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A Novel Hydrolase Identified by Genomic-Proteomic Analysis of Phenylurea Herbicide Mineralization by *Variovorax* sp. Strain SRS16▽†

Karolien Bers,[1] Baptiste Leroy,[2] Philip Breugelmans,[1] Pieter Albers,[1] Rob Lavigne,[3] Sebastian R. Sørensen,[4] Jens Aamand,[4] René De Mot,[5] Ruddy Wattiez,[2] and Dirk Springael[1]*

Division of Soil and Water Management, Katholieke Universiteit Leuven, Kasteelpark Arenberg 20, 3001 Leuven, Belgium[1]; Department of Proteomics and Protein Biochemistry, University of Mons-Hainaut, Av. du Champs de Mars 6, 7000 Mons, Belgium[2]; Laboratory of Gene Technology, Katholieke Universiteit Leuven, Kasteelpark Arenberg 21 Box 2462, 3001 Leuven, Belgium[3]; Department of Geochemistry, Geological Survey of Denmark and Greenland (GEUS), Øster Voldgade 10, 1350 Copenhagen K, Denmark[4]; and Centre of Microbial and Plant Genetics, Katholieke Universiteit Leuven, Kasteelpark Arenberg 20, 3001 Leuven, Belgium[5]

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The soil bacterial isolate *Variovorax* sp. strain SRS16 mineralizes the phenylurea herbicide linuron. The proposed pathway initiates with hydrolysis of linuron to 3,4-dichloroaniline (DCA) and *N*,*O*-dimethylhydroxylamine, followed by conversion of DCA to Krebs cycle intermediates. Differential proteomic analysis showed a linuron-dependent upregulation of several enzymes that fit into this pathway, including an amidase (LibA), a multicomponent chloroaniline dioxygenase, and enzymes associated with a modified chlorocatechol *ortho*-cleavage pathway. Purified LibA is a monomeric linuron hydrolase of ~55 kDa with a K_m and a V_{max} for linuron of 5.8 μM and 0.16 nmol min^{-1}, respectively. This novel member of the amidase signature family is unrelated to phenylurea-hydrolyzing enzymes from Gram-positive bacteria and lacks activity toward other tested phenylurea herbicides. Orthologues of *libA* are present in all other tested linuron-degrading *Variovorax* strains with the exception of *Variovorax* strains WDL1 and PBS-H4, suggesting divergent evolution of the linuron catabolic pathway in different *Variovorax* strains. The organization of the linuron degradation genes identified in the draft SRS16 genome sequence indicates that gene patchwork assembly is at the origin of the pathway. Transcription analysis suggests that a catabolic intermediate, rather than linuron itself, acts as effector in activation of the pathway. Our study provides the first report on the genetic organization of a bacterial pathway for complete mineralization of a phenylurea herbicide and the first report on a linuron hydrolase in Gram-negative bacteria.

The phenylurea herbicide linuron is a nonselective preemergent herbicide that acts as a photosystem II inhibitor. The herbicide is globally used to control a wide variety of annual and perennial broadleaf and grassy weeds in agricultural land. Microbial degradation is considered an important mechanism in the dissipation of linuron and other phenylurea herbicides in the environment. Several bacterial strains (39, 46), as well as consortia (5, 10), able to degrade and even use the compound as a sole source of carbon and nitrogen have been reported. Although derived from different geographical locations, most of the linuron-catabolizing isolates, either individual strains or key members of linuron-degrading consortia, belong to the genus *Variovorax*. This suggests that this genus plays an important role in linuron degradation in soil. The proposed pathway of linuron catabolism starts with amide hydrolysis to 3,4-dichloroaniline (DCA) and *N*,*O*-dimethylhydroxylamine (*N*,*O*-DMHA) (Fig. 1). DCA is harmful and recalcitrant, while *N*,*O*-DMHA is not and degraded easily. Several linuron-degrading *Variovorax* strains, in addition to mediating linuron hydrolysis, are able to use DCA as the sole carbon source and mineralize it. To date, little is known about the genes and enzymes responsible for linuron and DCA degradation. Engelhardt et al. (13) described an arylacyl amidase responsible for conversion of linuron to DCA in *Bacillus sphaericus* ATCC 12123. In addition, phenylurea hydrolase-encoding genes *puhA* and *puhB* were identified in the linuron-degrading actinomycetes *Arthrobacter globiformis* D47 (52) and *Mycobacterium brisbanense* JK1 (23), respectively. PuhA and PuhB form a novel branch within the metal-dependent amidohydrolase superfamily (23). Regarding the degradation of DCA, Dejonghe (9) and Breugelmans et al. (6) found indications for the involvement of a multicomponent aniline dioxygenase enzyme in DCA degradation in *Variovorax* sp. strain WDL1. However, the genes responsible for DCA degradation in linuron-mineralizing bacteria have not yet been identified.

We report here on the identification of the linuron and DCA degradation genes in the linuron-mineralizing strain *Variovorax* sp. strain SRS16 (46). The enzyme responsible for hydrolysis of linuron was purified and characterized. The expression of the catabolic genes under different conditions

* Corresponding author. Mailing address: Division of Soil and Water Management, Katholieke Universiteit Leuven, Kasteelpark Arenberg 20, 3001 Leuven, Belgium. Phone: 321 6321609. Fax: 321 6321997. E-mail: dirk.springael@ees.kuleuven.be.

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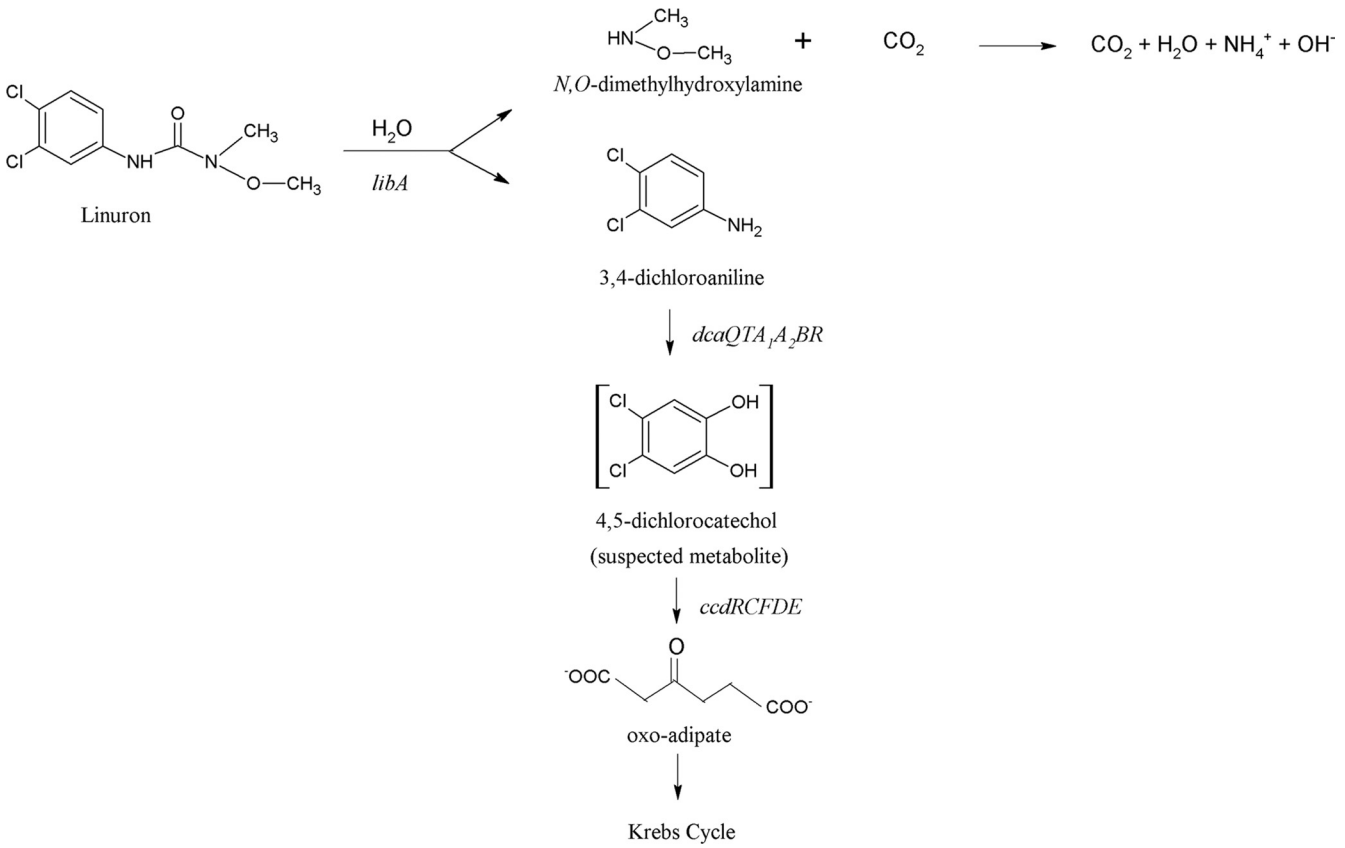

FIG. 1. Catabolic pathway of linuron degradation in *Variovorax* sp. SRS16. The catabolic steps specified by *libA*, *dcaQTA*$_1$*A*$_2$*B*, and *ccdCFDE* are indicated.

and their distribution among other linuron- and/or DCA-degrading strains was analyzed.

MATERIALS AND METHODS

Bacterial strains, cultivation conditions, and chemicals. *Variovorax* sp. strains SRS16 (46), WDL1 (10), PBS-H4, PBL-E5, and PBL-H6 and *Hydrogenophaga* sp. strain PBL-H3 (5) were routinely grown on R2A agar plates supplemented with 20 mg of linuron liter^{-1} at 26°C. *Variovorax* sp. strains PBD-E37, PBD-H1, and PBD-E5, *Cupriavidus* sp. strain PBS-E1, and *Afipia* sp. strain PBD-E87 (5), *Comamonas testosteroni* WDL7, and *Delftia acidovorans* WDL34 (10) were grown in R2A supplemented with DCA (20 mg liter^{-1}). *Hyphomicrobium sulfonivorans* strains WDL6 (10), PBN-E9, and PBN-H4 (5) were grown in MMO minimal medium supplemented with 1% methanol. R2A and MMO media were prepared as described previously (5, 10). Linuron [3-(3,4-dichlorophenyl)-1-methoxy-1-methyl urea] (99.5%), diuron [3-(3,4-dichlorophenyl)-1,1-dimethyl urea] (99.5%), isoproturon [3-(4-isopropylphenyl)-1,1-dimethyl urea] (99%), metobromuron [3-(4-bromophenyl)-1-methoxy-1-methyl urea] (99.9%), DCA (98%), and aniline were purchased from Sigma-Aldrich, Belgium.

HPLC analysis. Reverse-phase high-pressure liquid chromatography (HPLC; LaChrom; Merck Hitachi) was used to detect and quantify phenylurea herbicides and their metabolites in cultures containing initial concentrations of 20 to 50 mg liter^{-1}, as previously described (5).

Differential proteomic analysis using isotope-coded protein labeling (ICPL). SRS16 was cultured in MMO supplemented with succinate (0.2%) on a rotary shaker in the dark at 26°C until an optical density at 600 nm (OD_{600}) of 0.5 was reached. The culture was split in two and MMO, supplemented with either succinate (0.2%) or succinate (0.2%) and linuron (60 mg liter^{-1}), was added to reach a final medium composition of 0.1% succinate or 0.1% succinate and 30 mg of linuron liter^{-1}. The cultures were incubated at 26°C and every 90 min, samples were taken for HPLC-based linuron quantification and OD_{600} measurement. Degradation of linuron was observed immediately after addition of linuron, indicating that no catabolic repression of linuron degradation occurs when both succinate and linuron are available as carbon sources. After 6 h, when 50% of linuron was degraded and an OD_{600} of 0.5 was reached, the cultures were centrifuged (3,400 × *g*, 15 min, 4°C), and the pellets were stored at −80°C for protein extraction. Protein extraction, post-digest ICPL and MuDPIT (Multi-Dimensional Protein Identification Tool) were performed as described previously (27). For each experimental condition, duplicate cultures were analyzed.

Purification and characterization of linuron hydrolase. SRS16 was cultured in five replicates of 1 liter of MMO supplemented with succinate (0.2%) on a magnetic stirrer in the dark at 26°C until an OD_{600} of 0.7 was reached. The culture was amended with MMO containing succinate (0.2%) and linuron (60 mg liter^{-1}) to reach a final linuron concentration of 15 mg liter^{-1}. Every 90 min, samples were taken for HPLC-based linuron quantification and OD_{600} measurement. After 5 h, when 40% of the linuron was degraded and an OD_{600} of 0.6 was reached, the cultures were centrifuged (3,400 × *g*, 15 min, 4°C), and the pellets were washed with phosphate-buffered saline (150 mM NaCl, 7 mM K_2HPO_4, 2.35 mM KH_2PO_4), suspended in 10 ml of morpholinepropanesulfonic acid (MOPS) buffer (25 mM MOPS, 1 mM dithiothreitol, 5% glycerol; pH 7.6) supplemented with Complete, Mini, EDTA-free protease inhibitor cocktail (Roche Diagnostics GmbH, Germany), and stored at −80°C. The cells in the concentrated cell suspension were lysed by using a French press (Thermo). The crude cell extract was centrifuged (3,400 × *g*, 15 min, 4°C), and the cell-free supernatant was stored at −80°C. After a 35 to 50% ammonium sulfate precipitation and dialysis against MOPS buffer, the protein extract was loaded on an anion-exchange column (DEAE-Sepharose), equilibrated with MOPS buffer. The proteins were eluted over a linear gradient of 200 ml from 0 to 0.5 M KCl (flow rate, 1 ml min^{-1}). Active fractions were pooled and concentrated by ultrafiltration (molecular mass cutoff, 3 kDa [Millipore]; 4,500 × *g*, 4°C) before being subjected to size exclusion chromatography (column type G3000SW; Tosoh Corp., Japan). Elution was performed with 150 mM NaCl in MOPS buffer (flow rate, 1 ml min^{-1}), and the oligomeric state of the enzyme was estimated by using the following protein size standards: RNase A (13.7 kDa), ovalbumin (45 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (141 kDa), gamma globulin (160 kDa), and thyroglobulin (670 kDa). Protein concentrations

were estimated using a Bradford protein assay (Bio-Rad). Protein purity was assessed by means of SDS-PAGE and Coomassie brilliant blue staining. For low protein concentrations, silver staining was used (38). To identify proteins excised from polyacrylamide gels, liquid chromatography-electrospray ionization–tandem mass spectrometry (LC-ESI-MS/MS) was used, as previously described by Breugelmans et al. (6). The peptide sequences were analyzed by using BlastP/TBlastN to identify proteins with significant sequence similarity.

To assess linuron hydrolase activity, 10 μl of purified enzyme solution in MOPS buffer was supplemented with 140 μl of linuron solution (60 mg liter^{-1} [or smaller volumes with the same enzyme/linuron volume ratio]) and incubated at room temperature (15°C) for 60 min. The disappearance of linuron and appearance of DCA was monitored to quantify linuron hydrolase activity. The production of DCA was either detected by a colorimetric reaction or quantified by HPLC. The colorimetric detection of DCA consisted of a diazotization-coupling reaction according to the method of Pease (34) with the absorption measured at 500 nm. In the case of HPLC-based measurement of linuron and DCA, 50 μl of 4 M HCl was added to stop the reaction after 1 h of incubation. Corrections were made for background hydrolysis or absorption. The kinetic parameters K_m and V_{max} of the purified enzyme solution were determined for linuron by determining the hydrolysis activity by HPLC in the presence of a range of linuron concentrations (200, 120, 80, 40, 32, 24, 16, 8, and 4 μM). The substrate specificity of the linuron hydrolase and the crude protein extract of a linuron-amended *Variovorax* sp. strain SRS16 culture was examined by HPLC for diuron (50 mg liter^{-1}), isoproturon (50 mg liter^{-1}), and metobromuron (50 mg liter^{-1}) after 24 h of incubation. The activity of the linuron hydrolase at different temperatures (4, 22, 30, 37, and 60°C) was analyzed by HPLC using a linuron concentration of 50 mg liter^{-1}. All tests were performed in duplicate.

***De novo* sequencing and sequence analysis.** *De novo* sequencing of the genome of SRS16 was performed by BaseClear (Netherlands) on an Illumina GAIIx platform. CLC Bio Genomic Workbench 3.7 was used to assemble the 50-bp paired-end reads into 354 contigs with an average length of 21.5 kb. The microbial genome annotation system Magnifying Genomes (MaGe) (53) was used to annotate the contigs, while BLAST analyses were used to identify translated open reading frames (ORFs) with a sequence similarity to known proteins in databases (July 2011) (1). Peptide sequences of the amino group transfer protein component of the multicomponent aniline dioxygenase, the maleylacetate reductase, and the suspected linuron hydrolase were used to screen the contigs for the corresponding genes by using a local TBlastN search. For each gene, one contig was identified. The contig containing the multicomponent aniline dioxygenase gene and the contig containing the maleylacetate reductase gene could be assembled based on the sequences of eight fosmid clones selected from an SRS16 genome fosmid library constructed using the CopyControl HTP fosmid library production kit with pCC2FOS and phage T1-resistant EPI300-T1 *Escherichia coli* plating strain (Epicentre Biotechnologies) according to the recommendations of the manufacturer. These fosmid clones were identified by means of PCR screening of colonies carrying the SRS16 fosmid library using degenerate primer pair AGT-F/AGT-R (see Table S1 in the supplemental material) targeting the gene encoding the amino group transfer protein of the multicomponent aniline dioxygenase. Prior to PCR, a copy-control induction solution was used to induce a high copy number of the fosmids in the clones. Fosmid DNA was isolated with a FosmidMAX DNA purification kit (Epicentre Biotechnologies). The eight fosmid clones were sequenced as a mixture by BaseClear, as reported above for the *de novo* SRS16 genome sequencing. Mascot 2.2 was used to identify *Variovorax* sp. SRS16 genes encoding proteins with potential involvement in linuron catabolism.

Southern blotting. The primer pair LinAmidRF/LinAmidRR was used to synthesize an *libA*-specific gene probe from genomic SRS16 DNA using a digoxigenin (DIG) labeling mix (Roche Applied Science, Germany). Genomic DNA (10 μg) of *Variovorax* sp. strains SRS16, PBL-H6, PBL-E5, WDL1, and DSM66 was obtained from pure cultures (OD_{600} = 0.7) using a Puregene Core Kit A (Qiagen, Belgium) and digested by PstI (New England BioLabs, United Kingdom). Restriction fragments were separated by agarose gel electrophoresis (1.5%, 120 V, 1.5 h). Blotting, hybridization, and chemiluminescent detection were performed as recommended by Roche Applied Science. Details of the procedure are provided as supplemental material.

Transcription analysis. *Variovorax* sp. SRS16 was grown in R2A until an OD_{600} of 0.3 was reached. R2A or R2A supplemented with either linuron, DCA, or aniline was added to the culture at a final concentration of 17 mg liter^{-1} (0.2, 0.3, and 0.54 mM, respectively). At different time points after addition of the compounds (10, 20, 30, 40, 50, 60, and 120 min), samples were taken for RNA extraction. Degradation of linuron, DCA, and aniline was assessed and confirmed by HPLC analysis. RNA extraction was performed with the RNeasy minikit (Qiagen, Belgium), and contaminating DNA was removed with Turbo DNA-free (Ambion). The RNA concentration in the extracts was determined (Nanodrop), and the RNA extracts were diluted to equal concentrations. RNA was converted to cDNA by reverse transcription using a reverse transcription system (Promega). The cDNA was assessed for the presence of the respective gene sequences by PCR as described below. All experiments were performed in duplicate.

PCR analysis. Codehop (37) was used to design degenerate primers based on protein sequences. Primer BLAST was used to design primers based on known gene sequences. BLAST and MaGe (53) were used to confirm the selected primer specificity (July 2011). Primers (see Table S1 in the supplemental material) were obtained from Thermo Fisher Scientific (Belgium). PCR was performed in an Eppendorf MasterCycler (Eppendorf, Germany). The PCR mixture compositions and reaction conditions are provided in Table S1 in the supplemental material. Visualization of PCR amplicons was performed by means of agarose gel electrophoresis (1.5% agarose gel, 0.02% ethidium bromide, 90 V for 75 min).

Expression of *libA* in *E. coli*. The *libA* gene was optimized for *E. coli* gene expression and cloned into expression vector pJexpress 416 (pJexpress416_libA) by DNA2.0, Inc. A poly-His tag was added at the C-terminal end. As a negative control, the RFP gene was cloned into pJexpress 416 (pJexpress416_RFP_ctrl). Electroporation was used to transform *E. coli* BL21(DE3) with pJexpress416_libA and pJexpress416_RFP_ctrl. *E. coli* BL21(DE3) without expression vector, *E. coli* BL21(DE3) containing pJexpress416_libA, and *E. coli* BL21(DE3) containing pJexpress416_RFP_ctrl were grown in 50 ml of LB with kanamycin (50 mg liter^{-1}) at 37°C until an OD_{600} of 0.8 was reached. At this point, the cultures were split into two portions of 25 ml. In one of the portions, expression was induced by adding 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). After another 20 h of incubation at 20°C, 1 ml of each bacterial culture was taken for SDS-PAGE analysis, and linuron was added at a concentration of 10 mM. Linuron degradation activity was monitored by HPLC. Then, 1 ml of bacterial culture was prepared for SDS-PAGE by centrifugation (10,000 rpm, 1 min), after which the bacterial pellet was dissolved in 100 μl of SDS-PAGE loading buffer and boiled at 100°C for 3 min. After centrifugation (10,000 rpm, 1 min), 10 μl of the supernatants was used for SDS-PAGE. All treatments were performed in triplicate.

Phylogenetic analysis. Phylogenetic analysis was performed with Geneious Pro (version 5.4.4) (11) using PHYML (JTT matrix) (19).

GenBank accession numbers. The nucleotide sequences of contig 1 and contig 2 of the genome of SRS16, of *libA* in *Variovorax* sp. strains PBL-H6 and PBL-E5, and of *libA* in *Hydrogenophaga* sp. strain PBL-H3 were deposited in GenBank under accession numbers JN104632, JN104633, JN104629, JN104631, and JN104630, respectively.

RESULTS AND DISCUSSION

Differential proteomic analysis using ICPL. Isotope-coded protein labeling (ICPL) was used to detect differentially expressed proteins in *Variovorax* sp. SRS16 when grown in the presence of linuron compared to growth without linuron. The linuron-supplemented cultures showed active linuron degradation at the time point of analysis. A higher number of differentially downregulated proteins compared to upregulated proteins was observed in the linuron-amended cultures. Of the downregulated proteins, 45% were membrane proteins. Moreover, upregulation of stress-related proteins such as chaperones GroEL and DnaK was observed in linuron-amended SRS16 cultures (data not shown) as reported in *Variovorax* sp. strain WDL1 (6). Up- and downregulation of membrane proteins have been previously associated with a stress response (60, 62). As discussed by Breugelmans et al. (6), linuron exposure appears to induce a stress situation in *Variovorax*.

Interestingly, several upregulated proteins in the linuron-amended culture are associated with catalytic proteins of the proposed linuron degradation pathway. The most strongly upregulated protein in the linuron-amended culture (Table 1) showed significant homology with (putative) amidases, which catalyze the hydrolysis of amide or ester functional groups at

TABLE 1. Overview of the ORFs and their annotations as identified in contigs 1 and 2 (see Fig. 3) and their differential proteomic expression[a]

ORF	Name	Position[b]	Possible function	Closest homologous protein (GenBank accession no./% identity)	Organism	Biological replicate 1		Biological replicate 2	
						Score	L/C [SD(geo)]	Score	L/C [SD(geo)]
Contig 1									
1		3–515	Cyclase	HMPREF0005_01234 (EFV81793.1/61)	*Achromobacter xylosoxidans* C54				
2		515–1024	Acyl dehydratase	Rmet_4072 (YP_586209/75)	*Cupriavidus metallidurans* CH34				
3		1021–1944	Dihydropicolinate synthase	mll1010 (NP_102694.1/52)	*Mesorhizobium loti* MAFF303099				
4		2154–3113	Extracytoplasmic solute receptor	Reut_A1674 (YP_295884.1/60)	*Ralstonia eutropha* JMP134				
5	*ccdE*	3151–3873	Dienelactone hydrolase	Carboxymethylenebutenolidase (YP_001632346.1/68)	*Bordetella petrii* DSM 12804	134	2.26	216	6.37
6	*ccdD*	3870–5015	Chloromuconate cycloisomerase	TfdD (AATPP367/53)	*Sphingomonas* sp. strain tfd44				
7	*ccdF*	5041–6108	Maleylacetate reductase	TfdF (NP_990894.1/73)	*Achromobacter denitrificans*	ND	ND	52	3.08
8		6120–6455	Hypothetical protein	Bpet3729 (YP_001632340.1/85)	*Bordetella petrii* DSM 12804				
9		6601–6837*	FAD-dependent pyridine nucleotide-disulfide oxidoreductase	FAD-dependent pyridine nucleotide-disulfide oxidoreductase (YP_298914.1/56)	*Ralstonia eutropha* JMP134				
10		6825–7124*	Aromatic ring hydroxylating dioxygenase	AndAc (YP_556337.1/79)	*Burkholderia xenovorans* LB400				
11	*ccdC*	7229–7975	Chlorocatechol dioxygenase	Chlorocatechol dioxygenase DccAII (CAF32822.1/50)	*Sphingobium herbicidovorans*	182	**3.98** [1.96]	203	**3.98** [1.25]
12	*ccdR*	8108–9076	LysR family transcriptional regulator	LysR family transcriptional regulator Mpe_A3313 (YP_001022501.1/46)	*Methylibium petroleiphilum* PM1				
13		9585–10547	Extracytoplasmic solute receptor	Extracytoplasmic solute receptor h16_A1254 (YP_725762.1/45)	*Ralstonia eutropha* H16	ND	ND	161	2.49 [1.60]
14	IS*3 orfA*	10659–10925	Transposase	Putative transposase (NP_395222.1/68)	*Yersinia pestis* CO92				
15	IS*3 orfB*	10958–11776	Transposase	Integrase catalytic region AnaeK_1549 (YP_002133907.1/55)	*Anaeromyxobacter* sp. strain K				
16		11905–12399	IclR-type transcriptional protein	Transcriptional regulator Adeg_1855 (YP_003239789.1/31)	*Ammonifex degensii* KC4				
17	*istB*	12443–13237	IstB-like ATP-binding protein transposase	IstB protein 22 (YP_195848.1/100)	*Achromobacter xylosoxidans* A8				
18	*istA*	13227–14783	IstA transposase	Transposase IstA protein 59 (YP_195883.1/99)	*Achromobacter xylosoxidans* A8				
19	*dcaR*	14935–15852	LysR family transcriptional regulator	LysR-type regulator DanR (ABI20712.1/98)	*Delftia* sp. strain AN3	88	3.57	ND	ND
20	*dcaB*	15885–16892	Aniline dioxygenase reductase	Aniline dioxygenase reductase DanB (ABI20711.1/99)	*Delftia* sp. strain AN3				
21	$dcaA_2$	16909–17553	Aniline dioxygenase small subunit	Small subunit of dioxygenase DanA2 (ABI20710.1/100)	*Delftia* sp. strain AN3	ND	ND	128	2.5
22	$dcaA_1$	17550–18896	Aniline dioxygenase large subunit	Large subunit of dioxygenase TdnA1 (AA038208.1/99)	*Delftia acidovorans*	195	**4.20** [2.23]	201	**2.83** [1.44]
23	*dcaT*	18941–19705	Glutamine amido transferase	Glutamine amido transferase DanT (ABI20709.1/99)	*Delftia* sp. strain AN3	ND	ND	46	**1.73**
24	*dcaQ*	19725–21230	Glutamine synthetase	Putative glutamine synthetase DanQ (ABI20708.1/99)	*Delftia* sp. strain AN3	42	**2.46**	ND	ND
25		21515–21658*	Transposase	IS*4* family transposase (BAH90225.1/100)	Uncultured bacterium				
26	IS*66 orfA*	21742–22083	Transposase	Transposase IS*3*/IS*911* (YP_003643234/32)	*Thiomonas intermedia* K12				
27	IS*66 orfB*	22080–22421	Transposase	Transposase (BAH90226.1/92)	Uncultured bacterium				
28	IS*66 orfC*	22501–24084	Transposase	Transposase ISThsp3 IS*66* family (CAZ88005.1/60)	*Thiomonas* sp. strain sp3As				
29	*tnpA*	24388–27300	Transposase	Transposase TnpA (YP_001967688.1/100)	*Comamonas* sp. strain CNB-1				
30		27378–27842	Hypothetical protein	MYG1 (XP_002538853.1/56)	*Ricinus communis*				
31		28171–28611	LysR-type transcriptional regulator	LysR-type transcriptional regulator Dtpsy_1784 (YP_002553241/31)	*Acidovorax ebreus* TPSY				
32		28717–29250		No significant match					
33		29568–30488*	Transcriptional regulator	Putative transcriptional regulator THI_0620 (CAZ87353.1/57)	*Thiomonas* sp. strain sp3As				
Contig 2									
41		623–1444	LuxR family transcriptional regulator	Putative transcriptional regulator (YP_789362.1/26)	*Pseudomonas aeruginosa* UCBPP-PA14				
42	*libA*	1531–2958	Linuron hydrolase	Putative amidase (YP_002234156.1/52)	*Burkholderia cenocepacia* J2315	2,706	**13.00**	3,033	**8.73** [2.55]

[a] The last four columns show the genes which were upregulated in *Variovorax* sp. SRS16 cultures when amended with linuron compared to non-linuron-amended cultures as determined by post-digest ICPL analysis of two biological replicates. For each biological replicate the score is a confidence index of the identification of the peptide sequences with a predicted protein of the *Variovorax* sp. SRS16 genome. L/C represents the average ratio of the quantity of the protein in the crude protein extract of the linuron-amended culture to the quantity of the protein in the non-linuron-amended culture, calculated as the average of the ratios for each peptide detected for this protein. When L/C is significantly different from 1, the value is marked in boldface. SD(geo), geometrical standard deviation; ND, no data.

[b] *, truncated ORF.

carbon and phosphorus centers. In addition, as observed by Breugelmans et al. (6) in *Variovorax* sp. WDL1, upregulation of several proteins homologous to components of multicomponent aniline dioxygenases involved in (chloro)aniline degradation (amino group transfer protein, glutamine amidotransferase and the large subunit of the dioxygenase) was observed in cultures amended with linuron (Table 1). Expression of the small subunit of a multicomponent aniline dioxygenase was

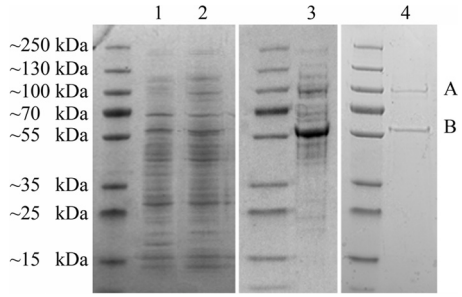

FIG. 2. SDS-PAGE analysis of protein extracts showing linuron hydrolase activity, from linuron-amended *Variovorax* sp. SRS16 cultures at different purification steps. Lane 1, crude cell-free protein extract; lane 2, after 35 to 50% ammonium sulfate precipitation; lane 3, after anion-exchange chromatography; lane 4, after size exclusion chromatography. Band A was identified as transcription elongation factor G, while band B was identified as the translation product of ORF 42 (*libA*).

also observed, although it was not significantly different from the control culture. These results imply the involvement of a similar multicomponent dioxygenase in the degradation of DCA in *Variovorax* sp. SRS16. Furthermore, three upregulated proteins showed significant amino acid similarity to enzymes involved in the modified *ortho*-cleavage pathway for chlorocatechol degradation, i.e., chlorocatechol dioxygenase responsible for *ortho*-ring fission of dichlorocatechols to dichloromuconate, carboxymethylenebutenolidase (a dienelactone hydrolase performing dichloromuconate cycloisomerization to chloromaleylacetate) and maleylacetate reductases (responsible for reduction of chloromaleylacetate to oxoadipate via maleylacetate), in chloroaromatic-degrading bacteria (29, 48, 56). These data suggest the involvement of a typical modified *ortho*-cleavage pathway for chlorocatechol degradation in the further mineralization of DCA by strain SRS16. Other than the discussed proteins, no up- or downregulation of proteins linked to linuron catabolism could be observed.

Purification and characterization of a linuron hydrolase. An enzyme with linuron hydrolase activity was purified from a cell extract of linuron-degrading SRS16 cells. The final purified protein solution with linuron hydrolase activity contained two proteins (~100 and ~55 kDa) (Fig. 2). Tandem mass spectrometry showed that the protein of ~55 kDa corresponded to the upregulated amidase in the linuron-amended SRS16 cultures. The protein of ~100 kDa was identified as the ribosomal protein elongation factor G (62% sequence coverage). The pI values of both proteins are identical, which explains the difficulty in separating them. Translation elongation factor G, however, is not expected to have amido hydrolase activity. Moreover, no upregulation of this housekeeping protein was observed in the differential proteomic approach. This indicates that the amidase is responsible for linuron hydrolysis in SRS16, and we designated this as LibA (Linuron biodegradation protein A). LibA is a monomeric enzyme with a K_m for linuron of 5.8 μM, a V_{max} of 0.16 nmol min^{-1}, and a maximum hydrolase activity between 22 and 30°C (see Fig. S1 in the supplemental material). The hexameric phenylurea hydrolase proteins PuhA and PuhB, previously identified in Gram-positive bacteria, show very similar K_m values (6.8 and 7.6 μM, respectively) and a maximal linuron hydrolase activity between 30 and 35°C (23). LibA of *Variovorax* sp. SRS16 showed activity toward linuron but no detectable activity toward other tested phenylurea herbicides such as the *N*-methoxy-*N*-methyl phenylurea herbicide metobromuron and the *N*,*N*-dimethyl phenylurea herbicides isoproturon and diuron. Sørensen et al. (46) reported diuron mineralization by *Variovorax* sp. SRS16, but only in case the organism was cultured in media containing growth substrates and nitrogen sources additional to diuron. Based on competition experiments with linuron, the authors of that study concluded that diuron was not hydrolyzed in SRS16 by the linuron hydrolase but instead by a broad-spectrum hydrolase present during active growth The observed inactivity of LibA toward diuron in our study seems to confirm this hypothesis. The narrow phenylurea herbicide specificity of the monomeric LibA enzyme is in contrast with the phenylurea herbicide spectrum of PuhA and PuhB, which show activity toward a wide range of phenylurea herbicides, including *N*-methoxy-*N*-methyl phenylureas (underlined) and *N*,*N*-dimethyl phenylureas: linuron, chlorbromuron > metobromuron, monolinuron, diuron, chlortoluron > fluomethuron, metoxuron, isoproturon, fenuron (arranged in decreasing order of PuhA/PuhB catalytic efficiency) (23). Also, total protein extracts from the linuron-degrading *Bacillus sphaericus* ATCC 12123 showed, in addition to linuron hydrolysis, activity toward other phenylurea herbicides (12).

Genetic backbone of linuron mineralization by strain SRS16. The peptide sequences showing similarity to the chloroaniline and chlorocatechol degradation enzymes in the differential proteomic analysis and those obtained from purified LibA could be assigned to ORFs present on two contigs in the draft SRS16 genome sequence, i.e., contig 1 of ±54 kb and contig 2 of ±3.6 kb (Fig. 3 and Table 1).

Within contig 1, ORFs 19 to 24 compose a cluster of six genes that show more than 98% nucleotide and amino acid similarity with the respective gene product(s) in the *danQTA*$_1$*A*$_2$*BR* gene cluster, a multicomponent aniline dioxygenase in the aniline-degrading strain *Delftia* sp. AN3. Five of these ORFs (ORFs 19 and 21 to 24) were linuron-dependent upregulated in the differential proteomic analysis. Moreover, the organization of ORFs 19 to 24 is identical to those in strain AN3 and other (chloro)aniline-degrading bacteria. This gene cluster is therefore proposed to be responsible for the dioxygenation of DCA to a chlorocatechol compound in SRS16 and was named *dcaQTA*$_1$*A*$_2$*BR* (Fig. 1). The protein product of *dcaR* shows significant similarity with LysR-type transcriptional regulators (LTTR), including the protein product of regulatory genes of aniline dioxygenation gene clusters such as *tadR* in *Delftia tsuruhatensis* AD9 (16). Therefore, *dcaR* is expected to perform a similar regulatory role in strain SRS16. *dcaQTA*$_1$*A*$_2$*BR* is surrounded by insertion sequence (IS) elements (i.e., ORFs 14, 15, 17, 18 and 25 to 29) of which one (ORF 14) appears to be truncated. The other eight ORFs belong to four different families of IS elements, i.e., IS*66*, IS*3*, IS*21* (i.e., IS*1600*) and Tn*3* (i.e., IS*1071*). This indicates that the *dcaQTA*$_1$*A*$_2$*BR* gene cluster is located within a transposon-like structure and that it was acquired by horizontal gene transfer (HGT) (49). The G+C content of the *dcaQTA*$_1$*A*$_2$*R* gene cluster (65.88%) is similar to the overall G+C content of the SRS16 genome (66.51%), suggesting that in case HGT was

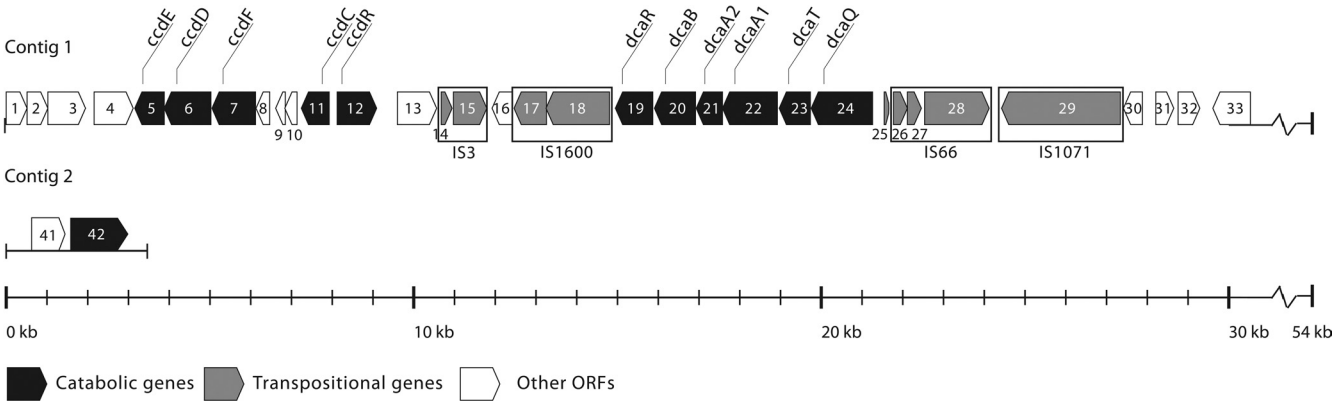

FIG. 3. Genetic organization of contigs 1 and 2 containing the identified genes involved in linuron and DCA catabolism in *Variovorax* sp. SRS16. Contig 1 contains a 54-kb DNA fragment including *dcaQTA*$_1$*A*$_2$*BR* and *ccdRCFDE*. Upstream of ORF 33, contig 1 includes another 24-kb-long sequenced nucleotide segment. The ORFs of this part of the contig are represented in the corresponding GenBank file JN104632. Contig 2 consists of a 3.5-kb genome fragment containing the linuron hydrolase gene *libA* (ORF 42). Further information about the different ORFs is shown in Table 1.

involved, it was not acquired recently or the origin of this gene cluster lies in a related bacterium.

Downstream of the *dcaQTA*$_1$*A*$_2$*BR* cluster, a second gene cluster (ORFs 5 to 12) was found which encoded the peptide sequences related to chlorocatechol-degrading proteins. The translation products of ORFs 11, 7, 6, and 5 show significant similarity with a chlorocatechol-1,2-dioxygenase, maleylacetate reductase, chloromuconate cycloisomerase, and dienelactone hydrolase, respectively. Moreover, ORFs 5, 7, and 11 showed linuron-dependent upregulation in the differential proteomic analysis (Table 1). As outlined above, these enzymes compose a classic modified *ortho*-cleavage pathway for the degradation of chlorocatechols to β-ketoadipate (54) (Fig. 1). Upstream of ORFs 11, 7, 6, and 5, the translation product of ORF 12 showed significant similarity with an LTTR and shows a typical opposite orientation to the operon it regulates (51). LTTRs have been reported to be regulators of chlorocatechol degradation previously (51) (Fig. 4). Therefore, the gene cluster composed of ORFs 11, 7, 6, 5, and 12 is proposed to be responsible for the degradation of DCA to β-ketoadipate and was named *ccdRCFDE*. This cluster as such complements the *dcaQTA*$_1$*A*$_2$*BR* gene cluster to enable complete degradation of DCA to Krebs cycle intermediates. Interestingly, in the aniline catabolic *Delftia* sp. strain AN3, the association of an aniline multicomponent dioxygenase with a catechol *meta*-cleavage pathway (61), typical for degradation of nonchlorinated aromatics (31), was reported. The organization of the *ccdRCFDE* cluster shows some significant differences with those of gene clusters encoding modified chlorocatechol *ortho*-cleavage pathways in other organisms (Fig. 4). Most strikingly, in between *ccdC* and *ccdF*, three ORFs (ORFs 8, 9, and 10) are located that cannot be directly linked to chlorocatechol degradation and that are not present in most other isofunctional gene clusters (13, 49, 54). The exception is ORF 8, whose translation product shows similarity with a hypothetical protein encoded by a gene present in a chlorocatechol degradation pathway gene cluster in *Bordettela petrii* (14, 19, 50, 55). ORFs 9 and 10 appear to be truncated genes. Their putative translation products show similarity with a pyridine nucleotide disulfide oxidoreductase in *Variovorax paradoxus* S110 (20) and an aromatic ring dioxygenase in *Burkholderia xenovorans* LB400 (7), respectively (Table 1). The *ccd* gene cluster is flanked by two ORFs (ORF 13 and ORF 4) of which both translation products show similarity with extra-cytoplasmic solute receptors, belonging to the Bug family. Interestingly, ORFs encoding similar hypothetical proteins of unknown function are present in other modified chlorocatechol *ortho*-cleavage pathway gene clusters (14, 18, 55). A wide range of orthologous *bug* genes have been identified in the genomes of various *Betaproteobacteria*, but the function is often unknown (3). Some *bug* homologues are involved in periplasmic uptake of substrates (3) and a regulatory role has been suggested (2, 35).

The peptide sequences of LibA could be assigned to ORF 42 in contig 2. ORF 42, which is absent in the genomes of *V. paradoxus* S110 (20) and *V. paradoxus* EPS (NC_014931), was named *libA*. Besides *libA*, contig 2 contains one ORF (ORF 41) directly upstream of *libA*. Its translation product contains a carboxy-terminal DNA-binding motif typical for regulators of the LuxR superfamily. The absence of a characteristic domain for interaction with a sensor kinase, a quorum-sensing molecule or multiple ligands, suggests that ORF 41 encodes a protein that belongs to the autonomical effector domain regulators, which activate gene expression in the presence of an effector molecule. Possibly, ORF 41 is involved in the regulation of *libA*.

Although LibA, PuhA, and PuhB all perform linuron hydrolysis, LibA lacks homology with PuhA/PuhB, which both belong to the metal-dependent amidohydrolase superfamily (23), while LibA shows highest homology with members of the amidase signature (AS) family (Fig. 5) that is part of the GGCT-like superfamily (Fig. 5). Although no sequence data of the linuron hydrolase identified in *Bacillus sphaericus* is available, it was previously proposed to belong to a different family than PuhA/PuhB based on differences in substrate specificity and physicochemical characteristics (23). LibA shows ~50% amino acid identity with the ω-octalactam hydrolase of *Rhodococcus* sp. Oct1 (Aaa R-Oct1 in Fig. 5) and orthologues from *Nocardia* strains (14), as well as with hypothetical proteins from

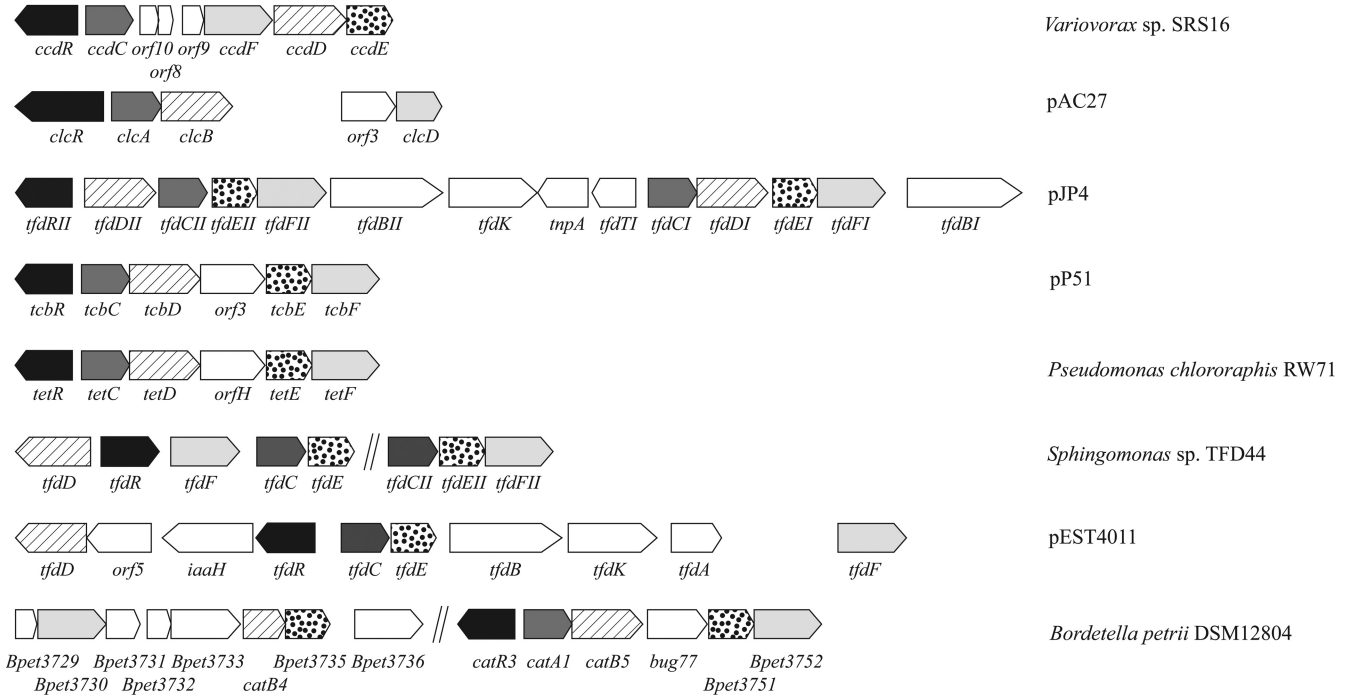

FIG. 4. Organization of the modified chlorocatechol *ortho*-cleavage pathway gene cluster in chloroaromatic-degrading bacterial strains (14, 18, 36, 48, 50, 55, 56). The arrows indicate the localization, size, and direction of transcription of the genes. Similar shaded patterns indicate isofunctional genes. The following enzymes are shown (the coding genes are in parentheses): 2,4-D transport protein (*tfdK*), chlorophenol hydroxylases (*tfdB*), chlorocatechol 1,2-dioxygenases (*tfdC*, *ccdC*, *clcA*, *tcbC*, *tetC*, and $catA_1$), chloromuconate cycloisomerases (*tfdD*, *ccdD*, *clcB*, *tcbD*, *tetD*, $catB_4$, and $catB_5$), dienelactone hydrolases (*tfdE*, *ccdE*, *tcbE*, *tetE*, *Bpet3735*, and *Bpet3751*), maleylacetate reductases (*tfdF*, *ccdF*, *clcD*, *tcbF*, *tetF*, and *Bpet3730*), transposases (*tnpA*, *Bpet3733*, and *Bpet3736*), and indole acetamide hydrolase (*iaaH*). The ORFs *Bpet3729*, *Bpet3731*, and *Bpet3732* code for hypothetical proteins. *bug77* codes for a Bug family protein.

another actinomycete (*Pseudonocardia dioxanivorans*) and from alpha- and betaproteobacteria (*Ruegeria* and *Burkholderia* spp., respectively) The hydrolase of *Rhodococcus* sp. Oct1, which is the most closely related biochemically characterized homolog of LibA, also shows high activity toward nitroacetanilides (15), which have a linuron-like chemical structure. Many other characterized members of the AS family show substrate specificity toward linuron-resembling molecules (Fig. 5), i.e., mostly aromatic substrates with a side chain containing an amide bond (Fig. 5). Based on the lack of amino acid sequence similarity and the functional and structural differences between LibA and the other phenylurea hydrolase protein families (PuhA/PuhB and linuron hydrolase of *Bacillus sphaericus*), we can conclude that in *Variovorax* sp. SRS16 a third phenylurea hydrolyzing protein family has evolved independently.

Together with the $dcaQTA_1A_2BR$ gene cluster encoding conversion of DCA to chlorocatechol and the *ccdRCFDE* gene cluster encoding a modified chlorocatechol *ortho*-cleavage pathway for further metabolism of chlorocatechol, *libA* provides all of the necessary genetic information for mineralization of linuron in *Variovorax* sp. SRS16. As such, strain SRS16 is another example of a patchwork assembly of catabolic gene modules (*libA*, $dcaQTA_1A_2B$, and *ccdCDEF*) in order to use a xenobiotic compound as the sole source of carbon and energy. A similar modular genetic arrangement involving a multicomponent aniline dioxygenase was recently described in the diphenylamine-degrading *Burkholderia* sp. strain JS667 (44).

Expression of *libA* in *E. coli*. The linuron hydrolysis activity of LibA was confirmed using a recombinant *E. coli* BL21(DE3) strain containing *libA* under the control of an IPTG-inducible promoter. In contrast to *E. coli* BL21(DE3) control strains, IPTG-induced cells of *E. coli* containing pJexpress416_libA showed linuron degradation (80% after 7 h; 100% in <20 h) and the formation of DCA. Without IPTG induction, a slow linuron degradation was observed (50 and 60% after 24 and 48 h, respectively [data not shown]). SDS-PAGE profiles confirmed the increased expression of a protein of ~55 kDa in the IPTG-supplemented cultures compared to noninduced cultures (data not shown).

Transcriptomic analysis of linuron degradation genes in *Variovorax* sp. SRS16. Transcription analysis was performed to gain a first insight in the regulation of *libA*, $dcaQTA_1A_2B$, and *ccdCFDE* and their mode of induction. RNA extracts were collected from a *Variovorax* sp. SRS16 culture which was unamended or supplemented with either linuron, DCA or aniline. Background expression of *libA* and *dca* was observed (Fig. 6). Linuron-amended cultures showed a significant increased expression of *libA* and genes of the *dca* cluster at all sampling times, as observed in the differential proteomic analysis. Moreover, crude protein extracts from linuron-amended SRS16 cultures degraded 70.45% ± 2.24% of linuron after 18 h of incubation while protein extracts recovered from non-linuron-amended cultures degraded only 10.19% ± 3.26%. Also, DCA and especially aniline caused increased expression of *libA* and

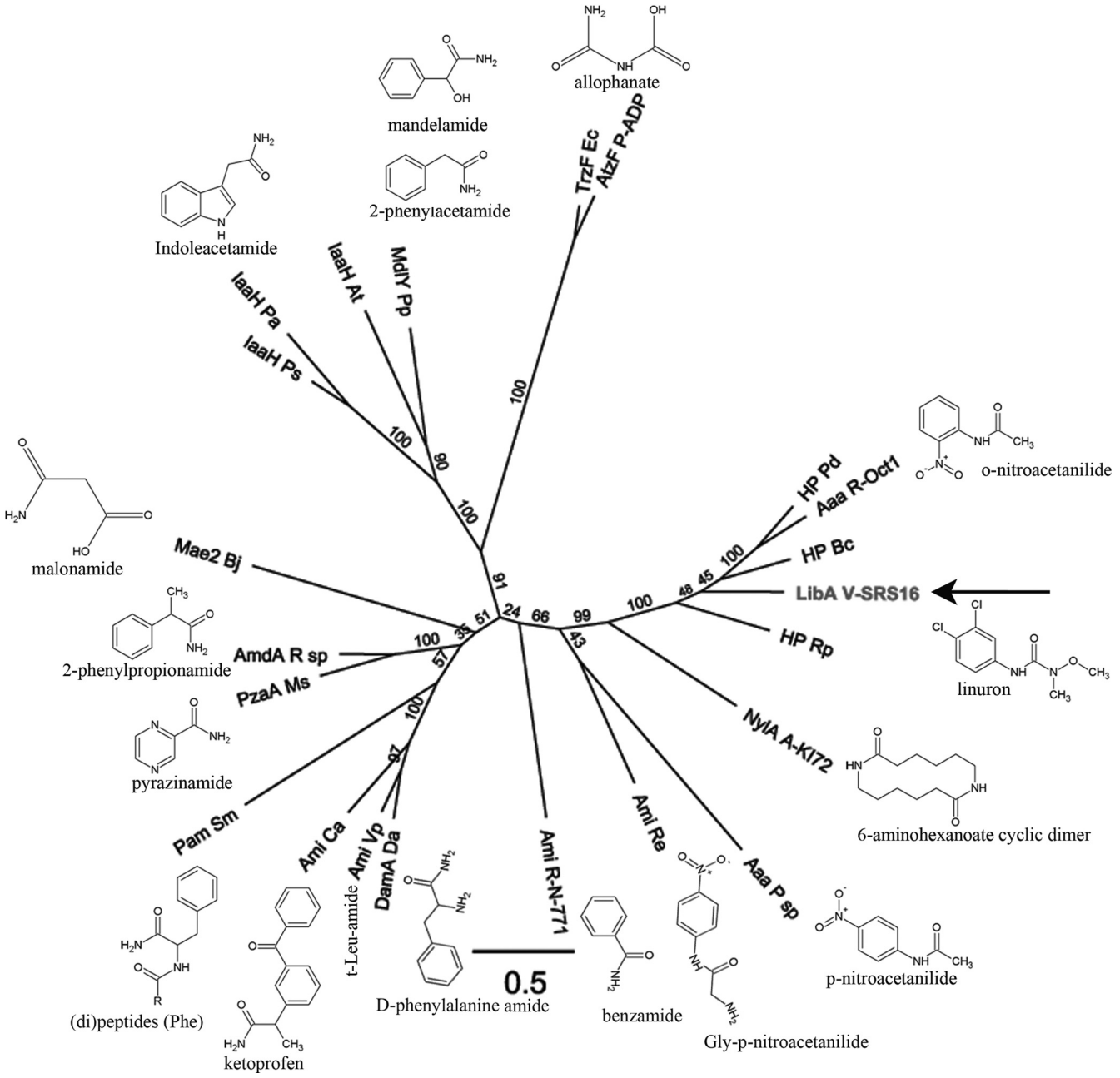

FIG. 5. Phylogenetic analysis of LibA and selected members of the amidase signature family. A multiple amino acid alignment of the respective Pfam domains (PF01425) was used to construct a maximum-likelihood tree. The AS domain of LibA (residues 27 to 453) was aligned with the corresponding domain of the following enzymes, with their (preferred) substrates indicated: Aaa R-Oct1 (*Rhodococcus* sp. strain Oct1, *o*-nitroacetanilide [15]), Aaa P sp (*Pseudomonas* sp., *p*-nitroacetanilide [24]), AmdA R sp (*Rhodococcus* sp., 2-phenylpropionamide [28]), Ami Ca (*Comamonas acidovorans*, ketoprofen [58]), Ami Re (*Rhodococcus erythropolis* PR4, Gly-*p*-nitroacetanilide [26]), Ami R-N-771 (*Rhodococcus* sp. strain N-771, benzamide [32]), Ami Vp (*Variovorax paradoxus*, *t*-Leu-amide [25]), AtzF P-ADP (*Pseudomonas* sp. ADP, allophanate [43]), DamA Da (*Delftia acidovorans*, D-phenylalanine amide [22]), IaaH At (*Agrobacterium tumefaciens*, indole acetamide [57]), IaaH Pa (*Pantoea agglomerans*, indole acetamide [8]), IaaH Ps (*Pseudomonas savastanoi*, indole acetamide [57]), Mae2 Bj (*Bradyrhizobium japonicum*, malonamide [45]), MdlY Pp (*Pseudomonas putida*, mandelamide/2-phenylacetamide [17]), NylA (*Arthrobacter* sp. strain KI72, 6-aminohexanoate cyclic dimer/β-laurolactam [59]), Pam Sm (*Stenotrophomonas maltophila*, [di]peptides [Phe] [30]), PzaA Ms (*Mycobacterium smegmatis*, pyrazinamide [4]), and TrzF Ec (*Enterobacter cloacae*, allophanate [42]). Three hypothetical proteins most similar to LibA were included: HP Bc (*Burkholderia cenocepacia* J2315 [YP_002234156]), HP Pd (*Pseudonocardia dioxanivorans* [YP_004335959]), HP Rp (*Ruegeria pomeroyi* DSS-3 [YP_167742]). The scale bar represents 0.5 substitutions per site. Bootstrap values are represented at the branches.

the *dca* cluster. Previously transcriptional activation of the aniline multicomponent dioxygenase gene cluster $tadQTA_1A_2BR$ by aniline and several chloroanilines was reported (*16*). However, the more pronounced increase in expression in the cultures amended with aniline (compared to the cultures supplemented with linuron and DCA) can, in part, be due to the difference in molar concentrations of the added compounds (184 mM aniline, 68 mM linuron, and 102 mM DCA). Different expression

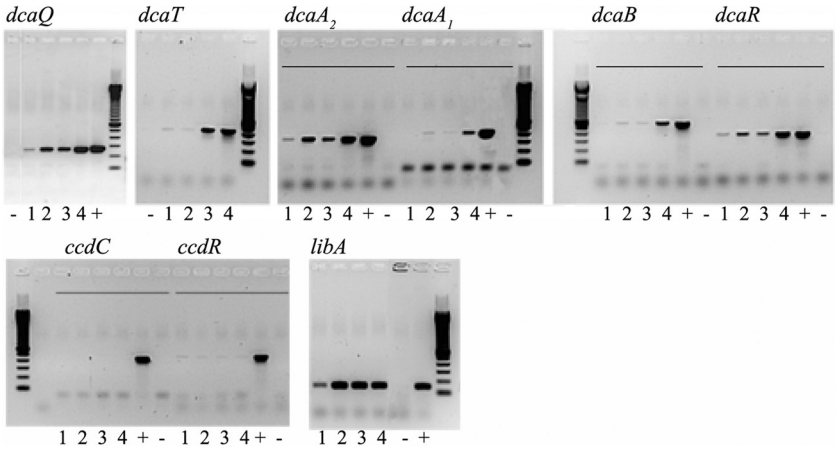

FIG. 6. Transcription analysis of linuron degradation genes in *Variovorax* sp. SRS16. RNA sampled after 30 min of growth on R2A without (lane 1) or with linuron (lane 2), DCA (lane 3) or aniline (lane 4). A "–" indicates a blank, while "+" indicates a positive control (genomic DNA of SRS16).

intensities as a response to linuron, DCA and aniline can also be due to differences in uptake. Although *libA* and the *dca* genes are clearly upregulated in the presence of linuron, the upregulation of *libA* and the *dca* cluster in the presence of DCA suggest that DCA or its metabolites, rather than linuron, act as effector(s) of both *libA* and the *dca* gene cluster in *Variovorax* sp. SRS16. Positive regulation of the degradation pathway of (chloro)aromatic compounds by its pathway intermediates has been described previously (33, 41). As such, previously reported linuron induction of linuron mineralization by SRS16 in cultures containing mg-liter^{-1} linuron concentrations but not in cultures containing μg-liter^{-1} concentrations (47) can be explained by the observed background expression of *libA* and its downstream genes, which would allow linuron mineralization at low concentrations even without pre-exposure to linuron. At high concentrations, DCA or other metabolites are produced, resulting in increased expression of *libA* and *dca*. A basal enzyme activity at low concentrations versus an enhanced activity at elevated concentrations has been described previously in other xenobiotic-degrading bacteria (21, 40).

No transcripts were detected for the genes of the *ccd* cluster (Fig. 6) and ORFs 8, 9, and 10 (data not shown) within the sampling time frame, except for *ccdR*, which showed background expression in each condition. The nondetectable expression of most of the *ccd* cluster genes is in contrast with the results from the differential proteomic analysis. However, samples for the differential proteomic approach were taken 6 h after addition of linuron, while for the transcription analysis RNA samples were only collected until 2 h after the addition of linuron, DCA, or aniline. In case a metabolite of DCA is the effector for activating transcription of the *ccd* gene cluster, an increased expression of these genes might only become apparent after longer incubation times, when more of the effector has accumulated.

Presence of *libA*, *dcaQ*, and *ccdC* in other linuron- and/or DCA-degrading bacteria. Different linuron- and/or DCA-degrading bacterial isolates were screened by PCR for the presence of the linuron and DCA catabolic genes *libA*, *dcaQ* (as representative for *dcaQTA*$_1$*A*$_2$*B*), and *ccdC* (as representative for *ccdCDEF*) (Table 2). A false-negative result for the presence of a gene may result from differences in nucleotide sequence at the primer annealing sites. Only two other linuron-mineralizing *Variovorax* strains showed the presence of all three genes (*Variovorax* sp. strains PBL-H6 and PBL-E5). In contrast, all linuron-degrading strains that can also degrade DCA contained the *libA* gene, including *Hydrogenophaga* sp. strain PBL-H3, suggesting that *libA* has distributed over other (betaproteo)bacteria. The only linuron-mineralizing strain which did not contain *libA* was *Variovorax* sp. WDL1, indicating that WDL1 contains a linuron hydrolase gene substantially different from SRS16. This is in agreement with the previously reported absence of an upregulated protein resembling LibA in a linuron-amended *Variovorax* sp. WDL1 culture (6). The absence of LibA was confirmed by Southern blotting and a local BLAST search in its draft genome sequence (unpublished data). In contrast to SRS16, WDL1 can only efficiently degrade linuron in synergy with other DCA-degrading bacteria (10). The *libA* amplicons recovered from strains PBL-H6 (100% sequenced), PBL-E5 (97% sequenced), and PBL-H3 (100% sequenced) showed 100% nucleotide identity with *libA* of SRS16.

dcaQ was detected in all linuron-degrading strains and in all DCA-degrading *Variovorax* strains. Local BLAST search confirmed the presence of a homologous *dcaQTA*$_1$*A*$_2$*BR* gene cluster in the draft genome sequence of WDL1. Some non-*Variovorax* strains which do not degrade linuron but degrade DCA did not show the presence of *dcaQ*, whereas *dcaQ* was detected in *Variovorax* sp. PBS-H4, which does not degrade DCA. This strain possibly lost the ability to degrade DCA but still contains part of the catabolic pathway.

ccdC was only detected in some of the linuron or DCA-degrading strains. Although strain WDL1 yielded no amplicon with the *ccdC* primers, a local BLAST search showed the presence of a *ccdRCFDE*-like gene cluster in strain WDL1. The gene cluster shows a configuration similar to this in SRS16, but the region between *ccdF* and *ccdC* differs substantially. In WDL1, this region contains ORF8, but ORF9 and ORF10 are replaced by other unknown genes. Differences in nucleotide sequence at the elongation ends of primers target-

TABLE 2. Distribution of selected *Variovorax* sp. SRS16 degradation genes in other relevant bacterial strains[a]

Organism	Degradation capacity			PCR screening		
	Linuron	DCA	N,O-DMHA	*dcaQ*	*ccdC*	*libA*
Variovorax sp. strain SRS16	+	+	−	+	+	+
Variovorax sp. strain WDL1	+	+	−	+	−	−
Variovorax sp. strain PBL-H6	+	+	−	+	+	+
Hydrogenophaga sp. strain PBL-H3	+	+	−	+	−	+
Variovorax sp. strain PBL-E5	+	+	−	+	+	+
Variovorax sp. strain PBS-H4	+	−	−	+	−	−
Comamonas testosteroni WDL7	−	+	−	+	−	−
Delftia acidovorans WDL34	−	+	−	+	−	−
Variovorax sp. strain PBD-E5	−	+	−	+	−	−
Variovorax sp. strain PBD-E37	−	+	−	+	+	−
Cupriavidus sp. strain PBS-E1	−	+	−	−	−	−
Afipia sp. strain PBD-E87	−	+	−	−	−	−
Variovorax sp. strain PBD-H1	−	+	−	+	−	−
Hyphomicrobium sp. strain PBN-H4	−	−	−	−	−	−
Variovorax paradoxus DSM66	−	−	−	−	−	−
Hyphomicrobium sp. strain PBN-E9	−	−	+	−	−	−
Hyphomicrobium sulfonivorans WDL6	−	−	−	−	−	−

[a] The results are based on PCR analysis. The identity of *libA* amplicons was confirmed by sequencing.

ing *ccdC* explain the negative result for PCR detection of *ccdC* in WDL1.

Our data suggest that in the tested strains, the linuron and DCA degradation genes can differ substantially from those in SRS16, including strains originating from the same environmental sample.

Conclusions. The linuron catabolic pathway in *Variovorax* sp. SRS16 is encoded by a patchwork of catabolic gene modules which together allow complete mineralization of linuron. The first gene involved in the pathway encodes a novel phenylurea hydrolase, designated LibA, which converts linuron to DCA, with a high specificity toward linuron. It is the first phenylurea hydrolase reported in Gram-negative bacteria and the first coupled in the same organism with other gene clusters specifying further degradation of DCA. The data suggest that, in accordance with the catabolism of other xenobiotic compounds, the assembly of well-specified existing gene modules in the same organism resulted in the emergence of a novel catabolic pathway. The absence of *libA* in another linuron-degrading *Variovorax* strain, i.e., strain WDL1, suggests furthermore that acquisition of linuron degradation by different *Variovorax* strains involves substantially different linuron hydrolase genes. Current research is focusing on the genes involved in linuron degradation in *Variovorax* sp. WDL1.

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