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Unraveling an FNR based regulatory circuit in *Paracoccus denitrificans* using a proteomics-based approach

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ABSTRACT

The switch from aerobic to anaerobic respiration in the bacterium *Paracoccus denitrificans* is orchestrated by the action of three FNR-type transcription regulators FnrP, NNR and NarR, which are sensors for oxygen, nitric oxide and nitrite, respectively. In this work, we analyzed the protein composition of four strains (wild type, FnrP-, NNR- and NarR-mutant strains) grown aerobically, semiaerobically and semiaerobically in the presence of nitrate to discover the global role of FNR-family transcription regulators using proteomics, with data validation at the transcript and genome levels. Expression profiles were acquired using two-dimensional gel electrophoresis for 737 protein spots, in which 640 proteins were identified using mass spectrometry. The annotated 2-D proteome map provided the most comprehensive coverage of *P. denitrificans* proteome available to-date and can be accessed on-line at http://www.mpiib-berlin.mpg.de/2D-PAGE/. Our results revealed several types of regulation under the conditions tested: (1) FnrP-controlled regulation of nitrous oxide reductase, UspA and OmpW as confirmed at protein, transcript and DNA level (position of FNR boxes). (2) Proteins regulated via additional regulators, including proteins involved in NNR and NarR regulons: nitrate reductase β-subunit, TonB-dependent receptors, nitrite reductase, a TenA-type transcription regulator, and an unknown protein with an alpha/beta hydrolase fold. (3) Proteins whose expression was affected mainly by the growth condition. This group contains SSU ribosomal protein S305 / σ^{54} modulation protein, and two short-chain reductase–dehydrogenase proteins.

1. Introduction

Paracoccus denitrificans is a non-fermentative, facultatively autotrophic soil bacterium often studied in the field of bioenergetics, particularly due to the resemblance of its aerobic respiratory chain to that of mitochondria. This bacterium responds to a decreasing level of oxygen and the presence of nitrate in the environment by a switch from aerobic to a denitrification growth mode. The first step of denitrification is the reduction of nitrate to nitrite, which is then sequentially reduced via nitric oxide (NO) and nitrous oxide to dinitrogen gas [1]. *P. denitrificans* is one of the organisms that has the genetic potential to express all four key enzymes of this process, *i.e.* the nitrate, nitrite, nitric oxide and nitrous oxide reductases [2]. Expression of these enzymes is tightly controlled on the transcription level not only globally according to an energetic hierarchy but also on the level of the individual genes to ensure a proper balance in the concentration and activity of the reductases since the intermediate compounds of denitrification, nitrite and nitric oxide, are cytotoxic [3]. The major players in this regulatory network are three members of the FNR (fumarate and nitrate reductase regulatory protein) family of transcription regulators, which upon activation by their corresponding signals bind to specific sites (FNR boxes) in target promoters upstream of the σ factor binding site to stabilize the RNA-polymerase transcription initiation complex. One of the paralogues is FnrP which has a [4Fe–4 S] cluster for oxygen sensing, the second is 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 [4,5]. All three have dedicated tasks in gene regulation and cannot take over each other's role [6]. In response to oxygen deprivation, FnrP controls expression of the *nar* gene cluster encoding nitrate reductase, the *cco*-gene cluster encoding a cbb_3-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

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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 [4,7]. 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 is scarce.

The major aim of this study was to provide a more fundamental and global view on the regulatory network that orchestrates this important metabolic switch in *P. denitrificans* along with the role of the three FNR-type transcription regulators therein. A set of four *P. denitrificans* strains (wild type strain and the three mutant strains, FnrP-, NNR- and NarR-) was cultivated under three different growth conditions, aerobically, semiaerobically and semiaerobically with nitrate. The total cell lysates obtained were analyzed for their protein and mRNA profiles. Such an integrative approach enabled us to reveal expression differences at both protein and mRNA levels between strains. In addition, the *P. denitrificans* genome sequence was scanned for potential binding sequences for FNR-type regulators (FNR box) in DNA regions preceding all the genes. From the methodological point of view, our study provides the most comprehensive experimental information on *P. denitrificans* proteome via on-line accessible 2-D map.

2. Materials and methods

2.1. Bacteria and culture conditions

Four strains of *P. denitrificans* were used in the study: Pd1222 (wild type), Pd2921 (FnrP mutant [5]), Pd7721 (NNR mutant [8]) 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 till an optical density of 0.6, (ii) semiaerobically at 100 rpm till an optical density of 1.0 and (iii) semiaerobically with nitrate at 100 rpm till 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 (6200 × *g*, 30 min), washed with 50 mM TRIS/HCl pH 7.3 and stored as a pellet at −80 °C.

2.2. Sample preparation and two-dimensional gel electrophoresis

After cultivation, the cells were disrupted by sonicating 15 mg (wet weight) of pellet for 30 × 0.1 s (50 W output) in 300 µL of lysis buffer containing 7 M urea, 2 M thiourea, 1% (w/v) C7BzO (3-((4-Heptyl)phenyl-3-hydroxypropyl)dimethylammoniopropanesulfonate), 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 (16,000 × *g*, 20 min, 15 °C) and the supernatant (total cell lysate) was stored at −80 °C. The protein content was determined by RC-DC Protein Assay (Bio-Rad) with BSA as a standard.

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 (16,000 × *g*, 20 min, 15 °C) before loading by in-gel rehydration on 18 cm nonlinear immobilized pH gradients (IPG) 3–10 (Bio-Rad, Hercules, CA). Two-dimensional gel electrophoresis (2-DE) standards (Bio-Rad) were used for determination of approximate M_r and p*I*.

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 IPGs were stored frozen at −80 °C ([9] with several modifications).

Equilibration of the IPGs and SDS-PAGE protein separation in the 2nd dimension was performed using PROTEAN PLUS DodecaCell for total cell lysate proteins as described previously [10] except that the equilibration step was prolonged for 2 × 12 min. The gel patterns were visualized by tetrathionate-silver nitrate staining [11] 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.

2.3. Statistical analysis of 2-DE 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 [12]) was performed if there were at least 3 replicates in each of the compared groups. Proteins were considered as significantly differentially expressed if the false discovery rate (FDR) did not exceed 10% and if the mean quantitative change was higher than 2 (upregulation) or lower than 0.5 (downregulation). 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 [13]. For SAM the “samr” package was used, and clustering was performed using package “cluster”.

2.4. Mass spectrometric analysis

Sypro Ruby stained protein spots selected for MS analysis were excised from 2-DE gels. After destaining, the proteins in the gel pieces were incubated with trypsin (sequencing grade, Promega) at 37 °C for 2 h [14]. Matrix-assisted laser desorption-ionization mass spectrometry (MALDI-MS) and tandem mass spectrometry (MS/MS) analyses were performed on an Ultraflex III mass spectrometer (Bruker Daltonik, Bremen, Germany).

In case of insignificant or negative results of the MS/MS ion search, tryptic digests were subjected to liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis. LC–MS/MS experiments were accomplished on an 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 [15]. See Supplementary Table 1 for detailed conditions of the mass spectrometric analysis.

2.5. MS data processing

MASCOT 2.2 (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*