Elucidation of the Indole-3-acetic Acid Biosynthetic Pathway
via Indole-3-pyruvic acid in Enterobacter cloacae.

根圏微生物エンテロバクター・クロアカにおけるインドールピルビン酸
を経由するIAA生合成経路の解明

Jinichiro Koga

Elucidation of the Indole-3-acetic Acid Biosynthetic Pathway

via Indole-3-pyruvic Acid in <u>Enterobacter</u> <u>cloacae</u>.

Jinichiro Koga

Bio Science Laboratories, Meiji Seika Kaisha, Ltd.

CONTENTS

List of Abbreviations

DNA	deoxyribonucleic acid
FPLC	first protein liquid chromatography
HPLC	high performance liquid chromatography
IAA	indole-3-acetic acid
IAAld	indole-3-acetaldehyde
IAAm	indole-3-acetamide
IAn	indole-3-acetonitrile
ILA	indole-3-lactic acid
IPDC	indolepyruvate decarboxylase
IPyA	indole-3-pyruvic acid
kb	kilobase pair
NTG	N-methyl-N'-nitro-N-nitrosoguanidine
ODS	octadesyl silanized silica gel
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Tam	tryptamine
Ti plasmid	tumor-inducing plasmid
T-DNA	DNA transferred from the Ti plasmid to a plant cell
Tol	tryptophol
TPP	thiamine pyrophosphate
L-Trp	L-tryptophan
D-Trp	D-tryptophan

GENERAL INTRODUCTION

1. Interactions between microorganisms and their host plants

There have been several reports that plant hormones produced by bacteria can increase the growth rates and improve the yields of their host plants (Barea and Brown 1974; Brown 1976; Tien et al. 1979). <u>Azospirillum</u> <u>brasilense</u> is able to produce plant hormones, such as indole-3-acetic acid (IAA), cytokinin and gibberellin, and the stimulatory effects of <u>A.</u> <u>brasilense</u> on plant growth seem to involve the effects of these plant hormones on root growth.

It has been shown that bacterial production of IAA is involved in the pathogenic effects of several interactions between microorganisms and plants (Morris 1986). For example, two well studied phytopathogens, <u>Pseudomonas</u> <u>syringae</u> pv. <u>savastanoi</u> and <u>Agrobacterium</u> <u>tumefaciens</u>, induce hyperplasias on susceptible plant hosts. Gall formation on olive oleander, caused by <u>P.</u> <u>savastanoi</u>, has been shown to be dependent on bacterial synthesis of IAA (Smidt and Kosuge 1978). <u>A.</u> <u>tumefaciens</u> causes crown gall disease in dicotyledonous hosts. The formation of crown galls is associated with the presence of a portion of the Ti plasmid (Liu et al. 1982), the T-DNA, which is transferred to plant cells and integrated into the plant nuclear DNA. Two of the genes on T-DNA encode for enzymes involved in the IAA biosynthesis (Schröder et al. 1984; Thomashow et al. 1984; Follin et al. 1985).

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2. General review of IAA biosynthetic pathway in plants and bacteria

IAA is a typical plant hormone that controls several aspects of plant growth and development. Although much is known about the effects of IAA and its role in the control of growth and differentiation, little is known about the biochemistry of the IAA synthesis and its regulation since the main biosynthetic pathway has not been fully elucidated in plants (Sheldrake 1973).

In general, the following are the best characterized IAA biosynthetic pathways leading from L-tryptophan (L-Trp) to IAA (Sheldrake 1973): indole-3-pyruvic acid (IPyA) pathway (L-Trp $\rightarrow$ IPyA $\rightarrow$ indole-3-acetaldehyde [IAAld] $\rightarrow$ IAA), tryptamine (Tam) pathway (L-Trp $\rightarrow$ Tam $\rightarrow$ IAAld $\rightarrow$ IAA) and indole-3-acetamide (IAAm) pathway (L-Trp $\rightarrow$ IAAm $\rightarrow$ IAA).

The IAAm pathway has been studied in detail in P. savastanoi (Kosuge et al. 1966; Comai and Kosuge 1980; Comai and Kosuge 1982), in A. tumefaciens (Schröder et al. 1984; Thomashow et al. 1984; Follin et al. 1985; Thomashow et al. 1986) and in Bradyrhizobium japonicum (Sekine et al. 1988; Sekine et al. 1989). In the IAAm pathway, tryptophan 2-monooxygenase converts L-Trp to IAAm and then indole-3-acetamide hydrolase catalyzes the conversion of IAAm to IAA. Tryptophan 2-monooxygenase in P. savastanoi has been purified and characterized in an attempt to determine its role in the IAA biosynthesis. This enzyme has high affinity for L-Trp and its activity is inhibit-

ed by IAAm and IAA. Thus, it appears that the IAAm pathway in
P. savastanoi is regulated by tryptophan 2-monooxygenase
(Kosuge et al. 1966; Hutcheson and Kosuge 1985).

The genes coding for the enzymes in the IAAm pathway have
been isolated and characterized in detail. The genes for tryp-
tophan 2-monooxygenase and indole-3-acetamide hydrolase in P.
savastanoi are functional only in bacteria (Comai and Kosuge
1980, 1982), whereas the comparable genes in A. tumefaciens are
functional only in plants (Schröder et al. 1984; Thomashow et
al. 1984; Follin et al. 1985). Furthermore, the nucleotide
sequences of these genes from P. savastanoi and B. japonicum
exhibit significant homology to the sequences from A. tumefaci-
ens (Yamada et al. 1985; Sekine et al. 1990).

Although the IAAm pathway was thought to exist only in
bacteria, it was recently identified in Trifoliata Orange
(Kawaguchi et al. 1993). Since the rate-limiting step in this
pathway is the conversion of $_L$-Trp to IAAm, which is catalyzed
by tryptophan 2-monooxygenase, it is difficult to detect IAAm.
Therefore, Kawaguchi established an assay system for the detec-
tion of the conversion of $_L$-Trp to IAAm using α-naphthalene
acetamide as a competitor of indole-3-acetamide hydrolase.

3. The IAA biosynthetic pathway from $_L$-Trp via IPyA and the aim of this study

Although the IPyA pathway is thought to be the main IAA
biosynthetic pathway in plants, the exact mechanism of this

pathway has not been unequivocally demonstrated yet (Sheldrake 1973).

The first step in this pathway is the conversion of L-Trp to IPyA, which is catalyzed by L-tryptophan aminotransferase. This enzyme, has been found in many species of plants and bacteria (Gunsalus and Stamer 1955; Truelsen 1973; Paris and Magasanik 1981), is nonspecific for amino acid substrates, and has a very high K_m value for L-Trp (Gamborg and Wetter 1963; Truelsen 1972). The IAA concentration in plants is maintained in the nanomolar range, and it is unlikely that such low levels of IAA are regulated by L-tryptophan aminotransferase with its low affinity for L-Trp (Law 1987).

The third step in the IPyA pathway is the conversion of IAAld to IAA, which is catalyzed by indole-3-acetaldehyde oxidase. Kenten and Mann suggested that IAAld is oxidized to IAA by an aldehyde oxidase in plants (Kenten and Mann 1952). The majority of bacteria produce little IAA, but they are capable of converting IAAld to IAA (Koga et al. 1991). Thus, in plants and bacteria, there is no evidence to suggest that the first and third enzymes in the IPyA pathway are involved in the production of IAA from L-Trp.

Little is known about the second enzyme in the IPyA pathway, indolepyruvate decarboxylase, which catalyzes the conversion of IPyA to IAAld. Both IPyA and IAAld are unstable compounds and difficult to isolate (Moore and Shaner 1968; Gibson et al. 1972; Garcia-Tabares et al. 1987). Furthermore, since

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IPyA is degraded nonenzymatically into IAA, it is difficult to demonstrate that the second reaction is catalyzed enzymatically in biological systems (Kaper and Verdstra 1958; Sheldrake 1973). In consequence, it seems likely that the control of the IPyA pathway does not involve any very specific regulatory mechanisms at the enzymatic level.

The aim of this study has been to elucidate the mechanism of the IPyA pathway in E. cloacae at the enzymatic and genetic level. Our efforts will facilitate elucidation of the IPyA pathway in plants and may provide some insight into the bio-chemistry of the IAA synthesis and its regulation.

References

Barea, J.M. and Brown, M.E. (1974) Effects on plant growth produced by Azotobacter paspali related to synthesis of plant growth regulating substances. J. Appl. Bacteriol. **40**: 583-593.

Brown, M.E. (1976) Role of Azotobacter paspali in association with Paspalum notatum. J. Appl. Bacteriol. **40**: 341-348.

Comai, L. and Kosuge, T. (1980) Involvement of plasmid deoxyribonucleic acid in indoleacetic acid synthesis in Pseudomonas savastanoi J. Bacteriol. **143**: 950-957.

Comai, L. and Kosuge, T. (1982) Cloning and characterization of iaaM, a virulence determinant of Pseudomonas savastanoi. J. Bacteriol. **149**: 40-46.

Follin, A., Inzé, D., Budar, F., Genetello, C., Van Montagu, M. and Schell, J. (1985) Genetic evidence that the tryptophan 2-monooxygenase gene of Pseudomonas savastanoi is function-ally equivalent to one of the T-DNA genes involved in plant tumor formation by Agrobacterium tumefaciens. Mol. Gen. Genet. **201**: 178-185.

Gamborg, O.L. and Wetter, L.R. (1963) An aromatic amino acid transaminase from mung bean. Can. J. Biochem. Physiol. **41**: 1733-1740.

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Garcia-Tabares, F., Herraiz-Tomico, T., Amat-Guerri, F. and Garcia-Bilbao, L.J. (1987) Production of 3-indoleacetic acid and 3-indolelactic acid in <u>Azotobacter</u> <u>vinelandii</u> cultures supplemented with tryptophan. Appl. Microbiol. Biotechnol. **25**: 502-506.

Gibson, R.A., Schneider, E.A. and Wightman, F. (1972) Biosynthesis and metabolism of indole-3yl-acetic acid. II. <u>In</u> <u>vivo</u> experiments with ^{14}C-labelled precursors of IAA in tomato and barley shoots. J. Exp. Bot. **23**: 381-399.

Gunsalus, I.C. and Stamer, J.R. (1955) Transaminases in bacteria. In: Colowick, S.P., Kaplan, N.O. (eds) Methods in Enzymolgy, Vol. 2. Academic Press, New York, pp 170-177.

Hutcheson, S.W. and Kosuge, T. (1985) Regulation of 3-indoleacetic acid production in <u>Pseudomonas</u> <u>syringae</u> pv. <u>savastanoi</u>. J. Biol. Chem. **260:** 6281-6287.

Kaper, J.M. and Verdstra, H. (1958) On the metabolism of tryptophan by <u>Agrobacterium</u> <u>tumefaciens</u>. Biochim. Biophys. Acta. **30:** 401-420.

Kawaguchi, M., Fujioka, S., Sakurai, A., Yamaki, Y.T. and Syōno, K. (1993) Presence of a pathway for the biosynthesis of auxin via indole-3-acetamide in Trifoliata Orange. Plant Cell

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Physiol. **34:** 121-128.

Kenten, R.H. and Mann, P.J.G. (1952) The oxidation of amines by extracts of pea seedlings. Biochem. J. **50:** 360-369.

Koga, J., Adachi, T. and Hidaka, H. (1991) Molecular cloning of the gene for indolepyruvate decarboxylase from <u>Enterobacter cloacae</u>. Mol. Gen. Genet. **226:** 10-16.

Kosuge, T., Heskett, M.G. and Wilson, E.E. (1966) Microbial synthesis and degradation of indole-3-acetic acid. J. Biol. Chem. **241:** 3738-3744.

Law, D.M.(1987) Gibberellin-enhanced indole-3-acetic acid biosynthesis: D-Tryptophan as the precursor of indole-3-acetic acid. Physiol. Plant. **70:** 626-632.

Liu, S.T., Perry, K.L., Schardl, C.L. and Kado, C.I. (1982) <u>Agrobacterium</u> Ti plasmid indoleacetic acid gene is required for crown gall oncogenesis. Proc. Natl. Acad. Sci. USA **79:** 2812-2816.

Moore, T.C. and Shaner, C.A. (1968) Synthesis of indoleacetic acid from tryptophan via indolepyruvic acid in cell free extracts of pea seedlings. Arch. Biochem. Biophys. **127:** 613-621.

Morris, R.O. (1986) Genes specifying auxin and cytokinin biosynthesis in phytopathogens. Annu. Rev. Plant Physiol. **35**: 509-538.

Paris, C.G. and Magasanik, B. (1981) Tryptophan metabolism in Klebsiella aerogenes: regulation of the utilization of aromatic amino acids as sources of nitrogen. J. Bacteriol. **145**: 257-265.

Schröder, G., Waffenschmidt, S., Weiler, E.W. and Schröder, J. (1984) The T-region of Ti plasmid codes for an enzyme synthesizing indole-3-acetic acid. Eur. J. Biochem. **138**: 387-391.

Sekine, M., Ichikawa, T., Kuga, N., Kobayashi, M., Sakurai, A and Syōno, K. (1988) Detection of the IAA biosynthetic pathway from tryptophan via indole-3-acetamide in Bradyrhizobium spp. Plant Cell Physiol. **29**: 867-874.

Sekine, M., Watanabe, K. and Syōno, K. (1989) Molecular cloning of a gene for indole-3-acetamide hydrolase from Bradyrhizobium japonicum. J. Bacteriol. **171**: 1718-1724.

Sekine, M., Watanabe, K. and Syōno, K. (1990) Nucleotide sequence of a gene for indole-3-acetamide hydrolase from Bradyrhizobium japonicum. Nucleic. Acids. Res. **17**: 6400-6400.

Sheldrake, A.R. (1973) The production of hormones in higher plants. Biol. Rev. Camb. Philos. Soc. **8:** 510-515.

Smidt, M. and Kosuge, T. (1978) The role of indole-3-acetic acid accumulation by alpha methyl tryptophan-resistant mutants of <u>Pseudomonas</u> <u>savastanoi</u> in gall formation on oleanders. Physiol. Plant Pathol. **13:** 203-214.

Thomashow, L.S., Reeves, S and Thomashow, M.F. (1984) Crown gall oncogenesis: evidence that a T-DNA gene from the <u>Agrobacterium</u> Ti plasmid pTiA6 encodes an enzyme that catalyzes synthesis of indoleacetic acid. Proc. Natl. Acad. Sci. USA **81:** 5071-5075.

Thomashow, M.F., Hugly, S., Buchholz, W.G. and Thomashow, L.S. (1986) Molecular basis for the auxin-independent phenotype of crown gall tumor tissues. Science **231:** 616-618.

Tien, T.M., Gaskins, M.H. and Hubbell, D.H. (1979) Plant growth substances produced by <u>Azospirillum</u> <u>brasilense</u> and their effect on the growth of Pearl Millet. Appl. Environ. Microbiol. **37:** 1016-1024.

Truelsen, T.A. (1972) Indole-3-pyruvic acid as an intermediate in the conversion of tryptophan to indole-3-acetic acid. I. Some characteristics of tryptophan transaminase from mung bean

seedlings. Physiol. Plant. **26**: 289-295.

Truelsen, T.A. (1973) Indole-3-pyruvic acid as a intermediate in the conversion of tryptophan to indole-3-acetic acid. II. Distribution of tryptophan transaminase activity in plants. Physiol. Plant. **28**: 67-70.

Yamada, T., Palm, C.J., Brooks, B. and Kosuge, T. (1985) Nucleotide sequences of the <u>Pseudomonas</u> <u>savastanoi</u> indoleacetic acid genes show homology with <u>Agrobacterium</u> <u>tumefaciens</u> T-DNA. Proc. Natl. Acad. Sci. USA. **82**: 6522-6526.

CHAPTER 1

Effects of <u>Enterobacter</u> <u>cloacae</u> on the Growth of Cucumber
and Rice Plants.

Abstract

A microorganism, strain SAI-6010, was isolated from the
rhizosphere of actively growing cucumber plants. From physio-
logical properties, strain SAI-6010 was identified as a strain
of <u>Enterobacter</u> <u>cloacae</u>. Shoots and roots of both cucumber and
rice plants treated with <u>E. cloacae</u> were significantly larger
than those of plants without addition of <u>E. cloacae</u>, suggesting
that <u>E. cloacae</u> promotes the growth of these plants.

Introduction

There have been several reports that microorganisms iso-
lated from the rhizosphere of plants can increase growth rates
and the yields of host plants (Barea and Brown 1974; Brown
1976; Tien et al. 1979). For the efficiency of crop production
by utilizing such microorganisms, we have screened a large
number of microorganisms promoting plant growth. We found that
<u>Enterobacter</u> <u>cloacae</u> isolated from the rhizosphere of actively
growing cucumber plants promotes the growth of agriculturally
useful plants.

Materials and Methods

Bacterial culture and plant culture

E. cloacae was grown for 24 hours at 30° C in liquid medium that contained 1.0% glucose, 0.5% peptone, 0.1% KH_2PO_4 and 0.05% $MgSO_47H_2O$ (pH 6.8), with shaking on an orbital shaker. The resulting culture was added to soil sterilized by autoclaving for 30 min at 120° C (10^7 cfu per g of soil). Seeds of cucumber (Cucumis sativus L. 'Kifujin') and rice (Oryzae sativa L. 'Japonica') were then planted in the soil that had been inoculated with E. cloacae. For control experiments, seeds were planted in sterilized soil without addition of E. cloacae. After cultivation for 20 days at 25° C, the growth of cucumber and rice plants was assessed by measuring the fresh weights of shoots and roots.

Results and Discussion

A microorganism, strain SAI 6010, was isolated from the rhizosphere of actively growing cucumber plants. As shown in Table 1.1, the strain is a Gram-negative and facultatively anaerobic bacteria. It does not form spores and has peritrichous flagella. In terms of its physiological properties, it is negative to oxidase activity and positive for nitrate reduction. From these properties, this strain appears to belong to the family Enterobacteriaceae. Furthermore, from various other physiological properties, strain SAI-6010 has been identified as a strain of Enterobacter cloacae. The strain has been

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Table 1.1 Morphological and physiological characteristics of strain SAI-6010[a].

Characteristics	Strain SAI-6010
Morphological characteristics	
Shape and size	Straight rods, 0.8 to 1.0 μm wide x 1.5 to 3.0 μm long
Polymorphism	None
Motility	Motile by peritrichous flagella
Spores	None
Gram's stain	-
Acid-fast staining	-
Physiological characteristics	
β-Galactosidase	+
Arginine dihydrolase	+
Lysine decarboxylase	-
Ornithine decarboxylase	+
Citric acid utilization	+
Hydrogen sulfide production	-
Urease	-
Oxidase	-
Tryptophan deaminase	-
Indole production	-
Denitrification reaction	-
Nitrate reduction	+

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Inorganic nitrogen utilization	Nitrates and ammonium salts are utilized as sources of nitrogen
Gelatin liquefaction	Extremely slow liquefaction occurs
MR test	−
VP test	+
O-F test	F type
Starch hydrolysis	−
DNase production	−
Aesculin decomposition	−
Pigment production	−
Growth range	Growth temperature ranges from 15° C to 45° C, with optimal growth from 28° C to 37° C, and the pH for optimal growth is in the vicinity of neutrality.
Requirement for oxygen	Facultative anaerobe
Gas from D-glucose	+
Acid from:	+
D-glucose, sucrose, L-arabinose,	
D-xylose, D-mannose, D-fructose,	
maltose, lactose, D-mannitol,	
D-sorbitol	
Acid from:	
D-adonitol, glycerol	−

ᵃ Symbols: +, positive; −, negative.

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deposited at the Fermentation Research Institute, Agency of Industrial Science and Technology of Japan, Tsukuba-shi, Ibaraki, Japan, with the accession number FERM BP-1529.

As shown in Table 1.2, shoots of both cucumber and rice plants treated with E. cloacae were 5 to 7% heavier than control shoots. Furthermore, roots of both cucumber and rice plants treated with E. cloacae were significantly 15-21% heavier than those of control plants. These results indicate that E. cloacae isolated from actively growing cucumber plants promotes the growth of cucumber and rice plants by stimulating the roots of plants.

E. cloacae is known to produce indole-3-acetic acid which is a typical plant hormone (Koga et al. 1991), and it is also known as a nitrogen-fixing bacterium (Ladha et al. 1983; Neilson and Allard 1985). Therefore, it is possible that E. cloacae promotes plant growth by both hormonal stimulation and enhancement of the supply of nitrogen.

Table 1.2 Effects of <u>Enterobacter</u> <u>cloacae</u> on the growth of cucumber and rice plants[a].

| Condition | Fresh weight of plants (g/plant) | | | |
| | Cucumber | | Rice | |
	Shoot	Root	Shoot	Root
Soil only (control)	3.958(100[b])	0.596(100)	0.259(100)	0.073(100)
Soil + E. cloacae	4.233(107)	0.721[*c](121)	0.272(105)	0.084[*](115)

[a] Twenty cucumber plants and two hundred rice plants were cultivated in plastic pots with <u>E. cloacae</u> for 20 days at 25° C. Similar numbers were used as controls in each case.

[b] Relative rate of fresh weight of plants. A value of 100 was assigned to the fresh weight of plants without addition of <u>E. cloacae</u>.

[c] *Significantly different from control (P<0.05).

References

Barea, J.M. and Brown, M.E. (1974) Effects on plant growth produced by Azotobacter paspali related to synthesis of plant growth regulating substances. J. Appl. Bacteriol. **40**: 583-593.

Brown, M.E. (1976) Role of Azotobacter paspali in association with Paspalum notatum. J. Appl. Bacteriol. **40**: 341-348.

Koga, J., Adachi, T. and Hidaka H. (1991) IAA biosynthetic pathway from tryptophan via indole-3-pyruvic acid in Entero-bacter cloacae. Agric. Biol. Chem. **55**: 701-706.

Ladha, J.K., Barraquio, W.L. and Watanabe, I. (1983) Isolation and identification of nitrogen-fixing Enterobacter cloacae and Klebsiella planticola associated with rice plants. Can. J. Microbiol. **29**: 1301-1308.

Neilson, A.H. and Allard, A.-S. (1985) Acetylene reduction (N_2-fixation) by Enterobacteriaceae isolated from industrial wastewaters and biological treatment systems. Appl. Microbiol. Biotechnol. **23**: 67-74.

Tien, T.M., Gaskins, M.H. and Hubbell, D.H. (1979) Plant growth substances produced by Azospirillum brasilense and their effect on the growth of Pearl Millet. Appl. Environ. Microbiol. **37**: 1016-1024.

CHAPTER 2

IAA Biosynthetic Pathway from Tryptophan via Indole-3-pyruvic
Acid in Enterobacter cloacae.

Abstract

Indole-3-acetic acid (IAA) was identified as a L-tryptophan
(L-Trp) catabolite in the cultures of Enterobacter cloacae
isolated from the rhizosphere of well-grown cucumbers. This
strain produced IAA in a tryptophan-supplemented liquid medium
at levels of up to 1 mg/ml, and indole-3-lactic acid (ILA) and
tryptophol (Tol) were also produced. Under aerobic conditions,
IAA was produced in higher concentrations than ILA and Tol, but
under less aerobic conditions, ILA and Tol were produced in
higher concentrations than IAA. In metabolic studies with eight
kinds of indole substrates, E. cloacae converted L-Trp, indole-
3-pyruvic acid (IPyA), and indole-3-acetaldehyde (IAAld) to
IAA. These results strongly suggest that the biosynthesis of
IAA in E. cloacae involves the pathway of L-Trp to IAA via IPyA
and IAAld.

Introduction

There have been several reports that the plant hormones
produced by bacteria could increase growth rates and improve
yields of the host plants (Barea and Brown 1974; Brown 1976;
Tien et al. 1979). On the other hand, it has been shown that
bacterial production of IAA is involved in the virulence of

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several interactions between microorganisms and plants (Morris 1986). For example, two well-studied phytopathogens, _Pseudomonas syringae_ pv. _savastanoi_ and _Agrobacterium tumefaciens_, incite hyperplasias on susceptible plant hosts. Gall formation on olive oleander caused by _P. savastanoi_ has been shown to be dependent on the bacterial IAA synthesis (Smidt and Kosuge 1978). _A. tumefaciens_ causes crown gall disease in dicotyledonous hosts. The formation of crown galls is associated with the presence of a portion of the Ti plasmid (Liu et al. 1982), the T-DNA, which is transferred and integrated into the plant nuclear DNA. Two of the T-DNA genes encode for IAA biosynthetic enzymes (Schröder et al. 1984; Thomashow et al. 1984; Follin et al. 1985).

In general, the following are the three most characterized pathways for the conversion of $_L$-Trp to IAA: (a) the IPyA pathway ($_L$-Trp $\rightarrow$ IPyA $\rightarrow$ IAAld $\rightarrow$ IAA), (b) the tryptamine (Tam) pathway ($_L$-Trp $\rightarrow$ Tam $\rightarrow$ IAAld $\rightarrow$ IAA), and (c) the indole-3-acetamide (IAAm) pathway ($_L$-Trp $\rightarrow$ IAAm $\rightarrow$ IAA). The IAAm pathway has been studied in detail in _P. savastanoi_ (Kosuge et al. 1966; Comai and Kosuge 1980; Comai and Kosuge 1982), in _A. tumefaciens_ (Schröder et al. 1984; Thomashow et al. 1984; Follin et al. 1985), and in _Bradyrhizobium japonicum_ (Sekine et al. 1988; Sekine et al. 1989). In these pathways, tryptophan 2-monooxygenase converts $_L$-Trp to IAAm and then indole-3-acetamide hydrolase catalyzes the conversion of IAAm to IAA. However, the IPyA pathway, considered the main pathway

in plants, has yet to be elucidated, since IPyA and IAAld, intermediates in the IPyA pathway, are such unstable compounds that it is difficult to isolate them (Moore and Shaner 1968; Gibson et al. 1972; Sheldrake 1973; Garcia-Tabares et al. 1987). Furthermore, IPyA is degraded spontaneously to give various products including IAA, and IAAld is oxidized spontaneously to give IAA. Against these background of spontaneous degradations, it is difficult to obtain evidence for the participation of enzymes in the IPyA pathway (Kaper and Verdstra 1958; Sheldrake 1973).

It has been found that E. cloacae isolated from the rhizosphere of actively growing cucumbers accelerates growth of various kinds of agriculturally useful plants (Koga et al. 1991). This report describes an identification of IAA produced by E. cloacae and its biosynthetic pathway from L-Trp via IPyA and IAAld.

Materials and Methods

Bacterial strains and cultures

The Enterobacter cloacae strain FERM BP-1529 was originally isolated from the rhizosphere of cucumbers (Koga et al. 1991). The strain was maintained on LB medium (Maniatis et al. 1982). For analysis of endogenous indoles, the strain was grown for 2 days at 30° C in liquid LB medium containing 1.0% glucose and 0.2% L-Trp (LBGT medium), shaken with an orbital shaker.

Extraction and purification procedure of endogenous IAA, Tol, and ILA.

Bacterial cultures were centrifuged at 9,000 x g for 20 min at 4°C. The supernatant was adjusted to pH 10 with Na_2CO_3 and extracted with ethylacetate (Tol fraction). The aqueous phase was adjusted to pH 3 with HCl and extracted with ethylacetate (IAA and ILA fractions). The two ethylacetate extracts were dried in vacuo. The residues were dissolved in ethanol and analyzed by HPLC. The HPLC analysis was done by a Toso model CCPM under the following conditions: column, Toso TSK gel-ODS-120A (4.6 by 250 mm) (Toyo Soda Co., Ltd., Tokyo); mobile phase, ethanol-water-acetic acid (30:65:5 vol/vol/vol); flow rate, 0.7 ml/min; and detector, Toso FS-8000 spectrofluorometer (excitation wavelength of 280 nm and emission wavelength of 350 nm). For the ^{1}H-NMR analysis, the fractions corresponding to the retention times of authentic IAA, Tol, and ILA were collected and dried in vacuo to give purified samples.

TLC

The TLC chromatogram was run on silica gel 60 plates (Merck), developed with chloroform-acetic acid (95:5 vol/vol). IAA was detected on TLC plate by spraying with Ehrlich reagent (Bentley 1962).

^{1}H-NMR

^{1}H-NMR spectra were recorded on a JOEL FX-200 in $CDCl_3$

with tetramethylsilane as an internal standard or in D_2O adjusting the signal of H_2O to 4.83 ppm. For each spectrum, the HPLC purified samples obtained from peaks X and Y were dissolved in 0.5 ml $CDCl_3$, and that from peak Z was dissolved in 0.5 ml D_2O.

Conversion of eight kinds of indole substrates to IAA, Tol, and ILA

The $\underline{E.\ cloacae}$ strain was grown for 2 days at 30° C in 10 ml of liquid M9 minimal medium (Maniatis et al. 1982) and 1 ml of 10 mM phosphate buffer (pH 6.5) containing 0.01% $_L$-Trp, IPyA, IAAld, ILA, Tol, Tam, IAAm, or indole-3-acetonitrile (IAn) was added to the cultures. To avoid the decomposition of IPyA, IPyA solution was added to the cultures as soon as possible after it was made. After further incubation for 10 hr at 30° C, the cultures were centrifuged and the supernatants were analyzed by HPLC as described previously.

Effects of oxygen on indole production

We developed a simple bacterial culture method to maintain various oxygen conditions. Glass bottle cultures (200 ml) containing various volumes (from 5 to 80 ml) of LBGT medium were sealed with butyl rubber caps and $\underline{E.\ cloacae}$ was incubated for 2 days at 30° C in the cultures with shaking. After incubation, the cultures were diluted at a rate of 1:10 in a 0.1 N HCl solution and the supernatants were analyzed by HPLC as

described previously.

Results

Identification and measurement of endogenous indoles

An HPLC chromatogram of the supernatant of a culture in a
tryptophan-supplemented medium showed three main peaks (Fig.
2.1). Main peak X, having the same retention time (16.0 min) as
that of authentic IAA, was supposed to correspond to IAA. For
the identification of the structure, a separated sample of peak
X was analyzed by TLC and ^{1}H-NMR. The TLC chromatogram of the
sample showed a clear red spot at the Rf corresponding to
authentic IAA by spraying with Ehrlich reagent (data not
shown), and the ^{1}H-NMR spectrum was identical with that of
authentic IAA (Fig. 2.2). The second main peak Y (retention time,
17.7 min) and third main peak Z (retention time, 12.5 min) were
supposed to correspond to authentic Tol and ILA, respectively.
By the ^{1}H-NMR analysis, samples obtained from peaks Y and Z
were identical with authentic Tol and ILA, respectively (Fig.
2.2). The production of IAA increased with increasing
concentration of $_L$-Trp from 0.001 to 1 mg/ml in culture medium,
resulting in an extracellular level of about 1 mg/ml. No
appreciable amount of IPyA, IAAld, Tam, IAAm, or IAn, was
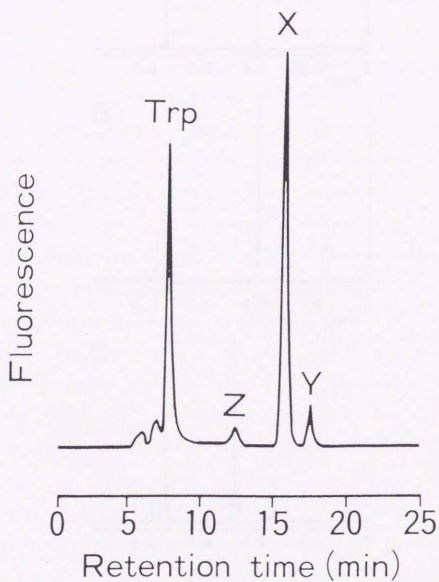detected as a catabolite of $_L$-Trp in the cultures.

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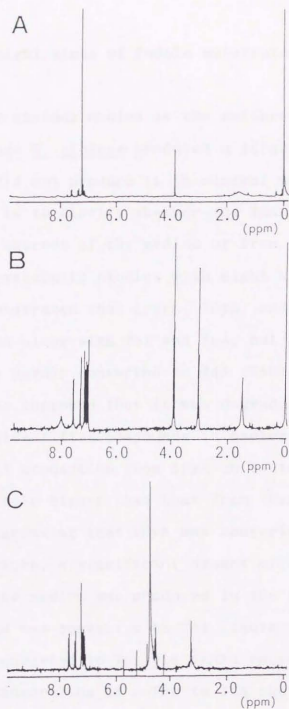Fig. 2.1 HPLC chromatogram of extracts from E. cloacae cultures in LBGT medium.

Fig. 2.2 ¹H-NMR analysis of endogenous indoles obtained from
E. cloacae cultures. (A) Purified sample obtained from peak X.
(B) Purified sample obtained from peak Y. (C) Purified sample
obtained from peak Z.

Conversion of eight kinds of indole substrates to IAA, Tol, and ILA

We used M9 minimal medium as the culture medium in this experiment, since _E. cloacae_ produced a large amount of IAA in LB medium but did not produce it in minimal medium. The purpose of this method is to clarify whether the IAA production was derived from N sources of the medium or from the indole substrate. The metabolic studies with eight kinds of indole substrates demonstrated that L-Trp, IPyA, and IAAld were converted to IAA along with Tol and ILA, but the other five substrates were hardly converted to IAA (Table 2.1). IPyA was such an unstable compound that it was degraded spontaneously into IAA and unidentified compounds in bacteria-free medium. However, the IAA production from IPyA in bacterial culture medium was ten-fold higher than that from IPyA in the bacteria-free medium, suggesting that IPyA was converted to IAA enzymatically. Furthermore, a significant amount of Tol undetected in the bacteria-free medium was produced in the bacterial culture medium and IAAld was converted to Tol (Table 2.1), suggesting that IPyA was converted to Tol _via_ IAAld enzymatically. In the same way, the conversions of L-Trp to ILA and IPyA to ILA suggest that L-Trp was converted to ILA _via_ IPyA. In conclusion, these results indicate that L-Trp was converted to IAA _via_ IPyA and IAAld in this pathway.

Table 2.1 Conversion of eight kinds of indole substrates to
IAA, Tol, and ILA.

Indole substrate[a]	Indole production (μg/ml)		
	IAA	Tol	ILA
L-Trp	8.5	0.8	0.3
IPyA	7.8	0.5	0.3
IAAld	4.5	4.4	ND[b]
ILA	0.3	ND	—
Tol	0.9	—	ND
Tam	ND	ND	ND
IAAm	0.4	ND	ND
IAn	ND	ND	ND
None	ND	ND	ND

[a] Indole substrate was added to the cultures
(10 μg/ml).

[b] ND, Not detectable.

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Effects of oxygen on indole production

The production of IAA, Tol, and ILA by E. cloacae was examined in various volumes of liquid medium. Since total volumes of oxygen are limited in the butyl rubber capped cultures, the growth conditions of a large volume of culture medium become less aerobically. As shown in Fig. 2.3, under more aerobic conditions, IAA was produced in higher concentrations than ILA and Tol. However, under less aerobic conditions, ILA and Tol were produced in higher concentrations than IAA, and total production of the indoles decreased. These results suggest that the composition of the indole metabolites depends on the amount of oxygen in the cultures. Metabolic studies show that IPyA and IAAld brought about corresponding reduction products, ILA and Tol, respectively (Table 2.1). Furthermore, under less aerobic conditions, ILA and Tol were produced in higher concentrations than those under aerobic conditions (Table 2.2). These results indicate that the IAA production is regulated by oxidative-reductive reactions. Under more aerobic conditions, IAA is produced by oxidative reactions, and under less aerobic conditions, ILA and Tol are reductively produced from IPyA and IAAld, respectively.

Discussion

This is the first report on the detection of the IAA bio-synthetic pathway from L-Trp via IPyA and IAAld in E. cloacae. HPLC, TLC, and ^{1}H-NMR analysis showed that E. cloacae produced
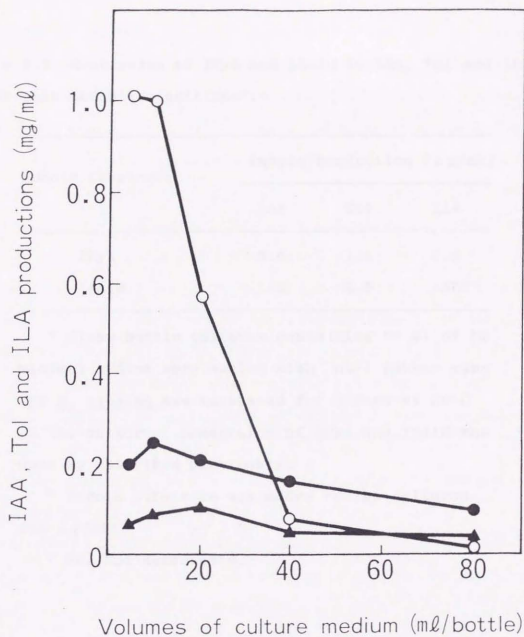
Fig. 2.3 Effects of oxygen on production of IAA, Tol, and ILA
in various volumes of liquid medium. Symbols: ○, production of
IAA; ●, production of Tol; ▲, production of ILA.

Table 2.2 Conversion of IPyA and IAAld to IAA, Tol and ILA
under less aerobic conditions[a].

Indole substrate[b]	Indole production (μg/ml)		
	IAA	Tol	ILA
IPyA	3.0	1.4	0.6
IAAld	1.9	6.5	ND[c]

[a] Glass bottle cultures containing 20 ml of M9
minimal medium were sealed with butyl rubber caps
and E. cloacae was incubated for 2 days at 30° C
in the cultures. Conversion of IPyA and IAAld was
done as described previously.

[b] Indole substrate was added to the cultures
(10 μg/ml).

[c] ND, Not detectable.

a large amount of IAA. Metabolic studies, showing the major
conversion (50-90%) of ʟ-Trp, IPyA, and IAAld to IAA, suggested
that the main biosynthesis of IAA in E. cloacae involves the
IPyA pathway, which is different from that found in Pseudomonas
savastanoi and Agrobacterium tumefaciens. However, the fact
that a small quantity of IAAm was converted to IAA (Table 2.1)
suggests the simultaneous presence of the IAAm pathway. We
could not detect IPyA and IAAld as catabolites of ʟ-Trp in E.
cloacae, since IPyA and IAAld are such unstable compounds that
they are supposed to be reduced to ILA and Tol, respectively.
In cultural experiments by controlling the amount of oxygen,
IAA was produced in higher concentrations than ILA and Tol
under aerobic conditions. Under less aerobic conditions,
however, ILA and Tol were produced in higher concentrations
than IAA, and total production of the indoles decreased. This
experiment indicates that the metabolism of ʟ-Trp was affected
by the amount of oxygen in the cultures.

A scheme of the IAA biosynthetic pathway deduced from
these results is shown in Fig. 2.4. The IAA production in E.
cloacae is supposed to be regulated by oxidative-reductive
reactions as follows: while under oxidative conditions, a large
amount of IAA is produced by the main IPyA pathway (ʟ-Trp →
IPyA → IAAld → IAA), under reductive conditions, IAA produc-
tion decreases, and ILA and Tol are accumulated. It is because
the enzymes involved in the main pathway are inactivated, and
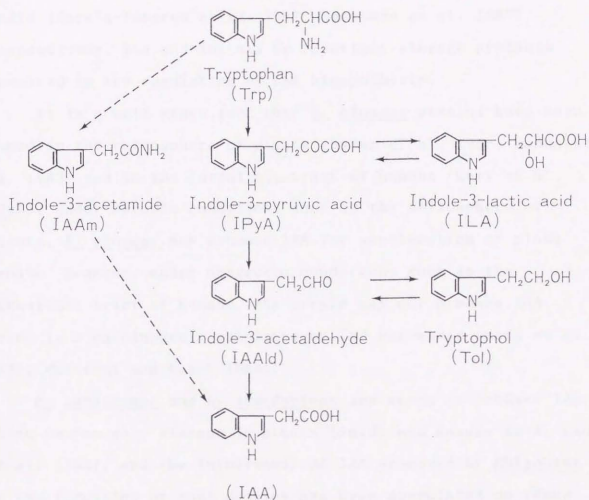indolelactate dehydrogenase (Kaper and Verdstra 1958; Jean and

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Fig. 2.4 A scheme of the IAA biosynthetic pathway in
E. cloacae.

De Moss 1968; Garcia-Tabares et al. 1987) and indoleacetalde-
hyde dehydrogenase (Kaper and Verdstra 1958; Ernstsen et al.
1987) which catalyze the conversion of IPyA to ILA and IAAld to
Tol, respectively, are activated (Table 2.2). Since it was
reported that ILA and Tol were reversibly formed from IPyA and
IAAld (Garcia-Tabares et al. 1987; Ernstsen et al. 1987),
respectively, ILA and Tol may be important storage products
involved in the regulation of IAA biosynthesis.

It is a well known fact that E. cloacae strains have been
found in the rhizosphere of plants (Hadar et al. 1983; Ladha et
al. 1983) and in the intestinal tract of humans (Levy et al.
1985). Under aerobic conditions such as the rhizosphere of
plants, E. cloacae may produce IAA for acceleration of plant
growth. However, under anaerobic conditions such as the
intestinal tract of humans, the strain may not produce IAA
which is a carcinogenic substance by the Rec-assay (Kada et al.
1972; Morotomi and Mutai 1986).

P. savastanoi and A. tumefaciens are known to produce IAA,
which causes gall disease in plants (Smidt and Kosuge 1978; Liu
et al. 1982), and the involvement of IAA produced by Rhizobium
in the formation of root nodules has been speculated on (Wang
et al. 1982). The main biosynthesis of IAA in these bacteria
appears to involve the IAAm pathway, which is different from
the IPyA pathway. Our experiments do not identify the reason
why E. cloacae has the IPyA pathway, which is considered the
main pathway for IAA production in plants. Since E. cloacae

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produces IAA as a secondary metabolic product, the genes coding for this pathway may be derived from plants. To identify the role of IAA produced by E. cloacae and the mechanism of the IPyA pathway, we are proceeding to isolate the genes involved in the IAA biosynthetic pathway.

References

Barea, J.M. and Brown, M.E. (1974) Effects on plant growth
produced by Azotobacter paspali related to synthesis of plant
growth regulating substances. J. Appl. Bacteriol. **40:** 583-593.

Bentley, J.A. (1962) Analysis of plant hormones. Methods
Biochem. Anal. **9:** 75-124.

Brown, M.E. (1976) Role of Azotobacter paspali in association
with Paspalum notatum. J. Appl. Bacteriol. **40:** 341-348.

Comai, L. and Kosuge, T. (1980) Involvement of plasmid
deoxyribonucleic acid in indoleacetic acid synthesis in
Pseudomonas savastanoi J. Bacteriol. **143:** 950-957.

Comai, L. and Kosuge, T. (1982) Cloning and characterization
of iaaM, a virulence determinant of Pseudomonas savastanoi.
J. Bacteriol. **149:** 40-46.

Ernstsen, A., Sandberg, G. Crozier, A. and Wheeler C.T. (1987)
Endogenous indoles and the biosynthesis and metabolism of
indole-3-acetic acid in cultures of Rhizobium phaseoli. Planta.
171: 422-428.

Follin, A., Inzé, D., Budar, F., Genetello, C., Van Montagu, M.
and Schell, J. (1985) Genetic evidence that the tryptophan 2-

monooxygenase gene of _Pseudomonas savastanoi_ is functionally
equivalent to one of the T-DNA genes involved in plant tumor
formation by _Agrobacterium tumefaciens_. Mol. Gen. Genet. **201:**
178-185.

García-Tabares, F., Herraiz-Tomico, T., Amat-Guerri, F. and
García-Bilbao, L.J. (1987) Production of 3-indoleacetic acid
and 3-indolelactic acid in _Azotobacter vinelandii_ cultures
supplemented with tryptophan. Appl. Microbiol. Biotechnol.
25: 502-506.

Gibson, R.A., Schneider, E.A. and Wightman, F. (1972) Biosyn-
thesis and metabolism of indole-3yl-acetic acid. II. _In vivo_
experiments with ^{14}C-labelled precursors of IAA in tomato and
barley shoots. J. Exp. Bot. **23:** 381-399.

Hadar, Y., Harman, G.E., Taylor, A.G. and Norton, J.M. (1983)
Effects of pregermination of pea and cucumber seeds and of
seed treatment with _Enterobacter cloacae_ on rots caused by
Pythium spp. Phytopathology **73:** 1322-1325.

Jean, M. and De Moss R.D. (1968) Indolelactate dehydrogenase
from _Clostridium sporogenes_. Can. J. Microbiol. **14:** 429-435.

Kada, T., Tutikawa, K. and Sadaie, Y. (1972) _In vitro_ and host-
mediated rec-assay procedures for screening chemical mutagens;

and phloxine, a mutagenic red dye detected. Mutation Res. **16:** 165-174.

Kaper, J.M. and Verdstra, H. (1958) On the metabolism of tryptophan by <u>Agrobacterium</u> <u>tumefaciens</u>. Biochim. Biophys. Acta. **30:** 401-420.

Koga, J., Ishii, T., Miyadoh, S. and Adachi, T. (1991) Effects of <u>Enterobacter</u> <u>cloacae</u> on the growth of cucumber and rice plants. Sci. Reports Meiji Seika Kaisha **30:** 35-37.

Kosuge, T., Heskett, M.G. and Wilson, E.E. (1966) Microbial synthesis and degradation of indole-3-acetic acid. J. Biol. Chem. **241:** 3738-3744.

Ladha, J.K., Barraquio, W.L. and Watanabe, I. (1983) Isolation and identification of nitrogen-fixing <u>Enterobacter</u> <u>cloacae</u> and <u>Klebsiella</u> <u>planticola</u> associated with rice plants. Can. J. Microbiol. **29:** 1301-1308.

Levy, S.B., Hedges, R.W., Sullivan, F., Medeiros, A.A. and Sosroseputro, H. (1985) Multiple antibiotic resistance plasmids in Enterobacteriaceae isolated from diarrhoeal specimens of hospitalized children in Indonesia. J. Antimicrob. Chemother. **16:** 7-16.

Liu, S.T., Perry, K.L., Schardl, C.L. and Kado, C.I. (1982)
Agrobacterium Ti plasmid indoleacetic acid gene is required
for crown gall oncogenesis. Proc. Natl. Acad. Sci. USA
79: 2812-2816.

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular
cloning: a laboratory manual. Cold Spring Harbor Laboratory,
Cold Spring Harbor, N.Y.

Moore, T.C. and Shaner, C.A. (1968) Synthesis of indoleacetic
acid from tryptophan via indolepyruvic acid in cell free
extracts of pea seedlings. Arch. Biochem. Biophys. 127: 613-
621.

Morotomi, M. and Mutai, M. (1986) Intestinal microflora and
metabolism of chemical carcinogens in vivo, pp. 145-172. In T.
Mitsuoka (ed.), Metabolism in intestinal flora. Japan
Scientific Societies Press, Tokyo.

Morris, R.O. (1986) Genes specifying auxin and cytokinin
biosynthesis in phytopathogens. Annu. Rev. Plant Physiol. 35:
509-538.

Schröder, G., Waffenschmidt, S., Weiler, E.W. and Schröder, J.
(1984) The T-region of Ti plasmid codes for an enzyme synthe-
sizing indole-3-acetic acid. Eur. J. Biochem. 138: 387-391.

Sekine, M., Ichikawa, T., Kuga, N., Kobayashi, M., Sakurai, A
and Syōno, K. (1988) Detection of the IAA biosynthetic pathway
from tryptophan via indole-3-acetamide in Bradyrhizobium spp.
Plant Cell Physiol. 29: 867-874.

Sekine, M., Watanabe, K. and Syōno, K. (1989) Molecular
cloning of a gene for indole-3-acetamide hydrolase from
Bradyrhizobium japonicum. J. Bacteriol. 171: 1718-1724.

Sheldrake, A.R. (1973) The production of hormones in higher
plants. Biol. Rev. Camb. Philos. Soc. 8: 510-515.

Smidt, M. and Kosuge, T. (1978) The role of indole-3-acetic
acid accumulation by alpha methyl tryptophan-resistant mu-
tants of Pseudomonas savastanoi in gall formation on olean-
ders. Physiol. Plant Pathol. 13: 203-214.

Thomashow, L.S., Reeves, S and Thomashow, M.F. (1984) Crown
gall oncogenesis: evidence that a T-DNA gene from the Agro-
bacterium Ti plasmid pTiA6 encodes an enzyme that catalyzes
synthesis of indoleacetic acid. Proc. Natl. Acad. Sci. USA
81: 5071-5075.

Tien, T.M., Gaskins, M.H. and Hubbell, D.H. (1979) Plant growth
substances produced by Azospirillum brasilense and their effect
on the growth of Pearl Millet. Appl. Environ. Microbiol. 37:

1016-1024.

Wang, T.L., Wood, E.A. and Brewin, N.J. (1982) Growth regulators, _Rhizobium_ and nodulation in peas. Indole-3-acetic acid from the culture medium of nodulating and non-nodulating strains of _R. leguminosarum_. Planta **155**: 345-349.

CHAPTER 3

Molecular Cloning of the Gene for Indolepyruvate Decarboxylase
from <u>Enterobacter</u> <u>cloacae</u>.

Abstract

Although indole-3-acetic acid (IAA) is a well known
plant hormone, the main IAA biosynthetic pathway from $_L$-tryp-
tophan ($_L$-Trp) via indole-3-pyruvic acid (IPyA) has yet to
be elucidated. Previous studies suggested that IAA was pro-
duced by <u>Enterobacter</u> <u>cloacae</u> isolated from the rhizosphere
of cucumbers and its biosynthetic pathway may possibly be the
same as that in plants. To elucidate this pathway, the IAA
biosynthetic gene was isolated from a genomic library of <u>E.</u>
<u>cloacae</u> by assessing the ability for converting $_L$-Trp to IAA.
The DNA sequence analysis showed that this gene codes for one
enzyme alone and its predicted protein sequence has extensive
homology with pyruvate decarboxylase in yeast and <u>Zymomonas</u>
<u>mobilis</u>. Cell-free extracts prepared from <u>Escherichia</u> <u>coli</u>
harboring this gene could convert IPyA to indole-3-acetalde-
hyde (IAAld). These results clearly show that this pathway is
regulated by only indolepyruvate decarboxylase, which cata-
lyzes the conversion of IPyA to IAAld.

Introduction

IAA is a well known plant hormone that controls various
aspects of plant growth and development, but the biochemistry

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of IAA synthesis and its regulation are not well understood (Sheldrake 1973) since the IAA biosynthetic enzymes and the genes coding for them have not been isolated.

In general, the following are the most characterized IAA biosynthetic pathways leading from $_L$-Trp to IAA (Fig. 3.1): indole-3-pyruvic acid (IPyA) pathway ($_L$-Trp $\rightarrow$ IPyA $\rightarrow$ IAAld $\rightarrow$ IAA), tryptamine (Tam) pathway ($_L$-Trp $\rightarrow$ Tam $\rightarrow$ IAAld $\rightarrow$ IAA) and indole-3-acetamide (IAAm) pathway ($_L$-Trp $\rightarrow$ IAAm $\rightarrow$ IAA). The IAAm pathway has been studied in detail in <u>Pseudomonas savastanoi</u> (Kosuge et al. 1966; Comai and Kosuge 1980; Comai and Kosuge 1982; Yamada et al. 1985) and <u>Agrobacterium tumefaciens</u> (Schröder et al. 1984; Thomashow et al. 1984; Follin et al. 1985; Thomashow et al. 1986). In this pathway, tryptophan 2-monooxygenase converts $_L$-Trp to IAAm and then indole-3-acetamide hydrolase catalyzes the conversion of IAAm to IAA. However, the IPyA pathway, thought to be the main pathway in plants, has yet to be fully elucidated (Sheldrake 1973), since IPyA and IAAld, intermediates in this pathway, are such unstable compounds that it is difficult to isolate them (Moore and Shaner 1968; Gibson et al. 1972; Garcia-Tabares et al. 1987). Furthermore, since IPyA is nonenzymatically degraded into IAA, it is difficult to obtain evidence that the second and third steps of the IPyA pathway occur enzymatically (Kaper and Verdstra 1958; Sheldrake 1973). In particular, there is little known about the presence of the second step enzyme which catalyzes the conversion of IPyA to IAAld.
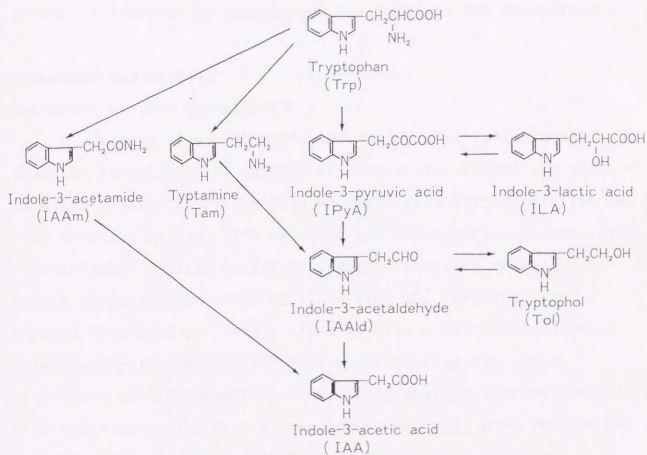
Fig. 3.1 Pathways in the biosynthesis of IAA. The most
characterized pathways in plants and bacteria are shown.

IAA was found to be produced by E. cloacae isolated from the rhizosphere of actively growing cucumbers and its biosynthesis may possibly involve the IPyA pathway (Koga et al. 1991b). In the present study, to elucidate the mechanism of the IPyA pathway, the IAA biosynthetic gene was isolated from a genomic library of E. cloacae and its function was determined.

Materials and methods
Bacterial strains and plasmids

E. cloacae strain FERM BP-1529 was originally isolated from the rhizosphere of cucumbers (Koga et al. 1991a) and used as a source of DNA. Strain FERM BP-1529R is a Rif^r derivative of FERM BP-1529. Strains G140 and G438 are IAA-negative mutants of FERM BP-1529R. Strain 12348R is a Rif^r derivative of Entero-bacter aerogenes IAM 12348 obtained from the Institute of Applied Microbiology. Strain 1236R is also a Rif^r derivative of Enterobacter agglomerans JCM 1236 obtained from the Japan Collection of Microorganisms. These Enterobacter strains were used for triparental mating experiments. E. coli DH5α and JM109 were used for expression of the IAA biosynthetic gene and for nucleotide sequencing experiments, respectively.

Plasmid vectors pUC19 and pUC119 were purchased from Takara Shuzō Co., and pKK223-3 was purchased from Pharmacia LKB biotechnology. Plasmid vectors pHC79 was kindly provided by H. Anzai.

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Genomic DNA library construction and screening

Chromosomal DNA was isolated from E. cloacae FERM BP-1529 as described (Sone et al. 1987). Partially PstI digested 30-40 kilobases (kb) chromosomal DNA of E. cloacae was ligated to the PstI site of cosmid pHC79. The ligated DNA was in vitro packaged in the lambda phage and transfected E. coli. For assessment of the enzymatic conversion of L-Trp to IAA, E. coli harboring a recombinant plasmid was incubated for 2 days at 37°C in liquid LB medium (Maniatis et al. 1982) containing 0.2% L-Trp and 0.2 M potassium phosphate buffer (pH 6.5) followed by extraction at pH 2 with 50% ethanol. Extracts of the reaction products were analyzed by C-18 HPLC. The column was eluted with 30% ethanol in 5% acetic acid at a flow rate of 0.7 ml/min. The effluents were monitored using an ultraviolet detector (absorbance at 280 nm).

Nucleic acid sequencing

DNA from the IAA positive clone was subcloned into pUC119, and overlapping pUC119 deletion clones were generated by the unidirectional deletion procedure with exonuclease III and mung bean nuclease using a kit from Takara Shuzo, according to the manufacturers' instructions. Sequencing was performed on single-strand DNA derived from M13KO7 helper phage superinfection of plasmid-bearing E. coli JM109 by dideoxy-termination methods (Sanger et al. 1977) using an ABI 370A automated DNA sequencer.

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SDS-polyacrylamide gel electrophoresis

E. coli DH5α was grown at 37° C in LB medium containing 0.2 M potassium phosphate buffer (pH 6.5) to an optical density at 550 nm of 0.8. The bacterial cells were harvested by centrifugation and resuspended in 50 mM potassium phosphate buffer (pH 6.5). For protein isolation, the cells were disrupted by sonication and centrifuged at 10000 x g for 20 min. Samples of the supernatant were subjected to SDS-polyacrylamide gel electrophoresis as described (Laemmli 1970).

Indolepyruvate decarboxylase activity in a cell-free system of E. coli

E. coli cell-free extracts were prepared as previously described for SDS-PAGE analysis. IPyA was incubated with E. coli cell-free extracts containing 10 mM potassium phosphate buffer (pH 6.5), 5 mM $MgCl_2$ and 0.1 mM thiamine pyrophosphate for 10 min at 37° C. Extraction with ethanol and HPLC analysis for IPyA and IAAld were conducted as described previously. The HPLC fraction corresponding to the IAAld peak was collected and re-extracted at pH 8 with CH_2Cl_2 (Atsumi 1980). Extracts were dried in vacuo at room temperature and the residue was dis-solved in [$^2H_6$]dimethyl sulfoxide. The structure of the ex-tracted compound was confirmed by ^{1}H-NMR as described (Koga et al. 1991b).

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Construction of IAA-negative mutants

N-methyl-N'-nitro-N-nitrosoguanidine (NTG) mutagenesis was performed as described (Adelberg et al. 1965). E. cloacae strain FERM BP-1529R log-phase cells were treated with NTG (400 μg/ml) in Tris-maleic buffer (pH 6.0) for 20 min. Individual colonies on LB agar plates were suspended in 1 ml of liquid M9 minimal medium (Maniatis et al. 1982) containing 0.2% L-Trp and grown for 48 hr at 30°C. Samples (0.1 ml) of each culture were mixed with 1 ml of 0.1 N HCl. IAA production by each mutants were analyzed by C-18 HPLC as previously described for IAA analysis.

Bacterial matings

The IAA biosynthetic gene was subcloned into pHC79 and mobilized from E. coli DH5α into E. aerogenes 12348R, E. agglomerans 1236R and E. cloacae IAA-negative mutants G140 and G438 by triparental matings using pRK2013 as a helper plasmid (Ditta et al. 1980). Transconjugants were selected on LB agar plates containing Rif (100 μg/ml) and tetracycline (Tc) (20 μg/ml). Single colonies were then picked and used to analyze the ability for converting L-Trp to IAA.

Results
Isolation of the IAA biosynthetic gene

To isolate the gene coding for the IAA biosynthetic enzyme from E. cloacae, a cosmid library was constructed in E. coli.

One transductant out of 500 was isolated as conferring the
ability for converting ʟ-Trp to IAA on E. coli. This clone,
designated pHE82, contained a 40kb insert of chromosomal
DNA. Subcloning of plasmid pHE82 into appropriate sites in
pUC19 indicated that the gene coding for the conversion of ʟ-
Trp to IAA was located on a 3.8 kb BamHI fragment, designated
the B4K gene. To more precisely locate the DNA region, deletion
mutants were constructed using exonuclease III and mung bean
nuclease. A 1.7 kb fragment, designated as the IPDC gene, was
found sufficient to confer the ability for converting ʟ-Trp to
IAA on E. coli. When the IPDC gene was oriented in the opposite
direction in pUC19 plasmid vector, the activity for converting
ʟ-Trp to IAA was not detected in E. coli transformants. Howev-
er, when the B4K gene was oriented in the opposite direction in
pUC19, this activity was detected. These results indicate that
the E. cloacae promoter for IAA biosynthesis is recognized by
E. coli, but the IPDC gene does not contain its own promoter.

Sequence analysis of the IPDC gene

The nucleotide sequencing of the IPDC gene was deter-
mined and is shown in Fig. 3.2. There is a single open reading
frame that predicts the translation of a 552-residue protein
with a molecular weight of 60,020. Based upon sequence simi-
larities with E. coli, a possible ribosomal binding sequence,
AGGA, was found 12 bp upstream from the start codon at posi-
tion 31, however, no such sequences were present within 30 bp

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   1   AGTGAGGTCAACGTCAGAAGGACACCTGTTATGCGAACCCCATACTGCGTCGCCGATTACCTGCTGGACCGTCTTACA      78
   1                               M  R  T  P  Y  C  V  A  D  Y  L  L  D  R  L  T       16

  79   GATTGTGGTGCCGATCATCTGTTTGGCGTGCCGGGCGACTATAACCTGCAGTTTCTCGACCACGTAATAGACAGCCCG     156
  17    D  C  G  A  D  H  L  F  G  V  P  G  D  Y  N  L  Q  F  L  D  H  V  I  D  S  P      42

 157   GATATTTGTTGGGTGGGCTGTGCCAATGAGCTGAACGCATCCTATGCCGCTGACGGATACGCCCGATCTGTAAGGGCTTT     234
  43    D  I  C  W  V  G  C  A  N  E  L  N  A  S  Y  A  A  D  G  Y  A  R  C  K  G  F      68

 235   GCCGCGCTACTGACCACATTCGGCGTTGGGGAGTTAAGTGCCATGAACGGCATTGCCGGCAGCTATGCCGAGCATGTC     312
  69    A  A  L  L  T  F  G  V  G  E  L  S  A  M  N  G  I  A  G  S  Y  A  E  H  V       94

 313   CCGGTTTTACATATTGTGGGGGCGCGGGTACGGGCGGCACGACAAAGGGGAGAGTTGCTGCATCATACGTTGGGGGAT     390
  95    P  V  L  H  I  V  G  A  P  G  T  A  A  Q  Q  R  G  E  L  L  H  H  T  L  G  D     120

 391   GGGGAGTTCCGTCACTTTTATCATATGAGCGAGCCGATCACCGTCGCACAGGCGGTCCTTACCGAACAAAATGCCTGT     468
 121    G  E  F  R  H  F  Y  H  M  S  E  P  I  T  V  A  Q  A  V  L  T  E  Q  N  A  C     146

 469   TATGAAATCGACCGTGTGTTGACAACCATGCTTCGGGAACGCCGCCCCGGTTATCTGATGTTACCCGCCGATGTGGCA     546
 147    Y  E  I  D  R  V  L  T  T  M  L  R  E  R  R  P  G  Y  L  M  L  P  A  D  V  A     172

 547   AAAAAAGCCGCCACGCCGCCTGTAAACGCTCTCACTCATAAGCAGGCTCATGCCGATAGCGCCTGCCTGAAAGCGTTC     624
 173    K  K  A  A  T  P  P  V  N  A  L  T  H  K  Q  A  H  A  D  S  A  C  L  K  A  F     198

 625   CGGGATGCTGCTGAGAACAAACTGGCGATGAGCAAACGTACCGCGCTGCTGGCCGACTTCCTTGTTCTGCGCCATGGC     702
 199    R  D  A  A  E  N  K  L  A  M  S  K  R  T  A  L  L  A  D  F  L  V  L  R  H  G     224

 703   CTGAAACATGCGCTACAGAAGTGGGTGAAAGAGGTACCGATGGCCCATGCCACCATGCTGATGGGGGAAAGGGATATTC     780
 225    L  K  H  A  L  Q  K  W  V  K  E  V  P  M  A  H  A  T  M  L  M  G  K  G  I  F     250

 781   GACGAGCGTCAGGCGGGTTTTTACGGCACATACAGTGGTTCAGCCAGCACTGGCGCGGTAAAAGAGGCGATTGAAGGG     858
 251    D  E  R  Q  A  G  F  Y  G  T  Y  S  G  S  A  S  T  G  A  V  K  E  A  I  E  G     276

 859   GCTGACACGGTATTGTGTGTGGCACGCGTTTTACCGATACCCTGACGGCCGGGTTCACGCACCAGCTTACCCCGGCCG     936
 277    A  D  T  V  L  C  V  G  T  R  F  T  D  T  L  T  A  G  F  T  H  Q  L  T  P  A     302

 937   CAGACCATTGAAGTTCAGCCGCATGCCGCACGCGTCGGGGGATGTCTGGTTTACCGGCATCCCAATGAACCAGGCGATT    1014
 303    Q  T  I  E  V  Q  P  H  A  A  R  V  G  D  V  W  F  T  G  I  P  M  N  Q  A  I     328

1015   GAGACGCTGGTCGAACTCTGCAAACAGCACGTGCATGCTGGCCTTATGTCGTCATCATCCGGCGCAATACCGTTCCCG    1092
 329    E  T  L  V  E  L  C  K  Q  H  V  H  A  G  L  M  S  S  S  S  G  A  I  P  F  P     354

1093   CAGCCGGACGGTTCGCTTACCCAGGAGAATTTCTGGAGAACGTTGCAAACCTTTATTCGCCCGGGGGACATTATCCTT    1170
 355    Q  P  D  G  S  L  T  Q  E  N  F  W  R  T  L  Q  T  F  I  R  P  G  D  I  I  L     380

1171   GCCGACCAGGGAACATCGGCCTTCGGCCGCGATTGATCTGCGTTTACCGGCTGATGTGAACTTTATCGTCCAGCCGCTG    1248
 381    A  D  Q  G  T  S  A  F  G  A  I  D  L  R  L  P  A  D  V  N  F  I  V  Q  P  L     406

1249   TGGGGCTCGATTGGTTACACGCTGGCGGCCGGCGTTTGGTGCACAAACCGCATGCCCGAACCGGCGCGTGATTGTGCTG    1326
 407    W  G  S  I  G  Y  T  L  A  A  A  F  G  A  Q  T  A  C  P  N  R  R  V  I  V  L     432

1327   ACGGGGGATGGCGCTGCCCAGCTCACTATTCAGGAACTAGGCTCGATGCTGCGTGATAAACAGCACCCCATTATTCTG    1404
 433    T  G  D  G  A  A  Q  L  T  I  Q  E  L  G  S  M  L  R  D  K  Q  H  P  I  I  L     458

1405   GTGCTCAACAACGAAGGTTACACGGTTGAGAGGGTATCCACGGGGCGGAGCAGCGGTATAACGACATTGCTTTGTGG    1482
 459    V  L  N  N  E  G  Y  T  V  E  R  A  I  H  G  A  E  Q  R  Y  N  D  I  A  L  W     484

1483   AACTGGACGCACATTCCGCAGGCGTTGAGCCTCGATCCTCAGTCTGAGTGCTGGCGGGTCAGTGAAGCGGAACAGCTG    1560
 485    N  W  T  H  I  P  Q  A  L  S  L  D  P  Q  S  E  C  W  R  V  S  E  A  E  Q  L     510

1561   GCGGACGTACTTGAAAAAGTGGCGCACCACGAGCGGCTCTCGTTGATTGAGGTGATGCTCCCGAAAGCGGATATCCCG    1638
 511    A  D  V  L  E  K  V  A  H  H  E  R  L  S  L  I  E  V  M  L  P  K  A  D  I  P     536

1639   CCGCTGCTCGGGGCGCTTACCAAGGCTCTGGAAGCGTGTAATAACGCCTGATTACTTGTCGTTTCGCCAGGCCATCAT    1716
 537    P  L  L  G  A  L  T  K  A  L  E  A  C  N  N  A  *                               553

1717   CATCGGGTTGCCCGCCA                                                                 1733
```

Fig. 3.2 Nucleotide sequence and deduced amino-acid sequence

of the IPDC gene. The nucleotide sequence has been deposited in

the DDBJ, EMBLE and GenBank data bases, accession number

D90214.

upstream of the other start codons, suggesting that the IPDC
gene is translated from the start codon at position 31.

To provide some clarification of the function of the
IPDC gene product, a search for homology to known proteins
was carried out using the protein sequence databases of the
Protein Identification Resource and SWISS-PROT. As shown in
Fig. 3.3, the predicted amino-acid sequence (552 amino acids)
of the IPDC gene showed considerable homology with pyruvate
decarboxylase in yeast (Kellermann et al. 1986) (36%) and
Zymomonas mobilis (Neale et al. 1987b) (32%).

Expression of the IPDC gene into protein in E. coli

Nucleotide sequence data indicates that the IPDC gene
codes for one peptide alone. However, three enzymes are
thought to be involved in the metabolism of $_L$-Trp to IAA in
the IPyA pathway. To determine whether the IPDC gene codes
for one enzyme, SDS-polyacrylamide gel electrophoresis was
carried out on total protein from E. coli. It is evident that
abundant new protein of about M_r 60,000 is present only in E.
coli harboring the IPDC gene and appears identical in size to
that of the protein predicted from the open reading frame
(Fig. 3.4).

**Indolepyruvate decarboxylase activity of the IPDC gene
products**

Amino acid sequence homology between the predicted IPDC

```
Yeast PDC     MSEITLGKYLFERLKQVNVNTVFGLPGDFNLSLLDKIYEVEGMRWAGNANELNAAYAADGYARIKGMSCI
                 :: ::       :        :  :         :        :::    :::  ::: :: ::
IPDC          MRTPYCVADYLLDRLTDCGADHLFGVPGDYNLQFLDHVIDSPDICWVGCCANELNASYAADGYARCKGFAAL
                  :  ::      :   :   ::  :  :: ::       :        :::      ::  :: ::
Zymomonas PDC SYTVGTYLAERLVQIGLKHHFAVAGDYNLVLLDNLLLNKNMEQVYCCNELNCGFSAEGYARAKGAAAA


Yeast PDC     ITTFGVGELSALNGIAGSYAEHVGVLHVVGVPSISSQAK-QLLLHTLGNGDFTVFHRMSANISETTAMITD
              :::::::::::: ::::::::::::::::::: :::::     :   :     :  :::   :  ::
IPDC          LTTFGVGELSAMNGIAGSYAEHVPVLHIVGAPGTAAQQRGELLHHTLGDGEFRHFYHMSEPITVAQAVLTE
               : :: ::  :  : : :  : ::  ::: ::       :  ::    : : :::   ::  :  :
Zymomonas PDC VVTYSVGALSAFDAIGGAYAENLPVILISGAPNNNDHAAGHVLHHALGKTDYHYQLEMAKNITAAAEAIYT


Yeast PDC     ICTPQAEIDRCIRTTYVTQRPVYLGLPANLVDLNVPAKLLQTPIDMSLKPNDAESEKEVIDTILVLVKDAK
               :     :       :   :: : :::      ::  :   :   ::  ::    :  :: :  :: ::
IPDC          Q-NACYEIDRVLTTMLRERRPGYIMLPADVAK-KAATPPVN-ALTHKQAHADSACLKAFRDAAENKLAMSK
                  :::   :::        :   ::  :    :::         :  ::   :::: ::  : ::
Zymomonas PDC PEEAPAKIDHVIKTALREKPVYLEIACNIASMPCAAPGPASALFNDEAS-DEASLNAAVEETLKFIANRD


Yeast PDC     NPVILADACCSRHDVKAETKKLIDLTQFPAFVTPMGKGSISEQHPRYGGVYVGTLSKPEVKEAVESADLIL
              : :     ::::    :  :: :    ::    ::   :: ::   :::        :     :::
IPDC          RTALLADFLVLRHGLKHALQKWVKEVPMAHATMLMGKGIFDERQAGFYGTYSGSASTGAVKEATEGADTVL
                 : :::  :  ::   :   :  :  : ::   :  :::  :  ::  : : :   :::  ::
Zymomonas PDC KVAALVGSKLRAAGAEEAAVKFADALGGAVATMAAAKSFFPEENPHYIGTSWGEVSYPGVEKTMKEADAVI


Yeast PDC     SVGALLSDFNTGSFSYSYKTKNIVEFHSDHMKIRNATFPGVQMKFVLQKLL-TNIADAAKGYKPVAVPART
              : : :: :::   : : :::  :::   :  ::  ::    : :: ::        :::::  :  ::
IPDC          CVGTRFTDTLTAGFTHQLTPAQTIEVQPHAARVGDVWFTGIPMNQAIETLV-----ELCKQHVHAGLMSSS
                :   ::   ::   ::    ::    : :     :  :   :: :            : ::::::
Zymomonas PDC ALAPVFNDYSTTGWTDIPDPKLVLAEPRSVVVNGIRFPSVHLKDYLTRLAQKVSKKTGALDFFKSLNAGE


Yeast PDC     PANAAVP-ASTPLKQEWMWNQLGNFLQEGDVVIAETGTSAFGINQTTFPNNTYGISQVLWGSIGFTTGATL
              :   ::    :: :       :::   :::::::::::: ::     :::     :::   :: :
IPDC          SGAIPFFQPDGSLTQENFWRTLQTFIRPGDILADQGTSAFGAIDLRLPADVNPIVQPLWGSIGY----TL
               : : ::    :     :    :   ::: :::: :::::   :  : :    : :::::
Zymomonas PDC LKKAAPADPSAPLVNAEIARQVEALLTPNTTVIAETGDSWFNAQRMKLPNGARVEYEMQWGHIGW----SV


Yeast PDC     GAAFAAEEIDPKKRVILFIGDGSLQLTVQEISTMIRWGLKPYLFVLNNDGYTIEKLIHGPKAQYNEIQGWD
              ::: :::::::  ::::::::::: ::: :  :: :::   :: :::::: ::   ::    ::   :::::
IPDC          AAAFGAQTACPNRRVIVLTGDGAAQLTIQELGSMLRDKQHPIILLVNNEGYTVERAIHGAEQRYNDIALWN
               :::: ::  :: ::: :::::  :::  ::  ::  :   ::: :::::: ::  :::    ::   :::
Zymomonas PDC PAAFGYAVGAPERRNILMVGDGSFQLTAQEVAQMVRLKLPVIIFLINNYGYTIEVMIH---DGPYNNIKNWD


Yeast PDC     HLSLLPTFG-AKDYETHRVATTGEWDKLTQDKSFNDNSKIRMIEVMLPVFDCSTKLG
              : :: ::::  :::      ::  ::: ::      ::   :: : :::: :
IPDC          WTHIPQALSLDPQSECWRVSEAEQLADV-LEK-VAHHERLSLIEVMLPKADIPPLLGALTKALEACNNA
               :    ::  :::  :   ::   :::       :: :::: ::::: :: ::: : :::  :
Zymomonas PDC YAGLMEVFNGNGGYDSGAGKGLKAKTGGELAEAIKVALANTDGPTLIECFIGREDCTEELVKWGKRVAAA
```

Fig. 3.3 Comparison of the deduced amino-acid sequence of
the IPDC gene with pyruvate decarboxylase in yeast and
Zymomonas mobilis. Hyphens are introduced to maximize
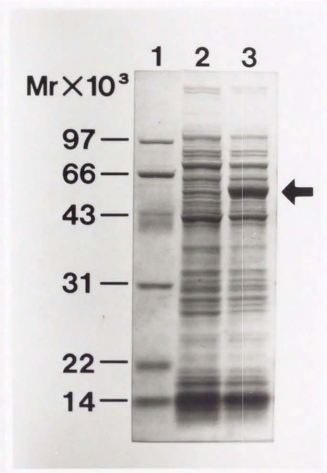homology. Colons indicate identical amino-acids.

Fig. 3.4 Expression of the IPDC gene into protein in E. coli. Lane 1, standard protein; lane 2, vector plasmid pUC19 alone; lane 3, the IPDC gene in pUC19. The position of M_r 60,000, the IPDC gene product is shown by the arrow.

gene product and pyruvate decarboxylase indicates that the IPDC gene codes for indolepyruvate decarboxylase, which catalyzes the conversion of IPyA to IAAld in the IPyA pathway.

To determine the function of the IPDC gene, IPyA conversion was carried out in a cell-free system prepared from _E. coli_ harboring the IPDC gene. When IPyA was incubated with cell-free extracts, IAAld could be detected in extracts from _E. coli_ harboring the IPDC gene but not in extracts from _E. coli_ lacking the IPDC gene (Fig. 3.5). IAAld production was increased 20-fold by the addition of $MgCl_2$ and thiamine pyrophosphate (Table 3.1), a co-factor in decarboxylation (Gounaris et al. 1971; Neale et al. 1987a). The conversion of IPyA to IAAld thus occurs enzymatically and the IPDC gene codes for indolepyruvate decarboxylase.

Transfer of the IPDC gene into _Enterobacter_ strains

Starting with the Rif^r _E. cloacae_ strain FERM BP-1529R, IAA-negative mutants were constructed by NTG mutagenesis to obtain two successive mutants G140 and G438. Under $_L$-Trp-enriched culture conditions, the levels of production by the two IAA-negative mutants were 20 times lower than that of the wild type (Table 3.2), suggesting that they were defective in IAA biosynthesis but not in $_L$-Trp uptake.

To transfer the IPDC gene into _Enterobacter_ strains, this fragment was ligated with the _tac_ promoter and Tc resistance gene since the IPDC gene does not contain the promoter region

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Fig. 3.5 Indolepyruvate decarboxylase activity in a cell-free
system of E. coli. (A) Vector plasmid pUC19 alone. (B) The
IPDC gene in pUC19.

Table 3.1 Effect of Mg^{2+} and thiamine pyrophosphate on indolepyruvate decarboxylase activity.

Conditions	Enzyme activity in 0.1 ml extract[a] (nmol/min)
Control	0.8
+ 5 mM $MgCl_2$	2.4
+ 0.1 mM thiamine pyrophosphate	4.5
+ 5 mM $MgCl_2$ + 0.1 mM thiamine pyrophosphate	16.3

[a] Enzyme activity was measured as nanomoles of IAAld produced per minute per 0.1 ml of E. coli cell-free extracts.

Table 3.2 IAA production in <u>Enterobacter</u> strains harboring the IPDC gene.

Enterobacter strain	IAA production (mg/ml)[a]	
	- the IPDC gene	+ the IPDC gene
E. cloacae FERM BP-1529R	0.89	0.87
G140	0.04	0.40
G438	0.05	0.90
E. aerogenes 12348R	0.02	0.28
E. agglomerans 1236R	0.02	0.79

[a] IAA was measured in the supernatant of LB medium containing 0.2% L-Trp and 0.2 M potassium phosphate buffer (pH 6.5), using the HPLC analysis.

and Enterobacter strains are sensitive to Tc but not ampicillin. The IPDC gene was treated with various amounts of mung bean nuclease, and subcloned into the SmaI site of pKK223-3 containing the tac promoter. This plasmid, designated pIP27, was digested with BamHI, and the DNA fragment containing the IPDC gene and tac promoter was ligated into the BglII site of the pHC79 containing Tc resistance gene. This plasmid, designated pIP79, contains the Tc resistance gene and the IPDC gene preceded by the tac promoter.

Furthermore, triparental matings were carried out to transfer plasmid pIP79 into E. aerogenes 12348R, E. agglomerans 1236R and E. cloacae IAA-negative mutants G140 and G438. As shown in Table 3.2, all the strains harboring pIP79 could convert L-Trp to IAA, but the strains lacking pIP79 were essentially incapable of converting L-Trp to IAA. Specifically, E. agglomerans 1236R and E. cloacae IAA-negative mutant G438 harboring pIP79 produced the same amount of IAA as did the wild type E. cloacae strain.

Discussion

We have isolated the gene coding for indolepyruvate decarboxylase, which is a novel enzyme in IAA biosynthesis, and elucidated the mechanism of the IPyA pathway.

To isolate the gene coding for the IAA biosynthetic enzyme from E. cloacae, a cosmid library was constructed in E. coli. First, for assessment of the enzymatic conversion of L-Trp to

IAA, E. coli harboring a recombinant plasmid was incubated in
LB medium or LB medium containing glucose (LBG medium).
However, no IAA positive clone was isolated (data not shown),
suggesting that E. coli harboring the IPDC gene could not
produce IAA in these media. On the other hand, E. cloacae
produced high amounts of IAA from ʟ-Trp in LBG medium (Koga et
al. 1991b). In LBG medium, the low quantities of acid formed
from glucose by E. cloacae caused the final pH of the culture
to be pH 6.8, whereas the high amounts of acid formed from
glucose by E. coli caused the final pH of the culture to be pH
4.0. Furthermore, in LB medium, pH of the final culture with E.
coli was 8.9 (data not shown). In view of the difference in
glucose metabolism between E. cloacae and E. coli (Brenner et
al. 1984), the E. coli culture medium was buffered with
potassium phosphate at the optimal pH 6.5 and an IAA positive
clone was subsequently obtained. These results indicate that
the optimal pH range of indolepyruvate decarboxylase is 6.5 to
6.8.

DNA sequence analysis showed that the IPDC gene codes
for one enzyme alone and its predicted protein sequence has
extensive homology with pyruvate decarboxylase in yeast and
Zymomonas mobilis. Pyruvate decarboxylase in yeast has 549
amino acids and catalyzes the decarboxylation of pyruvic acid
to produce acetaldehyde and carbon dioxide (Gounaris et al.
1971; Kuo et al. 1986; Kellermann et al. 1986). If the second
step of the IPyA pathway occurs enzymatically, indolepyruvate

decarboxylase may be expected to catalyze the decarboxylation of IPyA to produce IAAld and carbon dioxide. The similarity between these two enzymatic reactions indicates that the IPDC gene codes for indolepyruvate decarboxylase.

To determine whether the IPDC gene codes for indolepyruvate decarboxylase, cell-free extracts from E. coli harboring the IPDC gene were assayed for enzymatic conversion of IPyA to IAAld. When IPyA was incubated with cell-free extracts, high amounts of IAAld were directly detected in extracts from E. coli harboring the IPDC gene, suggesting that the conversion of IPyA to IAAld occurs enzymatically and the IPDC gene codes for indolepyruvate decarboxylase. To date, no reports have appeared on the direct detection of IAAld formed from IPyA by decarboxylase activity, since IAAld is such an unstable compound that it is readily converted to IAA and Tol. Furthermore, in consideration of the nonenzymatic degradation of IPyA to IAA, it is difficult to obtain evidence for the participation of indolepyruvate decarboxylase in the IPyA pathway. Nevertheless, we could directly detect IAAld in E. coli cell-free system since indolepyruvate decarboxylase activity was higher than the degradation of IAAld by the addition of $MgCl_2$ and thiamine pyrophosphate and overexpression from the lac promoter.

Triparental mating experiments indicate that only indolepyruvate decarboxylase is necessary for the conversion of $_L$-Trp to IAA in Enterobacter strains as well as E. coli.

$_L$-Trp could be converted to IPyA in vitro by a purified trans-aminase from E. coli (Gunsalus and Stamer 1955), and many species of bacteria and plants have been found to produce IPyA and indole-3-lactic acid from $_L$-Trp (Jean and De Moss 1968; Truelsen 1973; Aragozzini et al. 1979; Paris and Magasanik 1981). Transaminase, which catalyzes the conversion of $_L$-Trp to IPyA, thus appears to be nonspecific for substrates. IAAld was also oxidized to IAA nonspecifically by an aldehyde oxidase in plants (Kenten and Mann 1952). The majority of bacteria, including E. coli hardly produce any IAA, but are capable of converting IAAld to IAA (data not shown). It thus follows that the rate limiting step in the IPyA pathway is the conversion of IPyA to IAAld and the ability for IAA production depends on the presence of indolepyruvate decarboxylase.

The present study on IAA biosynthesis in E. cloacae will facilitate elucidation of the metabolism of the IPyA pathway in plants, and may provide some insight into the biochemistry of IAA synthesis and its regulation as a plant hormone.

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References

Adelberg, E.A., Mandel, M. and Chen, G.C.C. (1965) Optimal conditions for mutagenesis by N-methyl-N'-nitro-N-nitrosoguanidine in Escherichia coli K12. Biochem. Biophys. Res. Commun. **18:** 788-795.

Aragozzini, F., Ferrari, A., Pacini, N. and Gualandris, R. (1979) Indole-3-lactic acid as a tryptophan metabolite produced by Bifidobacterium spp. Appl. Environ. Microbiol. **38:** 544-546.

Atsumi, S. (1980) Studies on the production of indole-3-acetic acid with auxin-heterotrophic mutants derived from cultured crown gall cells. Plant Cell Physiol. **21:** 1227-1232.

Brenner, D.J., Farmer, J.J., Frederiksen, W., Le Minor, L. and Sakazaki, R. (1984) Facultatively anaerobic gram-negative rods. In: Krieg, N.R. (ed) Bergey's manual of systematic bacteriology, Vol. 1. Williams & Wilkins, Baltimore Md, pp 408-516.

Comai, L. and Kosuge, T. (1980) Involvement of plasmid deoxyribonucleic acid in indoleacetic acid synthesis in Pseudomonas savastanoi. J. Bacteriol. **143:** 950-957.

Comai, L. and Kosuge, T. (1982) Cloning and characterization of iaaM, a virulence determinant of Pseudomonas savastanoi. J. Bacteriol. **149:** 40-46.

Ditta, G., Stanfield, S., Corbin, D. and Helinski, D.R. (1980) Broad host range DNA cloning system for gram-negative bacteria: Construction of a gene bank Rhizobium meliloti. Proc. Natl. Acad. Sci. USA. **77**: 7347-7351.

Follin, A., Inzé, D., Budar, F., Genetello, C., Van Montagu, M. and Schell, J. (1985) Genetic evidence that the tryptophan 2-monooxygenase gene of Pseudomonas savastanoi is functionally equivalent to one of the T-DNA genes involved in plant tumor formation by Agrobacterium tumefaciens. Mol. Gen. Genet. **201**: 178-185.

Garcia-Tabares, F., Herraiz-Tomico, T., Amat-Guerri, F. and Garcia- Bilbao, J.L. (1987) Production of 3-indoleacetic acid and 3-indolelactic acid in Azotobacter vinelandii cultures supplemented with tryptophan. Appl. Microbiol. Biotechnol. **25**: 502-506.

Gibson, R.A., Schneider, E.A. and Wightman, F. (1972) Biosynthesis and metabolism of indole-3yl-acetic acid. II. In vivo experiments with ^{14}C-labelled precursors of IAA in tomato and barley shoots. J. Exp. Bot. **23**: 381-399.

Gounaris, A.D., Turkenkopf, I., Buckwald, S. and Young, A. (1971) Pyruvate decarboxylase. I. Protein dissociation into subunits under conditions in which thiamine pyrophosphate is

released. J. Biol. Chem. **246**: 1302-1309.

Gunsalus, I.C. and Stamer, J.R. (1955) Transaminases in bacteria. In: Colowick, S.P., Kaplan, N.O. (eds) Methods in Enzymology, Vol. 2. Academic Press, New York, pp 170-177.

Jean, M. and De Moss R.D. (1968) Indolelactate dehydrogenase from Clostridium sporogenes. Can. J. Microbiol. **14**: 429-435.

Kaper, J.M. and Verdstra, H. (1958) On the metabolism of tryptophan by Agrobacterium tumefaciens. Biochim. Biophys. Acta. **30**: 401-420.

Kellermann, E., Seeboth, P.G. and Hollenberg, C.P. (1986) Analysis of the primary structure and promoter function of a pyruvate decarboxylase gene (PDC1) from Saccharomyces cerevisiae. Nucleic. Acids. Res. **14**: 8963-8977.

Kenten, R.H. and Mann, P.J.G. (1952) The oxidation of amines by extracts of pea seedlings. Biochem. J. **50**: 360-369.

Koga, J., Ishii, T., Miyadoh, S. and Adachi, T. (1991a) Effects of Enterobacter cloacae on the growth of cucumber and rice plants. Sci. Reports Meiji Seika Kaisha **30**: 35-37.

Koga, J., Adachi, T. and Hidaka H. (1991b) IAA biosynthetic

pathway from tryptophan _via_ indole-3-pyruvic acid in Entero-
bacter cloacae. Agric. Biol. Chem. **55**: 701-706.

Kosuge, T., Heskett, M.G. and Wilson, E.E. (1966) Microbial
synthesis and degradation of indole-3-acetic acid. J. Biol.
Chem. **241**: 3738-3744.

Kuo, D.J., Dikdan, G. and Jordan, F. (1986) Resolution of
Brewers' yeast pyruvate decarboxylase into two isozymes. J.
Biol. Chem. **261**: 3316-3319.

Laemmli, U.K. (1970) Cleavage of structural proteins during the
assembly of the head of bacteriophage T4. Nature **227**: 680-
685.

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular
cloning: a laboratory manual. Cold Spring Harbor Laboratory,
Cold Spring Harbor, N.Y.

Moore, T.C. and Shaner, C.A. (1968) Synthesis of indoleacetic
acid from tryptophan via indolepyruvic acid in cell free
extracts of pea seedlings. Arch. Biochem. Biophys. **127**: 613-
621.

Neale, A.D., Scopes, R.K., Wettenhall, R.E.H. and Hoogenraad,
N.J. (1987a) Pyruvate decarboxylase of Zymomonas mobilis:

Isolation, properties, and genetic expression in *Escherichia coli*. J. Bacteriol. **169**: 1024-1028.

Neale, A.D., Scopes, R.K., Wettenhall, R.E.H. and Hoogenraad, N.J. (1987b) Nucleotide sequence of the pyruvate decarboxylase gene from *Zymomonas mobilis*. Nucleic. Acids. Res. **15**: 1753-1761.

Paris, C.G. and Magasanik, B. (1981) Tryptophan metabolism in *Klebsiella aerogenes*: regulation of the utilization of aromatic amino acids as sources of nitrogen. J. Bacteriol. **145**: 257-265.

Sanger, F., Nicklen, S. and Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA. **74**: 5463-5467.

Schröder, G., Waffenschmidt, S., Weiler, E.W. and Schröder, J. (1984) The T-region of Ti plasmid codes for an enzyme synthesizing indole-3-acetic acid. Eur. J. Biochem. **138**: 387-391.

Sheldrake, A.R. (1973) The production of hormones in higher plants. Biol. Rev. Camb. Philos. Soc. **8**: 510-515.

Sone, H., Fujii, T., Kondo, K. and Tanaka, J. (1987) Molecular cloning of the gene encoding α-acetolactate decarboxylase from *Enterobacter aerogenes*. J. Biotechnol. **5**: 87-91.

Thomashow, L.S., Reeves, S and Thomashow, M.F. (1984) Crown
gall oncogenesis: evidence that a T-DNA gene from the Agro-
bacterium Ti plasmid pTiA6 encodes an enzyme that catalyzes
synthesis of indoleacetic acid. Proc. Natl. Acad. Sci. USA
81: 5071-5075.

Thomashow, M.F., Hugly, S., Buchholz, W.G. and Thomashow, L.S.
(1986) Molecular basis for the auxin-independent phenotype of
crown gall tumor tissues. Science **231**: 616-618.

Truelsen, T.A. (1973) Indole-3-pyruvic acid as a intermediate
in the conversion of tryptophan to indole-3-acetic acid. II.
Distribution of tryptophan transaminase activity in plants.
Physiol. Plant. **28**: 67-70.

Yamada, T., Palm, C.J., Brooks, B. and Kosuge, T. (1985)
Nucleotide sequences of the Pseudomonas savastanoi indoleacetic
acid genes show homology with Agrobacterium tumefaciens T-DNA.
Proc. Natl. Acad. Sci. USA. **82**: 6522-6526.

CHAPTER 4

Purification and Characterization of Indolepyruvate
Decarboxylase, a Novel Enzyme for Indole-3-acetic Acid
Biosynthesis in Enterobacter cloacae.

Abstract

Indolepyruvate decarboxylase, which is a key enzyme in
indole-3-acetic acid biosynthesis, was found in extracts of
Enterobacter cloacae. The enzyme catalyzes the decarboxylation
of indole-3-pyruvic acid to yield indole-3-acetaldehyde and
carbon dioxide. The enzyme purified to apparent homogeneity
from Escherichia coli cells harboring the genetic locus for
this enzyme obtained from Enterobacter cloacae. The results of
gel filtration experiments showed that indolepyruvate decar-
boxylase is a tetramer with an M_r of 240,000. In the absence of
thiamine pyrophosphate and Mg^{2+}, the active tetramers dissoci-
ate into inactive monomers and dimers. However, the addition of
thiamine pyrophosphate and Mg^{2+} to the inactive monomers and
dimers results in the formation of active tetramers. These
results indicate that the thiamine pyrophosphate-Mg^{2+} complex
functions in the formation of the tetramer which is the enzy-
matically active holoenzyme.

The enzyme exhibited decarboxylase activity with indole-3-
pyruvic acid and pyruvic acid as substrates, but no decarboxy-
lase activity was apparent when L-tryptophan, indole-3-lactic
acid, β-phenylpyruvic acid, oxalic acid, oxaloacetic acid, and

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acetoacetic acid. The $\underline{K_m}$ values for indole-3-pyruvic acid and pyruvic acid were 15 μM and 2.5 mM, respectively. These results indicate that the IAA biosynthesis in Enterobacter cloacae is mediated by indolepyruvate decarboxylase, which has a high specificity and affinity for indole-3-pyruvic acid.

Introduction

Indole-3-acetic acid (IAA) is a plant hormone which controls several aspects of plant growth and development. Although much is known about the effect of IAA and its role in the control of growth and differentiation, little is known about the biochemistry of IAA synthesis and its regulation since the IAA biosynthetic enzymes have not been fully characterized (Sheldrake 1973).

In plants, the main IAA biosynthetic pathway (the IPyA pathway) leading from L-tryptophan (L-Trp) to IAA is as follows (Sheldrake 1973):

L-Trp $\rightarrow$ indole-3-pyruvic acid (IPyA) $\rightarrow$ indole-3-acetaldehyde (IAAld) $\rightarrow$ IAA

The first step in this pathway is the conversion of L-Trp to IPyA, which is catalyzed by L-tryptophan aminotransferase. This enzyme, which has been found in many species of plants and bacteria (Gunsalus and Stamer 1955; Truelsen 1973; Paris and Magasanik 1981), is nonspecific with respect to amino acid substrates (Truelsen 1972). The third step in this pathway is the conversion of IAAld to IAA, which is catalyzed by indole-3-

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acetaldehyde oxidase. Kenten and Mann suggested that IAAld is oxidized to IAA by an aldehyde oxidase in plants (Kenten and Mann 1952). The majority of bacteria produce little IAA, but are capable of converting IAAld to IAA (Koga et al. 1991c). Thus, in plants and bacteria, there is no evidence to suggest that the first and third enzymes in the IPyA pathway are involved in the production of IAA from L-Trp.

Little is known about the second enzyme in the IPyA pathway, indolepyruvate decarboxylase, which catalyzes the conversion of IPyA to IAAld. Both IPyA and IAAld are unstable compounds and it is difficult to isolate them (Moore and Shaner 1968; Gibson et al. 1972; Garcia-Tabares et al. 1987). Furthermore, since IPyA is nonenzymatically degraded into IAA, it is difficult to demonstrate that the second reaction is catalyzed enzymatically (Kaper and Verdstra 1958; Sheldrake 1973). In consequence, it seems likely that the control of the IPyA pathway does not involve any very specific regulatory mechanisms at the enzymatic level.

In a previous study, IAA was found to be produced by Enterobacter cloacae isolated from actively growing cucumbers, and its biosynthesis utilized the IPyA pathway (Koga et al. 1991b). To elucidate this pathway, the IAA biosynthetic gene was isolated from a genomic library of E. cloacae by assessing the ability of clones to convert L-Trp to IAA. Molecular-cloning experiments showed that this gene (the IPDC gene) codes for only one protein and E. coli, harboring the IPDC gene, are

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able to convert IPyA to IAAld. Furthermore, Enterobacter aerogenes, Enterobacter agglomerans, and IAA-negative mutants of Enterobacter cloacae, into which the IPDC gene was transferred, were able to convert ʟ-Trp to IAA (Koga et al. 1991c). These findings showed that the IPyA pathway is mediated only by indolepyruvate decarboxylase.

In the present study, to further elucidate the regulation of the IAA biosynthesis in E. cloacae, we purified indolepyruvate decarboxylase and characterized its physical and regulatory properties.

Materials and Methods
Bacterial strain and culture conditions

E. cloacae strain FERM BP-1529 was originally isolated from the rhizosphere of cucumbers (Koga et al. 1991a). E. coli strain DH27 is an E. coli DH5α strain harboring plasmid pIP27, which contains the IPDC gene inserted into the SmaI site of the plasmid pKK223-3 (Koga et al. 1991c).

For preparation of large amounts of bacteria, 5-liter cultures of strains FERM BP-1529 and DH27 were grown for 24 h at 30 ˚C in a medium containing 2% Bacto tryptone, 1% yeast extract, 0.5% NaCl, 0.1 mM thiamine pyrophosphate (TPP), 0.01% ampicillin, and 0.15 M potassium phosphate (pH 6.5), respectively. Cells were harvested by centrifugation and were stored at -80 ˚C until needed.

Buffers

The buffers used were as follows; Buffer A, 50 mM potassium phosphate (pH 6.5); Buffer B, 50 mM potassium phosphate and 5 mM $MgCl_2$ (pH 6.5); Buffer C, 50 mM potassium phosphate and 1 mM TPP (pH 6.5); Buffer D, 50 mM potassium phosphate, 5 mM $MgCl_2$, and 1 mM TPP (pH 6.5); Buffer E, Buffer D plus 0.4 M NaCl (pH 6.5); Buffer F, Buffer D plus 0.5 M $(NH_4)_2SO_4$ (pH 6.5); Buffer G, 10 mM potassium phosphate, 5 mM $MgCl_2$, and 0.1 mM TPP (pH 6.5); and Buffer H, 50 mM potassium phosphate and 150 mM NaCl (pH 6.6).

Reagents

Pyruvate decarboxylase (EC 4.1.1.1) from brewers' yeast and IPyA were purchased from Sigma, St. louis MO, USA, as were all other reagents.

Indolepyruvate decarboxylase assay

When the crude enzyme from <u>E. cloacae</u> was subjected to the indolepyruvate decarboxylase assay, IAA, IAAld, and tryptophol (Tol) are formed from IPyA as reaction products. IPyA is non-enzymatically degraded into various products, including IAA (Kaper and Verdstra 1958). However, IPyA is not degraded nonenzymatically to IAAld and Tol (Kaper and Verdstra 1958; Koga et al. 1991b). Indolepyruvate decarboxylase catalyzes the decarboxylation of IPyA to yield IAAld, and 25 % of the IAAld produced is readily reduced to Tol and oxidized to IAA (Koga et

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al. 1991b). These results indicate that the majority of IAA is
a product of the nonenzymatic degradation of IPyA, and IAAld
and Tol are products of the enzymatic decarboxylation of IPyA.
Therefore, indolepyruvate decarboxylase activity was assayed by
monitoring the formation of IAAld and Tol.

The reaction mixture, 3 ml, contained Buffer G and the
preparation of enzyme to be tested. The reaction was started
by the addition of 30 μl of 20 mM IPyA, which was dissolved in
ethanol, and incubated at 25 °C for 10 min. After incubation,
the reaction was stopped by the addition of 6 ml of 0.1 N HCl
containing 50 % ethanol, and the amounts of IAAld and Tol
formed from IPyA were determined by HPLC on a C-18 column as
described (Koga et al. 1991b, 1991c). The column was eluted
with 30% ethanol in 5% acetic acid at a flow rate of 0.7
ml/min. The effluent was monitored with a spectrofluorometer
(excitation wavelength, 280 nm, emission wavelength, 350 nm).
One unit of activity is defined as the amount of enzyme that
catalyzes the production of 1 μmole of the total amount of
IAAld plus twice the amount of Tol per min under the above
conditions.

Protein concentrations were determined by Bradford's
method using bovine serum albumin as a standard (Bradford
1976).

Other decarboxylase assays

All assays were performed at 25 °C in the presence of

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Buffer G. Activities of L-tryptophan decarboxylase, indolelactate decarboxylase and phenylpyruvate decarboxylase were determined by estimating the amount of the reaction products, namely, tryptamine, tryptophol, and phenylacetaldehyde, respectively. These reaction products were quantitated by HPLC on a C-18 column as described (Koga et al. 1991b, 1991c). For quantitative determination of tryptamine, the column was eluted with 5% acetic acid at a flow rate of 1.0 ml/min. The effluent was monitored with a spectrofluorometer (excitation wavelength, 280 nm, emission wavelength, 350 nm). For quantitative determination of phenylacetaldehyde, the column was eluted with 50% ethanol in 5% acetic acid at a flow rate of 1.0 ml/min. The effluent was monitored with an ultraviolet detector (absorbance at 280 nm). Pyruvate decarboxylase activity was determined by estimating the amount of acetaldehyde produced. The estimation was made by measuring the oxidation of NADH in the presence of alcohol dehydrogenase. Activities of oxalate decarboxylase (Shimazono and Hayaishi 1957), oxaloacetate decarboxylase (Schmitt et al. 1966), and acetoacetate decarboxylase (Fridovich 1963) were assayed as described.

Indolepyruvate decarboxylase purification

Step 1. **Crude extract preparation** — Samples of 45 g of cell paste, obtained from either E. cloacae strain FERM BP-1529 or E. coli strain DH27 were suspended in 600 ml of Buffer D and the cells were disrupted by sonication. The mixture was cen-

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trifuged at 18000 x g for 40 min and the pellet was discarded.

Step 2. Fractionation with $(NH_4)_2SO_4$ — The enzymatically active protein fraction was obtained in the 38% (230 g/l) and 55% (351 g/l) ammonium sulfate precipitate fraction at 4 °C. After centrifugation of the 55% ammonium sulfate solution at 18000 x g for 20 min, the precipitate was dissolved in 100 ml of Buffer D and the solution was dialyzed against 20 l of Buffer D.

Step 3. FPLC on a Q Sepharose column — A Q Sepharose High Performance 60/100 column (6 x 10 cm; Pharmacia LKB Bio-technology, S-75182 Uppsala, Sweden) was equilibrated with Buffer D and the protein solution from Step 2 was applied to the column. The column was eluted with a linear gradient of 900 ml each of Buffer D and Buffer E. The active fractions were pooled and concentrated to 100 ml by ultrafiltration.

Step 4. FPLC on a Phenyl Sepharose column — A Phenyl Sepharose High Performance 60/100 column (6 x 10 cm; Pharmacia LKB Biotechnology) was equilibrated with Buffer F. The active fraction from Step 3 was applied to the column and the protein was eluted with a linear gradient of 800 ml each of Buffer F and Buffer D. The active fractions were pooled and concentrated to 100 ml by ultrafiltration.

Molecular weight determination

For the M_r determination of indolepyruvate decarboxylase from E. cloacae strain FERM BP-1529, a Superose 6 HR 10/30 gel

filtration column (1 x 30 cm; Pharmacia LKB Biotechnology) was equilibrated with Buffer D. The active protein fraction of strain FERM BP-1529 from Step 4 was applied to the column. The column was eluted with Buffer D at a flow rate of 0.4 ml/min at 25 ˙ C. The M_r of the enzyme was determined by measuring the active fractions.

The M_r of indolepyruvate decarboxylase from E. coli strain DH27 was determined by a Superose 6 HR 10/30 gel filtration column (Fig. 4.2). The column was eluted with Buffer H at a flow rate of 0.4 ml/min at 25 ˙ C and the effluent was monitored using an ultraviolet detector (absorbance at 280 nm). Under this conditions, it is confirmed that the tetrameric structure of indolepyruvate decarboxylase does not dissociate during the time it takes to pass the enzyme through the column. The following proteins were used as M_r standards: thyroglobulin (670,000), gamma globulin (158,000), ovalbumin (44,000), myoglobin (17,000), and vitamin B-12 (1,350).

Results
Stabilization of IPyA

To establish an indolepyruvate decarboxylase assay, it is essential to determine conditions under which IPyA is stable, since IPyA spontaneously degrades into various products, including IAA.

For accurate quantitative determinations of IPyA, analysis was carried out by HPLC. To solution of IPyA, 2 equal volumes

of 0.1N HCl containing 50% ethanol was added. This solution was analyzed by HPLC on a C-18 column and eluted isocratically with 30% ethanol in 5% acetic acid at a flow rate of 0.7 ml/min. The effluent was monitored with an ultraviolet detector (absorbance at 280 nm).

Although IPyA was less stable in aqueous solution, it was rather stable in organic solvents (Table 4.1). Especially, IPyA was less stable at high potassium phosphate concentration and high pH in aqueous buffers (Table 4.1). In consideration of these results, indolepyruvate decarboxylase assays were performed in 10 mM potassium phosphate buffer (pH 6.5). Furthermore, stock solutions of IPyA were made by dissolving IPyA in ethanol.

Identification of TPP and Mg^{2+} as cofactors of indolepyruvate decarboxylase

To investigate the cofactor requirements of indolepyruvate decarboxylase, we studied the effects of TPP and Mg^{2+} on the decarboxylase reaction using the crude enzyme from E. cloacae strain FERM BP-1529, since pyruvate decarboxylase requires TPP and Mg^{2+} as cofactors (Green et al. 1941; Singer and Pensky 1952). An 8-fold increase in activity was found in the presence of 0.1 mM TPP and a 10-fold increase in activity was found in the presence of 5 mM $MgCl_2$. Furthermore, a 39-fold increase in activity was found in the presence of 0.1 mM TPP and 5 mM $MgCl_2$. In our experiments, pyridoxal phosphate and lipoic acid

Table 4.1 Stability of indole-3-pyruvic acid dissolved in
various solutions

    To examine the stability of IPyA, IPyA was dissolved at
0.2 mM in various solutions and the resultant solutions were
incubated at 25 ˚C for 180 min. The residual IPyA was then
analyzed by HPLC on a C-18 column, as described.

Solution	pH	Amount of indole-3-pyruvic acid remaining
		%
100 mM potassium phosphate buffer	6.5	7
30 mM potassium phosphate buffer	6.5	21
10 mM potassium phosphate buffer	6.5	68
10 mM potassium phosphate buffer	8.5	8
0.1 N HCl containing 50% ethanol	1.0	95
Ethanol	–	100
Ethyl acetate	–	100

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had no effect on the activity of the enzyme.

Stabilization of indolepyruvate decarboxylase

Indolepyruvate decarboxylase is an unstable enzyme in the absence of any additions. For example, when the crude enzyme was incubated at 30 °C for 24 h, 95% of the original enzymatic activity was lost. Since indolepyruvate decarboxylase requires TPP and Mg^{2+} for activity, these cofactors were included in the enzyme solutions to stabilize the enzyme.

The crude enzyme from E. cloacae strain FERM BP-1529 was stabilized by the addition of 1 mM TPP and was slightly stabilized by the addition of 5 mM $MgCl_2$ (Table 4.2). Other tested compounds had little or no effect on the stability of the enzyme. Furthermore, in the presence of 1 mM TPP and 5 mM $MgCl_2$, the enzyme was stable to heating at 50 °C for 30 min (data not shown), suggesting that the cofactors (TPP and $MgCl_2$) effectively stabilized the enzyme against denaturation by heat.

pH optimum

The effect of pH on the catalytic activity of the crude enzyme from E. cloacae strain FERM BP-1529 was investigated in 10 mM potassium phosphate buffer from pH 5.5 to pH 7.5. Under the assay conditions, the enzyme has a sharp pH optimum at 6.4-6.6 (Fig. 4.1).

Table 4.2 Effects of various compounds on the stability of
indolepyruvate decarboxylase

To examine the stability of indolepyruvate decarboxylase,
the enzyme prepared from the crude extract of E. cloacae strain
FERM BP-1529 was incubated at 30 °C for 12 hours in Buffer A
containing various compounds, and the residual activity was
measured as described.

Compound	Residual activity
	%
None	13
0.1 mM indole-3-acetic acid	11
0.1 mM indole-3-pyruvic acid	14
0.1 mM indole-3-acetaldehyde	7
1.0 mM pyridoxal phosphate	11
1.0 mM thiamine pyrophosphate	96
5.0 mM $MgCl_2$	21

Fig. 4.1 The pH optimum for the reaction catalyzed by indole-
pyruvate decarboxylase prepared from a crude extract of
Enterobacter cloacae.

Establishment of an assay for indolepyruvate decarboxylase

In consideration of the above results, the reaction mixture for the assay of indolepyruvate decarboxylase activity, 3 ml, contained Buffer G and the preparation of enzyme to be tested. The reaction was started by the addition of 30 μl of 20 mM IPyA and incubated at 25 °C for 10 min. After incubation, the reaction was stopped by the addition of 6 ml of 0.1 N HCl containing 50 % ethanol, and the amounts of IAAld and Tol formed from IPyA were determined by HPLC on a C-18 column. The HPLC fractions corresponding to the peaks of IAAld and Tol were collected and extracted as described (Atsumi 1980; Koga et al. 1991b). The structures of the extracted compounds were confirmed by ^{1}H-NMR as described previously (Koga et al. 1991b, 1991c).

Indolepyruvate decarboxylase purification

By $(NH_4)_2SO_4$ fractionation, Q Sepharose, and Phenyl Sepharose, indolepyruvate decarboxylase from E. cloacae strain FERM BP-1529 was purified 100-fold with a recovery of 11% of the initial activity. Specific activity of the purified enzyme was 0.0775 units/mg protein. The enzyme was extremely stable at the early stages of purification, but after chromatography on Phenyl Sepharose, the recovery was quite unsatisfactory.

The M_r of indolepyruvate decarboxylase from E. cloacae strain FERM BP-1529 was estimated to be 240,000 by gel filtration on Superose 6. Since the monomer has an M_r of 60,000, as

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determined by SDS-PAGE of the product of the IPDC gene and by
DNA sequence analysis of the gene (Koga et al. 1991c), it is
proposed that indolepyruvate decarboxylase is a tetramer com-
posed of four identical monomers.

The purification of indolepyruvate decarboxylase from _E.
cloacae_ strain FERM BP-1529 to homogeneity was difficult be-
cause of the low level of activity in cell lysates and the
instability of the enzyme. Therefore, we purified the enzyme
from the _E. coli_ strain DH27 harboring the cloned IPDC gene
(Koga et al. 1991c). The purification is summarized in Table
4.3. The preparation of purified enzyme was essentially homoge-
neous, as determined by SDS-PAGE. SDS-PAGE of the purified
enzyme gave a single band with a mobility that corresponded to
an M_r of 60,000 (Fig. 4.2). Gel filtration chromatography on
Superose 6 in the presence of TPP and Mg^{2+} resulted in a single
peak that corresponded to an M_r of 240,000 (Fig. 4.3, sample
A). This enzyme is, therefore, present as a tetramer composed
of four identical monomers in _E. coli_, as it is in _E. cloacae_.
The remaining studies were performed with this purified enzyme.

Effect of ligands on the quaternary structure of indolepyruvate decarboxylase

A solution of the purified enzyme (sample A, Fig. 4.3) was
dialyzed against Buffer A (no TPP or $MgCl_2$) at 4 ˚C for 24 h
(sample B, Fig. 4.3). After dialysis, cofactors were added to
sample B to give final concentrations of 1 mM TPP and 5 mM

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Table 4.3 Purification of indolepyruvate decarboxylase from
E. coli strain DH27 harboring the IPDC gene.

Step	Total protein	Total activity	Specific activity	Purification	Yield
	mg	units[a]	units/mg protein	fold	%
Crude extract[b]	9450	83.3	0.00881	1.0	100
$(NH_4)_2SO_4$ fractionation	2990	54.0	0.0181	2.0	65
Q Sepharose	315	35.6	0.113	12.8	43
Phenyl Sepharose	176	29.6	0.168	19.1	36

[a] units = the total amount of indole-3-acetaldehyde plus twice
   the amount of tryptophol produced per min, in μmol.
[b] 45 g (fresh weight) of cell paste were used as starting
   material.

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Fig. 4.2 SDS-polyacrylamide gel electrophoresis of indolepyr-
uvate decarboxylase from E. coli strain DH27 after each step of
the purification procedure. Lane S, prestained protein stand-
ards; lane 1, crude extract; lane 2, fraction precipitated by
38-55% $(NH_4)_2SO_4$; lane 3, active fractions from the Q Sepharose
column; lane 4, active fractions from the Phenyl Sepharose
column. Proteins were visualized by Coomassie Blue.

Fig. 4.3 Determination of the $\underline{M}_r$ of indolepyruvate decarboxylase by gel filtration chromatography on Superose 6 HR 10/30.

MgCl₂, and the mixture was incubated at 4 °C for 24 h (sample
C, Fig. 4.3). Next, sample A was incubated in 0.5 M Tris-HCl,
pH 8.5, at 4 °C for 48 h (sample D, Fig. 4.3). After incuba-
tion, sample D was dialyzed against Buffer D (1 mM TPP and 5 mM
MgCl₂) at 4 °C for 24 h (sample E, Fig. 4.3). Aliquots of each
sample were both applied to a Superose 6 HR 10/30 gel filtra-
tion column for estimation of the M_r of the enzyme (Fig. 4.3)
and assayed for indolepyruvate decarboxylase activity. At
temperature of 25 °C, the dimeric form of the enzyme associates
rapidly (6 min) to form tetramers in the presence of 1 mM TPP
and 5 mM MgCl₂. However, at temperature of 4 °C, the dimers
associate slowly (10 h) to form tetramers (Table 4.5). There-
fore, the assay of the enzyme for each sample was performed at
4 °C to determine the enzymatic activity of oligomer form.

The results of gel filtration chromatography of sample B
showed that the M_r of the enzyme that corresponded to the main
peak was 120,000, and the enzymatic activity was 10-fold lower
than that in sample A. However, the results of gel filtration
chromatography of sample C showed that the M_r of the enzyme
that corresponded to the main peak was 240,000, and the enzy-
matic activity was the same as that in sample A. These results
indicate that, in the absence of TPP and Mg²⁺, active tetramers
dissociate into inactive dimers, while in the presence of TPP
and Mg²⁺, inactive dimers associate to form active tetramers.

The results of gel filtration chromatography of sample D
showed that the M_r of the enzyme that corresponded to the main

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peak was 60,000, and sample D exhibited no enzymatic activity. However, the results of gel filtration chromatography of sample E showed that the M_r of the enzyme that corresponded to the two main peaks were 60,000 and 240,000, respectively, and the enzymatic activity was 2-fold lower than that in sample A. These results indicate that, at alkaline pH, active tetramers dissociate into inactive monomers, while in the presence of TPP and Mg^{2+}, inactive monomers can partially associate to form inactive dimers and active tetramers. It appears that the association of monomers to dimers occurs more slowly than that of dimers to tetramers. A scheme for the dissociation and association of the subunits of indolepyruvate decarboxylase is shown in Fig. 4.4.

The role of Mg^{2+} in the formation of oligomers

To clarify the roles of TPP and Mg^{2+} in the formation of oligomers, aliquots of sample B, which contained no TPP or $MgCl_2$, were dialyzed separately against Buffers B, C, and D at 4 °C for 24 h, and the resultant respective samples, designated F, G, and H, were subjected to the Superose 6 HR 10/30 gel filtration column for the estimation of the M_r of the enzyme in each sample. The gel filtration chromatography revealed that the enzyme in samples F (5 mM $MgCl_2$) and G (1 mM TPP) was dimeric, and that the enzyme in sample H (1 mM TPP and 5 mM $MgCl_2$) was tetrameric. The monomers associated to form tetramers only upon dialysis against Buffer D, as in the case of the
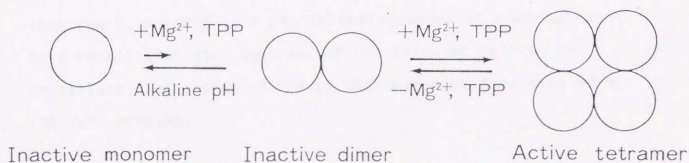
Inactive monomer   Inactive dimer   Active tetramer

Fig. 4.4 A representation of the subunit structure and
dissociation behavior of indolepyruvate decarboxylase.

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association of dimers to tetramers. The monomers and dimers
were not able to associate to form tetramers in the absence of
either TPP or Mg^{2+}, suggesting that both TPP and Mg^{2+} are
involved in the formation of oligomers.

To examine the correlation between the roles of Mg^{2+} and
TPP, the K_m values for Mg^{2+} and TPP of the purified enzyme from
E. coli strain DH27 were determined. The K_m value for Mg^{2+} was
2.5 mM and it was independent of the concentration of TPP.
However, the K_m value for TPP varied significantly with the
concentration of Mg^{2+} (Table 4.4). The K_m for TPP in the
presence of 10 μM $MgCl_2$ was 6 mM which is 10,000-fold higher
than the K_m for TPP (0.6 μM) in the presence of 5 mM $MgCl_2$.
This result indicates that one of the roles of Mg^{2+} is to
facilitate the binding of TPP to the enzyme by formation of a
TPP-Mg^{2+} complex.

The rate of interconversion of oligomers

TPP and Mg^{2+} were added to preparations of either the
monomeric or dimeric form of the enzyme and we monitored the
rate at which the catalytic activity returned. This system
appears to provide a straightforward method for determining the
rate of formation of tetramers, since only the tetramer has
catalytic activity. Next, tetramers were passed rapidly through
a small gel filtration column to remove TPP and Mg^{2+} and we
examined the rate at which the catalytic activity was lost. In
this way, we were able to get some idea of the rate at which

Table 4.4 .Effect of the concentration of Mg^{2+} on the affinity of indolepyruvate decarboxylase for TPP.

The purified enzyme was dialyzed against Buffer A at 4 °C for 24 h. The resultant solution was incubated at 25 °C in buffer A containing various concentrations of TPP and $MgCl_2$, and the decarboxylase activity was measured as described under "Materials and Methods". The values of apparent Michaelis-Menten constants (K_m) for TPP of the enzyme were obtained from linear plots of the initial rates of reactions by the method of Lineweaver and Burke (Lineweaver and Burk 1934).

Concentration of $MgCl_2$	K_m for TPP
mM	mM
0.00	10.0
0.01	6.0
0.20	2.1×10^{-3}
0.50	1.1×10^{-3}
1.00	1.0×10^{-3}
2.00	8.0×10^{-4}
5.00	6.0×10^{-4}

tetramers dissociate into dimers. As shown in Table 4.5, it appears that the association of monomers to tetramers occurs more slowly than that of dimers to tetramers, and that the interconversion of oligomers occurs more slowly at 4 °C than at 25 °C.

Substrate specificity

A number of indole derivatives and acids were tested as substrates for the purified enzyme from E. coli strain DH27 (Table 4.6). Among the compounds tested, pyruvic acid was decarboxylated at a rate of 19% of the decarboxylation of IPyA. However, L-Trp, indole-3-lactic acid, β-phenylpyruvic acid, oxalic acid, oxaloacetic acid, and acetoacetic acid were hardly decarboxylated at all. Substrate specificity of the purified enzyme from E. cloacae strain FERM BP-1529 was the same as that of the purified enzyme from E. coli strain DH27.

To examine whether other decarboxylation enzymes have non-specific indolepyruvate decarboxylase activity, pyruvate decarboxylase from Brewers' yeast was subjected to the indolepyruvate decarboxylase assay that is described above. This enzyme catalyzed the decarboxylation of IPyA at a rate of 0.1% of that of pyruvic acid. This result indicates that a highly specific enzyme is necessary for the decarboxylation of IPyA.

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Table 4.5 The rate of interconversion of oligomers.

To examine the rate of association of monomers and dimers to tetramers, 1 mM TPP and 5 mM $MgCl_2$ were added to samples B (dimers) and C (monomers), which contained no TPP or $MgCl_2$, and the mixtures were incubated at 4 °C or 25 °C in Buffer A. After incubation for various times, the decarboxylase activity of each sample was measured at 4 °C and the time required for restoration of 100 % of the catalytic activity was determined.

To examine the rate of dissociation of tetramers into dimers, sample A (tetramers) was passed rapidly through a NICK™ gel filtration column (0.9 x 2.0 cm; Pharmacia LKB Bio-technology) at 4 °C to remove TPP and Mg^{2+}, and then the mixtures were incubated at 4 °C or 25 °C in Buffer A. After incubation for various times, the decarboxylase activity of each sample was measured at 4 °C and the time required for loss of 70 % of the catalytic activity was determined.

Interconversion of oligomers	Temperature	Time required for dissociation or association
	°C	h
Monomers to tetramers	4	50.0
	25	0.3
Dimers to tetramers	4	10.0
	25	0.1
Tetramers to dimers	4	6.0
	25	1.0

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Table 4.6 Substrate specificity of indolepyruvate decarboxylase.

The purified indolepyruvate decarboxylase from E. coli strain DH27 harboring the IPDC gene was incubated at 25 °C in Buffer G containing various substrates, and the decarboxylation activity was measured as described. A value of 100 was assigned to the decarboxylation activity with IPyA as substrate.

Substrate	Concentration	Relative activity
	mM	%
Indole-3-pyruvic acid	0.1	100
L-Tryptophan	1.0	ND[a]
Indole-3-lactic acid	1.0	ND
β-Phenyl pyruvic acid	1.0	ND
Pyruvic acid	0.1	19
Oxalic acid	2.0	ND
Oxaloacetic acid	1.0	ND
Acetoacetic acid	3.0	ND

[a] ND, not detectable.

Substrate affinity

To examine the difference in substrate affinity of indole-pyruvate decarboxylase for IPyA and pyruvic acid, the K_m values for these substrates were determined. The K_m values for IPyA and pyruvic acid of the purified enzyme from E. coli strain DH27 were 15 μM and 2.5 mM, respectively. This result indicates that this enzyme has a high affinity for IPyA.

The K_m values for IPyA and pyruvic acid of the purified enzyme from E. cloacae were the same as that of the purified enzyme expressed in E. coli strain DH27.

Inhibition of indolepyruvate decarboxylase activity

To investigate the inhibition of indolepyruvate decarboxy-lase activity, three pyruvic acid derivatives and six indole derivatives were examined for their effects on the purified enzyme from E. coli strain DH27. As shown in Table 4.7, the pyruvic acid derivatives inhibited indolepyruvate decarboxylase activity, but the indole derivatives had no effect on the enzymatic activity at the concentration tested. Further investigation showed β-phenylpyruvic acid to function as a competitive inhibitor of indolepyruvate decarboxylase. The K_i for β-phenylpyruvic acid was calculated to be 50 μM.

Table 4.7 Inhibition of indolepyruvate decarboxylase by
various indole derivatives.

The purified indolepyruvate decarboxylase from E. coli
strain DH27 harboring the IPDC gene was incubated at 25 °C in
Buffer G containing various indole derivatives and IPyA, and
the amounts of IAAld produced were measured as described. A
value of 100 was assigned to the enzymatic activity in the
absence of any additions.

Indole compound	Concentration	Relative activity
	mM	%
None	-	100
Pyruvic acid	0.5	86
α-Ketoglutaric acid	0.5	64
β-Phenylpyruvic acid	0.5	7
L-Tryptophan	1.0	100
Indole	1.0	100
Indole-3-lactic acid	1.0	100
Indole-3-acetaldehyde	0.5	100
Tryptophol	1.0	100
Indole-3-acetic acid	1.0	100

Discussion

We report here for the first time the identification, purification, and characterization of indolepyruvate decarboxylase, which is a novel enzyme in IAA biosynthesis.

Little is known about the indolepyruvate decarboxylase in plants since the enzyme may be unstable and present at only low levels. Therefore, using $\underline{E.\ cloacae}$ isolated from the rhizosphere of cucumbers, we examined indolepyruvate decarboxylase in an effort to elucidate details of the IPyA pathway.

The results of gel filtration experiments revealed that indolepyruvate decarboxylase is a tetramer with an M_r of 240,000, and that TPP and Mg^{2+} function in the formation of the tetramer which is the enzymatically active holoenzyme. The monomers and dimers were not able to form tetramers in the absence of either TPP or Mg^{2+}. It appears, therefore, that both TPP and Mg^{2+} are involved in the formation of oligomers by formation of a TPP-Mg^{2+} complex. Furthermore, the K_m value for TPP of the enzyme increased significantly with the addition of Mg^{2+}, suggesting that TPP and Mg^{2+} bind at the same site of the enzyme, and that one of the roles of Mg^{2+} is to facilitate the binding of TPP to the enzyme by formation of the TPP-Mg^{2+} complex. A possible mechanism for the activation of the apoenzyme by the cofactors is as follows. First, TPP and Mg^{2+} bind at the same site of the enzyme by formation of the TPP-Mg^{2+} complex. Second, inactive monomers or dimers, being equivalent to the apoenzyme, associate to form active tetramers by binding

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of the TPP-Mg^{2+} complex to the enzyme.

It has been suggested that Mg^{2+} in pyruvate carboxylase plays two roles (Keech and Barritt 1967; Cazzulo and Stoppani 1969; McClure et al. 1971; Barden et al. 1972; Bais and Keech 1972): (a) Mg^{2+} forms a complex with ATP^{4-} to form the true $ATP-Mg^{2-}$ substrate; and (b) free Mg^{2+} forms a complex with the enzyme to potentiate the catalytic activity. The $\underline{K_m}$ value for Mg^{2+} of indolepyruvate decarboxylase (2.5 mM) was higher than that for TPP (0.6 μM) and excess Mg^{2+} induced a significant stimulation of indolepyruvate decarboxylase activity at saturating levels of the TPP-Mg^{2+} complex (data not shown), suggesting that free Mg^{2+} can also potentiate the catalytic activity.

Indolepyruvate decarboxylase from E. cloacae required TPP and Mg^{2+} as cofactors and it was stabilized by the presence of TPP and Mg^{2+}. The enzyme was a holoenzyme composed of four monomers. Pyruvate decarboxylase from yeast also requires TPP and Mg^{2+} as cofactors (Green et al. 1941), and TPP has been found to stabilize the enzyme during purification and storage (Juni and Heym 1968). Furthermore, pyruvate decarboxylase is a holoenzyme composed of four monomers (Gounaris et al. 1971; Gounaris et al. 1975). These results indicate that the physical characteristics of indolepyruvate decarboxylase from E. cloacae are very similar to those of pyruvate decarboxylase from yeast. However, there are significant differences between the two enzymes. While pyruvate decarboxylase from yeast has a low

specificity and affinity for pyruvic acid (K_m = 30 mM) (Green
et al. 1941), indolepyruvate decarboxylase from E. cloacae has
a high specificity and affinity for IPyA (K_m = 15 μM). Further-
more, pyruvate decarboxylase from yeast only slowly catalyzed
the decarboxylation of IPyA. Indolepyruvate decarboxylase from
E. cloacae was able to catalyze the decarboxylation of pyruvic
acid but the K_m for pyruvic acid (2.5 mM) is evidence of a
lower affinity for pyruvic acid than for IPyA (K_m, 15 μM).
These results indicate that indolepyruvate decarboxylase from
E. cloacae is a highly specific enzyme, which has a high affin-
ity for only IPyA. Although E. cloacae produces IAA as a sec-
ondary metabolic product, why does this bacterium has such a
highly specific enzyme? In view of the isolation of E. cloacae
from the rhizosphere of cucumbers, it is possible that the gene
for this enzyme is derived from plants.

In plants, L-tryptophan aminotransferase, which catalyzes
the first step in the IPyA pathway, is nonspecific for amino
acid substrates, and has a very high K_m value (Gamborg and
Wetter 1963; Truelsen 1972). The IAA concentration in plants is
maintained in the nanomolar range, and it is unlikely that such
low levels of IAA are regulated by L-tryptophan aminotransfer-
ase with its low affinity for L-Trp. From these consideration,
the following new pathway for the IAA biosynthesis is proposed:

L-Trp $\rightarrow$ D-Trp $\rightarrow$ IPyA $\rightarrow$ IAAld $\rightarrow$ IAA

Law suggested that tryptophan racemase might regulate the
conversion of L-Trp to IAA in dwarf pea plants (Law 1987).

McQueen-Mason and Hamilton proposed that D-tryptophan amino-transferase is important in the IAA biosynthesis in tall pea plants (McQueen-Mason and Hamilton 1989).

In bacteria, it was suggested that the rate-limiting step in the IPyA pathway is the conversion of IPyA to IAAld, and that indolepyruvate decarboxylase is a key enzyme in this pathway (Koga et al. 1991c). Furthermore, the present study indicates that the synthesis of the secondary metabolite, IAA, is regulated at the enzymatic level by indolepyruvate decarboxylase, which is highly specific for IPyA. Therefore, it is possible that indolepyruvate decarboxylase regulates the IAA biosynthesis in plants if the gene for indolepyruvate decarboxylase in E. cloacae is derived from plants.

References

Atsumi, S. (1980) Studies on the production of indole-3-acetic acid with auxin-heterotrophic mutants derived from cultured crown gall cells. Plant Cell Physiol. **21**: 1227-1232.

Bais, R. and Keech, B. (1972) The magnesium ion (Mg^{2+}) activation of sheep kidney pyruvate carboxylase. J. Biol. Chem. **247**: 3255-3261.

Barden, R.E., Fung, C-H., Utter, M.F., and Scrutton, M.C. (1972) Pyruvate carboxylase from chicken liver. J. Biol. Chem. **247**: 1323-1333.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of dye binding. Anal. Biochem. **72**: 248-254.

Cazzulo, J.J. and Stoppani, A.O.M. (1969) Effects of magnesium, manganese and adenosine triphosphate ions on pyruvate carboxylase from Baker's yeast. Biochem. J. **112**: 747- 754.

Fridovich, I. (1963) Inhibition of acetoacetic decarboxylase by anions. J. Biol. Chem. **238**: 592-598

Gamborg, O.L. and Wetter, L.R. (1963) An aromatic amino acid transaminase from mung bean. Can. J. Biochem. Physiol. **41**:

1733-1740.

Garcia-Tabares, F., Herraiz-Tomico, T., Amat-Guerri, F. and
Garcia-Bilbao, J.L. (1987) Production of 3-indoleacetic acid
and 3-indolelactic acid in Azotobacter vinelandii cultures
supplemented with tryptophan. Appl. Microbiol. Biotechnol.
25: 502-506.

Gibson, R.A., Schneider, E.A. and Wightman, F. (1972) Biosyn-
thesis and metabolism of indole-3yl-acetic acid. II. In vivo
experiments with ^{14}C-labelled precursors of IAA in tomato and
barley shoots. J. Exp. Bot. 23: 381-399.

Gounaris, A.D., Turkenkopf, I., Buckwald, S. and Young, A.A.
(1971) Pyruvate decarboxylase I. Protein dissociation into
subunits under conditions in which thiamine pyrophosphate is
released. J. Biol. Chem. 246: 1302-1309.

Gounaris, A.D., Turkenkopf, I., Civerchia, L.L. and Greenlie,
J. (1975) Pyruvate decarboxylase III. Specificity restric-
tions for thiamine pyrophosphate in the protein association
step, sub-unit structure. Biochim. Biophys. Acta 405: 492-499.

Green, D.E., Herbert, D. and Subrahmanyan, V. (1941)
Carboxylase. J. Biol. Chem. 138: 327-339.

Gunsalus, I.C. and Stamer, J.R. (1955) Transaminases in bacteria. In: Colowick, S.P., Kaplan, N.O. (eds) Methods in Enzymology, Vol. 2. Academic Press, New York, pp 170-177.

Juni, E. and Heym, G.A. (1968) Properties of yeast pyruvate decarboxylase and their modification by proteolytic enzymes. I. Stability of decarboxylases from wild-type and mutant strains. Arch. Biochem. Biophys. **127:** 79-88.

Kaper, J.M. and Verdstra, H. (1958) On the metabolism of tryptophan by Agrobacterium tumefaciens. Biochim. Biophys. Acta. **30:** 401-420.

Keech, B. and Barritt, G.J. (1967) Allosteric activation of sheep kidney pyruvate carboxylase by the magnesium ion (Mg^{2+}) and the magnesium adenosine triphosphate ion ($MgATP^{2-}$). J. Biol. Chem. **242:** 1983-1987.

Kenten, R.H. and Mann, P.J.G. (1952) The oxidation of amines by extracts of pea seedlings. Biochem. J. **50:** 360-369.

Koga, J., Ishii, T., Miyadoh, S. and Adachi, T. (1991a) Effects of Enterobacter cloacae on the growth of cucumber and rice plants. Sci. Reports Meiji Seika Kaisha **30:** 35-37.

Koga, J., Adachi, T. and Hidaka H. (1991b) IAA biosynthetic

pathway from tryptophan via indole-3-pyruvic acid in Entero-
bacter cloacae. Agric. Biol. Chem. **55**: 701-706.

Koga, J., Adachi, T. and Hidaka, H. (1991c) Molecular cloning
of the gene for indolepyruvate decarboxylase from Enterobacter
cloacae. Mol. Gen. Genet. **226**: 10-16.

Law, D.M.(1987) Gibberellin-enhanced indole-3-acetic acid
biosynthesis: D-Tryptophan as the precursor of indole-3-acetic
acid. Physiol. Plant. **70**: 626-632.

Lineweaver, H. and Burk, D. (1934) The determination of enzyme
dissociation constants. J. Am. Chem. Soc. **56**: 658-666.

McClure, W.R., Lardy, H.A. and Kneifel, H.P. (1971) Rat liver
pyruvate carboxylase. I. Preparation, properties, and cation
specificity. J. Biol. Chem. **246**: 3569-3578.

McQueen-Mason, S.J. and Hamilton, R.H. (1989) The biosynthe-
sis of indole-3-acetic acid from D-tryptophan in Alaska pea
plastids. Plant Cell Physiol. **30**: 999-1005.

Moore, T.C. and Shaner, C.A. (1968) Synthesis of indoleacetic
acid from tryptophan via indolepyruvic acid in cell free
extracts of pea seedlings. Arch. Biochem. Biophys. **127**: 613-
621.

Paris, C.G. and Magasanik, B. (1981) Tryptophan metabolism in Klebsiella aerogenes: regulation of the utilization of aromatic amino acids as sources of nitrogen. J. Bacteriol. **145**: 257-265.

Schmitt, A., Bottke, I. and Siebert, G. (1966) Eigenschaften einer oxalacetat-decarboxylase aus dorschmuskulatur. Hoppe Seyler's Z. Physiol. Chem. **347**: 18-34.

Sheldrake, A.R. (1973) The production of hormones in higher plants. Biol. Rev. Camb. Philos. Soc. **8**: 510-515.

Shimazono, H. and Hayaishi, O. (1957) Enzymatic decarboxylation of oxalic acid. J. Biol. Chem. **227**: 151-159.

Singer, T.P. and Pensky, J. (1952) Isolation and properties of the α-carboxylase of wheat germ. J. Biol. Chem. **196**: 375-388.

Truelsen, T.A. (1972) Indole-3-pyruvic acid as an intermediate in the conversion of tryptophan to indole-3-acetic acid. I. Some characteristics of tryptophan transaminase from mung bean seedlings. Physiol. Plant. **26**: 289-295.

Truelsen, T.A. (1973) Indole-3-pyruvic acid as a intermediate in the conversion of tryptophan to indole-3-acetic acid. II.

Distribution of tryptophan transaminase activity in plants.
Physiol. Plant. **28**: 67-70.

CHAPTER 5

Involvement of $_L$-Tryptophan Aminotransferase in the Indole-3-acetic Acid Biosynthesis in Enterobacter cloacae.

Abstract

$_L$-Tryptophan aminotransferase ($_L$-tryptophan:2-oxoglutarate aminotransferase; EC 2.6.1.27) from Enterobacter cloacae was purified 62-fold and characterized to determine its role in indole-3-acetic acid biosynthesis. The enzyme reversibly catalyzed the transamination of $_L$-tryptophan with a-ketoglutarate as the amino acceptor to yield indole-3-pyruvic acid and $_L$-glutamate, and the K_m values for $_L$-tryptophan and indole-3-pyruvic acid were 3.3 mM and 24 μM, respectively. In the indole-3-acetaldehyde synthesis experiments in vitro, 94% of $_L$-tryptophan was efficiently converted to indole-3-acetaldehyde by the purified $_L$-tryptophan aminotransferase plus indolepyruvate decarboxylase. Furthermore, the amounts of $_L$-tryptophan decreased with increases in the indolepyruvate decarboxylase activity, while the amounts of indole-3-acetaldehyde increased with increases in this activity. In genetic experiments, the amounts of $_L$-tryptophan produced by Enterobacter and Pseudomonas strains harboring the gene for indolepyruvate decarboxylase were lower than those produced by these strains lacking the gene, while the amounts of IAA produced by Enterobacter and Pseudomonas strains harboring the gene for indolepyruvate decarboxylase were higher than those produced by these strains

lacking the gene. These results clearly show that L-tryptophan
aminotransferase is involved in the IAA biosynthesis and that
this pathway is regulated by indolepyruvate decarboxylase
alone.

Introduction

Indole-3-acetic acid (IAA) was the first plant hormone
identified, and much has been learned about its biosynthesis.
Considering the important roles of IAA in plant growth and
development, the regulation of its biosynthesis is of consider-
able interest.

L-Tryptophan (L-Trp) is generally accepted as the major
precursor of IAA in plants. On the basis of potential interme-
diates isolated from higher plants, the main IAA biosynthetic
pathway (the indole-3-pyruvic acid [IPyA] pathway) leading from
L-Trp is thought to be as follows (Sheldrake 1973):

L-Trp $\rightarrow$ IPyA $\rightarrow$ indole-3-acetaldehyde (IAAld) $\rightarrow$ IAA
However, the exact mechanism of the IPyA pathway has not yet
been unequivocally demonstrated. Since the intermediary com-
pound, IPyA, is unstable and is nonenzymatically degraded into
IAA, it is difficult to demonstrate that the second and third
steps in this pathway occur under enzymatic control (Kaper and
Verdstra 1958; Moore and Shaner 1968; Gibson et al. 1972;
Sheldrake 1973; Garcia-Tabares et al. 1987).

In a previous study, it was shown that IAA was produced by
Enterobacter cloacae isolated from actively growing cucumbers

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(Koga et al. 1991a), and that its biosynthesis followed the
IPyA pathway (Koga et al. 1991b). Genetic and enzymatic experi-
ments showed that indolepyruvate decarboxylase, which catalyzes
the conversion of IPyA to IAAld at the second step in this
pathway, is a key enzyme and that it has high specificity and
affinity for IPyA (Koga et al. 1991c, 1992). However, important
questions about the involvement of L-tryptophan aminotransfer-
ase in the IPyA pathway have not yet been answered. The amounts
of IAA produced from L-Trp by <u>Enterobacter</u> strains harboring
the gene for indolepyruvate decarboxylase were higher than
those produced by these same strains lacking the gene (Koga et
al. 1991c). Since indolepyruvate decarboxylase catalyzes the
second step in the IPyA pathway, the amounts of IAA produced
from L-Trp should not be increased without the activation of L-
tryptophan aminotransferase, as well as of indolepyruvate
decarboxylase.

L-tryptophan aminotransferase catalyzes the conversion of
L-Trp to IPyA, the first step in the IPyA pathway. This enzyme,
which has been found in many species of plants and bacteria
(Gunsalus and Stamer 1955; Truelsen 1973; Paris and Magasanik
1981), is nonspecific for amino acid substrates, and has a very
high K_m value for L-Trp (Gamborg and Wetter 1963; Truelsen
1972). The IAA concentration in plants is maintained in the
nanomolar range, and it is unlikely that such low levels of IAA
are regulated by L-tryptophan aminotransferase with its low
affinity for L-Trp (Law 1987).

Only a small proportion of ʟ-Trp supplied is converted to
IAA in plant tissues, and the rate of conversion from ʟ-Trp is
far lower than would be expected for a direct precursor (Lib-
bert et al. 1966; Libbert and Silhengst 1970; Black and Hamil-
ton 1971). From these considerations, a hypothetical pathway
for IAA biosynthesis without ʟ-Trp as a precursor has been
proposed (Winter 1966; Wright et al. 1991).

Therefore, in an effort to answer the various questions
related to the IAA biosynthetic pathway, we have purified ʟ-
tryptophan aminotransferase from E. cloacae and characterized
its physical and regulatory properties.

Materials and Methods
Bacterial strains, plasmids, and culture conditions

Strain FERM BP-1529R is a rifampicin resistant (Rif^r)
derivative of E. cloacae FERM BP-1529, isolated from the rhizo-
sphere of cucumbers (Koga et al. 1991a). Strain G438 is an IAA-
negative mutant of FERM BP-1529R (Koga et al. 1991c). Strain
1236R is a Rif^r derivative of Enterobacter agglomerans JCM
1236, obtained from the Japan Collection of Microorganisms,
Wako-shi, Saitama, Japan. Strain 14164R is a Rif^r derivative of
Pseudomonas putida IFO 14164, obtained from the Institute for
Fermentation, Yodogawa-ku, Osaka, Japan. Strain 14160R is also
a Rif^r derivative of Pseudomonas fluorescens IFO 14160, ob-
tained from the Institute for Fermentation. These Enterobacter
and Pseudomonas strains were used for triparental mating exper-

iments.

Plasmid vectors pUC19 and pBI121 were purchased from Clontech Laboratories, Inc., 4030 Fabian Way, Palo Alto, CA 94303-4607, USA.

For purification of L-tryptophan aminotransferase from E. cloacae, 2.2-liter cultures of strain FERM BP-1529 were grown for 24 h at 30 °C in a medium containing 2% tryptone (Difco), 1% yeast extract (Difco), 1 mM pyridoxal phosphate, and 0.1 M potassium phosphate (pH 6.5). Cells were harvested by centrifugation and were stored at -80 °C until needed.

To examine the production of L-Trp and IAA by bacterial strains harboring the gene for indolepyruvate decarboxylase, E. cloacae FERM BP-1529R, E. cloacae G438, E. agglomerans 1236R, P. putida 14164R, and P. fluorescens 14160R were grown for 24 h at 30 °C in a medium containing 1% tryptone, 0.5% yeast extract, 0.5% NaCl, and 0.15 M potassium phosphate (pH 6.5).

Buffers

The buffers used were as follows: Buffer A, 50 mM potassium phosphate and 1 mM pyridoxal phosphate (pH 6.5); Buffer B, 50 mM potassium phosphate, 1 mM pyridoxal phosphate, and 0.5 M NaCl (pH 6.5); Buffer C, 10 mM potassium phosphate, 0.5 mM α-ketoglutarate, 0.05 mM pyridoxal phosphate, 5 mM $MgCl_2$, and 0.1 mM thiamine pyrophosphate (pH 6.5); Buffer D, 10 mM potassium phosphate, 10 mM L-glutamate, and 0.05 mM pyridoxal phosphate (pH 6.5); and Buffer E, 10 mM potassium phosphate, 0.5 mM α-

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ketoglutarate, 1.0 mM $_L$-glutamate, 0.05 mM pyridoxal phosphate, 5 mM $MgCl_2$, and 0.1 mM thiamine pyrophosphate (pH 6.5).

Reagents

Indolepyruvate decarboxylase was purified to apparent homogeneity from <u>Escherichia coli</u> harboring plasmid pIP27, which was generated by inserting the gene for indolepyruvate decarboxylase into the <u>Sma</u>I site of the plasmid pKK223-3 as described (Koga et al. 1992). $_L$-Trp, IPyA, and IAAld were purchased from Sigma, as were all other reagents.

$_L$-Tryptophan aminotransferase assay

To date, a spectrophotometric method, that is based on the absorbance of the enol form of IPyA in neutral solution at 305 nm, has been used for this assay (Tangen et al. 1965; O'Neil and DeMoss 1968; Speedie et al. 1975). However, IPyA is an unstable compound and difficult to quantitate accurately. Therefore, $_L$-tryptophan aminotransferase activity was determined by estimating the amount of IAAld produced from IPyA in the presence of indolepyruvate decarboxylase.

The reaction mixture, 3 ml, contained Buffer C, 10 mM $_L$-Trp, 0.1 units of the purified indolepyruvate decarboxylase, and the preparation of enzyme to be tested, was incubated at 25 ˙C for 10 min. After incubation, the reaction was stopped by the addition of 6 ml of 0.1 N HCl containing 50% ethanol, and the amount of IAAld was determined by HPLC on a C-18 column as

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described (Koga et al. 1991c). The column was eluted with 30% ethanol in 5% acetic acid at a flow rate of 0.7 ml/min. The effluent was monitored with a spectrofluorometer (excitation wavelength, 280 nm, emission wavelength, 350 nm). One unit of activity was defined as the amount of enzyme that catalyzed the production of 1 μmole of IAAld per min under the above conditions.

The reverse reaction, with IPyA as amino acceptor and L-glutamate as donor, was performed by estimating the amount of L-Trp produced. The reaction mixture, 3 ml, contained Buffer D and the preparation of enzyme to be tested. The reaction was started by the addition of 30 μl of 4 mM IPyA, which was dissolved in ethanol, and incubated at 25 °C for 10 min. After incubation, the reaction was stopped by the addition of 6 ml of 0.1 N HCl, and the amount of L-Trp produced was determined by HPLC on a C-18 column. For quantitative determination of L-Trp, the column was eluted with 5% acetic acid at a flow rate of 1.0 ml/min. The effluent was monitored with a spectrofluorometer (excitation wavelength, 280 nm; emission wavelength, 350 nm). The HPLC fraction corresponding to the L-Trp peak was collected and the structure of the compound was confirmed by ^{1}H-NMR as described (Koga et al. 1991b).

Protein concentrations were determined by Bradford's method using bovine serum albumin as a standard (Bradford 1976).

L-Tryptophan aminotransferase purification

Step 1. Crude extract preparation — A total of 22 g of cell paste, prepared from a culture of E. cloacae strain FERM BP-1529, was suspended in 90 ml of Buffer A and the cells were disrupted by sonication. The mixture was centrifuged at 40,000 x g for 40 min and the supernatant was diluted with 810 ml of Buffer A.

Step 2. Fractionation with $(NH_4)_2SO_4$ — Enzymatically active protein was obtained in the fractions precipitated between 50% (313 g/l) and 60% (390 g/l) ammonium sulfate saturation at 4 °C. After centrifugation of the 60% ammonium sulfate solution at 40,000 x g for 40 min, the precipitate was dissolved in 80 ml of Buffer A and the solution was dialyzed against 10 liters of Buffer A.

Step 3. Fast Protein Liquid Chromatography on a Q-Sepharose FF Column — A Q-Sepharose FF column (2.6 x 40 cm; Pharmacia LKB Biotechnology) was equilibrated with Buffer A and the protein solution from Step 2 was applied to the column. The column was eluted with a linear gradient of 1,000 ml each of Buffer A and Buffer B. The active fractions were pooled and concentrated to 2 ml by ultrafiltration.

Step 4. Fast Protein Liquid Chromatography on a Superose 12 Gel Filtration Column — A Superose 12 Prep Grade gel filtration column (1.6 x 50 cm; Pharmacia LKB Biotechnology) was equilibrated with Buffer A. The active fraction from Step 3 was applied to the column and the column was eluted with Buffer A

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at a flow rate of 0.5 ml/min at 4 °C. The active fractions were
pooled and concentrated to 10 ml by ultrafiltration.

Molecular weight determination

The M_r of ʟ-tryptophan aminotransferase was estimated by
fast protein liquid chromatography on a Superose 12 HR 10/30
gel filtration column (1 x 30 cm; Pharmacia LKB Biotechnology).
The column was equilibrated with Buffer A. The active protein
fraction from Step 4 was applied to the column. The column was
eluted with Buffer A at a flow rate of 0.4 ml/min. The M_r of
the enzyme was determined by monitoring the elution of active
fractions. The following proteins were used as M_r standards:
thyroglobulin (670,000), γ-globulin (158,000), bovine serum
albumin (67,000), ovalbumin (44,000), myoglobin (17,000), and
vitamin B-12 (1,350).

Reconstitution of the IAAld synthesis from ʟ-Trp *in vitro*

Each reaction mixture, 1 ml, contained Buffer E, 0.4 mM ʟ-
Trp, 0.007 units of the purified ʟ-tryptophan aminotransferase,
and the various amounts (from 0 to 0.1 units) of the purified
indolepyruvate decarboxylase, was incubated at 37 °C for 240
min. After incubation, the amounts of ʟ-Trp and IAAld in reac-
tion mixtures were determined by HPLC on a C-18 column as
described above.

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Results

Purification of ʟ-tryptophan aminotransferase

As a result of $(NH_4)SO_4$ fractionation and chromatography on Q-Sepharose FF and Superose 12, ʟ-tryptophan aminotransferase was purified 62-fold, with recovery of 4.1% of the initial activity (Table 5.1). The specific activity of the purified enzyme was 1.41 units/mg protein. The enzyme was stable at the early stages of purification, but after chromatography on Superose 12, the recovery was quite unsatisfactory. As a consequence, the purification of ʟ-tryptophan aminotransferase to homogeneity was difficult because of the instability of the enzyme. The remaining studies were performed with this purified enzyme.

The M_r of ʟ-tryptophan aminotransferase was estimated to be 70,000 by gel filtration on Superose 12.

pH optimum

The effect of pH on the catalytic activity of the purified enzyme was investigated in 20 mM potassium phosphate buffer from pH 5.5 to pH 8.0. Under the assay conditions, the enzyme showed a broad pH optimum with a peak around pH 6.5.

Cofactor requirements of ʟ-tryptophan aminotransferase

To investigate the cofactor requirements of ʟ-tryptophan aminotransferase, we studied the effects of the coenzyme pyridoxal phosphate and of the amino acceptor α-ketoglutarate.

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Table 5.1 Summary of the purification procedure for
L-tryptophan aminotransferase.

Step	Total protein	Total activity	Specific activity	Purification	Yield
	mg	units[a]	units/mg protein	fold	%
Crude extract[b]	1780	40.8	0.0229	1.0	100
$(NH_4)SO_4$ fractionation	420	13.9	0.0331	1.4	34
Q Sepharose FF	23.3	6.00	0.258	11.3	15
Superose 12	1.20	1.69	1.41	61.6	4.1

[a] One unit of activity was defined as the amount of enzyme that
   catalyzed the production of 1 μmole of indole-3-acetaldehyde
   per min at 25 °C.

[b] 22 g (fresh weight) of cell paste were used as starting
   material.

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A solution of the purified enzyme was dialyzed against 50 mM potassium phosphate, pH 6.5, at 4 °C for 24 h. After dialysis, cofactors were added to the enzyme to give a final concentration of 0.05 mM pyridoxal phosphate or of 0.5 mM α-ketoglutarate, and the ʟ-tryptophan aminotransferase activity was examined. A 10-fold increase in activity was found in the presence of 0.05 mM pyridoxal phosphate. A 39-fold increase in activity was found in the presence of 0.5 mM α-ketoglutarate, while higher concentrations inhibited the enzymatic activity. Furthermore, a 68-fold increase in activity was found in the presence of 0.05 mM pyridoxal phosphate and 0.5 mM α-ketoglutarate together. These results indicate that the ʟ-tryptophan aminotransferase from E. cloacae belongs to the pyridoxal phosphate dependent class of aminotransferases.

Reverse reaction of ʟ-tryptophan aminotransferase with IPyA as amino acceptor

To investigate the reverse reaction catalyzed by ʟ-tryptophan aminotransferase, the purified enzyme was subjected to the assay of the reverse reaction as described above. The purified ʟ-tryptophan aminotransferase catalyzed the transamination of IPyA with ʟ-glutamate as amino donor to yield ʟ-Trp. This result suggests that this enzyme can catalyze a reverse aminotransferase reaction.

A highest activity was found in the presence of 40 μM IPyA as amino acceptor, while higher concentrations (above 40 μM)

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inhibited the enzymatic activity.

Kinetic properties of L-tryptophan aminotransferase

The values of the apparent Michaelis-Menten constants (K_m) of the purified L-tryptophan aminotransferase were obtained from linear plots of the initial rates of reactions by the method of Lineweaver and Burk (Lineweaver and Burk 1934). The K_m values for L-Trp and α-ketoglutarate of L-tryptophan amino-transferase in the direction of IPyA formation were 3.3 mM and 45 μM, respectively. The K_m values for IPyA and L-glutamate of L-tryptophan aminotransferase in the direction of L-Trp forma-tion were 24μM and 1.8 mM, respectively. The K_m value for IPyA is 138-fold lower than that for L-Trp, suggesting that the reaction in the direction of L-Trp formation is greater than that in the opposite direction.

IAAld synthesis from L-Trp in vitro by the purified L-trypto-phan aminotransferase and indolepyruvate decarboxylase

The details of the IPyA pathway remain the subject of controversy. Therefore, to demonstrate the involvement of L-tryptophan aminotransferase in this pathway, we examined wheth-er the conversion of L-Trp to IAAld is possible in vitro in a combined reaction with the two purified enzymes that are thought to catalyze reactions in the IPyA pathway. 94% of L-Trp was efficiently converted to IAAld by L-tryptophan aminotrans-ferase plus 0.1 U of indolepyruvate decarboxylase, which cata-

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lyzes the conversion of IPyA to IAAld at the second step in the IPyA pathway (Fig. 5.1). This result indicates that ʟ-tryptophan aminotransferase is involved in the IPyA pathway and that ʟ-Trp is a precursor to IAA.

ʟ-Tryptophan aminotransferase from E. cloacae catalyzes a reverse reaction, and it appears that this enzyme is regulated by indolepyruvate decarboxylase. Therefore, holding the ʟ-tryptophan aminotransferase activity constant, we examined the effect of indolepyruvate decarboxylase activity on the synthesis of ʟ-Trp and IAAld. The amounts of ʟ-Trp decreased with increases in the indolepyruvate decarboxylase activity, while the amounts of IAAld increased with increases in this activity (Fig. 5.1). It appears, therefore, that indolepyruvate decarboxylase controls the levels of ʟ-Trp, as well as that of IAAld. Only 30% of ʟ-Trp was converted to IPyA by ʟ-tryptophan aminotransferase alone (Fig. 5.1). However, 94% of ʟ-Trp was converted to IPyA by ʟ-tryptophan aminotransferase plus 0.1 U of indolepyruvate decarboxylase, since ʟ-tryptophan aminotransferase activity in the direction of ʟ-Trp formation was decreased by the competition with indolepyruvate decarboxylase.

Effect of the gene for indolepyruvate decarboxylase on the production of ʟ-Trp and IAA in various bacterial strains

The plasmid pIP121 was constructed for expression of the gene for indolepyruvate decarboxylase in Enterobacter and Pseudomonas strains (Fig. 5.2). This plasmid was mobilized from

Fig. 5.1 Effects of the indolepyruvate decarboxylase activity on the synthesis of ʟ-tryptophan and indole-3-acetaldehyde.

Each reaction mixture, 1 ml, contained Buffer E, 0.4 mM ʟ-Trp, 0.007 units of the purified ʟ-tryptophan aminotransferase, and the various amounts (from 0 to 0.1 units) of the purified indolepyruvate decarboxylase, was incubated at 37 °C for 240 min. After incubation, the amounts of ʟ-Trp and IAAld in reaction mixtures were determined by HPLC on a C-18 column.
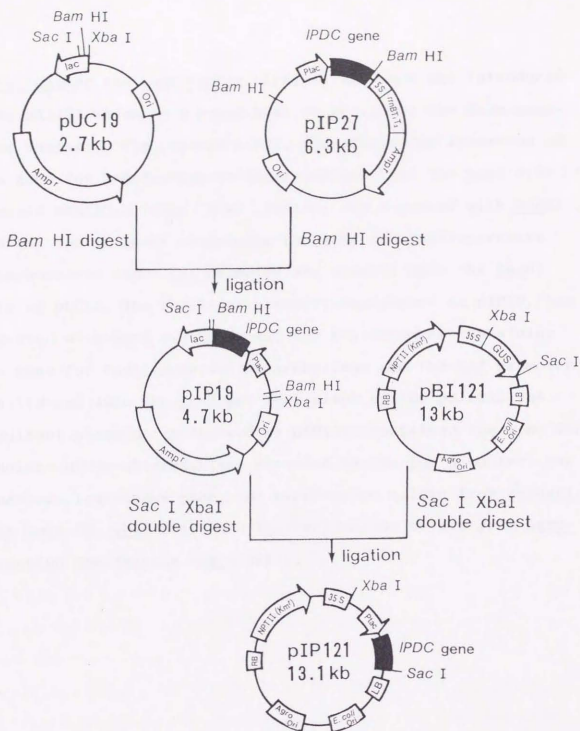
Fig. 5.2 Plasmid pIP121 construction for expression of the gene for indolepyruvate decarboxylase in Enterobacter and Pseudomonas strains.

To transfer the gene for indolepyruvate decarboxylase into

Enterobacter and Pseudomonas strains, the gene was introduced
into pBI121 which is a broad host range vector for Gram-nega-
tive bacteria. The plasmid pIP27, constructed by insertion of
the gene for indolepyruvate decarboxylase into the SmaI site of
plasmid pKK223-3 (Koga et al. 1991c), was digested with BamHI,
and the DNA fragment containing the gene for indolepyruvate
decarboxylase and a tac promoter was ligated into the BamHI
site of pUC19. The resultant plasmid, designated as pIP19, was
digested with SacI and XbaI, and the DNA fragment containing
the gene for indolepyruvate decarboxylase and the tac promoter
was ligated into the SacI and XbaI sites of the pBI121. The
resultant plasmid, designated as pIP121, contained the gene for
indolepyruvate decarboxylase preceded by the tac promoter, the
kanamycin resistance gene, the replication origin from Escheri-
chia coli (E. coli Ori), and the replication origin from Agro-
bacterium tumefaciens (Agro ori).

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E. coli DH5α into E. cloacae G438, E. agglomerans 1236R, P. putida 14164R, and P. fluorescence 14160R by triparental matings using pRK2013 as a helper plasmid (Ditta et al. 1980). To examine the effect of the gene on the production of L-Trp and IAA, each strain was grown for 24 h at 30 °C in liquid culture medium and the amounts of L-Trp and IAA produced by these strains were examined as described.

The amounts of IAA produced by the wild-type strain of E. cloacae were 41 times higher than those produced by an IAA-negative mutant E. cloacae G438, and were the same as those produced by E. cloacae G438 harboring the gene for indolepyruvate decarboxylase (Table 5.2), suggesting that E. cloacae G438 was defective in the pathway from IPyA to IAAld. The amounts of L-Trp produced by the wild-type strain of E. cloacae and E. cloacae G438 harboring the gene for indolepyruvate decarboxylase were very much lower than those produced by E. cloacae G438 (Table 5.2). This genetic experiment clearly showed that the synthesis of L-Trp and IAA in E. cloacae is regulated by indolepyruvate decarboxylase alone.

Furthermore, the amounts of L-Trp produced by Enterobacter and Pseudomonas strains harboring the gene for indolepyruvate decarboxylase were lower than those produced by these strains lacking the gene, while the amounts of IAA produced by Enterobacter and Pseudomonas strains harboring the gene for indolepyruvate decarboxylase were higher than those produced by these strains lacking the gene (Table 5.2). It appears, therefore,

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Table 5.2 Effects of the gene for indolepyruvate decarboxy-
lase on the production of ʟ-tryptophan and indole-3-acetic acid
in variousbacterial strains.

Bacterial strains	Production of[a] (μM):	
	ʟ-Tryptophan	IAA
Enterobacter cloacae		
FERM BP-1529R (wild-type)	ND	409
G438	404	10
G438 + the indolepyruvate decarboxylase gene	ND	408
Enterobacter agglomerans		
1236R	387	5
1236R + the indolepyruvate decarboxylase gene	28	360
Pseudomonas putida		
14164R	403	4
14164R + the indolepyruvate decarboxylase gene	11	386
Pseudomonas fluorescens		
14160R	374	ND
14160R + the indolepyruvate decarboxylase gene	161	207

[a] Each strain was grown for 24 h at 30 ˚C in a medium contain-
  ing 1% tryptone, 0.5% yeast extract, 0.5% NaCl, and 0.15 M
  potassium phosphate (pH 6.5). The amounts of ʟ-Trp and
  IAA produced by each strain were quantitated by HPLC on a
  C-18 column. The HPLC fractions corresponding to the
  peaks of ʟ-Trp and IAA were collected and the structures of
  the compounds were confirmed by ^{1}H-NMR as described previ-
  ously (Koga et al. 1991b).

that in the majority of bacteria, the synthesis of $_L$-Trp and IAA can be regulated by indolepyruvate decarboxylase alone and that these bacteria have an aminotransferase that catalyzes both directions of reversible reaction.

Discussion

We report here for the first time the identification, purification, and characterization of an $_L$-tryptophan aminotransferase from E. cloacae that catalyzes both directions of a reversible reaction. The majority of aminotransferases, such as alanine aminotransferases and tyrosine aminotransferases, have also been found to act reversibly (Kenney 1959; Hopper and Segal 1962; De Rosa et al. 1979). In general, the K_m value for the amino acceptor is lower than that for the amino donor. However, the K_m value for IPyA of $_L$-tryptophan aminotransferase (24 μM) was very much lower than that of other known aminotransferases. The high affinity of the enzyme for IPyA may reflect the need to prevent the build-up of high intracellular concentrations of this unstable substrate.

In experiments to examine the synthesis of IAAld in vitro, most of the $_L$-Trp in the reaction mixture was efficiently converted to IAAld by the purified $_L$-tryptophan aminotransferase plus indolepyruvate decarboxylase. This result provides evidence that $_L$-Trp is the natural precursor in the IPyA pathway. Furthermore, the amounts of $_L$-Trp decreased with increases in the indolepyruvate decarboxylase activity, while the amounts

of IAAld increased with increases in this activity. This result indicates that the conversion of L-Trp to IAAld is regulated by indolepyruvate decarboxylase alone. Considering the reversibility of the reaction catalyzed by L-tryptophan aminotransferase, it seems likely that the transaminase activity from L-Trp to form IPyA is regulated by the indolepyruvate decarboxylase activity. A scheme for the regulation of the IAA biosynthetic pathway, deduced from our results, is shown in Fig. 5.3. When indolepyruvate decarboxylase is activated, the transaminase activity in the direction of IPyA formation is increased, since the transaminase activity in the direction of L-Trp formation is decreased by the competition with indolepyruvate decarboxylase. When indolepyruvate decarboxylase is inactivated, the transaminase activity in the direction of IPyA formation disappears, since the transaminase activity in the direction of L-Trp formation is greater than that in the opposite direction.

In plants, L-tryptophan aminotransferase is nonspecific for amino acid substrates, and has a very high K_m value for L-Trp (Gamborg and Wetter 1963; Truelsen 1972). The IAA concentration in plants is maintained in the nanomolar range, and it is unlikely that such low amounts of IAA are regulated by L-tryptophan aminotransferase with its low affinity for L-Trp. From these considerations, the following new pathway for the IAA biosynthesis is proposed:

L-Trp $\rightarrow$ D-Trp $\rightarrow$ IPyA $\rightarrow$ IAAld $\rightarrow$ IAA

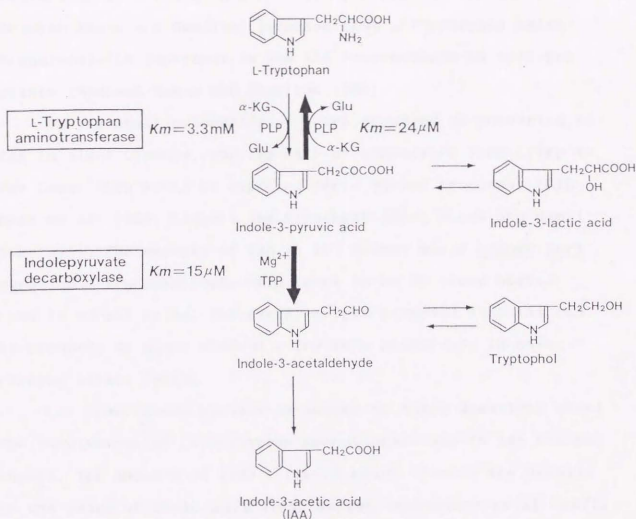Law suggested that tryptophan racemase might regulate the

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Fig. 5.3 A scheme for the indole-3-acetic acid biosynthesis in
Enterobacter cloacae.

The reactions that function efficiently are indicated by
the bold arrows. The abbreviations used are: α-KG, α-ketogluta-
rate; Glu, L-glutamate; PLP, pyridoxal phosphate; and TPP,
thiamine pyrophosphate. The $\underline{K_m}$ values for L-Trp and IPyA of L-
tryptophan aminotransferase are 3.3 mM and 24 μM, respectively.
The $\underline{K_m}$ value for IPyA of indolepyruvate decarboxylase is 15 μM.

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conversion of $_L$-Trp to IAA in dwarf pea plants (Law 1987).
McQueen-Mason and Hamilton proposed that $_D$-tryptophan amino-
transferase is important in the IAA biosynthesis in tall pea
plants (McQueen-Mason and Hamilton 1989).

Only a small proportion of $_L$-Trp supplied is converted to
IAA in plant tissues, and the rate of conversion from $_L$-Trp is
far lower than would be expected for a direct precursor (Lib-
bert et al. 1968; Libbert and Silhengst 1970; Black and Hamil-
ton 1971). The amounts of IAA in the mutant maize orange peri-
carp, an $_L$-Trp auxotroph, were found to be 50 times higher
than in normal maize. Therefore, Wright proposed that IAA can
be produced de novo, without $_L$-Trp as a precursor, in maize
(Wright et al. 1991).

Our experiments provide an answer to these questions about
the involvement of $_L$-tryptophan aminotransferase in IAA biosyn-
thesis. The amounts of free $_L$-Trp in plant tissues are usually
in the range of 10-50 μg/g fresh weight (Schneider et al. 1972;
Sheldrake 1973). Although $_L$-tryptophan aminotransferase has a
very high K_m value, in consideration of the large size of the
$_L$-Trp pool, we can anticipate that $_L$-tryptophan aminotransfer-
ase provides sufficient IPyA for the production of IAA. Fur-
thermore, when indolepyruvate decarboxylase is activated, the
IPyA produced should be efficiently converted to IAA by this
enzyme, with its high affinity for IPyA. Therefore, a second
question arises. IPyA is an unstable compound and is nonenzy-
matically degraded into IAA, suggesting that IAA can be pro-

duced simply as a consequence of $_L$-tryptophan transamination in the absence of indolepyruvate decarboxylase. However, in consideration of the high affinity for IPyA of $_L$-tryptophan aminotransferase for the reaction in the opposite direction ($K_m = 24$ μM), it is unlikely that free IPyA is produced from $_L$-Trp by $_L$-tryptophan aminotransferase alone _in vivo_. This hypothesis can also explain why only a small proportion of $_L$-Trp supplied is converted to IAA in plant tissues. Since the $_L$-tryptophan aminotransferase activity in the direction of $_L$-Trp formation is greater than that in the opposite direction, $_L$-tryptophan aminotransferase will provide very small amounts of IPyA for the production of IAA. Therefore, we can anticipate from these results that $_L$-Trp is a precursor in the IAA biosynthetic pathway in plants and the low levels of IAA are regulated by indolepyruvate decarboxylase exclusively, with its high affinity for IPyA.

The IPyA pathway has been poorly understood and it has been the subject of much investigation and controversy. However, the results of our experiments clearly show that $_L$-tryptophan aminotransferase is involved in the IPyA pathway in _E. cloacae_ and that this pathway is regulated by indolepyruvate decarboxylase alone. The present study on IAA biosynthesis in _E. cloacae_ may provide some insight into the biochemistry of IAA synthesis and its regulation in plants.

References

Black, R.C. and Hamilton, R.H. (1971) Plant Physiol. Indoleacetic acid biosynthesis in _Avena_ coleoptile tips and excised bean shoots. **48**: 603-606.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of dye binding. Anal. Biochem. **72**: 248-254.

De Rosa, G., Burk, T.L. and Swick, R.W. (1979) Isolation and characterization of mitochondrial alanine aminotransferase from porcine tissue. Biochim. Biophys. Acta **567**: 116-124.

Ditta, G., Stanfield, S., Corbin, D. and Helinski, D.R. (1980) Broad host range DNA cloning system for gram-negative bacteria: Construction of a gene bank _Rhizobium meliloti_. Proc. Natl. Acad. Sci. USA. **77**: 7347-7351.

Gamborg, O.L. and Wetter, L.R. (1963) An aromatic amino acid transaminase from mung bean. Can. J. Biochem. Physiol. **41**: 1733-1740.

Garcia-Tabares, F., Herraiz-Tomico, T., Amat-Guerri, F. and Garcia-Bilbao, J.L. (1987) Production of 3-indoleacetic acid and 3-indolelactic acid in _Azotobacter vinelandii_ cultures supplemented with tryptophan. Appl. Microbiol. Biotechnol.

25: 502-506.

Gibson, R.A., Schneider, E.A. and Wightman, F. (1972) Biosynthesis and metabolism of indole-3yl-acetic acid. II. _In vivo_ experiments with ^{14}C-labelled precursors of IAA in tomato and barley shoots. J. Exp. Bot. **23**: 381-399.

Gunsalus, I.C. and Stamer, J.R. (1955) Transaminases in bacteria. In: Colowick, S.P., Kaplan, N.O. (eds) Methods in Enzymology, Vol. 2. Academic Press, New York, pp 170-177.

Hopper, S. and Segal, H.L. (1962) Kinetic studies of rat liver glutamic-alanine transaminase. J. Biol. Chem. **237**: 3189-3195.

Kaper, J.M. and Verdstra, H. (1958) On the metabolism of tryptophan by _Agrobacterium tumefaciens_. Biochim. Biophys. Acta. **30**: 401-420.

Koga, J., Ishii, T., Miyadoh, S. and Adachi, T. (1991a) Effects of _Enterobacter cloacae_ on the growth of cucumber and rice plants. Sci. Rep. Meiji Seika Kaisha **30**: 35-37.

Koga, J., Adachi, T. and Hidaka H. (1991b) IAA biosynthetic pathway from tryptophan _via_ indole-3-pyruvic acid in _Enterobacter cloacae_. Agric. Biol. Chem. **55**: 701-706.

Koga, J., Adachi, T. and Hidaka, H. (1991c) Molecular cloning of the gene for indolepyruvate decarboxylase from Enterobacter cloacae. Mol. Gen. Genet. **226:** 10-16.

Koga, J., Adachi, T. and Hidaka, H. (1992) Purification and characterization of indolepyruvate decarboxylase, a novel enzyme for indole-3-acetic acid biosynthesis in Enterobacter cloacae J. Biol. Chem. **267:** 15823-15828.

Law, D.M.(1987) Gibberellin-enhanced indole-3-acetic acid biosynthesis: ᴅ-Tryptophan as the precursor of indole-3-acetic acid. Physiol. Plant. **70:** 626-632.

Libbert, E., Wichner, S., Schiewer, U., Risch, H. and Kaiser, W. (1966) The influence of epiphytic bacteriae on auxin metabolism. Planta **68:** 327-334.

Libbert, E. and Silhengst, P. (1970) Interactions between plants and epiphytic bacteria regarding their auxin metabolism. Physiol. Plant. **23:** 480-487.

Lineweaver, H. and Burk, D. (1934) The determination of enzyme dissociation constants. J. Am. Chem. Soc. **56:** 658-666.

McQueen-Mason, S.J. and Hamilton, R.H. (1989) The biosynthesis of indole-3-acetic acid from ᴅ-tryptophan in Alaska pea

plastids. Plant Cell Physiol. **30**: 999-1005.

Moore, T.C. and Shaner, C.A. (1968) Synthesis of indoleacetic acid from tryptophan via indolepyruvic acid in cell free extracts of pea seedlings. Arch. Biochem. Biophys. **127**: 613-621.

O'Neil, S.R. and DeMoss, R.D. (1968) Tryptophan transaminase from Clostridium sporogenes. Arch. Biochem. Biophys. **127**: 361-369.

Paris, C.G. and Magasanik, B. (1981) Tryptophan metabolism in Klebsiella aerogenes: regulation of the utilization of aromatic amino acids as sources of nitrogen. J. Bacteriol. **145**: 257-265.

Schneider, E.A., Gibson, R.A. and Wightman, F. (1972) Biosynthesis and metabolism of indol-3yl-acetic acid I. The native indoles of barley and tomato shoots. J. Exp. Bot. **23**: 152-170.

Sheldrake, A.R. (1973) The production of hormones in higher plants. Biol. Rev. Camb. Philos. Soc. **8**: 510-515.

Speedie, M.K., Hornemann, U. and Floss, H.G. (1975) Isolation and characterization of tryptophan transaminase and

indolepyruvate C-methyltransferase. J. Biol. Chem. **250**: 7819-7825.

Tangen, O., Fonnum, F. and Haavaldsen, R. (1965) Separation and purification of aromatic amino acid transaminases from rat brain. Biochim. Biophys. Acta **96**: 82-90.

Truelsen, T.A. (1972) Indole-3-pyruvic acid as an intermediate in the conversion of tryptophan to indole-3-acetic acid. I. Some characteristics of tryptophan transaminase from mung bean seedlings. Physiol. Plant. **26**: 289-295.

Truelsen, T.A. (1973) Indole-3-pyruvic acid as an intermediate in the conversion of tryptophan to indole-3-acetic acid. II. Distribution of tryptophan transaminase activity in plants. Physiol. Plant. **28**: 67-70.

Winter, A. (1966) A hypothetical route for the biogenisis of IAA. Planta **71**: 229-239.

Wright, A.D., Sampson, M.B., Neuffer, M.G., Michalczuk, L., Slovin, J.P. and Cohen, J.D. (1991) Indole-3-acetic acid biosynthesis in the mutant maize orange pericarp, a tryptophan auxotroph. Science **254**: 998-1000.

GENERAL DISCUSSION

1. IAA biosynthetic pathway and its regulation in Enterobacter cloacae

In the present study, we found that IAA was produced by E. cloacae isolated from actively growing cucumber plants, and its biosynthesis occurred via the IPyA pathway. Under aerobic conditions, IAA was produced at a higher rate than ILA and Tol, but under less aerobic conditions, ILA and Tol were produced at higher rates than IAA. Since it has been reported that ILA and Tol are formed reversibly from IPyA and IAAld (Garcia-Tabares et al. 1987; Ernstsen et al. 1987), respectively, ILA and Tol may be important storage products that are involved in the regulation of the IAA biosynthesis. Strains of E. cloacae have been found in the rhizospheres of plants (Hadar et al. 1983; Ladha et al. 1983) and in the intestinal tracts of humans (Levy et al. 1985). Under aerobic conditions such as the rhizosphere of plants, E. cloacae may produce IAA for acceleration of plant growth. However, under anaerobic conditions such as the intestinal tract of humans, it may not produce IAA, which has been judged to be a carcinogenic substance by the Rec-assay (Kada et al. 1972; Morotomi and Mutai 1986).

2. Elucidation of the IPyA pathway in Enterobacter cloacae at the molecular level

Considerable efforts have been expended to elucidate the

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details of the IPyA pathway in plants and bacteria but the IAA biosynthetic enzymes have not been fully characterized since the intermediates, IPyA and IAAld, are labile and difficult to isolate (Kaper and Verdstra 1958; Moore and Shaner 1968; Gibson et al. 1972; Sheldrake 1973). It seems likely that it would be difficult to purify the enzymes in the IPyA pathway from E. cloacae. Therefore, to characterize this pathway, we tried to isolate an IAA biosynthetic gene from a genomic library of E. cloacae by monitoring the ability of E. coli clones to convert L-Trp to IAA.

To isolate the gene for the IAA biosynthetic enzyme from E. cloacae, a cosmid library was constructed in E. coli. First, to monitor the enzymatic conversion of L-Trp to IAA, E. coli harboring a recombinant plasmid was incubated in LB medium containing glucose (LBG medium). Although E. cloacae produced large amounts of IAA from L-Trp in LBG medium, no IAA positive clone was isolated from the cosmid library (data not shown), suggesting that E. coli harboring the IPDC gene could not produce IAA in this medium. In LBG medium, the low levels of acid formed from glucose by E. cloacae resulted in a final pH of the culture medium of pH 6.8, whereas the high levels of acid formed from glucose by E. coli resulted in a final pH of pH 4.0. In view of the differences in glucose metabolism between E. cloacae and E. coli (Brenner et al. 1984), the culture medium for E. coli was buffered with potassium phosphate at the optimal pH of 6.5 and an IAA positive clone was subsequently

obtained. These results indicate that the optimal pH range for
the IAA biosynthetic enzyme is 6.5 to 6.8. E. cloacae belongs
to the family Enterobacteriaceae and is negative for production
of indole derivatives in terms of its generally assessed physi-
ological properties (Brenner et al. 1984). However, at pH 6.5,
E. cloacae can produce IAA as a indole derivative, suggesting
that the biosynthesis of the secondary metabolic product is
influenced by the pH of the culture medium.

All strains of Enterobacter harboring the IPDC gene were
capable of converting L-Trp to IAA. Thus, it appears that the
strains of Enterobacter originally have two enzymes, which
catalyze the conversion of L-Trp to IPyA and that of IAAld to
IAA, respectively. Many species of bacteria and plants have
been found to produce IPyA from L-Trp (Truelsen 1973;
Aragozzini et al. 1979; Paris and Magasanik 1981). The majority
of bacteria, including E. coli, hardly produce any IAA, but
they are capable of converting IAAld to IAA (data not shown).
It follows, therefore, that the rate-limiting step in the IPyA
pathway is the conversion of IPyA to IAAld and the ability to
produce IAA depends on the presence of indolepyruvate decar-
boxylase.

3. Physical properties of indolepyruvate decarboxylase, a key enzyme for the IPyA pathway

Indolepyruvate decarboxylase from E. cloacae required TPP
and Mg^{2+} as cofactors and it was stabilized by the presence of

TPP and Mg^{2+}. The enzyme was a holoenzyme composed of four identical monomers. Pyruvate decarboxylase from yeast also requires TPP and Mg^{2+} as cofactors (Green et al. 1941), and TPP has been found to stabilize the enzyme during purification and storage (Juni and Heym 1968). Pyruvate decarboxylase is also a holoenzyme composed of four identical monomers (Gounaris et al. 1971; Gounaris et al. 1975). DNA sequence analysis showed that the amino acid sequence predicted from the IPDC gene has extensive homology to that of pyruvate decarboxylase from yeast. These results indicate that the physical characteristics of indolepyruvate decarboxylase from E. cloacae are very similar to those of pyruvate decarboxylase from yeast. Furthermore, indolepyruvate decarboxylase exhibited decarboxylase activity with pyruvic acid as substrate, as well as with IPyA. If a nonspecific pyruvate decarboxylase is also able to catalyze the decarboxylation of IPyA, there is a possibility that indolepyruvate decarboxylase from E. cloacae may be a type of pyruvate decarboxylase and that all bacteria that synthesize pyruvate decarboxylase may be able to produce IAA.

However, there are significant differences between the two enzymes. While pyruvate decarboxylase from yeast has a low specificity and affinity for pyruvic acid (K_m = 30 mM) (Green et al. 1941), indolepyruvate decarboxylase from E. cloacae has a high specificity and affinity for IPyA (K_m = 15 μM). Furthermore, pyruvate decarboxylase from yeast only slowly catalyzed the decarboxylation of IPyA. Indolepyruvate decarboxylase from

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$\underline{E.\ cloacae}$ was able to catalyze the decarboxylation of pyruvic acid but the $\underline{K_m}$ for pyruvic acid (2.5 mM) is evidence of a lower affinity for pyruvic acid than for IPyA ($\underline{K_m}$, 15 μM). These results indicate that indolepyruvate decarboxylase from $\underline{E.\ cloacae}$ is a highly specific enzyme, which is different from pyruvate decarboxylase.

$\underline{E.\ cloacae}$ produces IAA as a secondary metabolic product, and we can ask why this bacterium has such a highly specific enzyme. In view of the isolation of $\underline{E.\ cloacae}$ from the rhizo- sphere of cucumber plants, it is possible that the gene for this enzyme is derived from plants. If so, indolepyruvate decarboxylase may, in fact, regulate the IAA biosynthesis in plants.

4. Involvement of ʟ-tryptophan aminotransferase in the IPyA pathway and the regulation of this pathway by indolepyruvate decarboxylase

In plants, ʟ-tryptophan aminotransferase is nonspecific for amino acid substrates, and has a very high $\underline{K_m}$ value for ʟ- Trp (Gamborg and Wetter 1963; Truelsen 1972). The IAA concen- tration in plants is maintained in the nanomolar range, and it is unlikely that such low amounts of IAA are regulated by ʟ- tryptophan aminotransferase with its low affinity for ʟ-Trp (Law 1987).

Only a small proportion of ʟ-Trp supplied is converted to IAA in plant tissues, and the rate of conversion from ʟ-Trp is

far lower than would be expected for a direct precursor (Libbert et al. 1966; Libbert and Silhengst 1970; Black and Hamilton 1971). From these considerations, a hypothetical pathway for IAA biosynthesis without $_L$-Trp as a precursor has been proposed (Winter 1966; Wright et al. 1991).

Our studies provide an answer to these questions about the involvement of $_L$-tryptophan aminotransferase in IAA biosynthesis. The amounts of free $_L$-Trp in plant tissues are usually in the range of 10-50 μg/g fresh weight (Schneider et al. 1972; Sheldrake 1973). Although $_L$-tryptophan aminotransferase has a very high $\underline{K_m}$ value, in consideration of the large size of the $_L$-Trp pool, we can anticipate that $_L$-tryptophan aminotransferase provides sufficient IPyA for the production of IAA. Furthermore, when indolepyruvate decarboxylase is activated, the IPyA produced should be efficiently converted to IAA by this enzyme, with its high affinity for IPyA. Therefore, a second question arises. IPyA is an unstable compound and is nonenzymatically degraded into IAA, suggesting that IAA can be produced simply as a consequence of $_L$-tryptophan transamination in the absence of indolepyruvate decarboxylase. However, in consideration of the high affinity for IPyA of $_L$-tryptophan aminotransferase for the reaction in the opposite direction ($\underline{K_m}$ = 24 μM), it is unlikely that free IPyA is produced from $_L$-Trp by $_L$-tryptophan aminotransferase alone $\underline{in}$ $\underline{vivo}$. This hypothesis can also explain why only a small proportion of $_L$-Trp supplied is converted to IAA in plant tissues. Since the $_L$-tryptophan

aminotransferase activity in the direction of $_L$-Trp formation is greater than that in the opposite direction, $_L$-tryptophan aminotransferase will provide very small amounts of IPyA for the production of IAA. Therefore, we can anticipate from these results that $_L$-Trp is a precursor in the IAA biosynthetic pathway in plants and the low levels of IAA are regulated by indolepyruvate decarboxylase exclusively, with its high affinity for IPyA.

The IPyA pathway has been poorly understood and it has been the subject of much investigation and controversy. However, the results of our experiments clearly show that $_L$-tryptophan aminotransferase is involved in the IPyA pathway in E. cloacae and that this pathway is regulated by indolepyruvate decarboxylase alone. The present study on the IAA biosynthesis in E. cloacae will help us to elucidate the IPyA pathway in plants and may provide some insight into the biochemistry of the IAA synthesis and its regulation.

References

Aragozzini, F., Ferrari, A., Pacini, N. and Gualandris, R.
(1979) Indole-3-lactic acid as a tryptophan metabolite produced
by Bifidobacterium spp. Appl. Environ. Microbiol. **38**: 544-546.

Black, R.C. and Hamilton, R.H. (1971) Plant Physiol.
Indoleacetic acid biosynthesis in Avena coleoptile tips and
excised bean shoots. **48**: 603-606.

Brenner, D.J., Farmer, J.J., Frederiksen, W., Le Minor, L. and
Sakazaki, R. (1984) Facultatively anaerobic gram-negative rods.
In: Krieg, N.R. (ed) Bergey's manual of systematic bacteriology,
Vol. 1. Williams & Wilkins, Baltimore MD, pp 408-516.

Ernstsen, A., Sandberg, G. Crozier, A. and Wheeler C.T. (1987)
Endogenous indoles and the biosynthesis and metabolism of
indole-3-acetic acid in cultures of Rhizobium phaseoli. Planta.
171: 422-428.

Gamborg, O.L. and Wetter, L.R. (1963) An aromatic amino acid
transaminase from mung bean. Can. J. Biochem. Physiol. **41**:
1733-1740.

Garcia-Tabares, F., Herraiz-Tomico, T., Amat-Guerri, F. and
Garcia-Bilbao, J.L. (1987) Production of 3-indoleacetic acid
and 3-indolelactic acid in Azotobacter vinelandii cultures

supplemented with tryptophan. Appl. Microbiol. Biotechnol. 25: 502-506.

Gibson, R.A., Schneider, E.A. and Wightman, F. (1972) Biosynthesis and metabolism of indole-3yl-acetic acid. II. *In vivo* experiments with ^{14}C-labelled precursors of IAA in tomato and barley shoots. J. Exp. Bot. 23: 381-399.

Gounaris, A.D., Turkenkopf, I., Buckwald, S. and Young, A.A. (1971) Pyruvate decarboxylase I. Protein dissociation into subunits under conditions in which thiamine pyrophosphate is released. J. Biol. Chem. 246: 1302-1309.

Gounaris, A.D., Turkenkopf, I., Civerchia, L.L. and Greenlie, J. (1975) Pyruvate decarboxylase III. Specificity restrictions for thiamine pyrophosphate in the protein association step, sub-unit structure. Biochim. Biophys. Acta 405: 492-499.

Green, D.E., Herbert, D. and Subrahmanyan, V. (1941) Carboxylase. J. Biol. Chem. 138: 327-339.

Hadar, Y., Harman, G.E., Taylor, A.G. and Norton, J.M. (1983) Effects of pregermination of pea and cucumber seeds and of seed treatment with *Enterobacter* *cloacae* on rots caused by *Pythium* spp. Phytopathology 73: 1322-1325.

Juni, E. and Heym, G.A. (1968) Properties of yeast pyruvate
decarboxylase and their modification by proteolytic enzymes. I.
Stability of decarboxylases from wild-type and mutant strains.
Arch. Biochem. Biophys. **127**: 79-88.

Kada, T., Tutikawa, K. and Sadaie, Y. (1972) In vitro and host-
mediated rec-assay procedures for screening chemical mutagens;
and phloxine, a mutagenic red dye detected. Mutation Res. **16**:
165-174.

Kaper, J.M. and Verdstra, H. (1958) On the metabolism of
tryptophan by Agrobacterium tumefaciens. Biochim. Biophys.
Acta. **30**: 401-420.

Ladha, J.K., Barraquio, W.L. and Watanabe, I. (1983) Isola-
tion and identification of nitrogen-fixing Enterobacter
cloacae and Klebsiella planticola associated with rice plants.
Can. J. Microbiol. **29**: 1301-1308.

Law, D.M.(1987) Gibberellin-enhanced indole-3-acetic acid
biosynthesis: ᴅ-Tryptophan as the precursor of indole-3-acetic
acid. Physiol. Plant. **70**: 626-632.

Levy, S.B., Hedges, R.W., Sullivan, F., Medeiros, A.A. and
Sosroseputro, H. (1985) Multiple antibiotic resistance plasmids
in Enterobacteriaceae isolated from diarrhoeal specimens of

hospitalized children in Indonesia. J. Antimicrob. Chemother.
16: 7-16.

Libbert, E., Wichner, S., Schiewer, U., Risch, H. and Kaiser,
W. (1966) The influence of epiphytic bacteriae on auxin
metabolism. Planta **68:** 327-334.

Libbert, E. and Silhengst, P. (1970) Interactions between
plants and epiphytic bacteria regarding their auxin metabolism.
Physiol. Plant. **23:** 480-487.

McQueen-Mason, S.J. and Hamilton, R.H. (1989) The biosynthe-
sis of indole-3-acetic acid from ᴅ-tryptophan in Alaska pea
plastids. Plant Cell Physiol. **30:** 999-1005.

Moore, T.C. and Shaner, C.A. (1968) Synthesis of indoleacetic
acid from tryptophan via indolepyruvic acid in cell free ex-
tracts of pea seedlings. Arch. Biochem. Biophys. **127:** 613-
621.

Morotomi, M. and Mutai, M. (1986) Intestinal microflora and
metabolism of chemical carcinogens in vivo, pp. 145-172. In T.
Mitsuoka (ed.), Metabolism in intestinal flora. Japan
Scientific Societies Press, Tokyo.

Paris, C.G. and Magasanik, B. (1981) Tryptophan metabolism

in <u>Klebsiella</u> <u>aerogenes</u>: regulation of the utilization of
aromatic amino acids as sources of nitrogen. J. Bacteriol.
145: 257-265.

Schneider, E.A., Gibson, R.A. and Wightman, F. (1972)
Biosynthesis and metabolism of indol-3yl-acetic acid I. The
native indoles of barley and tomato shoots. J. Exp. Bot. **23:**
152-170.

Sheldrake, A.R. (1973) The production of hormones in higher
plants. Biol. Rev. Camb. Philos. Soc. **8:** 510-515.

Truelsen, T.A. (1972) Indole-3-pyruvic acid as an intermediate
in the conversion of tryptophan to indole-3-acetic acid. I.
Some characteristics of tryptophan transaminase from mung bean
seedlings. Physiol. Plant. **26:** 289-295.

Truelsen, T.A. (1973) Indole-3-pyruvic acid as a intermediate
in the conversion of tryptophan to indole-3-acetic acid. II.
Distribution of tryptophan transaminase activity in plants.
Physiol. Plant. **28:** 67-70.

Winter, A. (1966) A hypothetical route for the biogenisis of
IAA. Planta **71:** 229-239.

Wright, A. D., Sampson, M. B., Neuffer, M. G., Michalczuk, L.,

Slovin, J. P. and Cohen, J. D. (1991) Indole-3-acetic acid biosynthesis in the mutant maize orange pericarp, a tryptophan auxotroph. Science **254**: 998-1000.

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