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Identification of inducible protein complexes in the phenol degrader *Pseudomonas* sp. strain phDV1 by blue native gel electrophoresis and mass spectrometry

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Summary. *Pseudomonas* sp. strain phDV1, being a phenol degrading bacterium, has been found to utilize phenol as sole carbon source via the *meta* pathway. Blue native polyacrylamide gel electrophoresis (BN-PAGE) is widely used for the analysis of oligomeric state and molecular mass non-dissociated protein complexes. In this study, a number of proteomic techniques were used to investigate the oligomeric state enzymes involved in the aromatic degradation pathway. In particular, the *Pseudomonas* sp. strain phDV1 proteome was monitored under two different growth substrate conditions, using glucose or phenol as sole carbon source. The protein complexes map was compared by BN-PAGE after fractionation by sucrose density centrifugation of the cell extracts. Multiple differences were detected. Further, analysis and identification of the subunit composition of these complexes was carried out using MALDI-TOF MS, allowing the identification of 49 proteins. Additionally, functional information regarding protein–protein interactions was assembled, by coupling 2-D BN-PAGE with MALDI-TOF MS. Application of this functional proteomics method resulted in an higher number of the identified proteins.

Keywords: MALDI – Phenol degradation – *Pseudomonas* sp. strain phDV1 – Protein complex – Blue native

Abbreviations: MALDI-TOF, matrix assisted laser desorption ionization – time of flight; MS, mass spectrometry; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; BN, blue native; C23O, catechol 2, 3 dioxygenase

Introduction

Organic pollutants in the environment are biodegraded by a range of naturally occurring microorganisms. The extensive use of phenolic compounds in various industrial operations has led to widespread environmental pollution (Annachhatre and Gheewala, 1996). The most important phenol-degrading microorganism that showed high biodegradability belongs to the genus of *Pseudomonas*. A strain of these species with high phenol removal efficiency is *Pseudomonas* sp. strain phDV1, which was isolated from enriched mixed culture from samples of petroleum-contaminated soil in Denmark. A number of studies have shown that phenol is metabolised as sole carbon-energy source for the growth of *Pseudomonas* sp. strain phDV1 (Tsirogianni et al., 2005). Application of mass spectroscopy revealed that the aromatic molecule is being degraded via the "*meta*" pathway (Fig. 1) (Tsirogianni et al., 2004, 2005). The importance of examining the degrative pathways with which the bacteria catabolize aromatic compounds is evident, considering the environmental pollution of soil and water caused by agricultural and industrial activities, or by accidental contamination with fuels, oils and solvents.

Having recently entered the postgenome era (Shouse, 2001), the new challenge is to assign a function to each protein and elucidate its interaction with other proteins and macromolecules in the cell (Wilkins et al., 1997). Proteins are not solitary molecules; on the contrary their function is most often mediated by their interactions with other molecules. In this way, enables different steps in a biological process may be combined. By keeping the enzymes in the form of a complex, a series of enzymatic reactions is more efficient and also any toxic intermediates can be kept within the protein complex, limiting possible damage to other parts of the cell.

BN-PAGE is widely used for the analysis of non-dissociated protein complexes with respect to their

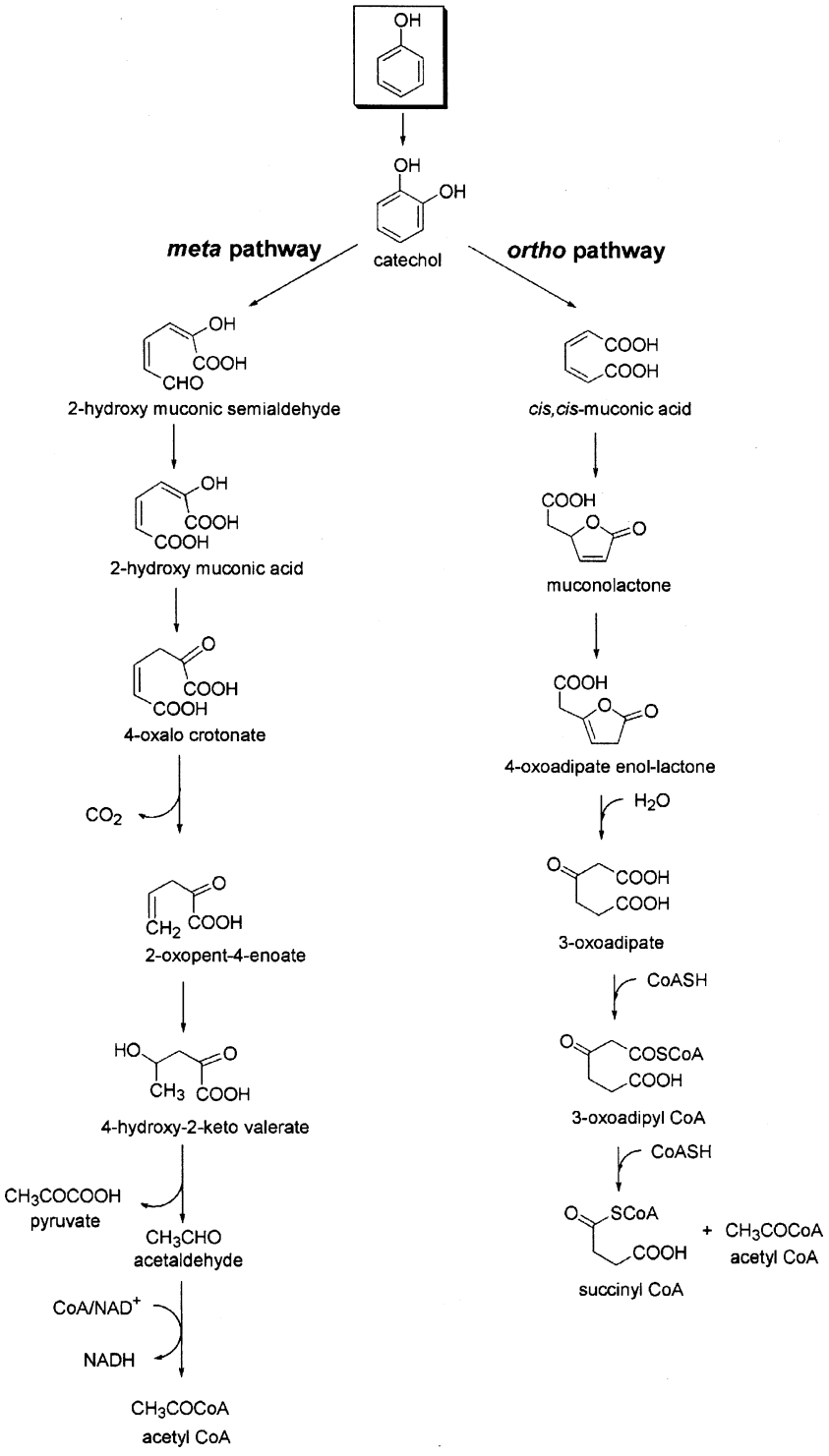

Fig. 1. Scheme of the conversion of phenol via the *ortho*- and *meta*-pathway

composition, oligomeric state and molecular mass (Schaegger and von Jagow, 1991). This technique separates complexes without dissociating them into their constituent polypeptides. High-throughput mass spectrometry (MS), is a basic technology in proteomics due to its unrivalled speed and sensitivity in the identification and sequencing of proteins in complex biological mixtures and at low concentrations (Shouse, 2001). MS can be applied for the identification of the proteins which form the BN-PAGE isolated complexes either with or without a second dimensional analysis by Tricine-SDS PAGE (Nijtmans et al., 2002).

In the present study, 2-D BN PAGE and peptide mass fingerprinting (PMF) of the gel – digested protein spots by

MALDI-TOF MS has been applied in order to examine the functional supramolecular arrangement of the metabolic enzymes extracted from a new phenol degrader, *Pseudomonas* sp. strain phDV1. Cell extracts fractionation using sucrose density gradient followed by 2-DE (1-D: BN-PAGE and 2-D: Tricine-SDS-PAGE) and PMF by MALDI-TOF MS has proven to be an effective tool for the identification of supramolecular interactions of proteins involved in the metabolic pathway.

Material and methods

1 Growth conditions

The bacterium was cultured in a minimal medium (Kunz and Chapman, 1981). Solution A contains 0.211 M Na_2HPO_4, 0.108 M KH_2PO_4, 0.042 M NaCl, 0.093 M NH_4Cl, pH = 7.4, solution B contains 10% $MgSO_4 \cdot 7H_2O$ and 1.0% $FeSO_4 \cdot 7H_2O$ and solutions of 1 M $MgSO_4$ and 1 M $CaCl_2$. 1 liter medium prepared by mixing 200 ml solution A, 7 ml solution B, 1 ml of 1 M $MgSO_4$, 0.1 ml of 1 M $CaCl_2$ and 790 ml water. The carbon source (phenol or glucose) was added, to a final concentration of 200 mg/l. The bacterium was grown at 30°C with 1% inoculum. The growth was monitored by measuring the optical density at 600 nm and the activity of catechol 2,3 dioxygenase (C23O).

2 Preparation of enzyme extracts

The purification procedure was carried out as described in (Tsirogianni et al., 2005). Briefly, cells (5 gr wet weight) were suspended in 10 ml 50 mM Tris-H_2SO_4, pH 8.0. The cells were then broken by sonication using a Broxon ultrasonic processor at the maximum power (20 times, 15 sec with 45 sec intervals), making sure that the temperature was maintained below 10°C, with a freezing bath. Unbroken cells and cell debris were removed from the resulting suspension by centrifugation at 10,000 g for 15 min at 4°C. The supernatant was then centrifuged at 100,000 g for 1 h at 4°C to pellet the membrane fraction. The supernatant obtained after the later centrifugation was loaded on a sucrose density gradient. The steps were 30% (1 ml), 20% (4 ml) and 10% (4 ml) 6 sucrose. The sucrose solution were prepared in 50 mM Tris-H_2SO_4 buffer, pH 8.0 containing 10 mM acetone (Kobayashi et al., 1995). After centrifugation in a Sorvall TH-641 rotor (210,000 g, 10 h) the yellow band located in 10% sucrose step, indicating C23O activity, was collected.

3 Enzyme assay

C23O activity was measured spectrophotometrically following the formation of 2-hydroxymuconate semialdehyde at 375 nm and 25°C ($\varepsilon_{375} = 4.4 \times 10^4$ mole^{-1} cm^{-1}) by the procedure of (Briganti et al., 1997). The reaction was initiated by catechol addition. The reaction mixture consisted of 0.1 mM catechol, 50 mM Tris/HCl at pH 7.5 and 10 μl enzyme extract, in a total volume of 2 ml. A control reaction which did not contain catechol was similarly inducted.

4 Gel electrophoresis

4.1 First-dimensional electrophoresis (BN-PAGE)

The BN-PAGE was carried out in a 8% polyacrylamide gel (2.5% bis-acrylamide), as described in (Schaegger and von Jagow, 1991). In brief prior to electrophoresis, glycerol was added to the samples at a 20% v/v final concentration. Electrophoretic run was inducted at 10°C, initially at 80 V until the entry of the protein sample into the stacking gel, a subsequently at 150 V. The run was terminated as soon as the dye front run off from the gel. The high molecular weight calibration kit for native electrophoresis from Amersham Biosciences, containing thyroglobulin 669 kDa, ferritin 440 kDa, catalase 232 kDa, lactate dehydrogenase 140 kDa and albumin 66 kDa was used for the molecular weight estimation of the separated complexes.

4.2 Second-dimensional electrophoresis (Tricine SDS-PAGE)

Tricine-SDS-PAGE was performed according to (Schaegger and von Jagow, 1987) using a self-built system with 12% acrylamide gels (26 × 24 × 0.1 cm). The excised lane was transferred to a 26 × 24 cm glass plate at the position of the sample gel and soaked with 1% SDS and 1% mercaptoethanol for 2 h. One millimeter spacers were positioned, a glass plate was placed above the gel and clamps were fixed. While still horizontal, mercaptoethanol was soaked off with a paper towel. The spacers and glass plates were aligned and vertically placed in the casting stand. The acrylamide solution was then injected between the glass plates up to 3 cm from the first-dimensional gel strip. The freshly poured gel was overlaid with a small amount of water. After polymerization, the water was decanted and a 2 cm layer of 3% T. Tricine-SDS-gel mixture was added. Electrophoresis was performed at room temperature, starting for 3 h at 75 V, the voltage was raised to 150 V overnight (max. 50 mA).

Staining was carried out with 0.02% Coomassie Brilliant Blue G-250 in 10% acetic acid or colloidale Coomassie solution (Lauber et al., 2001). SDS-PAGE standards (Fluka Laboratories, USA) were used for molecular weight estimation which contained α-lactalbumin 14.2 kDa, trypsin inhibitor 20 kDa, trypsinogen 24 kDa, carbonic anhydrase 29 kDa, glyceraldeyde-3-phosphate dehydrogenase 36 kDa,

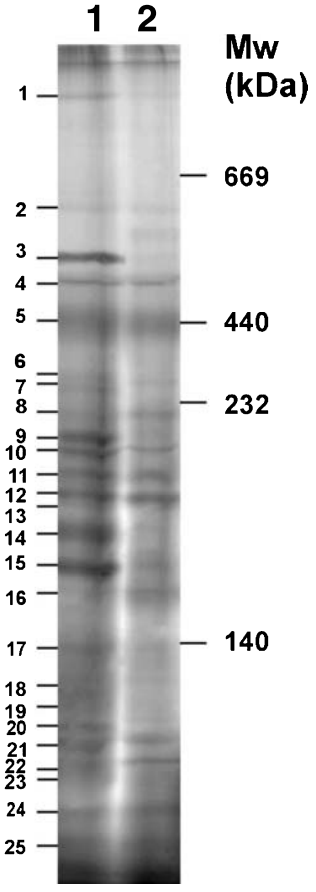

Fig. 2. 1-D BN electrophoresis of cell extract of the fraction with C23O activity. Cells grown using phenol as sole carbon source (lane 1) and glucose (lane 2)

Table 1. Identity, molecular mass and criteria for the identification of matched peptides of protein bands in Fig. 2

Band Nr	Protein description	Gene/Species	pI/MW(kD)	Peptides matched	Coverage (%)
1	DNA-directed RNA polymerase, alpha subunit	rpoA/Pseudomonas putida KT2440	4.9 /36.73	9	19
	DNA-directed RNA polymerase, beta subunit	rpoB/Pseudomonas putida KT2440	5.6 /151.55	30	23
2	DNA gyrase subunit A	gyrA/Pseudomonas aeruginosa PA01	4.9/ 101.23	22	21
	putative TraC protein	traC/ Pseudomonas putida	9.4/108.95	21	29
	type III helper protein HrpK (Pto)	hrpK/Pseudomonas syringae pv. tomato str. DC3000	5.3 /81.08	15	27
	ATP-dependent protease, putative	PSPTO4585/ Pseudomonas syringae pv. tomato str. DC3000	4.8 /89.98	13	19
3	4-oxalocrotonate decarboxylase	dmpH/Pseudomonas putida	5.1 / 28.52	6	30
	2-hydroxypent -2,4 dienoate hydratase	dmpE/Pseudomonas putida	5.2 / 28.13	9	51
4	polyribonucleotide nucleotidyltransferase	pnp/Pseudomonas syringae pv. tomato str. DC3000	5.2 /75.14	13	24
	isocitrate dehydrogenase, NADP-dependent	PSPTO3356/Pseudomonas syringae pv. tomato str. DC3000	5.5/ 81.05	15	25
	sulfate adenylate transferase, subunit 1/adenylylsulfate kinase	cysNC/Pseudomonas syringae pv. tomato str. DC3000	5.5/ 69.21	15	28
5	aldehyde dehydrogenase family protein	PP2680/Pseudomonas putida KT2440	5.5/ 55.23	13	31
6	isocitrate dehydrogenase	idh/Pseudomonas aeruginosa PA01	5.8 /81.84	15	30
	pyruvate dehydrogenase, E1 component	aceE-2/Pseudomonas syringae pv. tomato str. DC3000	5.5/ 99.53	15	21
	phenol 2-monooxygenase P3 component	dmpN/Pseudomonas Putida	6.0 / 60.90	8	23
	oxidoreductase, FMN-binding	PP0310/Pseudomonas putida KT2440	5.8/ 76.37	13	30
7	aspartyl-tRNA synthetase	Q87Y31/ Pseudomonas syringae pv. tomato str. DC3000	5.3 /66.62	12	25
	non-ribosomal peptide synthetase, initiating component	Q87WM8/Pseudomonas syringae pv. tomato str. DC3000	5.9 /196.28	14	14
	phenol 2-monooxygenase P3 component	dmpN/Pseudomonas Putida	6.0 / 60.90	7	19
8	phosphoenolpyruvate synthase	PpsA/Pseudomonas putida KT2440	5.0/86.02	21	32
	benzene monooxygenase oxygenase subunit	bmoA/Pseudomonas aeruginosa	4.9 /58.17	13	34
	pyruvate dehydrogenase, E1 component	aceE-2/Pseudomonas syringae pv. tomato str. DC3000	5.5 /99.53	17	28
	translation elongation factor G	fusA-1/Pseudomonas putida KT2440	5.1 /79.08	19	37
9	2-hydroxymuconic semialdehyde dehydrogenase	dmpC/Pseudomonas Putida	5.4 /52.06	14	34
	COG1112: Superfamily I DNA and RNA helicases and helicase subunits	Paer033801/Pseudomonas aeruginosa UCBPP-PA14	5.6 /230.29	15	11
	DNA polymerase III, alpha chain	dnaE/Pseudomonas aeruginosa PA01	5.3/ 131.63	12	14
10	benzene monooxygenase oxygenase subunit	bmoA/Pseudomonas aeruginosa	4.9 /58.17	26	43
	phenol 2-monooxygenase P3 component	dmpN/Pseudomonas Putida	6.0 / 60.90	13	36
	phosphoenolpyruvate synthase	PpsA/Pseudomonas putida KT2440	86017/5.0	21	29
	probable polyketide synthase type I	pltB/Pseudomonas fluorescens	5.3 /265.17	22	14
	oxaloacetate decarboxylase, alpha subunit	oadA/Pseudomonas syringae pv. tomato str. DC3000	5.5 /65.81	13	37
11	oxaloacetate decarboxylase, alpha subunit	oadA/Pseudomonas syringae pv. tomato str. DC3000	5.5 /65.81	16	27
	succinyl-CoA synthetase, beta subunit	sucC/Pseudomonas putida KT2440	5.8 /41.51	14	40
	succinyl-CoA synthetase, alpha subunit	sucD/Pseudomonas putida KT2440	5.9 /30.49	9	57
	aconitate hydratase, putative	PP2336/Pseudomonas putida KT2440	5.4/ 94.46	14	26
	clpB protein	clpB/Pseudomonas syringae pv. tomato str. DC3000	5.4 /95.28	11	17
12	hypothetical protein	Psyr1985/Pseudomonas syringae pv. syringae B728a	8.7 /198.23	28	24
	glutamate synthase large subunit	gltB/Pseudomonas aeruginosa	5.8 /162.77	22	30
	exodeoxyribonuclease V, gamma subunit	recC/Pseudomonas putida KT2440	5.3 /131.00	19	19
	DNA polymerase III, alpha chain	dnaE/Pseudomonas aeruginosa PA01	5.3 /131.63	15	14
	clpB protein	clpB/Pseudomonas syringae pv. tomato str. DC3000	5.4/ 95.28	19	29
	translation elongation factor G	fusA-1/Pseudomonas putida KT2440	5.1 /79.08	14	31
	pyruvate dehydrogenase, E1 component	aceE-2/Pseudomonas syringae pv. tomato str. DC3000	5.5 /99.53	11	16
13	probable polyketide synthase type I	pltB/Pseudomonas fluorescens	5.3 /265.17	17	13
	DNA polymerase III, alpha chain	dnaE/Pseudomonas aeruginosa PA01	5.3 /131.63	13	13
	2-oxoglutarate dehydrogenase	kdgA/Pseudomonas putida	6.2 /103.87	15	23
	phenol 2-monooxygenase P3 component	dmpN/Pseudomonas Putida	6.0 / 60.90	10	35
	glutamate synthase large subunit	gltB/Pseudomonas aeruginosa	5.8 /162.77	13	16
	clpB protein	clpB/Pseudomonas syringae pv. tomato str. DC3000	5.4 /95.28	13	23
14	acetaldehyde dehydrogenase (acetylating)	dmpF/Pseudomonas putida	5.9/ 32.78	17	60
	phenol 2-monooxygenase P3 component	dmpN/Pseudomonas Putida	6.0 / 60.90	10	20
	4-hydroxy-2-oxovalerate aldolase	dmpG/Pseudomonas Putida	5.9 /36.49	11	46
	ATP-dependent helicase HrpA	hrpA/Pseudomonas putida KT2440	8.5 /147.50	14	17
	prolyl-tRNA synthetase	proS/Pseudomonas putida KT2440	5.2 /63.78	11	33
	clpB protein	clpB/Pseudomonas aeruginosa PA01	5.9 /32.78	19	28
15	acetaldehyde dehydrogenase (acetylating)	dmpF/Pseudomonas putida	5.9 /32.78	16	61
	4-hydroxy-2-oxovalerate aldolase	dmpG/Pseudomonas Putida	5.9 /36.49	12	48
	benzene monooxygenase oxygenase subunit	bmoA/Pseudomonas aeruginosa	4.9/ 58.17	9	19
16	benzene monooxygenase oxygenase subunit	bmoA/Pseudomonas aeruginosa	4.9/ 58.17	12	24
	lysyl-tRNA synthetase	lysS/Pseudomonas syringae pv. tomato str. DC3000	5.0 /57.04	11	25

(*continued*)

Table 1. (*continued*)

Band Nr	Protein description	Gene/Species	pI/MW(kD)	Peptides matched	Coverage (%)
17	hypothetical protein	ZP_00125692 [Pseudomonas syringae pv. syringae B728a]	8.7 /198.23	34	28
	benzene monooxygenase oxygenase subunit	bmoA/Pseudomonas aeruginosa	4.9/ 58.17	17	32
	pyruvate dehydrogenase, E1 component	aceE-2/Pseudomonas syringae pv. tomato str. DC3000	5.5 /99.53	21	30
18	benzene monooxygenase oxygenase subunit	bmoA/Pseudomonas aeruginosa	4.9 /58.17	15	32
	catechol 2,3 dioxygenase	dmoB/Pseudomonas Putida	5.6/ 35.58	13	48
	phenol 2-monooxygenase P3 component	dmpN/Pseudomonas Putida	6.0 / 60.90	8	25
19	catechol 2,3 dioxygenase	dmpB/Pseudomonas Putida	5.6 /35.58	24	68
	benzene monooxygenase oxygenase subunit	bmoA/Pseudomonas aeruginosa	4.9 /58.17	22	41
	phenol 2-monooxygenase P3 component	dmpN/Pseudomonas Putida	6.0 / 60.90	14	39
	2-oxoglutarate dehydrogenase (E1 subunit)	sucA/ Pseudomonas aeruginosa PA01	6.1 /106.43	11	20
20	benzene monooxygenase oxygenase subunit	bmoA/Pseudomonas aeruginosa	4.9 /58.17	17	33
	catechol 2,3 dioxygenase	dmoB/Pseudomonas Putida	5.6 /35.58	13	48
	phenol 2-monooxygenase P3 component	dmpN/Pseudomonas Putida	6.0 / 60.90	14	36
	Phenol 2-monooxygenase P1 component	dmpL/Pseudomonas Putida	5.2 /38.30	10	36
	phosphoribosylformylglycinamidine synthase	purL/Pseudomonas syringae pv. tomato str. DC3000	5.0/ 141.43	14	19
21	benzene monooxygenase oxygenase subunit	bmoA/Pseudomonas aeruginosa	4.9 /58.17	20	31
	phenol 2-monooxygenase P3 component	dmpN/Pseudomonas Putida	6.0 / 60.90	10	28
	translation elongation factor G	fusA-1/Pseudomonas putida KT2440	5.1 /79.08	20	36
	phosphoribosylformylglycinamidine synthase	purL/Pseudomonas syringae pv. tomato str. DC3000	5.0 /141.43	9	12
22	benzene monooxygenase oxygenase subunit	bmoA/Pseudomonas aeruginosa	4.9 /58.17	25	45
	phenol 2-monooxygenase P3 component	dmpN/Pseudomonas Putida	6.0 / 60.90	12	33
	Phenol 2-monooxygenase P1 component	dmpL/Pseudomonas Putida	5.2/ 38.30	9	35
23	benzene monooxygenase oxygenase subunit	bmoA/Pseudomonas aeruginosa	4.9 /58.17	30	52
	phenol 2-monooxygenase P3 component	dmpN/Pseudomonas Putida	6.0 / 60.90	13	31
	translation elongation factor G	fusA-1/Pseudomonas putida KT2440	5.1 /79.08	11	26
24	benzene monooxygenase oxygenase subunit	bmoA/Pseudomonas aeruginosa	4.9 /58.17	22	37
	phenol 2-monooxygenase P3 component	dmpN/Pseudomonas Putida	6.0 / 60.90	11	27
	Phenol 2-monooxygenase P1 component	dmpL/Pseudomonas Putida	5.2 /38.30	12	44
	pyruvate dehydrogenase, E1 component	aceE-2/Pseudomonas syringae pv. tomato str. DC3000	5.5 /99.53	16	24
	sigma-54 dependent transcriptional regulator	PP3467/Pseudomonas putida KT2440	6.3/ 69.56	14	36
25	benzene monooxygenase oxygenase subunit	bmoA/Pseudomonas aeruginosa	4.9 /58.17	18	36
	phosphoribosylformylglycinamidine synthase	purL/Pseudomonas syringae pv. tomato str. DC3000	5.0 /141.43	15	20
	DNA-directed RNA polymerase, beta subunit	rpoB/Pseudomonas putida KT2440	5.6 /151.55	16	18
	pyruvate dehydrogenase, E1 component	aceE-2/Pseudomonas syringae pv. tomato str. DC3000	5.5 /99.53	13	24

albumin from chicken eggs 45 kDa and albumin from bovine serum 67 kDa.

5 Mass spectrometry for protein identification

Individual bands or proteins spots were excised from the Coomassie-stained gels, digested with trypsin (Roche, sequence grade) and PMFs generated by a Voyager-DE STR MALDI-TOF instrument (Applied Biosystems) as earlier described (Tsirogianni et al., 2004). MALDI-TOF mass spectra were externally calibrated with a peptide mixture containing der-Arg1-Bradykinin (904.47 Da), angiotensin I (1296.69 Da) glu1-fibrinopeptide B (1570.68 Da), ACTH (2093.0, 2465.20, 3657.93 Da) and analyzed using the software Data Explorer 3.0 (Applied Biosystem). The resulting peptide mass fingerprints were searched using the program Spectrum Mill MS Proteomics Workbench (Agilent Technologies). The Spectrum Mill workbench includes both Manual PMF and PMF Search. Manual PMF is a version of MS-Fit that allows search in a list of masses. The mass list must be entered or copied in manually. The search criteria for the manual PMF search were: 50 ppm mass tolerance, 4 matching peptides minimum, 1–2 maximum missed cleavage sites, oxidation of methionine as a possible modification, carbamidomethylation of cysteines as a fixed modification. Database search was performed on the subset database of bacteria and *Pseudomonas aeruginosa* (Stover et al., 2000). Positive identification were considered the ones with $p < 10^{-1}$ using the program Spectrum Mill MS Proteomics Workbench (Agilent Technologies).

Results

Several aromatic substrates were studied, such as benzene, toluene, phenol, m-cresol, nitrobenzene and bromobenzene (Tsirogianni et al., 2005). Phenol, when being the only carbon and energy source, can be degraded by *Pseudomonas* sp. within the range of 1–1200 mg L^{-1}. The catabolic pathway of phenol degradation was studied using culture containing 200 mg L^{-1} of phenol after for 16–20 h, at which time maximum activity of the enzyme was detected.

Starting material for the further analysis on the metabolic enzymes of the *Pseudomonas* sp. strain phDV1 was extracted from bacterium cells grown with either phenol or glucose as sole carbon source. The purification procedure involved a sucrose density fractionation of the crude cell

extract, which has proven to be useful in reducing the complexity of the total protein mixture as well as in collecting and concentrating the cell fraction exhibiting C23O activity (Tsirogianni et al., 2005). These fractions were immediately subjected to BN-PAGE. It clearly appeared that phenol as sole carbon energy source induces the production of protein bands in the BN-PAGE (Fig. 2, lane 1, 2). The molecular weights were determined by comparing their electrophoretic mobility with protein standards. Their molecular weight varies from 850 kDa to 100 kDa. In addition, no further change in the BN-PAGE profile was detected after freezing and thawing the sample (data not shown).

The separated by BN-PAGE bands, were digested with trypsin and the extracted peptides mixtures were analysed with MALDI-TOF MS and PMF search. Application of this methodology led to the identification of 49 gene products present in the protein bands of the BN gels (Table 1). In all inducible protein complexes, enzymes involved in the phenol degradation were identified (Fig. 1, Table 1). On the contrary, proteins of general metabolic pathways or of other cell function were identified, from the constitutive protein 10 bands present in cell extracts from bacteria growth using either phenol or glucose as sole carbon source (Table 1; Spot, 5, 6, 11 and 12).

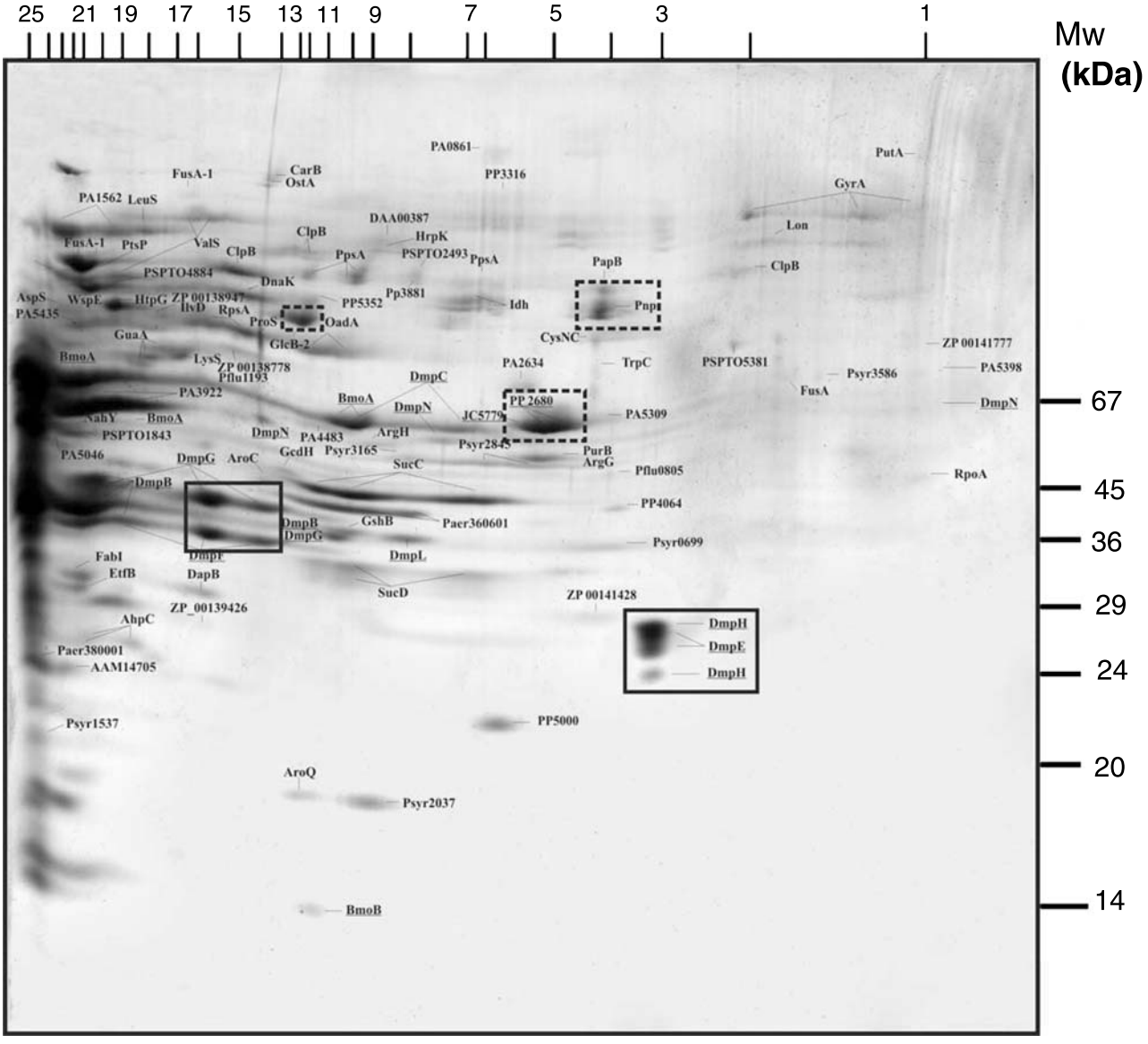

Fig. 3. 2-D BN-PAGE functional proteomic map of the cell extracts grown using phenol as sole carbon source (~300 μg protein). Arabic numerals along top of the gel indicate the position of the twenty five complexes in the first dimension. Spots were cut where indicated and identified by MALDI-TOF. Proteins involved in the phenol degradation are underline. Frames with continuous lines indicate proteins complexes in the phenol degradation and with dotted lines of other metabolic pathways

Table 2. Identity, molecular mass and criteria for the identification of matched peptides of the protein spots after the 2-D BN-PAGE (Fig. 3)

No	Protein description	Gene/Species	pI/MW(kD)	Peptides matched	Coverage (%)
1	4 -oxalocrotonate decarboxylase	dmpH/Pseudomonas putida	5.1/28.52	7	31
2	2-hydroxypent -2,4 dienoate hydratase	dmpE/Pseudomonas putida	5.2/28.13	11	56
3	phenol 2-monooxygenase P3 component	dmpN/Pseudomonas Putida	6.0/60.90	10	23
4	acetaldehyde dehydrogenase (acetylating) (EC 1.2.1.10)	dmpF/Pseudomonas putida	5.9/32.78	13	44
5	2-hydroxymuconic semialdehyde dehydrogenase	dmpC/Pseudomonas Putida	5.4 / 52.06	8	26
6	catechol 2,3 dioxygenase	dmoB/Pseudomonas Putida	5.6 / 35.58	7	39
7	4-hydroxy-2-oxovalerate aldolase	dmpG/Pseudomonas Putida	5.9 / 36.49	8	30
8	Phenol 2-monooxygenase P1 component	dmpL/Pseudomonas Putida	5.2 / 38.30	16	51
9	b moB	bmoB/Pseudomonas aeruginosa	6.4 / 98.10	4	47
10	benzene monooxygenase oxygenase subunit	bmoA/Pseudomonas aeruginosa	4.9/ 58.17	19	35
11	4-carboxy-2-hydroxymuconate-6-semialdehyde dehydrogenase	JC5779/Pseudomonas sp	4.8 / 51.95	15	39
12	aldehyde dehydrogenase family protein	PP2680/Pseudomonas putida KT2440	5.5 / 55.23	13	44
13	probable FMN oxidoreductase	PA5398/Pseudomonas aeruginosa PA01	5.6 / 76.38	14	31
14	COG0210: Superfamily I DNA and RNA helicases	ZP_00141777/Pseudomonas aeruginosa UCBPP-PA14	5.7 / 76.09	11	22
15	DNA gyrase subunit A	gyrA/Pseudomonas aeruginosa PA01	4.9 / 101.23	19	24
16	proline dehydrogenase	PutA /Pseudomonas aeruginosa PA01	5.8 / 11.62	15	27
17	COG1157: Flagellar biosynthesis/type III secretory pathway ATPase	Psyr3586/Pseudomonas syringae pv. syringae B728a	8.8 / 50.86	16	34
18	translation elongation factor G	fusA/Pseudomonas syringae pv. tomato str. DC3000	5.2 / 77.44	18	33
19	biotin carboxylase/biotin carboxyl carrier protein	PSPTO5381/Pseudomonas syringae pv. tomato str. DC3000	5.3 / 62.16	9	20
20	PapB protein	papB/Pseudomonas aeruginosa	5.0 / 73.65	24	42
21	polyribonucleotide nucleotidyltransferase	pnp/Pseudomonas putida KT2440	5.1 / 75.22	22	39
22	sulfate adenylyltransferase, subunit 1/adenylylsulfate kinase	cysNC/Pseudomonas putida KT2440	5.5 /69.20	14	28
23	probable oxidoreductase	PA5309/Pseudomonas aeruginosa PA01	6.9 / 48.08	9	24
24	COG2130: Putative NADP-dependent oxidoreductases	Psyr0699/Pseudomonas syringae pv. syringae B728a	5.9 / 36.30	11	46
25	COG2897: Rhodanese-related sulfurtransferase	ZP_00141428/Pseudomonas aeruginosa UCBPP-PA14	5.3 / 29.48	9	47
26	hypothetical protein	PA0861/Pseudomonas aeruginosa PA01	5.2 / 91.70	11	18
27	chaperone-associated ATPase, putative	PP3316/Pseudomonas putida KT2440	5.8 / 103.92	24	26
28	phosphoenolpyruvate synthase	ppsA/Pseudomonas putida KT2440	5.0 / 86.02	19	29
29	isocitrate dehydrogenase	idh/Pseudomonas aeruginosa PA01	5.8 / 81.84	16	27
30	probable isocitrate lyase	PA2634/Pseudomonas aeruginosa PA01	5.7 / 59.33	13	34
31	heat shock protein HslV	PP5000/Pseudomonas putida KT2440	5.8 / 18.81	8	64
32	adenylosuccinate lyase	purB/Pseudomonas syringae pv. tomato str. DC3000	5.6 / 50.98	10	28
33	argininosuccinate synthase	argG/Pseudomonas syringae pv. tomato str. DC3000	5.3 / 45.51	9	30
34	COG0105: Nucleoside diphosphate kinase	Psyr2037/Pseudomonas syringae pv. syringae B728a	5.4 / 14.93	5	29
35	3-dehydroquinate dehydratase, type II	AROQ/Pseudomonas syringae pv. tomato str. DC3000	6.1 / 16.40	6	76
36	argininosuccinate lyase	argH/Pseudomonas aeruginosa PA01	5.5 / 51.91	17	37
37	COG2718: Uncharacterized conserved protein	Psyr3165/Pseudomonas syringae pv. syringae B728a	6.8 / 48.77	15	48
38	COG0137: Argininosuccinate synthase	gil23471208/Pseudomonas syringae pv. syringae B728a	5.3 / 45.49	22	53
39	phage terminase, large subunit, putative	PP3881/Pseudomonas putida KT2440	5.4 / 67.58	9	27
40	glycosyl hydrolase, family 15	PSPTO2493/Pseudomonas syringae pv. tomato str. DC3000	5.1 / 69.91	12	28
41	HrpK [Pseudomonas syringae pv. syringae]	HrpK/Pseudomonas syringae pv. Syringae	5.1 / 79.82	15	34
42	TPA: putative type III effector HolPsyAG	DAA00387/Pseudomonas syringae pv. syringae B728a	9.8 / 79.28	12	22
43	phosphoenolpyruvate synthase	ppsA/Pseudomonas aeruginosa PA01	5.0 / 86.26	30	43
44	carbamoyl-phosphate synthase, large subunit	carB/Pseudomonas syringae pv. tomato str. DC3000	5.2 / 118.77	15	19
45	organic solvent tolerance protein OstA precursor	ostA/Pseudomonas aeruginosa PA01	5.4 / 104.60	10	16
46	ClpB protein	ClpB/Pseudomonas aeruginosa PA01	5.3 / 95.10	27	30
47	DNA helicase II	PP5352/Pseudomonas putida KT2440	5.9 / 81.79	16	25
48	oxaloacetate decarboxylase, alpha subunit	OadA/Pseudomonas putida KT2440	5.4 / 65.89	21	39
49	Glu-tRNA(Gln) amidotransferase subunit A	PA4483/Pseudomonas aeruginosa PA01	5.5 / 52.07	9	28
50	malate synthase G	GlcB-2/Pseudomonas syringae pv. tomato str. DC3000	5.9 / 80.38	14	23
51	glutaryl-CoA dehydrogenase	gcdH/Pseudomonas aeruginosa PA01	5.8 / 43.64	13	51
52	chorismate synthase	aroC/Pseudomonas putida KT2440	6.1 / 39.14	12	44
53	succinyl-CoA synthetase, beta subunit	sucC/Pseudomonas putida KT2440	5.8 / 41.51	24	63
54	hypothetical protein	Paer360601/Pseudomonas aeruginosa UCBPP-PA14	5.9 / 35.59	11	38
55	glutathione synthetase	gshB/Pseudomonas syringae pv. tomato str. DC3000	5.2 / 35.55	10	51
56	succinyl-CoA synthetase alpha chain	sucD/Pseudomonas aeruginosa PA01	5.8 / 30.65	15	56
57	Dihydrodipicolinate reductase (DHPR)	dapB/Pseudomonas struzeri	6.2 / 28.16	9	33
58	COG4067: Uncharacterized protein conserved in archaea	ZP_00139426/Pseudomonas aeruginosa UCBPP-PA14	9.7 / 24.57	9	52
59	alkyl hydroperoxide reductase subunit C	ahpC/Pseudomonas aeruginosa PA01	5.9 / 20.64	9	44
60	enoyl-(acyl-carrier-protein) reductase	fabI/Pseudomonas syringae pv. tomato str. DC3000	5.4 / 28.07	9	29
61	electron transfer flavoprotein beta-subunit	EtfB/Pseudomonas aeruginosa PA01	9.0 / 26.42	11	45
62	valyl-tRNA synthetase	valS/Pseudomonas syringae pv. tomato str. DC3000	5.4 / 107.45	15	16
63	lysyl-tRNA synthetase	lysS/Pseudomonas syringae pv. tomato str. DC3000	5.0 / 57.04	17	43
64	GMP synthase	GuaA/Pseudomonas syringae pv. tomato str. DC3000	5.4 / 58.56	19	44
65	COG1960: Acyl-CoA dehydrogenases	ZP 00138778/Pseudomonas aeruginosa UCBPP-PA14	8.8 / 51.22	14	36
66	COG3200: 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthase	Pflu1193/Pseudomonas fluorescens PfO-1	6.0 / 51.07	11	46
67	ribosomal protein S1	gil28868956/Pseudomonas syringae pv. tomato str. DC3000	4.9 / 62.43	12	24
68	Chaperone protein dnaK (Heat shock protein 70) (Heat shock 70 kDa protein)	dnaK/Pseudomonas aeruginosa PA01	4.9 / 68.99	14	20
69	dihydroxy-acid dehydratase	IlvD/Pseudomonas aeruginosa PA01	5.7 / 65.88	13	32
70	heat shock protein HtpG	HtpG/Pseudomonas putida KT2440	5.2 / 71.59	12	20
71	WspE	WspE/Pseudomonas fluorescens	5.0 / 83.50	19	39
72	phosphoenolpyruvate-protein phosphotransferase	PtsP/Pseudomonas putida KT2440	5.4 / 83.49	15	30
73	hypothetical protein	PSPTO4884/Pseudomonas syringae pv. tomato str. DC3000	8.6 / 85.91	17	30
74	malate synthase G	GlcB-2/Pseudomonas syringae pv. tomato str. DC3000	5.9 / 80.38	19	32
75	leucyl-tRNA synthetase	leuS/Pseudomonas aeruginosa PA01	5.5 / 98.31	24	31
76	probable transcarboxylase subunit	PA5435/Pseudomonas aeruginosa PA01	5.6 / 66.31	21	39
77	aspartate kinase, monofunctional class	gil28869048/Pseudomonas syringae pv. tomato str. DC3000	5.2 / 44.50	10	33
78	malic enzyme	PA5046/Pseudomonas aeruginosa PA01	5.1 / 45.75	11	26
79	COG4067: Uncharacterized protein conserved in archaea	Paer380001/Pseudomonas aeruginosa UCBPP-PA14	9.7 / 24.57	8	43
80	COG0360: Ribosomal protein S6	Psyr1537/Pseudomonas syringae pv. syringae B728a	4.9 / 16.38	9	59
81	aspartyl-tRNA synthetase	aspS/Pseudomonas syringae pv. tomato str. DC3000	5.3 / 66.62	19	34
82	methyl-accepting chemotaxis protein	NahY/Pseudomonas putida	5.0 / 58.97	12	38

The subunit composition of the protein bands separated by BN-PAGE was determinated by a Tricine-SDS-PAGE (2-D BN-PAGE) of the individual lanes (Fig. 3). A total of approximately 200 spots were visualized out of which, 82 different gene products were identified by PMF. Table 2 lists the proteins that were identified by MS. All inducible spots from bacteria using phenol as sole carbon source contained proteins that are involved in phenol degradation. Evidently, the enzymes dmpH and dmpE (Spot Nr. 3), form multimeric complexes with a apparent molecular weight of 570 kDa. Additionally, there is indication of the formation of a dmpG and dmpF complex (Spot Nr. 14, 15) with a apparent molecular weight of 180 kDa. Based on the Coomassie stain intensity a 1:1 stoichiometry was obtained for the dmpG and dmpF proteins. Apart from the inducible protein complexes, the presence of multimeric complex with an apparent molecular weight of 500 kDa of the aldehyde dehydrogenase family protein (Q88JG9) (Spot Nr. 5), of 530 kDa of the polyribonucleotide nucleotidyltransferase (Pnp, Spot Nr. 4) and of 200 kDa of malate synthase (GlcB/Q87Z72, spot nr. 13) were also detected.

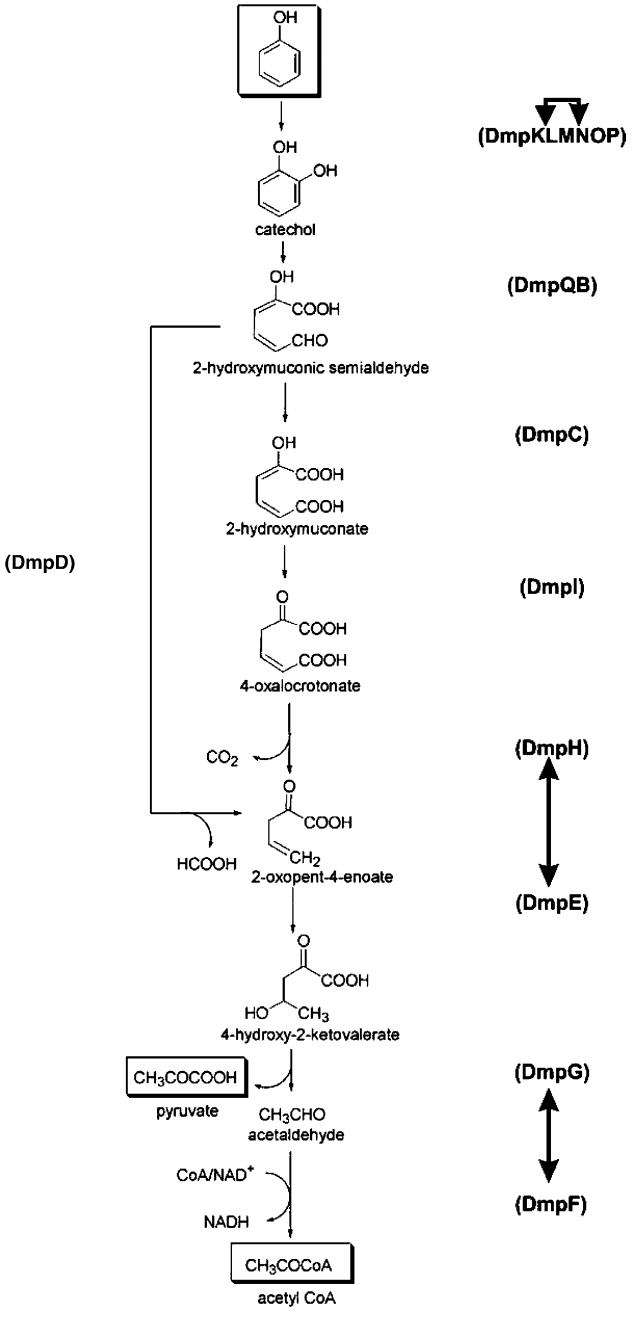

Fig. 4. Identified protein interactions in the metabolic pathway of phenol

Discussion

The capability of the bacterium *Pseudomonas* sp. strain phDV1 to degrade phenol has already been studied and most of the metabolic enzymes have been identified. Previous studies indicate that the strain possesses the *meta* cleavage pathway of catechol for degradation of phenol (Tsirogianni et al., 2004, 2005). However, to the best of our knowledge, this is the first report dealing with study of enzymatic complexes during aromatic growth from crude extracts of *Pseudomonas* sp. strain phDV1. Protein complexes, and those in particular that are formed by non-covalent interactions, generally require electrophoretic native conditions at pH values that are compatible with the stability of the association. BN-PAGE performed at a pH of 7.5 in the presence of Coomassie has been developed to study protein complexes (Schaegger and von Jagow, 1991).

In our study, the complexes separated by BN-PAGE have different sizes and the apparent molecular mass varies from 800 kDa to 100 kDa. As for molecular sieving, matrices like non-denaturing polyacrylamide gels, are limited to about molecular mass 10000 kDa (Tulp et al., 1999). The subsequent application of proteolysis of the protein complexes has enabled the identification of the proteins in the inducible protein complexes by MALDI-TOF MS and PMF search. Analysis of peptides from protein mixture will not achieve statistically significant scores because the more complex the protein mixture is, the more non-matching (noise) peptides for any given protein will appear. The search using Spectrum Mill workbench with manual PMF is limited to the analysis of digests from protein mixtures consisting of a maximum of five proteins. Using the proper search criteria described in the experimental session it was possible to identify more than 5 proteins in single band from BN-PAGE. When examining the results of BN-PAGE that caused

the separation of various complexes, the presence of proteins involved in the metabolic pathway was identified in most of those complexes (Table 1). Furthermore, in spot 3 (dmpH, dmpE), spot 15 (dmpF, dmpG, bmoA), spot 18 (dmpN, dmpB, bmoA) and spot 22 (dmpL, dmpN, bmoA), the identified proteins belong to the gene products of the dmp operon (Powlowski and Shingler, 1994). This correlates with the presence of these protein complexes in the protein fraction of the bacteria grown in phenol but not glucose (Fig. 2).

Phenol hydroxylase is a multicomponent that contains six proteins (dmpK-dmpP subunits). It has been found to be essential for growth of bacterial strain on phenol (Nordlund et al., 1990, 1993). The purification of the dmpL-dmpO subunits resolved these proteins in two components, one consisting of dmpM alone, and the other a complex of dmpL, dmpN and dmpO (Powlowski and Shingler, 1994). The identification of dmpL, dmpN and dmpP has been reported recently using 1-D Tricine-SDS-PAGE in combination with MALDI-TOF MS (Tsirogianni et al., 2004). BN-PAGE and PMF search allowed the identification of only two subunits namely the dmpL and dmpN in the obtained BN-PAGE protein complexes bands. The dmpN subunit is present in different bands but only in spot 22 (Fig. 2, lane 1) was in the presence of the dmpL and the bmoA. Unfortunately, the poor resolution of the BN-PAGE in this region, along with the high protein concentration, did not allow a significant identification of the proteins.

Most often, the product of one catalytic reaction is the substrate for a subsequent reaction. By holding the enzymes in a complex, a series of enzymatic reactions becomes more efficient (substrate channeling). One of the interesting properties of some of the *meta*-pathway cleavage enzymes is the formation of multienzyme complexes (Harayama et al., 1989; Powlowski and Shigler, 1994). The results of the current study are in correlation with these observations and suggest that metabolic channeling may be important for pathway efficiency. Based on the estimated molecular weight and the spot identity after 2-D BN-PAGE, the complex consists of two dmpG and two dmpF subunits. In contrast, the dmpE and dmpH form a higher numbered complex, which suggests the presence of at least five subunits of each gene product.

Except the inducible proteins the presence of a multimeric complex with a molecular weight of 500 kDa, which is a member of the aldehyde dehydrogenase family protein (Q88JG9/PP2680 gene product) was observed, correlating with the observation of a homomultimeric complex in higher plants (Yamada et al., 1999; Tang et al., 2002). Further, the presence of the Pnp in a native form at a molecular weight of 530 kDa is a first indication that the polyribonucleotide nucleotidyltransferase, which is involved in mRNA degradation is a homohexameric and not a homotrimeric (Stover et al., 2000; Nelson et al., 2002). Finally, the malate synthase G (Q87Z72), involved in the formation of S-malate from acetyl-CoA and glyoxylate, exist at least as dimer in contrast to its suggested monomer presence (Artiguenave and Gheewala, 1997).

The PMF spectra of the digested spots after 2-D BN-PAGE had generally a better quality compared to the 1D-BN having increased signal to noise ratio and better resolved peptides, allowing a more significant identification of the proteins. Furthermore, 2-D BNPAGE stained with Coomassie can provide information concerning the stoichiometry of protein in complexes, which is impossible on native gels. The example application of 2-D BN-PAGE presented here, shows that this technique is a useful tool for ''functional proteomics''.

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