Cloning and Characterization of Indolepyruvate Decarboxylase from *Methylobacterium extorquens* AM1

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Abstract—For the first time for methylotrophic bacteria an enzyme of phytohormone indole-3-acetic acid (IAA) biosynthesis, indole-3-pyruvate decarboxylase (EC 4.1.1.74), has been found. An open reading frame (ORF) was identified in the genome of facultative methylotroph *Methylobacterium extorquens* AM1 using BLAST. This ORF encodes thiamine diphosphate-dependent 2-keto acid decarboxylase and has similarity with indole-3-pyruvate decarboxylases, which are key enzymes of IAA biosynthesis. The ORF of the gene, named *ipdC*, was cloned into overexpression vector pET-22b(+). Recombinant enzyme IpdC was purified from *Escherichia coli* BL21(DE3) and characterized. The enzyme showed the highest k_{cat} value for benzoylformate, albeit the indolepyruvate was decarboxylated with the highest catalytic efficiency (k_{cat}/K_m). The molecular mass of the holoenzyme determined using gel-permeation chromatography corresponds to a 245-kDa homotetramer. An *ipdC*-knockout mutant of *M. extorquens* grown in the presence of tryptophan had decreased IAA level (46% of wild type strain). Complementation of the mutation resulted in 6.3-fold increase of IAA concentration in the culture medium compared to that of the mutant strain. Thus involvement of IpdC in IAA biosynthesis in *M. extorquens* was shown.

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Aerobic methylotrophic bacteria (methylotrophs), utilizing methane and its oxidized or substituted derivatives as the carbon and energy sources, are tightly associated with plants and considered as phytosymbionts [1]. On one hand, the basis for methylotroph phytosymbiosis is formation by plants various C_1-metabolites (methane, methanol, methylamines, formaldehyde, formate, methylhalides, sulfur-containing C_1-compounds) [2, 3]. On the other hand, methylotrophs can participate in plant development via biosynthesis of phytohormones [4, 5], nitrogen fixation [6], or by suppression of ethylene biosynthesis in plants [7]. Methylotroph inoculation stimulates the plant growth and development [8, 9], which can be a result of bacterial formation of the main plant phytohormones − auxins. Previously, we have

shown that methylotrophs are capable of synthesis of indole-3-acetic acid (IAA) [4], but its biosynthetic pathways in this group of bacteria remain uncertain so far.

Pink-pigmented bacteria from genus *Methylobacterium* are common inhabitants of the great majority of plants [1]. Recently, the complete genome of *Methylobacterium extorquens* AM1, the model organism of this genus, was sequenced [10], which offers new challenges for study of IAA biosynthetic pathways. IAA is the product of tryptophan oxidation in the microorganisms, which is facilitated by several pathways. The pathway via indole-3-pyruvate (tryptophan → IPyr → IAAld → IAA) is considered the major pathway in bacteria and plants (Fig. 1) [11]. The tryptamine (TAM) pathway (tryptophan → TAM → IAAld → IAA) occurs in plants but its existence in bacteria is not demonstrated yet (Fig. 1) [12]. The indole-3-acetamide (IAM) pathway (tryptophan → IAM → IAA) was found in plant-associated bacteria (*Agrobacterium*, *Burkholderia*, *Pseudomonas*, *Rhizobium*, *Pantoea agglomerans*) and plants as well (Fig. 1) [11, 12].

Abbreviations: IAA, indole-3-acetic acid; IAAld, indole-3-acetaldehyde; IAM, indole-3-acetamide; ILA, indole-3-lactic acid; IPyr, indole-3-pyruvic acid; ThDP, thiamine diphosphate.

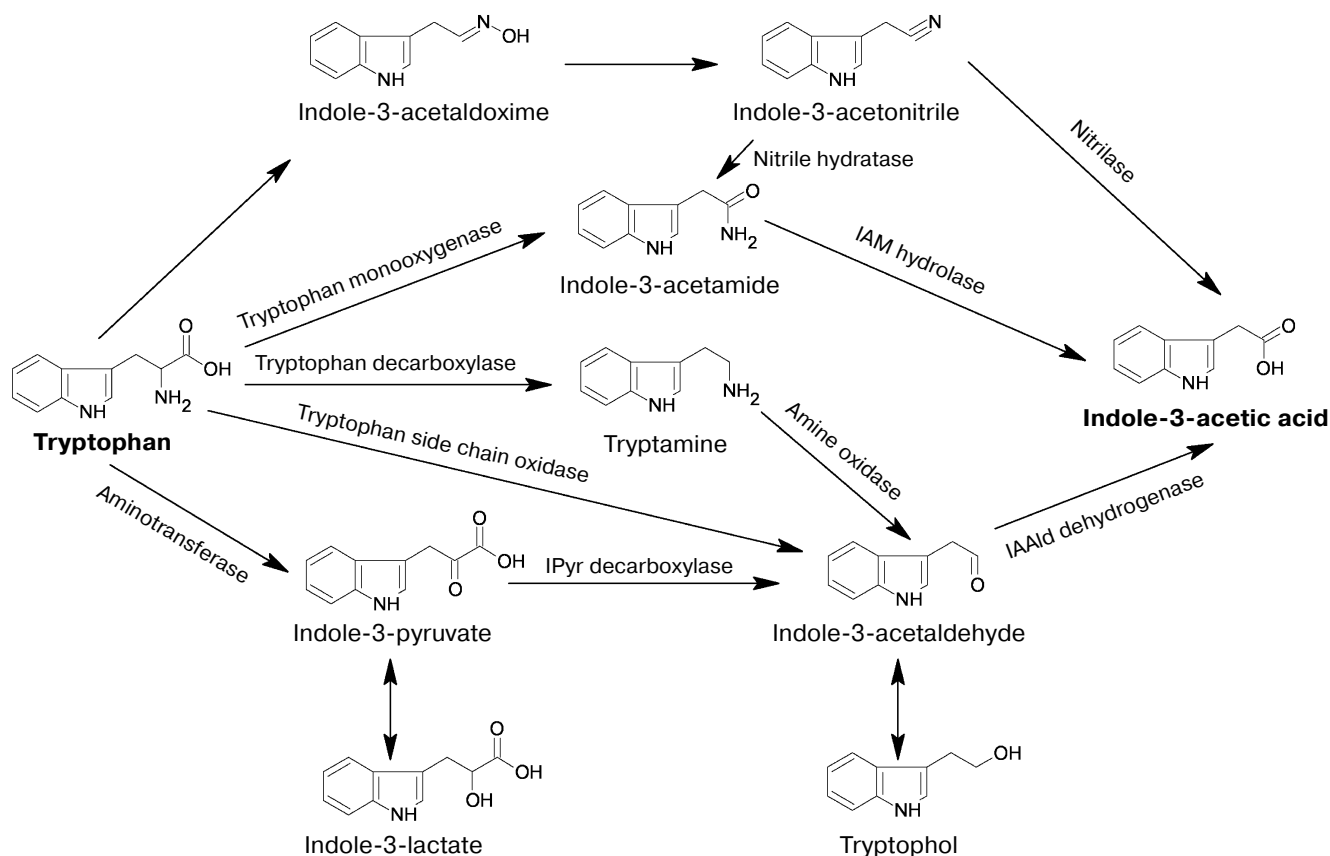* To whom correspondence should be addressed.

Fig. 1. Pathways of indole-3-acetic acid biosynthesis from tryptophan [12]. Designations: IPyr decarboxylase, indole-3-pyruvate decarboxylase; IAM hydrolase, indole-3-acetamide hydrolase; IAAld dehydrogenase, indole-3-acetaldehyde dehydrogenase.

The key enzyme of the indole-3-pyruvic acid (IPyr) pathway is thiamine diphosphate (ThDP)-binding indolepyruvate decarboxylase (EC 4.1.1.74) catalyzing formation of indole-3-acetaldehyde from indolepyruvate [13]. There are some bacterial genes encoding indolepyruvate decarboxylases and homologous enzymes in GenBank, but only indolepyruvate decarboxylase from *Enterobacter cloacae*, phenylpyruvate decarboxylase (EC 4.1.1.43) from *Azospirillum brasilense*, 2-keto acid decarboxylases (EC 4.1.1.72) from *Mycobacterium tuberculosis* and *Lactococcus lactis*, benzoylformate decarboxylases (EC 4.1.1.7) from *Pseudomonas putida* and *Pseudomonas stutzeri*, and pyruvate decarboxylase (EC 4.1.1.1) from *Zymomonas mobilis* have been well characterized [14-19]. These enzymes can decarboxylate a number of 2-keto acids, including indolepyruvate, benzoylformate, pyruvate, and phenylpyruvate with different catalytic efficiencies. However, the involvement of these enzymes in IAA biosynthesis was only shown for *A. brasilense*, *E. cloacae*, and *P. agglomerans* [14, 15].

The aim of the present study was to clone and characterize the indolepyruvate decarboxylase from *M. extorquens* AM1, one of the key enzymes of IAA biosynthesis.

MATERIALS AND METHODS

Reagents. Analytical grade reagents from domestic suppliers were used for the preparation of the growth mineral media. Biochemical reagents (Fluka, Switzerland), horse liver alcohol dehydrogenase, and yeast alcohol dehydrogenase (Serva, Germany), Sephacryl S-200 column for size-exclusion chromatography (Pharmacia, Sweden), agarose for metal-chelate affinity chromatography (Qiagen, USA), restriction endonucleases, PCR reagents, and bacteriophage T4 DNA-polymerase (Fermentas, Lithuania), thermostable DNA-polymerases and bacteriophage T4 DNA-ligase (SibEnzyme, Russia), reagents for polyacrylamide gel electrophoresis (Sigma, USA), and reagents for HPLC and TLC (Fluka and Sigma) were used in the study. Oligonucleotide synthesis was performed in the Syntol company (Russia).

Bacterial strains, vectors, and growth conditions. The bacterial strains and plasmids used are presented in Table 1. *Methylobacterium extorquens* wild-type strain AM1 and Δ*ipdC* mutant were maintained in a minimal K medium [4] at 28°C. The medium K supplemented with 1 mM L-tryptophan as the precursor of indole synthesis was used in some experiments. The *E. coli* strains, listed in Table 1,

Table 1. Bacterial strains and plasmids

Strain or plasmid	Characteristics	Reference or source
Methylobacterium extorquens AM1	wild-type strain	VKM B-2067 = NCIMB 9133 = ATCC 14718
Δ*ipdC*	derivative AM1, Δ*ipdC*::*aacC1*, Gm^r	this work
Escherichia coli XL1-Blue	*recA1 endA1 gyrA96 thi*-1 *hsdR17 supE44 relA1 lac* [F' *proAB lacI^{q}Z*ΔM15 Tn10(Tet^r)]	Stratagene
BL21(DE3)	F$^-$ *dcm ompT hsdS*(r$_B$- m$_B$-) *gal* λ(DE3)	Novagen
S17-1	F$^-$ *thi pro recA hsdR* [RP4-2Tc::Mu-Km::Tn7] Tp^r Sm^r	[20]
Plasmids pUC18	general cloning vector, Ap^r	[21]
pET-22b(+)	vector for expression of recombinant proteins under control of strong promoter of bacteriophage T7, Ap^r	Novagen
pK18mob	mobilizable multi-purpose cloning vector, Km^r	[22]
p34S-Gm	source of Gm^r-cassette, Ap^r, Gm^r	[23]
pT-GroE	vector expressing chaperone GroESL from *E. coli* under control of promoter of bacteriophage T7, Cm^r	[24]
pCM160	vector for expression of proteins in *M. extorquens* AM1 under control of methanol dehydrogenase large subunit gene (*mxaF*) promoter, Km^r	[25]
p7A-2	pUC18 containing EcoRI/HindIII fragment with *ipdC* gene from *M. extorquens* AM1	this work
p7A-3	pET-22b(+) containing *ipdC* ORF from *M. extorquens* AM1	−"−
p7A-4	p7A-2 derivative containing Gm^r-cassette from p34S-Gm cloned into blunted NcoI sites (Δ*ipdC*::*aacC1*)	−"−
p7A-5	pK18mob containing 1.9 kb EcoRI/HindIII fragment from p7A-4	−"−
p7A-9	pCM160 containing *ipdC* ORF	−"−

were cultured at 37°C in a Luria−Bertani (LB) medium [26] with appropriate antibiotics added as follows (μg/ml): ampicillin, 100; chloramphenicol, 50; gentamicin, 2.5; kanamycin, 50. Antibiotics for *M. extorquens* were added at following concentrations (μg/ml): gentamicin, 20; kanamycin, 50.

General techniques. Isolation of chromosomal and plasmid DNA, cloning procedures, and transformations were carried out according to the standard procedures [26]. Polymerase chain reaction (PCR) mixture in 30 μl 1× PCR buffer contained 150 μM (each) deoxyribonucleotide triphosphates, 200 nM of appropriate primers, 100 ng of genomic DNA, 3% (v/v) dimethylsulfoxide (Sigma), and 2 U Pfu-DNA-polymerase. The PCR conditions for all combinations of primers were as follows: 3 min at 96°C, followed by 30 cycles of 20 sec at 94°C, 20 sec at 60°C, and 4 min at 72°C, and a final extension of 5 min at 72°C. All amplicons were gel purified with a Wizard SV Gel and PCR Clean-up System (Promega, USA).

Phylogenetic analysis. For identification of a putative *ipdC* in the *M. extorquens* AM1 chromosome (GenBank accession No. CP001510), protein BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was applied using the amino acid sequences of *E. cloacae* indolepyruvate decarboxylase and phenylpyruvate decarboxylase from *A. brasilense*. Multiple sequence alignment of 41 amino acid sequences

of different decarboxylases was carried out with ClustalW [27]. A phylogenetic tree based on the multiple sequence alignment was constructed using a UPGMA (unweighted-pair group method with average linkages) model and the MEGA software [28]. Bootstrap values were used for reliability testing (1000 replicates).

Construction of plasmid for expression of recombinant IpdC protein. The ORF of the *M. extorquens ipdC* gene (1.7 kb) was amplified from strain AM1 genomic DNA by PCR using the forward primer 5′-TTCTA<u>CATATG</u>ACG-GTCACGACACTCGA-3′ and the reverse primer 5′-TAT<u>AAGCTT</u>CGCGGCGCTGACGACCACT-3′ containing *Nde*I and *Hind*III sites, respectively (underlined). To generate the *ipdC* recombinant gene, the *Nde*I-*Hind*III-digested PCR product was cloned into the *Nde*I-*Hind*III-cleaved vector pET-22b(+) yielding p7A-3.

Generation of *M. extorquens* strain with *ipdC*-deletion mutation. For deletion of the *ipdC* gene, the 2.36 kb PCR-fragment (containing the full length *ipdC* flanked by approximately 0.3 kb from both termini) was amplified using the forward primer 5′-TAT<u>GAATTC</u>ACG-GCTTCGGCTGGATGAC-3′ and the reverse primer 5′-TTA<u>AAGCTT</u>CGGAACGATTGCGAACACA-3′, containing *EcoR*I and *Hind*III sites, respectively (underlined). The *EcoR*I-*Hind*III-digested PCR-fragment was first cloned into the *EcoR*I-*Hind*III-cleaved vector pUC18 yielding p7A-2. The *Sma*I-fragment (855 bp) with gentamicin-resistance cassette (*aacC1*) from p34S-Gm was introduced at the completely filled-in with bacteriophage T4 DNA-polymerase *Nco*I sites of p7A-2. The resulting vector p7A-4 contained an artificial allele of *ipdC*, the major part of which (1.3 kb) was replaced with the gentamicin resistance marker (*aacC1*). The 1.87 kb *EcoR*I-*Hind*III fragment from p7A-4 was inserted at *EcoR*I-*Hind*III sites of the suicidal mobilizable vector pK18mob yielding p7A-5.

Plasmid p7A-5 was mobilized into *M. extorquens* AM1 in two-way cross with *E. coli* S17-1 (p7A-5) as a donor of the plasmid. Early exponential growth phase *M. extorquens* AM1 cells were concentrated and mixed with the overnight culture of *E. coli* S17-1 (p7A-5) and washed in a K medium (donor to recipient ratio of 1 : 5). The cell mixture was filtered onto a 0.45-μm membrane filter (Millipore, France) and incubated at 28°C on K agar medium supplemented with 0.02% (w/v) of casamino acids and 0.5% (v/v) of methanol for 24 h. Then the cells were washed out from the filter, and serial dilutions of cell suspension were plated onto selective medium K with 0.5% methanol, 20 μg/ml gentamicin, and 80 μg/ml nalidixic acid. All putative transconjugants were purified by a single-colony transfer to selective media with appropriate antibiotics. Only double recombinants (gentamicin resistant and kanamycin sensitive) were used. All recombinants were additionally tested by PCR for the presence of mutant allele.

Complementation of Δ*ipdC*::*aacC1* mutant. *Methylobacterium extorquens ipdC* gene ORF with Shine—Dalgarno sequence was amplified using the forward primer 5′-ATC<u>TCTAGA</u>**CTAA**GGTGCGGAAGGAG-CAGG-3′ and the reverse primer 5′-GCT<u>GAATTC</u>GT-**CA**CGCGGCGCTGACGAC-3′, containing *Xba*I and *EcoR*I sites, respectively (underlined), and stop-codons (bold). The 1.7 kb *EcoR*I-*Xba*I-digested fragment was cloned into the *EcoR*I-*Xba*I-cleaved vector pCM160 yielding p7A-9. *Escherichia coli* S17-1 (p7A-9) was used for trans-conjugation of p7A-9 into Δ*ipdC*::*aacC1* mutant in a similar manner as described in the mutant generation section. Expression of IpdC was monitored by SDS-PAGE [29].

Enzyme assay. The indolepyruvate decarboxylase activity was followed as NADH oxidation in the coupled optical test with horse liver alcohol dehydrogenase [14]. In the reaction with pyruvate, yeast alcohol dehydrogenase was used. Each reaction mixture (1 ml, pH 6.5) contained 10 mM Mes-NaOH, 0.2 mM NADH, and 1-5 U of the appropriate alcohol dehydrogenase. To avoid interference with substrates and products, the oxidation of NADH in the reactions with indolepyruvate, benzoylformate, and 4-hydroxyphenylpyruvate was measured at 366 nm using a Shimadzu PharmaSpec UV-1700 spectrophotometer. The oxidation of NADH in the reaction with pyruvate was monitored at 340 nm. IpdC was preincubated with 15 mM ThDP/Mg^{2+} in 10 mM Mes-NaOH at 25°C for 30 min to saturate the enzyme with cofactors. The enzyme concentration was 0.1 mg/ml for indole-3-pyruvate and pyruvate decarboxylation, 0.035 mg/ml for benzoylformate, and 0.08 mg/ml for 4-hydoxyphenyl-pyruvate.

Kinetic characteristics of the enzyme were obtained using the Michaelis—Menten equation ((1) in the case of pyruvate) and equation for uncompetitive substrate inhibition ((2) in case of benzoylformate, indolepyruvate, 4-hydroxyphenylpyruvate) using the Enzyme Kinetics 1.3 module of SigmaPlot 10.0 (Systat Software):

$$V = \frac{V_{\max} [S]}{K_{\mathrm{m}} + [S]}, \qquad (1)$$

$$V = \frac{V_{\max}}{1 + \dfrac{K_{\mathrm{m}}}{[S]} + \dfrac{[S]}{K_{\mathrm{i}}}}. \qquad (2)$$

Protein expression and purification. For expression of tagged IpdC protein, the cells of recombinant *E. coli* BL21(DE3, p7A-3, pT-GroE) were grown in 2 liters of LB medium with ampicillin (100 μg/ml) and chloramphenicol (50 μg/ml) at 28°C to $A_{600} = 0.6$-0.7. Protein synthesis was induced by addition of 0.2 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG); the cells were incubated overnight in a shaker at 28°C. IpdC-His$_6$-tag was isolated from *E. coli* superproducer as described by the Qiagen protocols with minor changes [30]. The cells were

precipitated by centrifugation at 7000g for 15 min at 4°C, resuspended in 30 ml of 10 mM Mes-NaOH, pH 7.4, containing 0.5 M NaCl, 10 mM imidazole, and lysozyme (10 mg/ml) and disrupted using an MSE ultrasonic disintegrator (England) (150 W, 10 Hz, 6 times for 15 sec with 1 min intervals). The cell lysate was centrifuged (10,000g, 20 min at 4°C), and the supernatant was tested for IpdC activity and then applied onto a column with 1 ml of Ni^{2+}-nitrilotriacetate (NTA) agarose. After intensive washing with 10 mM Mes-NaOH (pH 7.4) containing 0.5 M NaCl and 60 mM imidazole, the bound IpdC-His$_6$-tag was eluted with a buffer containing 0.5 M NaCl and 200 mM imidazole. The protein spectrum of the collected fractions (0.5 ml volume) was analyzed by 10% SDS-PAGE [29]. The fractions containing IpdC-His$_6$-tag were combined. Protein concentration was determined by direct optical measurement using the molar extinction coefficient ε_{280} = 50,900 M^{-1}·cm^{-1}, calculated by Peptide Property Calculator (http://www.basic.northwestern.edu/biotools/proteincalc.html), or by the Lowry method using BSA as a standard [31].

Molecular mass determination. The molecular mass of the holoenzyme was estimated by size exclusion chromatography on a Sephacryl S-200 column (1.5 × 60 cm), equilibrated with 10 mM Mes-NaOH, pH 6.5, 2.5 mM MgSO$_4$, 0.5 mM ThDP, 150 mM KCl (flow rate 0.2 ml/min). Ferritin (450 kDa), catalase (240 kDa), γ-globulin (158 kDa), BSA (68 kDa), and ovalbumin (45 kDa) were used as the molecular mass standards.

Analyses of indole compounds. *Methylobacterium extorquens* strains were grown in 1 liter of K medium, supplemented with 1 mM L-tryptophan, and the spent media were collected by centrifugation at 6000g for 30 min and freeze-dried. Auxins were extracted with 0.5 volume of ethyl acetate from the culture liquid at pH 7.0 and acidified to pH 2.8 with HCl. The extracts were dried, and the residues were dissolved in methanol. Purified auxins were separated on a reversed-phase Shim-pack XR-ODS (75 × 3 mm, pore size 2.2 μm) column (Shimadzu, Japan). Gradient elution from 10 to 50% methanol in 20 mM tetramethylammonium hydrochloride buffer, acidified with trifluoroacetic acid to pH 2.8, at a flow rate of 0.5 ml/min was performed using a Shimadzu LC-20AD gradient high-performance liquid chromatograph pump with a Shimadzu DGU-20A3 degasser. Auxins were detected at 280 nm using a Shimadzu SPD-20A UV detector. The column was calibrated using indole-3-acetic acid, indole-3-lactic acid, indole-3-acetaldehyde, indole-3-acetamide, tryptamine, and tryptophol. TLC was performed on TLC Silica gel 60 F$_{254}$ plates (Merck, Germany) in benzene–acetone (3 : 1) solvent system. After separation the indoles were revealed with Ehrlich reagent (2% 4-(dimethylamino)benzaldehyde in mixture of HCl–ethanol (1 : 4)). The amount of indole compounds in the culture fluid of *M. extorquens* was determined in the late stationary phase with the Salkowski reagent [4].

RESULTS AND DISCUSSION

Identification of candidate gene encoding indole-3-pyruvate decarboxylase in the genome of *M. extorquens* AM1. When grown on a minimal medium with tryptophan, methylotrophs from the genus *Methylobacterium* can synthesize IAA [32]. Analysis of the spent medium of *M. extorquens* AM1 revealed indole-3-lactic acid, the product of the indolepyruvate reduction, thus suggesting that IAA is synthesized by the IPyr pathway. The key enzyme of the IPyr pathway is indole-3-pyruvate decarboxylase, and there are several enzymes capable of decarboxylating various 2-keto acids with different catalytic efficiencies (k_{cat}/K_m).

The search in the genome of *M. extorquens* AM1 for the gene encoding indolepyruvate decarboxylase identified gene (GenBank locus tag: MexAM1_META1p2494; GeneID: 7992400), encoding protein (Accession numbers: ACS40273, YP_002963550) similar to the bacterial ThDP-dependent 2-keto acid decarboxylases. The multiple sequence alignments showed that this gene has rather low amino acid identity with all of the known enzymes. For example, the highest identity was with putative benzoylformate decarboxylases from *Rhodopseudomonas palustris* (40%), *Burkholderia mallei* (35%), *Bradyrhizobium japonicum* (34%), and only 32% with characterized enzyme from *P. putida*. The identity with other well-characterized ThDP-dependent 2-keto acid decarboxylases was 15.5% with pyruvate decarboxylase of *Z. mobilis*, 15.7% with phenylpyruvate decarboxylase of *A. brasilense*, and 17% with indolepyruvate decarboxylases of *E. cloacae* as well as with 2-keto acid decarboxylases of *L. lactis* and *M. tuberculosis*. Based on the multiple sequence alignment, a phylogenetic tree of 2-keto acid decarboxylases was constructed (Fig. 2). The sequences were clustered into several groups: the first one contained indolepyruvate decarboxylases and 2-keto acid decarboxylases, the second clade included phenylpyruvate decarboxylases, the third contained benzoylformate decarboxylases, while several uncharacterized decarboxylases and well-studied pyruvate decarboxylase from *Z. mobilis* were out of these clades. Amino acid sequences of the methylobacterial decarboxylases, including *M. extorquens* AM1, formed a separate group inside the benzoylformate decarboxylases clade. Thus, based on the amino acid sequence and phylogenetic analysis, *M. extorquens* AM1 decarboxylase is closely related to benzoylformate decarboxylases. It is well known that all characterized enzymes from the 2-keto acid decarboxylases family presented in the phylogenetic tree (Fig. 2) are capable of decarboxylating indole-3-pyruvate [14-17].

Heterologous expression, purification, and size-exclusion chromatography of indolepyruvate decarboxylase. To prove that the gene called *ipdC* (indole-3-pyruvate decarboxylase) encodes indolepyruvate decarboxylase, the recombinant enzyme IpdC was obtained by cloning the gene ORF into a C-terminal His$_6$-tag-attach-

ing over-expression vector. To reduce the formation of "inclusion bodies", conditions favorable for the proper protein folding were selected. Heterologous overexpression in *E. coli* and subsequent Ni²⁺ chelate affinity chromatography resulted in isolation of the recombinant

indolepyruvate decarboxylase of *M. extorquens* AM1. The maximum specific activity of the enzyme was 1.6 μmol/min per mg protein with benzoylformate as a substrate. Beside benzoylformate, the enzyme decarboxylated indole-3-pyruvate, 4-hydroxyphenylpyruvate, and pyru-
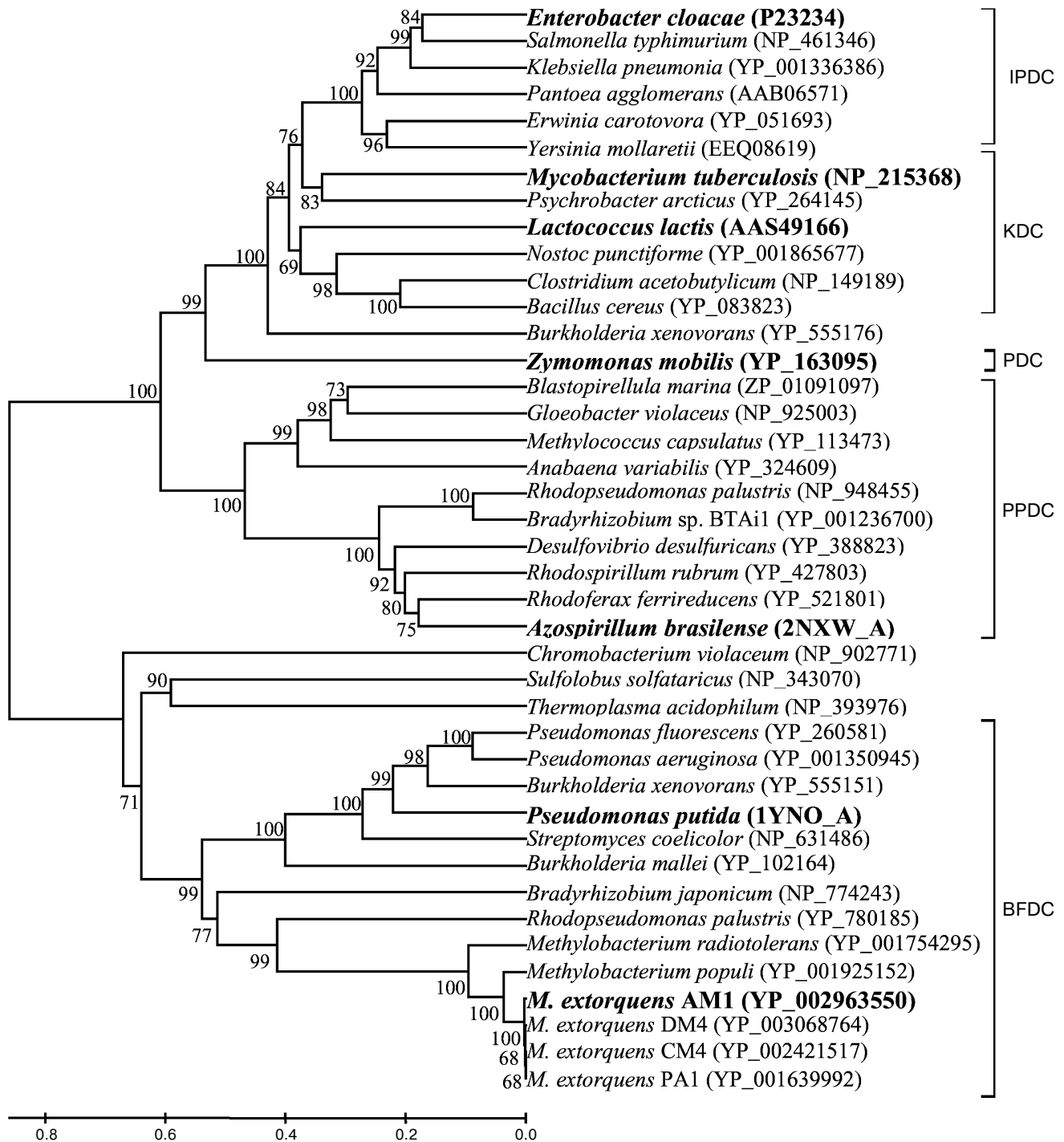

Fig. 2. Phylogenetic tree of various 2-keto acid decarboxylases. The values shown at the branches are bootstrap values for 1000 replicates. Accession numbers for appropriate sequences are shown in brackets. Species highlighted in bold indicate well-studied decarboxylases. BFDC, benzoylformate decarboxylases; IPDC, indolepyruvate decarboxylases; KDC, 2-keto acid decarboxylases; PDC, pyruvate decarboxylases; PPDC, phenylpyruvate decarboxylases.

Table 2. Kinetic parameters of indole-3-pyruvate decarboxylase from *M. extorquens* AM1

Substrate	K_m, mM	K_i, mM	k_{cat}*, sec^{-1}	k_{cat}/K_m, mM^{-1}·sec^{-1}
Indole-3-pyruvate	0.025 ± 0.008	0.73 ± 0.28	0.64 ± 0.09	25.6
Benzoylformate	7.3 ± 5.3	3.4 ± 2.5	31.4 ± 18.2	4.3
4-Hydroxyphenylpyruvate	5.86 ± 1.42	22.11 ± 8.14	1.65 ± 0.27	0.28
Pyruvate	43.3 ± 5.6	n.d.	0.89 ± 0.06	0.021

Note: Kinetic parameters of pyruvate decarboxylation were obtained using the Michaelis–Menten equation (1), and indole-3-pyruvate, benzoyl-formate, and 4-hydroxyphenylpyruvate decarboxylation using Eq. (2); n.d., not detected.

* k_{cat} corresponds to tetrameric enzyme.

vate. The enzyme was quite stable in 20% (v/v) glycerol with addition of ThDP/Mg^{2+} for six months at −20°C. A molecular mass of about 60 kDa per subunit was in good agreement with the value calculated from the nucleotide sequence of the structural gene.

A molecular mass of 245 kDa corresponding to a tetramer was determined for the native enzyme by size-exclusion chromatography. Notably, all characterized ThDP-dependent 2-keto acid decarboxylases except the enzyme from *L. lactis* are homotetramers [14-16].

Catalytic properties of indolepyruvate decarboxylase. Purified IpdC catalyzed the decarboxylation of indolepyruvate, benzoylformate, 4-hydroxyphenylpyruvate, and pyruvate. In Table 2 the catalytic constants of the enzyme for decarboxylation of these substrates are given. As shown i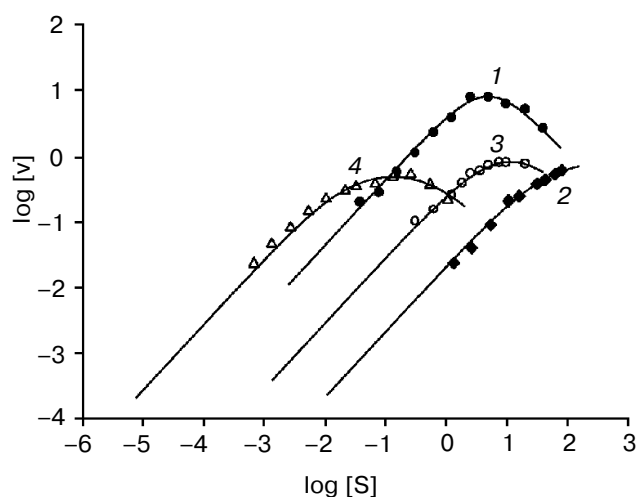n Fig. 3, with the substrates indole-3-pyruvate, benzoylformate, and 4-hydroxyphenylpyruvate excess substrate inhibited the enzyme. The strongest substrate inhibition of the enzyme was with indolepyruvate ($K_i = 0.73 \pm 0.28$ mM) while the inhibition with benzoylformate and 4-hydroxyphenylpyruvate was considerably weaker (Table 2). Weak inhibition of benzoylformate decarboxylase of *Pseudomonas putida* with benzoylformate was found, but in contrast to *M. extorquens* AM1 enzyme, the K_i value was 42 times higher [33]. It should be noted that indolepyruvate decarboxylase from *E. cloacae* is also inhibited by substrate excess in the reaction with pyruvate [14]. Moreover, due to symmetrical right and left branches of the curves displaying the dependence of enzyme activity on indole-3-pyruvate and benzoylformate concentrations in logarithmic coordinates, there is a complete inhibition of the enzyme by these substrates (Fig. 3). Because of the high affinity (K_m 25 ± 8 μM) to indolepyruvate, the *M. extorquens* decarboxylase had the highest catalytic efficiency (k_{cat}/K_m) to this substrate (Table 2). In consequence of close phylogenetic relation of IpdC amino acid sequence with benzoylformate decarboxylases, benzoylformate seemed to be a natural substrate for the enzyme. However, despite the highest reaction rate with benzoylformate among all tested substrates, the K_m value was 2-fold higher than in the reaction with indolepyruvate (7.3 ± 5.3 mM), thus the catalytic efficiency of benzoylformate decarboxylation was considerably lower (Table 2). Generally, kinetic constants of the enzyme determined for indolepyruvate and benzoylformate were quite similar to that of the *E. cloacae* indolepyruvate decarboxylase [14] despite the distinct phylogenetic position of the proteins. Conversely, the kinetic characteristics of benzoylformate decarboxylases from *Pseudomonas* spp. considerably differed from IpdC [19, 33]. For example, the K_m value of the *M. extorquens* AM1 decarboxylase was 10-17 times higher, whereas k_{cat} and k_{cat}/K_m were much lower (7-42 and 75-560 times, respectively) than the enzymes from *Pseudomonas* [19, 33].

Indoles and IAA synthesis in different strains of *M. extorquens* (AM1, strain with deletion in *ipdC* gene, and complemented mutant strain). For estimation of the

Fig. 3. Dependences of *M. extorquens* AM1 indole-3-pyruvate decarboxylase reaction velocity on concentrations of benzoylfor-mate (*1*), pyruvate (*2*), 4-hydroxyphenylpyruvate (*3*), and indole-3-pyruvate (*4*) in {log[*v*] − log[S]} coordinates. The Michaelis–Menten equation (1) was used for curve fitting in the case of pyruvate, and Eq. (2) was used in the cases of benzoylfor-mate, indole-3-pyruvate, and 4-hydroxyphenylpyruvate.

involvement of indolepyruvate decarboxylase in IAA biosynthesis by *M. extorquens* AM1, the mutant (with deletion in the *ipdC* gene) and complemented strains (mutant Δ*ipdC*, carrying constitutively expressing *ipdC* gene on the plasmid p7A-9) were generated. IpdC activity in the crude protein extracts of certain strains was 2 ± 0.5 nmol/min per mg protein in strain AM1, 13 ± 1 in the complemented strain, and in the mutant strain the activity was undetectable.

It should be noted that deletion did not affect the bacterial growth, thus suggesting that *ipdC* is not an essential gene for the bacterium. Nevertheless, *ipdC* inactivation resulted in the 2.5-fold decrease of total indole compounds concentration in the culture liquid of the mutant strain grown in mineral medium with tryptophan as a precursor of indoles compared to the wild type strain *M. extorquens* AM1 (Table 3). Complementation of the mutation restored the indoles biosynthesis level to the wild type as well as resulted in 2.4 times increase due to constitutive *ipdC* expression (Table 3).

HPLC and TLC analyses of culture fluids of the studied strains revealed that IAA concentration well correlated with the indoles concentration (Fig. 4 and Table
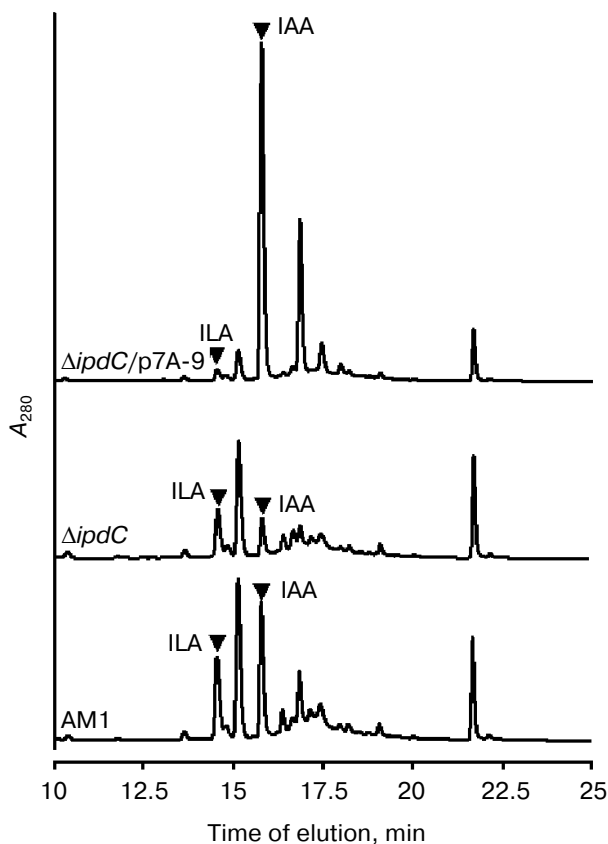
Table 3. Concentrations of indole-3-acetic acid and total indoles in culture media of various *M. extorquens* strains grown in the minimal medium in the presence of 1 mM L-tryptophan

Strain	IAA concentration, μg/ml	Total indoles concentration, μg/ml
AM1	6.13 ± 0.33	10.1 ± 0.7
Δ*ipdC*	2.84 ± 0.02	4.0 ± 0.4
Δ*ipdC*/p7A-9	18.06 ± 0.99	24.1 ± 1.2

3). As seen in Table 3, deletion of *ipdC* did not completely abolish IAA biosynthesis, since 46% of the strain AM1 level was found in the culture fluid of the mutant. On the other hand, *ipdC* expression in the complemented strain resulted in the 6.3 times increase of the IAA concentration in the spent medium compared to that of the mutant strain. It was 3 times higher than in strain AM1, thus additionally indicating involvement of IpdC in IAA biosynthesis by *M. extorquens* AM1 (Fig. 4 and Table 3).

These results are in disagreement with experiments with *Azospirillum brasilense*, where inactivation of *ipdC* resulted in 90% decrease of the IAA concentration. This fact suggested that IpdC is the key and, probably, single enzyme of auxin biosynthesis in this bacterium [35, 36]. In contrast, mutational analysis of the phytopathogenic bacterium *P. agglomerans* revealed the simultaneous operation of two IAA biosynthesis pathways via indolepyruvate and tryptamine [37]. Apparently, *M. extorquens* AM1 has several IAA biosynthesis pathways as well, and the qualitative analysis of indoles from culture fluid proves it. Interestingly, the products of indolepyruvate decarboxylation—indole-3-acetaldehyde and tryptophol—were not found using the chromatography methods even in the spent medium extract of the strain overproducer of IpdC. This is a consequence of high activity of indole-3-acetaldehyde dehydrogenase/oxidase. Conversely, indolelactic acid, the product of indolepyruvate reduction, was found in all extracts. Moreover, ILA concentration in the mutant showed some decrease instead of its accumulation, which indicates the operation of the alternative IAA biosynthetic pathway in *M. extorquens* (Fig. 4). Indeed, protein extracts of all three *M. extorquens* strains reduced NAD^+ after addition of indolepyruvate in the reaction mixture (2 nmol/min per mg protein). This activity appears to be coupled with the decrease of indolepyruvate and, as a consequence, the indolelactate concentrations in spent medium of the mutant strain as well as IAA accumulation up to 46% of wild type strain AM1 level. In addition, there were several paralogs of IpdC encoded in the *M. extorquens* AM1 genome. These are acetolactate syn-

Fig. 4. Reversed-phase chromatography of ethyl acetate extracts of spent medium of wild type strain *M. extorquens* AM1, mutant Δ*ipdC*, and complemented mutant Δ*ipdC*/p7A-9. IAA, indole-3-acetic acid; ILA, indole-3-lactic acid.

thase large subunit (ACS42655, ACS42922), pyruvate decarboxylase/oxidase (ACS39317), and oxalyl-CoA decarboxylase (ACS38885) that can be involved in IAA biosynthesis. Eventually, other key enzymes of auxin biosynthesis (tryptophan decarboxylase, tryptophan 2-monooxygenase) in the *M. extorquens* AM1 genome were not detected and, respectively, alternative IAA biosynthetic pathways in *M. extorquens* are not known yet.

Thus, for the first time for methylotrophs we have showed that the *ipdC* gene encodes indolepyruvate decarboxylase by using the recombinant enzyme IpdC as well as the mutant with deletion in the *ipdC* gene and the complemented strain. Although the protein from *Methylobacterium* phylogenetically belongs to the benzoylformate decarboxylases, the enzyme has catalytic properties of indolepyruvate decarboxylase, which was first demonstrated for this group of enzymes. Finally, mutational analysis also implied an alternative pathway of IAA biosynthesis operates in *M. extorquens* AM1, which is a subject of our further investigation.

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