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Proteomic study of linuron and 3,4-dichloroaniline degradation by *Variovorax* sp. WDL1: evidence for the involvement of an aniline dioxygenase-related multicomponent protein

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Abstract

A proteomic approach was used to explore the metabolism of the phenylurea herbicide linuron and 3,4-dichloroaniline (3,4-DCA) in *Variovorax* sp. WDL1. This bacterium grows on linuron as sole source of carbon, nitrogen and energy, while it transiently accumulates 3,4-DCA as a metabolite. Differential protein expression analysis of *Variovorax* sp. WDL1 grown in a heterotrophic medium in the presence and absence of linuron or 3,4-DCA was conducted using 2-D PAGE. Selected up- and downregulated proteins were identified with nanoLC-ESI-MS/MS. In the 3,4-DCA-supplemented culture, upregulation of several proteins showing high amino acid sequence similarity to different components of the multicomponent aniline dioxygenase in aniline-degrading *Proteobacteria* was observed. For one of the components, multiple variant proteins were detected, suggesting that strain WDL1 harbors several copies of the aniline dioxygenase (AD) gene cluster which are simultaneously expressed in the presence of 3,4-DCA. A number of unidentifiable proteins, which were upregulated in the linuron- and/or 3,4-DCA-supplemented cultures, might represent up to now uncharacterized proteins with a role in linuron and/or 3,4-DCA degradation in strain WDL1. In addition, several stress-related proteins were differentially expressed.
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Keywords: *Variovorax*; Biodegradation; Linuron; 3,4-Dichloroaniline; Aniline dioxygenase; Proteome

1. Introduction

Phenylureas such as linuron and diuron are commonly applied herbicides in agriculture, and their extensive use has resulted in substantial environmental contamination of surface water and groundwater (Batisson et al., 2007; Caux et al., 1998). The potential deleterious effects of linuron on ecosystem and human health, due to its toxicity towards aquatic organisms (Kegley et al., 2008) and its potential endocrine-disrupting properties (McIntyre et al., 2002), has stimulated research on its natural dissipation processes in soil. Several linuron-degrading bacteria have been isolated from long-term linuron-treated soils. *Bacillus sphaericus* ATCC 12123 and *Arthrobacter globiformis* D47 degrade linuron into the toxic 3,4-dichloroaniline (3,4-DCA), which accumulates as a dead-end product (Cullington and Walker, 1999; Wallnöfer, 1969). Recently, several bacteria which degrade and mineralize linuron beyond 3,4-DCA have been reported (Breugelmans et al., 2007; Dejonghe et al., 2003; Sørensen

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et al., 2005). All those isolates belong to a specific cluster within the genus *Variovorax* and it was suggested that members of this cluster fulfill a crucial role in linuron degradation in the environment (Breugelmans et al., 2007).

The main bacterial degradation route of linuron is initiated with direct hydrolysis of the amide bond resulting in the metabolites 3,4-DCA and *N,O*-dimethylhydroxylamine (Dejonghe et al., 2003; Engelhardt et al., 1972). Further degradation of 3,4-DCA can proceed via different possible routes, which include transformation to catechol-based moieties. However, knowledge about the pathways, catabolic genes and enzymes involved in linuron and 3,4-DCA degradation is scarce. Thus far, two enzymes which catalyze the hydrolysis of the amide bond in linuron have been identified, i.e., an aryl acylamidase in *B. sphaericus* ATCC 12123 showing activity towards methoxy-methyl-substituted phenylureas (Engelhardt et al., 1973) and a phenylurea hydrolase (PuhA) in *A. globiformis* D47 transforming methoxy-methyl- and dimethyl-substituted phenylureas (Turnbull et al., 2001). No reports exist on the identification of linuron hydrolysis enzymes in linuron-degrading *Variovorax*.

Regarding the metabolism of 3,4-DCA, You and Bartha (1982) reported that in the presence of aniline, a *Pseudomonas putida* strain degraded 3,4-DCA to the metabolite 4,5-dichlorocatechol, which was further transformed through *ortho* ring cleavage. On the other hand, 3-chloro-4-hydroxyaniline was detected during degradation of 3,4-DCA by *Pseudomonas fluorescens* 26-K, suggesting that 3,4-DCA degradation in this strain is initiated with the removal of a chloride substituent (Travkin et al., 2003). In cultures of the 3,4-DCA-degrading strains *P. fluorescens* 26-K and *Pseudomonas* sp. KB35B, grown in the presence of 3,4-DCA, high levels of chlorocatechol-2,3-dioxygenase activity were observed, indicating degradation of a 3,4-DCA-derived catechol-based moiety through a meta-cleavage pathway in those strains (Kim et al., 2007; Travkin et al., 2003). Overall, current data suggest that oxidative deamination with production of chlorinated catechols plays an important role in the degradation of 3,4-DCA. In this context, it should be mentioned that many monochloroaniline-degrading bacteria contain genes encoding proteins that are homologous to components of the multicomponent enzyme aniline dioxygenase (AD), which is responsible for oxidative deamination of aniline in aniline-degrading bacteria (e.g. Boon et al., 2001; Dejonghe, 2003; Liang et al., 2005; Takeo et al., 1998; Vangnai and Petchkroh, 2007). Yet the involvement of such AD enzymes in the degradation of chlorinated anilines has not yet been shown (Boon et al., 2001). Furthermore, no information exists on proteins involved in 3,4-DCA degradation in linuron-degrading *Variovorax* strains.

In this study, we used 2-D PAGE (2-dimensional polyacrylamide gel electrophoresis) to study the differential protein expression patterns exhibited by *Variovorax* sp. WDL1 when cultivated in a defined heterotrophic broth supplemented with linuron or 3,4-DCA, or in a medium without supplements, in order to identify proteins involved in linuron/3,4-DCA degradation. Selected up- and downregulated proteins were sequenced and their putative function was identified by homology searching.

2. Materials and methods

2.1. Bacteria and growth media

Variovorax sp. WDL1 has been described previously (Dejonghe et al., 2003). WDL1 was routinely grown on R2A agar medium supplemented with 10 mg L^{-1} linuron. R2A is composed of 0.5 g tryptone, 0.5 g yeast extract, 0.5 g casein hydrolysate, 0.5 g glucose D+, 0.5 g soluble starch, 0.3 g sodium pyruvate, 0.3 g KH_2PO_4 and 0.05 g $MgSO_4$ per liter distilled water (pH 7.0). Liquid and solid R2A media were prepared as described previously (Breugelmans et al., 2007).

2.2. Sample preparation and protein extraction

WDL1 was precultured in liquid R2A medium supplemented with 10 mg L^{-1} linuron. Cells were harvested during exponential growth and washed three times with 0.9% w/v NaCl. After adjusting the optical density (OD) to 600 nm (OD_{600}) to 0.1, 1 mL was inoculated in 250 mL Erlenmeyer flasks containing 100 mL R2A medium supplemented with either 50 mg L^{-1} (0.2 mM) linuron or 32 mg L^{-1} (0.2 mM) 3,4-DCA, and in R2A medium without supplements. R2A was selected as background medium, since it allows efficient growth of WDL1 for producing sufficient biomass for protein analysis. Linuron (99.5%, Sigma Aldrich) and 3,4-DCA (99.3%, Sigma Aldrich) were added as 20 g L^{-1} acetone solutions to the Erlenmeyer flasks and the solvent was allowed to evaporate prior to addition of the medium. Duplicate cultures were incubated in the dark on a rotary shaker (120 rpm) at 25 °C. The OD_{600} of the cultures were measured regularly and linuron and 3,4-DCA concentrations were monitored using HPLC analysis as described previously (Breugelmans et al., 2007).

Linuron-supplemented and unsupplemented R2A cultures of WDL1 were harvested for protein extraction when linuron and 3,4-DCA concentrations fell below the HPLC detection limit in the linuron-supplemented WDL1 cultures. Similarly, proteins were extracted from 3,4-DCA-supplemented cultures when 3,4-DCA was completely degraded. Then, 50 mL of the cultures was centrifuged (1400 × *g*, 15 min, 20 °C). Pellets were washed once with PBS buffer (7.1 mM K_2HPO_4, 2.9 mM KH_2PO_4 and 151 mM NaCl in double distilled water; pH 7.0) and subsequently re-suspended in 1 mL SDS-buffer (100 mM Tris pH 9.5 and 1% w/v SDS). Cells were mechanically lysed with a probe sonifier (B. Braun Labsonic U; 3 times for 20 s with 0.9 s pulses at full power with intermittent cooling on ice) and proteins were extracted using a phenol-based procedure as described previously (De Mot and Vanderleyden, 1989). Protein concentrations were determined by using a 2-D Quant Kit with bovine serum albumin (BSA) as protein standard following the manufacturer's guidelines (GE Healthcare). Protein extracts were stored at −20 °C until use.

2.3. Protein separation and identification

Protein separation was performed as described previously (De Mot et al., 2007). Initially, extracted protein samples from

linuron-supplemented and control cultures were analyzed on IPG strips with a non-linear pH gradient ranging from 3 to 10 (Immobiline DryStrips, 24 cm, GE Healthcare). To increase resolution, electrophoresis was additionally performed with IPG strips with a linear pH gradient ranging from 4 to 7 (Immobiline DryStrips, 24 cm, GE Healthcare) for the control and the linuron- and 3,4-DCA-grown samples. Following isoelectric focusing, proteins were separated in the second dimension in an Ettan DALT*twelve* gel system (Amersham Biosciences). After staining with Sypro Ruby Protein gel stain (Invitrogen), protein patterns were visualized with a high-resolution scanner (Typhoon 9400 Variable Mode Imager, Amersham Biosciences) and analyzed with the Imagemaster 2D platinum software package (Amersham Biosciences). For each treatment, two gels of two independently grown biological samples were processed. After normalization of spot intensities to total intensity within a gel, spots having ratios larger than 1.20 and gaps larger than 0.02 vol% were selected. Finally, spots which showed more than 100% up- or down-regulation in the linuron- or 3,4-DCA-supplemented cultures compared to the control cultures grown in R2A (as determined by the ratio of the averaged center values of corresponding protein spots in gels of the supplemented vs. control cultures) were retained for further analysis. Selected protein spots were excised from the gels using an automated Ettan Spot Picker (Amersham Biosciences) and subjected to trypsinolysis in 1.5 mL polypropylene tubes as described previously (Noël-Georis et al., 2004). Peptides generated from digestions were extracted in two steps: first with an equal volume of 0.1% v/v trifluoroacetic acid (TFA) (50 µL) and vigorous vortexing for 15 min and subsequently with the same volume of 70% v/v acetonitrile/25% v/v H_2O/5% v/v TFA and vortexing. The collected peptide solution was dried in a Speed-Vac, re-suspended in 50 µL 0.1% v/v TFA and stored frozen until use.

Tryptic peptides were separated using a 75 µm × 15 cm reverse-phase C18 column (LC-Packing) at a flow rate of 0.3 mL min^{-1} and a 3–32% acetonitrile gradient in 0.1% v/v formic acid on an Ultimate 3000 NanoLC system (Dionex). The LC eluent was coupled to a nano-ionspray source attached to a high-capacity ion spectrometer (HCTultra Plus, Bruker Daltonics). Peptides were analyzed in positive ion mode. MS spectra were acquired on 200,000 targets in the trap. For each MS spectrum, the three most intense multiple charged peaks were selected for generation of subsequent collision-induced dissociation MS. A dynamic exclusion window was applied, which prevented peptides with the same mass/charge (m/z) from being selected for 1 min after the initial acquisition. MS and MS/MS data were processed and analyzed using the default method of DataAnalysis 3.4 and Biotools 3.1 (Bruker Daltonics). Mascot 2.2 (Matrix Science) was used as a database search engine (Perkins et al., 1999) in the NCBI database (NCBInr 20080108) with taxonomy restrictions set to *Bacteria*. One missed cleavage per peptide was allowed, with peptide tolerance of 1.2 Da and MS/MS tolerance of 0.6 Da. Peptide modifications allowed during the search were carbamidomethylation of cysteines (fixed) and oxidation of methionines (optional). Protein identification results including the molecular weight (MW) and isoelectric point (p*I*) of identified proteins were evaluated manually and verified relative to the electrophoretic mobility of the protein spot on the 2-D gels.

3. Results and discussion

3.1. Growth of Variovorax *sp. WDL1 in heterotrophic medium in the presence and absence of linuron or 3,4-DCA*

Growth of *Variovorax* sp. WDL1 in R2A broth was retarded in the presence of linuron and especially 3,4-DCA (Fig. 1A). Several studies have shown toxicity of 3,4-DCA and linuron towards the bioluminescent bacterium *Vibrio fischeri* in the Microtox® assay, reporting EC_{50} values for 3,4-DCA and linuron of 0.50 mg L^{-1} and 5.5 mg L^{-1}, respectively (Hernando et al., 2003; Tixier et al., 2002). In this study, we used 50 mg L^{-1} linuron in the linuron-supplemented cultures, which is about 9 times the reported EC_{50} (Hernando et al., 2003). The maximal accumulated 3,4-DCA concentration in those cultures was about 20 times the reported EC_{50} value (Tixier et al., 2002). Therefore, growth retardation of WDL1 in the linuron-supplemented cultures was probably due to the toxicity of 3,4-DCA. The transient accumulation of 3,4-DCA during growth on linuron is typical for strain WDL1 in both heterotrophic media and oligotrophic media (Dejonghe et al., 2003). In the 3,4-DCA-supplemented cultures 32 mg L^{-1} 3,4-DCA was present corresponding to about 60 times the reported EC_{50} (Tixier et al., 2002). This can explain the

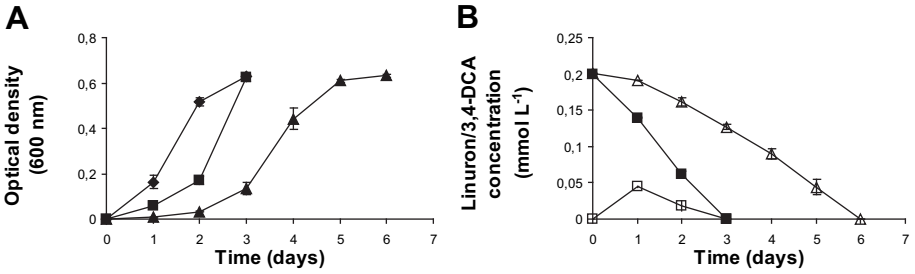

Fig. 1. (A) Growth of *Variovorax* sp. WDL1 in R2A liquid medium in the absence of linuron or 3,4-DCA (diamonds), in the presence of 50 mg L^{-1} linuron (squares) and in the presence of 32 mg L^{-1} 3,4-DCA (triangles). (B) Degradation of linuron (black squares) and transient accumulation of 3,4-DCA (open squares) in linuron-supplemented R2A cultures, and degradation of 3,4-DCA in 3,4-DCA-supplemented R2A cultures (open triangles). Data points and error bars represent the means and standard deviations of duplicate measurements. When error bars are not visible, they are hidden behind the symbols.

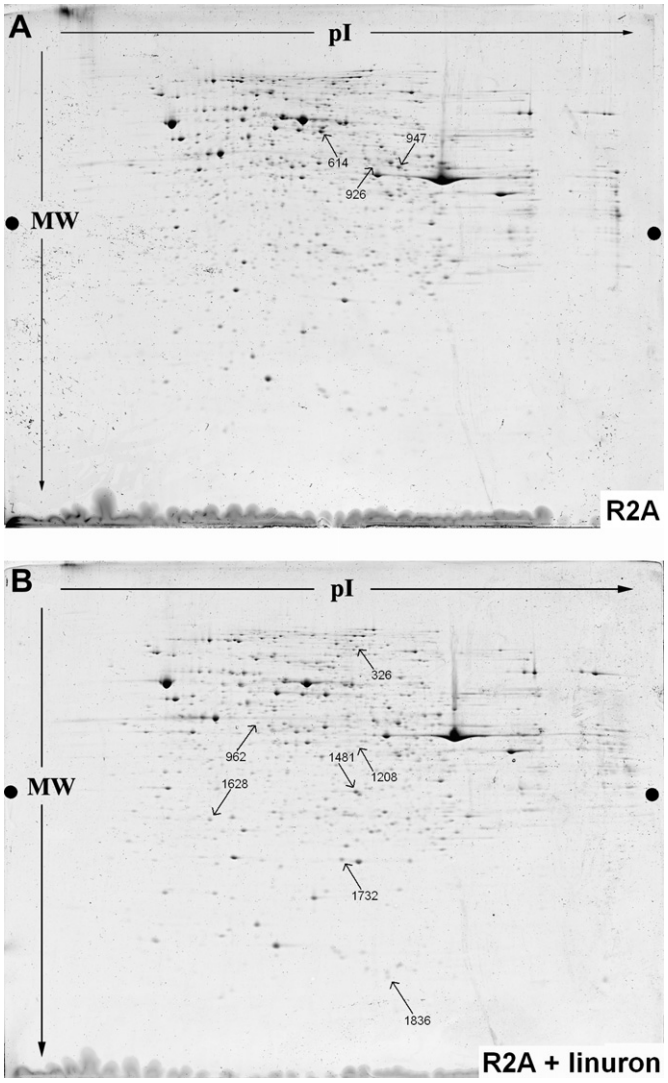

Fig. 2. Analytical 2-D gels of total cellular proteins of *Variovorax* sp. WDL1 focused using pH 3–10 IPG strips and stained with Sypro Ruby. Molecular weight (MW) ranges from 10 to 105 kDa. Protein extracts originated from control R2A cultures (A) and linuron-supplemented R2A cultures (B). Numbers correspond to identified proteins listed in Table 1.

observed differences in growth of WDL1 in the linuron- and 3,4-DCA-supplemented R2A cultures.

3.2. Upregulation of aniline dioxygenase-like components in Variovorax *sp. WDL1 grown in the presence of 3,4-DCA*

In the 3,4-DCA-supplemented cultures, several proteins (i.e. corresponding to spots 3267, 3268, 3299, 3407, 3513, 3568, 3570, 3610 and 3614; Fig. 3C & Table 1) were upregulated and showed significant similarity to different components of AD identified in aniline-degrading strains such as *P. putida* UCC22 and *Delftia tsuruhatensis* AD9. Both strains convert aniline through oxidative deamination to catechol (Fukumori and Saint, 1997; Liang et al., 2005). In *P. putida* UCC22 the AD gene cluster (i.e. *tdnQTA1A2B*) is located on

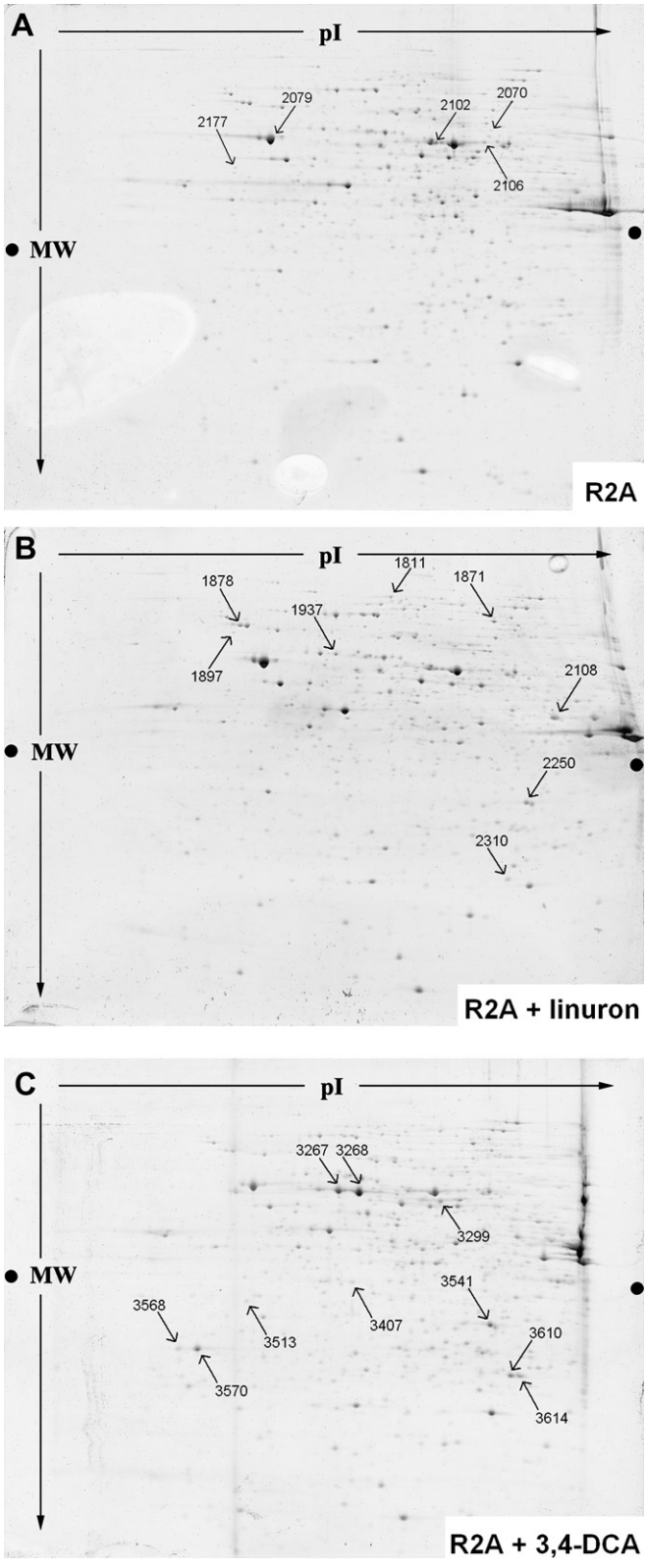

Fig. 3. Analytical 2-D gels of total cellular proteins of *Variovorax* sp. WDL1 focused using pH 4–7 IPG strips and stained with Sypro Ruby. Molecular weight (MW) ranges from 10 to 105 kDa. Protein extracts originated from control R2A cultures (A), linuron-supplemented R2A cultures (B), and 3,4-DCA-supplemented R2A cultures (C). Numbers correspond to identified proteins listed in Table 1.

Table 1
Overview of identified differentially expressed proteins from *Variovorax* sp. WDL1 grown in R2A medium in the presence and absence of linuron or 3,4-DCA.

2-D gel	Spot number[a]	Differential expression[b]	Protein description (NCBI accession number)[c]	Species	MASCOT score[f] ($p < 0.05$)	Queries matched[g]	Theoretical[h]		Experimental[h]	
							MW	p*I*	MW	p*I*
Fig. 2A	614	Lin (−12.9)	Aldehyde dehydrogenase (YP_346283)	*Pseudomonas fluorescens* PfO-1	125	2	54	5.39	52	5.64
	926	Lin (−5.8)	Acyl-CoA dehydrogenase domain protein (YP_970340)	*Acidovorax avenae* subsp. *citrulli* AAC00-1	128	3	43	6.18	46	6.20
	947	Lin (−3.0)	Acyl-CoA dehydrogenase domain protein (YP_987191)	*Acidovorax* sp. JS42	216	5	44	6.18	46	6.50
Fig. 2B	326	Lin (+2.4)	ATP-dependent Clp protease, ATP-binding subunit ClpA (YP_982165)	*Polaromonas naphthalenivorans* CJ2	381	6	86	6.22	89	5.89
	962	Lin (NS)	No significant similarity	–	–	–	–	–	46	5.19
	1208	Lin (NS)	No significant similarity	–	–	–	–	–	44	5.90
	1481	Lin (NS)	Tetrahydromethanopterin biosynthesis protein (YP_552900)	*Burkholderia xenovorans* LB 400	52	1	51	5.19	34	5.88
	1628	Lin (+3.9)	Hypothetical protein Bpro_0629 (YP_547486)	*Polaromonas* sp. JS666	88	1	27	5.46	31	4.73
	1732	Lin (NS)	Hypothetical protein Bpro_0629 (YP_547486)	*Polaromonas* sp. JS666	66	1	27	5.46	27	5.76
	1836	Lin (NS)	No significant similarity	–	–	–	–	–	10	6.27
Fig. 3A	2070	DCA (−5.4)	Pyruvate dehydrogenase subunit E1 (YP_549486)	*Polaromonas* sp. JS666	115	2	102	6.07	62	5.65
	2079	Lin (+2.2)	Chaperonin GroEL (YP_547613)[d]	*Polaromonas* sp. JS666	485	26	58	5.16	60	4.19
	2102	DCA (−5.3)	Chaperonin GroEL (YP_997330)[d]	*Verminephrobacter eiseniae* EF01-2	456	8	57	5.18	54	3.39
			Methylmalonate-semialdehyde dehydrogenase (YP_970950)	*Acidovorax avenae* subsp. *citrulli* AAC00-1	122	2	54	6.36	54	3.39
	2106	DCA (−2.0)	No significant similarity	–	–	–	–	–	54	5.63
	2177	DCA (−4.3)	Chaperone GroEL (YP_969317)[d]	*Acidovorax avenae* subsp. *citrulli* AAC00-1	148	3	57	5.13	48	4.12
Fig. 3B	1811	Lin (+3.0)	Formate dehydrogenase, alpha subunit (YP_987660)	*Acidovorax* sp. JS42	219	3	104	6.14	103	5.17
	1871	Lin (NS)/DCA (NS)	Molecular chaperone protein DnaK (YP_987417)[e]	*Acidovorax* sp. JS42	579	1	69	4.96	84	5.65
	1878	DCA (−2.0)	Probable chaperone protein DnaK (CAD16342)[e]	*Ralstonia solanacearum* GMI1000	167	2	70	5.04	81	4.14
	1897	Lin (+2.0)	No significant similarity	–	–	–	–	–	77	4.13
	1937	Lin (+2.1)	30S ribosomal protein S1 (YP_971619)	*Acidovorax avenae* subsp. *citrulli* AAC00-1	222	3	61	5.31	64	4.73
	2108	Lin (+2.4)	Molecular chaperone protein DnaK (YP_523225)[e]	*Rhodoferax ferrireducens* T118	71	1	70	5.08	44	6.31
	2250	Lin (NS)/DCA (NS)	Cpn60 (AAM88520)[d]	*Acidovorax facilis* ATCC 11228	114	1	20	5.04	35	5.79
			Tetrahydromethanopterin biosynthesis protein (YP_552900)	*Burkholderia xenovorans* LB 400	52	1	51	5.19	35	5.79
	2310	Lin (NS)	Hypothetical protein Bpro_0629 (YP_547486)	*Polaromonas* sp. JS666	66	1	27	5.46	27	5.71
Fig. 3C	3267	DCA (+5.3)	Amino group transfer protein (TadQ) (AAX47239)	*Delftia tsuruhatensis* AD9	680	12	55	5.52	58	4.83
	3268	DCA (+5.4)	Amino group transfer protein (TadQ) (AAX47239)	*Delftia tsuruhatensis* AD9	1018	19	55	5.52	57	5.07
	3299	DCA (+2.5)	Amino group transfer protein (TadQ) (AAX47239)	*Delftia tsuruhatensis* AD9	422	6	55	5.52	54	5.53
			Large subunit of terminal dioxygenase (TdnA1) (BAA12807)	*Pseudomonas putida* UCC22	395	6	51	5.65	54	5.53
	3407	DCA (+3.8)	Electron transfer protein (TdnB) (BAA12809)	*Pseudomonas putida* UCC22	601	8	36	5.41	40	5.00
			Small subunit of terminal dioxygenase (TdnA2) (BAA12808)	*Pseudomonas putida* UCC22	268	5	24	6.32	40	5.00
	3513	DCA (+3.7)	Large subunit of terminal dioxygenase (TdnA1) (BAA12807)	*Pseudomonas putida* UCC22	136	2	51	5.65	36	4.23
			Amino group transfer protein (TadQ) (AAX47239)	*Delftia tsuruhatensis* AD9	123	2	55	5.52	36	4.23

3541	Lin (NS)/DCA (NS)	No significant similarity	–	–	–	–	–	35	5.79
3568	DCA (NS)	Amino group transfer protein (TadT) (AAX47240)	*Delftia tsuruhatensis* AD9	309	6	27	4.97	33	4.00
3570	DCA (+4.5)	Amino group transfer protein (TadT) (AAX47240)	*Delftia tsuruhatensis* AD9	464	13	27	4.97	33	4.05
3610	DCA (+2.6)	Small subunit of terminal dioxygenase (TdnA2) (BAA12808)	*Pseudomonas putida* UCC22	445	13	24	6.32	30	6.10
3614	DCA (+4.1)	Small subunit of terminal dioxygenase (TdnA2) (BAA12808)	*Pseudomonas putida* UCC22	381	11	24	6.32	30	6.23

[a] Indicated spot numbers are corresponding to those marked on the 2-D gels in Figs. 2 and 3. When two matches are reported for the same protein spot, peptide sequences were recovered from that spot, which matched with either of the two indicated proteins.

[b] Upregulation (+) or downregulation (−) of proteins in the linuron (Lin)- and/or 3,4-DCA (DCA)-supplemented cultures relative to control cultures. Values represent the ratios of averaged center values of corresponding spots in 4 gels of the linuron- or 3,4-DCA-supplemented cultures vs. the averaged center values of corresponding spots in 4 gels of the control cultures. NS: new spot not observed in the R2A control cultures.

[c] When two matches are reported for the same protein spot, peptide sequences were recovered from that spot which matched significantly with either of the two indicated proteins.

[d] Chaperone GroEL and Cpn60 are synonyms of 60 kDa chaperonin (HSP60 family).

[e] Chaperone DnaK is a synonym of 70 kDa heat shock protein (HSP70 family).

[f] MASCOT score: probability score based on the MOWSE algorithm.

[g] Queries matched: indicates the number of MS/MS spectra that were matched to the corresponding protein found in the NCBI database.

[h] Theoretical MW and p*I* were calculated from protein sequence data found in the NCBI database. Experimental MW and p*I* were calculated from the position of the spots on the 2-D gels using Imagemaster.

MW: molecular weight (kDa); p*I*: isoelectric point.

plasmid pTDN1 (Fukumori and Saint, 1997), while in *D. tsuruhatensis* AD9 the AD genes (i.e. *tadQTA1A2B*) are located on the chromosome where they are flanked by two copies of IS*1071* (Liang et al., 2005). Gene clusters encoding AD have also been characterized in other aniline-degrading strains such as *Acinetobacter* sp. YAA (Fujii et al., 1997), *Delftia acidovorans* 51-N (Kim et al., 2003), *D. acidovorans* 7N (Urata et al., 2004) and *Frateuria* sp. ANA-18 (Murakami et al., 2003). All AD gene clusters identified so far in aniline-degrading bacteria show strong similarity in genetic organization and nucleotide sequence (Liang et al., 2005).

Aniline dioxygenases are Rieske non-heme iron dioxygenases composed of multiple components, i.e. a protein homologous to glutamine synthetase (GS), a protein homologous to glutamine amidotransferase (GA), a large (α) and a small (β) subunit of the terminal dioxygenase, and a reductase (electron-transferring) component (Fukumori and Saint, 1997; Liang et al., 2005). In *P. putida* UCC22/*D. tsuruhatensis* AD9 these components are encoded by, respectively, *tdnQ*/*tadQ*, *tdnT*/*tadT*, *tdnA1*/*tadA1*, *tdnA2*/*tadA2* and *tdnB*/*tadB*. GS proteins catalyze the condensation of glutamate and ammonia to glutamine, while GA proteins catalyze the removal of the ammonia group from a glutamine molecule and its subsequent transfer to a specific substrate. Therefore, it was suggested that the GS and GA homologues found in ADs of aniline-degrading strains are involved in the recognition and release of amino groups from aniline (Takeo et al., 1998). *Escherichia coli* cells expressing the GS homologue TdnQ of *P. putida* UCC22 showed no GS activity (Fukumori and Saint, 1997), indicating that AD GS-like proteins are not involved in glutamine biosynthesis in aniline-degrading strains. Takeo et al. (1998) reported that all components of the AD multicomponent enzyme are necessary to initiate aniline degradation in *Acinetobacter* sp. YAA. Recently, it has been shown that the α subunit of the terminal dioxygenase in strain YAA (i.e. AtdA3) plays a role in AD substrate specificity (Ang et al., 2007).

The peptide sequences determined from protein spots 3267, 3268, 3299 and 3513 matched most closely to amino acid (AA) sequences of the AD GS homologues TadQ of *D. tsuruhatensis* AD9 and TdnQ of *P. putida* UCC22 (Fig. 3C & Table 1). Phylogenetic analysis showed that the AD GS homologues form a group of proteins distant from GlnA of *E. coli*, indicating that AD GS homologues evolved from a common ancestor (Fig. 4A). In addition to GS homologues of ADs identified in aniline-degrading bacteria, this group also contains putative TdnQ-like proteins identified from translated DNA sequences of *tdnQ*-like gene fragments amplified by PCR from monochlorinated aniline-degrading *Delftia* and *Comamonas* strains (Boon et al., 2001). However, the involvement of those genes/proteins in degradation of chlorinated anilines has not been shown. All common peptide sequences obtained from spots 3267 and 3268 were identical. Moreover, both spots had the same MW and only a slightly different p*I*, indicating that these spots likely represent the same protein. When using 2-D PAGE, it is common to observe spots with identical peptide sequences but different mobility.

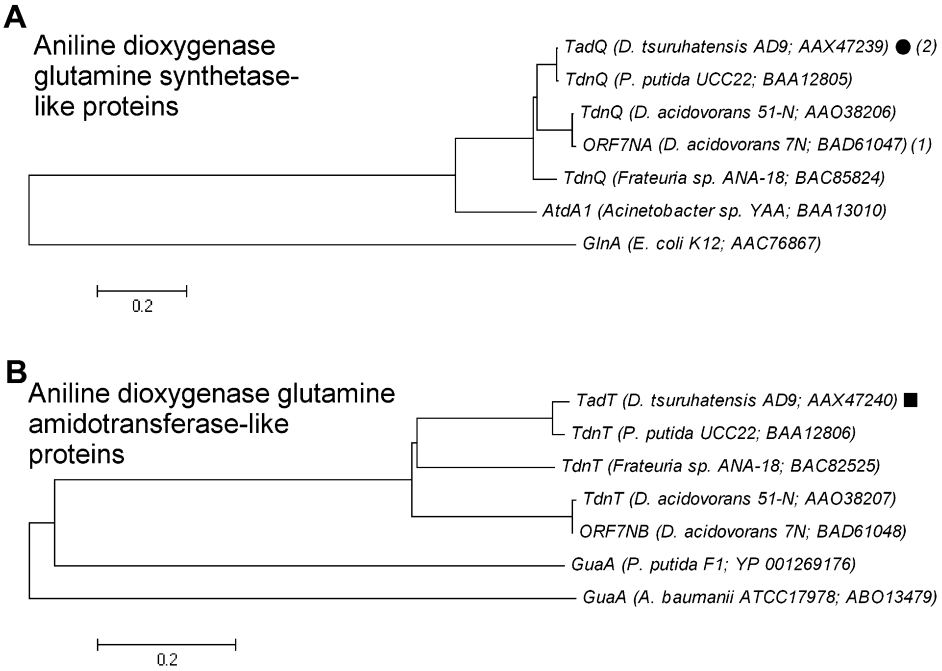

Fig. 4. Phylogenetic trees showing the evolutionary relationship among selected aniline dioxygenase (AD) glutamine synthetase (GS)-like proteins (based on Liang et al., 2005) (A) and AD glutamine amidotransferase (GA)-like proteins (B). Amino acid sequences, which were retrieved from the NCBI protein database were aligned with the ClustalW algorithm (Thompson et al., 1994) and trees were constructed using the NJ method with *MEGA* version 3.1 (Kumar et al., 2004). Strain designations and NCBI accession numbers are shown between brackets. The closest relative of protein spots 3267, 3268, 3299 and 3513 is marked with a black circle, while the closest relative of protein spots 3568 and 3570 is marked with a black square (based on partial AA sequences). In A, the TdnQ-like protein showing the most sequence identity to the deduced AA sequence from the PCR-derived *tdnQ* gene fragment of the aniline- and 3-chloroaniline (3-CA)-degrading strains *D. acidovorans* CA28 (AF315639), LME1 (AF315640) and B8c (AF315643), and *C. testosteroni* I2 (AF315641) is marked with (1) (Boon et al., 2001). Similarly, the closest relative of the AA sequence deduced from the PCR-derived *tdnQ* gene fragment of the aniline and 3-CA degrader *D. acidovorans* BN3.1 (AF315642) is marked with (2) (Boon et al., 2001). The scale bars represent a divergence equivalent to an average of a 20% change in amino acids.

The difference in mobility can be attributed either to post-translational protein modifications (Wilkins et al., 1999), to gel-induced protein modifications (Jeannot et al., 1999) or to the occurrence of isoforms/splicing variants for which sequence coverage was not sufficient to distinguish between them. On the other hand, spots 3299 and 3513 showed a MW and a p*I* different from those of spots 3267 and 3268. Spots 3299 and 3513 might represent fragments of the protein associated with spots 3267 and 3268, since (nearly) identical AA sequences were obtained. Alternatively, they represent different proteins encoding the same function. The latter seems to be the case for the protein associated with spot 3299, since from that spot, one peptide sequence was recovered with a slightly lower MW (i.e. 16 Da) than analogous peptides recovered from spots 3267 and 3268. As shown in Fig. 5, this peptide sequence matched exactly to a region in the AD GS homologue TdnQ (translated from PCR-derived DNA sequence data) of *Frateuria* sp. ANA-18, but differed at 3 positions from the corresponding region in the AD GS homologues TadQ of *D. tsuruhatensis* AD9 and TdnQ of *P. putida* UCC22. Two of those differences were conservative substitutions, i.e. a substitution with an amino acid of the same family (D ↔ E; acidic AA, and I ↔ L; hydrophobic AA).

From spots 3299 and 3513, in addition to peptides showing similarity to AD GS homologues, a number of peptide sequences were recovered showing significant similarity to proteins constituting the α-subunit of AD in aniline-degrading bacteria such as TdnA1. Since spot 3299 migrated very close to the calculated MW and p*I* of both TdnQ and TdnA1, it is likely that this spot consists of a mixture of both proteins. However, spot 3513 was located in a region which did not correspond to the theoretical position of either TdnQ or TdnA1. This can be explained by protein fragmentation/processing, but it is unlikely that independent processing results in exactly the same MW and p*I* shift. Another explanation is that gene rearrangements occurred in the AD gene cluster of *Variovorax* sp. WDL1, resulting in gene fusions. However, PCR-based analysis using multiple primer sets designed from the peptide sequences and targeting intergenic regions in the AD gene cluster did not support the idea of gene fusion events (data not shown).

The peptide sequences obtained from spots 3568 and 3570 matched AA sequences of the AD GA homologue in aniline-degrading bacteria (Table 1). Given their neighboring location in the 2-D gel (Fig. 3C) and the fact that all obtained AA sequences were identical, spots 3568 and 3570 probably represent the same protein. The AA sequences recovered from these spots are most similar to AA sequences of the GA homologues TadT present in the AD of *D. tsuruhatensis* AD9 and TdnT present in the AD of *P. putida* UCC22, and are less related to the AD GA homologues of *Frateuria* sp. ANA-18, *D. acidovorans* 51-N and *D. acidovorans* 7N. All AD GA homologues cluster in a different branch than GA proteins not implicated in aniline degradation (such as GuaA), indicating divergent evolution in response to (chloro)aniline (Fig. 4B).

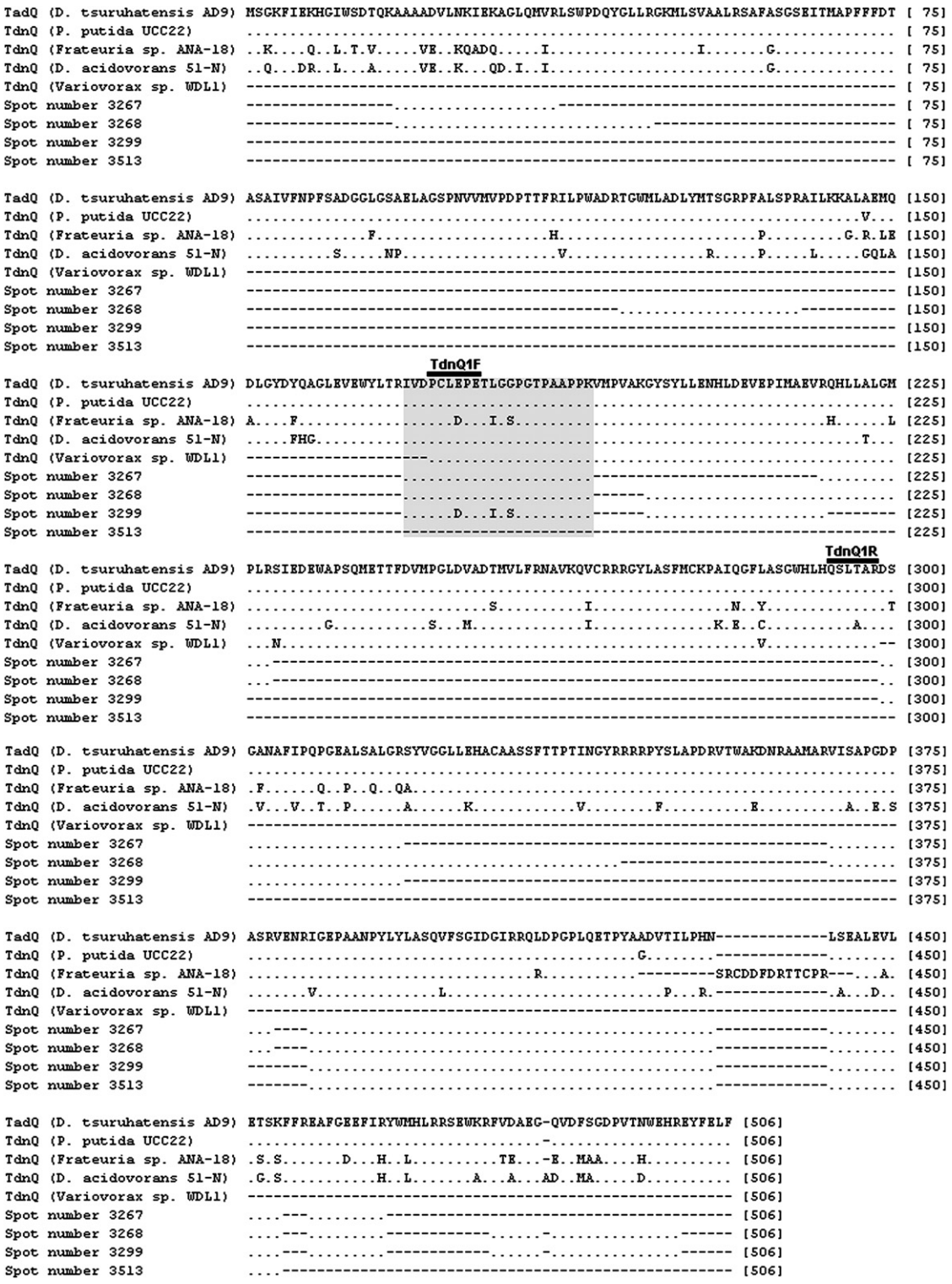

Fig. 5. ClustalW multiple protein sequence alignment performed with *MEGA* version 3.1 (Kumar et al., 2004) of AD GS-like sequences of *D. tsuruhatensis* AD9 (AAX47239), *P. putida* UCC22 (BAA12805), *Frateuria* sp. ANA-18 (BAC82524), *D. acidovorans* 51-N (AAO38206), and AA sequences obtained from spot numbers 3267, 3268, 3299 and 3513. TadQ and TdnQ AA sequences were translated from nucleotide sequences. The partial TdnQ sequence of *Variovorax* sp. WDL1 was deduced from a PCR-amplified *tdnQ* gene fragment (Genbank accession number FJ392118). The target regions of PCR primers TdnQ1F and TdnQ1R (Boon et al., 2001) are indicated. Dots represent identical AAs compared to the TadQ AA sequence of *D. tsuruhatensis* AD9 and dashes represent gaps or unidentified AAs. The gray shading highlights an AA segment with differences in the peptide sequences recovered from spots 3267, 3268 and 3299 (see text). The non-matching region at the C-terminal end of TdnQ of *Frateuria* sp. ANA-18 is probably due to frameshifts in the published DNA sequence.

Furthermore, proteins showing significant similarity to the terminal dioxygenase TdnA2 (spots 3610 and 3614; Fig. 3C & Table 1) and the electron transfer protein TdnB (spot 3407) of AD in *P. putida* UCC22 were found. Based on the partial AA sequences recovered from spots 3610 and 3614, the corresponding proteins were more similar to dioxygenase β-subunits of AD (i.e. TadA2 and TdnA2) than to dioxygenase β subunits involved in oxidation of other aromatic compounds (data not shown). From spots 3610 and 3614 identical peptide sequences were retrieved and these spots had the same MW and only a slightly different p*I*, indicating that they are probably derived from the same protein. Interestingly, from protein spot 3407, in addition to TdnB-related peptide sequences, several peptide sequences were derived which matched strongly with AA sequences of TdnA2 of AD in *P. putida* UCC22 and which were identical to AA sequences obtained from spots 3610 and 3614. In contrast with spots 3610 and 3614, spot 3407 was substantially distantly located from its theoretical migration point. The presence of peptides belonging to both TdnB and TdnA2 in spot 3407 and its particular location in the gel might again point to genetic rearrangements in the AD gene cluster of WDL1 compared to earlier described ADs, yet this could not be confirmed by a PCR-based analysis (data not shown).

Overall, our results show that expression of genes encoding different components of an AD-like multicomponent enzyme is enhanced in the presence of 3,4-DCA, strongly suggesting that an AD is involved in 3,4-DCA degradation in *Variovorax* sp. WDL1. Moreover, it seems that several AD GS homologues are upregulated in the presence of 3,4-DCA, indicating that several AD gene clusters are present in the strain. Preliminary Southern blot experiments indeed show that WDL1 harbors two copies of a *tdnQ*-like gene encoding such a GS homologue (data not shown). Unexpectedly, similar components of an AD were not upregulated in linuron-supplemented cultures. Possibly, this can be attributed to the limited amount of 3,4-DCA which accumulates during metabolism of linuron (Fig. 1B). The 3,4-DCA metabolic machinery might not be fully induced at low concentrations. Previously, we showed that complex interactions exist between expression of linuron and 3,4-DCA degradative genes in strain WDL1 (Breugelmans et al., 2008).

3.3. Up- and downregulation of stress-related proteins

In both the linuron- and the 3,4-DCA-supplemented cultures, stress-related proteins such as ClpA protease (Gottesman et al., 1997), 30S ribosomal protein S1 (De Mot et al., 2007) and chaperones GroEL and DnaK (Servant and Mazodier, 2001) were differentially expressed compared to the control cultures. Such proteins assist in protein folding, assembly, transport and degradation during normal growth, and their transcription is controlled by complex positive and negative regulatory mechanisms (Narberhaus, 1999). Under stress conditions, such as exposure to toxic substances, expression of those proteins is usually increased (e.g. Segura et al., 2005). Upregulation of such proteins was indeed observed in linuron- and 3,4-DCA-supplemented cultures (Table 1), which can be associated with the observed decreased growth rate of WDL1 in linuron- and 3,4-DCA-supplemented R2A cultures (see above). However, in the 3,4-DCA-supplemented cultures as well, downregulation of certain stress-related proteins was detected. These proteins belonged to the same protein families as those upregulated in the linuron- and/or 3,4-DCA-supplemented cultures but were recovered from different positions in the 2-D gels. For example, proteins related to chaperone DnaK were both up- and downregulated in 3,4-DCA-supplemented culture (Table 1: spots 1871 & 1878). Downregulation of stress proteins in response to exposure to xenobiotics such as *o*-xylene and toluene has been reported previously in *P. putida* KT2440 (Domínguez-Cuevas et al., 2006).

3.4. Assignment of other differentially expressed proteins

In the linuron-supplemented culture, the upregulation of two proteins was observed which showed similarity, based on a single peptide sequence, to the hypothetical protein Bpro_0629 of *Polaromonas* sp. JS666 (spots 1628 and 1732; Fig. 2B & Table 1). Spot 1732 was also sequenced as spot 2310 from another 2-D gel (Fig. 3B & Table 1). The experimental MW and p*I* of the proteins associated with spots 1732 and 2310 correspond to the theoretical MW and p*I* calculated for Bpro_0629, while the protein associated with spot 1628 had a MW and p*I* slightly different from the predicted values. Nevertheless, from all three spots, an identical peptide sequence of 14 AAs was obtained which matched exclusively with the AA sequence of Bpro_0629. Bpro_0629 is encoded by a gene located within 300 bp downstream of a gene encoding the α subunit of a protocatechuate-3,4-dioxygenase (3,4-PCD) (i.e. Bpro_0628) in *Polaromonas* sp. JS666. Therefore, it was hypothesized that Bpro_0629 is functionally linked to 3,4-PCD degradation in strain JS666 (von Mering et al., 2007). However, proteins related to Bpro_0629 in other bacteria, i.e. ReutA0056 of *Cupriavidus necator* JMP134 and Ajs_3716 of *Acidovorax* sp. JS42, show only about 50% AA sequence identity to Bpro_0629, and neither of them are associated with 3,4-PCD-like enzymes in the corresponding strains.

The protein associated with spot 1481 was upregulated in both the linuron- and 3,4-DCA-supplemented cultures (Fig. 2B & Table 1), indicating its involvement in linuron and/or 3,4-DCA degradation by WDL1. One of the AA sequences recovered from spot 1481 showed similarity to a peptide sequence of a tetrahydromethanopterin biosynthesis protein of *Burkholderia xenovorans* LB400 (Table 1). This protein is linked with C_1-transfer in methanogenesis and methylotrophy (Chistoserdova et al., 1998). However, the theoretical MW and p*I* of the protein does not correspond to the experimental MW and p*I*, and therefore, it is unlikely that the match is reliable. We also determined peptide sequences from spots associated with upregulated proteins and showing a similar position as spot 1481 in two other 2-D gels, i.e. spot 2250 (Fig. 3B) and spot 3541 (Fig. 3C). The peptide sequences obtained from spot

3541 showed no significant similarity to known proteins (Table 1), while those obtained from spot 2250 again showed similarity to the tetrahydromethanopterin biosynthesis protein of *B. xenovorans* LB 400, but also to chaperone Cpn60 of *Acidovorax facilis* (Table 1). The latter dual identification might be caused by the presence of two different proteins co-migrating in the gel.

In addition to the above mentioned proteins, some additional proteins could be recognized whose expression was positively affected or induced in the presence of linuron, such as proteins associated with spots 962, 1208, 1836 and 1897 (Figs. 2B and 3B; Table 1). However, for none of those proteins did we find peptide sequences which matched significantly ($p < 0.05$) with known proteins in the NCBI database or other protein databases up to now. As such, we detected several proteins whose expression seems to be affected by the presence of linuron or 3,4-DCA, but we were not able to hypothesize on their function in linuron/3,4-DCA degradation based on homology searching. Their actual involvement in linuron/3,4-DCA degradation in *Variovorax* sp. WDL1 awaits further genetic research.

Unexpectedly, we did not recover or identify enzymatic functions which appear to be responsible for the initial transformation of linuron to 3,4-DCA, such as enzymes related to the aryl acylamidase and phenylurea hydrolase identified in linuron-degrading Gram-positive bacteria (Engelhardt et al., 1973; Turnbull et al., 2001). It could be that this reaction is either catalyzed by novel enzymes or that the enzyme(s) was (were) not differentially expressed. Alternatively, the concentration of the enzyme(s) was below the detection limit of the used technique or we were unable to identify the proteins due to low MS spectra quality. Those reasons could also explain why no enzymes involved in the further mineralization of 3,4-DCA, such as enzymes involved in chlorocatechol degradation, were detected.

3.5. Conclusions

In this study, using a proteomic-based approach, we showed for the first time that an enzyme related to AD is involved in chloroaniline degradation in *Variovorax*. In addition, we have indications that multiple variants of the AD are expressed. However, no enzymes catalyzing the conversion of linuron to 3,4-DCA could be recognized, nor were enzymes detected as being involved in the degradation of chlorocatechols. On the other hand, both 3,4-DCA and linuron degradation appeared to be associated with a stress response, indicating toxicity of linuron and/or 3,4-DCA for the cells.

It is not clear whether the identified AD attacks 3,4-DCA directly or a dechlorinated product thereof. In theory, 3,4-DCA may undergo dechlorination prior to deamination and subsequent ring cleavage of the formed catechol, as an alternative to direct deamination of the dichlorinated aniline. Currently, we are characterizing the AD genes in *Variovorax* to elucidate the exact role of AD in 3,4-DCA degradation and are using alternative approaches to identify genes/proteins involved in the conversion of linuron to 3,4-DCA in *Variovorax*.

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