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2D-PAGE Database for Studies on Energetic Metabolism of the Denitrifying Bacterium *Paracoccus denitrificans*

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1. Introduction

The gram-negative soil bacterium *Paracoccus denitrificans* is a chemoorganotroph and a facultative chemolithotroph, capable of using the oxidation of molecular hydrogen, methanol or thiosulphate as sole source of energy for autotrophic growth. Many different organic compounds serve as sole carbon source, the metabolism is, however, always respiratory and never fermentative. *P. denitrificans* synthesizes three distinct terminal oxidases (aa_3-type and cbb_3-type cytochrome c oxidases and ba_3-type quinol oxidase) during aerobic growth (Fig. 1). Under limited oxygen concentration, it can produce four additional terminal oxidoreductases for stepwise anaerobic conversion of nitrate to nitrogen gas (denitrification): nitrate reductase, nitrite reductase, nitrous oxide reductase and nitric oxide reductase (Fig. 2). Synthesis of these enzymes is tightly controlled at the transcription level: (i) globally according to an energetic hierarchy and (ii) on the level of the individual genes. As a result, a proper balance in the concentration and activity of these reductases is achieved and the cytotoxicity of the toxic intermediates of denitrification, nitrite and nitric oxide, is eliminated (Zumft 1997). The major players in the mentioned regulatory network are three members of the FNR (fumarate and nitrate reductase regulatory) protein family of transcription regulators. Upon activation by their corresponding signals, they bind to specific sites (FNR boxes) in target promoters upstream/downstream of the σ factor binding site and destabilize/stabilize the RNA-polymerase transcription initiation complex. The first regulatory protein is FnrP which has a [4Fe-4S] cluster for oxygen sensing, the second is
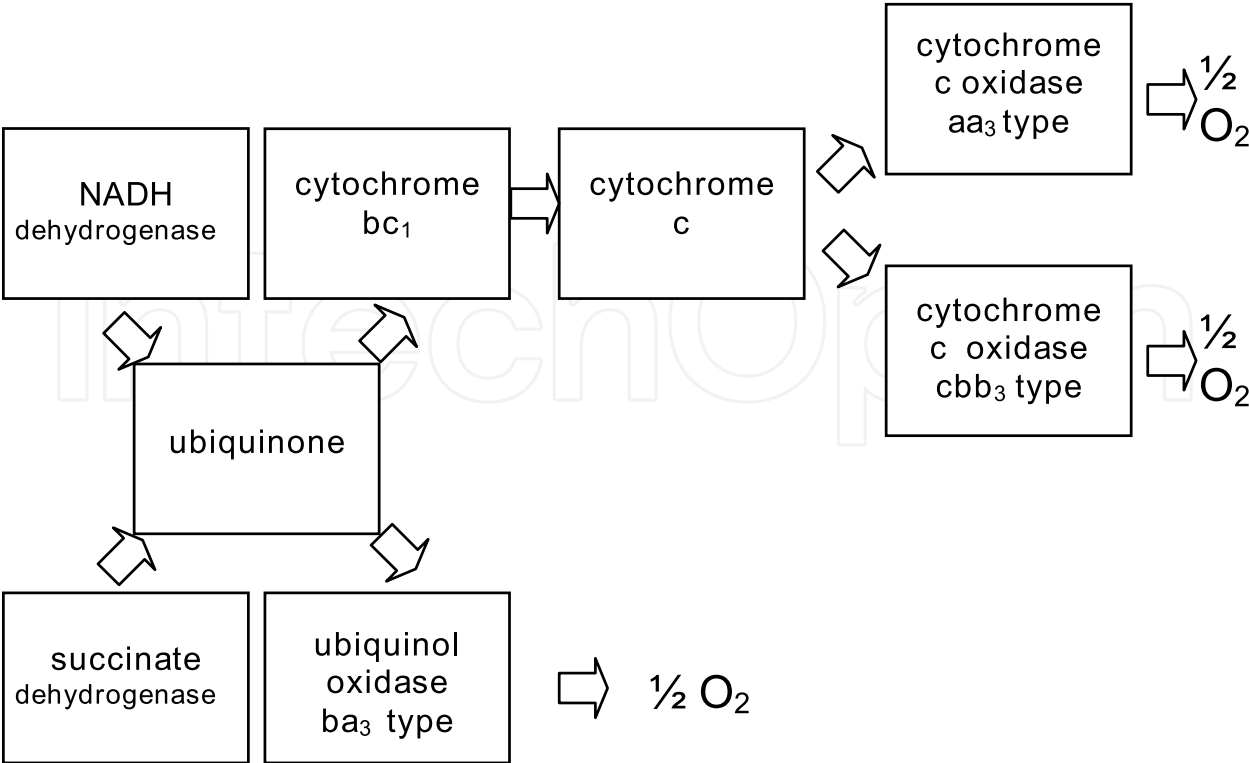
Fig. 1. Major components of *P. denitrificans* respiratory chain. Three distinct terminal oxidases are synthesized depending on environmental factors (*e.g.*, oxygen tension). The arrows indicate the electron flow. Inhibition of terminal oxidases with azide led to elevation of superoxide dismutase as revealed using a proteomic approach and confirmed at transcript and enzyme activity level (Bouchal *et al.* 2011).
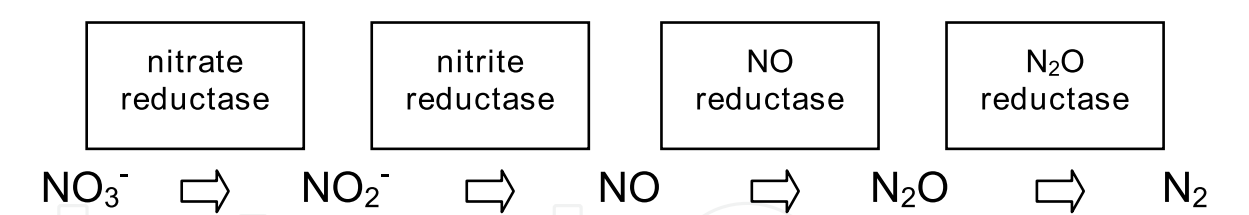

Fig. 2. Schema of *P. denitrificans* anaerobic denitrification pathway. Nitrate reductase β-subunit, nitrite reductase and nitrous oxide reductase can be detected and quantified using a proteomic approach (Bouchal *et al.* 2004; Bouchal *et al.* 2011). The expression of the denitrification enzymes is tightly controlled by FnrP, NNR and NarR transcription regulators at transcription level.

NNR, which has a heme for NO sensing and the third one is NarR which is poorly characterized and likely to be a nitrite sensor (Van Spanning *et al.* 1997; Wood *et al.* 2001). In response to oxygen deprivation, FnrP controls expression of the *nar* gene cluster encoding nitrate reductase, the *cco*-gene cluster encoding a *cbb₃*-type oxidase for respiration at low oxygen concentrations and the *ccp* gene encoding cytochrome *c* peroxidase. NNR specifically controls expression of the gene clusters encoding the nitrite (*nirS*), and nitric oxide (*norCB*) reductases and, to a certain extent, nitrous oxide (*nosZ*) reductase. NarR is required for transcription of the *nar* gene cluster in an unknown interplay with the FnrP protein (Wood *et al.* 2001; Veldman *et al.* 2006). These properties have been deduced from a

number of studies on each of these transcriptional activators, but knowledge on the interplay between these regulators along with their position in the complete regulatory network are scarce.

Given its metabolic versatility, this bacterium becomes an excellent model system to study the mechanisms of cellular responses to different environments.

2. *P. denitrificans* proteome analysis: Method development

Our group gained extensive experience in studying *P. denitrificans* physiology, reaching from measurement of enzyme activities to characterization of the role of individual proteins, *e.g.*, pseudoazurin (Koutny & Kucera 1999), nitrate transporter (Kucera 2003) and ferric iron reductases (Mazoch *et al.* 2004; Sedlacek *et al.* 2009). New proteomic technologies have been employed in our research since 2001 when we used a two-dimensional gel electrophoresis (2D-PAGE) with carrier ampholytes for the first time. Using this technique, we obtained good separations of about 150 proteins present in membrane fraction, allowing a comparison of its protein composition with the periplasmic fraction (Bouchal & Kucera 2004), see also Fig. 3. However, the capacity of original sample preparation procedure (Bouchal & Kucera 2004) led to difficulties with identification of less intensive protein spots.
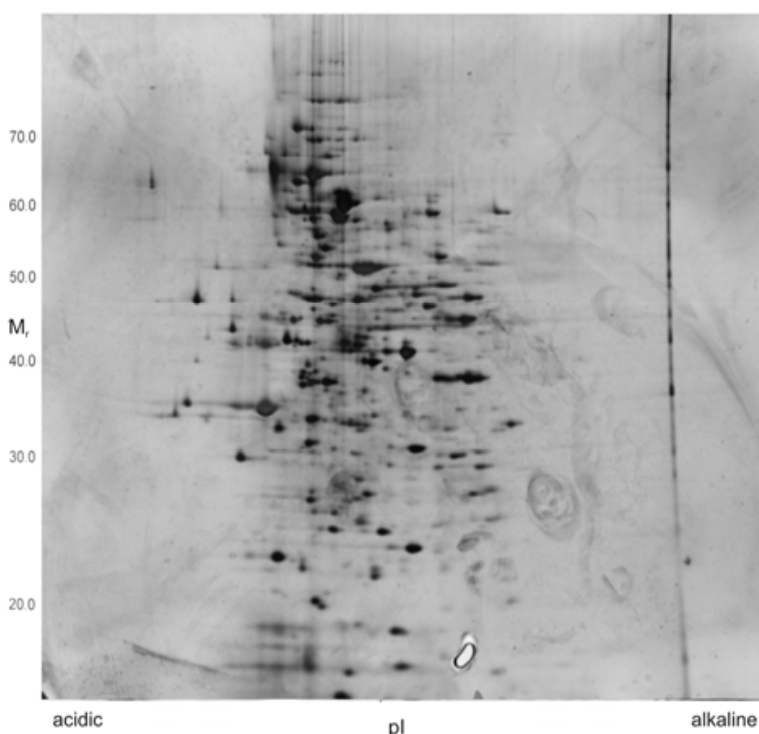

Fig. 3. A separation of *P. denitrificans* periplasmic proteins with 2D-PAGE based on carrier ampholytes–isoelectric focusing in the first dimension (Bouchal & Kucera 2002), with permission.

After establishing the mass spectrometry laboratory in 2002, we optimized new methods for the proteome analysis of *P. denitrificans* using immobilized pH gradients. See next paragraphs for complete protocols and Fig. 4 for a typical 2-D proteome map. Namely, the mass spectrometric analysis opened the way towards high-throughput and precise protein identification and valid conclusions made based on proteomics data. Matrix-Assisted Laser
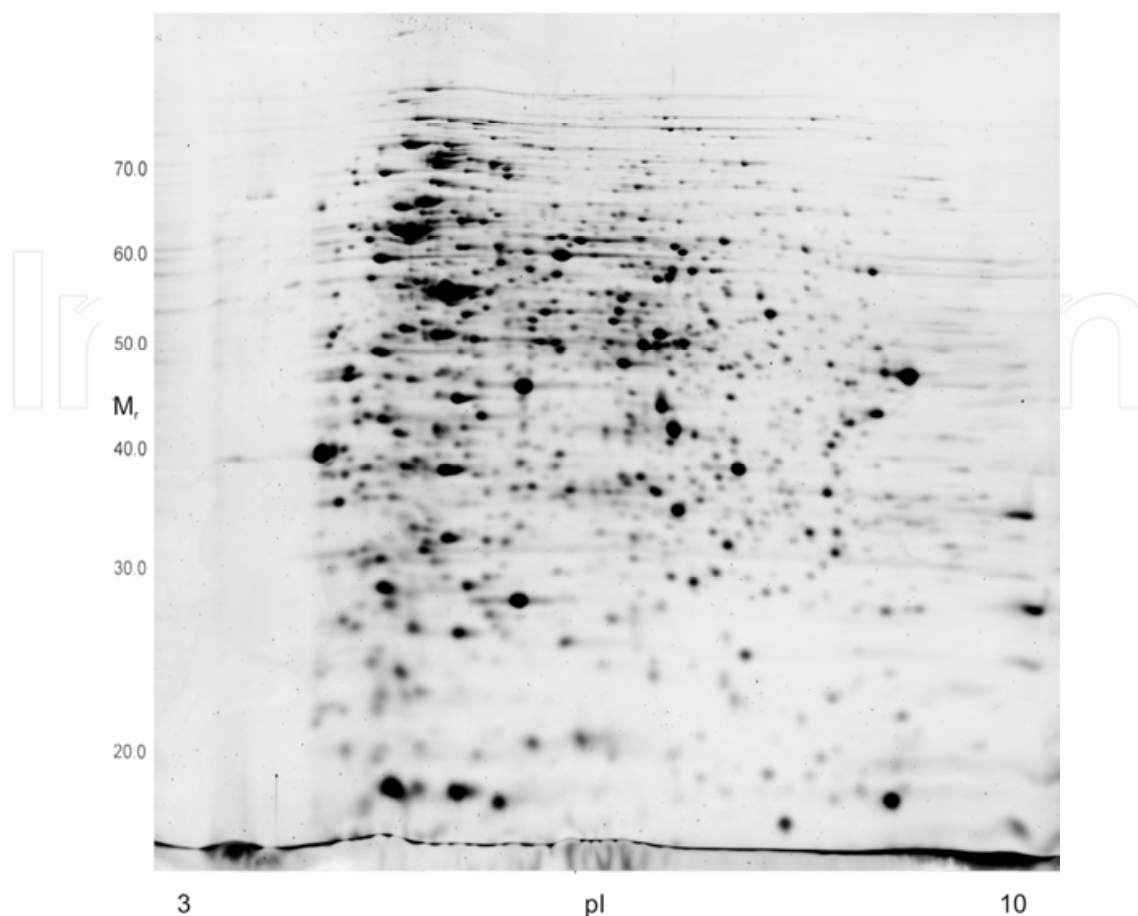
Fig. 4. Current stage of *P. denitrificans* proteomics approach: 2D-PAGE map (20 cm x 20 cm size) prepared with non-linear immobilized pH gradients (pH 3-10 NL) and Sypro Ruby staining has been used for both quantitative analysis and mass spectrometry protein identification.

Desorption-Ionization Mass Spectrometry (MALDI-MS) was used in initial proteomic studies identifying proteins exclusively by peptide mass fingerprinting. In addition, sensitivity of our MALDI-MS instrumentation of that time was not sufficient in case of weak protein spots. Since 2007, we have started to identify proteins using tandem mass spectrometric techniques, MALDI-MS/MS and ESI-MS/MS (concretely, capillary liquid chromatography – ion trap mass spectrometry with electrospray ionization), which resulted in more reliable protein identification based on MS/MS data. To improve the sensitivity of our LC-MS/MS system, we introduced nano-scale LC separation in 2008. At present, practically each analysis of protein spots leads to positive identification, involving sensitive fluorescent staining (Sypro Ruby). Subsequently, several comprehensive proteomic experiments were performed during the years using our proteomic platform in order to study the differences in protein composition caused by the growth on different terminal electron acceptors in both total cell lysates and membrane fractions (Bouchal *et al.* 2004). An additional large proteomic study with data confirmation at transcript level was performed to describe the regulons of three FNR-type transcription regulators FnrP, NNR and NarR at protein level (Bouchal *et al.* 2010). Quantitative and statistical image analysis primarily resulted in creation of local database files in a PDQUEST format. Subsequently, we decided to publish the 2-D maps in a web form to make all details accessible to other researchers on-

line *via* the "Proteome Database System for Microbial Research at Max Planck Institute for Infection Biology" in Berlin, Germany as described below.

3. Optimized methods used for *P. denitrificans* proteomics

3.1 Bacteria and culture conditions

Four strains of *P. denitrificans* were used in the published studies: Pd1222 (wild type), Pd2921 (FnrP mutant (Van Spanning *et al.* 1997)), Pd7721 (NNR mutant (Van Spanning *et al.* 1995)) and Pd11021 (NarR mutant, unpublished data). These four strains were cultivated at 30 °C in 1 l bottles filled with 0.5 l cultures with a starting optical density at 600 nm of 0.01, under the three following growth conditions: (i) aerobically at 250 rpm up to an optical density of 0.6, (ii) semiaerobically at 100 rpm up to an optical density of 1.0 and (iii) semiaerobically with nitrate at 100 rpm up to an optical density of 1.0. The minimal medium was composed of NH_4Cl (30 mM), sodium succinate (25 mM), Na_2MoO_4 (0.6 mM), $MgSO_4$ (0.4 mM), EDTA (0.25 mM), Lawford trace solution (1 mL/L) and potassium phosphate (65 mM, pH 7.0); KNO_3 (100 mM) was added in the case of cultivations in the presence of nitrate. Each culture was grown in three biological replicates, and as such we availed of a set of 36 independently grown *P. denitrificans* cultures. Cells were harvested by centrifugation (6 200 x g, 30 min), washed with 50 mM tris(hydroxymethyl)aminomethane/HCl (Tris/HCl) pH 7.3 and stored as a pellet at -80 ˚C.

3.2 Sample preparation and two-dimensional gel electrophoresis

After cultivation, the cells were disrupted by sonicating 15 mg (wet weight) of pellet for 30 x 0.1 s (50 W output) in 300 µL of lysis buffer containing 7 M urea, 2 M thiourea, 1% (w/v) (3-((4-Heptyl)phenyl-3-hydroxypropyl)dimethylammoniopropanesulfonate) (C7BzO), 40 mM Tris-base, 70 mM dithiothreitol (DTT), 2% (v/v) Pharmalyte 3/10, 5 mM NaF, 0.2 mM $NaVO_3$, CompleteMini Protease Inhibitor Cocktail (Roche, Penzberg, Germany, one tablet per 10 mL of lysis buffer) and 150 U of benzonase (Sigma-Aldrich, St. Louis, MO, USA). The cell extracts were incubated for 1.5 h at 20 ˚C. Cellular debris was then removed by centrifugation (16000 x g, 20 min, 15 ˚C) and the supernatant (total cell lysate) was stored at –80 ˚C.

For preparation of membrane fraction, cells were disintegrated and converted into membrane vesicles as previously described (Burnell *et al.* 1975) with several modifications. Briefly, the suspension of harvested cells was diluted with 5.7 volumes of a solution containing 0.5 M sucrose, 200 mM Tris/HCl pH 7.3, 0.5 mM EDTA and lysozyme (1 mg/ml of the total volume). After 45 min of enzymatic lysis at 30 ˚C, an osmotic lysis (45 min, 30 ˚C) was initiated by addition of equal volume of ice-chilled water. After centrifugation (4600 x g / 30 min/4 ˚C), the pellet (spheroplasts) was further lyzed (30 min/4 ˚C) in 7.5 volumes of chilled water containing a trace of DNAse and 14 mM $MgSO_4$. The unbroken spheroplasts were sedimented at 4600 x g (30 min/4 ˚C) and the supernatant from this step was subjected to ultracentrifugation at 184000 x g for 40 min (4˚C) using Beckman L8-55M Ultracentrifuge with 45 Ti rotor (Beckman, USA). The collected membranes were resuspended in 50 mM Tris/HCl pH 7.3, utracentrifuged again and resuspended in the same buffer. 150 µg of protein for analytical gels or 1 mg of protein for micropreparative separations, respectively, were extracted using sample solution containing 7 M urea, 2 M thiourea, 1 % (w/v) 3-[N,N-dimethyl(3-myristoylaminopropyl)ammonio]propanesulfonate (ASB 14), 1 % (v/v) TRITON X-100, 2 mM tributylphosphine, 15 mM Tris base, 1 % (v/v) Pharmalyte 3/10 and 0.5 % (v/v) Pharmalyte 8/10 for 1.5 h at 20 ᵒC.

The protein content was determined by RC-DC Protein Assay (Bio-Rad, Hercules, CA) with BSA as a standard. Bio-Rad 2-D standards were added for determination of approximate M_r and pI.

Aliquots containing 150 µg of protein for analytical purposes or 400 µg of protein for micropreparative separation, respectively, were precipitated overnight with 7.5 volumes of acetone containing 0.2 % (w/v) DTT at -20 ˚C. After washing the pellets again in the same solution, the samples were resolubilized in 350 µL of rehydration solution containing 7 M urea, 2 M thiourea, 1 % (w/v) C7BzO, 40 mM Tris-base, 70 mM DTT and 2 % (v/v) Pharmalyte 3/10 by incubating at 20 ˚C for 1 h. The samples were centrifuged again (16000 x g, 20 min, 15 ˚C) before loading on 18 cm nonlinear immobilized pH gradients (IPG) 3-10 (Bio-Rad, Hercules, CA) by in-gel rehydration.

Proteins were separated by isoelectric focusing using PROTEAN IEF Cell (Bio-Rad). The voltage was varied from 100 V (100 Vh, rapid), 500 V (500 Vh, linear), 1000 V (1000 Vh, linear) to 8000 V (95000 Vh, rapid), subsequently. The paper electrode wicks were changed 10 times during the first 10 kVh (the anodic wicks were soaked with water and the cathodic ones with 50 mM DTT). The IPGs were stored frozen at –80 ºC.

The IPG strips containing total cell proteins were equilibrated for 12 min in a solution containing 6 M urea, 30 % (v/v) glycerol, 2 % (w/v) SDS, 50 mM Tris/HCl pH 8.8, trace of bromphenol blue and 1 % (w/v) DTT and then for a further 12 min in the same buffer except that DTT was replaced with 2.5 % (w/v) iodoacetamide. The IPGs were then embedded onto SDS-PAGE gels (20 cm x 20 cm in size, 1 mm thick) using 0.5 % (w/v) low-melting agarose in Laemmli electrode buffer. In the second dimension, homogenous (12 % T, 1.07 % C) SDS-PAGE gels, Laemmli buffer system (Laemmli 1970) and PROTEAN Plus Dodecacell were used. After an initial ramp up period of 2 h at 50 V, the gels were run at 100 V for about 20 h at 4 ºC. The gel patterns were visualized by tetrathionate-silver nitrate staining (Rabilloud 1992) for analytical purposes or by SYPRO Ruby (Molecular Probes) in the case of micropreparative separations according to manufacturer's instructions. GS-800 and Pharos FX Pro instruments (Bio-Rad) were used for gel scanning. Spot detection, background subtraction, spot matching and data normalization using a local regression model method were performed using PDQUEST 8.0 software.

3.3 Statistical analysis of 2D-PAGE data

The normalized data exported from PDQUEST 8.0 were analyzed as follows: Values estimated by threshold level were excluded from the analysis. To reveal differences between groups, significance analysis of microarrays (SAM) (Tusher *et al.* 2001) was performed if there were at least 3 replicates in each of the compared groups. Proteins were considered as significantly differentially regulated if the false discovery rate (FDR) did not exceed 10 % and if the mean quantitative change was higher than 2 (up-regulation) or lower than 0.5 (down-regulation). In order to visualize the effect of selected proteins, hierarchical clustering based on Spearman correlation was performed. Data analysis was performed in a R-2.8.1 environment for statistical computing (R_Development_Core_Team 2008). For SAM the "samr" package was used, and clustering was performed using the package "cluster".

3.4 Mass spectrometry analyses

Sypro Ruby-stained protein spots selected for MS analysis were excised from 2D-PAGE gels. After destaining, the proteins in the gel pieces were incubated with trypsin (sequencing grade, Promega) at 37 °C for 2 h (Havlis *et al.* 2003). Peptide mass fingerprinting and tandem

mass spectrometry (MS/MS) analyses were performed by matrix-assisted laser desorption-ionization mass spectrometry (MALDI-MS) with an Ultraflex III mass spectrometer (Bruker Daltonik, Bremen, Germany).

Sample preparation protocol for MALDI-MS employing α-cyano-4-hydroxycinnamic acid solution prepared according to Havlis (Havlis *et al.* 2003) used as the matrix in combination with AnchorChip target was used to enhance measurement sensitivity. The sample (1 µl) was mixed with matrix solution on the target in a 2:1 ratio. Peptide maps were acquired in reflectron positive mode (25 kV acceleration voltage) with 800 laser shots. Twelve dominant peaks within 700 – 3600 Da mass range and minimum S/N 10 were picked for MS/MS analysis employing laser induced dissociation – "LIFT" arrangement with 600 laser shots for each peptide. Known autoproteolytic products of trypsin were used for internal calibration of digested peptides. In absence of these products, an external calibration procedure was employed, using a mixture of seven peptide standards (Bruker Daltonik) covering the mass range of 1000 – 3100 Da. The Flex Analysis 3.0 and MS Biotools 3.1 (Bruker Daltonik) software were used for data processing.

In case of insignificant or negative results of the MS/MS ion search, tryptic digests were subjected to electrospray ionization liquid chromatography-tandem mass spectrometry (ESI-LC-MS/MS) analysis. LC-MS/MS experiments were accomplished on a high performance liquid chromatography system consisting of a gradient pump (Ultimate), autosampler (Famos) and column switching device (Switchos; LC Packings, Amsterdam, The Netherlands) on-line coupled with an HCTultra PTM Discovery System ion trap mass spectrometer (Bruker Daltonik). The column used for LC separation was filled according to a previously described procedure (Planeta *et al.* 2003). Prior to LC separation, tryptic digests were concentrated and desalted using PepMap C18 trapping column (300 µm x 5 mm, LC Packings). Sample volume was 15 µl. After washing with 0.1 % formic acid, the peptides were eluted from the trapping column using an acetonitrile/water gradient (4 µL/min) onto a fused-silica capillary column (320 µm x 180 mm), on which peptides were separated. This column was filled with 4-µm Jupiter Proteo sorbent (Phenomenex, Torrance, CA). The mobile phase A consisted of acetonitrile/0.1 % formic acid (5/95 v/v) mixture and the mobile phase B consisted of acetonitrile/0.1 % formic acid (80/20 v/v) mixture. The gradient elution started at 5 % of mobile phase B, and after 4 minutes, it was increased linearly from 5 % to 50 % during 55 minutes. The analytical column outlet was connected to the electrospray ion source via a 50-µm-inner diameter fused-silica capillary. Nitrogen was used as nebulizing as well as drying gas. The pressure of nebulizing gas was 15 psi. The temperature and flow rate of drying gas were set to 300 ºC and 6 L/min, respectively, and the capillary voltage was 4.0 kV. The mass spectrometer was operated in the positive ion mode in an m/z range of 300 – 1500 for MS and 100 - 3000 for MS/MS scans. Extraction of the mass spectra from the chromatograms, mass annotation and deconvolution of the mass spectra were performed using DataAnalysis 4.0 software (Bruker Daltonik).

3.5 Mass spectrometry data processing

MASCOT 2.0 (MatrixScience, London, UK) search engine was used for processing the MS and MS/MS data. Database searches were done against the translated genome sequence data of *P. denitrificans* downloaded from http://genome.ornl.gov/microbial/pden/, the last sequence version released in 2006). A mass tolerance of up to 30 ppm was accepted during processing MALDI-MS data for PMF and 0.6 Da during processing laser-induced

dissociation -"LIFT" data for MS/MS ion searches. For ESI-MS/MS data, mass tolerances of peptides and MS/MS fragments for MS/MS ion searches were 0.5 Da. Oxidation of methionine and carbamidomethylation of cysteine as optional and fixed modifications, respectively, and one enzyme miscleavage were set for all searches. Gene annotations are consistent with *P. denitrificans* genome database at http://genome.ornl.gov/microbial/pden/. Note: In the time of preparation of this chapter, the *Paracoccus denitrificans* genome database was just in the process of moving to a new address: http://genome.jgi-psf.org/parde/parde.download.ftp.html.

4. Web accessible 2D-PAGE dataset of *P. denitrificans* proteome

PDQUEST 8.0 software was used for image analysis of 2D-PAGE gels and handling and keeping all operational data in a local file. This software also served for gel calibration, spot numbering and quantitation. Local files in PDQUEST format contain 2-D maps of total cell lysates and membrane fractions annotated with spot numbers, experimental Mr/pI, gel ID numbers, identification status, MS identification mode, protein name and UniProt accession number.

These data have been submitted to the "Proteome Database System for Microbial Research at Max Planck Institute for Infection Biology" in Berlin, Germany where they are now stored in the "Proteome 2D-PAGE Database" subsection (http://www.mpiib-berlin.mpg.de/2D-PAGE/) (Mollenkopf *et al.* 1999). The "Proteome 2D-PAGE Database" currently contains 11146 protein identifications from 10975 spots and 3124 mass peaklists in 55 reference maps representing experiments from 26 different organisms and strains. The data were submitted by 104 submitters from 30 institutes from 13 nations. The aim of the PDBS is to share proteomics information in a readily manner with the scientific community as an invitation for data mining. Showing experimental data like MS peaklists and raw spectra leads to more transparency of the results. In addition, protein identification data are integrated with genomic, metabolic and other biological knowledge sources to increase the value of the primary data.

The frontend of the "Proteome 2D-PAGE Database", i.e. the website, is dynamically generated mainly by a combination of PERL and CGI, but also JAVA, PHP and R (http://www.r-project.org/) are used. The data in the backend are organized in a relational database under the control of MySQL (http://www.mysql.com) as database management system.

The user of the *P. denitrificans* 2D-PAGE dataset can find three 2-D maps of *P.denitrificans* proteome: (i) Coomassie-stained 2-D map of total cell lysate (Fig. 2, 26 proteins identified) , (ii) silver-stained map of membrane fraction (Fig. 3, 14 proteins identified) and (iii) Sypro Ruby-stained total cell lysate map (Fig. 4, 640 proteins identified). The third Sypro Ruby total cell lysate map was prepared with the latest technologies and protocols and contains the most comprehensive annotation, including the possibility of downloading the complete list of ORFs identified at proteome level.

The user of the database has several possibilities to highlight the MS analyzed spots according to MS analysis results: He/she can highlight (i) only identified spots (ii) only non-identified spots, (iii) all spots, and (iv) none spots. The spots with significant identification are marked with a red cross while the spots without identification are labeled with a blue cross. A zoom function is available; a detailed map view can be thus obtained. If the user moves the cursor over a cross, spot number and protein name appears. If he clicks on the
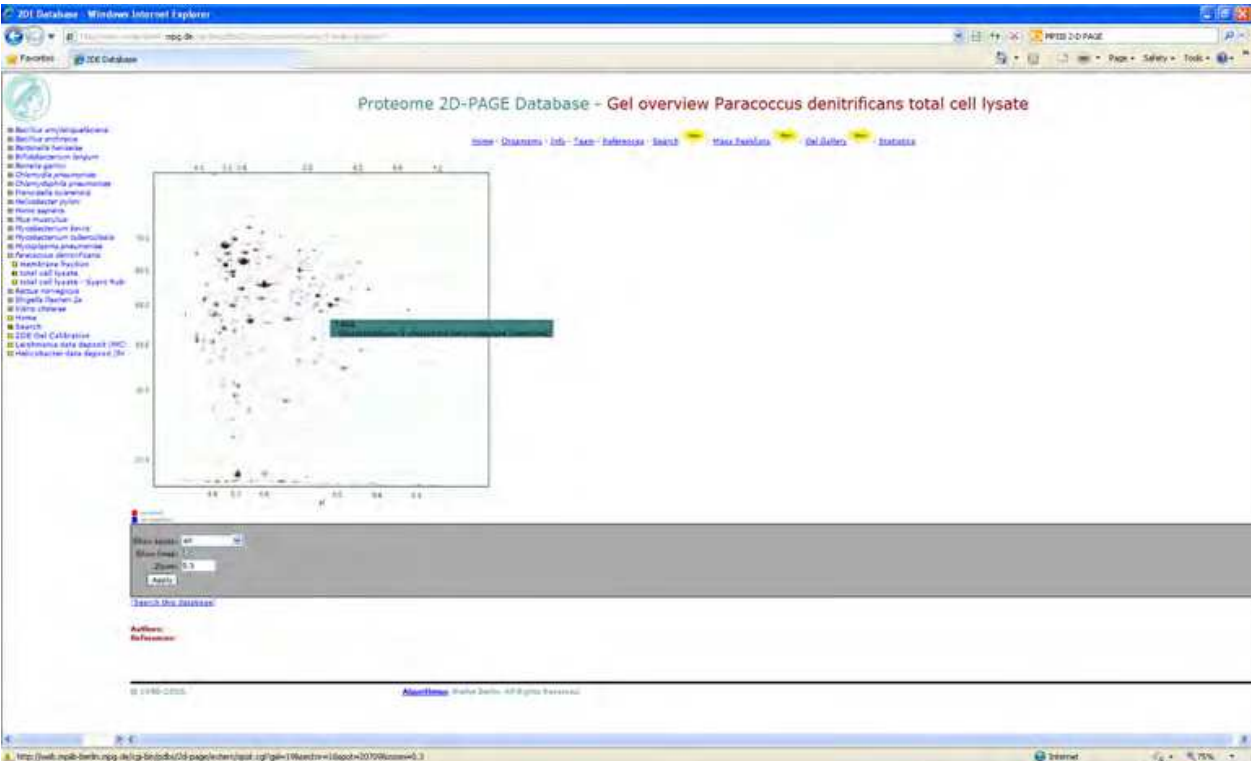
Fig. 5. The web browser window of *P. denitrificans* dataset in the 2D-PAGE database: total cell lysate 2D-PAGE map. The cursor-selected spot is annotated with its protein name.
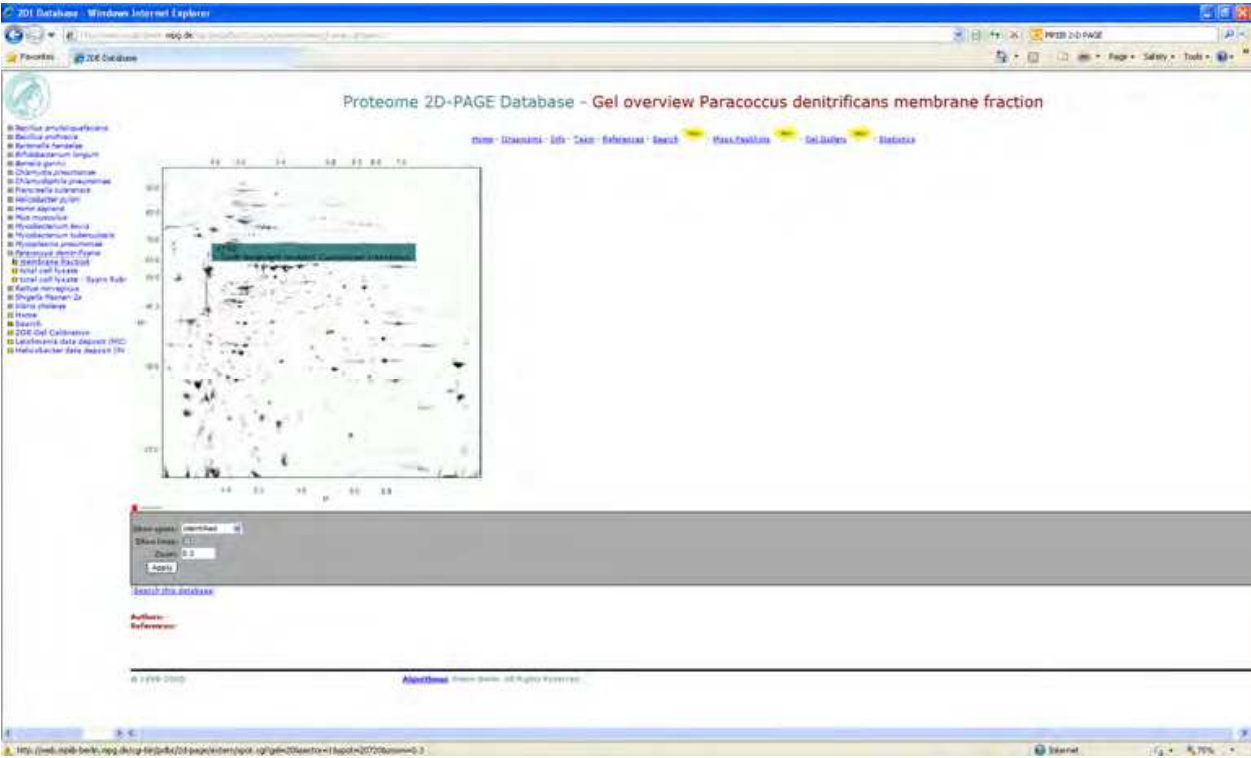

Fig. 6. The window of *P. denitrificans* dataset in the 2D-PAGE database: 2D-PAGE map of the membrane fraction.

Fig. 7. Sypro Ruby-stained total cell lysate map gel containing 640 identified proteins. The list of corresponding open reading frames identified by MS can be downloaded directly from this web page.

protein spot, the page with more detailed protein information is opened, including spot molecular weight, spot pI, UniProt accession number, gene locus, protein name, identification method, identification status and sequence coverage. If the visitor is interested in protein amount changes among different growth conditions, he can get detailed information in publicly accessible supplementary tables related to each published project.

Originally, only 8 proteins were identified by peptide mass fingerprinting from 49 analyzed spots within the first Coomassie-stained total cell lysate map and in membrane fraction map due to unknown sequence of *P. denitrificans* genome (Bouchal *et al.* 2004). Subsequent genome sequencing at Joint Genome Institute (http://www.jgi.doe.gov) resulted in public release of a *P. denitrificans* complete genome sequence in 2006. Thereafter the translated sequence data were transformed in the file applicable for MASCOT searches. Remaining 41 proteins were successfully identified using available MS spectra and the database gels were updated by new identifications. This substantial update underlines the significance of genome information on the effectivity of protein identification in proteomics.

5. *P. denitrificans* -omics projects facilitated *via* web accessible 2D-PAGE database

Because of the current progress in –omics methods and applications, it is expected that other laboratories interested in *P. denitrificans* biology will implement proteomic approach into their method toolboxes. The *P. denitrificans* web accessible dataset can help them with formulating their hypotheses, planning their experiments, orientation in their own proteome maps as well as facilitating communication among laboratories.

The perspective results coming from future studies on *P. denitrificans* are likely to be important from the bioenergetic point of view, providing a basis for understanding the well-known nutritional versatility of this bacterium. Since the rate of metabolic processes, in many cases depending on the substrate used, is often related to levels of involved proteins, identification of pivotal metabolic enzymes is one of the first tasks of proteomic research. From this point of view, the first important finding among the published results is the identification of proteins involved in denitrification. Nitrate reductase β-subunit, nitrite reductase and nitrous oxide reductase are the key enzymes of denitrification pathway and all of them were detected using proteomic approach in Sypro Ruby total cell lysate proteome maps as spots 5710 (nitrate reductase β-subunit), 1701 (nitrite reductase) and 2701 (nitrous oxide reductase).

Since the synthesis of denitrification enzymes is regulated by FNR-type transcription regulators (FnrP, NNR, NarR), looking for spot position of these regulatory proteins in 2-D maps is a next logical step of the expert user. Although their level is probably too low for detection on 2-D PAGE gels, an *UspA* gene product (gene 1849) as a direct neighbour of *fnrP* in the genome was detected in spot 3211 (Sypro Ruby 2-D map). Its expression profile was similar to *fnrP* gene product, suggesting their co-expression in single operone (Bouchal *et al.* 2010). Terminal oxidases (*aa3*, *cbb3* and *ba3* types in *P. denitrificans*, Fig. 4) are very hydrophobic proteins as most of their subunits contain more than one transmembrane domain (information obtained using PSORT algorithm, http://psort.nibb.ac.jp) and their identification on 2-D PAGE gels cannot be expected - for review, see (Santoni *et al.* 2000). On the other hand, we identified α, β and ε-subunits of F_0F_1-ATPase (spot 2103 in Coomassie-stained total cell lysate map and spots 0011, 0120, 0503, 1204, 1319, 5529 in a map of membrane fraction) being in many cases downregulated under anaerobic growth conditions as a probable result of general slowing down of the energetic metabolism. Among the proteins induced with azide (Bouchal *et al.* 2004; Bouchal *et al.* 2011) Fe/Mn superoxide dismutase (spot 6106 in Coomassie-stained total cell lysate map and spot 5001 in a map of membrane fraction) was identified, indicating generation of an increased amount of reactive oxygen species, possibly as a result of the increased degree of reduction of respiratory components. This was independently confirmed at transcript and enzyme activity level (Bouchal *et al.* 2011). Furthermore, synthesis of Fe/Mn superoxide dismutase is independent of FNR-type regulators (Bouchal *et al.* 2010). The only protein in membrane fraction induced synergically by nitrate and azide (spot 1702 in membrane fraction map) was TonB dependent receptor, a protein involved in iron transport. His ORF is located very close to preudoazurin gene, an alternative electron-transporter in a denitrification pathway. We also found glyceraldehyde-3-phosphate dehydrogenase (spot 7402 in Coommassie-stained total cell lysate map) non-affected with different growth conditions, so it could serve as an internal standard for gene expression comparison.

Our subsequent comprehensive study focused on FNR-type transcription regulators (Bouchal *et al.* 2010) revealed four significant protein clusters according to correlation of their levels under aerobic, semiaerobic and semiaerobic with nitrate growth conditions (see Fig. 8, spot numbers correspond to Sypro Ruby-stained proteome map): (i) The first cluster contains proteins involved in the FnrP regulon. It involves nitrous oxide reductase (spot 2701), UspA protein (spot 3211), and two OmpW proteins (spots 4107 and 5105) as well as two spots 501 and 8701 identified as unknown proteins. The direct regulation of nitrous oxide reductase, UspA and OmpW proteins by FnrP is a new finding from the mentioned study. (ii) Second cluster involves proteins regulated *via* additional regulators, including
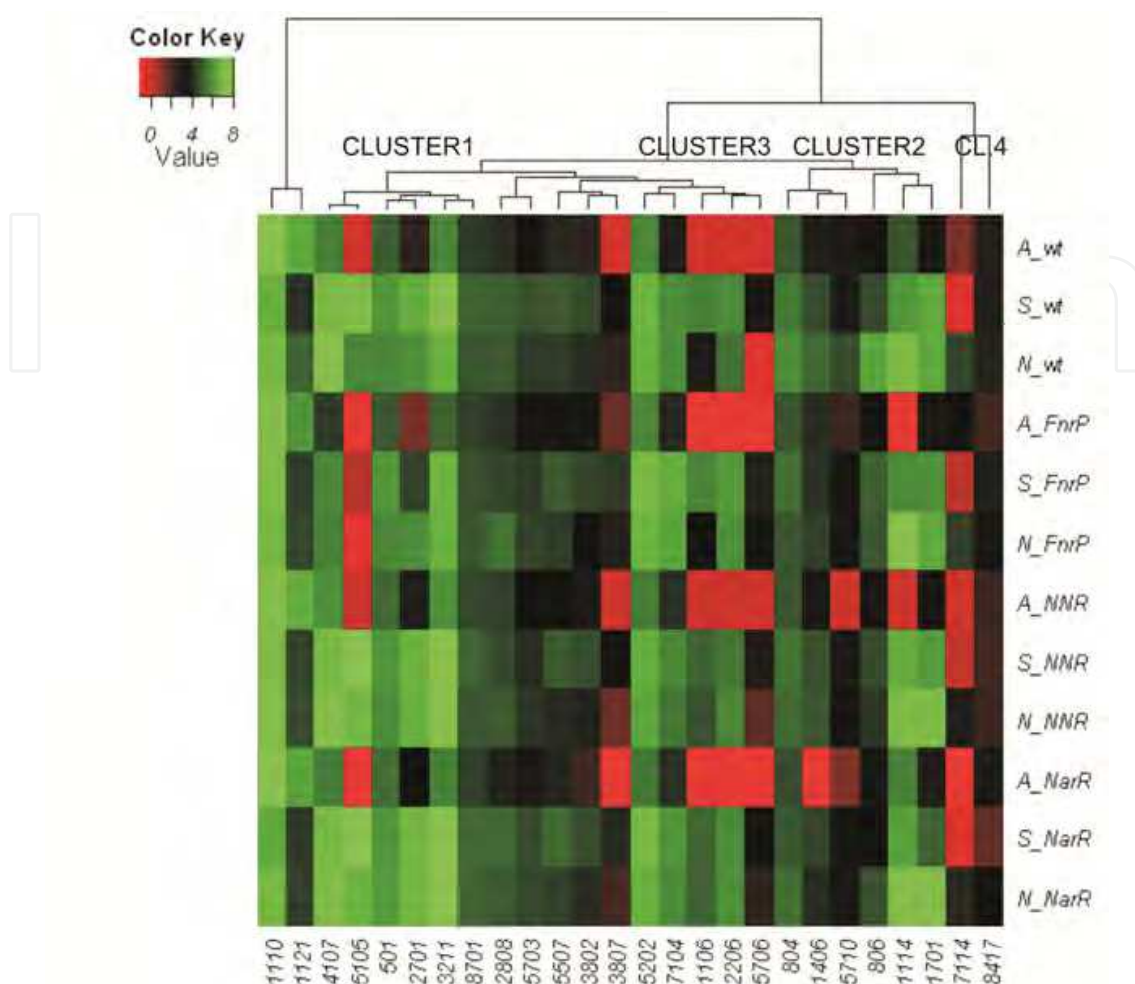
Fig. 8. The Spearman correlated clustering of the regulation profiles of proteins differentially regulated in response to mutation in FnrP, NNR and NarR transcription regulators and/or to oxygen and nitrate. See (Bouchal *et al.* 2010) for details.

proteins involved in NNR and NarR regulons. This cluster contains two TonB dependent receptors (spots 804, 806 and 1814), nitrate reductase β-subunit (spot 5710), a TenA-type transcription regulator (spot 1114), nitrite reductase (spot 1701) and an unknown protein with an alpha/beta hydrolase fold (spot 1406). The clustering of the TenA transcription regulator with nitrite reductase might well be indicative for the involvement of such an additional regulator. This clustering also indicates that ranking of the above mentioned proteins specifically under the NNR or the NarR regulon is less straightforward, probably since both regulators are activated by the reduction products of a common substrate, nitrate. (iii) The third cluster involves proteins whose amount is affected by the growth condition rather than by mutations in the FNR-type proteins. As such, these proteins may be part of a more global regulatory switch. This cluster contains SSU ribosomal protein S305 / σ54 modulation protein (spot 5202) and two SDR proteins (spots 2206 and 7104). (iv) The fourth cluster contains only the proteins specifically upregulated in cells grown semiaerobically in the presence of nitrate: one uncharacterized protein (spot 7114) and an ABC-type transporter of unknown function (spot 8417) (Bouchal *et al.* 2010).

6. Perspectives

In the upcoming time, development of the *P. denitrificans* 2-D PAGE dataset can continue in the following ways. (1) The identification of gel proteins spots not yet analyzed *via* mass spectrometry will continue, followed by immediate updates of the dataset. Resolution at the protein species level as obtained by 2D-PAGE-MS methods has in contrast to the bottom-up LC-MS approach the advantage to consider protein speciation (Jungblut *et al.* 2008) and will allow the analysis of protein species-specific regulation as already described within the phosphoproteome of *Helicobacter pylori* infected human stomach adenocarcinoma AGS cell line (Holland *et al.* 2011). (2) Implementation of new perspective analytical methods is in progress. 2DLC-MS/MS-based approaches with stable isotope labelling (iTRAQ, SILAC), or with label-free quantification can serve as a method complementary to 2D-PAGE-MS. These approaches are also helpful when identification of integral membrane proteins with non-membrane domains is required (Wu *et al.* 2003; Bouchal *et al.* 2009). (3) In the case of the most hydrophobic or low abundant proteins, non-proteomic approaches like qRT-PCR and cDNA chips are available for the study of gene expression. (4) Further progress of *P. denitrificans* genome information and annotation is expected. Direct accession from the *P. denitrificans* dataset in the 2D-PAGE database into *P. denitrificans* genome database would underline the integrity of genomic and proteomic data and facilitate finding the relations between protein level, gene location and gene function.

The data collection in the similar format raises the possibility of data comparison between different proteomes. During reading the communications about various microbial proteomic studies, one can be surprised how many identical (or very relative) proteins have been identified in different organisms, while other physiologically important proteins may be underrepresented in 2-D gels. It is more interesting if the number of theoretical ORFs in genomes is taken into account. It is obvious that protein hydrophobicity and solubility together with copy number plays an important role in these observations (Wilkins *et al.* 1998; Santoni *et al.* 2000; Jungblut *et al.* 2010). Using an integrated "Proteome 2D-PAGE Database" (http://www.mpiib-berlin.mpg.de/2D-PAGE/) covering a number of bacterial proteomes, it is easier to predict whether the protein of one's interest will, or will not be identified using proteomics approach and can be quantified this way. Such a tool is very useful for people making a choice of the best method for a screening of the gene(s) expression, or the protein(s) synthesis in *Paracoccus denitrificans* focused projects.

7. Conclusions

We feel that -omics approach is a powerful tool for a study of thousands of cellular genes and proteins in *P. denitrificans* and their variability between different growth conditions. Keeping in mind the principles of proteomics, it can be viewed as a screening tool able to reveal the specific changes, the detection of which would require significantly larger amount of work using classical approaches. With regard to nature of various environmental effects, „omics" approaches itself cannot provide final evidences for their mechanisms. However, the hypotheses obtained using this toolbox can provide a firm bases for targeted functional studies using integrated modern biochemical, bioinformatic and molecular-biological methodologies. We hope that our dataset within the 2D-PAGE database will be useful in designing such integrated *P. denitrificans* projects and in facilitating the international interlaboratory cooperation.

8. Acknowledgements

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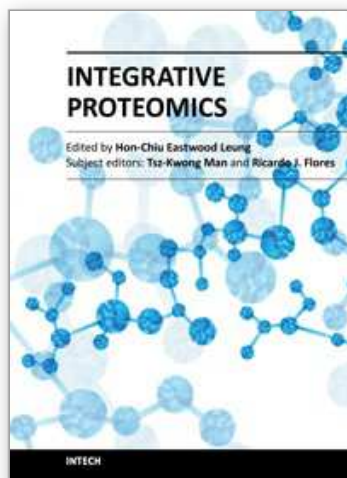
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Proteomics was thought to be a natural extension after the field of genomics has deposited significant amount of data. However, simply taking a straight verbatim approach to catalog all proteins in all tissues of different organisms is not viable. Researchers may need to focus on the perspectives of proteomics that are essential to the functional outcome of the cells. In Integrative Proteomics, expert researchers contribute both historical perspectives, new developments in sample preparation, gel-based and non-gel-based protein separation and identification using mass spectrometry. Substantial chapters are describing studies of the sub-proteomes such as phosphoproteome or glycoproteomes which are directly related to functional outcomes of the cells. Structural proteomics related to pharmaceutics development is also a perspective of the essence. Bioinformatics tools that can mine proteomics data and lead to pathway analyses become an integral part of proteomics. Integrative proteomics covers both look-backs and look-outs of proteomics. It is an ideal reference for students, new researchers, and experienced scientists who want to get an overview or insights into new development of the proteomics field.

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