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HylA, an Alternative Hydrolase for Initiation of Catabolism of the Phenylurea Herbicide Linuron in *Variovorax* sp. Strains

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***Variovorax* sp. strain WDL1, which mineralizes the phenylurea herbicide linuron, expresses a novel linuron-hydrolyzing enzyme, HylA, that converts linuron to 3,4-dichloroaniline (DCA). The enzyme is distinct from the linuron hydrolase LibA enzyme recently identified in other linuron-mineralizing *Variovorax* strains and from phenylurea-hydrolyzing enzymes (PuhA, PuhB) found in Gram-positive bacteria. The dimeric enzyme belongs to a separate family of hydrolases and differs in K_m, temperature optimum, and phenylurea herbicide substrate range. Within the metal-dependent amidohydrolase superfamily, HylA and PuhA/PuhB belong to two distinct protein families, while LibA is a member of the unrelated amidase signature family. The *hylA* gene was identified in a draft genome sequence of strain WDL1. The involvement of *hylA* in linuron degradation by strain WDL1 is inferred from its absence in spontaneous WDL1 mutants defective in linuron hydrolysis and its presence in linuron-degrading *Variovorax* strains that lack *libA*. In strain WDL1, the *hylA* gene is combined with catabolic gene modules encoding the downstream pathways for DCA degradation, which are very similar to those present in *Variovorax* sp. SRS16, which contains *libA*. Our results show that the expansion of a DCA catabolic pathway toward linuron degradation in *Variovorax* can involve different but isofunctional linuron hydrolysis genes encoding proteins that belong to evolutionary unrelated hydrolase families. This may be explained by divergent evolution and the independent acquisition of the corresponding genetic modules.**

Linuron [3-(3,4-dichlorophenyl)-1-methoxy-1-methyl urea] is a phenylurea herbicide widely used in agriculture to control germinating and newly emerging grasses and broad-leafed weeds. Biodegradation contributes largely to the dissipation of linuron in the environment. Several single bacterial strains (1, 2) and consortia (3, 4) that degrade (3, 5) or even mineralize and use linuron as the sole source of carbon, nitrogen, and energy have been reported (1–4). Bacterial degradation of linuron is initiated by amide hydrolysis of linuron to 3,4-dichloroaniline (DCA) and *N*,*O*-dimethylhydroxylamine (*N*,*O*-DMHA). In the case of linuron mineralization, DCA is further converted to water and carbon dioxide (Fig. 1). Bacteria belonging to the genus *Variovorax* appear to play a crucial role in linuron biodegradation. In linuron-degrading consortia, they are almost always responsible for at least the initial hydrolysis step in linuron degradation, and most linuron-mineralizing single-strain isolates are of the genus *Variovorax* (1, 2). The genetic basis of linuron degradation in the linuron-mineralizing *Variovorax* sp. strain SRS16 was recently elucidated (6) and involves three major catabolic gene modules. In strain SRS16, conversion of linuron to DCA is catalyzed by the hydrolase LibA, encoded by the *libA* gene. Further mineralization of DCA involves a multicomponent dioxygenase complex encoded by *dcaQTA*$_1$*A*$_2$*BR*, which degrades DCA to a chlorocatechol intermediate. The latter is further degraded by a modified *ortho*-cleavage pathway encoded by *ccdRCFDE*. Apparently, the acquisition of the ability to mineralize linuron by strain SRS16 involved patchwork assembly of these three catabolic gene modules. A survey of the occurrence of *libA* in other linuron-degrading *Variovorax* strains revealed that some strains do not carry a *libA* homologue, suggesting that alternative enzymes for the initial linuron hydrolysis step exist in *Variovorax* (6). However, until now, it is not known if, or to what extent, various linuron hydrolases in *Variovorax* are evolutionarily related. To determine this relationship is important in order to understand the evolutionary adaptation toward linuron degradation in the genus *Variovorax* and the ecology of linuron-degrading *Variovorax* in linuron-treated ecosystems.

This study reports on the identification of the enzyme and gene involved in linuron hydrolysis in the linuron-mineralizing strain *Variovorax* sp. WDL1, a *Variovorax* strain that carries no *libA* homologue. The enzyme responsible for linuron hydrolysis in strain WDL1 was purified and characterized, and the distribution of the corresponding gene in other linuron-degrading strains was analyzed.

MATERIALS AND METHODS

Bacterial strains, cultivation conditions, and chemicals. *Variovorax* sp. strains WDL-1 (4), PBS-H4 (3), SRS16 (2), PBL-H6 (3), and PBL-E5 (3) and *Hydrogenophaga* sp. strain PBL-H3 (3) were routinely cultivated on R2A medium (solid agar or broth) supplemented with 20 mg liter^{-1} of linuron at 25°C. Mutants of strain WDL1 lacking the capacity for linuron hydrolysis arose spontaneously when strain WDL1 was plated onto R2A medium without linuron. Degradation of linuron was assessed in MMO broth supplemented with 160 μM linuron. R2A and MMO media were

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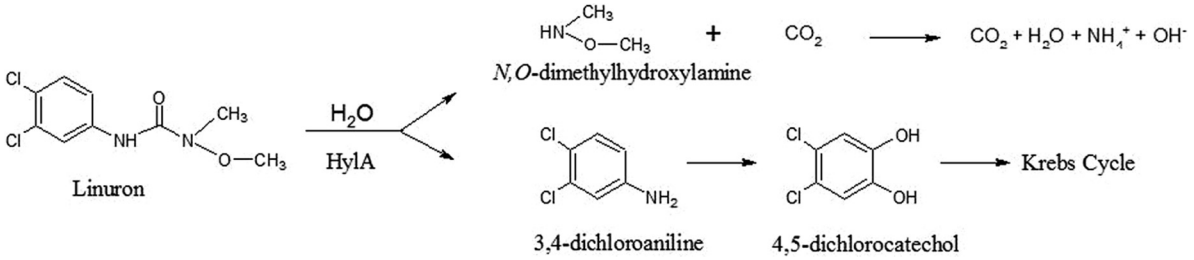

FIG 1 Proposed catabolic pathway of linuron degradation in *Variovorax* sp. WDL1. The catabolic step attributed to HylA is indicated.

prepared as described previously (3, 4). 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%), monolinuron [3-(4-chlorophenyl)-1-methoxy-1-methyl urea] (99%), and 3,4-dichloroaniline (DCA) (98%) were purchased from Sigma-Aldrich, Belgium.

HPLC analysis. Reverse-phase high-pressure liquid chromatography (HPLC) was used to detect and quantify phenylurea herbicides and their metabolites in the supernatants of bacterial cultures. HPLC analyses were performed at 21°C with a LaChrom (Merck Hitachi) HPLC system equipped with a reversed-phase C_{18} Alltima column (100 mm by 4.6 mm by 3 μm) using CH_3CN-H_2O (65:35, vol/vol) as the mobile phase at a flow rate of 0.8 ml min^{-1}. The injection volume was 20 μl (for concentrations of >100 μM) or 100 μl (for concentrations of <100 μM). UV absorption at a wavelength of 210 nm was used to detect the compounds that were identified and quantified based on retention times and peak areas derived from corresponding standard solutions of known concentrations.

Purification of the linuron hydrolase. Strain WDL1 was cultured in five replicates in 1 liter of R2A supplemented with 20 mg $liter^{-1}$ of linuron for 2 days under agitation in the dark at 25°C until an optical density at 600 nm (OD_{600}) of 4.5 was reached. After centrifugation of the cultures (15 min, 3,400 × *g*), the pellets were resuspended in the same volume of MMO containing linuron (40 mg $liter^{-1}$). Every 90 min, samples were taken for HPLC-based analysis of the residual linuron concentration. After 3 h, when about 65% of the linuron was degraded, the cultures were centrifuged (3,400 × *g*, 15 min, 4°C) and the pellets washed with phosphate-buffered saline (150 mM NaCl, 7 mM K_2HPO_4, 2.35 mM KH_2PO_4). The pellets were suspended in 10 ml of morpholinepropanesulfonic acid (MOPS) buffer (25 mM MOPS, 1 mM dithiothreitol, 5% glycerol [pH 7.6]) and stored at −80°C. The concentrated cell suspension was defrosted, and the cells were lysed in a French press (Thermo). The crude cell extract was centrifuged (8,000 × *g*, 30 min, 4°C) and concentrated by ultrafiltration on a Sartorius Stedim Vivaspin 6 column (molecular mass cutoff, 3 kDa; 4,000 × *g*, 4°C). The pH of the concentrated protein extract was adjusted to 7.6 by addition of Tris-HCl (20 mM, pH 7.6), and the solution was incubated with 1 μl of recombinant Benzonase nuclease (25 KU; Sigma) for 45 min at 37°C. The obtained protein extract was then concentrated as described above and loaded on an anion-exchange column (AIEX Q Sepharose HP), equilibrated with Tris-HCl buffer (20 mM, pH 7.6). The proteins were eluted over a linear gradient from 0 to 30% NaCl in an elution volume of 200 ml with a flow rate of 1 ml min^{-1}. Active fractions were pooled, concentrated by ultrafiltration as described above, and dissolved in a 1 M $(NH_4)_2SO_4$ solution in Tris-HCl (50 mM, pH 7) before being subjected to hydrophobic interaction chromatography (Phenyl Sepharose HP) [gradient from 0.7 M to 0 M $(NH_4)_2SO_4$; flow rate of 1 ml min^{-1}]. Finally, active fractions were subjected to gel permeation chromatography (column HiLoad Superdex 200 pg 16/60; flow rate of 1 ml min^{-1}) using an elution buffer consisting of 50 mM NaCl in Tris-HCl 20 mM (pH 7.2). Protein purity was assessed by means of 10% SDS-PAGE and Lumitein Protein gel staining (Biotium). To assess the linuron amidohydrolase activity of the different fractions at each purification step, first the production of DCA from linuron was measured using a colorimetric assay consisting of a diazotization-coupling reaction that detects DCA and measurement of absorption at 500 nm according to the method of Pease (7). Then, the activity was checked and quantified by monitoring the degradation of linuron and production of DCA by HPLC as described above. For both the colorimetric and HPLC-based assays, 3 μl (about 180 ng) of purified enzyme solution in MOPS buffer was supplemented with 140 μl of linuron solution (50 mg $liter^{-1}$ [i.e., 200 μM]) and incubated at room temperature for 60 min. 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.

Characterization of the linuron hydrolase. The oligomeric state of the enzyme was estimated by comparing the gel permeation chromatography elution time of active fractions with the elution times of the following proteins (size standards in parentheses): aprotinin (6.5 kDa), RNase A (13.7 kDa), carbonic anhydrase (29 kDa), conalbumin (75 kDa), and aldolase (158 kDa). Protein concentrations were estimated using a spectrophotometer (Nanodrop ND-1000). Tryptic peptides generated from the purified protein, excised from polyacrylamide gels, were sequenced using liquid chromatography-electrospray ionization–tandem mass spectrometry (LC-ESI-MS/MS), as previously described (8). N-terminal amino acid sequencing was performed as described previously (9). The kinetic parameters K_m, V_{max}, and k_{cat} of the purified enzyme for linuron were calculated by determining the hydrolytic activity in the presence of a range of linuron concentrations (80, 60, 45, 20, 10, and 5 μM). The amount of linuron hydrolyzed was determined after 20 min of incubation at 22°C. For each concentration, the linuron degradation rate (V) was calculated as moles of linuron degraded per time and volume unit (mol linuron min^{-1} ml^{-1}). These values were used to determine the kinetic parameters K_m and V_{max} of the purified enzyme for linuron based on the Michaelis-Menten kinetics. V_{max} was normalized for the enzyme concentration (mol linuron min^{-1} mol of $HylA^{-1}$), resulting in the turnover number, k_{cat}. The activity of the linuron hydrolase at different temperatures (10, 15, 20, 25, 30, 35, 40, 45, and 60°C) was analyzed after 1 h of incubation using a linuron concentration of 50 mg $liter^{-1}$ (200 μM). The substrate specificity of the purified linuron hydrolase was determined at 35°C by assessing the degradation of linuron, diuron, isoproturon, monolinuron, and metobromuron (100 μM each) after 1 h and 24 h of incubation. All above-mentioned tests were performed in a reaction volume consisting of 350 μl MOPS buffer and 350 μl of the herbicide of interest diluted in sterile water and using HPLC to determine residual phenylurea herbicide concentrations. All tests were performed in triplicate.

Sequence analyses. ORF finder (NCBI) and GeneMark (10) were used to identify open reading frames (ORFs) in contigs of the draft genome sequence of *Variovorax* sp. WDL1 (P. Albers and D. Springael, unpublished results). TBlastN search was applied to identify the gene coding for the HylA-derived peptide sequences in the draft WDL1 genome sequence. BLASTP was used to identify proteins with significant sequence similarity to the deduced gene product (11). The presence of a signal peptide was predicted with SignalP 4.0 (12). Phylogenetic analysis of HylA and related protein sequences was performed with Geneious Pro (version 5.6.3) (13) using PHYML (JTT matrix) (14).

DNA extraction. Extraction of genomic DNA from bacterial cultures was performed with the DNeasy blood and tissue kit (Qiagen) using the protocol provided by the manufacturer but with an extra mechanical lysis step after enzymatic lysis. Mechanical lysis was performed by adding 0.3 g of glass beads to the sample, vortexing at full speed for 30 s, and heating the sample for 1 h at 60°C.

PCR analysis. Primers for PCR detection of *hylA* were designed using Primer BLAST. BLAST and RDPII (http://rdp.cme.msu.edu/; July 2012) were used to confirm primer specificity. Primers were obtained from Thermo Fisher Scientific (Belgium). The presence of *hylA* in the different strains was examined by using two primer sets. The first primer set consisted of primers linhydrWDL1_S_Fw (5′-GACGCGACGTTCCGCCAC T-3′) and linhydrWDL1_S_Rv (5′-AGGAAGCGCCTGCCGCATTA-3′), which amplify a genomic fragment of 1,919 bp that contains the complete *hylA* gene (1,755 bp). The second primer set consisted of primers linhydrWDL1_comp_Fw (5′-TCATTTCGCCGGCTCGCCAA-3′) and linhydrWDL1_comp_Rv (5′-ATGCCGATGCATAGGGCCATAT-3′), which amplify the *hylA* gene from the start to the stop codons. In both cases, PCRs were performed in a total reaction volume of 25 μl containing 200 μM each deoxyribonucleotide triphosphate (dNTP), 0.5 μM each primer, 1× PCR buffer containing 1.5 mM $MgCl_2$, 0.625 U of *Taq* polymerase (Qiagen), and 1 μl of genomic DNA. PCRs were performed in an Eppendorf MasterCycler (Eppendorf, Germany) with the following reaction scheme: an initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, primer annealing at 60°C for 1 min, and elongation at 72°C for 1 min, and a final extension step at 72°C for 10 min. Amplification products were analyzed by electrophoresis in a 1.5% agarose gel (wt/vol) in 1× Tris-borate EDTA (TBE) containing 0.02% ethidium bromide (90 V, 75 min).

Sequencing of PCR amplicons. Amplicons were purified with the Qiaquick PCR purification kit (Qiagen) according to the manufacturer's instructions and sequenced using the BigDye Terminator cycle sequencing kit (Applied Biosystems) as described by the manufacturer. The sequencing reaction was performed on a thermocycler UNOII (Biometra, Germany). ClustalOmega (15) was used to align the obtained nucleotide sequences with the *hylA* sequence of *Variovorax* sp. WDL1.

Expression of *hylA* in *Escherichia coli*. The amidohydrolase gene was amplified with primers linhydrWDL1_comp_Nostop_Fw (5′-TTTCGCC GGCTCGCCAA-3′) and linhydrWDL1_comp_Rv, and the amplification product was cloned into expression vector pEXP5-CT/TOPO following the manufacturer's recommendations (Invitrogen). A poly-His tag was added at the C-terminal end. Electroporation was used to transform *E. coli* BL21(DE3)(pLys) with the recombinant vector (pEXP5-CT_HylA). The transformed *E. coli* BL21(DE3)(pLys) was grown in 50 ml of LB with ampicillin (100 μg ml^{-1}) at 37°C until an OD_{600} of 0.8 was reached. At this point, the cultures were split into two subcultures of 25 ml. In one subculture, 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to induce expression of the amidohydrolase. During the first 5 h of incubation, each hour two 1-ml samples were taken from each subculture to determine residual linuron concentrations by HPLC and for SDS-PAGE analysis. After centrifugation (10,000 × *g*, 5 min), the bacterial pellet (1 ml) was dissolved in 80 μl of SDS-PAGE loading buffer and boiled at 100°C for 5 min. After centrifugation (10,000 × *g*, 5 min), 5 μl of the supernatant was used for SDS-PAGE on a 10% polyacrylamide gel. In parallel, the linuron degradation capacity was evaluated et each sampling time on 1 ml of both cultures by adding linuron at a final concentration of 10 mg $liter^{-1}$ (40 μM) and incubation at 25°C. Linuron degradation was measured by HPLC after 1 h and after 24 h.

Nucleotide sequence accession numbers. The nucleotide sequences of contig 1, contig 10, and contig 100 of the draft genome of WDL1 and the nucleotide sequence of the *hylA* gene of *Variovorax* sp. PBS-H4 recovered by PCR were deposited in GenBank under accession numbers KC146403, KC146405, KC146404, and KC146406, respectively.

RESULTS

Purification and characterization of a linuron hydrolase in *Variovorax* sp. WDL1. A protein with linuron hydrolase activity was purified from a crude protein extract of linuron-degrading WDL1 cells (see Fig. S1A in the supplemental material). Based on gel permeation chromatography, the size of the purified protein was estimated to be ~118 kDa (data not shown). However, SDS-PAGE revealed a single protein of about ~56 kDa (see Fig. S1A in the supplemental material). This suggests that the linuron amidohydrolase from *Variovorax* sp. strain WDL-1 is a homodimer in its active form. Tandem mass spectrometry analysis showed that the peptide amino acid sequences of the purified protein have homology to the sequences of amidohydrolase-like proteins that catalyze the hydrolysis of amide or ester functional groups at carbon and phosphorus centers. The rate of degradation of linuron and production of DCA by the purified enzyme was determined at different initial linuron concentrations to determine kinetic parameters. Stoichiometric production of DCA was observed until a concentration of linuron of 45 μM, but at higher concentrations less DCA was produced than expected (see Fig. S2 in the supplemental material), which may have been due to impurities in the enzyme solution. K_m, V_{max}, and k_{cat} values were calculated based on the recorded linuron degradation rates (see Fig. S2 in the supplemental material). The novel amidase, designated HylA (hydrolase of linuron A), displayed a K_m for linuron of 14.97 ± 0.81 μM, a V_{max} of $2.66\ 10^{-4} \pm 7.6\ 10^{-6}$ μmol min^{-1} ml^{-1}, and a turnover number, k_{cat}, of $9.00\ 10^4 \pm 0.24\ 10^4$ min^{-1}. The temperature range for activity and the phenylurea herbicide substrate range of HylA were determined using a linuron concentration of 100 μM. As observed in the kinetic analysis, less DCA was produced than expected based on the linuron degradation extent. Maximum linuron hydrolase activity of HylA was around 35°C (see Fig. S3 in the supplemental material). The enzyme showed significant activity toward the *N*-methoxy-*N*-methyl phenylurea herbicides monolinuron and metobromuron, although at rates much lower than toward linuron (Fig. 2). On the other hand, HylA did not hydrolyze the *N*,*N*-dimethyl phenylurea herbicides diuron and isoproturon (Fig. 2), not even after prolonged incubation (for 24 h) (data not shown).

Identification of the *hylA* gene in *Variovorax* sp. WDL1. The identified peptide sequences of the purified HylA enzyme were assigned to ORF 1.1 on contig 1 (7,530 bp) in the draft genome sequence of strain WDL1 (Fig. 3; see Table S1 in the supplemental material), covering all together about 75% of the deduced amino acid sequence. The *hylA* gene is absent in the genome of *Variovorax paradoxus* S110 (16) and *V. paradoxus* EPS (NC_014931). HylA shows homology with members of the YtcJ-like family (Pfam PF07969, amidohydrolase_3 family) that is part of the metal-dependent amidohydrolase superfamily (see Fig. S4 in the supplemental material). Although HylA and LibA both perform linuron hydrolysis in two closely related linuron-mineralizing *Variovorax* strains, HylA lacks amino acid homology with LibA (Pfam PF01425, amidase family). Furthermore, no significant amino acid sequence homology was found with other linuron-degrading metal-dependent phenylurea hydrolases (Pfam PF01979, amidohydrolase_1 family), i.e., the orthologues PuhA and PuhB from the actinomycetes *Arthrobacter globiformis* D47 (17) and *Mycobacterium brisbanense* JK1 (12), respectively. These enzymes are most similar to the molinate hydrolase MolA (~50%

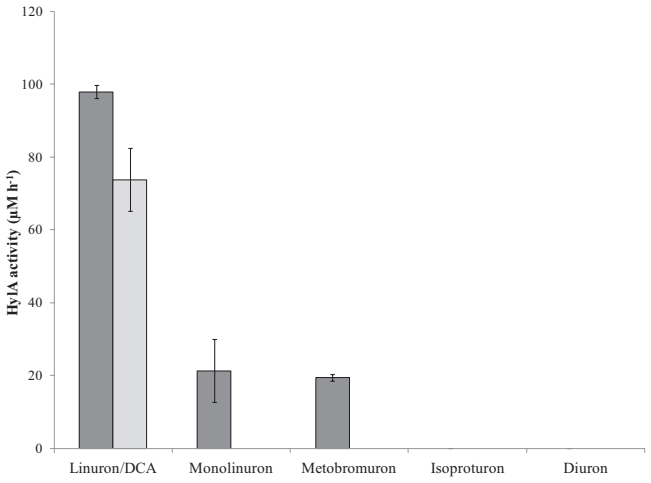

FIG 2 Phenylurea herbicide substrate specificity of HylA. The purified enzyme was incubated with different phenylurea compounds (100 μM) at 35°C, and the residual herbicide concentration was quantified by HPLC after 1 h. Only for linuron was the simultaneous production of the hydrolysis product (DCA) monitored. HylA activity is calculated as the amount of pesticide hydrolyzed per unit of time (dark gray) or the amount of degradation product formed per unit of time (pale gray). All tests were realized in triplicate ($n = 3$). Data shown are average values. Standard deviations are indicated.

amino acid sequence identity) mediating thiocarbamate herbicide degradation by the actinomycete *Gulosibacter molinativorax* ON4 (18). Until now, no enzymes closely related to HylA have been characterized. Among the characterized YctJ-like enzymes, the N-substituted formamide deformylase NfdA (19), which catalyzes the hydrolysis of some specific N-substituted formamides, showed some homology with HylA (only 25% amino acid identity). HylA showed higher amino acid identity (46 to 51%) with several hypothetical YtcJ-like amidohydrolases encoded in the *Sphingomonas wittichii* RW1 genome (see Fig. S4 in the supplemental material): Swit_0715 (51%), Swit_3245 (50%), Swit_3246 (49%), Swit_3242 (49%), Swit_3243 (47%), and Swit_2059 (46%). Two additional RW1 hypothetical proteins (Swit_3759 and Swit_3767) show lower similarity (29 to 30% amino acid identity) to HylA (see Fig. S4 in the supplemental material). SignalP prediction suggested the presence of an N-terminal signal peptide in HylA with a cleavage site between position 24 and position 25. N-terminal amino acid sequencing confirmed that this signal peptide is missing in the purified HylA. Moreover, although the molecular mass of the mature protein deduced from the nucleotide sequence (59,747 Da) is slightly higher than the value estimated from SDS-PAGE (~56 kDa) and the dimeric size measured in gel filtration (~118 kDa), it is smaller than the unprocessed form of HylA (62,380 Da).

Expression of *hylA* in *E. coli*. The linuron hydrolysis activity of HylA was confirmed by expression analysis in *E. coli* BL21(DE3)(pLys) carrying a vector containing *hylA* under the control of an IPTG-inducible promoter. The sequence encoding the N-terminal signal peptide was retained in this construct. In contrast to *E. coli* BL21(DE3)(pLys), for which no linuron degradation was observed, IPTG-induced cells of the *E. coli* containing pEXP5-CT_hylA showed complete linuron degradation in less than an hour (Fig. 4). Without IPTG induction, a significantly slower linuron degradation capacity was observed (10% after 1 h and 86% after 24 h) (Fig. 4). SDS-PAGE profiles showed two additional bands corresponding to the expected protein size of ~56 kDa in the IPTG-supplemented cultures compared to the noninduced cultures (see Fig. S1B in the supplemental material), which probably correspond to the processed and unprocessed forms of the protein, with and without the signal peptide cleaved off, respectively.

Presence of *hylA* in other linuron-degrading bacteria. Different linuron-degrading strains (*Variovorax* sp. strains SRS16, PBL-E5, PBL-H6, and PBS-H4 and *Hydrogenophaga* sp. strain PBL-H3) were examined by PCR for the presence of *hylA*. In addition to strain WDL1, *hylA* was detected only in strain PBS-H4, the only strain that does not contain *libA* (6). The *hylA* amplicon recovered from PBS-H4 showed 99.7% nucleotide identity with *hylA* of strain WDL1, with a different amino acid sequence at three positions. Spontaneous mutants of strain WDL1 that had lost the capability to degrade linuron after growth on a medium without

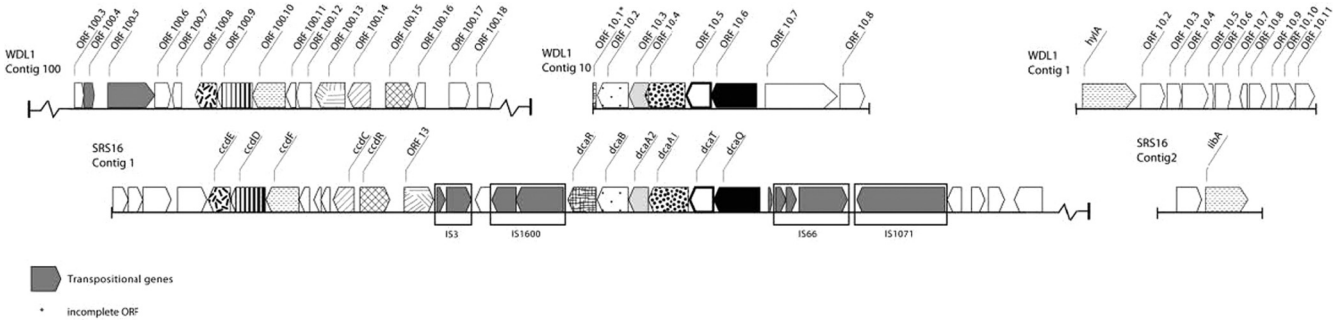

FIG 3 Organization of the genes involved in linuron and DCA metabolism in *Variovorax* sp. WDL1 compared to the corresponding genes in *Variovorax* sp. SRS16 (6). The arrows indicate the direction of transcription of the genes. Similar shaded patterns indicate isofunctional genes in WDL1 and SRS16. The following linuron/DCA catabolic functions correspond to the indicated genes (the coding genes in WDL1 and SRS16, respectively, are shown in parentheses): linuron hydrolase (*hylA*, *libA*), glutamine synthetase (*orf10.6*, *dcaQ*), glutamine amidotransferase (*orf10.5*, *dcaT*), chloroaniline dioxygenase large subunit (*orf10.4*, *dcaA*$_1$), chloroaniline dioxygenase small subunit (*orf10.3*, *dcaA*$_2$), chloroaniline dioxygenase reductase (*orf10.2*, *dcaB*), LysR family transcriptional regulator (*orf10.1*, *dcaR*), LysR family transcriptional regulator (*orf100.15*, *ccdR*), chlorocatechol 1,2-dioxygenase (*orf100.14*, *ccdC*), maleylacetate reductase (*orf100.10*, *ccdF*), chloromuconate cycloisomerase (*orf100.9*, *ccdD*), dienelactone hydrolase (*orf100.8*, *ccdE*), and the hypothetical Bug family protein (*orf100.13*, *orf13*). Further information about the different ORFs is shown in Table S1 in the supplemental material. The ORFs present on contig 100 in the genome of WDL1 upstream of ORF 100.3 and downstream of ORF 100.18 are represented in Table S1 in the supplemental material and the corresponding GenBank file (KC146404).

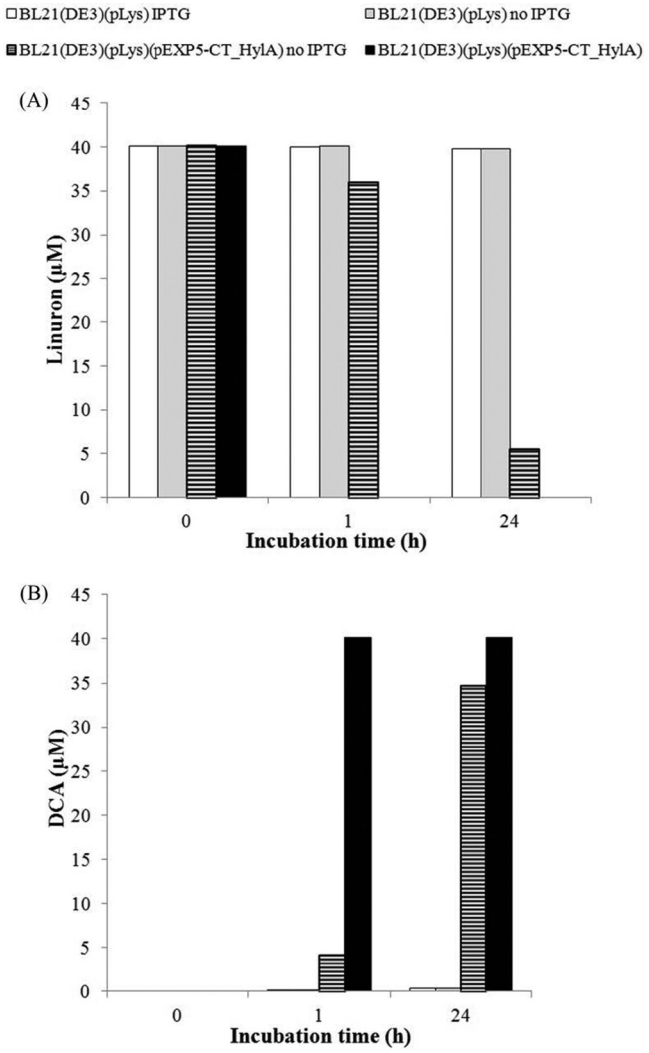

FIG 4 Linuron degradation by *E. coli* expressing recombinant HylA. Activity of BL21(DE3)(pLys) control cells was compared with that of cells carrying recombinant vector pEXP-CT_HylA. Linuron was added (final concentration of 40 μM) to both IPTG-induced and noninduced cultures 5 h after addition of IPTG (1 mM). Linuron degradation (A) and DCA production (B) were measured by HPLC after 1 h and after 24 h of incubation.

linuron did not show amplification with the *hylA*-specific primers (data not shown).

DISCUSSION

This paper reports on the identification of a novel enzyme, designated HylA, that hydrolyzes the phenylurea herbicide linuron to DCA in *Variovorax* sp. WDL1. The novel amidohydrolase differs from other previously reported and characterized linuron hydrolases, i.e., PuhA identified in *Arthrobacter globiformis* D47 (20), the PuhA orthologue PuhB in *Mycobacterium brisbanense* strain JK1 (21) and LibA in *Variovorax* sp. SRS16 (6). First, they belong to different protein families. HylA belongs to the YctJ-like family within the metal-dependent amidohydrolase superfamily. PuhA and PuhB are members of a separate family within this superfamily, while LibA belongs to the amidase signature (AS) family (6). Second, HylA shows different enzyme kinetics toward linuron. LibA, PuhA, and PuhB show a 2-fold lower K_m (~7 to 8 μM) than HylA (14.97 ± 0.81 μM), indicating that the former have a somewhat higher affinity for linuron. Conversely, PuhA and PuhB demonstrated lower turnover numbers (4.25 ± 0.22 s^{-1} and 4.3 ± 0.13 s^{-1}, respectively) than that of HylA (1.55 × 10^3 ± 0.04 × 10^3 s^{-1}). No k_{cat} data are currently available for LibA. LibA shows optimal linuron hydrolysis activity in a lower temperature range (22 to 30°C) than that of HylA. Maximum linuron hydrolysis by PuhA and PuhB takes place between 30°C and 35°C, which is comparable to the temperature range for HylA (see Fig. S3 in the supplemental material). Third, the phenylurea herbicide substrate range of HylA differs from those of LibA, PuhA, and PuhB. The substrate range of HylA is limited compared to those of PuhA and PuhB, which show activity toward a wide range of *N*-methoxy-*N*-methyl as well as *N*,*N*-dimethyl phenylurea herbicides, but its activity is less restricted than the one of LibA, which among all tested phenylurea herbicides hydrolyzes only linuron. Furthermore, in contrast to the monomeric linuron hydrolase LibA (6), HylA is a dimer in its active form. Other amidohydrolases have been shown to have a dimeric structure in solution (22, 23), and only the dimer is active in some cases (17). Finally, in contrast to LibA, PuhA, and PuhB, HylA contains an N-terminal signal sequence for secretion across the cytoplasmic membrane. This signal sequence was indeed absent in the mature enzyme isolated from whole cells. Apparently, the enzyme is exported to the periplasm to exert its function and HylA-mediated linuron hydrolysis is a periplasmic process in WDL1 (24).

Some observations indicate that HylA is responsible for the initial hydrolysis in the linuron degradation pathway in *Variovorax* sp. WDL1. The *hylA* gene is missing in spontaneous mutants of WDL1 that lack linuron hydrolysis activity but retain the capacity to degrade DCA. The appearance of mutants defective in xenobiotic degradation on nonselective medium is a common observation in organic xenobiotic-degrading bacteria (25). Strain WDL1 is able to degrade linuron to mineral products and to degrade DCA. As such, *hylA* is expected to compose a complete linuron mineralization pathway together with a multicomponent dioxygenase and a chlorocatechol cleavage pathway in WDL1. In the draft genome sequence of WDL1, both a gene cluster that accommodates putative proteins of a multicomponent chloroaniline dioxygenase (consisting of ORF 10.6 to ORF 10.1) and a gene cluster that encodes putative proteins linked to a modified chlorocatechol *ortho*-cleavage pathway (ORF 100.8 to ORF 100.10, ORF 100.14, and ORF 100.15) were identified (Fig. 3; see Table S1 in the supplemental material). ORF 10.1 to ORF 10.6 can be linked to the multicomponent aniline dioxygenase components that were identified by differential SDS-PAGE analysis by Breugelmans et al. (8) as being increasingly expressed when strain WDL1 was grown on linuron. In contrast to *hylA*, the translation products of these two gene clusters show high amino acid similarity with the corresponding proteins in SRS16 (see Table S1 in the supplemental material). Both gene clusters also show an organization highly similar to those in SRS16 (Fig. 3), with minor differences in the chlorocatechol catabolic gene cluster. In strain SRS16, chlorocatechol degradation appears to be encoded by a gene cluster (*ccdRC orf10 orf9 orf8 ccdFDE*) whose organization differs substantially from those encoding modified *ortho*-cleavage pathways in other organisms (6). Similar to SRS16, three ORFs that cannot directly be linked to chlorocatechol degradation (ORFs 100.11 to 100.13) are located within the gene cluster (Fig. 3; see Table S1). However, only ORF 100.11 is a putative orthologue of the corresponding

ORF_9 in SRS16 (82% amino acid identity; see Table S1). The deduced translation product of ORF 100.13 shows significant amino acid similarity (43% amino acid identity) to ORF 13, a hypothetical transporter component (periplasmic solute-binding protein) encoded by a gene flanking the chlorocatechol cleavage gene cluster in SRS16 (Fig. 3; see Table S1). Thus, as previously suggested for *Variovorax* sp. SRS16, these observations suggest a similar patchwork assembly of catabolic gene modules in *Variovorax* sp. WDL1 that allows the strain to use the xenobiotic compound linuron as the sole source of carbon and energy, but instead of *libA* in strain SRS16, *hylA* completes the linuron degradation pathway in WDL1. PCR analyses of all linuron-degrading strains in our collection show that they contain either *libA* or *hylA* but never both. This might indicate that linuron hydrolysis genes were acquired by horizontal gene transfer and that the evolvement of a linuron catabolic pathway in *Variovorax* can involve different nonrelated isofunctional linuron hydrolytic enzymes. This can be explained by independent acquisition of the corresponding gene modules and hence divergent evolution.

The identification of HylA as an alternative linuron hydrolase used by linuron-degrading *Variovorax* strains poses questions about its ecological function in environmental linuron degradation. In the past, ecological studies showed that the dynamics of linuron-degrading activity in the environment as a response to linuron application (i.e., in linuron-treated agricultural soil and on-farm biopurification systems), is not always explained by the abundance of *libA* (26). We are currently investigating whether *hylA* provides an alternative explanation in those case studies, whether organisms containing *hylA* or *libA* coexist or compete in linuron-treated and -contaminated environments, and whether still other linuron hydrolysis gene functions exist.

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