Biosynthesis of Indole-3-acetic Acid in Ginseng Growth-promoting *Pseudomonas fluorescens* KGPP 207

Leonid N. Ten[1], Mee-Kyoung Lee, Mi Ja Lee* and Hoon Park

Korea Ginseng and Tobacco Research Institute, Taejon 305-345, Korea,
[1]*National University of Uzbekistan, VUZgorodok, Tashkent, 700179, Uzbekistan*

The ginseng growth-promoting bacterium *Pseudomonas fluorescens* KGPP 207 synthesized indole-3-acetic acid (IAA) from *L*-tryptophan, indole-3-pyruvic acid (IPyA), and indole-3-acetaldehyde (IAAld), but not from indole-3-acetamide (IAM) and other intermediates of various IAA biosynthetic pathways in the experiment with indole compound supplemented cell suspensions. TLC, HPLC, and GC-MS analyses revealed the presence of IPyA, indole-3-ethanol, indole-3-lactic acid and its methyl ester, IAA and its methyl, and ethyl esters in the culture supernatant of the bacterium. IAAld was detected in the supernatant using sodium bisulfite and TLC. The results indicate that unlike gall-forming bacteria which can synthesize IAA by IAM, the indole-3-pyruvic acid pathway is the route for IAA biosynthesis in this beneficial strain of *P. fluorescens.*

Key words: *indole-3-acetic acid, Pseudomonas fluorescens, indole-3-pyruvic acid pathway.*

Production of the phytohormone IAA is widespread among microorganisms that are commonly found in plant surfaces. Plant growth-regulating substances produced by bacteria could increase growth rates and improve yields of the host plant.[1] On the other hand, bacterial production of IAA is involved with the virulence of several interactions between microorganisms and plants.[2] Many pathways for the biosynthesis of IAA were reported (Fig. 1). The production of IAA via IAM was well- described in the gall-forming bacteria, for example, *Pseudomonas syringae* pv. *savastanoi*[3], and in the bean leaf spot pathogen *P. syringae* pv. *syringae*[4]. The presence of this pathway, which determines the virulence of certain plant pathogenic bacteria causing hyperplasia[5], is uncommon in plants. The biosynthesis of IAA via IPyA and IAAld was proposed to occur in several bacteria including *Pseudomonas solanacearum* and *Azospirillium brasilense*, and this pathway is predominant in higher plants.[6-9] Tryptophan can also be converted to IAAld and IAA via intermediate TAM.[7] Though the production of IAA via IAN was identified in higher plants, it was mostly excluded in discussions of microbially produced IAA.[7] A biosynthetic pathway of IAA production might be important in determining the effect of a

bacterium on a plant.[8]

The ability of *P. fluorescens* KGPP 207 to promote the ginseng growth and to produce auxins and auxin-like compounds were reported.[10] In this paper, we describe the presence of the IPyA pathway for IAA biosynthesis in this strain of *P. fluorescens.*

Materials and Methods

Chemicals. Chemicals were purchased from Sigma Chemical Co. unless otherwise noted. Agar and proteose peptone were obtained from Difco Laboratories. Silica gel (70~230 mesh, CC grade) was purchased from Merck. All other reagents were reagent grade unless otherwise specified.

Bacterium strain and growth conditions. Strain KGPP 207 of *P. fluorescens* was isolated from the soil of productive ginseng field.[11,12] The bacterium was grown in a 1-*l* Erlenmeyer flask containing 200 m*l* of King B medium[13] on a shaker at 28°C for six days.

The feeding of bacterium cells by indole derivatives. The routes for IAA biosynthesis from Trp were investigated by feeding a concentrated bacterial cell suspension with Trp or other indole derivatives (IPyA, IAAld, ILA, IAM, IAN, TOL, TAM) and determining the metabolic intermediates released into the supernatant.[14] Bacterium was grown as described above. The cells were then centrifuged from the culture medium at 10,000 × g for 15 min at 4°C, washed with 0.01 M phosphate- buffered saline (PBS, pH 7.2), and resuspended in the feeding solution. The experiment was carried out in a 250-m*l* Erlenmeyer flask containing fresh cells (25 g) and feeding solution composed of 2 mM PBS,

*Corresponding author
Phone: 82-42-866-5552; Fax: 82-42-861-1949
E-mail: mjlee@gtr.kgtri.re.kr
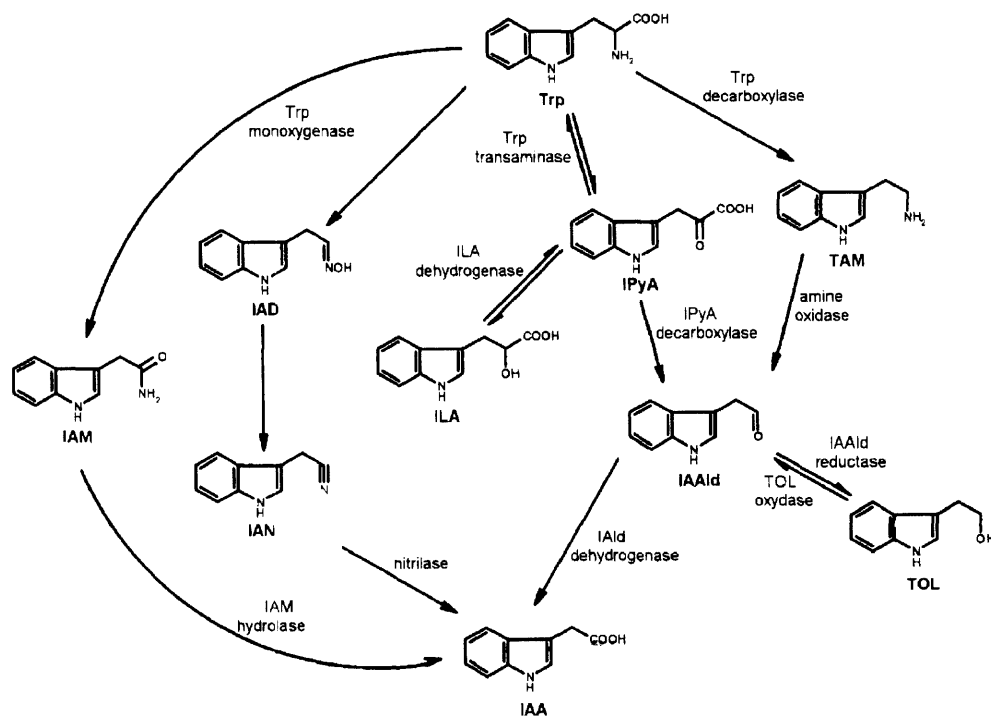
Fig. 1. Biosynthetic pathway of IAA formation. The pathways shown were implicated in both plant and microbial IAA biosyntheses.[9]

30 mM Tricine/KOH buffer(pH 8.3), 2.5 mM D-glucose, 0.2 mM MgSO$_4$, 50 μg/m*l* streptomycin, 50 μg/m*l* penicillin, 2.7 mM cetyltrimethylammonium bromide (Aldrich), and 2 mM Trp or other indole derivatives in a total volume of 90 m*l*. Flasks were incubated at 28°C for two days in the dark on a shaker. The cells were then removed by centrifugation at 10,000 × g for 15 min.

Extraction and separation of IAA and other indole compounds. The supernatant was acidified to pH 2.8 with 30% phosphoric acid and extracted twice with an equal volume of ethyl acetate (EtOAc). The EtOAc extracts were pooled and evaporated *in vacuo* at 30°C (acidic fraction). The supernatant was adjusted to pH 10 with sodium carbonate and extracted twice with EtOAc, and the solvent was evaporated (basic fraction). The acidic fraction was dissolved in 0.5 m*l* acetone containing 1% butylated hydroxytoluene (BHT) and chromatographed on a silica gel 60 column (2 × 12 cm). The column was washed three times with each of the following mixture of hexane/EtOAc [v/v: (I) 38 : 12, (II) 51 : 24, (III) 45 : 30, (IV) 33 : 42, (V) 21 : 54, and (VI) 12 : 60] with three parts of methanol and twice with 25 m*l* of methanol. The various fractions of each solvent were combined, reduced to dryness *in vacuo*, and dissolved in 0.3 m*l* acetone containing 1% BHT. The resultant solutions were analyzed for the presence of indole metabolites by GS-MS. IAAMe, IAAEt, and IAA were eluted in fraction I, ILAMe, ILA and IPyA in fraction IV, and Trp in fraction VI. Basic fraction was applied on the same silica gel column, and hexane/EtOAc (4 : 1, v/v, 100 m*l*) was used for washing. The eluent was analyzed by

GS-MS, and TOL was identified as the indole compound.

Thin layer chromatography. Initial identification of indole metabolites was carried out by TLC on silica gel-covered (0.25 mm) glass plates with authentic standards in two different solvents: (i) EtOAc-chloroform-formic acid (55 : 35 : 10, v/v) (ECF solvent) and (ii) EtOAc-isopropanol -ammonium hydroxide (45 : 35 : 20, v/v) (EIA solvent). The chromatograms were developed by spraying with Ehmann's reagent[15], followed by heating at 200°C.

Detection of IAAld. IAAld was trapped with sodium bisulfite (NaHSO$_3$) in dense suspensions of *P. fluorescens* KGPP 207 according to the method[16], with the exception that the cell suspension supernatant was adjusted to pH 2.5 following incubation and passed through a C$_{18}$ Sep-Pak Cartridge (Waters, Millipore) that was previously equilibrated with 10 m*l* methanol and washed with 10 m*l* of deionized water. After the supernatant was passed through the cartridge, it was rinsed with 10 m*l* of deionized water, and the indoles were then eluted in 2.5 m*l* methanol. A fraction of the purified sample was examined immediately for the presence of IAAld by TLC using the ECF solvent system.

High performance liquid chromatography. The complete HP1100 series HPLC system (Hewlett Packard) was used for quantitative analysis of IAA and the related compounds. HPLC was carried out on Spherisorb ODS-2 (3 μm, 150 mm × 4.6 mm i.d.) main column and Lichrosorb 5 RP-18 (5 μm, 60 mm × 10 mm i.d) guard column. The columns were eluted with water-acetic acid-acetonitrile (76 : 1 : 23) or water-acetic acid-acetonitrile (70.5 : 0.5 : 29)

Table 1. The conversion of indole compounds into IAA by cell suspensions of *P. fluorescens* KGPP 207.

Indole compounds[a]	Cell suspension[b]	IAA concentration (µg/m*l*)
No indole added	+	0.6-0.8
Trp	+	1.4-1.7
IPyA	+	2.4-2.8
IAAld	+	2.8-3.1
ILA	+	0.9-1.2
TOL	+	1.0-1.3
TAM	+	0.5-0.7
IAM	+	0.4-0.6
IAN	+	0.3-0.5
IPyA	-	0.1-0.2
IAAld	-	0.2-0.3
Trp	-	0.1-0.2

[a]2.7 mM Trp or 2 mM of each indole compound was in the assay solution.
[b]Cells were present (+) or absent (-) in the assay solution.

at a flow-rate of 0.6 m*l*/min. Detection was carried out by a photodiode array detector at 254 and 280 nm connected to a fluorometric detector (excitation, 280 nm; emission, 360 nm). The amount of sample injected into the column was 5-10 µ*l* of 50% acetonitrile. IAA and other indole compounds were quantified by reference to the peak area obtained for the respective authentic standards of known concentration.

GC-MS conditions. For the determination of the compounds from fractions I~VI, a GCQ GC/MS instrument with a Gateway 2000 Chemstation (Finnigan, San Jose, CA, USA) was used. MS and the column conditions were as previously described.[10]

Results

Detection of indole compounds in culture. Several indole compounds were produced in the culture of *P. fluorescens* KGPP 207 as described in a previous paper.[10] IAA, Trp, IPyA, ILA, ILAMe, IAAMe, and IAAEt were detected by GC-MS analysis of fractions I~VI from acidic extract, and TOL was identified in fraction I from basic fraction. The substances were identified on the basis of their mass spectra and retention time in accordance with those of the authentic standards. Other indole compounds such as IAM, IAN, and TAM were not detected. When uninoculated medium was incubated, extracted, separated, and derivatized in an identical manner to samples of the culture supernatant and analyzed by GC-MS, no indole compounds other than Trp could be detected.

Metabolism of indole intermediates by cell suspensions. The ability of concentrated bacterial cells to metabolize indole intermediates into IAA could provide evidence for the presence of a metabolic pathway for IAA

biosynthesis. These experiments were performed by permeating the cells with various indole derivatives and detecting the release of IAA into the supernatant. A substantial amount of IAA was produced by *P. fluorescens* KGPP 207 when Trp, IPyA, IAAld, ILA, or TOL were added to the suspended cells (Table 1). IPyA and IAAld were converted to IAA along with the 15~20% increase of TOL and ILA, but ILA and TOL were converted to IAA in a lower level. Incubation of the authentic IPyA or IAAld in the assay solution without suspended cells resulted in a rather low level of IAA, indicating that spontaneous breakdown of these compounds to IAA were minimal under these conditions. No substantial amounts of IAA were detected by TLC or HPLC, when IAM, TAM or IAN was supplied, and these intermediates were not identified in the culture when Trp was added to the cell suspensions. The conversion of Trp, IPyA, and IAAld to substantial amounts of IAA indicates that IPyA and IAAld are main precursors of IAA in the biosynthetic pathway.

Detection of IAAld in suspensions of *P. fluorescens* KGPP 207. IAAld, the precursor of IAA in the IPyA pathway, could not be detected in the supernatant either due to its inherent lability or low steady-state concentration. Therefore, IAAld was trapped with sodium bisulfite in dense suspensions of the bacterium. TLC analysis of the suspension extract revealed the presence of a compound with the same mobility ($R_f = 0.85$ in ECF solvent) and the same brown color upon reaction with Ehmann's reagent as an authentic IAAld standard.

Discussion

TLC, HPLC, and GC-MS analyses revealed the presence of IAA, IAAMe, IAAEt, IAAld, IPyA, ILA, ILAMe, and TOL in the supernatant of *P. fluorescens* KGPP 207. Indole compounds such as IAM, IAN, and TAM were not produced by this strain.

The detection of IPyA and IAAld among these substances and the bioassay with suspended cells are evidences that the IPyA biosynthetic pathway (Fig. 1) is the route for IAA synthesis in the investigated strain of *P. fluorescens*. There is no indication of the existence of the IAM pathway in the bacterium, since IAM-permeated cells did not produce IAA and IAM was not detected in the supernatant. It also seems very unlikely that IAA synthesis via pathways involving IAN or TAM are present in this bacterium based on the results of the indole compound determination and bioassays with the suspended cells. The release of ILA and TOL into the culture media of this strain of *P. fluorescens* supports the presence of IPyA biosynthetic pathway because ILA and TOL are reduced forms of IPyA and IAAld, respectively, and they are generally considered as storage products of these compounds. It is possible that IAAMe, IAAEt, and ILAMe identified in the culture of *P. fluorescens* are the storage forms of IAA and ILA, and they are involved in the

regulation of IAA production.

In contrast to certain plant pathogenic bacteria that synthesize IAA via IAM such as *P. syringae* pv. *savastanoi*[2] and *P. syringae* pv. *syringae*[3], *P. fluorescens* KGPP 207 produces IAA primarily via IPyA, a pathway found commonly in many plants but also in some plant-associated bacteria.[7] The fact that nonpathogenic bacteria produce IAA by IPyA and IAAld, and not by IAM like many IAA-producing phytopathogenic bacteria do, suggests that IPyA pathway has a role distinct from pathogenicity. Its presence in the plant-associated bacteria such as *P. fluorescens* is probably beneficial to their growth and interaction with plants.[5, 8] These results support the practical applicability of this bacterium for the promotion of ginseng root growth.

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