
169	Peptidyl-prolyl cis-trans isomerase; FKBP-type (FklB)	NP_743871	OM	4.3	6.0	3.6E-04
173	Peptidyl-prolyl cis-trans isomerase D, putative (SurA)	NP_744453	Multiple	4.1	4.9	1.2E-03
179	Lipoprotein releasing system, ATP-binding protein (LolD)	NP_744304	IM	1.70	3.5	5.3E-03
217	Heat shock protein (HtpG)	NP_746296	Multiple	0.2	0.2	6.5E-03
Regulatory functions						
70	DNA-Binding Response Regulator (GacA)	NP_746219	C	1.4	2.0	2.4E-03
83	Osmolarity Response Regulator (OmpR)	NP_742414	C	1.9	2.5	2.1E-02
Central intermediary metabolism						
Sulfur metabolism						
19	Sulfate adenylyltransferase subunit 2 (CysD)	NP_743463	C	0.3	0.3	2.1E-05
76	Sulphite reductase hemoprotein, beta subunit (CysL)	NP_744520	C	0.2	0.1	9.8E-03
Other						
74	Osmotically-Inducible Lipoprotein (OsmE)	NP_746960	Unknown	4.8	2.6	3.7E-02

93	Diaminobutyrate-2-Oxoglutarate Aminotransferase	NP_746340	Unknown	0.2	0.1	3.1E-03
236	SyrP protein, putative	NP_746339	C	0.2	0.2	2.6E-04
224	L-ornithine N5-oxygenase (PvdA)	NP_745926	C	0.2	0.1	2.7E-07
Unknown function						
3	Protein PP_0258 (LysM domain)	NP_742425	Unknown	6.9	58.1	7.6E-08
14	Protein PP_1392 (NAD-dependent epimerase/dehydratase)	NP_743551	C	1.3	3.3	3.4E-05
28	Protein PP_2729	NP_744873	Unknown	0.7	0.4	4.6E-05
33	Protein PP_0544 (GTP-binding protein TypA/BipA)	NP_747145	C	0.9	0.4	2.4E-03
34	Protein PP_1246	NP_743406	IM	0.8	0.3	1.9E-05
46	Protein PP_0765	NP_742926	Unknown	1.4	4.7	1.3E-04
56	Protein PP_3777	NP_745907	Unknown	1.0	0.4	2.3E-02
59	Protein PP_1737	NP_743893	C	2.6	3.2	2.8E-03
60	Protein PP_2449	NP_744597	C	2.6	2.1	7.4E-04
81	Protein PP_1357	NP_743516	Unknown	0.7	0.5	2.2E-02
85	Lipoprotein PP_4920	NP_747023	Unknown	2.1	2.7	3.2E-02
91	Protein PP_1752	NP_743908	Unknown	2.2	2.7	2.0E-03
106	Protein PP_4056	NP_746182	Unknown	0.5	0.2	1.1E-02
151	Protein PP_3154	NP_745298	Unknown	3.0	2.9	2.7E-03
158	Protein PP_0564	NP_742727	IM	15.0	28.0	2.9E-04
162	Protein PP_5353 (Tim44-Like Domain)	NP_747454	IM	13.0	43.0	9.8E-04
181	Protein PP_4939	NP_747042	IM	3.7	2.6	7.0E-04
182	Protein PP_2891 (Acetyltransferase, GNAT family)	NP_745035	Unknown	2.7	3.0	7.3E-03
189	Protein PP_0919	NP_743080	Unknown	2.3	5.3	1.1E-02
199	Protein PP_0913	NP_743074	OM	2.1	2.8	5.8E-03
220	Protein PP_4635	NP_746744	Unknown	0.2	0.2	1.5E-02
228	Protein PP_3785	NP_745915	C	0.3	0.2	1.2E-04
237	Protein PP_2418 (Lipase/esterase family protein)	NP_746335	C	0.2	0.2	6.6E-04

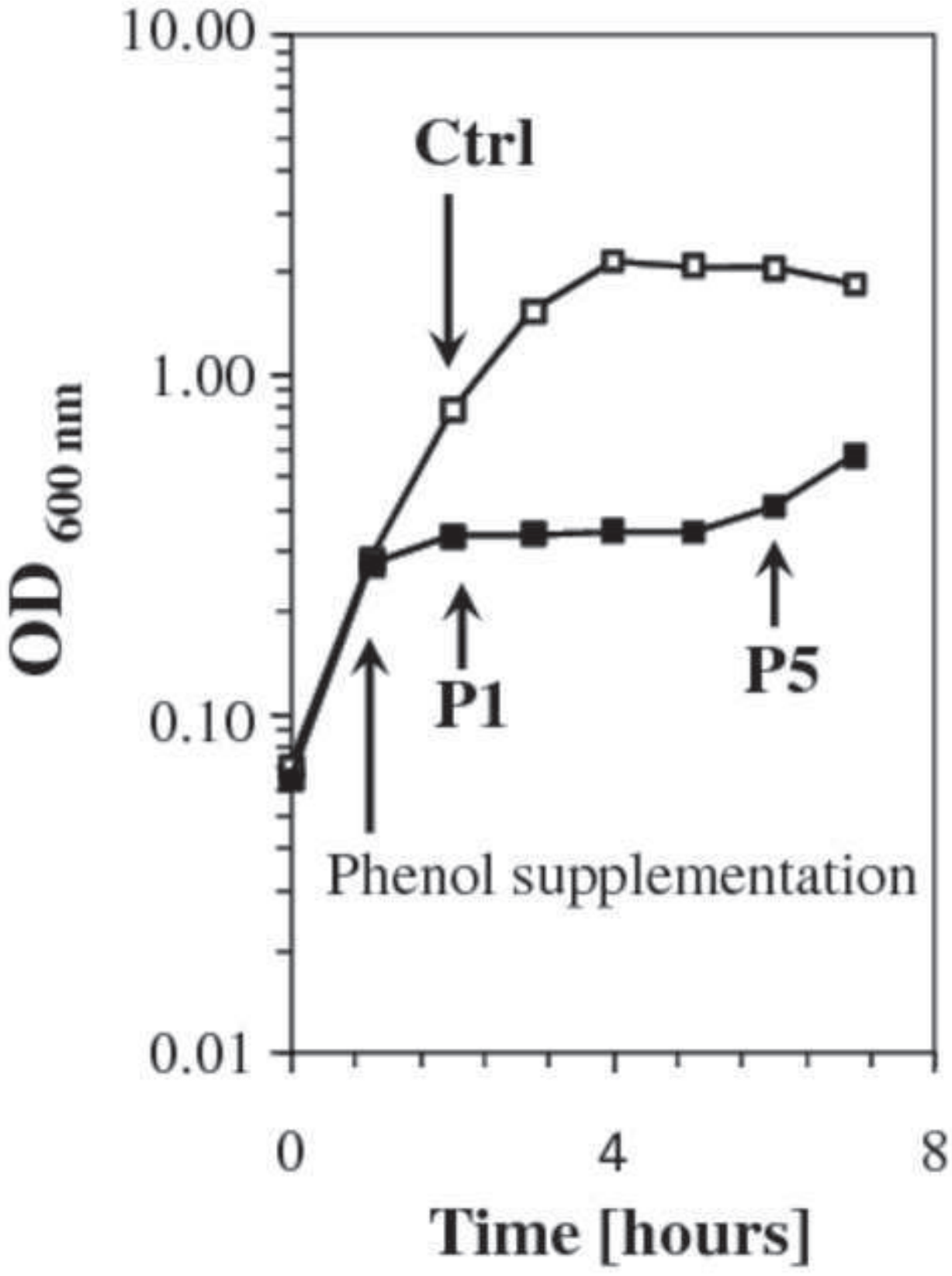
a) When the same protein was found in the three reference maps, the spot number of only one of them is referred (see Table D of Supplementary material). With exception for OprH porin, only the protein forms that exhibited different pattern profiles among the conditions examined were listed (see Table E of Supplementary material).

b) Protein localization was attributed according to the information provided in the Pseudomonas Genome database (http://www.pseudomonas.com) or from known localizations reported in scientific literature: IM – inner membrane; OM – outer membrane; P – periplasm; C – cytoplasm.

c) Values were calculated as the average data from, at least, three independent experiments and data were filtered to retain spots with ANOVA *p*. Fold changes correspond to ratio values of normalized protein spots intensities, in 2-DE gels, obtained using membrane fractions from cells of *P. putida* KT2440 after 1h (P1) and 5h (P5) of sudden exposure to 800 mg/L phenol *versus* cells grown in basal medium (C). The alterations of protein content above 2-fold are shaded in dark grey, while alterations below 0.5 are shaded in light grey.

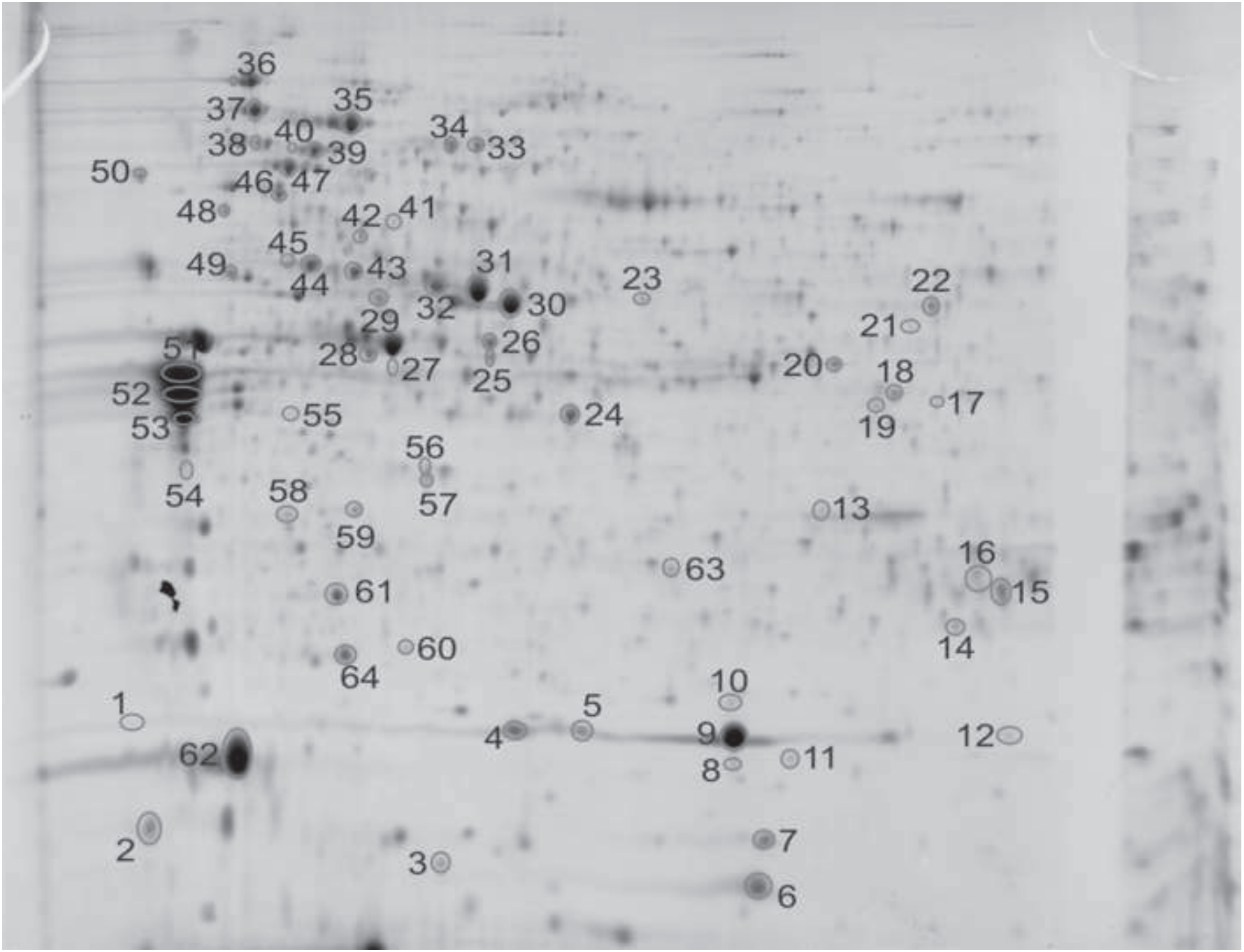
d) Values equal or below 0.05 were considered to ensure high statistical confidence of differential expression. In some specific cases results with ANOVA *p* values (underlined) up to 0.1 are also shown.

Figure
Click here to download high resolution image



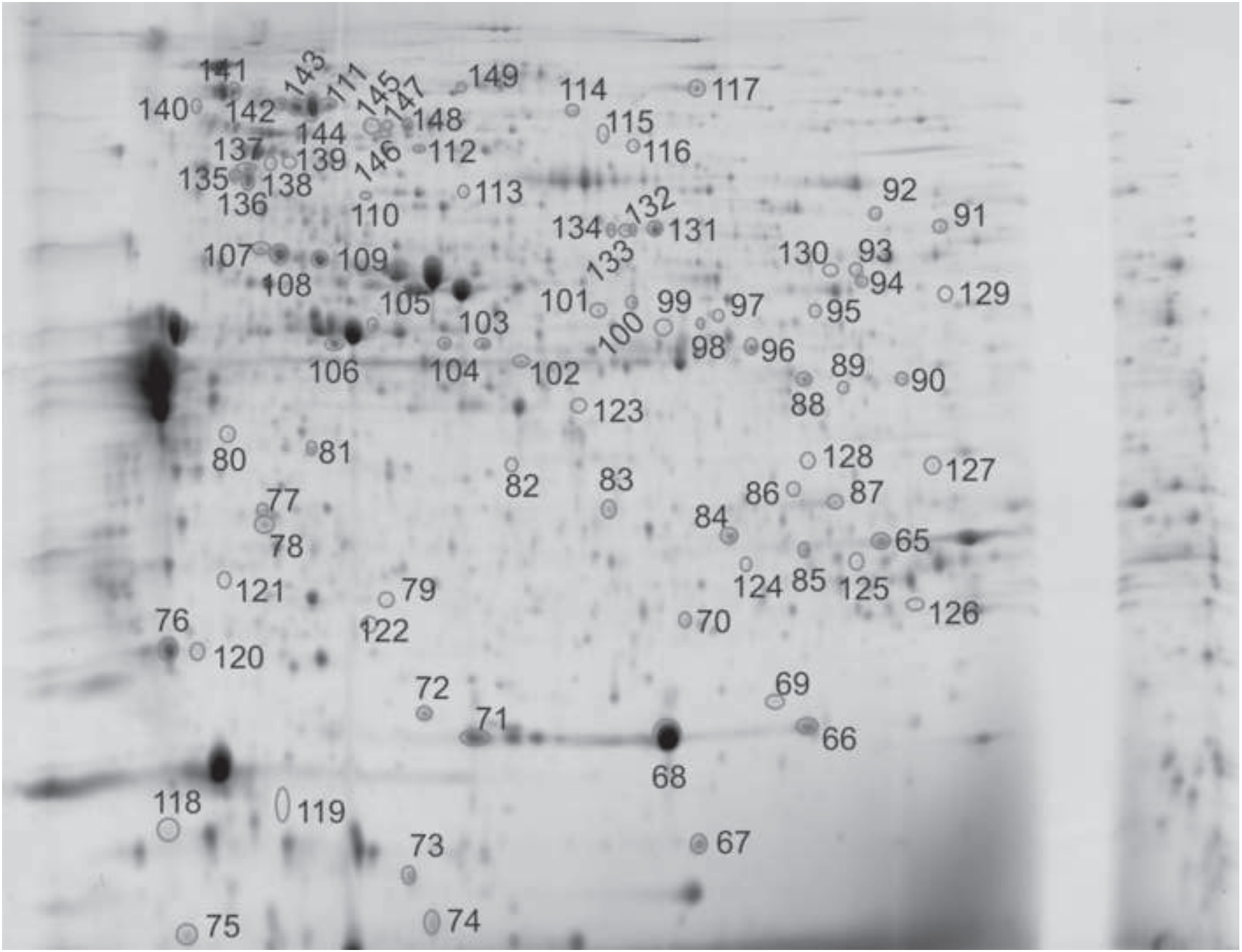
Figure

Click here to download high resolution image



Figure

Click here to download high resolution image



Figure

Click here to download high resolution image

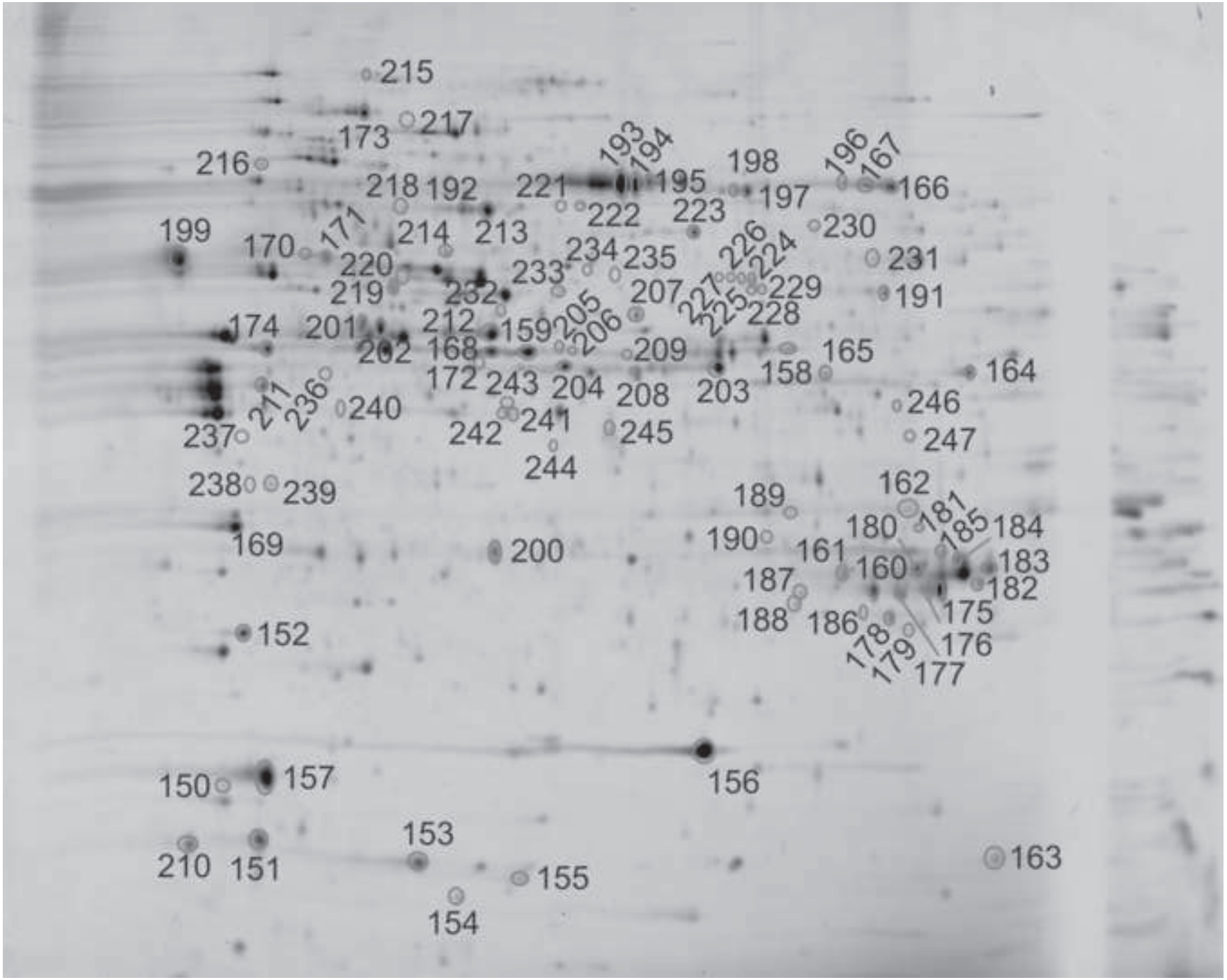


Figure
Click here to download high resolution image

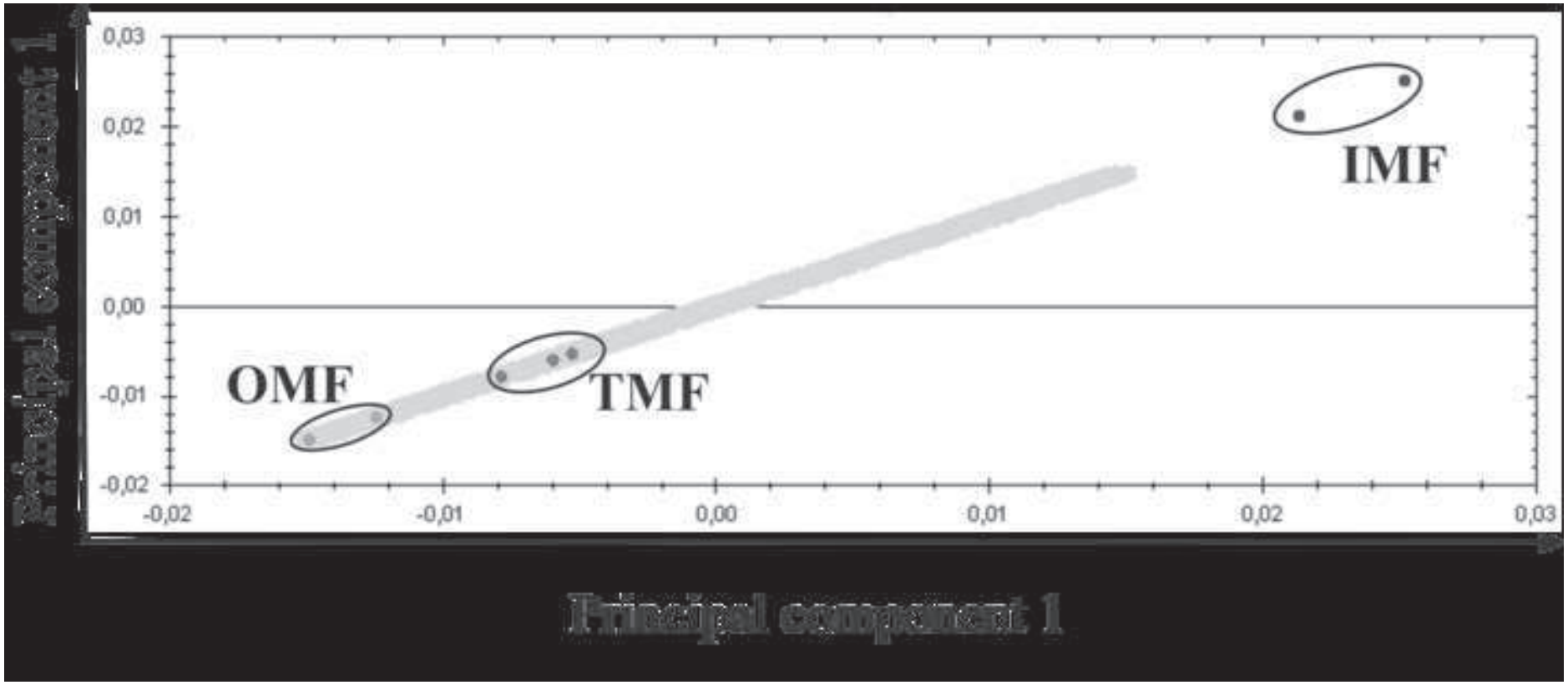
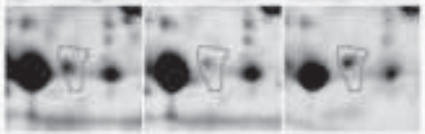
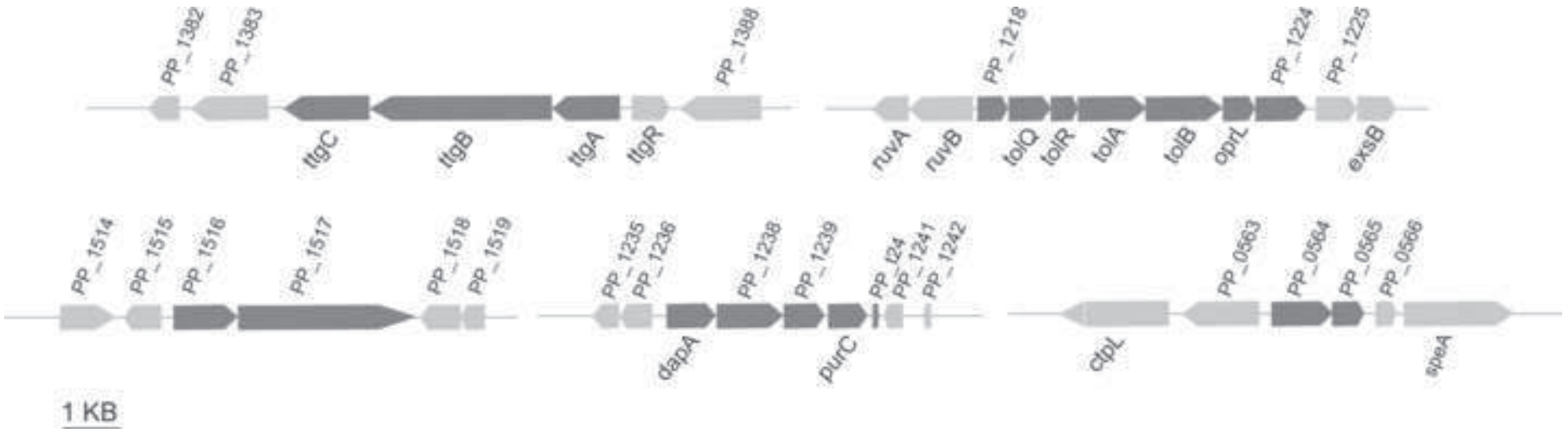


Figure
Click here to download high resolution image

Abundance variation pattern	Description	Number of proteins in pattern			Example			
		TMF	OMF	IMF	Protein name	Ctrl	P1	P5
Ctrl P1 P5								
	Ctrl = P1 < P5	50	35	25	OprH			
	Ctrl = P1 > P5	26	12	8	Protein PP_4056			
	Ctrl < P1 = P5	72	39	137	OmpA family protein			
	Ctrl < P1 < P5	27	32	47	TtgA			
	Ctrl > P1 = P5	64	64	168	IlvE			
	Ctrl > P1 < P5	54	6	10	OprB			
	Ctrl > P1 > P5	32	22	41	Protein PP_1357			

Figure

Click here to download high resolution image



Supplementary material

Click here to download Supplementary material: Supplementary material_Roma-Rodrigues_2009.doc