

## Functional Identification and Expression of Indole-3-Pyruvate Decarboxylase from *Paenibacillus polymyxa* E681

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Indole-3-acetic acid (IAA) is produced commonly by plants and many bacteria, however, little is known about the genetic basis involving the key enzymes of IAA biosynthetic pathways from *Bacillus* spp. IAA intermediates from the Gram-positive spore-forming bacterium *Paenibacillus polymyxa* E681 were investigated, which showed the existence of only an indole-3-pyruvic acid (IPA) pathway for IAA biosynthesis from the bacterium. Four open reading frames (ORFs) encoding indole-3-pyruvate decarboxylase-like proteins and putative indole-3-pyruvate decarboxylase (IPDC), a key enzyme in the IPA synthetic pathway, were found on the genome sequence database of *P. polymyxa* and cloned in *Escherichia coli* DH5 $\alpha$ . One of the ORFs, PP2\_01257, was assigned as probable indole-3-pyruvate decarboxylase. The ORF consisted of 1,743 nucleotides encoding 581 amino acids with a deduced molecular mass of 63,380 Da. Alignment studies of the deduced amino acid sequence of the ORF with known IPDC sequences revealed conservation of several amino acids in PP2\_01257, essential for substrate and cofactor binding. Recombinant protein, gene product of the ORF PP2\_01257 from *P. polymyxa* E681, was expressed in *E. coli* BL21 (DE3) as a glutathione S-transferase (GST)-fusion protein and purified to homogeneity using affinity chromatography. The molecular mass of the purified enzyme showed about 63 kDa, corresponding closely to the expected molecular mass of IPDC. The indole-3-pyruvate decarboxylase activity of the recombinant protein, detected by HPLC, using IPA substrate in the enzyme reaction confirmed the identity and functionality of the enzyme IPDC from the E681 strain.

**Keywords:** Indole-3-acetic acid, *Paenibacillus polymyxa*, indole-3-pyruvate decarboxylase

*Paenibacillus polymyxa* (previously *Bacillus polymyxa* [1]) is commonly found in soil, in the rhizospheres and rhizoplane of various plant species [11, 13, 26]. The beneficial effects of *P. polymyxa* on plant growth, including nitrogen fixation, plant-disease suppression, soil-phosphorous solubilization, increase of soil porosity, and production of antibiotics, hydrolytic enzymes, and phytohormones, have amply been demonstrated through studies to date [4, 5, 9, 10, 12, 13, 16–19, 23, 25, 26, 29, 31]. Auxin-related phytohormones, especially the most studied indole-3-acetic acid (IAA) produced by higher plants and microorganisms, have been reported to be produced by rhizobacteria [30]. IAA synthesized by plant-associated bacteria is implicated as one of the key determinants in association of plant-microbe interaction, stimulation of plant growth, and plant pathogenesis. In many cases, a single bacterial strain may possess more than one Trp-dependent biosynthetic pathway of IAA, in which the main precursors are indole-3-acetamide (IAM), indole-3-aldehyde (IAAld)/indole-3-ethanol (TOL), tryptamine (TRA), and indoleacetonitrile (INA) [22, 30]. The ability to produce IAA has been found in *P. polymyxa* isolates [17], and several IAA biosynthetic pathways, classified according to their intermediates, were reported in the bacteria [22, 30]. Some reports on the production of IAA by *Bacillus* species have been cited, but little is known about the IAA biosynthetic pathways in *Bacillus* spp. regarding the genetic basis involving the key enzymes of this process. By using HPLC analysis, the presence of IAA and IAA-related compounds in the growth medium supplemented with L-tryptophan (Trp) was shown for *P. polymyxa* strains isolated from different proximity to wheat roots. The detection of indole-3-lactic acid (ILA), TOL, and IAAld in almost all *P. polymyxa* cultures and IAM in some *P. polymyxa* cultures indicated the occurrence of indole-3-pyruvic acid (IPA) and IAM pathways of auxin biosynthesis in *P. polymyxa* depending on strains isolated from the rhizoplane, rhizosphere soil, and non-rhizosphere soil [17].

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The synthesis of IAA *via* IPA pathways, implicated by TOL, IAAld, and ILA, has been reported to occur in plants and bacteria. However, the key genes/enzymes have not yet been identified in plants. In the bacterium *Azospirillum brasiliense*, insertional inactivation of the IPA pathway resulted in lower IAA production, of up to 90% reduction, indicating the importance of the IPA pathway in auxin production [24]. The *ipdC* genes, encoding the key enzyme indole-3-pyruvate decarboxylase (IPDC) of the IPA pathway, have been isolated and characterized from several IAA-producing bacteria such as *A. brasiliense*, *Azospirillum lipoferum*, *Enterobacter cloacae*, *Pseudomonas putida*, *Erwinia herbicola*, and *Klebsiella aerogenes* [2, 6, 14, 32, 33, 34]. These genes code for IPDC polypeptides of about 550 amino acids (aa) in length, corresponding to a molecular mass of ~60 kDa per subunit. The deduced amino acid sequence shares extensive homology with those of pyruvate decarboxylase enzymes, which catalyze decarboxylation of pyruvate to acetaldehyde and CO<sub>2</sub>. In addition, the crystal structure of the IPDC from *E. cloacae* has recently been determined, and the native enzyme was estimated to be around 240 kDa, suggesting a homotetramer structure [27]. The enzyme is dependent on Mg<sup>2+</sup> and thiamine diphosphate (ThDP) as cofactors, and has higher affinity for the substrate IPA than for pyruvic acid, with a K<sub>m</sub> value of 15 μM for IPA [15]. These results indicate that IPDC from *E. cloacae* is a highly specific enzyme with a high affinity for IPA [5].

The *P. polymyxa* E681 used in this experiment was isolated from the rhizosphere of winter barley cultivated in Korea [25]. This strain is known to form endospores, suppress plant diseases, produce antimicrobial compounds, colonize on roots, secrete diverse degrading enzymes, and produce phytohormones [5, 25]. The full genome of *P. polymyxa* E681, about 5 Mb in size, has recently been sequenced by the Genome Research Center at Korea Research Institute of Bioscience and Biotechnology [21]. This advances a vast progress in molecular and genetic studies on bacterium-plant interactions. In this study, we investigated the auxin biosynthetic pathway and functional identification of an IPDC enzyme, a key enzyme responsible for this process, from *P. polymyxa* E681. To our best knowledge, there has been no study reported on the cloning and characterization of genes encoding IPDC from *P. polymyxa* and Gram-positive bacteria as well.

## MATERIALS AND METHODS

### Bacterial Strains and Culture Conditions

*P. polymyxa* E681 was previously isolated from the rhizosphere of winter barley cultivated in Korea [25]. The bacterium was grown at 30°C on tryptic soy agar (TSA; Difco, U.S.A.) medium and stored at -80°C in tryptic soy broth (TSB; Difco, U.S.A.) plus 15% glycerol. *Escherichia coli* strains were grown aerobically in Luria-

Bertani (LB) medium at 37°C. For IAA production, *P. polymyxa* strains were grown at 30°C in chemically defined medium (DM) supplemented with Trp to the final concentration of 0.5 mM. The DM medium included glucose (5 g/l), (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (1 g/l), NaCl (2 g/l), MgSO<sub>4</sub>·7H<sub>2</sub>O (0.2 g/l), MnSO<sub>4</sub>·4H<sub>2</sub>O (0.007 g/l), CaCl<sub>2</sub>·2H<sub>2</sub>O (0.008 g/l), L-arginine (0.7 g/l), L-glutamine (0.1 g/l), biotin (1 mg/l), thiamine (1 mg/l), and 50 mM phosphate buffer (K<sub>2</sub>HPO<sub>4</sub>, KH<sub>2</sub>PO<sub>4</sub>), pH 7.2.

Putative precursors of IAA, including Trp, IPA, IAM, TRA, and INA, were added from filter-sterilized 100X stock solutions to the final concentration of 0.5 mM.

### Quantification of Indolic Compounds by Using HPLC and TLC

For quantification of IAA intermediates produced by *P. polymyxa* E681, indolic compounds in the bacterial supernatant were assayed. Thus, the bacterium was precultured for 20 h in DM medium, and then a 400-μl aliquot was transferred into 100 ml of fresh DM medium supplemented with Trp, obtaining 10<sup>5</sup> CFU/ml of the final cell density. After 32 h of cultivation, the cell density of the culture was measured spectrophotometrically at 600 nm, and then bacterial cells were removed from the culture medium by centrifugation (5,000 ×g, 10 min). The supernatant of *P. polymyxa* E681 culture (25 ml) was adjusted to pH 2.5 using concentrated H<sub>3</sub>PO<sub>4</sub>, and then was extracted two times with equal volumes of ethyl acetate. The organic solvent was vacuum dried at 37°C, and compounds extracted were dissolved in 1 ml of 100% methanol. Twenty-μl aliquot of the filtered extract was injected into an Atlantis C18 reverse column (5 μm, 250 × 4 mm) (Atlantis Water, MA, U.S.A.) equipped with a differential UV detector at 280 nm. The mobile-phase solvent used for reverse-phase chromatography was 72% solvent A (1% acetic acid) and 28% solvent B (100% methanol) [7]. The flow rate was set to 0.6 ml/min, and the quantitative estimates of IAA were based on HPLC analysis. Authentic IAA intermediates (Sigma Chemical Co., St. Louis, MO, U.S.A.) were used as the standards. For TLC analysis, a volume of 20 μl of concentrated ethyl acetate extracts was spotted onto a silica gel 60 F254 plate (Merck, Darmstadt, Germany). The mobile phase was used to separate particular bands and confirmed the identities of indolic compounds as follows: n-hexane-ethyl acetate-isopropanol-acetic acid (40:20:5:1, v/v) as previously described [7]. After development, the plates were air-dried, and the bands were visualized using a short-wavelength (254 nm) hand-held UV lamp, after spraying with van Urk-Salkowski reagent (FeCl<sub>3</sub> 0.5 M : HClO<sub>4</sub> 35%, 2 : 100) [8] and heating to 90°C for 10 min. The R<sub>f</sub>s and colors of the bands were compared with the R<sub>f</sub>s and colors of standard IAA-related intermediates, including IAA, IPA, IAM, IAAld, TOL, TRA, INA, indole-3-carboxylic acid (ICA), and ILA [purchased from Sigma Chemicals Ltd. (U.S.A.) or Aldrich Chemicals (St. Louis, MO, U.S.A.)].

### Construction and Cloning of *ipdC* Candidates in pUC19 Vector

From computational whole genome and protein sequence analyses for *P. polymyxa* E681 (unpublished sequence) [21], we selected and cloned the four most-like *ipdC* gene candidates in the pUC19 vector to express in *E. coli* DH5α and test IPDC activity. The PCR products of the *ipdC* gene candidates, amplified by pairs of primers (Table 1), were cut with HindIII and EcoRI and inserted into the pUC19 vector cut with the same enzymes. The resulting plasmid was introduced into *E. coli* DH5α cells, and transformants were selected on ampicillin (Amp)-containing agar plates. Recombinant *E. coli* strains were grown in LB medium, supplemented with

**Table 1.** PCR primers used to amplify coding regions of the *ipdC* candidates from *P. polymyxa* E681.

Primer name	Primer sequence 5-3 <sup>a</sup>	Amplified ORF
ipdC1-F	GGC <u><i>AAGCTT</i></u> TACAGCCTTTCGTCCCTCAG	PP2_01257
ipdC1-R	CCG <u><i>GAATTC</i></u> CGCTCCAGGTTGTTTCATCC	
ipdC2-F	GGC <u><i>AAGCTT</i></u> CACCCATCCCCGAGACCTTG	PP2_01918
ipdC2-R	CCG <u><i>GAATTC</i></u> CAGGACGAATGGGCAGAAAC	
ipdC3-F	GGC <u><i>AAGCTT</i></u> ACTCGTGACGGCTTTATGAC	PP2_01914
ipdC3-R	CCG <u><i>GAATTC</i></u> TGGATCAGATCGAGGCATAG	
ipdC4-F	GGC <u><i>AAGCTT</i></u> TTTGGTATGACTGCGAGCTTC	PP2_00076
ipdC4-R	CCG <u><i>GAATTC</i></u> CACGTCTTCTCACTGTATCC	

<sup>a</sup>Restriction enzyme sites (HindIII and EcoRI) are italic and underlined. All DNA sequences are written in a 5' to 3' orientation.

0.1 mM thiamine diphosphate, 0.1 mM magnesium sulfate, and 100 µg/ml Amp, for 24 h at 30°C. The expression of IPDC was induced by addition of 1 mM IPTG at 4 h before harvesting cells. Cells were centrifuged, washed twice in potassium phosphate buffer (10 mM), resuspended to obtain 10<sup>8</sup> CFU/ml (measured by OD at 600 nm) in phosphate buffer (pH 6.5), containing Trp, 10 mM thiamine diphosphate, 0.1 mM magnesium sulfate and sodium bisulfite, and incubated at 28°C for 18 h as previously described [3], called buffer assay afterward. After reactions, indolic compounds were extracted by ethyl acetate and measured by HPLC and TLC.

#### Recombinant Vector Construction and *ipdC* Gene Cloning

A pair of primers, as forward primer: 5' CTCGATCCCCAATGAGTGCACAAATTC 3' and reverse primer: 5' GCGAATTCCTTACTCGTCCCCCATCAGC3', respectively, incorporating BamHI and EcoRI restriction sites was designed to allow amplification from the ATG codon to the downstream of the stop codon of the entire *ipdC* gene of *P. polymyxa* E681. The PCR product was isolated on agarose gel and cloned into pGEM-Teasy vector (Promega, Madison, WI, U.S.A.). The pGEM-Teasy:*ipdC* plasmid extracted from *E. coli* XL1-Blue was then digested with BamHI and EcoRI and ligated into the same restriction sites of pGEX-2T (Amersham Bioscience, Piscataway, NJ, U.S.A.). The ligation mixture was transformed into *E. coli* XL1-Blue to generate Amp-resistant colonies. Single colony was then cultured in LB medium supplemented with Amp. The correctly-constructed plasmid pGEX-2T-*ipdC* was transformed into competent *E. coli* BL21 (DE3) for protein expression.

#### Expression and Purification of Recombinant IPDC Protein

*E. coli* BL21 cells harboring plasmid pGEX-2T-*ipdC* were grown in LB medium containing 100 µg/ml Amp at 25°C until OD<sub>600</sub> reached 1.0 unit. The expression of fusion protein GST-IPDC was induced with isopropylthiogalactoside (IPTG) to obtain the final concentration of 0.05 mM and incubated under the same condition for three more hours. Cells were harvested by centrifugation at 4,500 ×g for 15 min at 4°C and resuspended in ice-cold phosphate-buffered saline (PBS), pH 7.3. After addition of 1% Triton X-100, the bacterial suspension was sonicated, and the fusion protein was extracted by centrifugation at 12,000 ×g, 15 min. The fusion protein GST-IPDC was purified using the bulk GST purification module kit (Amersham Biosciences, Piscataway, NJ, U.S.A.) according to the manufacturer's protocol, and the fusion protein was identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 10% polyacrylamide gel) after being stained with 0.003% Coomassie blue in 40% methanol-10% acetic acid. To remove the GST affinity tail from the fusion protein, thrombin protease cleavage was applied by incubation

at 4°C for 16 h. Cleaved GST was then combined with glutathione-Sepharose 4B, and the recombinant protein IPDC was eluted according to the manufacturer's instructions.

#### Enzyme Assay for Recombinant Proteins

To test the IPDC activity of proteins expressed in *E. coli*, the fusion protein and purified IPDC were mixed with substrate solution to obtain final protein concentrations of 265 µg/ml and 100 µg/ml, respectively, and reacted at 28°C for 3 h. Substrate solution was prepared by combining 0.2 mM indole-3-pyruvate as substrate, 15 mM ThDP, 0.1 mM magnesium sulfate, and 0.1 mM sodium bisulfite (to prevent the spontaneous breakdown of IAAld) in 50 mM phosphate buffer (pH 6.5) and the solution was preincubated for 20 min at room temperature. After reaction, the solution was adjusted to pH 2.5, extracted by two volumes of ethyl acetate, concentrated, and quantified by HPLC.

#### Nucleotide Sequence Accession Number

The sequence of the *P. polymyxa* E681 *ipdC* gene reported in this paper has been deposited in the GenBank database under the accession number EU126052.

## RESULTS AND DISCUSSION

#### Production of IAA Intermediates by *P. polymyxa* E681

The production of indolic compounds by strain *P. polymyxa* E681 was measured throughout the growth in DM medium supplemented with Trp. During the time-course experiment, the accumulation of indolic compounds in the supernatant was identified and quantified by HPLC at regular time-intervals and further analyzed by TLC. In the experimental scope, IAAld was only detected in the TLC analysis and could not be identified by HPLC, since compound was probably unstable and tended to spontaneously change into IAA and TOL. Analysis by HPLC and TLC revealed the presence of IPA, IAAld, ILA, IAA, and TOL in the ethyl acetate extract from *P. polymyxa* culture grown in Trp-containing DM medium (data not shown). Quantitative monitoring of main IAA intermediates, including IPA, IAA, and TOL, accumulated in bacterial supernatant during the time course, was determined (Fig. 1). However, there was no trace of IAM, TRA, INA, and other auxin-like IBA in the supernatant of the bacterial cultures. Feeding experiments

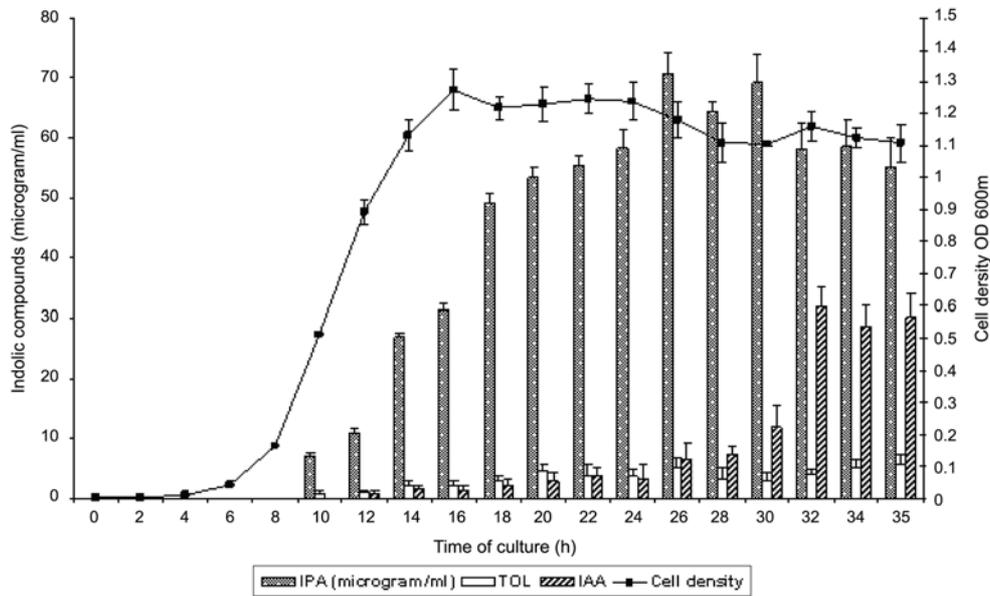


Fig. 1. Production of IAA intermediates by *P. polymyxa* E681.

were conducted with IAA precursors to determine the main IAA biosynthetic pathway of *P. polymyxa* E681. When IPA was added to the medium, the production of TOL, IAAld, IAA, and ILA was detected in the culture supernatant, whereas IAA was not detected in the cultures when growing the bacterium in medium feeding with IAM and INA, as

well in the non-Trp-defined culture supernatant of E681 strain. Feeding experiment with TRA led to the formation of a small amount of IAA similar to the IAA level formed in the culture fed with this compound without bacterial inoculant. This level of IAA might have been formed by spontaneous oxidation of TRA to IAA.

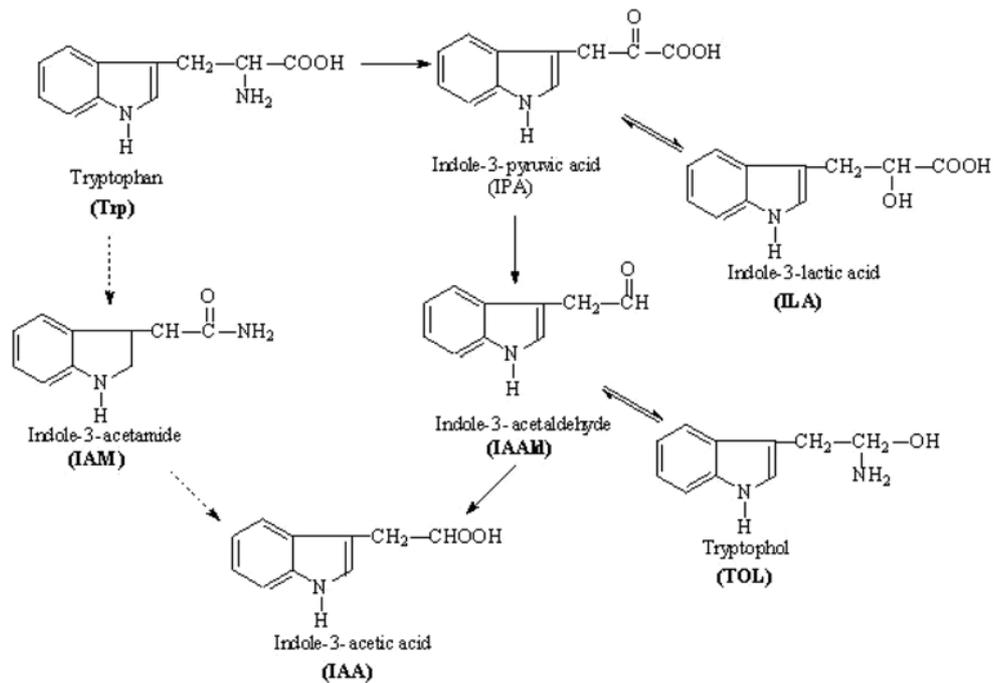


Fig. 2. Scheme of IAA biosynthetic pathways in *P. polymyxa* strains and *P. polymyxa* E681.

The IAA pathway in E681 strain is indicated by continuous lines, and some other *P. polymyxa* strains synthesize IAA via both IPA and IAM (discontinuous lines) pathways [17].

**Table 2.** Percent identity and homology between probable IPDC sequences from *P. polymyxa* E681 and ORFs from other sources with respect to their amino acid sequence.

ORF and putative annotation	E-value	With other pyruvate-derivative decarboxylases (%)		Protein	Microorganisms/ accession number
		Identities	Homology		
PP2_01257 (Indole-3-pyruvate decarboxylase, IpdC)	3e-180	56	72	Acetolactate synthase/ phosphonopyruvate decarboxylase	<i>Bacillus anthracis</i> str. A2012/ZP_00392128
PP2_01918 (pyruvate dehydrogenase)	0.0	56	73	Pyruvate decarboxylase	<i>Burkholderia cenocepacia</i> PC184/EAY65722
PP2_01914 (acetolactate synthase)	0.0	66	79	Pyruvate decarboxylase	<i>Exiguobacterium sibiricum</i> 255-15/ZP_00540212
PP2_00076 (acetolactate synthase)	1e-39	28	46	Pyruvate decarboxylase	<i>Burkholderia xenovorans</i> LB400/ABE36217

In this study, evidences of IAA intermediates of IPA, IAAld, ILA, IAA, and TOL in the culture supernatant and feeding experiments suggested the existence of the only IPA pathway for IAA biosynthesis of *P. polymyxa* E681 (Fig. 2). Results of the IAA intermediates accumulated in the E681 culture demonstrated the possibility that the IPA pathway plays a central role in the IAA production by *P. polymyxa* E681 in the presence of exogenous Trp (Fig. 2). Indeed, precursors of other IAA biosynthetic pathways responsible for IAA production in other plant-associated bacteria, such as IAM, INA, and TRA, were not detected. Feeding experiment with IAM, INA, and TRA precursors, resulting in insignificant production of IAA, indicated that the IAM, TRA, and INA pathways probably did not contribute to IAA production of *P. polymyxa* E681 strain. In the time-course of bacterial growth, TOL and IAA in low concentrations were detected in the bacterial culture of E681 strain during exponential growth, whereas IPA was increasingly accumulated. In the stationary phase, IPA remained at a constant level, and the remarkable increase of IAA and slight decrease of IPA accumulation in E681 supernatant from the 30th hour (late-stationary phase) might be due to formation of functional IPDC enzyme in the bacterial culture (Fig. 1). In a previous report, IAA biosynthesis and *ipdC* expression in *A. brasiliense* were found to be triggered by stress factors including exhaustion of carbon source, reduction in growth rate and oxygen stress [20]. The key enzyme IPDC in the IPA pathway, encoded by the *ipdC* gene from fungi and Gram-negative bacteria, has been well documented, but not much is known about the genetic basis involving the key enzyme IPDC from *Bacillus* spp. and other Gram-positive bacteria.

### Genome Search and Cloning of *ipdC* Candidates in pUC19 Vector

Metabolism of Trp by E681 strain, which apparently results in IAA intermediates including TOL, ILA, IPA, IAAld, and IAA, proved the IPA biosynthetic pathway and IPDC enzyme synthesized by the bacterium. Since *P. polymyxa*

E681 strain was previously found to be a plasmid-free bacterium [21], it is quite possible that IAA biosynthetic genes are located on the bacterial chromosome. The recently unpublished complete genome sequence of the bacterium *P. polymyxa* E681 provides new opportunities to identify proteins and to understand protein function. To identify the corresponding IPDC sequence(s) in the complete genome of *P. polymyxa* E681 available at <http://www.gem.re.kr>, a BLAST search was conducted using a default E-value cut-off of 10.0 against known amino acid sequences of IPDC from other sources. However, the known IPDC sequences did not show highly significant homology with the E681 genome. Such search revealed the most IPDC-probable four ORFs belonging to thiamine pyrophosphate (TPP)-dependent enzymes with similarity to pyruvate-derivative decarboxylases, the enzyme group having high homology with IPDCs from other microorganisms, and these ORFs were putatively annotated as probable indole-3-pyruvate decarboxylase (IPDC), pyruvate decarboxylase, and a couple of different acetolactate synthase enzymes

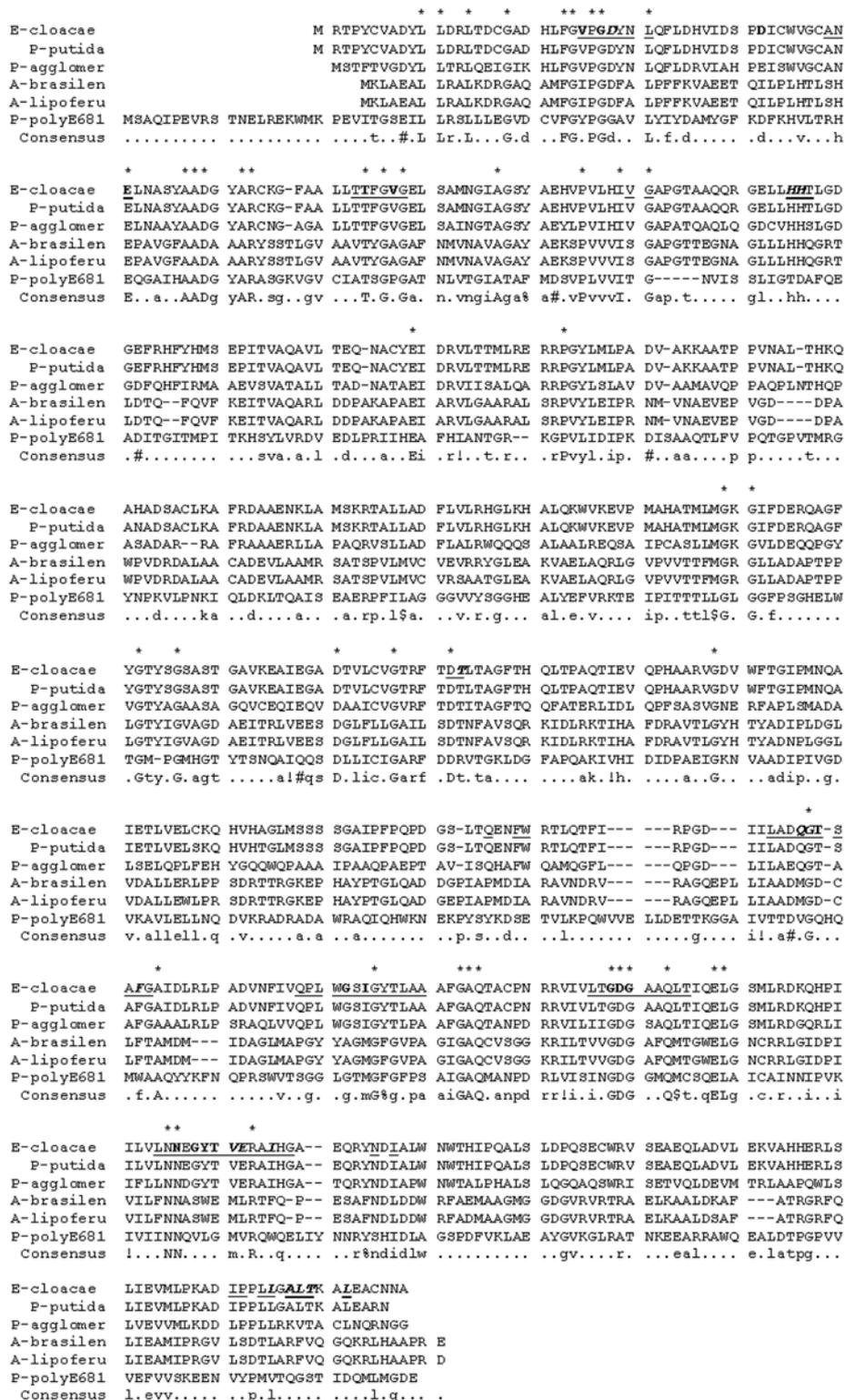
**Table 3.** Production of IAA intermediates by *P. polymyxa* E681 and *E. coli* harboring clones of the probable IPDCs from E681 strain.

Strain	IAAld <sup>b</sup>	TOL (µg/ml) <sup>c</sup>	IAA (µg/ml) <sup>d</sup>
No cells <sup>a</sup>	-	0.021	0.18
<i>E. coli</i> DH5α	-	0.021	0.213
<i>E. coli</i> (pP01257)	+	0.416	0.568
<i>E. coli</i> (pP01918)	-	0.021	0.203
<i>E. coli</i> (pP01914)	-	0.023	0.321
<i>E. coli</i> (pP00076)	-	0.024	0.352
<i>P. polymyxa</i> E681	+	2.303	11.842

<sup>a</sup> Assay of ethyl acetate extract of buffer assay without inoculum.

<sup>b</sup> IAAld was detected by TLC for corresponding samples. (+) detected; (-) not detected.

<sup>c,d</sup> TOL and IAA were assayed by quantifying indolic compounds in the ethyl acetate extracts from buffer assays mixed with 10<sup>8</sup> CFU/ml for *E. coli* strains; or from bacterial supernatant of cultures cultivated in Trp-supplemented DM medium at 30°C for 28 h for *P. polymyxa* E681.



**Fig. 3.** Multiple alignment of deduced amino acid sequence of the ORF PP2\_01257 with the known and biochemically characterized IPDC sequences from *Enterobacter cloacae* (Acc. No. P23234), *Pseudomonas putida* (Acc. No. AAG00523), *Pantoea agglomerans* (Acc. No. P71323), *Azospirillum brasiliense* (Acc. No. P51852), and *Azospirillum lipoferum* (Acc. No. Q93RB7).

The last line in each panel represents the consensus sequence, where amino acids among the aligned amino acid residues denoting identity are marked with an asterisk (\*). Based on the crystal structure analysis of IPDC from *Enterobacter cloacae*, residues lining the active site cavity are underlined, residues binding ThDP are shown in bold, and those involved in substrate binding are indicated by bold and italic letters.

(Table 2). Although one of the ORFs on the genome was annotated as a probable indole-3-pyruvate decarboxylase, three other ORFs also showed the C-terminal region of the deduced amino acid sharing partial similarity with known pyruvate decarboxylases (PDC)/IPDCs from other sequences. Search of the *P. polymyxa* E681 genome with known IPDC sequences was not able to detect any ORF with significant similarity to any of these IPDC sequences, suggesting that the IPDC sequence from E681 strain might be quite different from those from other sources. Therefore, bacterial expression and characterization of these ORF gene products might be an approach for identification of the ORF-coding IPDC protein.

Since the four such deduced amino acid sequences showed not much high similarity to annotated IPDC sequences on GenBank, genes coding for those proteins were amplified, cloned in pUC19 vector, forming recombinant plasmids pP01257, pP01918, pP01914, and p00076. The plasmids were then transformed into *E. coli* DH5 $\alpha$ , resulting in the transformants P01257, P01918, P01914, and P00076, respectively. For qualitative and quantitative analyses of IAA, TOL, and IAAld in the cultures of the transformants, *E. coli* DH5 $\alpha$  was used as the cloning host, because *E. coli* DH5 $\alpha$  did not produce TOL, IAA, and IAAld when cultured in minimal A medium supplemented with thiamine and Trp [3]. Of the four transformants tested by TLC and HPLC analyses of ethyl acetate extracts of buffer-reaction mixtures and Trp-containing minimal A culture, only one transformant (P01257) was found to significantly produce IAAld and TOL (Table 3). The confirmation of one cloned *E. coli* DH5 $\alpha$  conferring a gene (ORF PP2\_01257) from *P. polymyxa* E681 that showed the ability to synthesize significant amounts of IAAld and TOL supported the fact that there was genetic evidence for the existence of the IPA pathway for IAA biosynthesis in E681 strain.

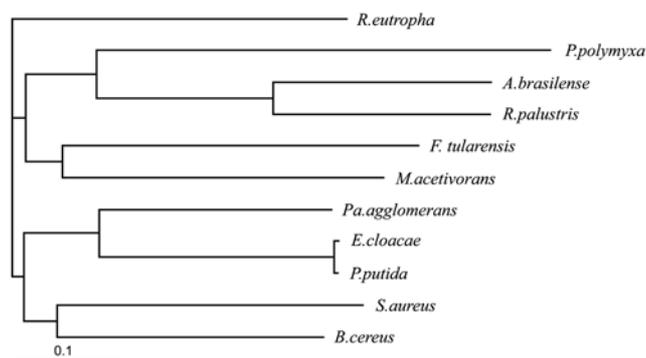
### Sequence Analysis of ORF PP2\_01257

Analysis of the ORF PP2\_01257 sequence from *P. polymyxa* E681 revealed an ORF, having 48.1% of GC content with 1,743 nucleotides that code for a protein of 581 amino acids with deduced molecular mass of 63,380 Da. A GenBank/EMBL search using BLAST found three putative conserved domains in the deduced amino acid sequence of PP2\_01257, in which a thiamine-pyrophosphate-binding domain and the center domain of TTP enzymes containing a 2-fold Rossmann fold showed high similarity with those of other thiamine pyrophosphate (TPP)-dependent proteins. However, the C-terminal domain of the deduced amino acids had a high identity (E-value of 2e-76) with that of the large subunit of acetolactate synthase (acetohydroxyacid synthase) from *Saccharomyces cerevisiae* (Acc. No. 1JSCA). Further search for homology of the C-terminal region amino acid sequence of PP2\_01257 of *P. polymyxa* E681 with the PDC/IPDC subfamily protein showed that this sequence is homologous

to the C-terminal sequence of PDC from *S. cerevisiae* (Acc. No. 1PVDA), IPDC from *Enterobacter cloacae* (Acc. No. 1OVMA), and PDC from *Zymomonas mobilis* (Acc. No. 1ZPDA) with the E-value of about 2e-11.

When a multiple sequence of PP2\_01257 translated with the cloned and characterized IPDC translated sequences was employed by using the CLUSTAL X and MULTALIN tool (<http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html>), several amino acids, which were found to be conserved in the *Enterobacter cloacae* IPDC sequence [27], were also completely conserved (marked \*) or conservatively replaced in the PP2\_01257 ORF sequence (Fig. 3). The multiple sequence alignment otherwise found the higher percentage of conserved homologous amino acids between the translated PP2\_01257 ORF sequence and IPDC from *A. brasiliense* than that between translated PP2\_01257 ORF sequence and IPDC from *E. cloacae*. The deduced amino acid sequence of the PP2\_01257 ORF had 21%, 17%, 20%, and 21% homologies with that from *A. brasiliense*, *E. cloacae*, *P. putida*, and *Bacillus cereus*, respectively.

Phylogenetical analysis showed that the IPDC from *P. polymyxa* E681 was more related closely to that of *A. brasiliense* and *Rhodopseudomonas palustris* (Fig. 4). Comparing the sequence similarities between IPDC characterized from *A. brasiliense* with that from other Gram-negative bacteria, the data showed not much high similarities; 21%, 22%, and 25% to that from *E. cloacae*, *P. agglomerans*, and *P. putida*, respectively. It is, therefore, probable that IPDC appears to be quite divergent among those of Gram-negative bacteria. Besides, this protein is about 543–565 aa in length, whereas that from *P. polymyxa* E681 showed 581 aa, being relatively similar to that from *Ralstonia eutropha* H16 with 583 aa (Acc. No.



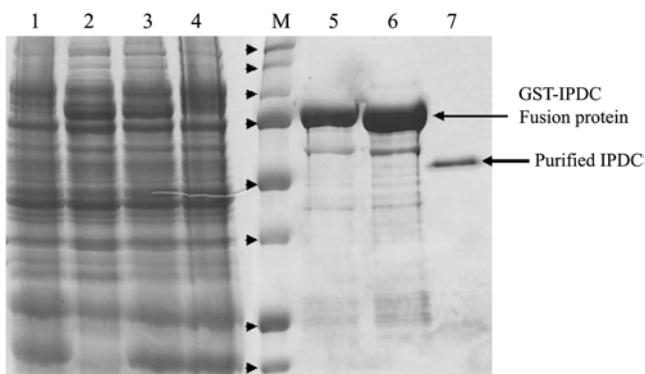
**Fig. 4.** Phylogram of indole-3-pyruvate decarboxylase sequences. The other sequences include *Azospirillum brasiliense* (Acc. No. ABF58692), *Ralstonia eutropha* (Acc. No. YP\_840918), *Francisella tularensis* (Acc. No. YP\_170639), *Rhodopseudomonas palustris* (Acc. No. NP\_948455), *Methanosarcina acetivorans* (Acc. No. AAM04038), *Enterobacter cloacae* (Acc. No. BAA14242), *Staphylococcus aureus* (Acc. No. YP\_001331167), *Bacillus cereus* (Acc. No. EAL11690), *Pseudomonas putida* (Acc. No. AAG00523), and *Pantoea agglomerans* (Acc. No. L80006).

YP\_840918). These evidences indicated that IPDCs may be even more divergent among Gram-positive and Gram-negative bacteria.

### Cloning and Expression of *ipdC* Gene in *E. coli* and Protein Purification

The DNA fragment coding for IPDC amplified from chromosomal DNA of E681 by PCR resulted in a single band of about 1.7 kb on agarose gel, matching with the expected value. After cloning in pGEM-Teasy vector, the insert was then subcloned into pGEX-2T, forming expression plasmid pGEX-2T-*ipdC*, and the correct gene insertion was confirmed by sequencing.

In an attempt to express fusion GST-IPDC protein in *E. coli* BL21 harboring pGEX-2T-*ipdC*, the *E. coli* was cultured at different conditions by changing the parameters of aeration, temperature, induction start time, IPTG concentration, and post-induction time. Almost all the experiments showed aggregation of the recombinant protein into inclusion bodies or low expression of soluble recombinant protein. The protein was finally expressed in *E. coli* at 25°C as a soluble form by induction with 0.05 mM IPTG until OD 600 nm reached to 1.0 (lane 2; Fig. 5), and cells were then harvested at 3 h after induction. Purified fusion protein was then analyzed on SDS-PAGE and showed a relatively pure band with molecular mass of approximately 89 kDa, consistent with that expected for glutathione-S-transferase-IPDC fusion (GST 26 kDa and IPTG 63.38 kDa) (Fig. 5). Enzymatic assay of the purified GST-tagged IPDC protein showed the production of IAA and TOL detectable on TLC and HPLC analyses, which proved this fusion protein as indole-3-pyruvate decarboxylase (data not shown). The purified protein recovered from the affinity column after



**Fig. 5.** SDS-PAGE analysis of the lysate of *E. coli* BL21 harboring pGEX-2T-*ipdC* after induction with IPTG at 25°C (lanes 1–4: IPTG concentration of 0.00, 0.05, 0.10, 0.50 mM, respectively). The fusion protein GST-IPDC was eluted from the Glutathione Sepharose bulk (lane 5 and lane 6), and IPDC was purified after thrombin cleavage (lane 7). Arrowheads indicate molecular mass standards (lane M) at 250, 150, 100, 75, 50, 37, 25, and 15 kDa (top to bottom).

**Table 4.** Enzymatic reactions of recombinant IPDC at various pHs.

pH	IAA <sup>a</sup>	TOL <sup>b</sup>
5.0	9.9±0.7	0.82±0.01
5.5	13.2±0.9	0.79±0.05
6.0	24.4±0.8	1.36±0.04
6.5	32.6±0.9	1.37±0.09
7.0	20.5±0.6	1.40±0.08
7.5	7.5±0.7	0.83±0.05
8.0	5.5±0.6	0.61±0.02

<sup>a,b</sup>Data are means±SD of three separate experiments of enzymatic batch reactions at 28°C for 3 h. Data in μM (calculated for 100 μg of recombinant IPDC protein).

thrombin cleavage showed a single band on SDS-PAGE gel with an apparent molecular mass of about 63 kDa, well corresponding to the one expected. Assay for decarboxylase activity of purified recombinant IPDC confirmed the identity and functionality of the cloned ORF PP2\_01257.

The expression of high-level fusion protein GST-IPDC in *E. coli* provided a feasibility of characterizing the protein of interest. However, a large part of the fusion protein GST-IPDC in this study was expressed in inclusion bodies. All efforts to express soluble and active IPDC in *E. coli* by changing the culture conditions obtained soluble fusion protein, which exhibited the activity of indole-3-pyruvate decarboxylation. Recombinant protein IPDC expressed heterologously in *E. coli* with IPDC activity confirmed the gene product of one ORF from *P. polymyxa* E681. When enzymatic assays were carried out for thrombin-cut purified IPDC in 50 mM phosphate buffer, ranging from pH 5.0 to 8.0, with substrate IPA at 28°C, the highest concentration of TOL and IAA was formed at pH 6.5 (Table 4). In the control without IPDC enzyme, a small amount of substrate IPA was spontaneously degraded to IAA at various pHs. However, the quantification of IAA spontaneously formed from IPA in phosphate buffer was much lower than IAA concentration formed in the reaction supplemented with recombinant IPDC enzyme, suggesting that the activity of recombinant IPDC had an optimal pH of 6.5. Although efforts to make the *ipdC* mutant strain by gene-knockout technique have not yet been successful, the confirmation of the *ipdC* gene product in *E. coli* proved that this gene product has the indole-3-pyruvate decarboxylase activity.

In conclusion, this study suggests that IAA is produced by *P. polymyxa* E681 via only the IPA pathway and is solely dependent on the presence of exogenous Trp. Heterologous expression of an ORF from *P. polymyxa* E681 in *E. coli* exhibited the activity of indole-3-pyruvate decarboxylase, a crucial enzyme in the IPA pathway, showing evidence of genetic material coding for a functional IPDC in the bacterium. Although the production of IPDC has not been found by proteome analysis to change in E681 strain under the conditions of bacterial growing with or without barley

[28], the confirmation of the *ipdC* gene in E681 strain might be helpful for further studies on the associative interactions between E681 and plants, especially the effects of IAA production by the bacterium on plant growth.

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