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Identification and Isolation of the Indole-3-Pyruvate Decarboxylase Gene from *Azospirillum brasilense* Sp7: Sequencing and Functional Analysis of the Gene Locus

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Abstract. The root-associated bacterium *Azospirillum brasilense* Sp7 produces the growth-stimulating phytohormone indole-3-acetic acid (=IAA) via the indole-3-pyruvate pathway. The DNA region containing *ipdC*, the structural gene for indole-3-pyruvate decarboxylase, was identified in a cosmid gene library of strain Sp7 by hybridization and has been sequenced. Upstream of the gene, two other ORF homologous to *gltX* and *cysS* were sequenced that are transcribed in the opposite direction. A functional analysis of the cloned *ipdC* region has been performed. To test the expression of the gene, a *lacZ-Km* cartridge was introduced into the gene. By this construct, tryptophan-dependent stimulation of gene expression in *A. brasilense* Sp7 was observed. Evidences for the existence of another copy of the *ipdC* gene in the *Azospirillum* genome are also reported.

[1]The phytohormone indole-3-acetic acid is one of the factors released by the root-associated bacterium *Azospirillum*, which might be responsible for the observed enhanced root growth of the host plants. However, the biosynthesis and its regulation in *Azospirillum* are only partially understood (for a review see [10]). In several *Enterobacteriaceae*, indole-3-acetic acid is synthesized from tryptophan via indole-3-pyruvate and indole-3-acetaldehyde [7, 18]. The first step of the pathway is catalyzed by an aromatic amino acid transferase, which was recently purified from *A. brasilense* UAP14 [13]. The enzyme catalyzes the transamination of tyrosine, phenylalanine, and tryptophan and is competitively inhibited by its product indole-3-pyruvate. The second enzyme of the pathway, the indole-3-pyruvate decarboxylase, was also shown to be present in *A. brasilense* Sp7 [1], and a gene could be isolated from strain Sp245 [5]. Sp245 has been described as an invading strain that can colonize the superficial layers of the root cortex in a more efficient way than strain Sp7, which essentially is a root surface colonizer. One of the reasons for the different behavior of the two strains might be the regulation and biosynthesis of phytohormones. Therefore, in the present investigation the isolation, genetic organization, and study of gene expression of the indole-3-pyruvate decarboxylase gene from *A. brasilense* Sp7 are described.

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Materials and Methods

Strains, plasmids, and bacteriophages. Derivatives of bacteriophage M13 were propagated in *Escherichia coli* TG1 (see [11]). The cosmids of the gene library were amplified in *E. coli* S17.1 [12].

Molecular cloning, hybridizations. Restriction enzymes, RNase, and T4-ligase (Gibco BRL, Berlin, Germany) were used in standard protocols [11]. Hybridization was performed at 68°C overnight without formamide, with the digoxigenin-labeled 0.45-kbp *Hin*cII/*Pvu*II internal segment of the *A. brasilense* Sp7 *ipdC* gene or a 1.6-kbp *Bam*HI/*Eco*RI segment of the *lacZ-Km* cartridge of pKOK6 [8] as probe. The hybridized nylon filters (Hybond N^+, Amersham, Braunschweig, Germany) were colored with a nonradioactive DNA labeling and detection kit (Boehringer-Mannheim, Germany).

Oligonucleotides and polymerase chain reaction. The oligonucleotides, synthesized by a Pharmacia LKB Gene Assembler Plus, were purified by separation on a 1.5-ml NAP™-10 Column (Pharmacia LKB, Uppsala, Sweden). PCR was performed in 30 circles (30 s denaturation at 93°C, 30 s annealing at 50°C, 60 s polymerization at 72°C) in a volume of 50 µl containing: Taq-polymerase (Gibco BRL), 2 U; 10 × Taq-polymerase buffer (Gibco BRL); $MgCI_2$, 0.25 µmol; oligonucleotides, 50 pmol of each; genomic DNA of *A. brasilense* Sp7, 50 ng. The following oligonucleotides were used: (1a) 5′ggc (gc)tg ct(cg) ct(cg)

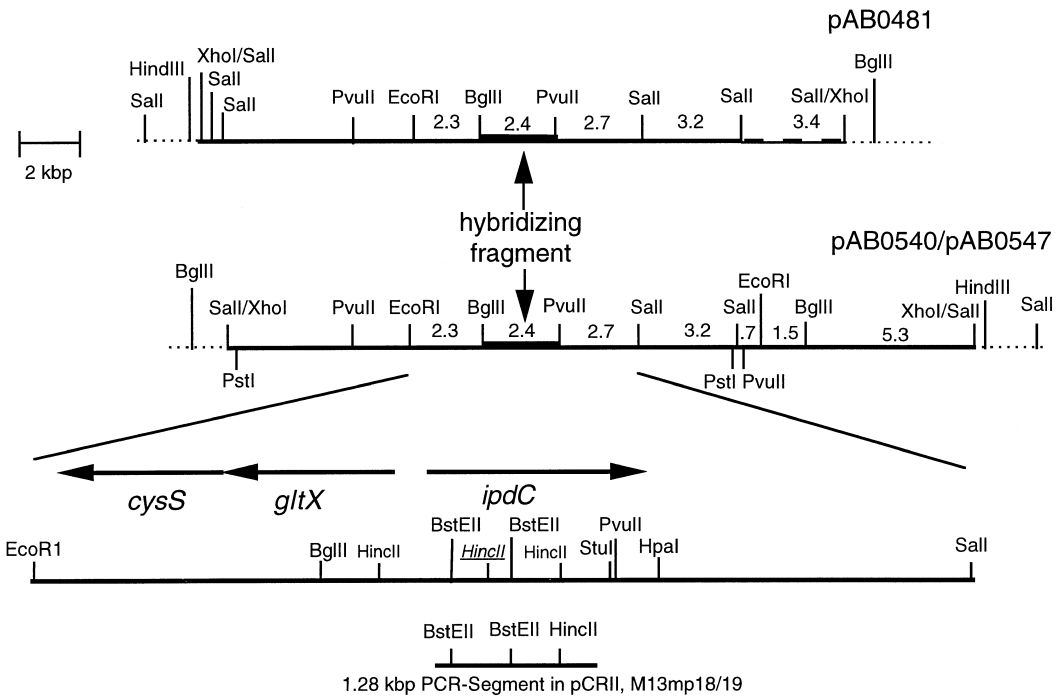

Fig. 1. Physical map of pAB0481, pAB0540, pAB0547, the subcloned fragment and of the amplified PCR segment. The sequenced genes and the identified fragment hybridizing with the PCR segment are indicated by arrows. The *Hinc*II site, which is missing in the PCR segment, is shown underlined in the enlarged map with the indicated gene positions. The adjacent parts of pAB0481, pAB0540, and pAB0547 that correspond to pVK100 are shown as dashed lines.

cac cac 3′; (1b) 3′ gcc (cg)tg gat gt(ac) (ag)cc gca cgc g 5′; (2a) 5′ ggc ttc gcg ggg gac gcg gcg gcg cgt tac 3′; (2b) 3′ ctt agc cgg aag ttg ctg gac ctg ctg acc 5′.

DNA sequencing. Overlapping restriction fragments (2.35 kbp *Eco*RI/*Bgl*II, 4.8 kbp *Eco*RI/*Pvu*II, 2.45 kbp *Bgl*II/*Pvu*II, 2.7 kbp *Stu*I/*Sal*I) of the 7.5 kbp *Eco*RI/*Sal*I region of pAB0540 were cloned into M13mp18 and M13mp19 (Fig. 1). PCR segments were cloned in both directions directly into M13mp18 as described [15]. Progressive deletions of these phages were performed with a deletion kit (Cyclone I Kit, International Biotechnologies, New Haven, CT, USA). The sequences of the 4933-bp *Eco*RI/*Hpa*I insert and of the 1.3-kbp PCR segment were determined on both strands by the dideoxy chain-termination technique. The EMBL, GenBank and DDBJ accession numbers are X99587 and X88853.

Construction of *ipdC-lacZ* fusions. For study of the expression of *ipdC*, an *Xho*I site was inserted into the cloned *ipdC* gene (168 bp downstream of the position of the ATG) by PCR with a sequence-specific oligonucleotide with an *Xho*I site. After controlling the integration of the *Xho*I site at the correct position by sequencing, a *lacZ-Km* cartridge [8] was inserted in frame and opposite to the frame of the *ipdC* gene in the generated *Xho*I site. The construct was transferred into *A. brasilense* Sp7 by the suicide vector pSUP202 [12]. The vectors with their inserts were completely integrated into the *Azospirillum* genome (see Fig. 2 B/D, lanes 3 and 4), resulting in an additional partial copy of the *ipdC* gene coupled to a *lacZ-Km* cartridge in frame or opposite to the *ipdC* promoter region.

Determination of indole-3-acetic acid production. Strains were grown at 30°C in 5 ml mineral malate containing medium supplemented with 5 mg/L tetracycline in case of the transconjugants. After 24 h, 50

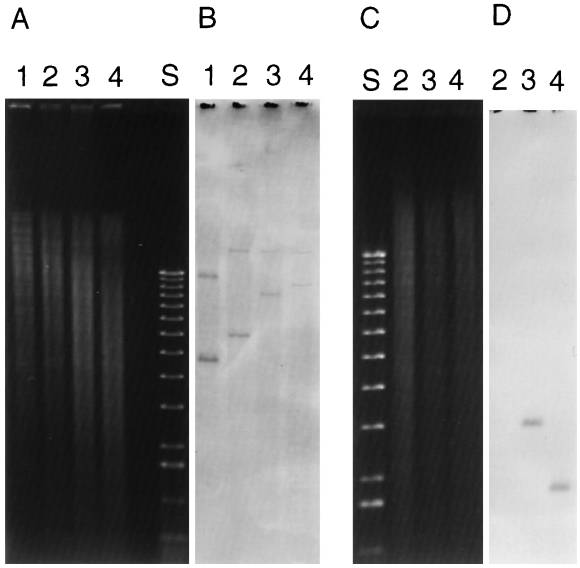

Fig. 2. Hybridization of an *ipdC* segment and of a segment of the *lacZ-Km* cartridge to digested DNA of *Azospirillum brasilense* Sp7 and derivative strains. (A) Agarose-gel of *Pvu*II-digested genomic DNA; (B) blot from (A) hybridized with a 0.45-kbp *ipdC* segment; (C) agarose gel of *Pvu*II/*Bgl*II-digested genomic DNA; (D) blot from (C) hybridized with a 1.65-kbp segment of the *IacZ-Km* cartridge. Lanes: S = 1-kbp ladder (Gibco BRL, Berlin, Germany); 1 = *A. brasilense* Sp245; 2 = *A. brasilense* Sp7; 3 = *A. brasilense* Sp7-01; 4 = *A. brasilense* Sp7-02.

mg/L L-tryptophan was added, and incubation was continued for 18 h. After centrifugation of the culture, the amount of tryptophan and indole-3-acetate was determined in the supernatant by reversed-phase HPLC (separation on a 5 µ Novapack C-18 column; methanol:1% H_3PO_4, 40:60 (vol/vol) as mobile phase, 1 ml flow rate, detection at 280 nm).

Determination of β-galactosidase activity. Precultures of strain Sp7 and its derivatives were grown in mineral malate containing medium (+ 25 mg/L kanamycin in case of the *lacZ-Km* insertion clones). In order to determine β-galactosidase activity, cells were inoculated (initial $O.D._{600nm} = 0.05$) into mineral medium ± tryptophan (0.1 mg/L) ± yeast extract (1 g/L). After 24 h of growth ($O.D._{600nm} = 1.3–1.5$) 1 ml of culture was used for determination of β-galactosidase activity [9].

Results and Discussion

Amplification of a segment of the *ipdC*-gene. Analysis of conserved regions in the *ipdC* genes from enteric bacteria [18] led to design of suitable oligonucleotides (1a, 1b; see Materials and Methods) to amplify a 445-bp segment of *A. brasilense* Sp7 DNA in a PCR reaction that shared 95% identity with the *ipdC* gene of *A. brasilense* Sp245 [5]. As the *ipdC* gene is 1.6 kbp in length, another set of primers was designed (2a, 2b), allowing amplification most of the gene as a 1.3 kbp segment from Sp7 genome. This segment was sequenced and deposited under the EMBL number X88853.

Construction of a cosmid gene library and identification of cosmids carrying the entire *ipdC* gene. The amplified 1.3-kbp PCR segment was used to screen a gene library of *Sal*I fragments of strain Sp7 in the broad host range vector pVK100 [19]. This enabled us to isolate three clones with the cosmids pAB0481, pAB0540, and pAB0547 (Fig. 1). Restriction and hybridization mapping of the cosmids revealed that all of them harbored the entire indole-3-pyruvate decarboxylase gene and neighboring regions. Whereas pAB0540 and pAB0547 had identical inserts, pAB0481 differed in the size of a lateral *Sal*I segment, which is probably not adjacent to the other segments in the host genome.

Sequencing of the locus and identification of three open reading frames. After subcloning the hybridizing area into M13mp18/19 phages (Fig. 1) a 4.93-kbp *Eco*RI/*Hpa*I fragment was sequenced. Three open reading frames, ORF1 to ORF3, could be identified with high G+C contents in the third codon position (95.4%, 91.7%, and 85.3%, respectively), typical for *Azospirillum* genes (for references see [16]). Putative ribosome binding sites were located 12–15 bp upstream of the ORFs ('G*G-GAGA*', '*GAA*', 'A*GGAGA*'). ORF1 starts with ATG at position 3153 and ends with TAA at position 4788 referred to the *Eco*RI site of the sequence at position 1. As the deduced amino acid sequence of ORF1 shared 28.3% identical (56.4% similar) amino acid residues with the indole-3-pyruvate decarboxylase of *Enterobacter cloacae* [7], and 95.1% identical (96.6% similar) amino acid residues with the sequence of the indole-3-pyruvate decarboxylase of *A. brasilense* Sp 245 [5] was detected, the ORF1 was named *ipdC*. However, upstream of position -26 referred to the ATG start codon of the gene and downstream position +12 referred to the TAA stop codon, there is no homology detectable between the DNA sequences of strain Sp7 and strain Sp245. ORF1 is followed by an inverted repeat (position 4793–4835), which can form a hairpin loop with a ΔG (25°C) of −35.2 kcal mol^{-1} after transcription [14], possibly causing termination of transcription. No adjacent ORFs were identified on the same DNA strand, suggesting that the *ipdC* gene is organized as a single gene operon. However, on the complementary strand, two ORFs could be identified in front of the *ipdC* gene. The deduced amino acid sequence (466 amino acid residues, ATG at position 2899 and TAA at position 1561) of ORF2 showed homology to the *glt*X gene product of *E. coli* (32.8% identity, 56.3% similarity, referred to the total length of the polypeptide) and *B. subtilis* (31.8% identity, 54.3% similarity) [3, 4], which is a glutamyl-tRNA synthase. Similarly, the deduced amino acid sequence of ORF3 (448 amino acid residues), starting with GTG at position 1524 and ending with TGA at position 180, showed significant homology to the *E. coli* (44.2% identity, 63.8% similarity, referred to the total length of the polypeptide) and *B. subtilis* (35.7% identity, 56.2% similarity) *cysS* gene product, the cysteinyl-tRNA synthetase [4, 6]. The homology to the *E. coli* and *B. subtilis* genes is in the same range as previously found for genes of the *trpGDC* operon of *A. brasilense* Sp7 (37.2–38.4% identity [17]). Obviously, both open reading frames on the complementary strand have nothing in common with the biosynthesis of auxins, but are essential genes for protein biosynthesis according to their homology to tRNA-synthetase genes. ORF3 is also followed by an inverted repeat that can form a hairpin loop (position 129–168) with a ΔG (25°C) of −39.4 kcal mol^{-1} after transcription [14]. No other reading frames were detected in the sequenced region upstream or downstream of the three ORFs.

Existence of more than one copy of the *ipdC* gene in *A. brasilense Sp7*. Hybridization of the internal 0.45-kbp *Hin*cII/*Pvu*II segment of *ipdC* of *A. brasilense* Sp7 with *Pvu*II-digested genomic DNA of *A. brasilense* Sp7 resulted in one fragment of 6.8 kbp as it is represented in pAB0540 and a second weaker fragment of about 15 kbp (Fig. 2B), which indicates a second copy of the gene. Also, in case of *A. brasilense* Sp245, two *Pvu*II fragments

Table 1. Tryptophan-dependent indole-3-acetic acid production by transconjugants of *Azospirillum brasilense* Sp 7 and *A. irakense* KA3. The transconjugants were grown aerobically in minimal medium with tetracycline at 30°C. After 48 h, 50 mg/L L-tryptophan was added, and the incubation was continued for 16 h. The concentration of tryptophan and indole-3-acetic acid was determined by HPLC as described in Materials and Methods. Data and standard deviations were calculated from three independently performed experiments

	A. irakense KA3		*A. brasilene* Sp7	
Plasmids [mg/L]	Trp consumption	IAA production	Trp consumption	IAA production
pVK100	10.9 ± 2.3	0.8 ± 0.2	3.6 ± 0.8	2.2 ± 0.5
pAB0481	11.7 ± 2.1	1.6 ± 0.4	7.2 ± 1.9	3.9 ± 1.0
pAB0540	12.2 ± 3.0	2.1 ± 0.6	10.7 ± 3.2	5.4 ± 1.5
pAB0547	12.7 ± 2.8	2.3 ± 0.5	10.4 ± 2.8	3.1 ± 0.8

Table 2. β-Galactosidase activity of *A. brasilense* Sp7 derivatives harboring a *lacZ-Km* cartridge in the *ipdC* gene. β-Galactosidase activity was determined after 24 h of growth (30°C) at the given conditions as described in Materials and Methods. Standard deviations were calculated from at least three determinations from independent experiments. These results are expressed as nmol o-nitrophenol produced per min per mg protein

	Strain		
Growth conditions	*A. brasilene* Sp7	*A. brasilense* Sp7-01 *lacZ* opposite to *ipdC*	*A. brasilense* Sp7-02 *lacZ* in frame to *ipdC*
Mineral-medium + malate	0.9 ± 0.3	3.9 ± 0.6	3.4 ± 0.5
Mineral-medium + malate + tryptophan	1.0 ± 0.4	3.7 ± 0.5	10.4 ± 1.8
Mineral-medium + malate + yeast extract	1.0 ± 0.3	5.8 ± 1.5	6.8 ± 1.3
Mineral-medium + malate + yeast extract + tryptophan	1.1 ± 0.4	4.3 ± 1.3	26.3 ± 4.2

(5 kbp and 13 kbp) hybridized with the 0.45-kbp *Hin*cII/*Pvu*II *ipdC* probe of strain Sp7, another strong hint for two copies of the gene in *A. brasilense*. In addition, the DNA sequence of the amplified 1.3-kbp PCR segment differed in 1.3% from the sequence derived from the pAB0540 locus. As this percentage is higher than the usual rate of Taq-polymerase mistakes in PCR, two copies of the gene probably exist in *A. brasilense*. Especially one *Hin*cII site is missing in the sequence of the PCR segment, which is present in the sequenced fragment of pAB0540 (Fig. 1). These observations are in good agreement with the described identification of two different hybridizing *Eco*RI fragments of a gene library of strain Sp245 (18 kbp and 6.5 kbp) [5], which was not further respected by the authors. The above-mentioned missing homology of the DNA sequences of strain Sp7 and Sp245 upstream and downstream of the *ipdC* gene could be because the isolated and sequenced genes from the two species corresponded to the two different copies of the gene locus. The existence of more than one copy of the indole-3-pyruvate decarboxylase gene in one strain could well explain the previously observed difficulty to isolate IAA-defective mutants of *A. brasilense* Sp7 or Sp245.

Functional analysis of the *ipdC*-gene from *A. brasilense* Sp7. To verify the involvement of the isolated gene locus in the biosynthesis of indole-3-acetic acid, we transferred the cosmids pAB0481, pAB0540, pAB0547, and the vector pVK100 by conjugation into the wild-type *A. brasilense* Sp7 and into *A. irakense* KA3, a strain known to produce only low amounts of indole-3-acetic acid [16]. In the transconjugants of strain KA3 and strain Sp7, the tryptophan-dependent indole-3-acetic acid production was increased by a factor of two for the cosmids pAB0481, pAB0540, and pAB0547 compared with the control cosmid pVK100 (Table 1). Thus, the identified gene locus is functionally involved in the IAA production of *Azospirillum*. As introduction of additional copies of the indole-3-pyruvate decarboxylase gene enhances the IAA production, there is apparently no inhibition of the gene product on its own transcription.

Study of *ipdC* expression. To study gene expression of the *ipdC* gene in *Azospirillum*, a *lacZ-Km* cartridge was cloned into a generated *Xho*I site 168 bp downstream of the ATG start codon of *ipdC* (see Materials and Methods), and the construct was integrated into the genome of *A. brasilense* Sp7 (see Fig. 2). Because indole acetic acid production in *Azospirillum* is known to be strictly dependent on tryptophan [1], the resulting strains (strain Sp7-01 with *IacZ* antiparallel to *ipdC*, strain Sp7-02 with *IacZ* parallel to *ipdC*) were grown in mineral medium in the presence or absence of tryptophan and/or yeast extract (Table 2). In case of strain Sp7-02 (*IacZ* parallel to the *ipdC* promoter), β-galactosidase activity in mineral medium and in yeast extract medium was significantly increased in the presence of tryptophan—3.1 fold and 3.9 fold respectively. In the control strain Sp7-01 (*lacZ* antiparallel to the *ipdC* promoter), the level of β-galactosidase activity with tryptophan remained at the low level observed for tryptophan-free medium for strains Sp7-01 and Sp7-02. The level of β-galactosidase activity in the wild-type strain Sp7 (without *IacZ-Km* cartridge) was quite low under all conditions tested, indicating that the observed β-galactosidase activity in

strain Sp7-01 and Sp7-02 was due mainly to the presence of the *lacZ-Km* cartridge. These experiments clearly show that the expression of *ipdC* in *Azospirillum* is induced by the presence of tryptophan. The previously observed, strict tryptophan-dependent indole-3-acetic acid production in *Azospirillum* can, therefore, be explained by the tryptophan-dependent induction of *ipdC* expression. In contrast, in the epiphytic *Erwinia herbicola* 299R, tryptophan does not influence *ipdC* expression [2]. The tryptophan-induced expression of *ipdC* observed in the present study has a fundamental advantage for the root-associated bacterium *Azospirillum*: it will prevent a loss of internal tryptophan in the free-living state and will enable the production of root growth-stimulating indole-3-acetic acid when tryptophan is available, e.g., at the root surface.

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