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The membrane complexome of a new *Pseudomonas* strain during growth on lysogeny broth medium and medium containing glucose or phenol

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ARTICLE INFO

Article history:
Received 12 December 2013
Received in revised form
8 April 2014
Accepted 9 April 2014
Available online 19 April 2014

Keywords:
Complexome
Membrane protein complexes
2-D native/SDS-PAGE
Mass spectrometry
Pseudomonas

ABSTRACT

In this study, we have performed a systematic analysis of *Pseudomonas* sp. *strain phDV1* membrane protein complexes by growing the strain in lysogeny broth medium, and medium containing glucose or phenol as sole carbon sources. In order to study the membrane complexome, we developed an approach for the extraction and the analysis of the membrane protein complexes in native conditions. Our strategy involves (a) enrichment of the membrane proteome from *Pseudomonas* sp. strain phDV1 by two washing steps; (b) solubilization using *n*-dodecyl-β-maltoside; (c) a combination of BN-PAGE with Tricine-SDS-PAGE; and (d) protein identification of tryptic peptides by mass spectrometry.

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] and using them as a carbon source [2]. A new *Pseudomonas* sp. strain phDV1 was isolated from enriched mixed culture from samples of petroleum contaminated soil in Denmark. The strain shows high phenol removal efficiency and proteomics studies of the water soluble sub-proteome of this strain have shown that phenol is metabolized via the *meta* cleavage pathway as a sole source of carbon and energy [3,4]. Recently, the profile of the membrane proteome was characterized by the analysis of soluble and insoluble sarcosyl fractions after growth with three different carbon sources [5]. The dSDS-PAGE map of the three carbon sources allowed the identification of inducible outer membrane proteins by growth in medium containing glucose or phenol.

Mass spectrometry based proteomics studies have emerged as a powerful tool to identify and characterize proteins on the individual amino acid level. Typically, the proteome is prefractionated by e.g. two-dimensional electrophoresis (2DE) and subsequently identified by mass spectrometry (MS), giving a two-dimensional map of soluble

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http://dx.doi.org/10.1016/j.euprot.2014.04.003

and membrane proteins and membrane protein complexes present in the sample representative for the growth conditions [6]. Although this strategy allows the identification of expressed proteins, only little information on the stoichiometry and functional composition of protein complexes can be derived. It is known that many cellular processes are carried out by multi-subunit protein machines. The identification and characterization of these multi subunit protein complexes, also termed complexome [7], is essential for comprehensive understanding of their functions in the cell [8]. This is particularly important in cellular membranes, where many well characterized proteins require assembly into functional complexes to perform important steps in energy metabolism, protein trafficking, and molecule transport. Our knowledge of protein complexes in cellular membranes is limited, mainly because membrane proteins are incompatible with commonly used protein interaction assays.

Protein complexes are maintained with weak bonds and need specific separation strategies to prevent protein dissociation. Blue native (BN)-PAGE has been established as an effective proteomics tool for the analysis of both soluble protein complexes and membrane protein complexes [9,10]. We used an anionic dye, Coomasie Blue (CBB G-250), that binds to the surface of all proteins and thus introduces a large number of negatively charged dye molecules to the proteins [9]. This binding facilitates the migration of the protein complexes in the first-dimensional electrophoresis (BN-PAGE). Due to the fact that the tendency of protein aggregation is reduced, the native protein complexes are separated according to their molecular weight. In a denatured, second-dimensional electrophoresis (SDS-PAGE) the interacting proteins of the native complexes can be visualized.

Although 2D BN/SDS-PAGE was developed for the separation of protein complexes belonging to the respiratory chain of mitochondria [9,11] and *Paracoccus denitrificans* cells [12,13], the method has been successfully applied to study the assembly of photosynthetic complexes of cyanobacteria [14], green sulfur bacteria [15], protein complexes of chloroplast membranes of plants [16] and green algae [17]. Furthermore the power of the method was demonstrated by the detection of protein complex deficiencies in mitochondrial membranes [14,18].

The aim of this study was to characterize the membrane complexome of a new *Pseudomonas* strain. In order to assess information about the protein composition of key membrane protein complexes involved in the biodegradation of aromatic compounds, we have grown the strain with different carbon sources. In this study, we present an optimized protocol for the isolation and solubilization of the membrane proteome. We analyzed the membrane complexome using 2DE (BN/Tricine-SDS-PAGE) and identified the protein subunits using a set of complementary MS-based techniques.

2. Materials and methods

2.1. Isolation and washing of membranes

The bacterium was cultured in LB medium or in a modified minimal medium as described in containing glucose or phenol at concentration of 200 mg/l [3]. Cultures were grown at 30 °C and the growth was followed by measuring the optical density at 600 nm [3,5]. The concentration of phenol in the culture was estimated by HPLC using a C18 columns at 254 nm after calibration with known concentrations. The isolation of the membrane proteome of *Pseudomonas* was performed according to Tsirogianni et al. [3]. Membrane-associated proteins were removed using 5 mM EDTA as described in [15]. To enrich further the membrane proteome, the resulted membranes were incubated with 0.1% (w/v) sodium lauryl sarcosinate solution at 4 °C for 20 min, followed by centrifugation at 100,000 × *g* for 1 h at 4 °C. The protein concentration in the resulted membrane fraction was determined according to Bradford [19].

2.1.1. Solubilization of membranes

Membranes were incubated for 30 min at 8 °C in solubilization buffer (50 mM Tris–H_2SO_4, pH 8.0) containing dodecyl-β-maltoside under gentle agitation. The detergent/protein ratio was 5:1, 10:1, and 15:1 and 30:1. Nonsolubilized proteins were sedimented at 50,000 × *g* for 90 min at 8 °C. Dodecyl-β-maltoside (DM) was obtained from Biomol (Hamburg, Germany).

2.2. Gel electrophoresis

2.2.1. First-dimensional electrophoresis (BN-PAGE)

The BN-PAGE was carried out in a gradient system 4–13% as described in Kantzilakis et al. [17]. The molecular weight estimation of the separated complexes was by using of the high molecular weight calibration kit for native electrophoresis (Amersham Biosciences), containing thyroglobulin 669 kDa, ferritin 440 kDa, catalase 232 kDa, lactate dehydrogenase 140 kDa, and albumin 66 kDa.

2.2.2. Second-dimensional electrophoresis (Tricine-SDS-PAGE)

Tricine-SDS-PAGE was performed according to Schaegger and von Jagow [20] using a self-built system with 10% acrylamide gels (26 cm × 24 cm × 0.1 cm) as described in [21]. Staining was carried out with 0.02% Coomassie Brilliant Blue G-250 in 10% acetic acid or colloidal Coomassie solution [22,23].

2.3. Protein Identification

2.3.1. In-gel digestion

The gel pieces were destained using sequentially 100 μL 50% ACN and 50 mM ammonium bicarbonate at least three times and subjected to the "in-gel digestion" protocol according to Shevchenko et al. [24]. The gel spots were reduced in 100 μL dithiothreitol (10 mM) at 56 °C for 1 h and then alkylated in 100 μL iodoacetamide (55 mM) at room temperature in the dark for 1 h. Subsequently the protein spots were digested overnight at 37 °C using a 10 mM ammonium bicarbonate/10% ACN buffer containing 0.125 μg of porcine pancreas trypsin (proteomics grade, Sigma, Steinheim, Germany). The extracts were collected in three pooling steps (1) nanopure water, (2) 50% ACN/0.1% TFA, and (3) 50% ACN. The samples were then dried and stored at −20 °C.

2.3.2. MALDI-TOF/TOF

MS and MS/MS experiments were performed using an ultrafleXtreme MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). Dissolved samples in 8 μL 50% acetonitrile, 0.1% trifluoroacetic acid were used for the co-crystallization with 8.33 mg/mL alpha-cyano-4-hydroxy-cinnamic acid at equal volume (1 μL). MALDI-TOF mass spectra were externally calibrated with a peptide mixture containing Bradykinin 1–7 (757.3992 Da), Angiotensin I (1296.6848 Da), Angiotensin II (1046.5418 Da), Substance P (1347.7454 Da), Bombensin (1619.8223 Da), Renin Substrate (1758.9326 Da), ACTH clip 1–17 (2093.0862 Da), ACTH clip 18–39 (2465.1983 Da), Somatostatin 28 (3147.4710 Da), yielding 30 ppm mass accuracy. MS measurements were performed applying the following criteria: 200 kHz smartbeam-II laser operated at 200 Hz repetition rate and accumulation of 1500 spectra in positive reflector mode by accelerating potential of 20.15 kV for the ion source 1 and 18.10 kV for the ion source 2 with 90 ns delayed extraction and output signal digitalization at 4 GHz. For measurements of MS/MS data, the LIFT mode was used employing standard manufacture's settings. Bruker Daltonics software "flexControl (v3.3)" was used for instrument operation and "flexAnalysis (v3.3)" for peak labeling. Resulting MS/MS spectra were used for automated protein identification by BioTools (v3.2) and a local MASCOT (v2.2) search engine using a data set containing the all *Pseudomonas* genomes download from NCBI on 01.06.2011. Matrix Sciences; parameters: enzyme: trypsin; modifications: carbamidomethylation of cysteins (fixed), oxidation of methionines (variable); missed cleavages: 1; peptide tolerance: ±30 ppm; mass values: MH+ and monoisotopic; only protein scores [P] as represented by the probability-based Mowse score were considered for significant protein identification (p value 0.05).

2.3.3. LC-nESI-MSn (Q-TOF)

For proteins not identified by MALDI-TOF/TOF, nano-HPLC-coupled ESI-MSn experiments were performed.

Proteolytic digests were loaded using a nano-HPLC (Proxeon easy-nLC) on reverse phase columns (trapping column: particle size 5 μm, C18, L = 20 mm; analytical column: particle size 3 μm, C18, L = 15 cm; NanoSeparations, Nieuwkoop, The Netherlands), and eluted in gradients of water (0.1% formic acid, buffer A) and acetonitrile (0.1% formic acid, buffer B). Typically, gradients were ramped from 5% to 55% B in 50 min at flowrates of 300 nl/min. Peptides eluting from the column were ionized online using a Advion Triversa NanoMate chip-based ion source (LC coupling mode, pos. ion mode, 2.5 μM nozzle size) and analyzed in a quadrupole time-of-flight mass spectrometer (Bruker maXis). Mass spectra were acquired over the mass range 50–2200 m/z, and sequence information was acquired by computer-controlled, data-dependent automated switching to MS/MS mode using collision energies based on mass and charge state of the candidate ions.

The data sets were processed using a standard proteomics script with the software Bruker DataAnalysis 4.0 Service Pack 1 Build 253 and exported as mascot generic files. Spectra were internally recalibrated on autoproteolytic trypsin fragments when applicable.

Proteins were identified by matching the derived mass lists against a *Pseudomonas* database (downloaded from http://www.ncbi.nlm.nih.gov/) on a local Mascot server (ver. 2.3, Matrix Science, UK). In general, a mass tolerance ±0.05 Da for parent ion and fragment spectra, two missed cleavages, oxidation of Met and fixed modification of carbamidomethyl cysteine were selected as matching parameters in the search program.

The 2D BN/SDS-PAGE and subsequent MS analyses were repeated twice with similar results.

3. Results and discussion

Bacterial biodegradation of hydrocarbons requires the passage of hydrophobic substrates across the cell membrane. The involvement of membrane proteins in the monoaromatic hydrocarbons uptake was demonstrated in vivo by the enhanced toluene metabolism upon expression of the *Pseudomonas putida* F1 TodX and *Ralstonia pickettii* PKO1 TbuX proteins [25,26]. Recently, proteomics studies indicate the presence of membrane proteins involved in the hydrocarbon degradation in a new *Pseudomonas* strain but until today no information are their protein interactions are available [5].

3.1. *Membrane sample preparation*

Pseudomonas cells were grown in LB and minimal medium containing glucose and phenol. The cells were harvested in the exponential growth phase. Similar to earlier reports in glucose, the cells have a faster growth-rate compared to phenol [3,21]. The bright-yellow coloration appeared in the culture containing phenol indicate the accumulation of 2-hydroxymuconic semialdehyde (HMSA). This coloration is typically observed upon phenol degradation via the catechol *meta*-cleavage pathway [3,4].

The first attempt in the present work was to develop an efficient method for the mapping of the membrane complexome from *Pseudomonas* using BN in the first dimension and Tricine-SDS-PAGE in the second dimension. To this end, first the membranes were treated with 5 mM EDTA in 50 mM Tris, pH 8.0, a method that had been successfully applied to remove ribosomal proteins in *Chlorobaculum tepidum* [15]. For further enrichment of the membrane proteome fraction we subsequently incubated this fraction with 0.1% lauryl sarcosinate, a concentration below its critical micelle concentration [27]. The purity of the treated membranes was investigated by separating the protein samples using Tricine-SDS-PAGE. As shown in Fig. 1, the protein patterns of the membrane fractions differ significantly depending on the growth conditions.

The washed membrane fraction was solubilized using a non-ionic, mild detergent compatible with BN-PAGE conditions [9]. The solubilization efficiency of DM was tested and the extracted membrane proteins were analyzed using Tricine-SDS-PAGE (Fig. 2A). The best result with respect to the amount of solubilized proteins was obtained in a ratio of 1:10 and 1:15 to the protein (Fig. 2A). In addition the solubilized membrane complexome was characterized by BN-PAGE (Fig. 2B). As Fig. 2B shows, distinct membrane protein complexes were only obtained by solubilizing the membrane with DM in a ratio of 1:15. In contrast, higher concentrations of DM

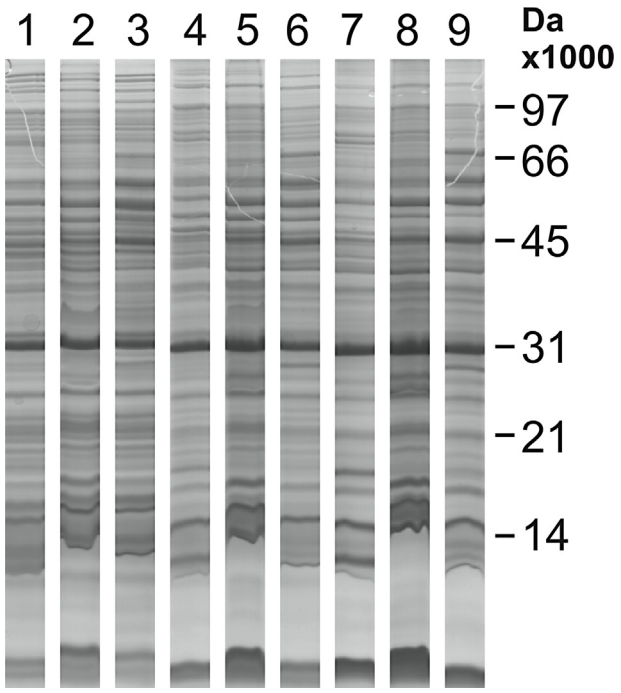

Fig. 1 – Tricine-SDS-PAGE. Membrane fraction of the bacterium grown in LB medium (lane 1), glucose (lane 2) and phenol (lane 3). Membrane fraction after treatment with 5 mM EDTA of the bacterium grown in LB medium (lane 4), glucose (lane 5) and phenol (lane 6). Membrane fraction after treatment with lauryl sarcosinate of the bacterium grown in LB medium (lane 7), glucose (lane 8) and phenol (lane 9).

interfered with the BN-PAGE separation, and no distinct bands were obtained. Thus, we employed DM in a 1:15 (1.5%, w/v) working ratio concentration according to its ability to resolve a satisfactory number of complexes in intact form. Further, it should be noted that each membrane protein complex may have different sensitivity to solubilization.

3.2. *Membrane protein complexes identification*

1-D BN-PAGE was combined with Tricine-SDS-PAGE to elucidate the identities and the subunit composition of the extracted membrane protein complexes (Fig. 3). For protein identification, gel spots were excised and identified by mass spectroscopy. From the three different carbon sources we identified 46 distinct proteins, which were categorized according to their predicted subcellular location: 50% cytoplasmic membrane proteins, 39.13% outer membrane proteins and only 6.52% cytoplasmic protein (Table 1). In a previous work on the same strain the rate of the cytoplasmic proteins was about 58% indicating the success of the membrane enrichment and the purification procedure [5]. In addition in this work the rate of the cytoplasmatic and outer membrane proteins was increase from 12% to 50% and from 12% to 39%, respectively [5].

3.2.1. *Common complexes*

The majority of the proteins complexes solubilized from the membrane proteome are involved in bioenergetic processes.

Succinate dehydrogenase is composed from the SdhA-D gene products and forms trimers with a predicted molecular mass of 355 kDa [28]. From the SDS gel, we identified the 66-kDa flavoprotein subunit SdhA (Q9I3D5) and the 26-kDa iron-sulfur protein SdhB (Q9I3D4). The two other subunits, SdhC (14 kDa) and SdhD (15 kDa) were not detected (Fig. 3). The SdhA and SdhB subunits were identified in the all carbon source in contrast to the previous study in which the SdhA was found only in phenol and the SdhB only in LB medium [5]. Based on the size of the succinate dehydrogenase complex in the BN gel, which indicates the presence of a complex of about 440 kDa, we conclude that the (SdhABCD) complex is intact.

The Na^+-translocating NADH:ubiquinone oxidoreductase (Na^+-NQR), analogous to mitochondrial Complex I, is involved

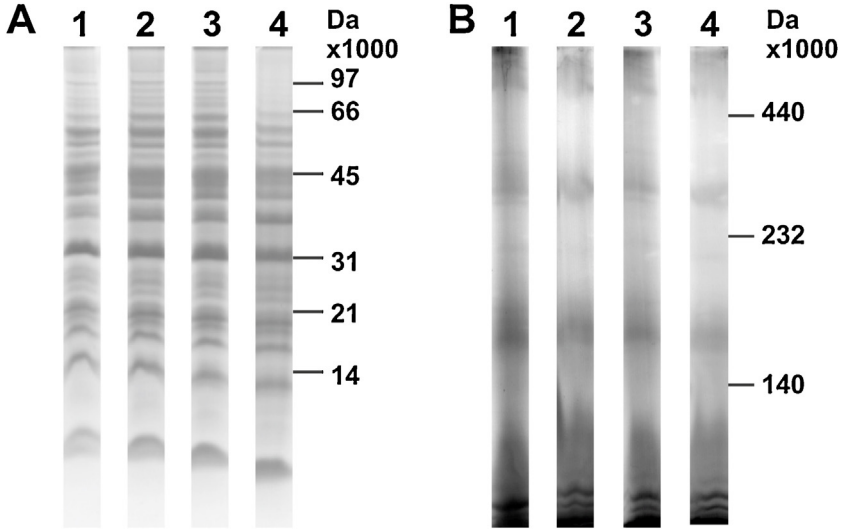

Fig. 2 – (A) Tricine-SDS-PAGE of the dodecyl maltoside soluble membrane protein fraction. (B) BN-PAGE of the dodecyl maltoside soluble membrane protein fraction. Ratios of detergent to protein: Lanes 1A and 1B 5:1. Lanes 2A and 2B 10:1. Lanes 3A and 3B 15:1. Lanes 4A and 4B 30:1.

Table 1 – Identified proteins. 1 LB, 2 carbon source glucose, 3 carbon source phenol. C, cytoplasmic; CM cytoplasmic membrane; OM, outer membrane.

	Accession no.	Identified protein accession no as in [5]	1	2	3	Method	MW	Localization
1	Q3KFU8	Dihydrolipoamide succinyltransferase	+			MALDI TOF/TOF	42251	C
2	A4XPY9	HflK protein	+	+	+	MALDI TOF/TOF	43072	CM
3	A4XPZ0	HflC protein	+	+	+	MALDI TOF/TOF	33007	C
4	Q88N55	Chaperonin GroEL (A4XYM0)	+	+	+	MALDI TOF/TOF	56765	C
5	A4XYP8	Hydrophobe/amphiphile efflux-1 (HAE1) family protein	+	+	+	MALDI TOF/TOF	113532	CM
6	Q9I3D5	Succinate dehydrogenase flavoprotein subunit	+	+	+	MALDI TOF/TOF	64062	CM
7	Q9I3D4	Succinate dehydrogenase iron-sulfur subunit (A4XV94)	+	+	+	MALDI TOF/TOF	26765	CM
8	A4XSP3	Na(+)-translocating NADH-quinone reductase subunit A	+	+	+	MALDI TOF/TOF	48406	CM
9	A4XSP8	Na(+)-translocating NADH-quinone reductase subunit F	+	+	+	MALDI TOF/TOF	46039	CM
10	A4XSP4	Na(+)-translocating NADH-quinone reductase subunit B	+	+	+	MALDI TOF/TOF	44120	CM
11	A4VMV2	Na(+)-translocating NADH-quinone reductase subunit C	+	+	+	MALDI TOF/TOF	28523	CM
12	A4XWT3	Surface antigen (D15)	+			MALDI TOF/TOF	87173	OM
13	A4XTL3	Acyl-CoA dehydrogenase	+	+	+	MALDI TOF/TOF	89054	CM
14	A4XU22	Hypothetical protein Pmen_2077	+			MALDI TOF/TOF	56668	CM
15	A4XNZ0	Protein of unknown function DUF513, hemX	+	+	+	MALDI TOF/TOF	41217	CM
16	A4XP25	Secretion protein HlyD family protein	+	+	+	MALDI TOF/TOF	38349	CM
17	A4XZJ1	Organic solvent tolerance protein	+	+	+	MALDI TOF/TOF	106928	OM
18	A4XU37	OmpF family protein	+	+	+	MALDI TOF/TOF	34795	OM
19	A4XVJ5	Cytochrome c oxidase, cbb_3-type, subunit II	+	+	+	MALDI TOF/TOF	22852	CM
20	Q883S8	Outer membrane lipoprotein OprI (O85420)	+	+	+	MALDI TOF/TOF	8865	OM
21	Q9HTV8	Hypothetical protein PA5232	+			MALDI TOF/TOF	38586	CM
22	A4XVR4	TonB-dependent siderophore receptor	+			MALDI TOF/TOF	87450	OM
23	A4VI97	Putative outer membrane receptor	+			MALDI TOF/TOF	80634	OM
24	A4XWV9	ABC-type uncharacterized transport system	+			MALDI TOF/TOF	67924	CM
25	Q4KAU8	Hypothetical protein PFL_3532	+	+	+	MALDI TOF/TOF	63925	OM
26	Q9HT18	F_0F_1 ATP synthase subunit alpha (A4Y189)	+	+	+	MALDI TOF/TOF	55486	CM
27	A4Y187	F_0F_1 ATP synthase subunit beta (A4Y187)	+	+	+	MALDI TOF/TOF	49641	CM
28	A4XZ52	MotA/TolQ/ExbB proton channel	+			MALDI TOF/TOF	48289	CM
29	Q4KF29	GGDEF domain-containing protein	+			MALDI TOF/TOF	58246	CM
30	A4XPB8	Amino acid carrier protein	+			MALDI TOF/TOF	53329	CM
31	A4XQQ7	Cytochrome c1-like protein	+			MALDI TOF/TOF	29304	CM
32	A4XS71	OmpA/MotB domain-containing protein	+	+	+	MALDI TOF/TOF	22156	OM
33	Q4KJP2	Outer membrane lipoprotein Blc	+			MALDI TOF/TOF	21974	OM
34	A4XRS7	OmpA domain-containing protein	+	+	+	MALDI TOF/TOF	17804	OM
35	A4VHP3	Outer membrane protein OprG	+	+	+	MALDI TOF/TOF	23928	OM
36	A4XWL6	Hypothetical protein Pmen_2977	+			MALDI TOF/TOF	21670	OM
37	A4XYP9	RND efflux system outer membrane lipoprotein		+		MALDI TOF/TOF	52181	OM
38	A4VFX8	TRAP-type mannitol/chloroaromatic compound transport system		+	+	MALDI TOF/TOF	43760	OM
39	A4VIG0	Ubiquinol—cytochrome c reductase, cytochrome b		+	+	MALDI TOF/TOF	45767	CM
40	A4XRF5	Porin, LamB type		+		MALDI TOF/TOF	45963	OM
41	A4XQ31	Hypothetical protein Pmen_0789			+	nLC ESI	78483	Unknown
42	A6EXX6	Hypothetical protein MDG893_16757			+	nLC ESI	50108	Unknown
43	A4VM31	Probable porin			+	nLC ESI	46953	OM
44	Q88GR2	Aromatic compound-specific porin, putative			+	nLC ESI	46672	OM
45	A4Y0D7	Outer membrane porin			+	nLC ESI	46667	OM
46	A4XQH7	Efflux transporter, RND family, MFP subunit (A4XYP7)			+	nLC ESI	41325	CM

in the respiratory chain of various bacteria [29]. The Na^+-NQR is composed of six structural genes (nqrA to nqrF). Four subunits were identified, namely nqrA (A4XSP3, 48 kDa), nqrB (A4XSP4, 44 kDa), nqrC (A4VMV2, 27.7 kDa) and nqrF (A4XSP8, 45.4 kDa). Two proteins in the same lane with a molecular weight in the range of 20 kDa were detected but not identified. Based on the size in the BN of about 240 kDa we conclude that the complex is an intact monomer. It should be mentioned that only the nqrF subunit (A4XSP8) was found only in glucose in the membrane subproteme analysis of the same strain [5].

Recently, the identification of the HflC (A4XPZ0) and HflK (A4XPY9) has been reported for the *Pseudomonas* strain growing in the three carbon source [5]. HflK and HflC are plasma membrane proteins of *Escherichia coli*. Both have a large C-terminal domain exposed to the periplasmic space and

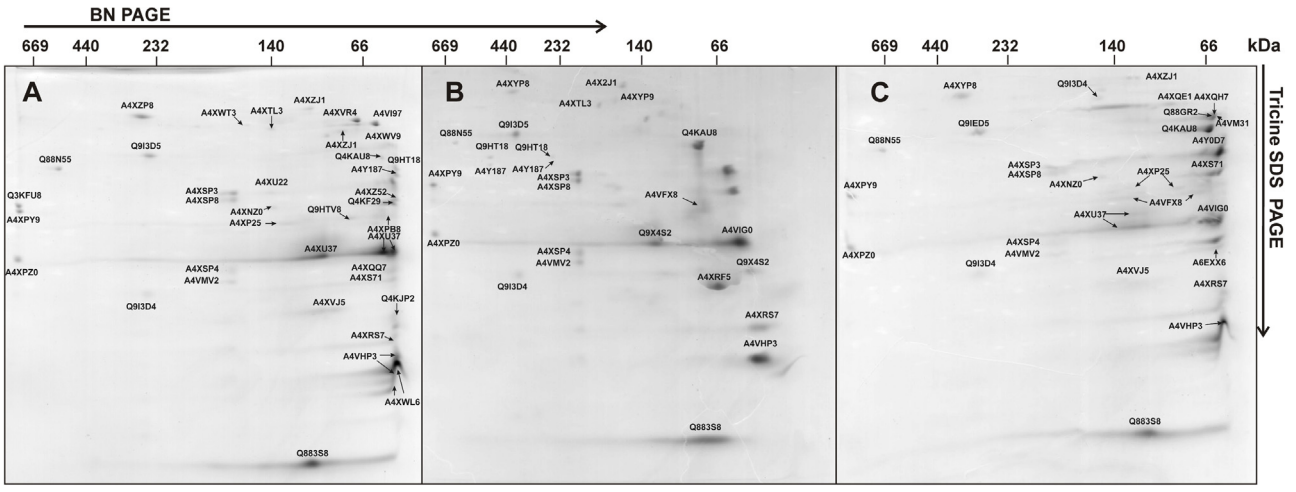

Fig. 3 – 2D BN/Tricine-SDS-PAGE pattern of the complexome of the bacterium grown in (A) LB, (B) glucose, and (C) phenol.

an N-terminally located transmembrane segment, which is thought to act as a signal anchor sequence for their biogenesis. They form a complex, HflKC, which acts as a 'modulator' of HflB. HflB, known as a 'quality control' protease, is an ATP-dependent metalloprotease and forms a ring-like complex. Six molecules of HflKC are presumably attached to HflB in a 1:1 molar ratio, forming a complex with an effective molecular weight of around 1000 kDa [30]. Although we have not detected HflB, based on the size of the HflKC complex in the BN gel (Fig. 3), we come to the conclusion that the $(HflKC)_6$–$HflB_6$ complex is formed.

The F_1–F_0 ATP synthase contains eight subunits arranged in two subcomplexes: the water soluble F_1 part (3α, 3β, γ, δ, ε) and membrane located F_0 part (a, b2, c10–14) [31]. Previous study has reported the identification of the α, β, γ and δ subunits from the F_1 subcomplex and the b subunit of the F_0 subcomplex [5]. Here, we detected two subunits of the F_1 complex, i.e. subunits α (Q9HT18) and β (A4Y187), but not γ, δ and ε. However, we did not detect any subunits of the F_0 complex. Based on the migration in the BN corresponding to a molecular mass of about 240 kDa, we assume that the F_1–F_0-ATP synthase was dissociated at the solubilization conditions and the complex at 240 kDa represents the F_1 complex without the F_0 complex.

In addition, we identified the chaperone GroEL (Q88N55) complex. This soluble complex is present in all growth conditions at a molecular weight of about 750 kDa, indicating the presence of the 14 subunit complex [32].

3.2.2. *Common proteins*

One of the most abundant protein in the outer membrane is the general diffusion porin, OmpF (A4XU37) [33]. OmpF (A4XU37) acts as a non-specific ion channel which allows small, polar molecules into the cell. Furthermore, OmpF acts as a porin and regulates osmotic pressure between the cell and its surroundings. We identified the 37-kDa OmpF (A4XU37) in a predominant complex that corresponds in molecular mass to a trimer in the BN gel (relative to the soluble markers), and we conclude that the native OmpF trimer is intact [33]. We found OmpF (A4XU37) in other bands in the SDS gel, but attribute this to potential dissociation, degradation or partial denaturation.

Another abundant membrane protein in the outer membrane is OmpA (A4XRS7), which is present at $>10^5$ copies per cell [34]. The main function of OmpA (A4XRS7) is to maintain the structural integrity of the cell surface. The OmpA (A4XRS7) protein was identified in the three carbon sources as a monomer which correlates to its reported monomeric form of this outer membrane protein. Additional to the OmpA protein an OmpA/MotB domain protein (A4XS71) has been identified in its monomeric state in the membrane proteome of the strain grown in LB and phenol (Fig. 3). In contrast in an earlier work the protein was found only in glucose [5]. This underlines the importance to apply complementary separation methods to study the same proteome.

OprG, a member of the OmpW family, is a major outer membrane protein [35]. Its expression is dependent on the growth conditions, suggesting a complex regulation. Recently, OprG expression was found to be increased under anaerobic conditions in the presence of iron [36]. On the other side, an *oprg* knockout strain showed that this protein is not involved in iron or antibiotics uptake [36]. Additionally, OprG from *P. putida*, was shown to have a high emulsifying activity and it was suggested that OprG plays a role for the utilization and uptake of hydrocarbons [37,38]. In our study we did not detect differential expression of OprG (A4VHP3) depending on the growth conditions. Furthermore, OprI (Q883S8), an outer membrane lipoprotein, has been identified in all carbon sources. It has been reported that OprI (Q883S8) is involved in the generation of large surface-exposed fusion proteins with enhanced immunogenicity, using its lipid tail [39].

The outer membrane protein LptD (A4XZJ1), an organic solvent tolerant protein, has an integral C-terminal β-barrel domain with a soluble N terminal domain [40]. LptD (A4XZJ1) is involved in membrane permeability and the assembly of LPS in the outer leaflet of the outer membrane. Although it has been suggested that LptD forms a high-molecular-weight complex in the outer membrane we found this protein in a monomeric form.

In addition, a putative uncharacterized protein (Q4KAU8) was identified in all carbon sources. Outer membrane proteins can be predicted using bioinformatics tools which allow the identification of a signal peptide sequence for translocation to the outer membrane, and the presence at the C-terminus of aromatic anchor residues [41,42]. Based on the presence of both in protein Q4KAU8, we suggest that this protein represents an outer membrane protein.

3.3. *Proteins found in two carbon sources*

3.3.1. *LB and phenol*

Cytochrome cbb_3 oxidases represent a distinctive class of proton-pumping respiratory heme-copper oxidases (HCO) and are found almost exclusively in proteobacteria. The proteins lack many of the structural features that contribute to the reaction cycle of the studied mitochondrial cytochrome c oxidase (CcO) [43]. Although the cbb_3-type cytochrome c oxidase is formed of CcoN, CcoO, CcoQ and CcoP subunits, in our study it was possible to identify only the CcoO (A4XVJ5) subunit (subunit II). This finding indicates the instability of the complex by the used solubilization conditions [44]. Another protein identified in both carbon sources is HlyD (A4XP25). The protein is a member of the Membrane Fusion Protein family and is proposed to span the periplasm, linking the inner and outer membranes [45]. Further, the HlyD (A4XP25) is involved in the export of a variety of compounds [46]. The obtained molecular weight of about 140 kDa in the BN is an indication that the protein exists not as monomer.

3.3.2. *Glucose and phenol*

Two proteins present in the glucose and phenol conditions belong to proteins involved in electron transport (A4VIG0) and in the TRAP-type transport system (A4VFX8).

3.4. *Proteins found in one carbon source*

Pseudomonas strains encode outer membrane channels involved in the uptake of aromatic substrates [47]. Recently, proteomic studies on the outer membrane subproteome allowed the identification of two outer membrane proteins, a membrane protein involved in aromatic hydrocarbon degradation (Q479D9) and a probable porin (A4VM31) [5]. In agreement with this study, an aromatic compound-specific porin Q88GR2 (Fig. 3C) has been identified after growing the strain in phenol.

Maltoporin (LamB), a β-barrel protein, assembles into a trimeric complex and allows permeation of sugars such as maltodextrins [48]. We identified the LamB protein (A4XRF5) only upon addition of glucose as carbon source and these results correlate to an earlier proteomics study of the outer membrane proteome of the same strain where the protein was identified only if glucose was provided as a carbon source [5]. The other protein present in the glucose only conditions belongs to proteins involved in the RND efflux system (A4XYP9). Furthermore, the proteins, which have been found only in phenol, are putative uncharacterized proteins and putative porins. We plan detailed biochemical analyses to understand the function of these proteins. In contrast, growing the bacterium in LB medium allowed the identification of twelve proteins involved in different metabolite transport and metabolic pathways. This finding indicates high metabolic activity of the strain in LB, which is directly supported by the high growth rates.

4. Conclusions

In conclusion, the method employed here for the characterization of the membrane complexome of *Pseudomonas* sp. strain phDV1 (BN/SDS-PAGE) is particularly suited to resolve the highly hydrophobic proteins which are notoriously underrepresented in the classical 2D-GE approaches. While full proteome analyses are possible now using nLC ESI MS or nLC MALDI MS, the isolation of functional membrane protein complexes needs to be achieved using biochemical methods. Although BN-PAGE is not capable of solving all problems when analyzing membrane proteins, it allows an efficient and reproducible separation of the membrane complexome. Here, we have detected a number of membrane protein complexes and described their subunit composition and stoichiometry. Furthermore, we were able to detect proteins which are only expressed in the presence of phenol or glucose. The presented 2D BN/SDS-PAGE gel pattern should facilitate future studies of the subunit composition and stoichiometry of *Pseudomonas* sp. strain phDV1 membrane protein complexes during growth in media containing different carbon sources.

Conflict of interest statement

The authors declare that they have no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

Acknowledgments

This research program was supported by the University of Crete (KA 3130), the Greek Ministry of Education, DAAD (grant A/11/15906-314 to G.T.), the European Union ESFRI Instruct initiative (Membrane Protein Core Center) A/11/15906-314 and the Max-Planck Gesellschaft A/11/15906-314. Prof. H. Michel, Max-Planck-Institute for Biophysics is gratefully acknowledged for his support. Infrastructure support from the European Community's Seventh Framework Programme (FP7) under Grand Agreement No. 229927, project BIOSOLENUTI (Bioinspired Solar Energy Utilization) is gratefully acknowledged.

REFERENCES

[1] Sardessai Y, Bhosle S. Tolerance of bacteria to organic solvents. Res Microbiol 2002;153:263–8.
[2] Sardessai YN, Bhosle S. Industrial potential of organic solvent tolerant bacteria. Biotechnol Prog 2004;20:655–60.
[3] Tsirogianni I, Aivaliotis M, Karas M, Tsiotis G. Mass spectrometric mapping of the enzymes involved in the

phenol degradation of an indigenous soil pseudomonad. Biochem Biophys Acta 2004;1700:117–23.
[4] Tsirogianni I, Aivaliotis M, Karas M, Tsiotis G. Detection and characterisation of catechol 2,3-dioxygenase in an indigenous soil Pseudomonad by MALDI-TOF MS using a column separation. Biodegradation 2005;16:181–6.
[5] Papasotiriou DG, Markoutsa S, Meyer B, Papadioti A, Karas M, Tsiotis G. Comparison of the membrane subproteomes during growth of a new pseudomonas strain on lysogeny broth medium, glucose, and phenol. J Proteome Res 2008;7:4278–88.
[6] Garbis S, Lubec G, Fountoulakis M. Limitations of current proteomics technologies. J Chromatogr A 2005;1077:1–18.
[7] Lin M, Zhou X, Shen X, Mao C, Chen X. The predicted Arabidopsis interactome resource and network topology-based systems biology analyses plant. Cell 2011;23:911–22.
[8] Alberts B, The. Cell as a collection of protein machines: preparing the next generation of molecular biologists. Cell 1998;92:291–4.
[9] Schaegger H, von Jagow G. Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. Anal Biochem 1991;199:223–31.
[10] Wittig L, Schagger H. Advantages and limitations of clear-native PAGE. Proteomics 2005;5:4338–46.
[11] Brookes PS, Pinner A, Ramachandran A, Coward L, Barnes S, Kim H, et al. High throughput two-dimensional blue-native electrophoresis: a tool for functional proteomics of mitochondria and signaling complexes. Proteomics 2002;2:969–77.
[12] Schagger H. Respiratory chain supercomplexes of mitochondria and bacteria. Biochim Biophys Acta 2002;1555:154–9.
[13] Stroh A, Anderka O, Pfeiffer K, Yagi T, Finel M, Ludwig B, et al. Assembly of respiratory complexes I, III, and IV into NADH oxidase supercomplex stabilizes complex I in *Paracoccus denitrificans*. J Biol Chem 2004;279:5000–7.
[14] Pineau B, Mathieu C, Gerard-Hirne C, De Paepe R, Chetrit P. Targeting the NAD7 subunit to mitochondria restores a functional complex I and a wild type phenotype in the Nicotiana sylvestris CMS II mutant lacking nad7. J Biol Chem 2005;280:25994–6001.
[15] Aivaliotis M, Karas M, Tsiotis G. An alternative strategy for the membrane proteome analysis of the green sulfur bacterium *Chlorobium tepidum* using blue native PAGE and 2-D PAGE on purified membranes. J Proteome Res 2007;6:1048–58.
[16] Eubel H, Braun HP, Millar AH. Blue-native PAGE in plants: a tool in analysis of protein–protein interactions. Plant Methods 2005;1.
[17] Kantzilakis K, Aivaliotis M, Kotakis C, Krasanakis K, Rizos AK, Kotzabasis K, et al. A comparative approach towards thylakoid membrane proteome analysis of unicellular green alga *Scenedesmus obliquus*. Biochim Biophys Acta 2007;1768:2271–9.
[18] Schaegger H. Quantification of oxidative phosphorylation enzymes after blue native electrophoresis and two-dimensional resolution: normal complex I protein amounts in Parkinson's disease conflict with reduced catalytic activities. Electrophoresis 1995;16:73–70.
[19] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantitites of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
[20] Schaegger H, von Jagow G. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal Biochem 1987;166:368–79.
[21] Tsirogianni E, Aivaliotis M, Papasotiriou DG, Karas M, Tsiotis G. Identification of inducible protein complexes in the phenol degrader *Pseudomonas* sp. strain phDV1 by blue native gel electrophoresis and mass spectrometry. Amino Acids 2006;30:63–72.
[22] Candiano G, Bruschi M, Musante L, Santucci L, Ghiggeri GM, Carnemolla B, et al. Blue Silver: a very sensitive colloidal Coomassie G-250 staining for proteome analysis. Electrophoresis 2004;25:1327–33.
[23] Lauber WM, Carroll JA, Dufield DR, Kiesel JR, Radabaugh MR, Malone JP. Mass spectrometry compatibility of two-dimensional gel protein stains. Electrophoresis 2001;22:906–18.
[24] Shevchenko A, Tomas H, Havli J, Olsen JV, Mann M. In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nat Protoc 2007;1:2856–60.
[25] Kahng HY, Byrne AM, Olsen RH, Kukor JJ. Characterization and role of tbuX in utilization of toluene by *Ralstonia pickettii* PKO1. J Bacteriol 2000;182:1232–42.
[26] Kasai Y, Inoue J, Harayama S. The TOL plasmid pWW0 xylN gene product from *Pseudomonas putida* is involved in m-xylene uptake. J Bacteriol 2001;183:6662–6.
[27] Venkataraman NI, Subrahmanyam VVR. Effect of structure on surfactance of sodium salts of N-acylamino acids in aqueous solutions. J Indian Chem Soc 1985;62:507–12.
[28] Yankovskaya V, Horsefield R, Tornroth S, Luna-Chavez C, Miyoshi H, Leger C, et al. Architecture of succinate dehydrogenase and reactive oxygen species generation. Science 2003;299:700–4.
[29] Verkhovsky MI, Bogachev AV. Sodium-translocating NADH:quinone oxidoreductase as a redox-driven ion pump. Biochim Biophys Acta 2010;1797:738–46.
[30] Saikawa N, Akiyama Y, Ito K, Fts H. exists as an exceptionally large complex containing HflKC in the plasma membrane of *Escherichia coli*. J Struct Biol 2004;146:123–9.
[31] Capaldi RA, Aggeler R. Mechanism of the F_1F_0-type ATP synthase, a biological rotary motor. Trends Biochem Sci 2002;27:154–60.
[32] Krishna KA, Rao GV, Rao KR. Chaperonin GroEL: structure and reaction cycle. Curr Protein Pept Sci 2007;8:418–25.
[33] Lugtenberg B, Van Alphen L. Molecular architecture and functioning of the outer membrane of *Escherichia coli* and other gram-negative bacteria. Biochim Biophys Acta 1983;737:51–115.
[34] Sugawara E, Nikaido H. Pore-forming activity of OmpA protein of *Escherichia coli*. J Biol Chem 1992;262:2507–11.
[35] Gensberg K, Smith A, Brinkman F, Hancock R. Identification of oprG, a gene encoding a major outer membrane protein of *Pseudomonas aeruginosa*. J Antimicrob Chemother 1999;43:607–8.
[36] McPhee J, Tamber S, Bains M, Maier E, Gellatly S, Lo A, et al. The major outer membrane protein OprG of *Pseudomonas aeruginosa* contributes to cytotoxicity and forms an anaerobically regulated, cation-selective channel. FEMS Microbiol Lett 2009;296:241–7.
[37] Roma-Rodrigues C, Santos PM, Benndorf D, Rapp E, Sá-Correia I. Response of *Pseudomonas putida* KT2440 to phenol at the level of membrane proteome. J Proteomics 2010;73:2461–78.
[38] Walzer G, Rosenberg E, Ron E. Identification of outer membrane proteins with emulsifying activity by prediction of [beta]-barrel regions. J Microbact Methods 2009;76:52–7.
[39] Cote-Sierra J, Bredan A, Toldos CM, Stijlemans B, Brys L, Cornelis P, et al. Bacterial lipoprotein-based vaccines induce tumor necrosis factor-dependent type 1 protective immunity against Leishmania major. Infect Immun 2002;70:240–8.
[40] Chng SS, Ruiz N, Chimalakonda G, Silhavy TJ, Kahne D. Characterization of the two-protein complex in *Escherichia*

coli responsible for lipopolysaccharide assembly at the outer membrane. Proc Natl Acad Sci U S A 2010;107:5363–8.
[41] Petersen NT, Brunak S, von Heijne G, Nielsen H. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat Methods 2011;8:785–6.
[42] Struyve M, Moons M, Tommassen J. Carboxy-terminal phenylalanine is essential for the correct assembly of a bacterial outer membrane protein. J Mol Biol 1991:218.
[43] Pitcher RS, Watmough NJ. The bacterial cytochrome cbb_3 oxidases. Biochim Biophys Acta 2004;1655:388–99.
[44] Urbani A, Gemeinhardt S, Warne A, Saraste M. Properties of the detergent solubilised cytochrome *c* oxidase (cytochrome cbb_3) purified from *Pseudomonas stutzeri*. FEBS Lett 2001;508:29–35.
[45] Schülein R, Gentschev I, Mollenkopf HJ, Goebel W. A topological model for the haemolysin translocator protein HlyD. Mol Gen Genet 1992;992:155–63.
[46] Pimenta AL, Racher K, Jamieson L, Blight MA, Holland IB. Mutations in HlyD, part of the type 1 translocator for hemolysin secretion, affect the folding of the secreted toxin. J Bacteriol 2005;187:7471–80.
[47] Cowles CE, Nichols NN, Harwood CS. BenR, a XylS homologue, regulates three different pathways of aromatic acid degradation in *Pseudomonas putida*. J Bacteriol 2000;182:6339–46.
[48] Ranquin A, Van Gelder P. Maltoporin: sugar for physics and biology. Res Microbiol 2004;155:611–6.