Characterization of Phenylpyruvate Decarboxylase, Involved in Auxin Production of *Azospirillum brasilense*[∇]

Stijn Spaepen,¹ Wim Versées,^{2,3} Dörte Gocke,⁴ Martina Pohl,⁴ Jan Steyaert,^{2,3} and Jos Vanderleyden^{1*}

Centre of Microbial and Plant Genetics and INPAC, K.U. Leuven, Kasteelpark Arenberg 20, 3001 Heverlee, Belgium¹;

Department of Ultrastructure, Vrije Universiteit Brussel, Pleinlaan 2, 1050 Brussel, Belgium²; Department of Molecular and

Cellular Interactions, VIB, Pleinlaan 2, 1050 Brussel, Belgium³; and Institute of Molecular Enzyme Technology,

Heinrich-Heine University Düsseldorf, Research Centre Jülich, 52426 Jülich, Germany⁴

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Azospirillum brasilense belongs to the plant growth-promoting rhizobacteria with direct growth promotion through the production of the phytohormone indole-3-acetic acid (IAA). A key gene in the production of IAA, annotated as indole-3-pyruvate decarboxylase (*ipdC*), has been isolated from *A. brasilense*, and its regulation was reported previously (A. Vande Broek, P. Gysegom, O. Ona, N. Hendrickx, E. Prinsen, J. Van Impe, and J. Vanderleyden, Mol. Plant-Microbe Interact. 18:311–323, 2005). An *ipdC*-knockout mutant was found to produce only 10% (wt/vol) of the wild-type IAA production level. In this study, the encoded enzyme is characterized via a biochemical and phylogenetic analysis. Therefore, the recombinant enzyme was expressed and purified via heterologous overexpression in *Escherichia coli* and subsequent affinity chromatography. The molecular mass of the holoenzyme was determined by size-exclusion chromatography, suggesting a tetrameric structure, which is typical for 2-keto acid decarboxylases. The enzyme shows the highest k_{cat} value for phenylpyruvate. Comparing values for the specificity constant k_{cat}/K_m , indole-3-pyruvate is converted 10-fold less efficiently, while no activity could be detected with benzoylformate. The enzyme shows pronounced substrate activation with indole-3-pyruvate and some other aromatic substrates, while for phenylpyruvate it appears to obey classical Michaelis-Menten kinetics. Based on these data, we propose a reclassification of the *ipdC* gene product of *A. brasilense* as a phenylpyruvate decarboxylase (EC 4.1.1.43).

Azospirillum brasilense is a gram-negative, nitrogen-fixing bacterium that lives in association with the roots of grasses and cereals (24). This bacterium belongs to the plant growth-promoting rhizobacteria with direct growth promotion. *Azospirillum* inoculation results in an increased number of plant root hairs and lateral roots, through which an improved uptake of water and nutrients can occur. This effect is primarily caused by the bacterial production of the phytohormone indole-3-acetic acid (IAA), the most abundant naturally occurring auxin (10). Tryptophan (Trp) is generally considered to be the precursor of IAA. The best-characterized pathways in bacteria for the conversion of Trp to IAA are the indole-3-acetamide pathway and the indole-3-pyruvate (IPyA) pathway (8, 25). In the IPyA pathway, Trp is first transaminated to IPyA, subsequently decarboxylated to indole-3-acetaldehyde, which can be oxidized to IAA. A gene (*ipdC*) encoding IPyA decarboxylase (IPDC) was isolated from *Enterobacter cloacae*, and a homologous gene has also been found in *A. brasilense* (7, 16). The characterization and structural analysis of IPDC from *E. cloacae* (IPDC_{Ec}) have been published by Schütz et al. (30, 31). IPDC_{Ec} shows the highest catalytic efficiency with the natural substrate IPyA and low or no activity with pyruvate and phenylpyruvate. In contrast to some other thiamine diphosphate (ThDP)-dependent 2-keto acid decarboxylases, there were no

indications for substrate activation. In *A. brasilense* an *ipdC*-knockout mutant was found to produce less than 10% of the level of wild-type IAA production, indicating that the *ipdC* gene product is a key enzyme for IAA biosynthesis in this bacterium (7, 28). Expression analysis of *ipdC* revealed a strict regulation. The gene is expressed only in the late exponential phase and is induced by auxins (37, 38). Recently, Somers et al. demonstrated that the *ipdC* gene product is also involved in the biosynthesis of phenylacetic acid (PAA), an auxin with antimicrobial activity (34). An *A. brasilense* *ipdC*-knockout mutant shows significantly lower production of PAA (34). Here we report the detailed characterization of the *A. brasilense* *ipdC* gene product, which suggests its reclassification as a phenylpyruvate decarboxylase (PPDC).

MATERIALS AND METHODS

Strains and growth media. The strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown in Luria-Bertani (LB) medium at 37°C. *A. brasilense* strains were grown at 30°C in LB medium supplemented with 2.5 mM CaCl₂ and 2.5 mM MgCl (LB* medium) or in *Azospirillum* minimal medium with 0.5% (wt/vol) malate (39), phenylalanine or tryptophan as a carbon source, and 20 mM NH₄Cl or 0.5% (wt/vol) phenylalanine or tryptophan as a nitrogen source. The antibiotic kanamycin was used when appropriate at 25 µg ml⁻¹.

Growth experiments under different carbon and nitrogen sources. Bacterial strains were grown in shaken culture overnight in LB* medium supplemented with the appropriate antibiotics. Cells were pelleted and washed twice with an 0.85% (wt/vol) sodium chloride solution. The optical density at 600 nm was adjusted to 1, and the cultures were diluted 1,500-fold in minimal medium with different carbon and nitrogen sources. The growth was monitored every 30 min (600 nm) in a Bioscreen C apparatus for 72 h. The growth characteristics were identified by the growth rate constant calculated on the exponential part of the

* Corresponding author. Mailing address: Centre of Microbial and Plant Genetics, K.U. Leuven, Kasteelpark Arenberg 20, 3001 Heverlee, Belgium. Phone: 32 16321631. Fax: 32 16321963. E-mail: jozef.vanderleyden@biw.kuleuven.be.

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TABLE 1. Strains and plasmids

Strain or plasmid	Relevant characteristic(s)	Reference or source
Strains		
<i>Escherichia coli</i>		
DH5 α	<i>hsdR17 endA1 thi-1 gyrA96 relA1 recA1 supE44 ΔlacU169(ϕ80lacZΔM15)</i>	33
BL21-CodonPlus(DE3)-RP	<i>ompT hsdS(r_B⁻ m_B⁻) dcm⁺ Tet^r gal λ(DE3) <i>endA</i> Hte [<i>argU proL</i> Cam^r]</i>	Stratagene
<i>Azospirillum brasilense</i>		
Sp245	Wild-type strain	5
FAJ0009	Sp245 <i>ipdC::Tn5</i>	7
Plasmids		
pET28a	Km ^r	Novagen
pCMPG9551	1.6-kb NdeI-XhoI fragment, carrying the open reading frame of the <i>ipdC</i> gene, in pET28a	This study

growth curve and defined by $\ln 2 g^{-1}$ (g , doubling time of an exponentially growing culture).

Construction of overexpression strain. The open reading frame of the *ipdC* gene of *A. brasilense* was amplified using PCR with the following primers: Azo-415 (5'-GATCCATATGAAGCTGGCCGAAGCCCTG-3') and Azo-416 (5'-CTAGTCTCAGTTACTCCCGGGGCGCGGCGTG-3'), containing NdeI and XhoI restriction sites, respectively, at the 5' end (underlined in nucleotide sequence). After digestion, the fragment was ligated in a pET28a vector (Novagen). *E. coli* DH5 α was transformed with this construct (called pCMPG9551). After selection of a correct clone by a restriction digest, the expression host *E. coli* BL21-CodonPlus(DE3)-RP (Stratagene) was transformed with the purified plasmid. The sequence was verified on an ALF Sequencer (Amersham).

Expression and purification of recombinant enzyme. Two hundred fifty milliliters LB medium was inoculated with 12.5 ml of an overnight culture of BL21-CodonPlus(DE3)-RP(pCMPG9551) and grown at 37°C till it reached a turbidity (595 nm) of 0.5 to 0.7 (2 to 3 h). Protein expression was induced by adding 1 mM isopropyl- β -D-thiogalactopyranoside. The cultures were grown at 30°C for a further 7 to 8 h. Cells were harvested by centrifugation at $4,000 \times g$ for 20 min (4°C), and the pellets were stored at -20°C. For cell disruption, the pellets were resuspended in binding buffer (10 mM 2-morpholinoethanesulfonic acid [MES], 2.5 mM MgSO₄, 0.1 mM ThDP, 0.5 M KCl, 20 mM imidazole, pH 7.4) supplemented with 1 mg lysozyme per ml binding buffer. After incubation at 37°C for 30 min, the cells were sonicated (200 W; eight times for 10 s each). The cell lysate was cleared by centrifugation ($10,000 \times g$; 25 min; 4°C), and the supernatant was applied to a HisTrap HP column (Amersham). After the column was washed with binding buffer, the recombinant protein was eluted using a linear gradient from 0 to 100% elution buffer (10 mM MES, 2.5 mM MgSO₄, 0.1 mM ThDP, 0.5 M KCl, 500 mM imidazole, pH 7.4). The protein eluted at an imidazole concentration of 250 mM. The elution fraction was dialyzed against the assay buffer (10 mM MES, 2.5 mM MgSO₄, 0.1 mM ThDP, pH 6.5), yielding a total dilution factor of 50,000. The protein-containing fractions, the molecular mass of the subunits, and the enzyme purity were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by Coomassie blue staining, and the protein concentration was determined by direct optical measurement ($\epsilon_{280} = 36,840 \text{ M}^{-1} \text{ cm}^{-1}$; determined using ProtParam tool; Swiss Institute of Bioinformatics [http://www.expasy.org/tools/protparam.html]) or by the Bradford method (6) using bovine serum albumin as a standard.

Size-exclusion chromatography. The molecular mass of the holoenzyme was estimated by size-exclusion chromatography on a Superdex G200 column, equilibrated with 10 mM MES, 2.5 mM MgSO₄, 0.5 mM ThDP, 150 mM KCl, pH 6.5 (flow rate of 1 ml min⁻¹). The molecular mass was estimated by comparing the elution volume (at least three repetitions) with those of known standard proteins (molecular mass in kDa is indicated in parentheses): RNase A (13.7), chymotrypsinogen A (25), ovalbumin (43), albumin (67), aldolase (158), catalase (232), ferritin (440), and thyroglobulin (669). Blue dextran 2000 was used to determine the void volume of the column.

Enzyme assay and kinetic analysis. The decarboxylation activity was measured using a coupled photometric assay with alcohol dehydrogenase as an auxiliary enzyme (43). A typical reaction mixture (pH 6.5) contained 10 mM MES, 2.5 mM MgSO₄, 0.1 mM ThDP, 0.35 mM NADH, and 0.125 U alcohol dehydrogenase from horse liver. To avoid interference with the substrates and products, the absorbance was measured at 366 nm ($\epsilon_{366, \text{NADH}} = 2.87 \text{ mM}^{-1} \text{ cm}^{-1}$) (30), using

a Cary 100 UV-Vis spectrophotometer (Varian Inc.). The reaction was followed for 15 min at 30°C. The reaction velocity was converted into the reaction rate (per monomer), and the data were plotted and fitted according to Michaelis-Menten kinetics or the simplified Hill equation using Origin software (version 7; OriginLab Corporation).

Phylogenetic analysis. The BLAST algorithm (1) with IPDC_{Ec} and PPDC of *A. brasilense* (PPDC_{Ab}) as baits was used to identify (putative) bacterial PPDCs and IPDCs. Thirty-three amino acid sequences were selected for multiple sequence alignment with ClustalW (36). The alignment was further adapted with the Genedoc program (23) for publication. A phylogenetic tree based on the multiple sequence alignment was constructed using an UPGMA (unweighted-pair group method using average linkages) model with the MEGA software (18), and bootstrap values were used for reliability testing (1,000 replicates).

RESULTS

Growth experiments. The *A. brasilense* wild-type strain and *ipdC*-knockout mutant (FAJ0009) were grown in minimal medium with different nitrogen sources (Fig. 1). The two strains show the same growth characteristics in minimal medium with ammonium as the sole nitrogen source. The growth rate of the *ipdC*-knockout mutant (growth rate constant, 0.087 h^{-1}) in minimal medium with phenylalanine as the sole nitrogen source is, however, impaired compared to the wild-type strain (0.102 h^{-1}). Tryptophan is not a nitrogen source for the *A. brasilense* strains: the wild-type and *ipdC*-knockout mutant strains did not show any significant growth. *A. brasilense* was also unable to grow on minimal medium with the aromatic amino acid phenylalanine or tryptophan as the sole carbon source (data not shown).

Heterologous overexpression, purification, and size-exclusion chromatography of PPDC_{Ab}. Recombinant PPDC_{Ab} was produced by cloning the open reading frame into an N-terminal His₆-tag-attaching overexpression vector. Heterologous overexpression in *E. coli* and subsequent Ni²⁺ chelate affinity chromatography resulted in isolation of recombinant PPDC_{Ab}. Expression level and purity were checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the recombinant enzyme was purified in high yield and purity. A subunit molecular mass of about 60 kDa could be estimated from comparison with the standard proteins.

A native molecular mass of 240.6 kDa was determined by size-exclusion chromatography for the recombinant PPDC_{Ab},

FIG. 1. Growth curves of *Azospirillum brasilense* and FAJ0009. Data represent means of five replicates of one experiment. Squares, *A. brasilense* Sp245; triangles, FAJ0009; open symbols, minimal medium for *Azospirillum* (MMAB) with ammonium as the sole nitrogen source; filled symbols, MMAB with phenylalanine as the sole nitrogen source.

which is consistent with the theoretical mass of a tetramer calculated from the amino acid sequence (240.0 kDa).

Substrate specificity of PPDC_{Ab}. The decarboxylase activity of the ThDP-dependent 2-keto acid decarboxylase PPDC_{Ab} was followed using a coupled optical test. For substrates showing significant activity, the steady-state parameters were determined. Depending on the reaction rate versus substrate concentration curve, different models were used to analyze the data. The aromatic 2-keto acids phenylpyruvate, IPyA, 4-phenyl-2-oxobutanoic acid, and 5-phenyl-2-oxopentanoic acid are decarboxylated by PPDC_{Ab} but with significantly different kinetic parameters (Table 2). No activity was observed with benzoylformate and indole-glyoxylic acid. The enzyme has a k_{cat}/K_m of 309 mM⁻¹ min⁻¹ for phenylpyruvate, showing apparently classical Michaelis-Menten kinetics (with a slight deviation from hyperbolicity: Hill coefficient [h] of 1.26 when fitted on the Hill equation). By contrast, the other aromatic substrates showed substrate activation, which is visible from the sigmoidal reaction rate-versus-substrate concentration curves and the deviation from linearity of Eadie-Hofstee plots

(Fig. 2). Therefore, the kinetic data were analyzed with the Hill model, yielding $k_{\text{cat}}/K_{0.5} = 32 \text{ mM}^{-1} \text{ min}^{-1}$ for IPyA, $7 \text{ mM}^{-1} \text{ min}^{-1}$ for 4-phenyl-2-oxobutanoic acid, and $339 \text{ mM}^{-1} \text{ min}^{-1}$ for 5-phenyl-2-oxopentanoic acid. Hill coefficients for these aromatic substrates were in the range of 1.8 to 1.9. The substrate specificity of PPDC_{Ab} was further tested for some commercially available aliphatic and branched-chain 2-keto acids. PPDC_{Ab} exhibited no significant activity towards 3-methyl-2-oxopentanoic acid and 4-methyl-2-oxopentanoic acid (maximum substrate concentration, 4 mM). The aliphatic 2-keto acid 2-ketohexanoic acid was a substrate for PPDC_{Ab} with $k_{\text{cat}}/K_{0.5} = 3 \text{ mM}^{-1} \text{ min}^{-1}$. 2-Keto acids with longer aliphatic chains (e.g., 2-keto-octanoic acid at 5 mM) were not converted.

Phylogenetic analysis. Sequences similar to PPDC_{Ab} and IPDC_{Ec} were extracted from the NCBI database using the amino acid sequence of these two well-characterized ThDP-dependent 2-keto acid decarboxylases as baits in a BLAST search (1). The multiple sequence alignment, generated by ClustalW, shows that most catalytically important residues are conserved among the 33 sequences, with representatives in

TABLE 2. Steady-state kinetic parameters of *A. brasilense* PPDC

Substrate ^d	h	K_m^a (mM)	k_{cat} (min ⁻¹)	k_{cat}/K_m (mM ⁻¹ min ⁻¹)	Relative activity (%) ^b
Phenylpyruvate	1	1.08 ± 0.09	333.7 ± 7.8	309 ± 27	100.0
IPyA	1.85	0.13 ± 0.01	4.1 ± 0.1	32 ± 3	10.4
4-Phenyl-2-oxobutanoic acid	1.79	1.91 ± 0.11	12.5 ± 0.4	7 ± 0	2.3
5-Phenyl-2-oxopentanoic acid	1.93	0.30 ± 0.01	101.8 ± 0.8	339 ± 12	110.0
2-Ketohexanoic acid ^c	2.23	5.51 ± 0.23	17.2 ± 0.6	3 ± 0	1.0

^a The parameter K_m is replaced by $K_{0.5}$ when using the Hill equation.

^b Based on $k_{\text{cat}}/K_{0.5}$ in comparison with k_{cat}/K_m of phenylpyruvate.

^c The enzyme activity of PPDC_{Ab} with 2-ketohexanoic acid as substrate was subjected to substrate inhibition. The data were analyzed for lower substrate concentrations using the Hill model without substrate activation.

^d The aromatic substrates, which have no activity towards PPDC_{Ab}, are benzoylformate and indole-glyoxylic acid. Most aliphatic 2-keto acids are also inactive, including the tested molecules 2-keto-octanoic acid, 3-methyl-2-ketopentanoic acid, and 4-methyl-2-ketopentanoic acid.

FIG. 2. Determination of the steady-state kinetic parameters of PPDC_{Ab}. (Top panels) Plots of initial reaction rates versus substrate concentrations. (Insets) Eadie-Hofstee plots. (Bottom panels) Hill plots of kinetic data. (Left panels) PPDC_{Ab} activity with phenylpyruvate. (Right panels) PPDC_{Ab} activity with IPyA. The reaction rate was determined as described in Materials and Methods. Data are shown in Table 2.

different subclasses of the proteobacteria, firmicutes, and cyanobacteria. In Fig. 3, the multiple sequence alignment of the region comprising the well-conserved ThDP-binding motif (GDGX₂₄NN [14]) is shown. However, the only nonconserved but catalytically important residue is glutamate 468 (numbering according to amino acid sequence of IPDC_{Ec}), which is exchanged by leucine in nine sequences (indicated with an arrow in Fig. 3). Based on the multiple sequence alignment, a phylogenetic tree was constructed (Fig. 4). The sequences fall into two clades: one contains IPDC_{Ec}, while the second contains PPDC_{Ab}. Eight sequences clustering with PPDC_{Ab} all have Leu in the position corresponding to Glu468 (sequences indicated with bracket designated "PPDC"). However, the other sequences in this clade (middle bracket) have the conserved Glu in position 468.

DISCUSSION

The *ipdC* gene and gene product from *A. brasilense* have been characterized in different ways during the past several years. The amount of IAA produced by a mutant with a knock-out mutation in the *ipdC* gene (FAJ0009) is less than 10% of that produced by the wild-type strain, indicating a central role of this gene in IAA biosynthesis (7, 28). Comparative in vitro growth tests with wheat seedlings inoculated with wild-type *A. brasilense* and FAJ0009 demonstrated a direct link between IAA production and root hair induction (10). The transcription of the *ipdC* gene is under tight control via a positive

feedback mechanism. The IAA production and *ipdC* gene induction are cell density regulated with a maximum in the stationary and late-exponential phases, respectively, and the end product IAA itself is responsible for the induction of the gene expression (37, 38). As this autoinduction is rather unusual for biosynthetic genes, the mechanism(s) for the *ipdC* gene regulation remains intriguing. A signaling role has been suggested for IAA both in bacterial and in bacterium-plant interactions (19, 29, 35). Recently, the production of PAA, a weak auxin with antimicrobial activity, was described by Somers et al. (34). The *ipdC* gene is also involved in the synthesis of PAA, as FAJ0009 produces significantly lower amounts of PAA. Furthermore, PAA can induce *ipdC* gene expression in a concentration range similar to that of IAA (34). Most results regarding PAA can be explained by the structural similarity between IAA and PAA (12). However, it is intriguing that, despite this similarity, IPDC_{Ec} does not show any activity towards phenylpyruvate (30). Therefore, it was reasoned that a biochemical and phylogenetic analysis of the *ipdC* gene product of *A. brasilense* could reveal its distinction from IPDC_{Ec}.

Inactivation of the *ipdC* gene has no effect on growth in medium with ammonium as a nitrogen source (Fig. 1), indicating that its activity is not required in the central ammonium metabolism of *A. brasilense*. Nevertheless, when growth of the wild type and that of the mutant were compared in minimal medium with phenylalanine as the sole nitrogen source, FAJ0009 was slightly impaired in growth compared to the wild-type strain. However, a similar growth experiment could

FIG. 3. Alignment of the amino acid sequences of the ThDP-binding domain. Details of the alignment of a set of (putative) PPDCs and IPDCs are shown. The ThDP-binding domain starts with GDG and ends with NN as defined in reference 14. The conserved glutamine residue, which is replaced by a leucine in (putative) PPDCs, is indicated with an arrow. Abbreviations: A, *Azoarcus* sp. strain EbN1; Ab, *Azospirillum brasilense* Sp245; Al, *Azospirillum lipoferum* FS; Av, *Anabaena variabilis* ATCC 29413; B, *Bradyrhizobium* sp. strain BTAi1; Bc, *Bacillus cereus* E33L; Bm, *Blastopirellula marina* DSM 3645; Bx, *Burkholderia xenovorans* LB400; Ca, *Clostridium acetobutylicum* ATCC 824; Dd, *Desulfovibrio desulfuricans* G20; Eca, *Erwinia carotovora* subsp. *atroseptica* SCRI1043; Ecl, *Enterobacter cloacae*; Gv, *Gloeobacter violaceus* PCC 7421; Hc, *Hahella chejuensis* KCTC 2396; Ll, *Lactococcus lactis* subsp. *lactis* IFPL730; Lp, *Legionella pneumophila* strain Lens; Mc, *Methylococcus capsulatus* strain Bath; Mt, *Mycobacterium tuberculosis* H37Rv; Np, *Nostoc punctiforme* PCC 73102; Pag, *Pantoea agglomerans*; Par, *Psychrobacter arcticus* 273-4; Pca, *Pelobacter carbinolicus* DSM 2380; Ppu, *Pseudomonas putida*; Ret, *Rhizobium etli* CFN 42; Reu, *Ralstonia eutropha* H16; Rf, *Rhodospirillum rubrum* ATCC 11170; Sa, *Staphylococcus aureus* RF122; Sm, *Sinorhizobium medicae* WSM419; St, *Salmonella enterica* serovar Typhimurium LT2; Sv, *Sarcina ventriculi*; Ym, *Yersinia mollaretii* ATCC 43969. Species names boxed in the figure contain IPDCs/PPDCs discussed in the text.

not be conducted with tryptophan as neither the wild type nor the mutant can grow with tryptophan as the sole ammonium source. Neither phenylalanine nor tryptophan can be used as a sole carbon/energy source by *A. brasilense*. This difference in growth could be partially linked to a difference in uptake of the amino acids. As *A. brasilense* cannot grow on minimal medium with phenylalanine as a carbon source, the generation of com-

pounds necessary for energy formation is insufficient probably due to either a low uptake of phenylalanine, slow conversion by different enzymes (e.g., PPDC_{Ab}), or the absence of enzymes necessary for the degradation of intermediates to coenzyme A derivatives. The small but significant effect of the *ipdC* mutation on growth with phenylalanine as nitrogen source could point to a partial role of the *ipdC* gene product in the Ehrlich pathway which involves transamination followed by decarboxylation. In *Saccharomyces cerevisiae* the Ehrlich pathway has been studied extensively for the production of fusel oils. Transcriptome analysis of yeast cells grown in glucose-limited chemostats with phenylalanine as the sole nitrogen source in comparison with cells grown in ammonium revealed that the transcript level of *ARO10/YDR380w* was increased 30-fold (42). Aro10p was first identified as a PPDC in *S. cerevisiae* (9, 42); later a broader substrate specificity was proposed (41). The yeast enzyme Aro10p itself has not yet been biochemically characterized.

In a second part of this study, PPDC_{Ab} was heterologously overexpressed in *E. coli* and subsequently purified via immobilized nickel chelate affinity chromatography. The recombinant protein appeared as a tetrameric form in size-exclusion chromatography, which is consistent with other ThDP-dependent 2-keto acid decarboxylases (2, 11, 13) and the recently determined crystal structure of PPDC_{Ab}, which shows the enzyme as a dimer of dimers (40). The kinetic analysis revealed quite surprising results. PPDC_{Ab} shows near Michaelis-Menten kinetics toward phenylpyruvate ($h = 1.26$) with the highest specificity constant of all tested substrates (except for 5-phenyl-2-oxopentanoic acid). For all other tested substrates with significant activity, the enzyme is subject to clear substrate activation ($h = 1.8$ to 1.9). To determine the kinetic parameters of these substrates, the kinetic data were analyzed with the Hill model. The structurally similar substrate IPyA has a 100-fold-lower k_{cat} than does phenylpyruvate. Due to a low $K_{0.5}$, the $k_{cat}/K_{0.5}$ value with IPyA is 10-fold lower. To broaden our understanding of the substrate specificity, phenyl-substituted 2-keto acids with a longer alkyl side chain were tested. Surprisingly, 4-phenyl-2-oxobutanoic acid has a 100-fold-lower $k_{cat}/K_{0.5}$ value, while 5-phenyl-2-oxopentanoic acid (with a side chain elongated by one methylene group) has a $k_{cat}/K_{0.5}$ value comparable to the k_{cat}/K_m value for phenylpyruvate. The reason for the difference between these two compounds could be the position of the keto acid group with respect to the phenyl moiety. Crystal structures of the enzyme cocrystallized with substrate (analogues) should shed further light on this. Benzoylformate (phenylglyoxylic acid), which has a shorter side chain than does phenylpyruvate, and indole-glyoxylic acid are not converted. The substrate activity profile of PPDC_{Ab} is clearly distinct from that of IPDC_{Ec}. IPDC_{Ec} shows activity towards IPyA, benzoylformate, and 4-substituted derivatives with no indication for substrate activation behavior by any of these compounds. Phenylpyruvate is not a substrate for IPDC_{Ec} (30). These differences in the substrate range of the *A. brasilense* protein and IPDC_{Ec} indicate an evolutionary difference and would allow the functional classification of the *A. brasilense* decarboxylase as a PPDC. In general the catalytic activity of PPDC_{Ab} is rather low compared to that of 2-keto acid decarboxylases but comparable to the catalytic activity of IPDC_{Ec}. As both enzymes are part of a pathway for the syn-

FIG. 4. Phylogenetic tree of (putative) PPDCs and IPDCs. ClustalW was used to align a set of (putative) PPDC and IPDC amino acid sequences. A phylogenetic tree was constructed based on the UPGMA method. The values shown at the branches are bootstrap values for 1,000 replicates. For species abbreviations, see the legend to Fig. 3. Boxed abbreviations indicate IPDCs/PPDCs discussed in the text.

thesis of the plant hormone IAA, to be considered as a secondary metabolite in microorganisms, their activity does not have to be as high as that of other enzymes, like pyruvate decarboxylases, which are involved in an important metabolic pathway (central intermediate metabolism).

Several ThDP-dependent 2-keto acid decarboxylases such as pyruvate decarboxylase from *Saccharomyces cerevisiae* (ScPDC) and from *Kluyveromyces lactis* are activated by their substrate (15, 17, 33). Most bacterial pyruvate decarboxylases, IPDC_{Ec}, and benzoylformate decarboxylase display hyperbolic

Michaelis-Menten kinetics without any indication of substrate activation (11, 27, 30). The combination of clear substrate activation for certain substrates and hyperbolic Michaelis-Menten kinetics (or only very weak substrate activation) for others in a single enzyme, as observed for the PPDC_{Ab}, is less common. The nature of this behavior is currently under investigation. The mechanism of substrate activation in ScPDC has in fact been the subject of discussion for quite a few years. In the most widely accepted mechanism, binding of the substrate to a cysteine residue (Cys221 in ScPDC) distant from the active site is proposed to be the first step in an information transfer cascade to the active site, leading ultimately to increased activity (3, 4). However, in a crystal structure cocrystallized with the substrate surrogate pyruvamide, the activator was found to bind to a site 10 Å away from the pivotal cysteine. Binding of the activator to ScPDC induces a conformational change in the enzyme: two (of the four) active sites transform from an open to a closed (=active) form, which might be the molecular explanation for the substrate activation behavior (21, 22). Since the pivotal cysteine in the proposed activation mechanism of ScPDC is not conserved in PPDC_{Ab} (40), it will be interesting to unravel the activation mechanism used by the latter.

Numerous sequences annotated as putative IPDCs and PPDCs are present in the NCBI database, but only for two has the activity of the purified enzymes been determined (PPDC_{Ab} [this study] and IPDC_{Ec}). To possibly distinguish between these two different kinds of enzyme specificities by in silico analysis, a multiple sequence alignment was performed and a phylogenetic tree was constructed. Strikingly, with the exception of one amino acid residue, all catalytically important residues are conserved in the aligned sequences, indicating the same catalytic mechanism suggested for other 2-keto acid decarboxylases. The multiple sequence alignment allows the division of the sequences into two groups based on the amino acid corresponding to position 468 in IPDC_{Ec} (477 in ScPDC numbering): those with Glu and those with Leu. Glu468 is a conserved catalytic residue in pyruvate decarboxylase and IPDC_{Ec}, which has been proposed to be involved in both pre- and postdecarboxylation steps. More specifically a role in protonation of the enamine-carbanion intermediate has been proposed (20, 26, 32). Enzymes with amino acid substitutions in this position showed an impaired release of the aldehyde product. In the benzoylformate decarboxylase of *Pseudomonas putida*, the glutamate residue is also replaced by Leu. Here, protonation of the enamine-carbanion intermediate is proposed to be mediated by a catalytic histidine residue in the active site (27). In PPDC_{Ab}, it was proposed that the leucine, which is located underneath the carboxylate group of the lactyl-ThDP intermediate, increases the hydrophobic character of the active site and consequently stabilizes the zwitterionic intermediates (40). In view of this clear partitioning, site-directed mutagenesis studies at this and other amino acid residue positions (see below) are ongoing, to provide information on their role in catalysis and substrate activation.

The phylogenetic tree based on the complete sequences (Fig. 4) also consists of two clades, indicating that the sequences have evolved from at least two different ancestors. For IPDC_{Ec} and PPDC_{Ab}, this evolutionary difference has been demonstrated via biochemical analysis: PPDC_{Ab} converts both

phenylpyruvate and IPyA, while IPDC_{Ec} shows no activity for phenylpyruvate. Based on the phylogenetic tree, it is tempting to speculate that all members indicated by the bracket "PPDC" can be classified as PPDCs as they cluster with PPDC_{Ab} and have a conserved Leu at position 468. However, it should be kept in mind that the biochemical activity of the purified enzyme has been determined for only one representative. The middle bracket of the phylogenetic tree, grouping four sequences, is part of the same clade, but these sequences have a conserved glutamate residue at position 468, in contrast to the other members of the clade. This implies that other amino acid residues are probably involved in determining the substrate specificity, unless a yet-unknown catalytic activity, different from that with IPyA or phenylpyruvate as a substrate, is represented by these four sequences.

Taking previous data and data presented in this study together, we propose the reclassification of the *ipdC* gene product of *A. brasilense* as a PPDC (EC 4.1.1.43).

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REFERENCES

1. Altschul, S. F., T. L. Madden, A. A. Schaffer, J. H. Zhang, Z. Zhang, W. Miller, and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**: 3389–3402.
2. Arjunan, P., T. Umland, F. Dyda, S. Swaminathan, W. Furey, M. Sax, B. Farrenkopf, Y. Gao, D. Zhang, and F. Jordan. 1996. Crystal structure of the thiamin diphosphate-dependent enzyme pyruvate decarboxylase from the yeast *Saccharomyces cerevisiae* at 2.3 angstrom resolution. *J. Mol. Biol.* **256**: 590–600.
3. Baburina, I., Y. Gao, Z. Hu, F. Jordan, S. Hohmann, and W. Furey. 1994. Substrate activation of brewers' yeast pyruvate decarboxylase is abolished by mutation of cysteine 221 to serine. *Biochemistry* **33**:5630–5635.
4. Baburina, I., H. J. Li, B. Bennion, W. Furey, and F. Jordan. 1998. Interdomain information transfer during substrate activation of yeast pyruvate decarboxylase: the interaction between cysteine 221 and histidine 92. *Biochemistry* **37**:1235–1244.
5. Baldani, V. L. D., M. A. D. Alvarez, J. I. Baldani, and J. Döbereiner. 1986. Establishment of inoculated *Azospirillum* spp. in the rhizosphere and in roots of field-grown wheat and sorghum. *Plant Soil* **90**:35–46.
6. Bradford, M. M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254.
7. Costacurta, A., V. Keijers, and J. Vanderleyden. 1994. Molecular cloning and sequence analysis of an *Azospirillum brasilense* indole-3-pyruvate decarboxylase gene. *Mol. Gen. Genet.* **243**:463–472.
8. Costacurta, A., and J. Vanderleyden. 1995. Synthesis of phytohormones by plant-associated bacteria. *Crit. Rev. Microbiol.* **21**:1–18.
9. Dickinson, J. R., L. E. Salgado, and M. J. Hewlins. 2003. The catabolism of amino acids to long chain and complex alcohols in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **278**:8028–8034.
10. Dobbelaere, S., A. Croonenborghs, A. Thys, A. Vande Broeck, and J. Vanderleyden. 1999. Phytostimulatory effect of *Azospirillum brasilense* wild type and mutant strains altered in IAA production on wheat. *Plant Soil* **212**:155–164.
11. Dobritzsch, D., S. König, G. Schneider, and G. Lu. 1998. High resolution crystal structure of pyruvate decarboxylase from *Zymomonas mobilis*. Implications for substrate activation in pyruvate decarboxylases. *J. Biol. Chem.* **273**:20196–20204.
12. Ferro, N., A. Gallegos, P. Bultinck, H. J. Jacobsen, R. Carbo-Dorca, and T. Reinard. 2006. Coulomb and overlap self-similarities: a comparative selectivity analysis of structure-function relationships for auxin-like molecules. *J. Chem. Inf. Model.* **46**:1751–1762.
13. Hasson, M. S., A. Muscate, M. J. McLeish, L. S. Polovnikova, J. A. Gerlt, G. L. Kenyon, G. A. Petsko, and D. Ringe. 1998. The crystal structure of benzoylformate decarboxylase at 1.6 angstrom resolution: diversity of catalytic residues in thiamin diphosphate-dependent enzymes. *Biochemistry* **37**: 9918–9930.
14. Hawkins, C. F., A. Borges, and R. N. Perham. 1989. A common structural motif in thiamin pyrophosphate-binding enzymes. *FEBS Lett.* **255**:77–82.

15. Hübner, G., R. Weidhase, and A. Schellenberger. 1978. The mechanism of substrate activation of pyruvate decarboxylase: a first approach. *Eur. J. Biochem.* **92**:175–181.
16. Koga, J., T. Adachi, and H. Hidaka. 1991. Molecular cloning of the gene for indolepyruvate decarboxylase from *Enterobacter cloacae*. *Mol. Gen. Genet.* **226**:10–16.
17. Krieger, F., M. Spinka, R. Golbik, G. Hübner, and S. König. 2002. Pyruvate decarboxylase from *Kluyveromyces lactis*. An enzyme with an extraordinary substrate activation behaviour. *Eur. J. Biochem.* **269**:3256–3263.
18. Kumar, S., K. Tamura, and M. Nei. 2004. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief. Bioinform.* **5**:150–163.
19. Lambrecht, M., Y. Okon, A. Vande Broek, and J. Vanderleyden. 2000. Indole-3-acetic acid: a reciprocal signalling molecule in bacteria-plant interactions. *Trends Microbiol.* **8**:298–300.
20. Lobell, M., and D. H. G. Crout. 1996. Pyruvate decarboxylase: a molecular modeling study of pyruvate decarboxylation and acyloin formation. *J. Am. Chem. Soc.* **118**:1867–1873.
21. Lu, G. G., D. Dobritzsch, S. Baumann, G. Schneider, and S. König. 2000. The structural basis of substrate activation in yeast pyruvate decarboxylase. A crystallographic and kinetic study. *Eur. J. Biochem.* **267**:861–868.
22. Lu, G. G., D. Dobritzsch, S. König, and G. Schneider. 1997. Novel tetramer assembly of pyruvate decarboxylase from brewer's yeast observed in a new crystal form. *FEBS Lett.* **403**:249–253.
23. Nicholas, K. B., H. B. Nicholas, and D. W. Deerfield II. 1997. GeneDoc: analysis and visualization of genetic variation. *EMBNET.news* **4**:1–4.
24. Okon, Y., and C. A. Labandera-Gonzalez. 1994. Agronomic applications of *Azospirillum*: an evaluation of 20 years worldwide field inoculation. *Soil Biol. Biochem.* **26**:1591–1601.
25. Patten, C. L., and B. R. Glick. 1996. Bacterial biosynthesis of indole-3-acetic acid. *Can. J. Microbiol.* **42**:207–220.
26. Pohl, M., P. Siegert, K. Mesch, H. Bruhn, and J. Grotzinger. 1998. Active site mutants of pyruvate decarboxylase from *Zymomonas mobilis*—a site-directed mutagenesis study of L112, I472, I476, E473 and N482. *Eur. J. Biochem.* **257**:538–546.
27. Polovnikova, E. S., M. J. McLeish, E. A. Sergienko, J. T. Burgner, N. L. Anderson, A. K. Bera, F. Jordan, G. L. Kenyon, and M. S. Hasson. 2003. Structural and kinetic analysis of catalysis by a thiamin diphosphate-dependent enzyme, benzoylformate decarboxylase. *Biochemistry* **42**:1820–1830.
28. Prinsen, E., A. Costacurta, K. Michiels, J. Vanderleyden, and H. Van Onckelen. 1993. *Azospirillum brasilense* indole-3-acetic acid biosynthesis: evidence for a non-tryptophan dependent pathway. *Mol. Plant-Microbe Interact.* **6**:609–615.
29. Remans, R., S. Spaepen, and J. Vanderleyden. 2006. Auxin signaling in plant defense. *Science* **313**:171.
30. Schütz, A., R. Golbik, K. Tittmann, D. I. Svergun, M. H. Koch, G. Hübner, and S. König. 2003. Studies on structure-function relationships of indolepyruvate decarboxylase from *Enterobacter cloacae*, a key enzyme of the indole acetic acid pathway. *Eur. J. Biochem.* **270**:2322–2331.
31. Schütz, A., T. Sandalova, S. Ricagno, G. Hübner, S. König, and G. Schneider. 2003. Crystal structure of thiamin diphosphate-dependent indolepyruvate decarboxylase from *Enterobacter cloacae*, an enzyme involved in the biosynthesis of the plant hormone indole-3-acetic acid. *Eur. J. Biochem.* **270**:2312–2321.
32. Sergienko, E. A., and F. Jordan. 2001. Catalytic acid-base groups in yeast pyruvate decarboxylase. 2. Insights into the specific roles of D28 and E477 from the rates and stereospecificity of formation of carboligase side products. *Biochemistry* **40**:7369–7381.
33. Sergienko, E. A., and F. Jordan. 2002. New model for activation of yeast pyruvate decarboxylase by substrate consistent with the alternating sites mechanism: demonstration of the existence of two active forms of the enzyme. *Biochemistry* **41**:3952–3967.
34. Somers, E., D. Ptacek, P. Gysegom, M. Srinivasan, and J. Vanderleyden. 2005. *Azospirillum brasilense* produces the auxin-like phenylacetic acid by using the key enzyme for indole-3-acetic acid biosynthesis. *Appl. Environ. Microbiol.* **71**:1803–1810.
35. Spaepen, S., J. Vanderleyden, and R. Remans. 2007. Indole-3-acetic acid in microbial and microorganism-plant signaling. *FEMS Microbiol. Rev.* **31**:425–448.
36. Thompson, J. D., D. G. Higgins, and T. J. Gibson. 1994. Clustal-W—improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**:4673–4680.
37. Vande Broek, A., P. Gysegom, O. Ona, N. Hendrickx, E. Prinsen, J. Van Impe, and J. Vanderleyden. 2005. Transcriptional analysis of the *Azospirillum brasilense* indole-3-pyruvate decarboxylase gene and identification of a cis-acting sequence involved in auxin responsive expression. *Mol. Plant-Microbe Interact.* **18**:311–323.
38. Vande Broek, A., M. Lambrecht, K. Eggermont, and J. Vanderleyden. 1999. Auxins upregulate expression of the indole-3-pyruvate decarboxylase gene in *Azospirillum brasilense*. *J. Bacteriol.* **181**:1338–1342.
39. Vanstockem, M., K. Michiels, J. Vanderleyden, and A. P. Van Gool. 1987. Transposon mutagenesis of *Azospirillum brasilense* and *Azospirillum lipof-erum*: physical analysis of Tn5 and Tn5-Mob insertion mutants. *Appl. Environ. Microbiol.* **53**:410–415.
40. Versées, W., S. Spaepen, J. Vanderleyden, and J. Steyaert. 2007. The crystal structure of phenylpyruvate decarboxylase from *Azospirillum brasilense* at 1.5 angstrom resolution: implications for its catalytic and regulatory mechanism. *FEBS J.* **274**:2363–2375.
41. Vuralhan, Z., M. A. Luttik, S. L. Tai, V. M. Boer, M. A. Morais, D. Schipper, M. J. Almering, P. Kottler, J. R. Dickinson, J. M. Daran, and J. T. Pronk. 2005. Physiological characterization of the *ARO10*-dependent, broad-substrate-specificity 2-oxo acid decarboxylase activity of *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* **71**:3276–3284.
42. Vuralhan, Z., M. A. Morais, S. L. Tai, M. D. Piper, and J. T. Pronk. 2003. Identification and characterization of phenylpyruvate decarboxylase genes in *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* **69**:4534–4541.
43. Weiss, P. M., G. A. Garcia, G. L. Kenyon, W. W. Cleland, and P. F. Cook. 1988. Kinetics and mechanism of benzoylformate decarboxylase using ¹³C and solvent deuterium isotope effects on benzoylformate and benzoylformate analogues. *Biochemistry* **27**:2197–2205.