# ISOLATION AND CHARACTERIZATION OF A BACTERIAL AUXIN GENE AND ITS PROMOTER

by

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## ABSTRACT

Bacteria that inhabit the rhizosphere may influence plant growth by modifying phytohormone levels in plants tissues, for example, by adding to a plant's pool of the hormone auxin. In phytopathogenic bacteria, the auxin indoleacetic acid (IAA) is produced mainly via the indoleacetamide pathway, and has been implicated in the induction of plant tumors. Beneficial bacteria synthesize IAA predominantly by the indolepyruvic acid pathway; however, the role of IAA in plant growth-promotion remains inconclusive.

The *ipdc* gene encoding indolepyruvate decarboxylase, which catalyzes a key step in the latter pathway, was isolated from the plant growth-promoting bacterium *Pseudomonas putida* GR12-2 by colony hybridization and PCR. The similarity of the amino acid sequences among other indolepyruvate decarboxylases, pyruvate decarboxylase, and acetolactate synthase suggests an evolutionary relationship among these proteins. Because IAA accumulates in the culture medium of *P. putida* GR12-2 grown in the presence of exogenous tryptophan, transcription of *ipdc* may be activated by tryptophan. To test this hypothesis, the *ipdc* promoter region was isolated by inverse PCR, and inserted upstream of the bioluminescent reporter gene *luxAB* on a plasmid in *P. putida* GR12-2. Activity of the *ipdc* promoter, measured by quantifying light production, increased dramatically in the presence of tryptophan, confirming that *ipdc* expression is induced by tryptophan. In addition, *ipdc* is regulated by the stationary phase sigma factor RpoS: the *ipdc* promoter contains a sequence similar to the RpoS recognition sequence, and transformation of *P. putida* GR12-2 with *rpoS* induced promoter activity before the onset of stationary phase when RpoS is not normally produced, and prolonged a higher level of transcription at the later stages of the cell cycle.

To determine if IAA is involved in the stimulation of plant growth by *P. putida* GR12-2, an IAA-deficient mutant was constructed by insertional mutagenesis of *ipdc*. The lengths of canola seedling primary roots from seeds treated with wild-type *P. putida* GR12-2 were on average 35-50% longer than roots from seeds treated with the IAA-deficient mutant, and roots from uninoculated seeds. In addition, exposure of mung bean cuttings to high levels of IAA by soaking them in a suspension of the wild-type strain, stimulated the formation of many, very small, adventitious roots. Fewer roots were initiated by the IAA-deficient mutant. These results suggest a major role for bacterial IAA in the development of the host plant root system.

## ACKNOWLEDGEMENTS

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Most of all, I must thank my husband, Patrick Patten. When he initially encouraged me to go back to school to finish my B.Sc., I'm sure that he did not forsee that I would be a student for most of the time that he's known me. Nevertheless, during the production of this thesis we have raised two children, had some fun, and remained poor, together. Throughout, his encouragement has not waivered. And now, I am told by my beautiful children, Russell and Adam, I must go out in the world and make my way, for Russell wants a BMW for his 16$^{th}$ birthday and Adam wants a cat.

TABLE OF CONTENTS

## LIST OF FIGURES

## LIST OF TABLES

# INTRODUCTION

In nature, plants do not grow in isolation. Rather, they are host to a complex community of organisms, including insects, fungi, and bacteria. Many of these organisms are pathogenic while others are beneficial to the host plant. In agriculture, the goal is to eliminate the pathogens and increase crop yield. To this end, hundreds of millions of tons of chemical pesticides and fertilizers are applied annually, and while they have effectively increased crop production, their success has come at a price to the environment and human health. Perhaps a better strategy to increase crop yields would be to encourage the natural, beneficial interactions between crop plants and associated microbes.

There exists in the rhizosphere (the area around the roots of plants), a group of bacteria known as plant growth-promoting rhizobacteria, more commonly known by the acronym PGPR. In exchange for a rich supply of nutrients provided by plant root exudates, these bacteria can promote seedling emergence, enhance root development or shoot elongation, increase plant vigor and provide protection from phytopathogens, particularly when plants are stressed (Boddey and Dobereiner, 1988). Members of the bacterial genera *Azotobacter*, *Bacillus*, *Pseudomonas*, *Enterobacter* and *Azospirillum*, are described as having plant growth-enhancing attributes (Brown, 1974; Kloepper et al., 1988; Koga et al., 1991b; Okon, 1994; Kloepper, 1994; Glick, 1995; Glick et al., 1999). The relationship between these free-living bacteria and their host plant is best described as mutualistic, distinct from that of the Rhizobiae which establish a unique symbiotic relationship with their host legumes, although these latter bacteria have been shown to promote the growth of non-leguminous plants in their free-living state. In order to encourage, or even improve, the natural influence of PGPR, it is first necessary to understand the mechanisms by which they promote plant

growth.

## Plant Growth-Promoting Mechanisms of Rhizobacteria

Plant growth-promoting bacteria may exert their beneficial effect by several means (Table 1). They may benefit the plant indirectly by inhibiting deleterious microorganisms, or directly by supplying the plant with elements essential for nutrition or by modifying phytohormone levels within plant tissues.

### Indirect Mechanisms

Some rhizobacteria are able to outcompete other soil microbes for colonization of roots by secreting antimicrobial compounds such as siderophores, antibiotics and other toxic metabolites. These bacteria are known as biocontrol PGPR and have great potential as a soil inoculant to control damage to crops by soil-bourne pathogens, often fungi.

Despite the abundance of iron on the earth's surface, soil organisms such as plants and microbes often have difficulty obtaining enough iron to support their growth because the iron is largely present as insoluble, ferric hydroxides, which cannot readily be transported into cells. To overcome this problem, a wide variety of organisms, including bacteria, secrete siderophores, low molecular weight molecules with a high affinity for ferric iron (Neilands, 1981; Neilands and Leong, 1986). Once it is formed, the iron-siderophore complex is taken up by specific receptors and, following reduction to the ferrous state, the iron is released into the cell. By scavenging iron required for its survival, a bacterium removes the iron from the soil and thereby makes it unavailable to other, perhaps phytopathogenic, organisms, preventing their proliferation.

Table 1. Some mechanisms used by plant growth-promoting rhizobacteria to enhance plant growth.

| |
|---|
| Indirect Mechanisms (Inhibition of Phytopathogens) |
| Depletion of iron from soil via siderophore secretion |
| Production of antibiotics |
| Production of hydrogen cyanide |
| Synthesis of cell wall-degrading enzymes |
| Induction of systemic resistance |
| Direct Mechanisms |
| Provision of essential elements |
| Reduction of ethylene levels |
| Production of cytokinins and auxins |

The effectiveness of siderophores in reducing fungal diseases of plants was demonstrated by three different types of experiments. Purified siderophores were shown to suppress proliferation of the phytopathogen *Erwinia carotovora* as effectively as the fluorescent *Pseudomonas* from which they were isolated (Kloepper et al., 1980). Siderophore synthesis is induced only under low iron conditions, therefore production can be inhibited by providing excess iron. Under these conditions, the ability of several PGPR to prevent plant disease was reduced (Elsherif and Grossman, 1994). A siderophore overproducing mutant of *Pseudomonas putida* was able to control the phytopathogenic fungus *Fusarium oxysporum* to a greater extent than the wild-type strain (Vanderburgh and Gonzalez, 1984). On the other hand, siderophore deficient mutants of biocontrol strains of *Pseudomonas aeruginosa* and *Alcaligenes* sp. were no longer able to suppress *Pythium* induced damping-off or *Fusarium oxysporum*, respectively (Buysens et al., 1996; Marinetti and Loper, 1992).

Production of antibiotics such as phenazines, pyoluteorin, pyrrolnitrin, agrocin and 2,4-diacetylphloroglucinol is common among biocontrol PGPR, especially species of *Pseudomonas* (O'Sullivan and O'Gara, 1992). The ability of these compounds to control fungal diseases in plants was established using mutants that no longer produce specific antibiotics. Loss of antibiotic production correlated with the loss of the capacity of these mutants to inhibit phytopathogenic fungi both *in vitro* and when associated with the plant (Tazawas-Isogami et al., 1997; Shoda et al., 1997; Hill et al., 1994; Keel et al., 1992). Antibiotic-overproducing mutants of *Pseudomonas fluorescens* CHA0 were able to protect cucumber plants against damping-off disease caused by *Pythium ultimum* to a greater extent than the wild-type strain (Maurhofer et al., 1992; Schnider et al., 1994).

Other toxic metabolites produced by PGPR such as hydrogen cyanide can interfere with the metabolism of soil microorganisms. While wild-type *Pseudomonas putida* CHA0 was able to effectively protect tobacco roots from black root rot caused by the fungus *Thielaviopsis basicola*, a hydrogen cyanide negative mutant was not (Voisard et al., 1989). Disease suppression was restored in the deficient strain and conferred on a non-producing strain by insertion of the genes for hydrogen cyanide biosynthesis. When the genes for hydrogen cyanide production were transferred to mutants of *P. putida* BK8661 unable to produce siderophores and antibiotics, the bacterium was better able to suppress wheat blotch caused by *Septoria tritici* (Flaishman et al., 1996).

Biocontrol PGPR can inhibit phytopathogenic fungi through the production of fungal cell wall degrading enzymes such as chitinases, β-glucanases, proteases and lipases (Chet and Inbar, 1994). Cucumber plants whose leaves were treated with *Erwinia ananas* genetically engineered to carry a chitinase gene were significantly protected against gray mold caused by *Botrytis cinerea* (Ui et al., 1997). Similarly, β-glucanase-producing *Pseudomonas cepacia* was able to reduce the damage to plants caused by several pathogenic fungi (Fridlender et al., 1993).

PGPR may outcompete phytopathogens for colonization of plant surfaces when they are better adapted to variable soil parameters such as temperature (Chiarini et al., 1994; Sun et al., 1995), moisture content (Hannusch and Boland, 1996) and soil composition (Heijnen and van Elsas, 1994; Bashan et al., 1995). The ability to utilize unusual compounds in plant root exudates may also provide these bacteria with a competitive advantage over other rhizosphere microorganisms. For example, many PGPR are able to hydrolyze the compound 1-aminocyclopropane-1-carboxylic acid (ACC), present in root exudates, as a unique source

of carbon and nitrogen (Glick et al., 1998).

A host plant may mount a systemic defense response, triggered by PGPR inoculation, effective against pathogenic fungi and bacteria. Several bacterial molecules have been shown to act as signals that induce systemic resistance including the outer membrane protein lipopolysaccharide O-antigenic side chain, siderophores and salicylic acid (Leeman et al., 1996; van Loon et al., 1997). By applying various purified bacterial compounds to the roots of *Arabidopsis* plants, or by treating roots with mutants deficient in specific traits, and subsequently challenging the leaves with a pathogen, Bakker et al. (2000) were able to implicate the siderophore pseudobactin, the lipopolysaccharide side chain and flagella in the induction of systemic resistance. While wild-type *Pseudomonas putida* 7NSK2 was able to induce resistance in tomatoes to *Botrytis cinerea* and in bean to anthracnose caused by *Colletotrichum lindemuthianum*, mutant strains unable to synthesize salicylic acid could not (Höfte et al., 2000).

Direct Mechanisms

PGPR may enhance plant growth directly by providing a host plant with nutrients that are otherwise difficult to obtain such as iron, nitrogen and phosphorous. The ability of plants to utilize microbial siderophore-iron complexes in order to obtain iron has been demonstrated by supplying radiolabeled ferric-siderophores to plants as a sole source of iron. Both monocots such as sorghum and oats (Crowley et al., 1988; Bar-ness et al., 1991) and dicots such as peanut, cotton, cucumber and sunflower (Cline et al., 1994; Jurkevitch et al., 1986; Bar-ness et al., 1991; Wang et al., 1993) were able to take up the labeled iron. Growth of cucumber in the presence of microbial siderophores resulted in increased plant biomass and

increased chlorophyll content (Wang et al., 1993). Chlorophyll concentration can be used as an indicator of plant iron health as iron is important in the biosynthesis of chlorophyll (Ismande, 1998).

Bacteria may stimulate plant growth by providing the host plant with fixed nitrogen. Particularly well known for this effect are the symbiotic diazotrophs of the family Rhizobiaceae which establish nitrogen fixing nodules on the roots of leguminous plants in exchange for photosynthetically fixed carbon. Although free-living PGPR do not form nodules on host plant roots, many are capable of fixing atmospheric nitrogen (Elmerich, 1984; Lifshitz et al., 1986; Davison, 1988). However, only about 5% of the nitrogen fixed by *Azospirillum brasilense* was taken up by a host plant (Boddey and Dobereiner, 1988), and a nitrogen-deficient mutant of a diazotrophic pseudomonad and a non-nitrogen fixing wild-type strain were both able to stimulate elongation of canola roots (Lifshitz et al., 1987). While PGPR are able to contribute some fixed nitrogen, it is not a primary mechanism of plant growth promotion. However, co-inoculation of *Azospirillum* with *Rhizobium* had a synergistic effect on nitrogen fixation and plant growth (Oliveira et al., 1997; Bashan and Holguin, 1997), suggesting that PGPR may indirectly contribute to a plant's nitrogen supply. Perhaps by providing free-living diazotrophs with an environment suitable for nitrogen fixation, such as decreased oxygen tension and increased access to plant nutrients, they may produce and transfer more fixed nitrogen to their host plants. Nodule-like structures, or para-nodules, on plant roots or stems, induced by application of synthetic auxin, and colonized by diazotrophs, have been shown to provide such an environment (Christiansen-Weniger, 1998).

Many rhizobacteria have the capacity to solubilze phosphates found in the soil by production of phosphatases or by secretion of acids. In a soluble form, phosphorous can be

taken up more readily by plants to be used in important processes such a photosynthesis, respiration and in the biosynthesis of nucleotides and membranes. Uptake of phosphates was significantly improved in several agriculturally important crop species following inoculation with *Azospirillum* species (Murty and Ladha, 1988) and *Rhizobium fredii* (Prévost et al., 2000). In addition, inoculation of plants with phosphate-solubilizing bacteria such as *Rhizobium leguminosarum* bv. *phaseoli* enhanced plant growth (Chabot et al., 1998).

PGPR may enhance plant growth by altering the levels of phytohormones within plant tissues, either by metabolizing the biosynthetic precursor of ethylene or by producing and secreting auxins or cytokinins. The phytohormone ethylene plays an important role in many aspects of a plant's normal development including vegetative growth, leaf and flower senescence, and fruit ripening. Less desirable from an agricultural point of view is its ability to inhibit plant growth when levels are elevated such as in response to pathogen attack or environmental stresses (Morgan and Drew, 1997). Plants synthesize ethylene from S-adenosylmethionine via the intermediate 1-aminocyclopropane-1-carboxylic acid (ACC) (Yang and Hoffman, 1984). Several PGPR produce the enzyme ACC deaminase that can reduce plant ethylene levels by hydrolyzing ACC and thereby, stimulate root elongation (Glick et al., 1998). *Pseudomonas putida* GR12-2, which possesses ACC deaminase activity, stimulated a significant increase in the length of canola roots (Caron et al., 1995) and a reduction in root and shoot levels of ACC (Penrose et al., 2001). In contrast, a mutant strain of *Enterobacter cloacae* UW4 that no longer produced ACC deaminase lost its ability to promote root growth (Li et al., 2000).

Cytokinins regulate many aspects of plant physiology and development including cell division, mobilization of nutrients, and chloroplast accumulation. Many of these effects are

the consequence of the interaction of cytokinins with other plant hormones. For example, exogenous auxin is required for cytokinin-induced cell division in callus tissue. The most prevalent cytokinin in plant tissues is zeatin, although other substituted aminopurines such as isopentenyladenine, dihyrozeatin and sugar derivatives have been found. Cytokinins have been identified in the culture media of several pathogenic bacteria including *Corynebacterium fascians* (Murai et al., 1980), *Agrobacterium tumefaciens* (Regier and Morris, 1982), *Agrobacterium rhizogenes* (Regier et al., 1989), and *Pseudomonas syringae* pv. *savastanoi* (Surico et al., 1975) and have been implicated in the induction of tumors by these bacteria (reviewed by Morris, 1986 and Gaudin et al., 1994). Beneficial bacteria, including *Bradyrhizobium japonicum*, *Rhizobium leguminosarum* (Sturtevant and Taller, 1989), *Azotobacter chroococcum* (Neito and Frankenberger, 1989), *Azotobacter vinelandii* (Taller and Wong, 1989) *Azosprillum brasilense* (Horemans et al., 1986) and several PGPR strains of *Pseudomonas* (de Salamone et al., 1997), also produce cytokinins.

A role for cytokinin in nodulation is indicated from the increased cytokinin content in root nodules, the induction of early nodulation genes by cytokinin (Dehio and deBruijin, 1992; Bauer et al., 1996) and the formation of bacteria-free nodules following treatment with either exogenous cytokinin (Arora et al., 1959) or with a *Rhizobium meliloti* nodulation-deficient mutant expressing the zeatin secretion gene of *Agrobacterium tumefaciens* (Cooper and Long, 1994). Cytokinins produced by free-living bacteria may also be involved in plant growth-promotion. Wild-type *Pseudomonas fluorescens* strain G20-18WT increased the fresh weight of tobacco callus almost five-fold, whereas insertional mutants with reduced capacity to produce cytokinin could no longer promote callus growth to the same extent (de Salamone and Nelson, 2000). Although these few studies suggest that bacterial cytokinin

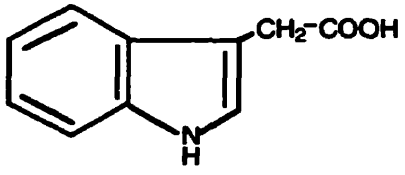
impacts positively on plant growth, most of the interest regarding the effects of bacterial phytohormone production has focused on auxin.

**Auxin in Plants**

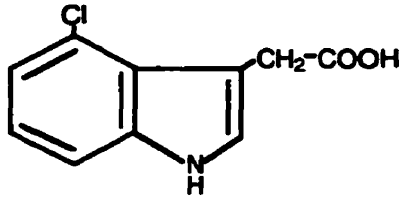
Plant tissues, like those in other multicellular organisms, use biochemical signals, or hormones, to induce cells to respond to internal or external cues. Members of one class of such phytohormones, the auxins, influence many cellular functions and therefore are important regulators of plant growth and development. Auxins have been implicated in the orientation of the growth of roots and shoots in response to light and gravity (Kaufman et al., 1995), in the differentiation of vascular tissue (Aloni, 1995), in apical dominance (Tamas, 1995), in the initiation of lateral and adventitious roots (Gaspar et al., 1996; Malamy and Benfey, 1997), in the stimulation of cell division (Kende and Zeevaart, 1997) and in elongation growth in stems and roots (Yang et al., 1993; Kende and Zeevaart, 1997).

Several naturally occurring auxins are known (Fig. 1); the most common is indole-3-acetic acid (IAA). Various derivatives of IAA, including halogenated compounds such as 4-chloroindole-3-acetic acid (Ernstsen and Sandberg, 1986; Reinecke et al., 1995; Antolíc et al., 1996) and conjugated forms in which IAA is covalently bonded through its carboxyl group with sugars, alcohols, amino acids and glycoproteins, as in IAA-glucose and IAA-aspartate, are found in plant tissues (Gaspar et al, 1996; Cohen and Bandurski, 1982). Precursors of IAA, for example, indole-3-acetonitrile, may also have weak auxin activity (Normanly et al., 1997). Other endogenous auxins include indole-3-butyric acid which is synthesized from IAA (Epstein and Ludwig-Müller, 1993; Ludwig-Müller et al., 1995) and phenylacetic acid (Wightman and Lighty, 1982).

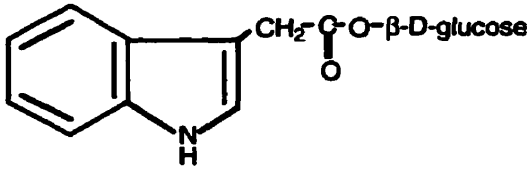
Figure 1. Some natural and synthetic auxins.



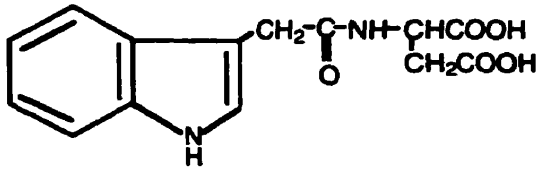

Indole-3-acetic acid



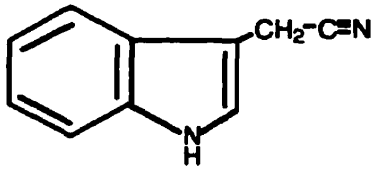

4-Chloroindole-3-acetic acid



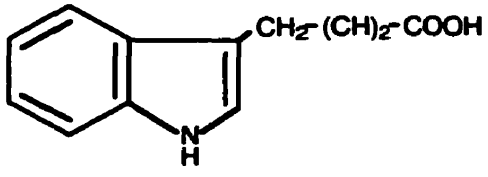

IAA-glucose



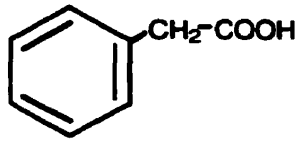

IAA-aspartate



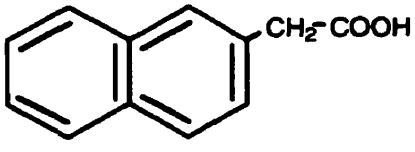

Indole-3-acetonitrile



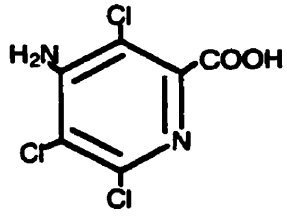

Indole-3-butyric acid



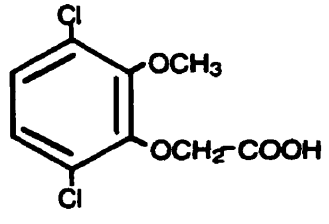

Phenylacetic acid



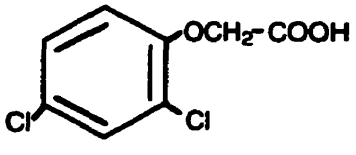

Naphthalenacetic acid



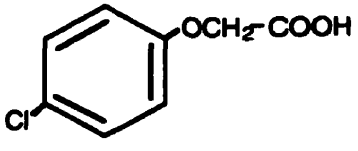

Pichloram



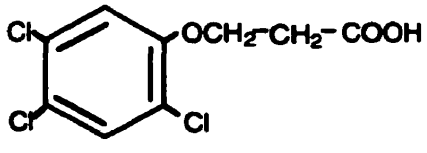

Dicambra




2,4-Dichlorophenoxy-acetic acid




4-Chlorophenoxy-acetic acid




2,4,5-Trichlorophenoxy-propionic acid

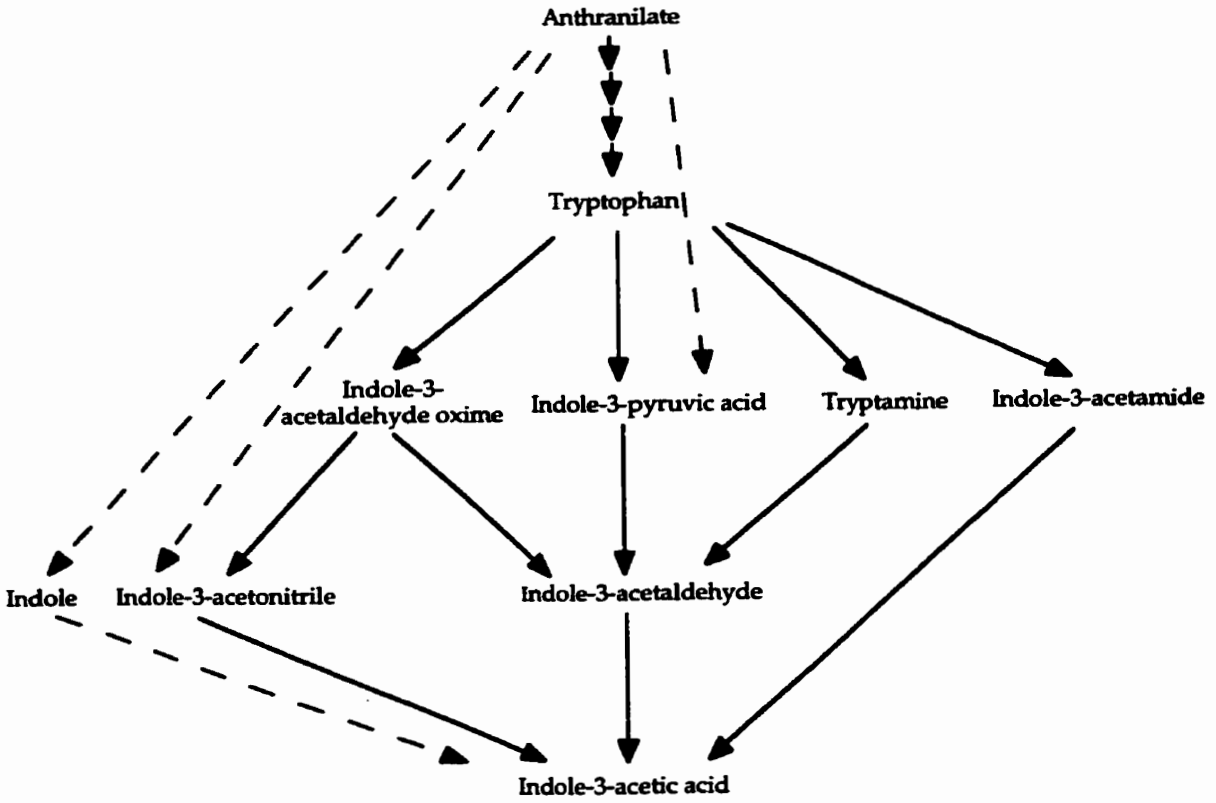
Synthetic auxins with enhanced efficacy or specialized application are commercially available (Fig. 1). 1-Naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) are often used in tissue culture to stimulate organogenesis from callus and in horticulture to promote rooting of cuttings (Gaspar et al., 1996). Auxin-like herbicides such as pichloram (4-amino-3,5,6-trichlorophyridine-2-carboxylic acid) and dicambra (3,6-dichloro-*o*-anisic acid) are effective inhibitors of growth at high concentrations (Gianfagna, 1995; Gaspar et al., 1996). Carefully timed application of 4-chlorophenoxyacetic acid (4-CPA) and 2,4,5-trichlorophenoxypropionic acid (2,4,5-TP) in commercial orchards can stimulate fruit set and prevent fruit abscission, respectively (Gianfagna, 1995).

Biosynthesis

Despite the recognition that IAA is an important regulator of plant growth, the sources of this compound in plants remains surprisingly elusive. With the advent of more sophisticated and sensitive analytical techniques, the long held notion of tryptophan as the predominant precursor is being challenged. Considering that plants cannot survive without IAA, and the inability of researchers to generate mutants completely deficient in auxin (Klee and Estelle, 1991), it is reasonable to expect that several IAA biosynthetic pathways exist, even within a single plant, the relative activities of which depend on the plant species, the plant tissue, and on the developmental state of the plant.

Several tryptophan-dependent biosynthetic routes have been identified in plants mainly from the presence of pathway intermediates and appropriate enzyme activity (Fig. 2); very little genetic evidence from plants is available. The so-called indole-3-pyruvic acid pathway involves the transamination of tryptophan to indole-3-pyruvic acid, followed by

Figure 2. Proposed pathways for indole-3-acetic acid biosynthesis in plants.


Anthranilate
Tryptophan
Indole-3-
acetaldehyde oxime
Indole-3-pyruvic acid
Tryptamine
Indole-3-acetamide
Indole
Indole-3-acetonitrile
Indole-3-acetaldehyde
Indole-3-acetic acid

decarboxylation to indole-3-acetaldehyde and further oxidation to IAA. Indole-3-pyruvic acid and the activity of enzymes involved in the pathway have been detected in pea and tomato plants (McQueen-Mason and Hamilton, 1989; Cooney and Nonhebel, 1991; Nonhebel et al., 1993). Tryptophan may also be converted to indoleacetaldehyde via indole-3-acetaldehyde oxime (Ludwig-Müller and Hilgenberg, 1988; Rajagopal et al., 1993) or through tryptamine. This latter pathway was suggested by the presence of tryptamine in tobacco (Phelps and Sequeira, 1967) and tomato (Cooney and Nonhebel, 1991), the isolation of a gene from periwinkle encoding tryptophan decarboxylase, which catalyzes the conversion of tryptophan to tryptamine (De Luca et al., 1989), and more recently, by the isolation of a gene (*yucca*) for a flavin monooxygenase that catalyzes the hydroxylation of tryptamine (Zhao et al., 2001). The common final step in these three pathways, the synthesis of IAA from indoleacetaldehyde, can occur via an aldehyde oxidase or an aldehyde dehydrogenase (Tsurusaki et al., 1997). The intermediate indoleacetaldehyde oxime can also be converted to IAA via indole-3-acetonitrile (Ludwig-Müller and Hilgenberg, 1990). Several differentially expressed genes for nitrilase, the enzyme that catalyzes the conversion of indoleacetonitrile to IAA, have been discovered in *Arabidopsis* (Bartling et al., 1994; Bartel and Fink, 1994; Hillebrand et al., 1996).

Convincing evidence for a tryptophan-independent pathway for IAA biosynthesis in plants has emerged from *in vivo* precursor labeling studies and from mutants deficient in tryptophan biosynthesis (Fig. 2). Even though a substantial portion of the endogenous tryptophan pool was enriched with heavy isotope from fed, labeled tryptophan, after several days very little of the isotope was incorporated into IAA in *Lemna gibba* (Baldi et al., 1991), carrot somatic embryos (Michalczuk et al., 1992) and maize seedlings (Wright et al., 1991), a

result that strongly rules out tryptophan as a primary precursor of IAA in these cells.

Mutants of *Arabidopsis* and maize, defective in various steps in the tryptophan biosynthesis pathway, produce very low levels of tryptophan compared to wild-type seedlings, but elevated levels of IAA (Wright et al., 1991; Normanly et al., 1993). The accumulation of pre-tryptophan metabolites in tryptophan deficient mutants concomitant with an increase in IAA has led to speculation that IAA is synthesized by a pathway branching off from an intermediate in the anthranilate to tryptophan biosynthesis route (Cooney and Nonhebel, 1991; Michalczuk et al., 1992; Normanly et al., 1993; Nonhebel et al., 1993; Rekoslavskaya and Bandurski, 1994; Bartling et al., 1994; Celenza et al., 1995; Bartel, 1997; Ouyang et al., 2000).

Metabolism

In addition to *de novo* synthesis, metabolic processes such as conjugate synthesis and hydrolysis, transportation and degradation can alter endogenous levels of plant IAA. Much of the newly synthesized IAA in a plant is stored in an inactive, conjugated form, covalently linked with various moieties such as sugars or amino acids. Many of the conjugate synthesis routes have been worked out (Szerszen et al., 1994; Bandurski et al., 1995) and some of the genes involved have been isolated (Szerszen et al., 1994). Hangarter and Good (1981) hypothesize that IAA is released from conjugate stores by slow hydrolysis to supply a constant, steady concentration of free, active IAA when needed. For example, ester conjugates stored in maize and legume seeds are mobilized to the developing shoot during germination where they are deconjugated to provide free IAA (Bandurski et al., 1995; Bialek et al., 1992). Because conjugates vary in the ease with which they are hydrolyzed by specific plant enzymes, the moiety to which IAA is conjugated is a major determinant in controlling

the available levels of free IAA (Hangarter and Good, 1981; Bartel, 1997). Conjugate-hydrolyzing enzymes, differing in their specificity and activity, have been identified and their genes isolated (Normanly, 1997; Bartel, 1997).

Transportation of indoleacetic acid from sites of synthesis to distant regions of the plant can be polar, passing from cell to cell, from apex to base, via protein carriers, or non-polar, moving passively up or down via the vascular tissue (Lomax et al., 1995). While polar auxin transport is the route for movement of free IAA, inactive IAA conjugates move through the phloem to sites where they are activated by enzyme hydrolysis. Conjugate hydrolases are not only specific with respect to substrate but also may be localized to specific tissues or to specific cellular locations (Normanly, 1997; Bartel, 1997). Isozymes of IAA conjugate hydrolases encoded by a six gene family in *Arabidopsis* have terminal sequences that may designate the final cellular location of the enzyme (Bartel, 1997).

Free IAA is readily inactivated by two enzymatic processes: peroxidase-catalyzed oxidative decarboxylation of the side chain and oxidation of the indole ring (Normanly, 1997). Protection of IAA against oxidation is provided by conjugation, although an exception to this may be IAA-aspartate as conjugation of IAA with aspartate is the first committed step in one IAA catabolic pathway (Catalá et al., 1992).

**Effect of Bacterial Auxin on Plants**

Typical of its hormonal nature, the concentration of auxin in plants is critical to the physiological response, with an excess or a deficiency having a characteristic effect. In addition to plant factors that influence the levels of available auxin, auxin secreted by microbes can contribute to a plant's endogenous pool. Production of auxin is widespread

among plant-associated bacteria (Gaudin et al., 1994; Costacurta and Vanderleyden, 1995; Patten and Glick, 1996; Glickmann et al., 1998). Estimates of the number of IAA producing organisms range as high as 80% of all soil bacteria, highlighting the enormous potential these organisms have to contribute to a plant's endogenous pool of IAA. Several of these microorganisms, such as *Agrobacterium tumefaciens*, *Agrobacterium rhizogenes*, *Erwinia herbicola* and pathovars of *Pseudomonas syringae*, are involved in plant pathogenesis, while others of the genera *Azotobacter*, *Bacillus*, *Pseudomonas*, *Enterobacter*, *Azospirillum*, *Rhizobium* and *Bradyrhizobium* are described as having plant growth enhancing attributes. Although tumor-inducing bacteria including *A. tumefaciens*, *E. herbicola* and *P. syringae* pv. *savastanoi*, and nodule-forming symbionts of the genera *Rhizobium* and *Bradyrhizobium* are not members of the group defined as plant growth-promoting rhizobacteria, or PGPR, they produce effects in host plants that can be considered as hypergrowth.

Why do so many rhizobacteria produce IAA? It may allow bacteria to detoxify excess tryptophan, although IAA-deficient mutants grow well in the presence of excess tryptophan, suggesting that tryptophan detoxification is not the function of IAA synthesis (Brandl and Lindow, 1998). Some IAA biosynthetic enzymes can convert methylated and halogenated tryptophan analogues to compounds that are less toxic to the bacterial cells (Hutcheson and Kosuge, 1985; Yamada et al., 1985; Bar and Okon, 1992). IAA is also suggested to regulate the synthesis of important compounds in bacteria such as cAMP and amino acids (Katsy, 1997). Perhaps the most obvious explanation for the prevalence of bacterial production of a phytohormone is that it provides bacteria with a mechanism to stimulate plant growth. In doing so, bacteria can increase production of plant metabolites that the bacteria can utilize for their own growth. Another consequence of enhanced plant

growth is a greater root surface area through which more of these metabolites can be exuded (Gaudin et al., 1994).

Tumor Formation

Consider the case of the soil bacterium, *Agrobacterium tumefaciens* which can transfer a specialized sequence of DNA, the so-called T-DNA, carrying auxin biosynthesis genes among others, into the genome of dicotyledonous plants. As a consequence of the genetic transfer and the ensuing unregulated overproduction of T-DNA encoded proteins, plant cells proliferate rapidly to form crown gall tumors and synthesize novel compounds known as opines which the inducing bacterium has the unique ability to metabolize (Nilsson and Olsson, 1997). Thus, *A. tumefaciens* not only enslaves plant cells to supply it with nutrients but expands its factory of productive cells. Although crown galls are considered disease symptoms, they are essentially a result of excessive growth, induced in part by IAA.

*Agrobacterium*-transformed crown gall cells synthesize levels of IAA much higher than those of untransformed plant cells and do not require the otherwise essential addition of auxin for *in vitro* propagation (Liu et al., 1982; Thomashow et al., 1984). When plant cells are transformed with T-DNA carrying insertionally inactivated IAA biosynthesis genes, both IAA levels and gall size are reduced (Garfinkel et al., 1981; Joos et al., 1983; Akiyoshi et al., 1983). Exogenous auxin can supply the missing component required for tumor formation in tissues carrying the mutant T-DNA (Ooms et al., 1981; Leemans, et al., 1982; Joos et al., 1983). In addition, virulence is restored to mutant cells by transformation with functional T-DNA-derived IAA biosynthesis genes (Inze et al., 1984).

Incorporation of IAA biosynthesis genes directly into the plant genome is not the only mechanism for tumor formation. Galls of oleander, olive and privet are induced by IAA

secreted by *Pseudomonas savastanoi* cells inhabiting the intercellular spaces in these plants. In *P. savastanoi* strains isolated from oleander, a plasmid is necessary for IAA biosynthesis and gall formation. Loss of the plasmid is associated with loss of IAA production and loss of tumor induction, while reintroduction of the plasmid restores both capabilities (Comai and Kosuge, 1980; Comai et al., 1982). Infection of susceptible plants with bacterial mutants with an increased level of auxin production induces the formation of larger galls, while plants inoculated with IAA deficient mutants failed to develop gall tumors (Smidt and Kosuge, 1978; Comai and Kosuge, 1983; Surico et al., 1984).

A plasmid carrying IAA biosynthesis genes is also necessary for gall induction by pathogenic strains of *Erwinia herbicola* pv. *gypsophilia* (Manulis et al., 1991a). Transfer of the plasmid to nonpathogenic strains of *E. herbicola* also transferred gall-forming ability (Yamada, 1993). Insertional inactivation of the plasmid IAA genes reduced virulence, although levels of IAA produced by the mutants in media supplemented with tryptophan, by a different, tryptophan-dependent pathway, were similar to the wild-type (Clark et al., 1993). This suggests that the pathway to IAA production may be important in determining the effect of a bacterium on a plant, a concept that will be explored further in a later section.

Nodulation

The role of bacterial IAA in plant pathogenesis has long been established, however a role for IAA in plant growth promotion as it is beneficial to the host plant remains to be clearly established. Members of the genera *Rhizobium* and *Bradyrhizobium* induce nodules on the roots of legumes through which they provide the host plants with fixed nitrogen in exchange for photosynthetically fixed carbon. Although not likely an essential factor for nodulation (Wang et al., 1982; Atzorn et al., 1988; Hirsch and Fang, 1994), there is some

evidence to suggest that IAA secreted by bacteria, as they move to the root cortex from the site of infection at the root hair, influences nodulation, at least for some rhizobial strains. In several legume species, the auxin content is higher in the root nodules than in the remainder of the root tissue and in ineffective nodules that do not fix nitrogen (Badenoch-Jones et al., 1983; Dangar and Basu, 1987). Nodules infected with *Bradyrhizobium japonicum* IAA-overproducing mutants contain elevated levels of free and conjugated IAA indicating that the nodule IAA is derived from the infecting bacterium rather than from the plant (Hunter, 1987).

Both nodulating and non-nodulating strains of *Rhizobium* produce and secrete IAA to the culture medium, although levels are low in the absence of tryptophan (Wang et al., 1982). With the addition of exogenous tryptophan, however, IAA production in the nodulating strains of *R. leguminosarum* was stimulated to a much greater extent than in the non-nodulating mutants (Wang et al., 1982). This suggests that at least two pathways for IAA biosynthesis exist in these strains – one pathway is constitutive and the other is induced by tryptophan. The inducible pathway, missing in the non-nodulating strain, may be involved in nodulation.

Inoculation of soybeans with spontaneous mutants of *R. japonicum* that overproduced IAA by a pathway not detectable in the wild-type bacterium resulted in a three-fold increase in the volume of root nodules (Kaneshiro and Kwolek, 1985). On the other hand, mutants of *Bradyrhizobium elkanii* deficient in IAA production induced fewer nodules on soybean roots than did the parental strain, and the normal number of nodules was re-established following application of exogenous IAA (Fukuhara et al., 1994). IAA derived from *B. elkanii* has been implicated as a causative agent in the swelling of outer cortical cells of soybean roots that

sometimes accompanies nodule formation and is suggested to give this and other such inducing strains a competitive advantage for nodulation (Yuhashi et al., 1995). Enlargement of cortical cells was not observed after inoculation with either IAA-deficient mutants of *B. elkanii* (Yuhashi et al., 1995) or wild-type *B. japonicum* strains that do not produce IAA (Minamisawa and Fukai, 1991).

Co-inoculation of legumes with *Rhizobium* and free-living IAA-producers such as *Azospirillum brasilense* (Yahalom et al., 1990), and several *Bacillus* species (Srinivasan et al., 1996) significantly increased the number of nodules on the host roots, and where measured, increased nodule fresh weight and nitrogenase activity, compared to inoculation with *Rhizobium* alone. In contrast, similar experiments using mutants of *Bacillus megaterium* with altered IAA production levels (overproducers and underproducers) had a negative effect on these parameters (Srinivasan et al., 1996). Although it is not clear how IAA is involved in this enhanced nodulation, Srinivasan et al. (1996) suggest that these free-living bacteria may increase the number of infection sites for *Rhizobium* on roots.

Application of exogenous auxins to the roots of graminaceous plants that do not normally form symbiotic relationships with Rhizobiaceae, induces the formation of tumor-like structures called para-nodules (reviewed by Christiansen-Weniger, 1998). Interestingly, free-living diazotrophs such as *Azospirillum* and *Azotobacter* were able to colonize these para-nodules and efficiently fix atmospheric nitrogen to the benefit of the host plant.

Root system development

Promotion of root growth is one of the major markers by which the beneficial effect of plant growth-promoting bacteria is measured. Rapid establishment of roots, whether by elongation of primary roots or by proliferation of lateral and adventitious roots, is

advantageous for young seedlings as it increases their ability to anchor themselves to the soil and to obtain water and nutrients from their environment, thus enhancing their chances for survival. It was soon recognized that most root promoting microbes synthesize IAA, and that their effect on plants mimics that of exogenous IAA. Establishing a direct relationship between root growth and bacterial IAA has proven to be more elusive, given the difficulty in isolating bacterial mutants completely deficient in IAA synthesis (Liu et al., 1982; Abdel-Salam and Klingmüller, 1987; Manulis et al., 1991b; Clark et al., 1993).

Plants generally put down one or more primary roots from which lateral roots emerge by division of specific pericycle cell (Lynch, 1995; Malamy and Benfey, 1997). Adventitious roots are a type of lateral root that arise from non-root tissue, such as at the base of the stem or on cuttings (Barlow, 1986). Whereas lateral and adventitious roots are induced by high concentrations of IAA, a feature exploited in horticulture by applying exogenous natural and synthetic auxins, primary root elongation is stimulated by relatively low levels of IAA, typically between $10^{-9}$ M and $10^{-12}$ M (Thimann, 1938; Pilet and Saugy, 1987; Alvarez et al., 1989; Meuwley and Pilet, 1991; Gaspar et al., 1996; Malamy and Benfey, 1997; Lloret et al., 1998).

The effect on root morphology seen following application of various concentrations of exogenous IAA is also seen following inoculation of plants with plant growth-promoting bacteria that produce different levels of IAA. For example, inoculation of canola seeds with wild-type *Pseudomonas putida* GR12-2, which produces relatively low levels of IAA (Xie et al., 1996), resulted in a two- to three-fold increase in the length of seedling primary roots compared to uninoculated controls (Glick, 1995; Caron et al., 1995). An IAA overproducing mutant of this bacterium stimulated extensive lateral root development on canola roots (Xie

et al., 1996) and adventitious roots on mung bean cuttings (Mayak et al., 1997). In both cases, it was impossible to tell whether the development of lateral roots was a consequence of IAA directly or of IAA-induced ethylene. The increase in the number of roots on the mung bean cuttings correlated with an increase in ethylene production.

The diazotroph, *Azospirillum brasilense*, naturally produces high levels of IAA and appears to positively influence root system development. Inoculation with *A. brasilense* increases the number and length of lateral roots in wheat (Barbieri et al., 1986; Barbieri and Galli, 1993) and pearl millet (Tien et al., 1979) similar to the application of exogenous IAA. That the plant response was due to IAA secretion rather than nitrogen fixation was confirmed using combinations of mutants unable to fix nitrogen or synthesize high levels of IAA. Loss of the capacity to fix nitrogen did not reduce the root promoting effect on wheat seedlings, whereas inoculation with a mutant that produced 70% less IAA did have this effect (Barbieri et al., 1986; Barbieri and Galli, 1993).

As previously mentioned, much of what is known about the role of auxin in plant growth and development was worked out from plant responses to the application of exogenous IAA. The underlying assumption of this approach is that the applied concentration is available to the target tissue, which may be erroneous in view of the fact that uptake, translocation and metabolism are seldom measured. Nevertheless, it was determined that only a small window of exogenous IAA concentration positively influences elongation growth (Thimann, 1938). The actual range of effective concentrations varies according to plant species or to the sensitivity of the plant organ to auxin, roots being more sensitive to, or stimulated by lower concentrations of exogenous auxin, than are shoots (Thimann, 1938; Evans et al., 1994). Levels below this range have no effect, whereas higher concentrations

inhibit elongation growth, likely via auxin induced ethylene (Peck and Kende, 1995). For example, Evans et al. (1994) found that only exogenous concentrations between $10^{-10}$ M and $10^{-12}$ M stimulated primary root elongation in *A. thaliana* seedlings.

Two different approaches have been taken to test for a similar trend in the effect of bacterial IAA on plant growth. One method compares the effects of inoculating roots with bacterial mutants that produce altered levels of IAA. A second approach varies the size of the inoculum for a single strain; the rationale here is that a higher inoculum density means more IAA is available to the plant. While *P. putida* GR12-2 stimulated elongation of roots of canola seedlings, an IAA-overproducing mutant significantly inhibited the length of canola primary roots (Xie et al., 1996). The deleterious effect on primary root growth has been demonstrated for many strains that synthesize high levels of IAA, for example, with a *P. fluorescens* CHA0 overproducing mutant added to autoclaved soil used to grow wheat and cucumber (Beyerler et al., 1997) and with *Enterobacter taylorae* which inhibited root growth in several weed species (Sawar and Kremmer, 1995). Loper and Schroth (1986) established a linear relationship between accumulation of IAA, above a threshold level in cultures of enterobacteria, and a negative effect on root elongation in sugar beet seedlings. Similarly, wild-type *P. syringae* pv. *savastanoi*, which produces high levels of IAA in culture, inhibited sugar beet root growth, whereas a spontaneous and an insertionally inactivated IAA-minus mutant did not (Loper and Schroth, 1986).

The inhibition of root elongation that follows inoculation of *Panicium miliaceum* plants with the culture extract of *A. brasilense* could be reversed by diluting the IAA-containing fraction (Harari et al., 1988). Moreover, greater dilutions of extracts from an IAA-overproducing strain of *A. brasilense* were required to achieve the same result, that is,

promotion rather than inhibition of root elongation. This confirms that the concentration of IAA secreted by a bacterium plays a role in the effect of that bacterium on a plant. Conversely, Selvadurai et al. (1991) showed that concentrating the indole fraction of culture supernatant from *Bacillus cereus*, a low IAA producer, shifted the effect of inoculation of wheat roots from growth promotion to growth inhibition. These latter observations are consistent with data obtained by varying the size of the inoculum, and therefore the concentration of IAA, of *Azospirillum* on various plant species (Bashan, 1986; Morgenstern and Okon, 1987; Harari et al., 1988; Fallik et al., 1988). Beyerler et al. (1997) found that decreasing the inoculum density of an IAA-overproducing mutant of *P. fluorescens* CHA0 resulted in elongation of cucumber and wheat roots that are inhibited by inoculation with a greater number of cells. That this beneficial effect was observed in non-sterile soil, suggests that competition may reduce the number of *P. fluoresens* CHA0 cells on a given host and consequently the level of IAA available to influence the host plant.

The level of auxin synthesized by the plant itself may be an important factor in determining whether bacterial IAA will stimulate or inhibit growth in a plant. In plant roots endogenous levels of IAA may be suboptimal or optimal for growth (Pilet and Saugy, 1987). Additional input into the IAA pool by bacteria could modify endogenous auxin to either optimal or supraoptimal levels, resulting in the induction or inhibition of plant growth, respectively. Dubeikovsky et al. (1993) inoculated two different species of plants with a recombinant strain of *P. fluorescens* that produced high levels of IAA. While this bacterium stimulated root development in blackcurrant cuttings, root development was suppressed in sour cherry cuttings. It could be argued that IAA contributions by this strain of *P. fluorescens* elevated endogenous IAA in blackcurrant to levels optimal for root growth.

Sour cherry, on the other hand, was already producing favorable concentrations of IAA, therefore additional input was inhibitory.

The auxin response may also depend upon the stage of plant root development. Application of low concentrations of IAA promoted growth in young maize roots, but inhibited growth in older roots (Pilet et al., 1979). This may indicate that with age, plant endogenous auxin content increases from suboptimal to almost supraoptimal levels or, alternatively, may reflect an altered sensitivity to auxin.

Also for consideration is the possibility that bacteria may alter the sensitivity of a plant to auxin rather than alter plant auxin levels directly. In a manner similar to *A. tumefaciens*, *Agrobacterium rhizogenes* transfers the T-DNA region of its Ri plasmid into the genome of wounded plants. However, rather than induce tumor formation, incorporation and expression of the Ri plasmid T-DNA genes leads to development of an extensive root system at the site of infection, a condition known as hairy root disease. In crown gall tumorigenesis, the expression of T-DNA encoded auxin biosynthesis genes induces rapid division of undifferentiated transformed and untransformed cells. In contrast, development of the highly branched lateral and adventitious roots characteristic of hairy root disease occurs by proliferation of differentiated cells from a single transformed phloem cell (Chilton et al., 1982; Nilsson and Olsson, 1997), and does not absolutely require T-DNA auxin biosynthesis genes. Neither free nor conjugated IAA levels are altered significantly in this root tissue (Hansen et al., 1991; Schaerer et al., 1993; Nilsson et al., 1993; Schmülling et al., 1993; Biondi et al., 1997). Rather, insertional mutagenesis and single gene transformation studies have shown that incorporation and expression of *A. rhizogenes rol* genes, particularly *rolB*, are necessary to induce hairy root disease symptoms (White et al., 1985; Spena et al., 1987).

The *rolB* gene products may increase the sensitivity of transformed cells to auxin, especially in those cells that are competent to become lateral root primordia (Shen et al., 1988; Spano et al., 1988; Maurel et al., 1991; Nilsson et al., 1997). Tobacco protoplasts expressing the *rolB* gene were able to form callus in the absence of exogenous auxin (Walden et al., 1993) and roots that developed from this callus were inhibited by much lower concentrations of auxin than their wild-type counterparts (Schmülling et al., 1993).

Not only are plant cells transformed with the *rolB* genes more sensitive to endogenous auxin, but co-transformation with microbial auxin biosynthesis genes enhances the effect of RolB activity (Cardarelli et al., 1987). Furthermore, expression of RolB is regulated by auxin (Maurel et al., 1990). Bacterial auxin genes seem to be involved in the induction of extensive root hairs, a characteristic for which the disease is named (Biondi et al., 1997).

**Regulation of Auxin Levels in Bacteria**

From the preceeding discussion it is evident that levels of IAA produced by a bacterium are at least partially responsible for the effect of the bacterium on a host plant. Thus, factors that regulate bacterial IAA synthesis must be considered, necessitating a knowledge of biosynthetic pathways and the regulation of IAA expression therefrom, the availability of precursors and inducers in the rhizosphere and the interaction of IAA with other plant hormones.

At least five different pathways for auxin biosynthesis have been identified in rhizobacteria (Patten and Glick, 1996). Some of these pathways are constitutively expressed, while others are inducible. Genes for the enzymes involved are found on plasmids as well as

in the bacterial genome. As is shown below, the manner and extent to which bacterial auxin impacts on the plant is governed by the number, type and genomic position of biosynthesis pathways and their regulation by genetic and environmental factors.

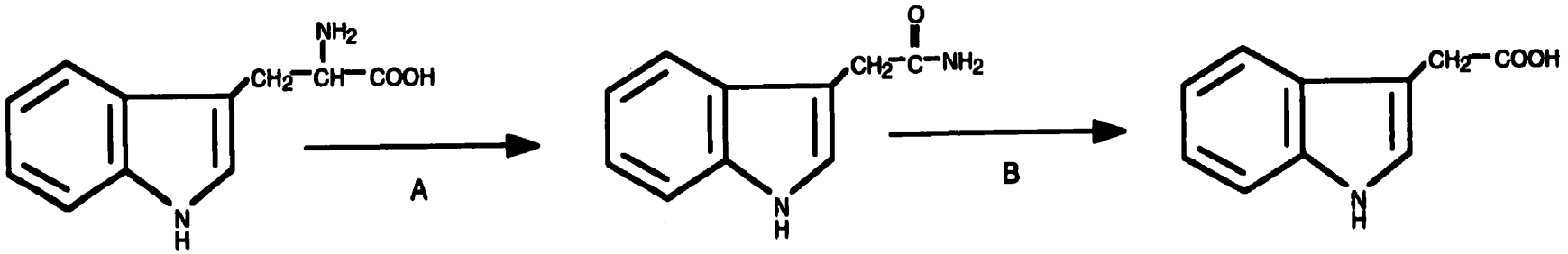
Biosynthesis

Several bacterial IAA biosynthesis pathways, classified in terms of their intermediates, are known, most proceeding from tryptophan as the precursor. Although the importance of bacterial IAA mutants as a tool for understanding growth promotion has long been recognized, attempts to isolate strains completely deficient in IAA synthesis have for the most part failed, probably due to the presence in many of these organisms of multiple routes to IAA production. In nature, two pathways appear to predominate, the indole-3-acetamide pathway and the indole-3-pyruvic acid pathway. Much of the available genetic and biochemical evidence for bacterial IAA biosynthesis centers on these two pathways, although documentation for the existence of other pathways is described.

*Indole-3-acetamide pathway.* Although the indoleacetamide pathway has been found in both tumorigenic and non-tumorigenic bacteria, it seems to be the primary route for IAA synthesis in strains classified as pathogenic. Two enzymes are responsible: tryptophan monooxygenase converts tryptophan to indole-3-acetamide which is then hydrolyzed by indoleacetamide hydrolase to IAA (Fig. 3).

Kuo and Kosuge (1970) determined that IAA biosynthesis in the pathogen *P. syringae* pv. *savastanoi* occurs predominantly via the indoleacetamide pathway. Essentially all exogenous radiolabelled tryptophan was converted to IAA through the intermediate indoleacetamide. The gene for tryptophan 2-monooxygenase (*iaaM*) was isolated by

Figure 3. Indole-3-acetamide pathway for indole-3-acetic acid biosynthesis in bacteria. A, tryptophan 2-monooxygenase; B, indoleacetamide hydrolase.




Tryptophan

Indole-3-acetamide

Indole-3-acetic acid

expression in *E. coli* of restriction enzyme digested fragments of the previously identified *P. syringae* pv. *savastanoi* 52 kb plasmid and identification of indoleacetamide-producing clones through Salkowski's chemical assay, thin layer chromatography, and tryptophan 2-monooxygenase activity (Comai and Kosuge, 1982). Organization of the IAA biosynthesis genes into a typical prokayotic operon was indicated by the loss of both tryptophan 2-monooxygenase and indoleacetamide hydrolase (encoded by the *iaaH* gene) activity upon insertion of a transposable element into the *iaaM* locus (Comai and Kosuge, 1983). The location of the promoter, 400 base pairs upstream from the *iaaM* gene, was determined by fusing various regions of the operon to the *E. coli lacZ* reporter gene (Gaffney et al., 1990).

Tryptophan 2-monooxygenase, purified from *P. syringae* pv. *savastanoi*, has been characterized as a somewhat unstable, 62 kDa protein that requires the catalytically active cofactor flavin adenine dinucleotide (FAD) to catalyze the oxidative decarboxylation of tryptophan to indoleacetamide (Hutcheson and Kosuge, 1985). It has broad substrate specificity for substituted tryptophan compounds, including a number of methylated and halogenated derivatives.

Mutagenesis of the Ti-plasmid T-DNA revealed that two genes were also involved in IAA biosynthesis in *A. tumefaciens* (Inzé et al., 1984; Klee et al., 1984; Schroeder et al., 1984). The product of "gene 2", overexpressed in *E. coli* cells, could convert supplied indole-3-acetamide to indole-3-acetic acid (Thomashow et al., 1984; Schroeder et al., 1984). Similarly, crown gall cells, transformed by *A. tumefaciens*, could also hydrolyze indoleacetamide to IAA whereas untransformed cells could not (Schroeder et al., 1984). "Gene 2", designated *tms-2*, therefore must encode indoleacetamide hydrolase.

Incubation of cell-free extracts from *E. coli* cells expressing "gene 1" with

[$^{14}$C]-tryptophan, resulted in the production of radiolabeled indole-3-acetamide, and when this product was added to cell-free extracts prepared from *E. coli* expressing the *tms-2* gene, IAA was produced (Thomashow et al., 1986). Accumulation of indoleacetamide in extracts of transformed tobacco cells expressing 'gene 1' but not *tms-2*, confirmed that T-DNA "gene 1", now known as *tms-1*, encodes the enzyme tryptophan 2-monooxygenase (Van Onckelen et al., 1986).

The monocistronic arrangement of the IAA genes of *A. tumefaciens* suggests adaptation for expression within plant cells. This is further supported by the observation that the *tms-1* and *tms-2* genes, located on opposite DNA strands (Inzé et al., 1984), are flanked by regulatory signals typical to eukaryotes including TATA and CAAT boxes in a promoter region recognized by RNA polymerase II, polyadenylation signals, and sequences recognized by plant transcription factors (Gaudin et al., 1994).

Nucleotide sequence comparison shows there is 54% identity (columns in an alignment of two sequences that contain identical amino acids) between *iaaM* from *P. syringae* pv. *savastanoi* and *tms-1* from *A. tumefaciens,* 38% identity between *iaaH* and *tms-2*, and 50% and 27% identity, respectively, at the amino acid level (Klee et al., 1984; Gielen et al., 1984; Yamada et al., 1985; Follin et al., 1985). Identity was strongest in a 25 amino acid region in both *iaaM* and *tms-1*, predicted to be a putative flavin adenine dinucleotide (FAD) binding site from the high degree of similarity to a known FAD site (Klee et al., 1984). The open reading frame for *iaaM* encodes a 557 amino acid protein with a molecular weight of 61,783 Da, which compares well with the estimated mass of 62,000 Da for purified tryptophan monooxygenase as determined by SDS-PAGE (Hutcheson and Kosuge, 1985). The larger *tms-1* open reading frame, indicating a 755 amino acid protein

with a molecular weight of 83,769 (Klee et al., 1984), may reflect requirements for expression in the plant genome. The 455 amino acid protein revealed by the *iaaH* open reading frame, is similar to the *tms-2* open reading frame that encodes a 473 residue protein with a molecular weight of 49,588 Da (Yamada et al., 1985).

Analysis of enzyme activity has revealed the *A. tumefaciens* indoleacetamide hydrolase to be a simple enzyme that functions in the absence of cofactors, with a high affinity for indoleacetamide ($K_m$ = 1 μM; Kemper et al., 1985). Other substrates can be hydrolyzed at a much slower rate, including indole-3-acetonitrile, phenylacetamide and IAA-ester conjugates such as IAA-glucose and IAA-myo-inositol, but not amide conjugates.

The *aux* genes of *A. rhizogenes*, located in the Ri-plasmid T-DNA region that is incorporated into the plant genome, are similar to the *tms* sequences of *A. tumefaciens*; *aux-1* and *tms-1* show 60% identity, while *aux-2* and *tms-2* show 71% identity. Regulation signals are also similar to those for *tms-1* and *tms-2* (Gaudin et al., 1994).

Similar to *P. syringae* pv. *savastanoi*, another pathovar of this species, *P. syringae* pv. *syringae*, produces IAA by the indoleacetamide pathway; however, in the latter case, the genes are present on the bacterial chromosome rather than a plasmid, and the consequences of IAA production are not clear (Fett et al., 1987). Rather than induce gall formation, *P. syringae* pv. *syringae* causes circular, brown necrotic lesions surrounded by a ring of chlorotic tissue (Mazzola and White, 1994). These symptoms of brown spot disease resulting from infection of bean correlate with high levels of IAA. The IAA biosynthesis genes involved were identified and cloned using the *iaaM* and *iaaH* genes of *P. syringae* pv. *savastanoi* as a probe (White and Ziegler, 1991). Although the amino acid sequences of *iaaM* and *iaaH* in the two pathovars share 90% identity, there are significant differences in the

nucleotide sequences flanking the two genes (Mazzola and White, 1994). Differences in the regulatory regions may account for the lower levels of IAA produced by *P. syringae* pv. *syringae* compared to *P. syringae* pv. *savastanoi* (White and Ziegler, 1991).

Biochemical evidence, including identification of pathway intermediates and production of IAA after treatment with intermediates, suggests that only pathogenic strains of *Erwinia herbicola* produce IAA via the indoleacetamide pathway (Manulis et al., 1991b). The genes involved were isolated from a plasmid of the *gypsophilia* pathovar of *E. herbicola* using *P. syringae* pv. *savastanoi iaaM* and *iaaH* genes as hybridization probes (Clark et al., 1993). Mutants of *E. herbicola* pv. *gypsophilia* containing insertionally inactivated *iaaM* and *iaaH* produce the same amount of IAA from tryptophan as the wild-type, indicating the presence of an additional pathway for IAA biosynthesis.

Sekine et al. (1988) have suggested that *Bradyrhizobium japonicum* and *Rhizobium fredii* synthesize IAA via the indoleacetamide pathway. These strains were able to convert an indoleacetamide analogue, naphthaleneacetamide (NAM) to naphthaleneacetic acid (NAA) suggesting that indoleacetamide hydrolase is produced. Sequence analysis of the *B. japonicum* gene (*bam*) responsible, isolated by mutant complementation (Sekine et al., 1989a), revealed an open reading frame of 465 amino acids corresponding to a 50,266 Da protein (Sekine et al., 1989b). A high degree of identity at the amino acid level was found only among the central regions of the *bam* gene, *iaaH* of *P. syringae* pv. *savastanoi* and *tms-2* of *A. tumefaciens* (Sekine et al., 1989b). Although both indoleacetamide and IAA were detected in *B. japonicum* cultures grown in tryptophan-supplemented media, regions upstream and downstream from the *bam* gene did not show similarity to *iaaM* or *tms-1*. The location of a tryptophan 2-monooxygenase gene in *B. japonicum* remains unknown.

There are indications that *Rhizobium* species may synthesize indoleacetamide hydrolase when associated with the host plant, but that this capacity is suppressed in the free-living state, making detection difficult. The high degree of similarity between the *bam* gene of *B. japonicum* and DNA sequences from a biovar of *Rhizobium leguminosarum* suggests that an indoleacetamide hydrolase gene is present in the latter bacterium; however, enzymatic activity could only be detected in the free-living state of spontaneous mutants (Kawaguchi et al., 1990). Host plant flavonoids appear to have a stimulating effect on IAA production in several *Rhizobium* species (Prinsen et al., 1991).

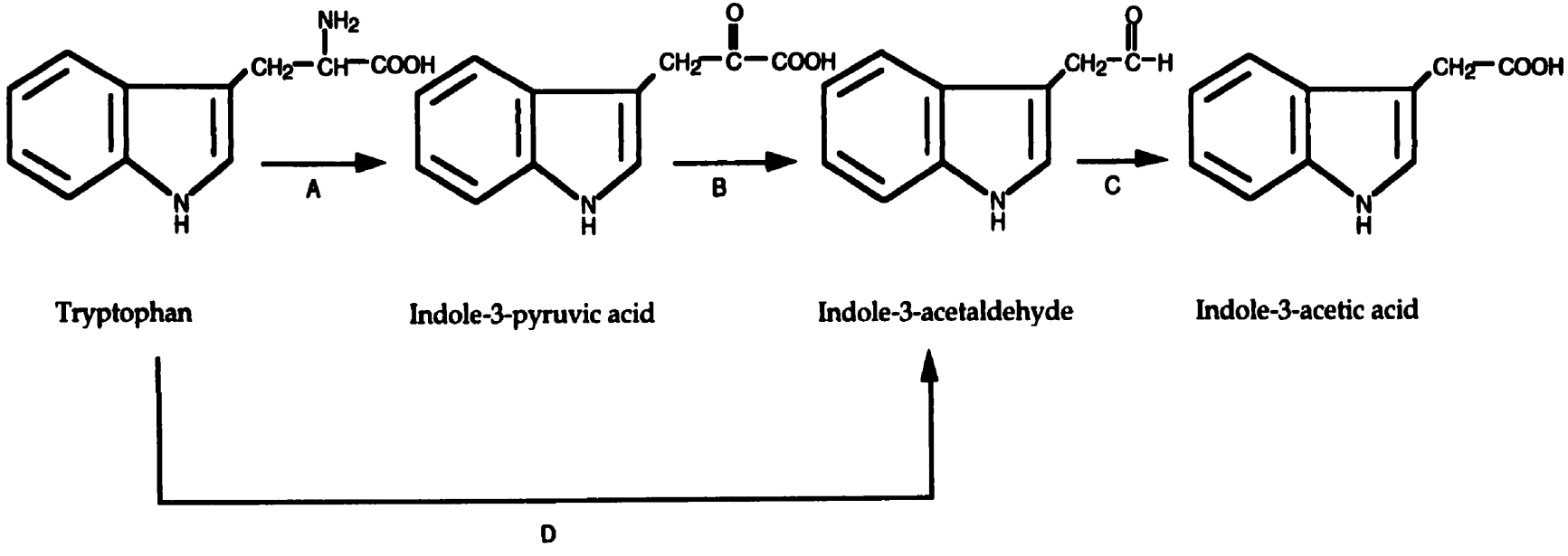
Although the principal IAA biosynthesis route in *Azospirillum* does not appear to proceed via indoleacetamide (Prinsen et al., 1993; Okon, 1994; Costacurta et al., 1994), this pathway does exist in at least some strains of *A. brasilense* (Bar and Okon, 1993). Indoleacetamide was identified in extracts of the culture supernatant of several *A. brasilense* strains and accumulated in mutants with a reduced capacity to synthesize IAA (Prinsen et al., 1993). Tryptophan 2-monooxygenase activity was detected in a 56 kDa protein on a non-denaturing gel and a probe containing *iaaM* and part of *iaaH* from *P. syringae* pv. *savastanoi* hybridized to *A. brasilense* total DNA (Bar and Okon, 1993).

*Indole-3-pyruvic acid pathway.* Although early work on IAA production in phytopathogens focused on the indoleacetamide pathway, it was recognized that IAA in bacteria can also be derived from indolepyruvic acid. This latter pathway however was believed to contribute relatively little to the measurable levels of IAA (Kuo and Kosuge, 1970). Later, when mutagenesis experiments designed to inactivate the indoleacetamide pathway failed to generate mutants completely deficient in the ability to synthesize IAA, interest in alternate pathways was renewed. An *A. tumefaciens* strain cured of its Ti-plasmid was able to

synthesize 50% of the level of IAA produced by the parental strain from genes located on the chromosome (Liu et al., 1982). After testing 5,000 Tn*5*-mutagenized colonies of *Azospirillum lipoferum* for decreased levels of IAA synthesis, Abdel-Salam and Klingmüller (1987) isolated eleven mutants that produced 9-55% of the IAA level of the wild-type. That the mutants still produced some IAA suggests that more than one biosynthesis pathway, or perhaps multiple gene copies for a single pathway, may be present. *E. herbicola* pv. *gypsophilae* strains containing insertionally inactivated *iaaM* and *iaaH* were still able to produce IAA from tryptophan (Manulis et al., 1991b; Clark et al., 1993). Chemical identification of intermediates, enzyme activity analysis, and, more recently, genetic evidence indicates that these strains can alternatively synthesize IAA via indolepyruvic acid.

This other major bacterial IAA biosynthesis pathway is believed to be similar to that suggested for plants; tryptophan is converted to indolepyruvic acid by an aminotransferase reaction, followed by decarboxylation to indole-3-acetaldehyde, which is then further oxidized by indoleacetaldehyde dehydrogenase to IAA (Fig. 4). Transamination to a keto acid by pyridoxal phosphate-dependent enzymes is a common first step in tryptophan metabolism. Aromatic amino acid transferase activity has been detected in *P. syringae* pv. *savastanoi* (Kuo and Kosuge, 1970), *A. tumefaciens* (Liu et al., 1982), *A. lipoferum* (Ruckäschel et al., 1988), *A. brasilense* (Soto-Urzua et al., 1996), *R. meliloti* (Kittel et al., 1989), *E. cloacae* (Koga et al., 1994) and *E. herbicola* (Brandl et al., 1996), although in all cases little substrate specificity was observed. In fact, in *P. syringae* pv. *savastanoi*, L-tryptophan was the poorest amino donor of the aromatic amino acids tested (Kuo and Kosuge, 1970) and, in *E. cloacae* and *A. brasilense*, the aminotransferase has a very low affinity for L-tryptophan (Koga et al., 1994; Soto-Urzua et al., 1996).

Figure 4. Indole-3-pyruvic acid pathway (A-C) and tryptophan side chain pathway (D) for indole-3-acetic acid biosynthesis in bacteria. A, tryptophan aminotransferase; B, indole-3-pyruvic acid decarboxylase; C, indole-3-acetaldehyde oxidase; D, tryptophan side chain oxidase


NH2
CH2—CH—COOH
A
O
CH2—C—COOH
B
O
CH2—C—H
C
CH2—COOH
N
H
Tryptophan
Indole-3-pyruvic acid
Indole-3-acetaldehyde
Indole-3-acetic acid
D

40

The genes for three of the four aromatic aminotransferases found in *R. meliloti* have been cloned (Kittel et al., 1989). Mutants with transposon insertions in one of these genes produced less IAA than the parental strain in the presence of tryptophan, and double mutants with disruptions in two of the genes had further decreased levels of IAA. Similarly, plasmid-cured *A. tumefaciens* cells with a mutation in a chromosomally located aromatic aminotransferase gene were able to produce some indolepyruvic acid from tryptophan, but with reduced efficiency (Liu et al., 1982). The presence of multiple aromatic aminotransferases in most of the bacteria studied may in some cases explain the inability to isolate true IAA-minus mutants.

Considering that tryptophan aminotransferase lacks substrate specificity and has a poor affinity for tryptophan, the key enzyme in the indolepyruvic acid pathway may be indole-3-pyruvate decarboxylase, which catalyzes the second step, the conversion of indole-3-pyruvic acid to indole-3-acetaldehyde. Tryptophan aminotransferase, in fact, has a much higher affinity (138-fold lower $K_m$ value) for indolepyruvic acid than for tryptophan (Koga, 1995), and may be involved in the synthesis of tryptophan. However, in the presence of indolepyruvate decarboxylase, indolepyruvic acid is efficiently converted to indoleacetaldehyde (Koga et al., 1994; Koga, 1995). Thus, intracellular indolepyruvic acid would be maintained at levels too low to contribute significantly to tryptophan biosynthesis, and the reaction equilibrium would be shifted to favour IAA production.

The presence of indolepyruvate decarboxylase has been detected in several plant growth-promoting bacterial strains. *Enterobacter cloacae* was able to convert tryptophan, indolepyruvic acid and indoleacetaldehyde to IAA, suggesting that this microbe synthesizes IAA via indolepyruvic acid (Koga et al., 1991b). An IAA biosynthesis gene was isolated by

screening a cosmid library, derived from *E. cloacae* chromosomal DNA, in *E. coli,* for the ability to convert tryptophan to IAA (Koga et al., 1991a). Cell-free extracts prepared from positive *E. coli* clones showed indolepyruvate decarboxylase activity; the presence of this enzyme was confirmed by nuclear magnetic resonance (NMR). Sequence analysis of the isolated *E. cloacae ipdc* gene indicates that it codes for a single, 60 kDa protein (Koga et al., 1991a). PCR primers designed from the *E. cloacae ipdc* sequence were able to amplify a fragment of predicted size from seven IAA producing enterobacteria, including strains of *E. cloacae*, *E. agglomerans* and *Klebsiella* sp. and from the plant *Zea mays* (Zimmer et al., 1994 and 1995).

Indolepyruvate decarboxylase from *E. cloacae* has been purified and characterized as having high specificity and high affinity for indolepyruvic acid (Koga et al., 1992; Koga, 1995). In its active form the enzyme exists as a homotetramer, requiring the cofactors $Mg^{2+}$ and thiamine diphosphate for activity and stability, and for formation of the tetramer. In the presence of these cofactors, cell-free extracts prepared from *Bradyrhizobium elkanii* cultures were able to produce high levels of indoleacetaldehyde from supplied indolepyruvic acid, strongly indicating that indolepyruvate decarboxylase is active in these cells (Minamisawa et al., 1996).

Indolepyruvate decarboxylase genes isolated from two *A. brasilense* strains are highly (97%) similar (the percentage of columns in an alignment of two sequences that contain amino acids that are either identical or have similar properties); however the flanking regions are not similar (Costacurta et al., 1994; Zimmer et al., 1998). Both appear to be monocistronic, although there is evidence for a second copy of the gene elsewhere in both chromosomes. DNA hybridization experiments indicate that the *A. brasilense* sequence is

present in *A. lipoferum* and *A. halopraeferens*, but not in *A. irakense*, a low IAA producer (Costacurta et al., 1994). Comparison of the deduced amino acid sequences with that from the *ipdc* gene sequence from *E. cloacae* shows 43% and 56% similarity (29% and 28% identity) between the *A. brasilense* Sp245 and *A. brasilense* Sp7 enzymes, respectively (Costacurta et al., 1994). Sequence analysis of the 1,635 nucleotide open reading frame from *A. brasilense* Sp245 shows that the 64 kDa protein shares 38% amino acid similarity with pyruvate decarboxylases from *Zymomonas mobilis* and *Saccharomyces cerevisiae*.

Tumorigenic and non-tumorigenic strains of *E. herbicola* synthesize IAA via the indolepyruvic acid pathway as determined by detection of pathway intermediates in cell cultures and production of IAA after incubation of cells in the presence of appropriate intermediates (Manulis et al., 1991b; Brandl et al., 1996). The gene for indolepyruvate decarboxylase was isolated from an *E. herbicola* pear isolate by screening a cosmid library of genomic DNA in *E. coli* for the ability to synthesize IAA (Brandl and Lindow, 1996). The open reading frame was characterized as a 1,650 base pair sequence encoding a 550 amino acid protein with 56% and 26% identity to the *ipdc* gene from *E. cloacae* and *A. brasilense*, respectively, and a high degree of similarity to pyruvate decarboxylases from several plant and fungal species. Mutants containing transposon insertions in the *ipdc* locus produced 90% less IAA than the wild-type and were unable to convert indolepyruvic acid to indoleacetaldehyde.

Almost nothing is known about bacterial indoleacetaldehyde oxidase that catalyzes the last step in the indolepyruvic acid pathway, the conversion of indoleacetaldehyde to IAA. Several aldehyde oxidase have been found in plants, each playing a unique role in the regulation of plant growth and development (Min et al., 2000; Sekimoto et al., 1997). An

aldehyde oxidase that preferentially utilizes indoleacetaldehyde as a substrate was identified in wild-type *Arabidopsis thaliana* and has higher activity in a *sur1* mutant that produces higher levels of endogenous IAA (Seo et al., 1998).

*Tryptophan side chain pathway.* An IAA biosynthesis route in which tryptophan is converted directly to indole-3-acetaldehyde, bypassing indole-3-pyruvic acid, has been demonstrated in *Pseudomonas fluorescens* (Narumiya et al., 1979; Oberhänsli et al., 1991). Indoleacetaldehyde is produced from tryptophan by the action of tryptophan side chain oxidase and is subsequently oxidized to IAA by aldehyde dehydrogenase (Fig. 4). Oberhänsli et al. (1991) present evidence that a biocontrol strain of *P. fluorescens* (CHA0), which can suppress some fungal borne root diseases including black root rot of tobacco, synthesizes tryptophan aminotransferase which is involved in the indolepyruvic acid pathway, as well as tryptophan side chain oxidase. Tryptophan monooxygenase and indoleacetamide hydrolase, both part of the indoleacetamide pathway, were not detected.

Tryptophan transaminase activity is constant throughout the exponential and stationary phases of bacterial growth, and is not significantly influenced by the addition of tryptophan to the culture medium. In contrast, tryptophan side chain oxidase activity is only observed during the stationary growth phase of *P. fluorescens* CHA0 (this was also observed by Narumiya et al. (1979) in another *P. fluorescens* strain) and is repressed four-fold in the presence of 10 mM tryptophan. In addition, in increasingly acidic media (from pH 7 to pH 5.5), IAA produced by the tryptophan side chain pathway increases, whereas that produced by the indolepyruvic acid pathway decreases. These observations support the suggestion that the pathway by which a microbe synthesizes IAA is dependent upon environmental conditions. Incidentally, mutations in tryptophan side chain oxidase did not

alter the ability of *P. fluorescens* CHA0 to protect wheat and tobacco against black root rot indicating that the tryptophan side chain pathway does not play an important role in disease suppression (Oberhänsli et al., 1991).

*Tryptamine pathway.* Tryptophan can also be converted to indole-3-acetaldehyde and IAA via the intermediate tryptamine. The initial metabolism of tryptophan to tryptamine is catalyzed by tryptophan decarboxylase followed by conversion of tryptamine to indoleacetaldehyde by amine oxidase (Fig. 5). Although this pathway appears to be present in plants (De Luca et al., 1989; Cooney and Nonhebel, 1991) and fungi (Frankenberger and Arshad, 1995), very little attention has been focused on tryptamine as a possible intermediate in bacterial IAA biosynthesis. However, *Bacillus cereus* has been reported to synthesize a decarboxylase which can act on tryptophan to produce tryptamine (Perley and Stowe, 1966) and *A. brasilense* could convert tryptamine, added to the culture medium, into IAA (Hartmann et al., 1983).

*Indole-3-acetonitrile pathway.* Although the pathway by which indole-3-acetonitrile is converted to indole-3-acetic acid has been identified in higher plants (Kobayashi et al., 1993; Kobayashi and Shimizu, 1994; Bartel and Fink, 1994; Bartling et al, 1994), its contribution to microbial IAA production tends to be overlooked. Nitriles can be hydrolyzed directly to their corresponding acids through the action of specific nitrilases, or can be catabolized via a two step process involving an initial conversion to an amide by nitrile hydratase followed by hydrolysis of the amide to an acid by amidase (Kobayashi et al., 1992; Fig. 6). Production of indoleacetonitrile for IAA synthesis by these reactions may be tryptophan-dependent, involving conversion of tryptophan to indoleacetaldoxime and then to indoleacetonitrile

Figure 5. Tryptamine pathway for indole-3-acetic acid biosynthesis in bacteria. A, tryptophan decarboxylase; B, amine oxidase; C, indole-3-acetaldehyde oxidase.

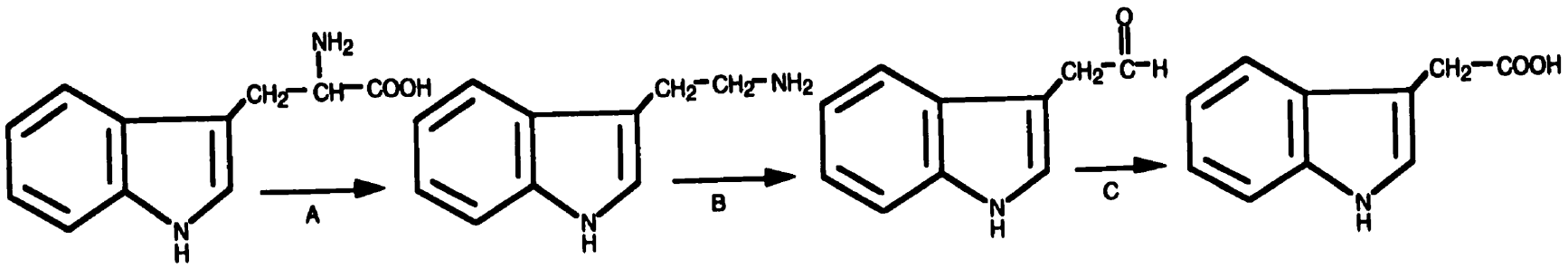
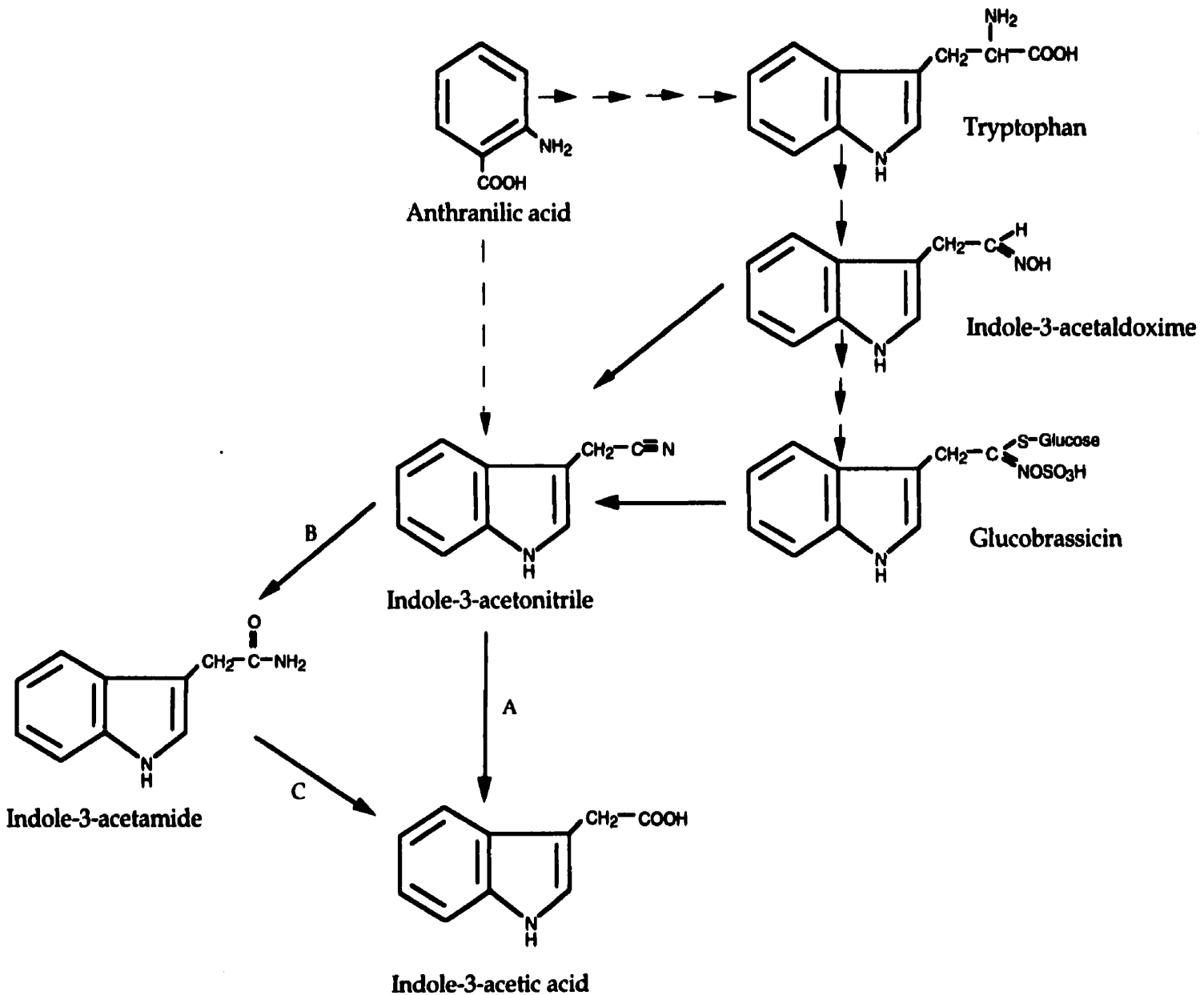

NH2
CH2 CH COOH
A
CH2 CH2 NH2
B
O
CH2 C H
C
CH2 COOH
N H
Tryptophan
Tryptamine
Indole-3-acetaldehyde
Indole-3-acetic acid

Figure 6. Bacterial synthesis of indole-3-acetic acid from indole-3-acetonitrile directly via nitrilase (A) or via a two step pathway catalyzed by the enzymes nitrile hydratase (B) and amidase (C). Production of indole-3-acetonitrile may involve the conversion of tryptophan to indoleacetaldoxime and then to indoleacetonitrile either directly or via glucobrassicin, or may occur from anthranilate without proceeding through tryptophan as an intermediate. Solid arrows represent a single enzymatic step; the broken arrow denotes that this pathway has not been fully characterized.


Anthranilic acid
Tryptophan
Indole-3-acetaldoxime
Glucobrassicin
Indole-3-acetonitrile
Indole-3-acetamide
Indole-3-acetic acid
A
B
C

either directly or via glucobrassicin (Kobayashi et al., 1993), or may be derived from the precursor anthranilate without proceeding through tryptophan as an intermediate (Normanly et al., 1993; Bartling et al., 1994).

Nitrilases are highly specific and are classified according to the substrate type upon which they act. Although several nitrilases have been found in microbes, of particular interest here are the arylacetonitrilases. Such a nitrilase, specific for indole-3-acetonitrile, was purified from *Alcaligenes faecalis* (Kobayashi et al., 1993) and the gene for this enzyme has been cloned and sequenced (Kobayashi et al., 1993). The deduced amino acid sequence shares only 27% identity with a nitrilase whose gene was cloned from the higher plant, *Arabidopsis thaliana*; however, residues in the active sites of these enzymes appear to be highly conserved (Bartling et al., 1994). In *Bradyrhizobium japonicum*, nitrilase activity catalyzes the conversion of indoleacetonitrile to IAA (Vega-Hernandez et al., 1999).

The conversion of indole-3-acetonitrile to IAA via indole-3-acetamide was demonstrated in *Agrobacterium* and *Rhizobium* species with nitrile hydratase and amidase activity (Kobayashi et al., 1995). The nitrile hydratase, which acts on indoleacetonitrile, was purified from the cell-free extract of *A. tumefaciens* and characterized. The molecular mass of the enzyme was determined to be 102 kDa, consisting of four subunits of identical mass.

The occurrence of nitrile hydratases and amidases is believed to be quite common in bacteria, although those identified in various species appear to be specific for different substrates (Nagasawa et al., 1987; Kobayashi et al., 1992; Duran et al., 1993). For example, the amino acid sequences of the nitrile hydratases from *Rhodococcus* sp. and *Rhodococcus erythropolis* are highly similar (95% identical), however their substrate preferences are different, suggesting that only a few amino acid residues are responsible for determining

substrate specificity (Duran et al., 1993). The arrangement of nitrile hydratase and amidase genes in an operon has been confirmed in several strains of *Rhodococcus* and in *Pseudomonas chlororaphis* (Duran et al., 1993; Kobayashi et al., 1992). Although nitrile hydratases and amidases appear to be widespread, the discovery of those specific for indoleacetonitrile is very recent, and much more study is required to determine their contribution to IAA biosynthesis in plant-associated bacteria.

Conjugation

In a manner similar to plants, microorganisms may also synthesize inactive IAA conjugates. IAA-lysine synthetase activity, which yields IAA-lysine and a derivative, acetyl-IAA-lysine, was discovered in oleander-derived strains of *P. syringae* pv. *savastanoi* (Glass and Kosuge, 1986). The gene for this enzyme (*iaaL*) was localized to a plasmid carrying the IAA biosynthesis genes *iaaM* and *iaaH*. The *iaaL* gene is not part of the IAA biosynthesis operon, but rather appears to be transcribed along with a gene encoding a hydrophobic protein that may function as a membane transport protein for IAA or IAA-lysine (Roberto et al., 1990).

Olive- and privet-derived strains of *P. syringae* pv. *savastanoi*, whose IAA genes are located on chromosomal DNA, could not convert IAA to IAA-lysine. These strains normally accumulate twice as much free IAA as their oleander counterparts. When the *iaaL* gene was introduced into olive isolates, not only could these strains convert fed, radiolabelled IAA to IAA-lysine, but IAA accumulation was reduced by one third to one half that of the wild-type strain after 72 hours (Glass and Kosuge, 1988). Similarly, oleander-derived strains with deletions in the IAA-lysine synthetase gene could no longer convert radiolabelled IAA to IAA-lysine and also accumulated five times more free IAA than the parental strains. These

results confirm that conversion of IAA to a conjugated form influences the pool size of free IAA.

Not only can some bacteria synthesize IAA conjugates but some can also hydrolyze conjugates to yield free IAA. Along with 18 other unidentified bacterial strains, an *Enterobacter agglomerans* strain was selected from sewage sludge by its ability to grow on IAA-aspartate as a sole carbon and nitrogen source (Chou et al., 1996). IAA-aspartate hydrolase was purified from this bacterium and found to consist of a 45 kDa monomer with a high specificity for converting IAA-aspartate to IAA.

Number and type of pathways

A survey of the pathways by which various bacterial strains synthesize IAA reveals an interesting pattern; one that suggests that the pathway to IAA production may be important in determining the effect of a bacterium on a plant (Table 2). Manulis et al. (199lb) have found that both pathogenic and non-pathogenic strains of *Erwinia herbicola* pv. *gypsophilae* synthesize IAA via the indolepyruvic acid pathway. However, only the tumorigenic strain can produce IAA via indoleacetamide. Inactivation of this latter pathway reduces virulence, while inactivation of the indolepyruvic acid pathway reduces the epiphytic fitness of *E. herbicola* pv. *gypsophilae* (Manulis et al., 1998).

Although rare incidence of the indoleacetamide pathway has been reported in plants (Saotome et al., 1993; Kawaguchi et al., 1993), it is generally believed that this is predominantly a microbial IAA biosynthesis route (Manulis et al., 199la; Gaudin et al., 1994). The uniqueness of this pathway to bacteria may allow them to evade plant regulatory signals that would normally keep plant IAA at non-toxic or physiologically appropriate levels, and thereby to induce uncontrolled growth in the plant tissues. Indeed, phytopathogens such as

Table 2. Indole-3-acetic acid biosynthesis pathway, location of biosynthesis genes, and regulation of expression of these genes for various pathogenic and beneficial plant-associated bacteria.

| Bacterium | Effect on Host Plant | IAA Biosynthesis Pathway Involved | Location of IAA Genes | Regulation | Reference |
|---|---|---|---|---|---|
| *Agrobacterium tumefaciens* | Tumor formation | Indoleacetamide | Ti plasmid | Constitutive (in plant) | Schroeder et al., 1984 |
| *Erwinia herbicola* pv.*gypsophilia* | Tumor formation | Indoleacetamide | Plasmid | Not determined | Manulis et al., 1991b |
| *Pseudomonas savastanoi* | Tumor formation | Indoleacetamide | Plasmid | Constitutive | Comai & Kosuge, 1980; Gaffney et al., 1990 |
| *Pseudomonas syringae* | Necrotic lesions | Indoleacetamide | Chromosome | Constitutive | Mazzola & White, 1994; Fett et al., 1987 |
| *Erwinia herbicola* 299R | Necrotic lesions | Indolepyruvic acid | Chromosome | Inducible | Brandl & Lindow, 1996 |
| *Pseudomonas putida* GR12-2 | Primary root elongation | Indolepyruvic acid | Chromosome | Inducible | Patten, 1996 |
| *Azospirillum brasilense* Sp245 | Lateral root development | Indolepyruvic acid | Chromosome | Inducible | Costacurta et al., 1994 |

*P. syringae* (Kuo and Kosuge, 1970; White and Ziegler, 1991; Mazzola and White, 1994), and *Agrobacterium*-transformed plant cells (Schroeder et al., 1984), synthesize IAA predominantly via the indoleacetamide pathway. Alternatively, because bacterial IAA synthesis by this route is generally constitutive, it may be that high levels of IAA exceed the capacity for IAA metabolism by a host plant (Sitbon et al., 1992). In contrast, plant growth-promoting bacteria such as *P. putida* GR12-2 (Patten, 1996), *E. cloacae* (Koga et al., 1991b) and *Azospirillum* spp. (Costacurta et al., 1994) synthesize IAA mainly via the indolepyruvic acid pathway, which may be subject to more stringent regulation by plant metabolites.

The position of IAA biosynthesis genes in the bacterial genome, that is, whether it is on a plasmid or in the chromosome, may also be important in specifying the effect of a bacterium on its host plant (Table 2). Transfer of the T-DNA region of a specialized plasmid carrying genes involved in the indoleacetamide pathway for IAA synthesis provides a unique mechanism by which *Agrobacterium* species can deliver high levels of IAA directly to plant cells and thereby induce tumor formation (Nilsson and Olsson, 1997). Similar genes are also carried on plasmids in tumorigenic strains of *P. syringae* pv. *savastanoi* (Comai and Kosuge, 1980) and *E. herbicola* (Manulis et al., 1991b), however, these are not transferred into the host plant genome. Perhaps significantly, *P. syringae* pathovars that induce bark and leaf lesions rather than tumorous growths also synthesize IAA via indoleacetamide, but from chromosomal genes (Mazzola and White, 1994). A non-tumorigenic *E. herbicola* isolate that induces lesions on pear fruit also carries IAA genes in the chromosome, although in this case the genes encode enzymes for the indolepyruvic acid pathway (Brandl and Lindow, 1996). Both pathogenic and nonpathogenic strains of *Xanthomonas campestris* pv. *glycines* can produce IAA; however IAA biosynthesis genes are found on a plasmid in the pathogenic

strains and in the chromosome of the nonpathogenic strains (Fett et al., 1987). Again, by comparison, the IAA biosynthesis genes of the strains that promote plant growth are found in the chromosome (Koga et al., 1991a; Costacurta et al., 1994; Patten, 1996), and thus IAA is more likely to be produced at lower levels in these bacteria than in bacteria whose IAA biosynthesis genes are expressed from multicopy plasmids.

Regulation of IAA expression

In general, the indoleacetonitrile and indolepyruvic acid pathways have been found to be inducible by pathway intermediates or precursors, whereas the indoleacetamide pathway is constitutive in most of the organisms studied. Nitrile hydratase, the enzyme that catalyzes the initial step in the conversion of indoleacetonitrile to IAA, is inducible by indoleacetamide, the intermediate in the pathway (Kobayashi et al., 1995). Tryptophan supplements have been shown to dramatically increase IAA biosynthesis by the indolepyruvic acid pathway in cultures of *Enterobacter cloacae* (Koga et al., 1991b), *Rhizobium phaseoli* (Ernstsen et al., 1987), *Bradyrhizobium japonicum* (Kaneshiro et al., 1983), *Pseudomonas putida* (Patten, 1996), *Erwinia herbicola* (Brandl and Lindow, 1996) and *Azospirillum brasilense* (Barbieri et al., 1986; Omay et al., 1993). In contrast, genes involved in the indoleacetamide pathway are transcribed constitutively in *Pseudomonas syringae* pv. *savastanoi* (Gaffney et al., 1990) and *Pseudomonas syringae* pv. *syringae* (Fett et al., 1987).

In addition to tryptophan, other amino acids are able to induce bacterial IAA synthesis. Of the amino acids detected in the root exudates of 15 different plants, alanine, lysine and asparagine were found to be the most abundant (Rovira, 1970). When bacteria isolated from the roots of lettuce were pre-grown in soil supplemented with any one of these

three amino acids, prior to addition of tryptophan, production of IAA increased (Martens and Frankenberger, 1993). Possibly by a similar mechanism, growth of *P. putida* GR12-2 in a rich medium results in substantial increases in IAA production compared with growth in minimal medium or minimal medium supplemented solely with tryptophan (Patten, 1996). This difference in the effect of rich versus minimal medium may also explain differences in the development of canola seedling roots after incubation of seeds in variously pre-treated bacterial inoculums. For example, it was found that inoculation of seeds with *P. putida* GR12-2 grown in rich medium resulted in root lengths that were on average double those from uninoculated seeds (Caron et al., 1995). Roots from canola seeds treated with the same bacterial strains, cultured in minimal medium with or without tryptophan, rarely achieved such lengths (Patten, 1996).

Few studies examining the promoters that govern production of IAA have been carried out. The promoter region for the *P. syringae* pv. *savastanoi* IAA operon, from which the constitutive expression of enzymes involved in the indoleacetamide pathway is initiated, was found to be similar to the -10 and -35 regions of the *E. coli* $\sigma^{70}$ consensus promoter (Gaffney et al., 1990). The region upstream of the indolepyruvate decarboxylase gene from *A. brasilense* contained a sequence similar to $\sigma^{54}$-dependant promoter (Costacurta et al., 1994). Vande Broek et al. (1999) fused the *A. brasilense ipdc* gene promoter to the *gus* reporter gene and found that the *ipdc* promoter is not regulated by tryptophan. Rather, expression was cell density dependent, being highest in the stationary phase, and was induced by IAA and other synthetic auxins. In addition, a sequence similar to the auxin-responsive element found in the promoters of some auxin-regulated plant genes was found in the *ipdc* promoter region (Lambrecht et al., 1999).

Brandl and Lindow (1997) fused a gene for an ice nucleation protein to the insertionally inactivated indolepyruvate decarboxylase gene in the chromosome of *E. herbicola* and examined factors that induced transcription of the latter gene by assaying for ice nucleation activity. In addition to several other factors that had no effect on promoter function, such as pH, oxygen and nitrogen availability, and variation of carbon source, addition of tryptophan did not promote reporter gene expression. Rather, in this bacterium, tryptophan appears to induce expression of tryptophan aminotransferase which catalyzes the first step in the indolepyruvic acid pathway for IAA synthesis (Brandl and Lindow, 1997). Also, because tryptophan aminotransferase prefers to convert indolepyruvic acid to tryptophan rather than the reverse reaction that yields IAA (Koga et al., 1994), excess tryptophan would be necessary to drive the reaction toward indolepyruvic acid, and subsequently IAA, production. On the other hand, tryptophan does induce the indolepyruvate decarboxylase gene promoter in *A. brasilense* Sp7 (Zimmer et al., 1998). This was assessed by replacing the *ipdc* sequence in the bacterial chromosome with the reporter gene *lacZ*, encoding β-galactosidase.

Interactions with other hormones

When considering the observations summarized in the preceeding discussion it must be borne in mind that many of the effects of auxin on plants, whether from application of synthetic or bacterial IAA, are a consequence of interactions between IAA and other phytohormones, especially ethylene and cytokinins. While it is likely that low levels of IAA enhance growth directly either by stimulating cell elongation or cell division (Theologis, 1986; Brummel and Hall, 1987), the inhibitory effects of high IAA levels are believed to be mediated by ethylene (Botella et al., 1992; Peck and Kende, 1995). The rate-limiting step in

ethylene biosynthesis, the conversion of S-adenosylmethionine (SAM) to 1-aminocyclopropane-1-carboxylic acid (ACC), is catalyzed by the enzyme ACC synthase. Expression and activity of this enzyme, and therefore ethylene production, are increased by exogenous IAA (Peck and Kende, 1995; Bekman et al., 2000).

Manipulation of the relative concentrations of auxin and cytokinin in order to induce root or shoot development in callus cultures is a longstanding practice in plant propagation (Skoog and Miller, 1957). It is now recognized that auxins and cytokinins interact to regulate a variety of physiological processes in plants and that these interactions may be antagonistic, as in the case of control of apical dominance, or synergistic, for example, in the activation of cell division (Coenen and Lomax, 1997). Furthermore, an increase in the concentration of either one of auxin or cytokinin, whether by endogenous overproduction or exogenous application, can decrease the levels of the other in plant tissues (Elköf et al., 1997). Our understanding of the effect of bacterial auxin on plants is thus further confounded by the production of cytokinins by many of these bacteria, including both tumorigenic (reviewed by Morris, 1986; Gaudin et al., 1994; Costacurta and Vanderleyden, 1995) and plant growth-promoting strains (Salamone et al., 1997).

**Objectives**

The rhizobacterium *Pseudomonas putida* GR12-2 is a strong candidate for development as a soil inoculant to enhance crop yields. Inoculation of canola, tomato, and other agriculturally important plants with this strain, results in substantial promotion of seedling root growth (Caron et al., 1995; Hall et al., 1996). This is of significant value as the early life of a plant is somewhat tenuous, and if roots could be established quickly the plant

would have an increased chance for survival. Characteristics that may contribute to the ability of *P. putida* GR12-2 to enhance plant growth include the capacity to synthesize siderophores (Hong et al., 1995), ACC deaminase (Jacobson et al., 1994; Glick et al., 1995) and IAA (Xie et al., 1996; Patten, 1996).

Prelimary evidence suggests that production of IAA by *P. putida* GR12-2 occurs via indolepyruvic acid. The lack of growth inhibition in the presence of the tryptophan analogue, α-methyltryptophan, whose toxicity seems to require tryptophan monooxygenase activity (Costacurta and Vanderleyden, 1995), and the inducibility of IAA by tryptophan (Patten, 1996), indirectly suggest that IAA is not synthesized via the indoleacetamide pathway. More direct support for this hypothesis can be found in Southern blots containing *P. putida* GR12-2 genomic DNA which indicate that sequences complementary to the genes encoding enzymes for the indoleacetamide pathway from *P. syringae* pv. *savastanoi* are not present, while sequences similar to the *ipdc* gene encoding indolepyruvate decarboxylase from the indolepyruvic acid pathway in *E. cloacae* FERM BP-1529 were detected (Patten, 1996).

The goal of the research described herein was to understand at the molecular level how IAA is synthesized by the PGPR *P. putida* GR12-2. The IAA biosynthesis pathway was confirmed by isolating the indolepyruvate decarboxylase gene (*ipdc*). The enzyme encoded by this gene catalyzes a key step in the indolepyruvic acid pathway and was chosen over the gene for tryptophan aminotransferase as the latter is neither specific for IAA synthesis nor does it prefer to catabolize tryptophan, the initial substrate in the pathway. In addition, it is likely that several genes for aromatic amino acid aminotransferases exist in a single bacterium. The *ipdc* promoter region was also isolated and analyzed in order to gain insight into how IAA synthesis is regulated in this bacterium.

The role of bacterial IAA in the mechanism by which *P. putida* GR12-2 influences its host plant was also investigated. By analogy to application of exogenous IAA, the level of IAA secreted by a bacterium may be important to the outcome of the interaction between a plant and a bacterium. A small window of IAA concentration positively influences plant growth, while levels below this range have no effect and levels above are inhibitory. To test this hypothesis, an IAA-deficient mutant of *P. putida* GR12-2 was created and characterized. Morphological changes in plants as a consequence of their interaction with this mutant were documented and compared to the effects of the wild-type strain.

# MATERIALS AND METHODS

## Bacterial Strains

*Pseudomonas putida* GR12-2 (Lifshitz et al., 1987) was kindly provided by Dr. G. Brown of Agrium, Inc. (Saskatoon, Sask.). *Escherichia coli* strains DH5α (Hanahan, 1983), S17.1 (Simon et al., 1983), and JM109 (Yanisch-Perron et al., 1985) were used as recipients for recombinant plasmids.

*P. putida* GR12-2 was routinely maintained in 3.6% (w/v) Tryptic Soy Broth (TSB; Difco), containing 1.8% (w/v) technical agar (Difco) as a solidifying agent when needed. The DF salts minimal medium of Dworkin and Foster (1958), used for propagation of *P. putida* GR12-2 where indicated, consisted of: $KH_2SO_4$, 4 g; $Na_2HPO_4$, 6 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $(NH_4)_2SO_4$, 2 g; D-glucose, 2 g; D-gluconic acid, potassium salt, 2 g; citric acid, monohydrate, 2 g; and trace elements, including $FeSO_4 \cdot 7H_2O$, 1 mg; $H_3BO_4$, 10 μg; $MnSO_4$, 10 μg; $ZnSO_4$, 70 μg; $CuSO_4$, 50 μg; and $MoO_3$, 10 μg; in 1 L of distilled $H_2O$. *E. coli* strains were grown in either 2% (w/v) Luria Broth (LB; Difco), or M9 minimal media (5X M9 salts ($Na_2HPO_4 \cdot 7H_2O$, 64 g; $KH_2PO_4$, 15 g; NaCl, 2.5 g; $NH_4Cl$, 5g; in 1 L of distilled, deionized water), 200 mL; 20% (w/v) glucose, 20 mL; in 1 L of distilled, deionized water).

## Isolation of the *ipdc* Gene

### Isolation of genomic DNA

Genomic DNA was isolated from bacterial cells by the method described by Sambrook et al. (1989). From a 5 mL overnight culture in TSB, 100 μL of *P. putida* GR12-2 were transferred into 50 mL TSB and incubated for an additional 20 h at 25°C in a shaking

water bath. Cells were collected by centrifugation in a Sorvall GSA rotor for 15 min at 5,000 x *g* and 4°C, washed once with TE buffer (10 mM Tris (tri(hydroxymethyl)aminomethane)·Cl; 1 mM EDTA (ethylenediaminetetraacetic acid); pH 8.0). Following resuspension in 20 mL TE buffer, cells were lysed with 1 mL of 20% (w/v) SDS (sodium dodecyl sulfate), pH 7.2, for 20 min at room temperature. DNA was then precipitated by addition of 2.4 mL of 3.0 M sodium acetate, pH 8.0, and 13 mL isopropanol, collected by spooling on a glass rod, and resuspended in 10 mL TE buffer. Proteinase K (Roche Diagnostics) was added to a final concentration of 50 μg/mL and incubated overnight at 37°C to digest any protein present. RNA was hydrolyzed with RNase A (Roche Diagnostics; 50 μg/mL). To purify the DNA, sequential extractions were carried out once each with equal volumes of phenol (equilibrated with 0.1 M Tris·Cl, pH. 8.0), phenol:chloroform:isoamylalcohol (24:24:1), and chloroform:isoamylalcohol (24:1). For each extraction the solution was mixed thoroughly by inverting slowly many times and then the aqueous phase was carefully collected after centrifugation (17,000 x *g* for 20 min at 4°C). The DNA pellet required 1.8 mL TE buffer for complete resuspension. The DNA was aliquoted into smaller volumes and stored at –20°C until needed.

Preparation of a clone bank

*P. putida* GR12-2 genomic DNA (0.5 – 1 μg) was digested with 0.125 U of *Sau*3AI (Gibco BRL) in 1X One-Phor-All™ Buffer (OPA; Pharmacia) restriction enzyme digestion buffer for 20, 30, 40 and 50 min at 37°C. The reaction was terminated by incubation at 65°C for 20 min and then stored on ice to prevent reactivation of the restriction enzyme. Half (10 μL) of the products from each digestion were run on a 0.8% agarose gel containing 0.5 μg/mL ethidium bromide in TBE buffer (45 mM Tris; 45 mM boric acid; 1 mM EDTA,

pH 8.0) for approximately 2 h at 80 V. Visualization under UV light revealed that the majority of products from the 30 min digestion were between 4 and 9 kb, large enough to carry a full length *ipdc* gene expected to be approximately 1.7 kb. Therefore, the remaining 30 min digestion products were used for subsequent ligation with pUC18.

Ten μL of the digested genomic fragments were mixed with 3 μL (150 ng) pUC18 (pre-cut with *Bam*HI and treated with alkaline phosphatase; Pharmacia), 1 μL T4 DNA ligase (400 U; New England Biolabs) and 1.7 μL 10X ligation buffer, and then incubated overnight at 14°C. Calcium chloride-competent *E. coli* DH5α cells were prepared for transformation with the recombinant plasmids as follows (Sambrook et al., 1989). Five mL LB medium were inoculated with a loopful of *E. coli* DH5α and incubated overnight in a 37°C shaking water bath. The next day, 300 μL of the overnight culture were transferred to 30 mL pre-warmed (37°C) LB medium and incubated again in a 37°C water bath until the culture reached an optical density at 600 nm of approximately 0.2. At this point, the culture was transferred to an ice-cold centrifuge tube and chilled on ice for 10 min. The cells were collected by centrifugation in a Sorvall centrifuge using an SS34 rotor at 5,000 rpm for 10 min. After decanting the supernatant, the cells were resuspended in 15 mL ice-cold 100 mM $CaCl_2$, stored on ice for 30 min, and then centrifuged again as above to collect the cells. The cells were finally resuspended in 3 mL ice-cold 100 mM $CaCl_2$.

After combining 200 μL of competent cells with 10 μL of ligation products, the mixture was stored on ice for 30 min and then the cells were treated with a mild heat shock (42°C) for 2 min. To allow the cells to recover and express the ampicillin resistance gene encoded on the plasmid, 1 mL LB medium was added and the culture was incubated at 37°C for 1 h. Serial dilutions ($10^{-1}$ to $10^{-4}$) were prepared in LB medium and 100 μL of each were

spread on LB agar containing ampicillin (100 μg/mL) for selection of transformed cells. For colony hybridization, 80 μL of undiluted cells were plated onto each of three LB plus ampicillin plates to generate approximately 1,000 CFU per plate.

Colony lifts

Bacterial colonies carrying the *P. putida* GR12-2 genomic library were transferred from agar plates to nylon membranes in order to screen for the clone carrying the indolepyruvate decarboxylase (*ipdc*) gene. Hybond™-N nylon membrane disks were cut just smaller than the plate, wetted with distilled, deionized water, and autoclaved between two pieces of Whatman 3MM paper wrapped in aluminium foil. Under aseptic conditions, a disk was carefully placed onto the surface of pre-cooled (4°C, 30 min) colonies on an agar plate, being careful to avoid air bubbles and to avoid moving the membrane once it had been applied. The membrane was marked in three places using a pencil in order to later orient the membrane to the plate to identify positive colonies. After approximately 1 min, the membrane was carefully removed from the agar plate and blotted briefly, colony side up, on dry Whatman 3 MM paper. Keeping the colony side up, the membrane was placed for 15 min on two layers of Whatman 3 MM paper pre-soaked in denaturation solution (0.5 N NaOH; 1.5 M NaCl; prepared fresh). The membrane was then transferred to two layers of Whatman 3 MM paper pre-soaked in neutralization solution (1.0 M Tris-HCl, pH 7.5; 1.5 M NaCl) for 15 min, and finally, to two layers of Whatman 3 MM paper saturated with 2X SSC buffer (prepared from 20X SSC (0.3 mM NaCl; 30 mM sodium citrate, pH 7.0)) for 10 min. The membrane was then placed colony side up on a piece of dry Whatman 3 MM paper and, while still damp, exposed to 150 mJoules of UV light in a GS Genelinker™ UV chamber (Bio-Rad) to fix the DNA to the membrane.

Cellular protein was digested by pipetting 750 µL of proteinase K (2 mg/mL in 2X SSC buffer) over the entire surface of each membrane, which were then wrapped in plastic wrap and incubated at 37°C for 1 h. To remove cellular debris, each membrane was blotted with a piece of Whatman 3 MM paper, fully wetted with distilled, deionized water, by applying pressure by rolling a bottle across the paper. This process was repeated several times, using fresh pieces of wetted 3 MM paper, until debris appeared to be completely removed.

Isolation and purification of probe fragment

The *ipdc* gene isolated from *Enterobacter cloacae* BP FERM-1529 (Koga et al., 1991) was used as a DNA hybridization probe and was kindly provided by Dr. Jinichiro Koga of Meiji Seika Kaisha, Ltd. (Saitama, Japan) on plasmid pIP27 as a 2.0 kb *Bam*HI fragment. This plasmid was maintained in *E. coli* DH5α following $CaCl_2$-mediated transformation and selection on LB plus ampicillin (100 µg/mL).

Approximately 15 µg of plasmid pIP27 DNA were digested simultaneously with *Pst*I (15 U; Gibco BRL) and *Bam*HI (15 U; Gibco BRL) in a final volume of 60 µL 1X OPA buffer at 37°C for 1 h. The digestion reaction was stopped with 2 µL 0.5 M EDTA and 12 µL of gel loading buffer (50% glycerol; 0.25% bromophenol blue; in distilled water). The products of two such digestions were combined, yielding a total volume of approximately 150 µL. Fifty µL were then loaded into each of three lanes in an 11 x 14 cm 0.7% low-melting-temperature agarose (Gibco BRL) gel and electrophoresed at 80 V for approximately 2.5 h at 4°C. While illuminated under UV light, the 1.7 kb *Pst*I-*Bam*HI fragments were cut from the gel using a scalpel and combined in a 12 mL plastic tube.

Gene Clean II®, a DNA purification kit purchased from Bio101, Inc. (La Jolla, CA,

USA), was used to purify the probe fragments from the surrounding agarose gel. The tube containing the probe fragments was incubated in a 55°C water bath until the agarose gel was melted, and then 0.5 volumes of TBE modifier™ and 4.5 volumes of NaI were added and the tubes were returned to the water bath for an additional 5 min. Ten µL of GLASSMILK® were mixed with the DNA solution and stored on ice, with periodic mixing, for 5 min to allow the DNA to bind to the silica matrix. The DNA/GLASSMILK® composite was recovered by pelleting in an IEC HN-S centrifuge at approximately 1,500 x *g* for 5 sec at 4°C, and the resulting pellet was washed by resuspension in 500 µL of New Wash buffer (a specially formulated solution containing NaCl, Tris, EDTA and ethanol; prepared by Biol 101, Inc.) followed by recentrifugation. A second resuspension in 500 µL of New Wash buffer was transferred to a 1.5 mL microcentrifuge tube before continuing with the next centrifugation step.

After a total of three washes in New Wash buffer, DNA was eluted into 5 µL of TE buffer by incubating in a 55°C water bath for 3 min. A brief spin at room temperature for 30 sec in an Eppendorf centrifuge separated the GLASSMILK® particles from the DNA, and the supernatant containing the DNA was transferred to a new microcentrifuge tube. Another 5 µL aliquot of TE buffer was added to the remaining pellet to extract any residual DNA and after centrifugation the supernatant was combined with that from the first extraction.

Labeling the probe

The protocol recommended by Boehringer Mannheim (now Roche Diagnostics) in their DIG DNA Labeling and Detection Kit was followed for tagging the purified *Pst*I-*Bam*HI probe fragment with digoxigenin (DIG). A 10 µL aliquot of template DNA (the 1.7 kb *Pst*I-*Bam*HI *ipdc* fragment prepared above) was diluted to a total volume of 15 µL

with TE buffer, and then denatured by boiling for 10 min in a water bath and immediately chilling in ice-water. While on ice, 2 μL of hexanucleotide reaction mix, 2 μL of dNTP labeling mixture (1 mM dATP; 1 mM dCTP; 1 mM dGTP; 0.65 mM dTTP; 0.35 mM DIG-11-dUTP (digoxygenin linked to the deoxyuridine nucleotide via a carbon chain); pH 7.5) and 1 μL of Klenow enzyme (2 U/μL; labeling grade from *E. coli*) were added and gently mixed. Incorporation of DIG molecules into the newly synthesized complementary DNA strands continued for 16 hours in a 37°C water bath and then the reaction was stopped with 1 μL of 0.5 M EDTA. Labeled DNA was collected by adding 2.5 μL of 4 M LiCl and 75 μL of ice-cold absolute ethanol and leaving the mixture for 30 min at –70°C before microcentrifuging for 15 min at 4°C. After a wash with 50 μL of ice-cold 70% ethanol, and a 10 min spin at 4°C, the pellet was dried under vacuum. Subsequently, the DNA was dissolved in 20 μl of TE buffer containing 0.1 % SDS by heating to 37°C for 5 min to help dissolve the somewhat insoluble DIG molecules.

To estimate probe yield, DIG-labeled, linearized pBR328 (control DNA supplied with the DIG DNA Labeling and Detection kit) of known concentration was used for comparison. Control DNA was sequentially diluted to the following concentrations (pg/μL): 1000, 100, 10, 1, 0.1, and 1 μL of each dilution was spotted onto a small piece of Hybond™-N nylon membrane. A 10-fold serial dilution in TE buffer (2 μl in 20 μL) was also prepared for the labeled probe to a maximum dilution of 1:10,000. Aliquots of 1 μL from each dilution were applied and then fixed to the membrane by exposure to 150 mJoules of UV light. The membrane was immersed briefly in 20 mL of maleic acid buffer (0.1 M maleic acid; 0.15 M NaCl; pH 7.5) and then incubated in 20 mL blocking buffer (1% w/v blocking reagent consisting of proteolytic fragments of casein dissolved in maleic acid buffer) for 10 min at

room temperature with gentle agitation (60 rpm). The blocking solution was replaced with 20 mL of blocking buffer containing sheep anti-DIG Fab fragments conjugated to alkaline phosphatase (diluted 1:10,000 from a 750 U/mL stock), incubated as above for 10 min, and then washed with 20 mL maleic acid buffer containing 0.3% (v/v) Tween® 20 for 10 min. The membrane was transferred to a 4" x 6" KAPAK heat sealable pouch (Fisher Scientific) containing 20 mL maleic acid buffer for a second 10 min wash. To adjust the pH to a level optimal for the anti-DIG/alkaline phosphatase detection reaction, the membrane was soaked for 5 min in 20 mL detection buffer (0.1 M Tris; 0.1 M NaCl; 50 mM $MgCl_2$; pH 9.5) prior to addition of the color development solution, which was prepared fresh by adding 45 µL of nitro blue tetrazolium salt (NBT; 75 mg/mL in 70% dimethylformamide) and 35 µL of 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt (BCIP, also known as X-phosphate; 50 mg/mL in 100% dimethylformamide) to 10 mL of detection buffer. The color was developed in the dark for 15 min and the probe concentration was estimated by comparing spot intensities with those for the labeled control DNA dilutions.

Hybridization

To prevent non-specific binding of the probes, three membranes were placed in a 6.5" x 8" KAPAK heat sealable bag and incubated in 60 mL prehybridization solution (5X SSC buffer (prepared from 20X SSC: 3 M NaCl; 0.3 M sodium citrate, pH 7.0); 0.1% (w/v) N-laurylsarcosine; 0.02% (w/v) SDS; 1% (w/v) blocking reagent dissolved in maleic acid; pre-warmed to 68°C) for 2 h with gentle agitation (80 rpm) at 68°C. The DIG-labeled *ipdc* probe, described above, was prepared for hyridization by boiling for 10 min, immediately immersing into ice-water, and then diluting into 5 mL of pre-warmed (68°C) prehybridization buffer for a final concentration of approximately 3.5 ng/mL. Where probes were re-used,

they were boiled for 10 min, rapidly cooled in ice-water, and then added directly to the membranes. The membranes were incubated in the buffer containing the probe for 17 h at 68°C while shaking gently.

After hybridization, the probe solution was poured off into a 15 mL polypropylene tube and stored at –20°C for subsequent re-use. The membranes were subjected to a series of high stringency washes as follows: two washes with 60 mL 2X SSC/0.1% SDS at room temperature for 5 min, followed by two washes with 60 mL 0.5X SSC/0.1% SDS at 68°C for 15 min.

Detection of probe hybridization

Binding of DIG-labeled probes to complementary sequences on the membranes was detected immunologically using anti-DIG antibodies coupled to an enzyme that, in the presence of an appropriate substrate, yields a characteristic colorimetric response. After the washes indicated above, residual SDS was removed from the membranes by briefly rinsing them in 50 mL maleic acid buffer. To prevent non-specific binding of the antibody, the membranes were blocked with 50 mL 1% (w/v) blocking reagent in maleic acid buffer in a new KAPAK pouch for 1 h at room temperature while rotating at 60 rpm. The blocking buffer was replaced with a 1:5,000 dilution of anti-DIG-alkaline phosphatase conjugate, prepared immediately before use by diluting 8 µL stock antibody (750 U/mL) in 40 mL blocking buffer, and the membranes were gently agitated for 30 min at room temperature. Two washes of 15 min each in 50 mL maleic acid buffer containing 0.3% (v/v) Tween® 20 followed; before each wash the membranes were transferred to a new Petri dish.

The membranes were equilibrated to pH 9.5 in detection buffer (described above) for 5 min before addition of 4 mL color substrate solution (prepared by diluting 54 µL NBT and

42 μL BCIP stock solutions in 12 mL detection buffer) to each membrane separately in a heat sealable bag. The color was developed in the dark for 30-45 min until spots of sufficient intensity were observed, at which time the reaction was terminated by washing the membranes for 5 min with 50 mL water. Results were documented by photography.

Isolation of plasmids from positive clones

Plasmids were routinely isolated from their host *E. coli* cells by modification of the alkaline lysis method described by Sambrook et al. (1989) and subsequent purification using polyethylene glycol (PEG). Five mL LB containing an appropriate antibiotic (e.g., ampicillin, 100 μg/mL; tetracycline, 10 μg/mL; kanamycin, 50 μg/mL) were inoculated with a single bacterial colony and grown overnight in a 37°C water bath with gentle shaking (300 rpm). The next morning, cells were collected from 1.5 mL of overnight culture by centrifugation at full speed in an Eppendorf Model 5414 microcentrifuge for 1 min at room temperature. After decanting the supernatant, another 1.5 mL aliquot of overnight culture was added to the same tube and again centrifuged as above. In this way, cells from a total of 4.5 mL of culture were collected in a single microcentrifuge tube. After the final centrifugation, the pellet was resuspended in 200 μL of GTE buffer (50 mM glucose; 25 mM Tris·Cl, pH 8.0; 10 mM EDTA, pH 8.0) and then lyzed with 300 μL of freshly prepared 0.2 M NaOH plus 1% SDS by gently mixing and incubating on ice for 5 min. The solution was neutralized with 300 μL of 3.0 M potassium acetate, pH 4.8, mixed by inversion, and again placed on ice for 5 min. Plasmid DNA was separated from cellular debris by spinning at full speed in an Eppendorf microcentrifuge for 10 min at room temperature. The supernatant was transferred to a 1.5 mL screw cap microfuge tube and treated with RNase A (20 μg/mL) for 20 min at 37°C, after which the DNA was extracted at least twice with

phenol:chloroform:isoamylalcohol (24:24:1). Each time the solution was mixed well by inverting the tube several times and the phases separated by microcentrifugation at room temperature for 1 min. The plasmid DNA was collected by precipitation for 1 h at –20°C with an equal volume of 100% isopropanol followed by centrifugation at 4°C for 15 min. The resultant pellet was rinsed with 500 µL of 70% ethanol, air dried, and then resuspended in 32 µL of distilled, deionized water. The plasmid DNA was purified by precipitation with 8.0 µL of 4 M NaCl and 40 µL of 13% PEG 4000 on ice for 20 min and then collected by centrifugation for 15 min at 4°C. Once again the pellet was rinsed with 500 µL of 70% ethanol and air dried, this time resuspending in 20 µL of water. Plasmid DNA was stored at -20°C until required.

Quantification of DNA preparations

To estimate the quantity of DNA present, 0.5 µL of undiluted and diluted (1:10) plasmid preparation were spotted onto the surface of a small piece of 1% agarose containing 0.5 µg/mL ethidium bromide. Around these were also spotted 0.5 µL aliquots of pBR328 DNA (Roche Diagnostics) of the following known concentrations (ng/µL): 200, 150, 100, 50, and 25. After allowing the DNA to bind to the ethidium bromide for 1 h at room temperature, the samples were viewed under UV light and the intensity of fluorescence of the samples of unknown concentration were compared to the intensities of the DNA of known concentration.

Analysis of plasmids by restriction enzyme digestion

The insert in the plasmid from the positive clone was mapped by restriction enzyme digestion. Approximately 200 ng of plasmid DNA were digested with each of the following

restriction endonucleases, either alone for single digestions or with a combination of two restriction endonucleases for double digestions: *Bam*HI (Gibco BRL), *Eco*RI (Roche Diagnostics), *Hin*dIII (Gibco BRL) and *Sca*I (Roche Diagnostics). Plasmid DNA was added to a volume of sterile distilled water calculated such that the final reaction volume, after addition of all reagents, would be 20 μL. Two μL of appropriate restriction enzyme digestion buffer (from a 10X stock) were added, followed by 5 U of restriction enzyme. The mixtures were incubated in a 37°C water bath for 1 h or overnight and then the reaction was stopped by addition of 0.5 M EDTA to a final concentration of 10 mM.

Fragment sizes were determined by comparison of migration distance with known DNA markers (usually bacteriophage λ DNA digested with *Hin*dIII (MBI Fermentas) or 1 kb ladder (MBI Fermentas)) following agarose gel electrophoresis. Four μL of loading buffer was mixed with 20 μL of digestion mix, and 12 μL was loaded into each well of a 0.7–1.0 % (depending on the expected fragment size) agarose gel prepared in 0.5X TBE buffer. Plasmid DNA was generally electrophoresced at 80-120 V. Staining with ethidium bromide was either through addition of 0.5 μg/mL ethidium bromide to the agarose gel or after electrophoresis. In the latter case, 10 μL of ethidium bromide (10 mg/mL) were added to 120 mL TE buffer in a large disposable weigh boat. The gel was carefully immersed in this solution for 10 min and then rinsed several times with water to remove residual stain.

Analysis of plasmids by Southern hybridization

Plasmid DNA from the positive clone was digested with *Bam*HI and *Hin*dIII as described above and then transferred to a nylon membrane by capillary action (Southern, 1975; Sambrook et al., 1989). After electrophoresis in a 0.8% agarose gel, the fragmented plasmid DNA was denatured to its single stranded from by submersing the gel in 1.5 M

NaCl, 0.5 M NaOH for 45 min while gently agitating on an orbital shaker (Lab-line) at 60 rpm. The gel was then neutralized by soaking in 1M Tris, pH 8.0, and 1.5 M NaCl for 45 min at room temperature, again with gentle shaking; the neutralization solution was changed once after 30 min.

A support for the gel was fashioned by inverting a gel casting tray, wrapped with a piece of Whatman 3MM paper, in a glass baking dish. Transfer buffer (20X SSC) was poured into the reservoir dish until the level of the buffer was just below the top of the support. After allowing the 3MM paper to become saturated with the transfer buffer, and removing any air bubbles present under the paper by rolling a test tube over the surface, the neutralized gel was inverted and placed on the wet 3MM paper in the center of the support. A piece of Hybond™-N nylon membrane (Amersham Life Sciences), cut just larger than the gel and wetted first in distilled water before saturating with 20X SSC, was positioned carefully over the gel and then overlaid with two pieces of 3MM paper, cut to the same size as the gel and soaked in 2X SSC. The orientation of the nylon membrane with respect to the gel was marked by clipping the bottom left hand corner from both. Each time a layer of this assemblage was added, trapped air bubbles were displaced by carefully rolling a test tube across the uppermost layer. An 8 cm stack of paper towels, cut only slightly smaller than the 3MM paper covering the membrane, was placed on top of the 3MM papers to draw the transfer buffer up through the gel and membrane. The whole apparatus was covered in plastic wrap to minimize evaporation and then topped with a glass plate and weighted down with a weight of at least 500 g.

Following transfer of the DNA for 19 h at room temperature, the nylon membrane was retrieved (the positions of the gel slots were marked with a pencil) and soaked in

6X SSC for 5 min at room temperature. The membrane was then removed from this solution, placed DNA side up on a piece of dry 3MM paper and, while still damp, exposed to 150 mJoules of UV light in a GS Genelinker™ UV chamber to fix the DNA to the membrane. The air-dried membrane was stored between two sheets of 3MM paper in an envelope at room temperature.

Hybridization with the recycled *ipdc* probe from the colony hybridization, and detection of bound probe were performed as described above for colony hybridization; volumes of the various buffers were adjusted to levels better suited to a smaller membrane, usually 20 mL. For detecting probes bound to Southern blots, membranes were blocked with blocking reagent overnight prior to addition of anti-DIG antibodies, rather than for 1 h as for detection of bound probes following colony hybridization. In addition, a more dilute concentration of anti-DIG-alkaline phosphate conjugate was used, 1:5,000 for Southern blots compared 1:10,000 for colony blots. Generally, a signal of sufficient intensity, indicating hybridization of the probe to a complementary sequence, was seen after incubation of the membrane in the dark for 15-30 min.

Sequence analysis of insert from positive clone

Restriction enzyme digestion and Southern hybridization of the *Bam*HI-digested 2.7 kb insert in the plasmid from the positive clone revealed that the *ipdc* gene was carried on a 1.1 kb *Bam*HI fragment. This fragment was therefore subcloned into the *Bam*HI site of pUC18 and introduced into *E. coli* DH5α. Restriction enzyme digestion of the plasmid, purification of the *ipdc* fragment from an agarose gel by Gene Clean II®, ligation with pUC18, and preparation and transformation of $CaCl_2$-competent cells were as described above. Colonies carrying plasmid pUC18 with an insert were selected by insertional

inactivation of the *E. coli lacZ* gene on pUC18 (Sambrook et al., 1989). To a pre-made LB agar plate containing an appropriate antibiotic, 40 μL of a stock solution of X-Gal (20 mg/mL in dimethylformamide; MBI Fermentas) and 4 μL of a stock solution of isopropylthio-β-D-galactoside (IPTG; 200 mg/mL in water; Bio-Rad) were added and spread over the entire surface using a sterile glass spreader. The plate was incubated at 37°C for approximately 3 h in order to allow absorption of the X-Gal and IPTG, and evaporation of the dimethylformamide. After inoculating the plate with 100 μL of transformation mixture and incubating overnight at 37°C, both white and blue colonies were seen on the plate; storing the plate for several hours at 4°C allowed the blue color to develop fully. White colonies indicated plasmids with the *lacZ* gene disrupted by an inserted fragment (designated *E. coli* DH5α/pUCIPDC), while blue colonies contained a functional *lacZ* gene and therefore active β-galactosidase capable of hydrolyzing X-Gal.

In order to obtain enough plasmid DNA in a purified form required for sequencing of the inserted fragment, plasmid DNA was isolated using a large-scale alkaline lysis method combined with a polyethylene glycol purification step outlined by Sambrook et al. (1989). A culture of *E. coli* DH5α/pUCIPDC was grown overnight from a single colony in 5 mL LB plus ampicillin in a shaking water bath at 37°C. From this culture, 2 mL was inoculated into 500 mL of pre-warmed (37°C) LB containing ampicillin and again incubated overnight in a shaking (300 rpm) water bath at 37°C. Bacterial cells were harvested by centrifugation in a Sorvall GSA rotor at 5,000 x *g* for 15 min at 4°C and resuspended in 100 mL of ice-cold STE buffer (0.1 M NaCl; 10 mM Tris·Cl, pH 8.0; 1 mM EDTA, pH 8.0). Cells were again collected by centrifugation as described above and this time resuspended in 10 mL of GTE buffer. Bacterial lysis was accomplished by addition of 1 mL freshly prepared lysozyme

(10 mg/mL in 10 mM Tris·Cl, pH 8.0; Roche Diagnostics), followed by 20 mL of 0.2 N NaOH/1% SDS, again prepared fresh. Several inversions of the tube allowed the contents to mix gently and the solution was then left at room temperature for 10 min. Following this, 15 mL of ice-cold 3.0 M potassium acetate, pH 4.8, was added and the mixture stored on ice for 10 min. Cellular debris was removed from the bacterial lysate by centrifugation in a Sorvall GSA rotor at 2,600 x *g* and 4°C for 15 min. The supernatant was passed through four layers of sterile cheesecloth, a 0.6 volume of 100% isopropanol was added to the filtrate, and DNA allowed to precipitate for 1 h at room temperature. The plasmid DNA was collected by centrifugation in a Sorvall SS34 rotor (3,000 x *g*, 4°C, 20 min), rinsed with 70% ethanol and air dried before dissolving in 3 mL TE buffer.

High molecular weight RNA was removed from the plasmid solution by precipitation with 3 mL of ice-cold 5 M LiCl in a 15 mL Corex™ tube followed by centrifugation in a Sorvall SS34 rotor at 12,000 x *g* for 10 min at 4°C. DNA was recovered, after decanting the supernatant into a clean tube, by addition of an equal volume of 100% isopropanol, precipitating at room temperature for 15 min and then centrifuging as described above. The pellet was rinsed with 70% ethanol and allowed to air dry for several minutes before dissolving in 320 µL TE buffer containing 20 µg/mL RNase A for 30 min at room temperature. The DNA was precipitated with 80 µL of 4 M NaCl plus 400 µL of 13% PEG 8000 (BDH) on ice for 20 min and then collected by centrifugation (full speed in Eppendorf Model 5414) for 5 min at 4°C. After dissolving the pellet in 400 µL of TE buffer, the solution was extracted once each with phenol, phenol:chloroform:isoamylalcohol, and chloroform:isoamylalcohol as previously described. DNA was again recovered from the aqueous phase by addition of 0.1 volume of 3 M sodium acetate, pH 8.0, and 2 volumes of

ice-cold 100% ethanol, storing at room temperature for 30 min, and centrifuging at full speed for 5 min at 4°C. The resulting pellet was rinsed with 200 μL of 70% ethanol, dried under vacuum for 10 min, and finally resuspended in 50 μL of TE buffer.

Because the plasmid preparation was found to be contaminated with residual RNA that might affect sequencing, the DNA preparation was again treated with lithium chloride to remove RNA (Sambrook et al., 1989). To the plasmid DNA, 300 μL of ice-cold 4 M LiCl was added and left at room temperature for 30 min. The mixture was then centrifuged at 13,000 rpm for 10 min at 4°C in a microcentrifuge, and the supernatant removed. The pellet was rinsed once with 70% ethanol, recentrifuged as above, dried, and finally resuspended in 20 μL of water.

Six μg of plasmid DNA dissolved in 30 μL of sterile, distilled, deionized water were sent to MOBIX (McMaster University, Hamilton, Ontario) for sequencing of the insert. M13 universal forward and reverse primers were used to sequence approximately 400 bp of the 5' end of each of the coding and non-coding strands. The initial sequence was analyzed for similarity to the probe *ipdc* sequence from *E. cloacae* FERM BP-1529 using ALIGN, an algorithm that aligns and compares two sequences, that can be found at the website for GeneStream (http://vega.igh.cnrs.fr).

Polymerase Chain Reaction

The following PCR primers were designed from the published *E. cloacae* FERM BP-1529 *ipdc* gene (Koga et al., 1991a) to span the entire open reading frame: forward primer, 5' GAAGGATCCCTGTTATGCGAACC 3'; reverse primer, 5' CTGGGGATCCGACAAGT-AATCAGGC 3' (MOBIX, McMaster University, Hamilton, Ontario). A *Bam*HI restriction site (underlined) was incorporated into the 5' end of both the forward and reverse primers in

order to facilitate subsequent subcloning of PCR products. These primers were used to amplify the *ipdc* gene from lysed cells of *P. putida* GR12-2 and from purified *P. putida* GR12-2 genomic DNA. The PCR reaction was also performed using pIP27 (that carries the *ipdc* sequence from *E. cloacae* FERM BP-1529) and water as templates, for a positive and negative control, respectively.

On ice, in a sterile 0.5 mL microcentrifuge tube, the following were mixed: dNTPs, 200 μM each (5 μL of a 2 mM stock; Roche Diagnostics); forward and reverse primers, 300 nM each (5 μL each from a 3 μM stock in 0.1 TE buffer, pH 8.0); template DNA, 100 ng genomic DNA or 100 pg plasmid DNA; and sterile water to a volume of 25 μL. The polymerase was prepared in a separate tube, on ice, by adding 3.5 U of Expand High Fidelity PCR System (Roche Diagnostics), containing a mixture of Taq polymerase and Pwo polymerase, to Expand High Fidelity PCR buffer with 15 mM $MgCl_2$ diluted to a 1X concentration in sterile water.

Twenty-five μL of the polymerase mixture were added to each of the tubes containing template DNA, mixed briefly by vortexing, and then centrifuged for a few seconds at 4°C to collect droplets from the side of the tube. The reaction mixture was overlaid with 30 μL of mineral oil (white, light; Mallinckrodt) to prevent evaporation, and incubated in a Perkin Elmer Cetus DNA Thermal Cycler as follows: one cycle of 94°C for 5 min; 30 cycles of 94°C for 30 s, 50°C for 30 s, 68°C for 2 min; and one cycle of 68°C for 2 min. PCR products were visualized by adding 3 μL of loading buffer to 15 μL of PCR reaction mixture and running on a 0.9% agarose gel, as described above.

Where whole cells were used as a template for PCR, the same procedure was followed except that all reagents, including the enzyme solution, were mixed on ice prior to

adding the template. Using a sterile toothpick, a small amount of a bacterial colony was aseptically transferred from an agar plate directly into the PCR reaction mixture. Tween® 20 (from a freshly prepared 10% v/v stock solution) was added to a final concentration of 0.5% (v/v) before the mixture was overlaid with mineral oil and incubated using the same cycles described above for purified DNA templates.

Sequence analysis of PCR products

PCR products were purified for sequencing by extraction with phenol:chloroform. The solution containing the PCR products was removed from below the mineral oil layer using a pipette and transferred to a clean 1.5 mL microfuge tube. Ten μL of 3 M sodium acetate, pH 8.0, was added followed by sterile water to a final volume of 100 μL. DNA was extracted twice with 100 μL of phenol:chloroform, precipitated with an equal volume of isopropanol and rinsed with 70% ethanol before final resuspension in 10 μL of sterile water. After quantification of DNA using the agarose plate method outlined above, the PCR products were ligated with pUC18 following digestion of both with *Bam*HI to generate pIPD2, and then transformed into *E. coli* DH5α as previously described. PCR products were also diluted to a final concentration of 500 ng in 20 μL of water and sent to MOBIX (McMaster University, Hamilton, Ontario) for determination of the nucleotide sequence.

Primers used for the initial sequencing reactions were the same as those used to amplify the sequence via PCR. Subsequent primers were designed from the returned sequences, until the complete sequence was obtained for both the coding and non-coding strands: for the second sequencing reactions, forward primer 5' CGCTCTCACTCATA-AGCAGGC 3', reverse primer 5' CTGGGTAAGCGAACCGTCCG 3'; for the third sequencing reactions, forward primer 5' CAGGGAACATCGGCCTTCG 3', reverse primer

5' GCCACATCGGCGGGTAACATC 3' (all synthesized by MOBIX, McMaster University, Hamilton, Ontario). The sequence (GenBank accession number AF285632) was analyzed using BLAST (Altschul et al., 1997), found at the website for the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov), to find similar nucleotide sequences of known identity in the databases, and by multiple alignment with known *ipdc* sequences available from GenBank (at NCBI) using the algorithm ClustalW, available from the website of the European Bioinformatics Institute (EMBL; http://www.ebi.ac.uk).

**Isolation of the *ipdc* Gene Promoter Sequence**

Inverse PCR

Two μg of *P. putida* GR12-2 genomic DNA was digested overnight at 37°C with 5 U *Kpn*I (Gibco BRL) in a total volume of 20 μL 1X OPA buffer (Pharmacia) diluted in water. *Kpn*I was chosen for genomic digestion because it would generate fairly large genomic fragments with cohesive ends that are relatively easy to ligate, and would cut once within the *ipdc* gene, at a known site near the 3' end, to avoid isolation of the 3' flanking region. The digestion reaction was stopped by addition of 1 μL of 0.5 M EDTA, and the DNA was extracted once with phenol:chloroform and once with chloroform before addition to the final aqueous phase of 10 μL 3 mM sodium acetate, pH 8.0, and sterile water to 100 μL. Following precipitation with isopropanol and rinsing with 70% ethanol, the pellet was air-dried and resuspended in 34 μL of water.

To circularize the *Kpn*I fragments, 4 μL of 10X ligation buffer (MBI Fermentas) and 2 μL of T4 DNA ligase (4 U; MBI Fermentas) were added to 34 μL of purified digestion products. Ligation was allowed to proceed overnight at 16°C before heat inactivation of the

ligase at 65°C for 15 min.

To amplify the region upstream of the *ipdc* gene, the following PCR primers were designed to bind within the *ipdc* sequence, approximately 50 bp from each end; however, rather than amplify the *ipdc* gene, the primers would initiate DNA synthesis in the opposite direction: forward primer (in this case, complementary to the 5' end of the non-coding strand just upstream of the *Kpn*I recognition site), 5' CAAACTGGCGATGAGCAAACG 3'; reverse primer (complementary to a region near the 5' end of the coding sequence), 5' GACGGTCCAGCAGGTAATCG 3' (MOBIX, McMaster University, Hamilton, Ontario). Twenty μL of inactivated ligation mixture were mixed on ice with 5 μL of each primer (from a 3 μM stock in 0.1X TE buffer) and 5 μL dNTPs (from a 2 mM stock; Roche Diagnostics). Expand High Fidelity PCR enzyme was prepared as before but with additional $MgCl_2$ (3 μL per PCR reaction from a 25 mM stock solution; Roche Diagnostics). PCR cycles were as previously described, except that annealing of the primers to the template was performed at 53°C.

Subcloning the *ipdc* upstream sequence

Because *Taq* polymerase, a component of the Expand High Fidelity PCR System used to amplify the *ipdc* upstream sequence, generates a single deoxyadenosine overhang on the 5' ends of PCR products, subcloning of PCR products is facilitated by vectors which have compatible thymidine overhangs in their cloning sites. One such vector is pGEM®-T, available in the kit pGEM®-T Vector System (Promega). PCR products were purified by phenol:chloroform extraction as described above and resuspended in sterile water to a final concentration of 5 ng/μL. Three μL (15 ng) PCR products were ligated to 1 μL (50 ng) pGEM®-T vector using 1 μL (3 U) T4 DNA ligase (Promega) in a total volume of 10 μL of

1X Rapid Ligation Buffer (Promega) diluted in sterile water.

The reaction was incubated overnight at 4°C before transforming *E. coli* JM109 High Efficiency Competent Cells (Promega) as follows. Note that these cells were recommended and supplied with the pGEM®-T Vector System because ligation of fragments with a single base overhang is inefficient and a high transformation efficiency is necessary to obtain a reasonable number of transformed colonies. Competent cells were removed from storage at 70°C and allowed to thaw on ice for about 5 min. Fifty μL of cells were gently mixed with 2 μL of ligation mixture in a microcentrifuge tube and placed on ice for 20 min. The cells were subjected to a mild heat shock in a 42°C water bath for 45 sec and then returned to ice for 2 min. To allow the cells to recover and express antibiotic resistance genes, 950 μL of SOC medium were added and cells were incubated for 1.5 h in a 37°C water bath with gentle shaking. SOC medium was prepared as follows: Bacto-tryptone, 2g; Bacto-yeast extract, 0.5 g; 1 M NaCl, 1 mL; 1 M KCl, 0.25 mL; were combined with 97 mL distilled deionized water and autoclaved. Once cooled, 1 mL 2 M glucose (filter sterilized) and 1 mL 2 M $Mg^{2+}$ stock ($MgCl_2 \cdot 6H_2O$, 20.33 g; $MgSO_4 \cdot 7H_2O$, 24.65 g in 100 mL water, filter sterilized) were added aseptically; the final pH is 7.0. Cells (100 μL) were spread onto LB agar plates containing ampicillin, IPTG and X-Gal, prepared as previously described. Successful transformants were identified as white colonies resistance to ampicillin and confirmed to carry a plasmid of the appropriate size.

Because the products of inverse PCR included part of the *ipdc* gene sequence, the positions of restriction enzyme recognition sites within the promoter sequence relative to the *ipdc* gene were known. Thus, it was possible to determine the orientation of the promoter fragment inserted into pGEM®-T by the position of restriction enzyme recognition sites

(e.g., an *Nco*I site) in the promoter region in relation to those on the vector (e.g., *Nco*I and *Sca*I). Knowledge of the orientation in pGEM®-T was important for subsequent subcloning of the promoter fragment into the reporter vector pQF70 in the proper orientation for transcription of the *luxAB* gene carried on the vector. pGEM®-T carrying the promoter region was digested with 10 U *Sal*I in 1X Buffer H (Roche Diagnostics) for 1 h at 37°C. The digestion reaction was heat inactivated for 10 min at 65°C, and the sticky ends made blunt using the Klenow fragment of DNA polymerase I as follows. Two µL Klenow mix (5U Klenow polymerase in Klenow buffer; Promega) were added to 18 µL digestion mix and incubated at 37°C for 3 min. Then, 2 µL dNTPs (0.125 mM each, Promega) were added and the mixture again incubated, this time at 30°C for 10 min. The polymerase was inactivated by incubation at 65°C for 10 min and the mixture stored on ice until the DNA was extracted once each with phenol:chloroform and chloroform, and precipitated with isopropanol as described previously. Similarly, vector pQF70 (Farinha and Kropinski, 1990) was digested with 10 U *Hin*dIII (Roche Diagnostics) in 1X Buffer B (Roche Diagnostics) for 1 h at 37°C, and then heat inactivated and treated with Klenow polymerase as above.

The purified linear plasmids were then digested (separately) with 10 U of *Nco*I (MBI Fermentas) in 1X $Y^+$ Tango buffer (MBI Fermentas) overnight at 37°C. The 0.5 kb *Nco*I-*Sal*I promoter fragment from pGEM®-T was recovered from a 0.9% (w/v) low-melting-temperature agarose (Gibco BRL) gel and purified as follows. The slices of agarose containing the desired fragments were melted in 5 volumes of 20 mM Tris·Cl, pH 8.0, 1 mM EDTA, pH 8.0, for 5 min at 65°C. After cooling to room temperature, the DNA was extracted from the solution once each with phenol, phenol:chloroform, and chloroform, precipitated with 0.1 volume of 3 M sodium acetate, pH 8.0, and 1 volume of isopropanol as

previously described, and finally, resuspended in 10 μL water.

To insert the *ipdc* promoter into pQF70 (to create pQFPROM), 25 ng of the gel-purified *Nco*I-*Sal*I promoter fragment and 50 ng of pQF70, digested with *Hin*dIII and then *Nco*I, were mixed with 2 U T4 DNA ligase (Roche Diagnostics) in 1X ligation buffer (Roche Diagnostics) and 2 μL 50% PEG 4000 (MBI Fermentas) in a total reaction volume of 20 μL overnight at 14°C. The ligation mixture was heat inactivated at 65°C for 10 min and then used to transform $CaCl_2$-competent *E. coli* DH5α. Ampicillin-resistant transformants (selected on LB agar containing 100 μg/mL ampicillin) were assayed for LuxAB activity, driven by the *ipdc* promoter, by spotting 5 μL n-decyl aldehyde (Sigma) on the lid of the inverted agar plate on which the cells were cultured, and checking for light emission by eye in a dark room.

To verify that the region isolated by inverse PCR was indeed the region upstream of the *ipdc* gene, a 5' (forward) PCR primer was designed from the putative upstream sequence (5' CCCACATGGTATAACCCGCTTTGG 3') and a 3' (reverse) PCR primer from the 3' end of the *ipdc* structural gene (5' CTGGGGATCCGACAAGTAATCA-GGC 3') such that amplification of the *P. putida* GR12-2 genome would yield a single fragment that would include approximately 500 bp of the promoter region and the entire *ipdc* gene. To a 0.5 mL microcentrifuge tube containing Ready-to-Go® PCR beads (consisting of 1.5 U dehydrated *Taq* DNA polymerase, buffer (10 mM Tris·Cl, pH 9.0; 50 mM KCl; 1.5 mM $MgCl_2$), 200 μM each dNTP; and BSA; Amersham-Pharmacia), on ice, approximately 100 ng of genomic DNA, 300 nM of each primer, and sterile water to a total volume of 25 μL were added. After vortexing to mix well, and centrifuging for about 5 sec to collect any droplets from the sides of the tube, the mixture was overlaid with 30 μL of mineral oil and then placed

in PTC-100™ Themal Cycler (MJ Research) for amplification using the cycles previously described (Ta = 53°C). Confirmation that the amplified fragments contained the expected sequence was obtained by restriction enzyme mapping.

**Characterization of *ipdc* Promoter Activity**

Introduction of pQFPROM-Kan into *P. putida* GR12-2 by electroporation

Because *P. putida* GR12-2 is somewhat resistant to ampicillin, it was necessary to insert the gene for kanamycin resistance into pQFPROM before introducing it into *P. putida* GR12-2. To this end, the 2.3 kb *Eco*RI fragment carrying the kanamycin resistance gene was isolated from pHP45Ω-Km (Fellay et al., 1987; Prentki and Krisch, 1984) and the ends made blunt by treatment with S1 nuclease as described below. Plasmid pQFPROM (200 ng) was digested with 10 U *Pvu*II in 1X buffer M (Roche Diagnostics) overnight at 37°C, extracted twice with 100 µL phenol:chloroform, precipitated with isopropanol, and resuspended in 10 µL water. The linear plasmid (approx. 100 ng) was then ligated overnight with 100 ng of the kanamycin resistance gene with 8 U T4 DNA ligase (MBI Fermentas) and 2 µL PEG 4000. The resultant plasmid, pQFPROM-Kan, was intitially introduced into $CaCl_2$-competent cells of *E. coli* DH5α, selected for LuxAB activity (i.e., light production in the presence of n-decyl aldehyde) and insertion of the kanamycin resistance gene in LB medium plus kanamycin, and then introduced into *P. putida* GR12-2 via electroporation.

Electrocompetent cells of *P. putida* GR12-2 were prepared by the method of Dower et al. (1988). A culture of *P. putida* GR12-2 was grown overnight in 5 mL TSB at 25°, and then 2.5 mL of the overnight culture was transferred to 250 mL TSB and incubated again at 25°C until the optical density at 600 nm reached 0.4. The culture was decanted into an ice-

cold centrifuge bottle and placed on ice for 10 min to chill. Cells were centrifuged in a Sorvall GSA rotor at 6,000 x *g* for 15 min at 4°C and resuspended in approximately 20 mL ice-cold MilliQ water. After a second centrifugation, cells were again suspended in ice-cold MilliQ water, this time in a volume of approximately 100 mL. These two cycles of centrifugation and suspension in first a small volume of MilliQ water and then a larger volume were repeated once more. The cells were next resuspended in 5 mL ice-cold 10% glycerol and centrifuged in a Sorval SS34 rotor for 15 min at 6,000 x *g* and 4°C, before finally being resuspended in 800 µL ice-cold 10% glycerol. Aliquots (40 µL) were dispensed into chilled 0.5 mL microcentrifuge tubes and flash-frozen in liquid nitrogen before being stored at -70°C.

Just prior to electroporation, the cells were thawed on ice, and then pQFPROM-Kan (5 ng suspended in water) was added, mixed gently, and the mixture stored on ice for a few minutes before being transferred to a chilled cuvette. Air bubbles were removed by gently tapping the cuvette and any moisture on the sides of the tube was removed. The cuvette was placed in the chilled cuvette holder and then in a Bio-Rad Gene Pulser™. The cells were pulsed under the following conditions: 2.5 KV, 25 µF capacitance, and 200 ohms. Immediately following electroporation, 1 mL SOC medium was added to the cells and mixed by pipetting up and down. The cells were transferred to a sterile 1.5 mL microcentrifuge tube and incubated at 25°C for 1 h with gentle shaking. *P. putida* GR12-2 cells carrying pQFPROM-Kan were selected by plating 100 µL of the transformation mixture on Tryptic Soy Agar (TSA) containing kanamycin (50 µg/mL).

Control cells, *P. putida* GR12-2 carrying pQF70-Kan, which is similar to pQFPROM-Kan except that it does not contain the *ipdc* promoter, were prepared similarly.

Introduction of *rpoS* into *P. putida* GR12-2/pQFPROM-Kan by triparental mating

To determine whether the stationary phase sigma factor RpoS ($\sigma^{38}$) regulates transcription from the *ipdc* promoter, the *rpoS* gene from *Pseudomonas fluorescens* Pf-5, carried on plasmid pJEL5649 (Sarniquet et al., 1995) kindly provided by Dr. J. Loper (USDA Agricultural Research Service, Corvallis, Oregon), was transferred from *E. coli* HB101 to *P. putida* GR12-2/pQFPROM-Kan by triparental mating using *E. coli* HB101/pRK2013 (Ditta et al., 1980) as a helper. Upregulation of the *ipdc* promoter by constitutively produced RpoS could then be measured by assaying for increased LuxAB activity. *P. putida* GR12-2/pQF70-Kan was similarly transformed with pJEL5649 and used as a negative control. *E. coli* HB101/pJEL5649 (donor), *E. coli* HB101/pRK2013 (helper), and either *P. putida* GR12-2/pQF70-Kan or *P. putida* GR12-2/pQFPROM-Kan (recipients) were grown overnight in 5 mL rich medium (LB plus 10 μg/mL tetracycline for donor cells, LB plus 50 μg/mL kanamycin for helper cells, and TSB plus 50 μg/mL kanamycin for recipient cells) at 37°C for *E. coli* cells and 27°C for *P. putida* GR12-2. Cells were washed once with 0.85% NaCl to remove antibiotics and then resuspended in 5 mL Nutrient Broth (8 g/L; Difco). The optical density of all of the cultures at 600 nm was adjusted to 1.2 with Nutrient Broth and then 1 mL donor cells was mixed with 1 mL helper cells and 5 mL recipient cells. Vacuum filtration was used to concentrate the mixed cells on a Millipore filter (0.45 μM nitrocellulose) which was then placed with the cells facing up on Nutrient Agar (23 g/L; Difco) and incubated at 30°C for 24 h to allow mating and homologous recombination to occur. To collect the cells, the filter was transferred to 1 mL 0.85% NaCl and incubated at 27°C for 1 h with gentle shaking. *P. putida* GR12-2 transconjugants were selected by spreading 100 μL of the collected cells onto DF salts minimal agar (on which *E. coli* cannot

grow) supplemented with tetracycline (10 μg/mL) to select for the presence of pJEL5649 and kanamycin (50 μg/mL) to select for the presence of pQF70-Kan or pQFPROM-Kan. To ensure that selected colonies were indeed *P. putida* GR12-2, these were subcultured on Simmon's Citrate agar (24.2 g/L; Difco) (on which *E. coli* cannot grow) containing tetracycline and kanamycin. That these cells carried both pJEL5649 and pQF70-Kan or pQFPROM-Kan was verified by isolation and restriction enzyme digestion of plasmids from selected transconjugants. Transconjugants carrying both plasmids were designated *P. putida* GR12-2/pQF70-Kan/pRpoS and *P. putida* GR12-2/pQFPROM-Kan/pRpoS.

Quantification of promoter activity in the presence of tryptophan

In addition to visualization by eye in the presence of n-decyl aldehyde as described above, Lux activity driven by the *ipdc* promoter in *P. putida* GR12-2/pQFPROM-Kan and in *P. putida* GR12-2/pQFPROM-Kan/pRpoS was quantified using a luminometer (Turner Design Model TD20/20). Five mL DF salts minimal medium, containing kanamycin for *P. putida* GR12-2/pQFPROM-Kan and kanamycin and tetracycline for *P. putida* GR12-2/pQFPROM-Kan/pRpoS, were inoculated from a single colony and grown for 24 h at 27°C. From the overnight culture, 200 μL were transferred into 50 mL DF salts minimal medium with and without tryptophan (200 μg/mL final concentration, prepared from a 5 mg/mL stock in warm water) and returned to the 27°C water bath.

At various times (8, 16, 20, 24, 27, 32 and 48 h), the optical density of the culture at 600 nm was determined (in duplicate), and then two 1 mL aliquots of each culture were transferred to 1.5 mL microcentrifuge tubes to measure Lux activity. In addition, the supernatant from 1 mL of culture was assayed for IAA content by reaction with Salkowski's reagent as described below. After centrifugation to remove the bacterial cells, 200 μL of the

culture supernatant was mixed with 800 µL of Salkowski's reagent in a disposable 1 mL cuvette (VWR) and left to stand at room temperature for 20 min. The absorbance at 535 nm was measured in a Novaspec II spectrophotometer; uninoculated DF salts minimal media with and without tryptophan were used as references.

The 1.5 mL microcentrifuge tubes containing 1 ml of culture were stored on ice until just before addition of n-decyl aldehyde when they were removed from the ice and warmed to room temperature for 5 min. Five µL n-decyl aldehyde were added to each tube, mixed well by vortexing, and the cells left at room temperature for an additional 5 min. Just before placing the tubes in the luminometer, the cells were again mixed briefly by vortexing, and then luminescence was measured immediately at the following luminometer settings: delay, 3 sec; integration, 15 sec; sensitivity, 20.1%. Light production by control cells, *P. putida* GR12-2/pQF70-Kan, and *P. putida* GR12-2/pQF70-Kan/pRpoS, was also measured to determine background levels of LuxAB activity.

Collection of canola seed exudate

To determine whether unidentified factors present in seed exudate can stimulate the *ipdc* promoter and IAA production in *P. putida* GR12-2, exudate was collected from imbibed canola seeds by the method of Penrose and Glick (2001) and added to the medium of *P. putida* GR12-2/pQFPROM-Kan cultures. Canola seeds (Hyola 401), kindly provided by Dr. J. Omielan (University of Guelph, Guelph, Ontario), were surface sterilized by soaking 100 seeds in 5 mL 10% $H_2O_2$ at room temperature for 2 min and then rinsing four times with sterile distilled water to remove residual $H_2O_2$. The seeds were then imbibed for 1 h in 5 mL 0.03 M $MgSO_4$ at room temperature, rinsed twice with sterile distilled water, and transferred to a 100 µm sterile nylon cell strainer (Becton Dickinson Labware, Franklin Lakes, N.J.) set

in to a sterile polypropylene Petri dish (60 x 15 mm) containing 1 mL of sterile distilled water. Four such Petri dishes containing 100 seeds each were placed in a plastic dish fitted with a lid to shade the seeds from light, and incubated in a growth chamber at 20°C. At 3, 6.5, and 10 h following imbibition, the exudate from four dishes was collected using a micropipette, and combined before filtering through a 0.2 μM syringe filter (Acrodisc®, Pall Gelman Laboratories) pre-wetted with sterile distilled water. Immediately following collection, the exudate was stored in 15 mL polypropylene tubes (Falcon) at –80°C.

Quantification of promoter activity in the presence of canola seed exudate

To measure the effect of canola seed exudate on IAA production and *ipdc* promoter activity, *P. putida* GR12-2/pQFPROM-Kan, and *P. putida* GR12-2/pQF70-Kan as a negative control, were first grown for 24 h at 27°C in 5 mL DF salts minimal medium containing kanamycin (50 μg/mL) before transferring 20 μL to 5 mL DF salts minimal medium to which either 400 μL water or 400 μL exudate (roughly equivalent to the exudate collected from 100 seeds) had been added. These cultures were incubated at 27°C for an additional 24 h, after which two 1 mL aliquots were transferred to cuvettes to measure the optical density of the cultures at 600 nm, and three 1 mL aliquots were transferred to 1.5 mL microcentrifuge tubes to measure promoter activity (LuxAB activity) and IAA production as described above. For each bacterial strain, the effect of exudate collected from Hyola 401 seeds after 3, 6.5, and 10 h was measured in duplicate and analyzed by two-way ANOVA; F-values shown in the text summarize the results of the analysis.

**Construction of an IAA-Deficient Mutant of *P. putida* GR12-2**

To construct a vector for insertional mutagenesis of the *ipdc* gene in *P. putida*

GR12-2, the *ipdc* sequence isolated above was first transferred from pIPD2 to the suicide vector pJQ200 (*sacB*, *oriT*, *mob* from RP4, $Gm^R$, *lacZ*; Quandt and Hynes, 1993) and then inserted with a gene for kanamycin resistance. Plasmid pJQ200 (150 ng) and pIPD2 (300 ng) were digested separately with 10 U of *Bam*HI (Roche Diagnostics) in a final volume of 20 μL of 1X Buffer B (Roche Diagnostics) overnight at 37°C. Digestion fragments of the appropriate size (4.9 kb for pJQ200 and 1.7 kb for pIPD2) were cut from a 0.8% agarose gel, purified using Gene CleanII®, and ligated using standard methods described previously. The resulting ligation products were transformed into *E. coli* JM109 and selected on LB agar containing gentamicin (30 μg/mL; Sigma), IPTG and X-Gal. After confirmation of plasmid size and the presence of expected restriction sites, one plasmid, pJQIPDC4, was chosen for further manipulation.

Construction of pJQIPDC4-Kn

The kanamycin resistance gene carried on a 2.3 kb *Eco*RI fragment was isolated from pHP45Ω-Km (Fellay et al., 1987; Prentki and Krisch, 1984) by digestion of 800 ng of plasmid with 10 U *Eco*RI (MBI Fermentas) in 20 μL of 1X *Eco*RI buffer (MBI Fermentas) overnight at 37°C. Following the digestion, *Eco*RI was heat inactivated by incubation at 65°C for 15 min. To purify the DNA from the mixture, 10 μL of 3 M sodium acetate, pH 8.0, and 70 μL of water were added to 20 μL of digestion mix, and the DNA was extracted twice with 100 μL of phenol:chloroform. Digestion products were collected by precipitation with 100 μL of isopropanol for 1 h at -20°C, centrifugation for 20 min at full speed in an Eppendorf microcentrifuge, followed by washing with 70% ethanol and resuspension of the air-dried pellet in 18.2 μL of water. The cut ends of the *Eco*RI fragments were made blunt by addition of 5 U of S1 nuclease (diluted in 1 μL of water from a 30 U/μL stock; Promega)

and 3 μL 7.4X S1 buffer (Promega) to the DNA suspension, and incubation at 30°C for 30 min. To inactivate the nuclease, 1 μL of S1 nuclease stop buffer (Promega) was added and the mixture was incubated at 65°C for 10 min. The DNA fragments were precipitated in 2 volumes of 100% ethanol and 2.2 μL of sodium acetate (3 M, pH 8.0) for 2 h at -20°C, collected by centrifugation and rinsed with 70% ethanol as described previously, and finally resuspended in 7.5 μL of water.

Plasmid pJQIPDC4 (100 ng) was digested at a unique site within the *ipdc* gene using *PmlI* (10 U; MBI Fermentas) in 10 μL of 1X $Y^+$ Tango buffer (MBI Fermentas) overnight at 37°C. Because *PmlI* generates blunt ends, S1 nuclease treatment was not required; therefore, following heat inactivation of the restriction enzyme, the digestion products were directly ligated with the blunt-ended kanamycin resistance gene fragment. To this end, 10 μL of the pJQIPDC4-*PmlI* digestion mixture was combined with 7.5 μL of S1 nuclease-treated pHP45Ω-Km *Eco*RI fragments, 2.5 μL 10X ligation buffer (MBI Fermentas), 2 μL PEG 4000 (MBI Fermentas) and 4 μL T4 DNA ligase (2 U/μL; MBI Fermentas), and incubated overnight at 20°C. Competent cells (200 μL) of *E. coli* S17.1 (carrying the RP4 *tra* genes required for transfer of the plasmid to *P. putida* GR12-2 via conjugation; Simon et al., 1983) were transformed with 12.5 μL of the ligation mixture and then selected for the presence of pJQIPDC4-Kn by resistance to gentamicin (30 μg/mL) and kanamycin (50 μg/mL).

Conjugation to transfer pJQIPDC4-Kn to *P. putida* GR12-2

Donor cells (*E. coli* S17.1/pJQIPDC4-Kn) and recipient cells (*P. putida* GR12-2) were grown overnight in 5 mL rich media (LB plus 30 μg/mL gentamicin and 50 μg/mL kanamycin, and TSB, respectively). Cells were washed once with 5 mL 0.85% (w/v) NaCl to remove antibiotics, and resuspended in 5 mL Nutrient Broth (8 g/L). The optical density

(at 600 nm) of each cell suspension was adjusted to 1.3 with Nutrient Broth. Two mL of donor cells were mixed with 8 mL of recipient cells and collected on a 0.45 μM Millipore filter by vacuum filtration. The filter containing the mixed cells was placed with the cells facing up on a Nutrient Agar (23 g/L) plate and incubated at 30°C for 24 h to allow mating and homologous recombination to occur. To collect the cells, the filter was transferred to 1 mL 0.85% NaCl and incubated at room temperature for 1 h with gentle shaking. Because *E. coli* donor cells cannot grow on Simmon's Citrate medium, and untransformed *P. putida* GR12-2 parental cells cannot grow on medium containing kanamycin, transconjugants were identified by growth on Simmon's Citrate agar (24.2 g/L) supplemented with kanamycin (50 μg/mL). Individual colonies were replica plated onto DF salts minimal medium agar (on which *E. coli* cannot grow) plus kanamycin to verify the identification of transconjugants. To select for transconjugants that had replaced the functional *ipdc* gene in their chromosome with the disrupted *ipdc* gene from the plasmid by double crossover between homologous *ipdc* sequences, colonies resistant to the lethal effects of the vector-encoded *sacB* gene product in the presence of sucrose were identified on TSA containing kanamycin and 5% (w/v) sucrose. In addition, these colonies were plated on TSA supplemented with gentamicin; lack of growth confirmed the absence of gentamicin acetyltransferase also encoded on the vector. Insertion of the kanamycin resistance gene into the chromosomal *ipdc* gene was substantiated by PCR using the primers used to isolate the *ipdc* gene, and by Southern hybridization. In the latter case, the probe was labeled by PCR using Ready-to-Go® PCR beads, 100 pg of pIPD2 as a template, 300 nM each of the primers used to isolate the *ipdc* gene, and 10 μL dNTP labeling mix (1 mM stock containing 1:2 DIG-dUTP:dTTP; Roche Diagnostics), in a total reaction volume of 25 μL. Amplification cycles were as described above.

## Characterization of IAA-Deficient Mutant of *P. putida* GR12-2

Quantification of IAA production

The chemical assay described by Gordon and Weber (1951) provides a reasonably specific and sensitive method for estimating the concentration of IAA in solution (Glickmann and Dessaux, 1995). The basis for the test is the reaction of Salkowski's reagent with IAA, resulting in a characteristic colorimetric change, the intensity of which can be measured spectrophotometrically.

Colonies of wild-type and IAA-deficient *P. putida* GR12-2 were propagated in 5 mL DF salts minimal medium overnight (17-20 h) at 30°C in a shaking water bath and then 20 µL were transferred into 5 mL DF salts minimal medium supplemented with the following concentrations of L-tryptophan (µg/mL; from a filter-sterilized 2 mg/mL stock prepared fresh in warm water; Sigma): 0, 50, 100, 200, 500. After incubation for 42 h at 30°C in a shaking water bath, the density of the culture was measured spectrophotometrically at 600 nm and then the bacterial cells were removed from the culture medium by centrifugation (IEC Model HN; 5,500 x *g*) at room temperature for 10 min. A 1 mL aliquot of the supernatant was mixed vigorously with 4 mL Salkowski's reagent (150 mL concentrated $H_2SO_4$, 250 mL distilled $H_2O$, 7.5 mL 0.5 M $FeCl_3 \cdot 6H_2O$) in a 13 x 100 mm test tube and allowed to stand at room temperature for 20 min. One mL was transferred to a disposable semi-micro cuvette (VWR), and the absorbance at 535 nm was measured immediately in a Shimadzu UV2102PC spectrophotometer; uninoculated medium, supplemented with the appropriate concentration of tryptophan, was used as a reference.

The concentration of IAA in each culture medium was determined by comparison

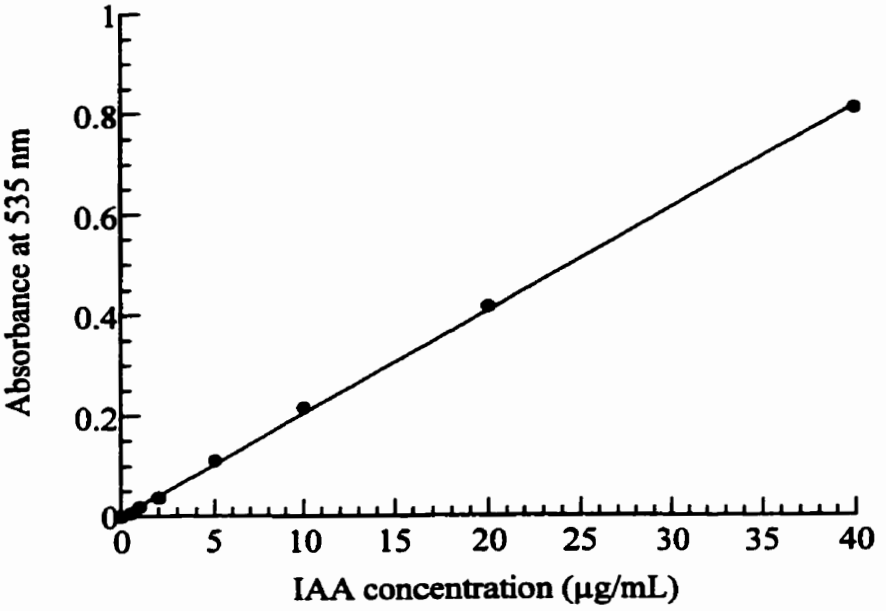
with a standard curve generated as follows. A 100 μg/mL stock solution of IAA (Sigma) was freshly prepared by dissolving 10 mg of IAA in 200 μL of 100% ethanol followed by 90 mL sterile distilled water. The solution was warmed slightly at 37°C to remove the ethanol and the volume adjusted to 100 mL with sterile distilled water. From this stock solution, dilutions were prepared to final concentrations of 0, 1, 2, 5, 10, 20, and 40 μg/mL. The absorbance of each concentration of IAA standard was measured using Salkowski's assay described above, and the values for absorbance at 535 nm versus IAA concentration (μg/mL) were plotted (Fig. 7).

To compare the reactions of IAA and indolepyruvic acid with Salkowski's reagent, the above procedure was modified slightly. Because indolepyruvic acid is highly unstable and difficult to dissolve in water, stock solutions and subsequent dilutions of both IAA and indolepyruvic acid were prepared in 100% ethanol. Although the concentrations of IAA are expressed elsewhere in μg/mL, for comparison of their reaction with Salkowski's reagent, the compounds were prepared in concentrations of 0, 0.005, 0.01, 0.025, 0.05, 0.125, 0.25, and 0.5 mM to account for differences in molecular weight between IAA (175 g/mole) and indolepyruvic acid (203 g/mole).

Gnotobiotic Root Elongation Assay

Cultures of wild-type and IAA-deficient strains of *P. putida* GR12-2 were grown overnight from a single colony in 5 mL DF salts minimal medium with and without kanamycin (50 μg/mL), respectively, at 30°C in a shaking water bath. After approximately 24 h, 20 μL of each culture was transferred to 5 mL DF salts minimal medium supplemented with tryptophan (200 μg/mL) to induce IAA production, and incubated for an additional 42 hours. The turbidity of the cultures was measured spectrophotometrically at 600 nm before

Figure 7. Standard curve for the spectrophotometric quantification of IAA in bacterial cultures using Salkowski's reagent.


Absorbance at 535 nm
1
0.8
0.6
0.4
0.2
0
0
5
10
15
20
25
30
35
40
IAA concentration (µg/mL)

separating the bacterial cells from the culture medium by centrifugation (5,500 x *g*) for 10 min at room temperature. The supernatant was immediately assayed for IAA concentration by Salkowski's assay as previously described. Cells were washed twice by suspension in 5 mL sterile 0.03 M $MgSO_4$ followed by centrifugation for 10 min. The final resuspension was adjusted to an absorbance of 0.5 at 600 nm with 0.03 M $MgSO_4$, and stored on ice while seeds were prepared.

Canola seeds (Hyola 401), kindly provided by Dr. J. Omielan, University of Guelph, were prepared and inoculated by the method outlined by Lifshitz et al. (1987) with some modifications (Caron et al., 1995). Approximately 300 seeds, previously stored in a dessicator at 4°C, were surface sterilized by soaking in 10 mL 70% ethanol for 1 min, followed by 10 mL 1% sodium hypochlorite (bleach) for 10 min in sterile polystyrene Petri dishes (100 x 15 mm). To remove residual bleach, the seeds were washed five times with sterile distilled water. For each treatment, approximately 100 seeds were transferred aseptically to sterile polystyrene Petri dishes (60 x 15 mm) and incubated with 5 mL of either the wild-type or the mutant bacterial suspension at room temperature for 1 h to allow the bacteria to bind to the seed coat and for seed imbibition. Seeds were also incubated in 5 mL 0.03 M $MgSO_4$ under the same conditions as a control.

Six seeds were aseptically placed in each growth pouch (Mega International, Minneapolis, Mn.) which had been previously filled with 10 mL distilled water and autoclaved. For each treatment, 10 pouches were prepared. Pouches were placed upright in metal racks, one treatment per rack to prevent cross-contamination, with two empty pouches at either end of the rack. Racks were set in a plastic bin containing about 3 cm of deionized water and covered loosely with plastic wrap to prevent dehydration. Pouches were incubated

in a growth chamber at 20°C with a cycle of 12 hours of dark followed by 12 hours of light (18 μmol photons/$m^2$/$sec^2$). For the first two days, seeds were kept in the dark by covering the pouches with aluminium foil. After five days, shoot and primary root length, and shoot and root fresh and dry weight were measured and analyzed by two-way ANOVA; F-values shown in the text summarize the results of the analysis. Seeds that failed to germinate three days after sowing were marked and shoots and roots that subsequently developed from these seeds were not measured.

Rooting Assay

The effect of wild-type and the IAA-deficient mutant of *P. putida* GR12-2 on the development of adventitious roots on mung bean cuttings was assessed using the method outlined by Mayak et al. (1999). Mung bean (*Vigna radiata*) seeds were surfaced sterilized in a manner similar to that used for canola seeds above, by soaking for 1 min in 70% ethanol and then for 10 min in 1% sodium hypochlorite (bleach) in a sterile polystyrene Petri dish, before rinsing five times with sterile distilled water. Seeds were imbibed for 1 h in sterile distilled water and then sown in sterile vermiculite in a flat equipped with a drainage tray and a transparent lid. Mung beans seedlings were grown in a growth chamber at 20°C with a cycle of 12 hours of dark followed by 12 hours of light (18 μmol photons/$m^2$/$sec^2$). After 7 days, the portion of the seedlings above the surface of the vermiculite was excised using a razor blade and placed immediately in either water or a bacterial suspension prepared as follows.

Five mL DF salts minimal medium was inoculated with wild-type or the IAA-deficient mutant of *P. putida* GR12-2 and incubated overnight at 27°C. Eighty μL of the overnight culture was transferred to 20 mL DF salts minimal medium containing tryptophan

(200 μg/mL) and incubated at 27°C for an additional 42 h. Cells were washed twice with sterile distilled water and resuspended in sterile water to an optical density of 0.5 at 600 nm. Three mL of each bacterial treatment were transferred to each of ten borosilicate glass tubes (10 x 75 mm), and ten additional tubes were filled with 3 mL sterile distilled water. One mung bean cutting was placed in each tube, for a total of ten cuttings per treatment, and these were placed in a rack, covered loosely with plastic wrap to prevent evaporation, and incubated in the growth chamber under the same conditions described above. After eight days, the cuttings were rinsed briefly with distilled water, and the number and lengths of adventitious roots were measured under a magnifying glass and analyzed by two-way ANOVA; F-values shown in the text summarize the results from the analysis.

# RESULTS

## Isolation of the Indolepyruvate Decarboxylase Gene

Hybridization of approximately 3,000 colonies of *E. coli* DH5α carrying fragments from the *P. putida* GR12-2 genome on plasmid pUC18, with the DIG-labeled indolepyruvate decarboxylase (*ipdc*) gene from *Enterobacter cloacae* FERM BP-1529 (Koga et al., 1991a) revealed a single colony carrying a 2.6 kb insert in pUC18 similar to the probe sequence (Fig. 8A). A secondary screen of this colony, in duplicate, using the same probe as above, confirmed hybridization (Fig. 8B). Digestion of the insert from the positive clone and Southern hybridization using the *E. cloacae ipdc* probe, showed that the *P. putida* GR12-2 *ipdc* sequence was present on a 1.1 kb *Bam*HI fragment (Fig. 9A). Sequence analysis of the 1.1 kb fragment indicated that only 200 bp of the *ipdc* sequence were contained on the fragment (Fig. 9B); however, this sequence was 98% identical at the nucleotide level to the 3' end of the *E. cloacae* FERM BP-1529 *ipdc* gene (Fig. 9C). The remainder of the 1.1 kb *Bam*HI fragment, which corresponds to the region downstream of the *ipdc* gene in the *P. putida* GR12-2 genome, carries a sequence 72% identical at the nucleotide level to an open reading frame in *E. coli* of unknown function (Accession No. AE000327) on the opposite strand (Fig. 10A). A putative transcription termination signal was also identified in the region immediately downstream of the *ipdc* gene by the presence of sequences capable of forming a stable stem-loop structure in the mRNA (Fig. 10B). A stable stem structure is characterized by 4-20 complementary nucleotide pairs with a high G+C content; the loop usually ranges from about 3-10 nucleotides (Ermolaeva et al., 2000).

Working on the assumption that the high degree of identity between the 3' ends of the

Figure 8. Hybridization of a colony of *E. coli* DH5α carrying a fragment of the *P. putida* GR12-2 genome with the DIG-labeled indolepyruvate decarboxylase (*ipdc*) gene from *E. cloacae* FERM BP-1529. A, primary screen; B, secondary screen.

A

B

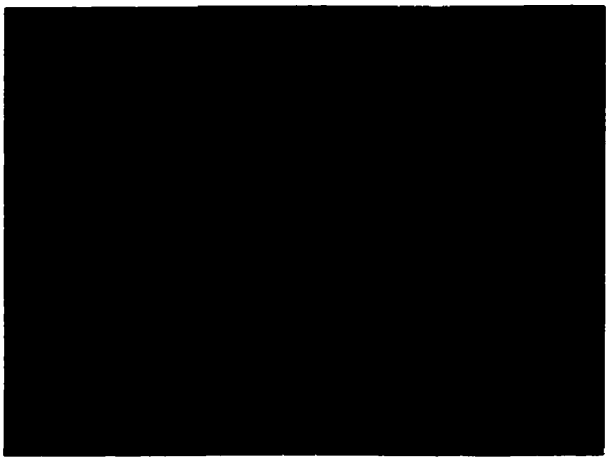
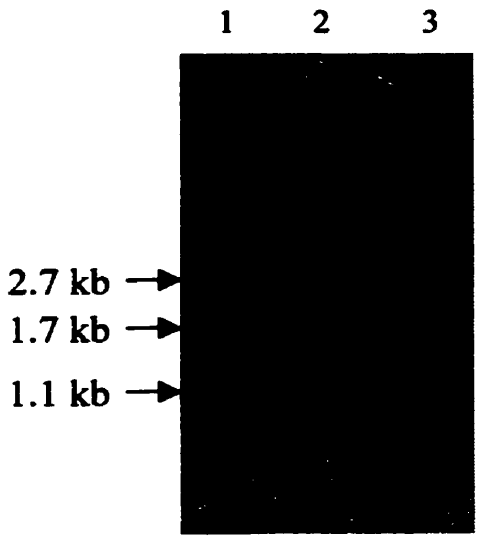
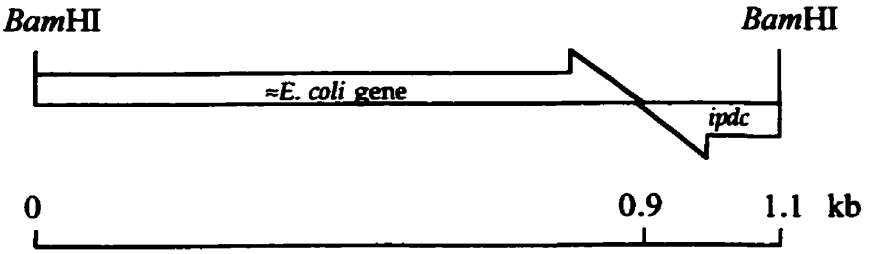


Figure 9. A 1.1 kb *Bam*HI fragment from the *P. putida* GR12-2 genome carries 200 bp of the 3' end of the indolepyruvate decarboxylase (*ipdc*) gene. A, Southern hybridization revealed that this fragment hybridized to the *E. cloacae* FERM BP-1529 *ipdc* gene probe (Lane 1, plasmid from positive clone revealed by colony hybridization of *P. putida* GR12-2 genomic clone bank (see Fig. 8), digested with *Bam*HI and *Hin*dIII; Lane 2, plasmid from the same positive clone digested with *Sca*I and *Hin*dIII; Lane 3, Plasmid pIP27 carrying the *E. cloacae* FERM BP-1529 *ipdc* gene on a 1.7 kb *PstI-BamHI* fragment); B, Map of the 1.1 kb fragment showing the 3' end of the *ipdc* gene and a sequence downstream similar to an *E. coli* gene of unknown function; C, The 3' end of the *P. putida* GR12-2 *ipdc* gene is 98% identical to the corresponding region of the *E. cloacae* FERM BP-1529 *ipdc* gene.

A




B




C

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```

Figure 10. The 1.1 kb *BamHI* fragment from the *P. putida* GR12-2 genome carries a sequence downstream of the *ipdc* gene that is 72% identical to an *E. coli* gene of unknown function (A) and, immediately downstream of the *ipdc* gene, a transcription termination signal (B, complementary sequences capable of forming a stem structure of a hairpin loop are underlined).

```
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               ||||||  |||  |  ||  ||  ||||||||||||  ||  ||  ||  ||  ||  |  ||  |  |  |
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Orf       365  R  D  G  W  L  S  L  F  M  A  A  V  V  V  P  N  T  T  L  L

Query:    901  ccctgctctgtatcgtgatgttgcccgcctggctcctgctggcgggcaaaccgatgatga 960
               |  ||||||||||||||  |||  |  ||  ||  |||||  |  |  ||  ||  ||  ||||||||||
Sbjct:   2462  cgctgctctgtatcgtcatgcttccggcatggctgttattagcaggtaagccgatgatga 2521
Orf       385  P  L  L  C  I  V  M  L  P  A  W  L  L  L  A  G  K  P  M  M

Query:    961  tgg 963
               |||
Sbjct:   2522  tgg 2524
Orf       405  M
```

**B**

```
gaagcgcgtaataacgcctgactacttgtcgtttcgccaggccatcatcatcggtttgcccgccagca
cttcgcgcattattgcggactgatgaacagcaaagcggtccggtagtagtagccaaacgggcggtcgt
E  A  R  N  N  A  *

ggagccaggcgggcaacatca
cctcggtccgcccgttgtagt
```

*P. putida* GR12-2 and *E. cloacae* FERM BP-1529 *ipdc* sequences could be extended to the entire gene, PCR primers were designed from the published *E. cloacae* FERM BP-1529 sequence in an attempt to amplify the entire open reading frame. Electrophoresis of the PCR products from purified genomic DNA and from whole cell lysates of *P. putida* GR12-2 revealed a single band of the expected size, about 1.7 kb (Fig. 11). Sequence analysis of the PCR products, and alignment of the nucleotide sequence with the *ipdc* gene from *E. cloacae* FERM BP-1529, confirmed that the *P. putida* GR12-2 *ipdc* gene had indeed been isolated (GenBank Accession No. AF285632) (Fig. 12). Furthermore, the translated amino acid sequence is similar to other known bacterial indolepyruvate decarboxylases (Fig. 13, Table 3).

The *P. putida* GR12-2 indolepyruvate decarboxylase is also similar to pyruvate decarboxylase from *Zymomonas mobilis* and *Saccharomyces cerevisiae*, sharing substantial identity (33% and 36%, respectively) and similarity (51% and 53%, respectively) in their amino acid sequences (Fig. 14), and to the C-terminal end of pyruvate oxidase from *Lactobacillus plantarum*. Four of the six residues believed to be involved in substrate binding and catalysis for pyruvate decarboxylase are conserved in the indolepyruvate decarboxylase sequence: $Asp^{29}$, $His^{115}$, $His^{116}$, and $Glu^{468}$ of indolepyruvate decarboxylase from *P. putida* GR12-2 correspond to residues $Asp^{27}$, $His^{113}$, $His^{114}$, and $Glu^{473}$ in the active site of *Zymomonas mobilis* pyruvate decarboxylase (Dobritzsch et al., 1998) (Fig. 14). In addition, most of the residues known to bind cofactors $Mg^{2+}$ and thiamine diphosphate in pyruvate decarboxylase are conserved in indolepyruvate decarboxylase including $Glu^{52}$, $Gly^{408}$, $Asp^{435}$, $Asn^{462}$, and $Gly^{464}$. Indolepyruvate decarboxylase is also similar to acetolactate synthase (large subunit), another member of the thiamine diphosphate-dependant

Figure 11. Agarose gel electrophoresis of PCR products amplified from purified genomic DNA (Lane 2) and whole cell lysates (Lane 3) from *P. putida* GR12-2 using primers designed from the *E. cloacae* FERM BP-1529 *ipdc* sequence to amplify the *ipdc* gene (Lane 1, PCR products from water as a template; Lane 4, DNA size markers).

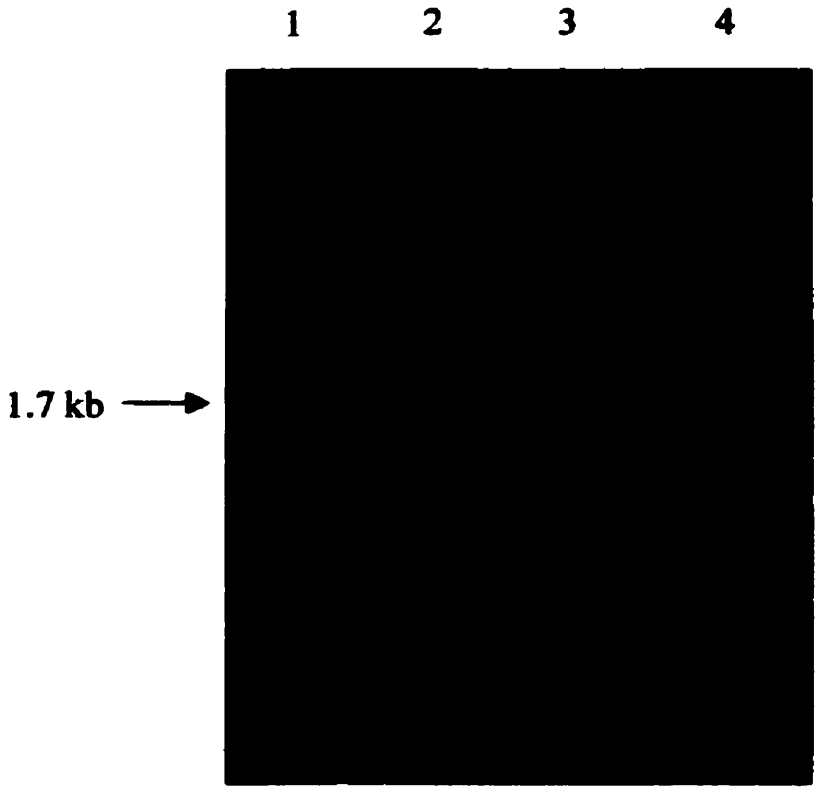

1
2
3
4
1.7 kb

Figure 12. Nucleotide and translated amino acid sequence of the *ipdc* gene from *P. putida* GR12-2 (GenBank Accession No. AF285632).

```
   1 ATGCGAACCCCATACTGGGTCGCCCATTACCTGCTGGACCGTCTTACAGATTGTGGTGCC  60
     M  R  T  P  Y  W  V  A  H  Y  L  L  D  R  L  T  D  C  G  A
  61 GATCATCTGTTTGGCGTGCCGGGCGACTATAACCTGCAGTTTCTCGACCACGTAATAGAC 120
     D  H  L  F  G  V  P  G  D  Y  N  L  Q  F  L  D  H  V  I  D
 121 AGCCCGGATATTTGTTGGGTGGGCTGTGCCAATGAGCTGAACGCATCCTATGCCGCTGAC 180
     S  P  D  I  C  W  V  G  C  A  N  E  L  N  A  S  Y  A  A  D
 181 GGATACGCCCGATGTAAGGGCTTTGCCGCGCTGCTGACCACATTCGGCGTTGGGGAGTTA 240
     G  Y  A  R  C  K  G  F  A  A  L  L  T  T  F  G  V  G  E  L
 241 AGTGCCATGAACGGCATTGCCGGCAGCTATGCCGAGCATGTCCCGGTTTTACATATTGTG 300
     S  A  M  N  G  I  A  G  S  Y  A  E  H  V  P  V  L  H  I  V
 301 GGGGCGCCGGGTACGGCGGCACAGCAAAGGGGAGAGTTGCTGCATCATACGTTGGGGGAT 360
     G  A  P  G  T  A  A  Q  Q  R  G  E  L  L  H  H  T  L  G  D
 361 GGGGAGTTCCGTCACTTTTATCATATGAGCGAGCCGATCACCGTCGCACAGGCGGTCCTT 420
     G  E  F  R  H  F  Y  H  M  S  E  P  I  T  V  A  Q  A  V  L
 421 ACCGAACAAAATGCCTGTTATGAAATCGACCGTGTGTTGACAACCATGCTTCGGGAACGT 480
     T  E  Q  N  A  C  Y  E  I  D  R  V  L  T  T  M  L  R  E  R
 481 CGCCCCGGTTATCTGATGTTACCCGCCGATGTGGCAAAAAAAGCCGCCACGCCGCCTGTA 540
     R  P  G  Y  L  M  L  P  A  D  V  A  K  K  A  A  T  P  P  V
 541 AACGCTCTCACTCATAAGCAGGCTAATGCCGATAGCGCCTGCCTGAAAGCGTTCCGGGAT 600
     N  A  L  T  H  K  Q  A  N  A  D  S  A  C  L  K  A  F  R  D
 601 GCTGCTGAGAACAAACTGGCGATGAGCAAACGTACCGCGCTGCTGGCCGACTTCCTTGTT 660
     A  A  E  N  K  L  A  M  S  K  R  T  A  L  L  A  D  F  L  V
 661 CTGCGCCATGGCCTGAAGCATGCGCTACAGAAGTGGGTGAAAGAGGTACCGATGGCACAT 720
     L  R  H  G  L  K  H  A  L  Q  K  W  V  K  E  V  P  M  A  H
 721 GCCACCATGCTGATGGGGAAAGGGATATTCGACGAGCGTCAGGCGGGTTTTTACGGTACA 780
     A  T  M  L  M  G  K  G  I  F  D  E  R  Q  A  G  F  Y  G  T
 781 TACAGTGGTTCAGCCAGCACCGGTGCGGTAAAAGAGGCGATTGAAGGGGCTGACACGGTA 840
     Y  S  G  S  A  S  T  G  A  V  K  E  A  I  E  G  A  D  T  V
 841 TTGTGTGTTGGCACGCGTTTTACCGATACCCTGACGGCCGGGTTCACGCACCAGCTTACC 900
     L  C  V  G  T  R  F  T  D  T  L  T  A  G  F  T  H  Q  L  T
 901 CCGGCGCAAACCATTGAAGTTCAGCCGCATGCCGCACGCGTCGGGGATGTCTGGTTTACC 960
     P  A  Q  T  I  E  V  Q  P  H  A  A  R  V  G  D  V  M  F  T
 961 GGCATCCCAATGAACCAGGCGATTGAGACGCTGGTCGAACTCAGCAAACAGCACGTGCAC 1020
     G  I  P  M  N  Q  A  I  E  T  L  V  E  L  S  K  Q  H  V  H
1021 ACTGGCCTTATGTCGTCATCATCCGGCGCAATACCGTTCCCGCAGCCGGACGGTTCGCTT 1080
     T  G  L  M  S  S  S  S  G  A  I  P  F  P  Q  P  D  G  S  L
1081 ACCCAGGAGAATTTCTGGAGAACGTTGCAAACCTTTATTCGCCCGGGGGACATTATCCTT 1140
     T  Q  E  N  F  W  R  T  L  Q  T  F  I  R  P  G  D  I  I  L
1141 GCCGACCAGGGAACATCGGCCTTCGGCGCGATTGATCTGCGTTTACCGGCTGATGTGAAC 1200
     A  D  Q  G  T  S  A  F  G  A  I  D  L  R  L  P  A  D  V  N
1201 TTTATCGTCCAGCCGCTGTGGGGCTCGATTGGTTACACGCTGGCGGCGGCGTTTGGTGCA 1260
     F  I  V  Q  P  L  W  G  S  I  G  Y  T  L  A  A  A  F  G  A
1261 CAAACCGCATGCCCGAACCGGCGCGTGATTGTGCTGACGGGGGATGGCGCGGCCCAGCTC 1320
     Q  T  A  C  P  N  R  R  V  I  V  L  T  G  D  G  A  A  Q  L
1321 ACTATTCAGGAACTAGGCTCGATGCTGCGTGATAAACAGCACCCCATTATTCTGGTGCTC 1380
     T  I  Q  E  L  G  S  M  L  R  D  K  Q  H  P  I  I  L  V  L
1381 AACAACGAAGGCTACACGGTTGAAAGGGCTATCCATGGGGCGGAGCAGCGGTATAACGAC 1440
     N  N  E  G  Y  T  V  E  R  A  I  H  G  A  E  Q  R  Y  N  D
1441 ATTGCTTTGTGGAACTGGACGCACATTCCACAGGCGTTGAGCCTCGATCCTCAGTCTGAG 1500
     I  A  L  W  N  W  T  H  I  P  Q  A  L  S  L  D  P  Q  S  E
1501 TGCTGGCGGGTCAGTGAAGCGGAACAGCTGGCGGACGTACTTGAAAAAGTGGCGCACCAC 1560
     C  W  R  V  S  E  A  E  Q  L  A  D  V  L  E  K  V  A  H  H
1561 GAGCGGCTCTCGTTGATTGAGGTGATGCTCCCGAAAGCGGATATCCCGCCGCTGCTCGGG 1620
     E  R  L  S  L  I  E  V  M  L  P  K  A  D  I  P  P  L  L  G
1621 GCGCTTACCAAAGCTCTGGAAGCGCGTAATAACGCCTGA 1657
     A  L  T  K  A  L  E  A  R  N  N  A  *
```

Figure 13. Alignment of bacterial indolepyruvate decarboxylase amino acid sequences from *Enterobacter cloacae* FERM BP-1529, *Pseudomonas putida* GR12-2, *Erwinia herbicola* 299R, and *Azospirillum brasilense* Sp245. The following symbols found below the sequences indicate conserved positions: *, columns that contain identical amino acid residues; :, columns that contain amino acids that have strongly similar properties (e.g., amino acids with similar charge); ., columns that contain amino acids that have weakly similar properties.

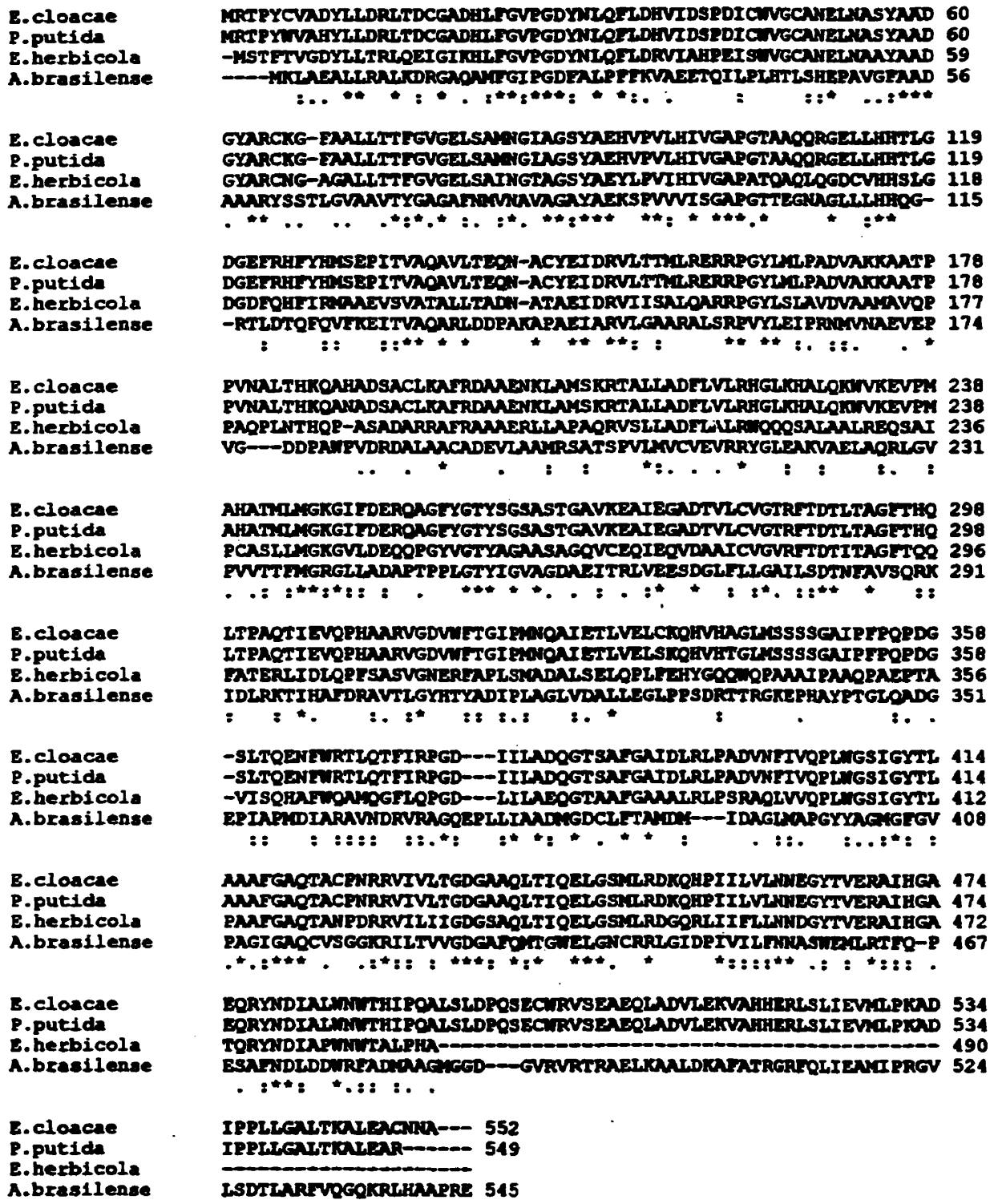

E.cloacae
P.putida
E.herbicola
A.brasilense

Table 3. Comparison of the *P. putida* GR12-2 indolepyruvate decarboxylase amino acid sequence with other bacterial indolepyruvate decarboxylases.

| Organism | Identity (%) | Similarity (%) |
|---|---|---|
| *Enterobacter cloacae* FERM BP-1529 | 99 | 99 |
| *Erwinia hericola* 299R | 57 | 71 |
| *Azospirillum brasilense* Sp245 | 29 | 44 |

Figure 14. Alignment of *P. putida* GR12-2 (P.p) indolepyruvate decarboxylase (IPDC) with pyruvate decarboxylase (PDC) from the yeast *Saccharomyces cerevisiae* (S.c), the bacterium *Zymomonas mobilis* (Z.mo), and the plant *Zea mays* (Z.ma), and with acetolactate synthase (ALS) from the chloroplast of *Brassica napus* (B.n), from the genome of *E. coli* (E.c), and from the mitochondrion of *Saccharomyces cerevisiae* (S.c). Conserved residues involved in substrate and cofactor binding are shown in bold. The following symbols found below the sequences indicate conserved positions: *, columns that contain identical amino acid residues; :, columns that contain amino acids that have strongly similar properties (e.g., amino acids with similar charge); ., columns that contain amino acids that have weakly similar properties.

```
P.p/IPDC ------------------------------------------------------------
S.c/PDC  ------------------------------------------------------------
Z.mo/PDC ------------------------------------------------------------
Z.mo/PDC ----------------------------------------------METLLAGNPANGVAKP 16
B.n/ALS  ---------MAAATSSSPISLTAKPSSKSPLPISRFSLP----FSLTPQKPSSRLHRPLA 47
E.c/ALS  ------------------------------------------------------------
S.c/ALS  MIRQSTLKNFAIKRCFQHIAYRNTPAMRSVALAQRFYSSSSRYYSASPLPASKRPEPAPS 60


P.p/IPDC ----------------------------MRTPYWVAHYLLDRLTDCGADHLFGVPGDYNLQ 33
S.c/PDC  ----------------------------MSEITLGKYLFERLKQVNVNTVFGLPGDFNLS 32
Z.mo/PDC ----------------------------MSYTVGTYLAERLVQIGLKHHFAVAGDYNLV 31
Z.mo/PDC TCNGVGALPVANSHAIIATPAAAAATLAPAGATLGRHLARRLVQIGASDVFAVPGDFNLT 76
B.n/ALS  ISAVLNSPVNVAPEKTDKIKTFISRYAPDEPRKGADILVEALERQGVETVFAYPGGASME 107
E.c/ALS  ----------------------------MNGAQWVVHALRAQGVNTVFGYPGGAIMP 29
S.c/ALS  FNVDPLEQPAEPSKLAKKLRAEPDMDTSFVGLTGGQIFNEMMSRQNVDTVFGYPGGAILP 120
                                                 .  .   :   .  .  *. .*.  :

P.p/IPDC FLDHVIDSPDICWVGCANELNASYAADGYARCKG-FAALLTTFGVGELSAMNGIAGSYAE 92
S.c/PDC  LLDKIYEVEGMRWAGNANELNARYAADGYARIKG-MSCIITTFGVGELSALNGIAGSYAE 91
Z.mo/PDC LLDNLLLNKNMEQVYCCNELNCGFSAEGYARAKG-AAAAVVTYSVGALSAFDAIGGAYAE 90
Z.ma/PDC LLDYLIAEPGLTLVGCCNELNAGYAADGYARSRG-VGACAVTFTVGGLSVLNAIAGAYSE 135
B.n/ALS  IHQALTRSSTIRNVLPRHEQGGVFAAEGYARSSGKPGICIATSGPGATNLVSGLADAMLD 167
E.c/ALS  VYDALYDGG-VEHLLCRHEQGAAMAAIGYARATGKTGVCIATSGPGATNLITGLADALLD 88
S.c/ALS  VYDAIHNSDKFNFVLPKHEQGAGHMAEGYARASGKPGVVLVTSGPGATNVVTPMADAFAD 180
         . : :     .      :* .    * **** *  .   .*   *  . .  :..:  :

P.p/IPDC HVPVLHIVGAPGTAAQQRGELLHHTLGDGEFRHFYHMSEPITVAQAVLTEQN-ACYEIDR 151
S.c/PDC  HVGVLHVVGVPSISSQAKQLLLHHTLGNGDFTVFHRMSANISETTAMITDICTAPAEIDR 151
Z.mo/PDC NLPVILISGAPNNNDHAAGHVLHHALGKTDYHYQLEMAKNITAAAEAIYTPEEAPAKIDH 150
Z.ma/PDC NLPVVCIVGGPNSNDYGTNRILHHTIGLPDFSQELRCFQTITCYQAIINNLDDAHEQIDT 195
B.n/ALS  SVPLVAITGQVPRRMIGTDAFQETPIVE--------VTRSITKHNYLVMDVDDIPRIVQE 219
E.c/ALS  SIPVVAITGQVSAPFIGTDAFQEVDVLG--------LSLACTKHSFLVQSLEELPRIMAE 140
S.c/ALS  GIPMVVFTGQVPTSAIGTDAFQEADVVG--------ISRSCTKWNVMVKSVEELPLRINE 232
          : :: . *           . .  :              :     :         :

P.p/IPDC VLTTMLRERR-PGYLMLPADVAKK--AATPP-VNALTHKQA-----NADSACLKAFRDAA 202
S.c/PDC  CIRTTYVTQR-PVYLGLPANLVDLNVPAKLL-QTPIDMSLK-----PNDAESEKEVIDTI 204
Z.mo/PDC VIKTALREKK-PVYLEIACNIA--SMPCAAPGPASALFNDE-----ASDEASLNAAVEET 202
Z.ma/PDC AIATALRESK-PVYISVSCNLAGLSHPTFSRDPVPMFISPR-----LSNKANLEYAVEAA 249
B.n/ALS  AFFLATSGRPGPVLVDVPKDIQQQLAIPNWDQPMRLPGYMS-----RLPQPPEVSQLGQI 274
E.c/ALS  AFDVASSGRPGPVLVDIPKDIQ--LASGDLEPWFTTVENEV-----TFPH----AEVEQA 189
S.c/ALS  AFEIATSGRPGPVLVDLPKDVTAAILRNPIPTKTTLPSNALNQLTSRAQDEFVMQSINKA 292
          :         *  : :. ::

P.p/IPDC ENKLAMSKRTALLADF-LVLRHGLKHALQKWVKEVPMAHATMLMGKGIFDERQAGFYGTY 261
S.c/PDC  LVLAKDAKNPVILADA-CCSRHDVKAETKKLIDLTQFPAFVTPMGKGSISEQHPRYGGVY 263
Z.mo/PDC LKFIANRDKVAVLVGS-KLRAAGAEEAAVKFADALGGAVATMAAAKSFFPEENPHYIGTS 261
Z.ma/PDC ADFLNKAVKPVMVGGP-KIRVAKAREAFAAVADASGYPFAVMPAAKGLVPEHHPRFIGTY 308
B.n/ALS  VRLISESKRPVLYVGG---GSLNSSEELGRFVELTGIPVASTLMGLGSYPCNDELSLQ-M 330
E.c/ALS  RQMLAKAQKPMLYVGGGV-GMAQAVPALREFLATTKMPATCTLKGLGAVEADYPYYLG-M 247
S.c/ALS  ADLINLAKKPVLYVGAGILNHADGPRLLKELSDRAQIPVTTTLQGLGSFDQEDPKSLD-M 351
                  .  :  .                        .       . .
```

```
P.p/IPDC SGSASTGAVKEAIEGADTVLCVGTRFTDTLTAGFTHQLTPAQ---------TIEVQPHAA 312
S.c/PDC  VGTLSKPEVKEAVESADLILSVGALLSDFNTGSFSYSYKTKN---------IVEFHSDHM 314
Z.mo/PDC WGEVSYPGVEKTMKEADAVIALAPVFNDYSTTGWTDIPDPKK---------LVLAEP-RS 311
Z.ma/PDC WGAVSTTFCAEIVESADAYLFAGPIFNDYSSVGYSLLLKREK---------AVIVQPDRM 359
B.n/ALS  LGMHGTVYANYAVEHSDLLLAFGVRFDDRVTGKLEAFASRAK---------IVHIDIDSA 381
E.c/ALS  LGMHGTKAANFAVQECDLLIAVGARFDDRVTGKLNTFAPHAS---------VIHMDIDPA 298
S.c/ALS  LGMHGCATANLAVQNADLIIAVGARFDDRVTGNISKFAPEARRAAAEGRGGIIHFEVSPK 411
          *  .       :: .*  :  .  : *  :                      :  .

P.p/IPDC RVGD-VWFTGIPMNQAIETLV----ELSKQ-----------HVHTGLMSSSS-GAIPFPQ 355
S.c/PDC  KIRN-ATFPGVQMKFVLQKLLTNIADAAKG-----------YKPVAVPARTP-ANAAVP- 360
Z.mo/PDC VVVNGIRFPSVHLKDYLTRLAQKVSKKTGA-----------LDFFKSLNAGELKKAAPAD 360
Z.ma/PDC VVGDGPAFGCILMPEFLRALAKRLRRNTTA-----------YDNYRRIFVPD-REPPNGK 407
B.n/ALS  EIGKNKTPHVSVCGDVKLALQGMNKVLENRAEELKLDFGVWRSELSEQKQKFPLSF--KT 439
E.c/ALS  EMNKLRQAHVALQGDLNALLPALQQPLN---------INDWQQYCAQLRDEHAWRY--DH 347
S.c/ALS  NINKVVQTQIAVEGDATTNLGKMMSKIFPVKE-----RSEWFAQINKWKKEYPYAYMEET 466
          : .                 *

P.p/IPDC PDGSLTQENFWRTLQTFIR--PGDIILADQG--TSAFGAIDLRLPADVNFIVQPLWGSIG 411
S.c/PDC  ASTPLKQEWMWNQLGNFLQ--EGDVVIAETG--TSAFGINQTTFPNNTYGISQVLWGSIG 416
Z.mo/PDC PSAPLVNAEIARQVEALLT--PNTTVIAETG--DSWFNAQRMKLPNGARVEYEMQWGHIG 416
Z.ma/PDC PNEPLRVNVLFKHIKGMLS--GDSAVVAETG--DSWFNCQKLRLPEGCGYEFQMQYGSIG 463
B.n/ALS  FGEAIPPQYAIQVLDELT---QGKAIISTGVGQHQMWAAQFYKYRKPRQWLSSSGLGAMG 496
E.c/ALS  PGDAIYAPLLLKQLSDRK---PADCVVTTDVGQHQMWAAQHIAHTRPENFITSSGLGTMG 404
S.c/ALS  PGSKIKPQTVIKKLSKVANDTGRHVIVTTGVGQHQMWAAQHWTWRNPHTFITSGGLGTMG 526
          .  :      . :           :::      . :                .   * :*

P.p/IPDC YT----LAAAFGAQTACPNRRVIVLTGDGAAQLTIQELGSMLRDKQHPIILVLNNE--GY 465
S.c/PDC  FTTGATLGAAFAAEEIDPKKRVILFIGDGSLQLTVQEISTMIRWGLKPYLFVLNND--GY 474
Z.mo/PDC WS----VPAAFGYAVGAPERRNILMVGDGSFQLTAQEVAQMVRLKLPVIIFLINNY--GY 470
Z.ma/PDC WS----VGATLGYAQAAKDKRVIACIGDGSFQVTAQDVSTMLRCGQKSIIFLINNG--GY 517
B.n/ALS  FG----LPAAIGASVANPDAIVVDIDGDGSFIMNVQELATIRVENLPVKILLLNNQHLGM 552
E.c/ALS  FG----LPAAVGAQVARPNDTVVCISGDGSFMMNVQELGTVKRKQLPLKIVLLDNQRLGM 460
S.c/ALS  YG----LPAAIGAQVAKPESLVIDIDGDASFNMTLTELSSAVQAGTPVKILILNNEEQGM 582
          :     : *:..       .   :   **.:  :.  ::.           :.:::*   *

P.p/IPDC TVERAIHGAEQRYNDIALWN-------WTHIPQALSLDP-----QSECWRVSEAEQLADV 513
S.c/PDC  TIEKLIHGPKAQYNEIQGWD-------HLSLLPTFGAK------DYETHRVATTGEWDKL 521
Z.mo/PDC TIEVMIH--DGPYNNIKNWD-------YAGLMEVFNGNGGYDSGAGKGLKAKTGGELAEA 521
Z.ma/PDC TIEVEIH--DGPYNVIKNWD-------YTGLVNAIHNS----EGNCWTMKVRTEEQLKEA 564
B.n/ALS  VMQWEDRFYKANRAHTYLGDPARENEIFPNMLQFAGACG------IPAARVTKKEELREA 606
E.c/ALS  VRQWQQLFFQERYSETTLTD-------NPDFLKLASAFG------IPGQHITRKDQVEAA 507
S.c/ALS  VTQWQSLFYEHRYSHTHQLN--------PDFIKLAEAMG------LKGLRVKKQEELDAK 628
          . :      .         :         :                  :     :

P.p/IPDC LEKVAHHER--LSLIEVMLPKADIPPLLGALTKALEARNNA------------------ 550
S.c/PDC  TQDKSFNDNSKIRMIEVMLPVFDAPQNLVEQAKLTAATNAKQ----------------- 563
Z.mo/PDC IK-VALANTDGPTLIECFIGREDCTEELVKWGKRVAAANSRKPVNKLL---------- 568
Z.ma/PDC IATVTGAKKDCLCFIEVIVHKDDTSKELLEWGSRVSAANSRPPNPQ------------ 610
B.n/ALS  IQTMLDTPG--PYLLDVICPHQEHVLPMIPSGGTFKDVITEGDGRTKY---------- 652
E.c/ALS  LDTMLNSDG--PYLLHVSIDELENVWPLVPPGASNSEMLEKLS--------------- 548
S.c/ALS  LKEFVSTKG--PVLLEVEVDKKVPVLPMVAGGSGLDEFINFDPEVERQQTELRHKRTGGK 686
                    ::.          :
```

protein family, involved in amino acid biosynthesis, from bacteria and plastids found in some yeast, plants and algae (Fig. 14).

**Isolation of the *ipdc* Promoter Region**

The promoter region for the *P. putida* GR12-2 *ipdc* gene was isolated by inverse PCR as outlined in Fig. 15. Briefly, *P. putida* GR12-2 genomic DNA was digested with *Kpn*I because it was known that this restriction enzyme would cut within the *ipdc* gene as well as at an unknown site somewhere upstream of the *ipdc* gene. This facilitated isolation of only the upstream region and avoided isolation of the downstream region which would have unnecessarily complicated the identification of the promoter sequence. Following circularization of the *Kpn*I genomic fragments, the region upstream of the *ipdc* gene was amplified using PCR primers designed to anneal to regions within the *ipdc* gene as illustrated in Fig. 16. Forward and reverse primers were chosen to bind just upstream of the *Kpn*I recognition site and slightly downstream of the start codon in the *ipdc* sequence, respectively, such that PCR products containing the *ipdc* upstream region could be confirmed by the presence of some *ipdc* coding sequence. Agarose gel electrophoresis of PCR products revealed a single band of about 700 bp (Fig. 17A). Subsequent sequence analysis of the PCR products suggested that a sequence recognized by the RNA polymerase sigma factor RpoD ($\delta^{70}$), characterized by the consensus sequence TTGACA and TATAAT at positions –35 and –10, respectively, and separated by a spacer of 16-18 nucleotides, was not present; however, three possible stationary phase sigma factor RpoS ($\delta^{38}$) recognition sequences (CTATACT) were apparent (Fig. 17B). In addition, two putative ribosome binding sites were revealed (Fig. 17B). Interestingly, the region further upstream from the binding site for the

Figure 15. Strategy to isolate the *P. putida* GR12-2 *ipdc* promoter region using inverse PCR. *P. putida* GR12-2 genomic DNA was digested with *Kpn*I which is known to cut within the *ipdc* gene. *Kpn*I fragments were circularized by ligation with T4 DNA ligase and the region upstream of the *ipdc* gene was amplified using PCR primers designed to anneal to regions within the *ipdc* gene.

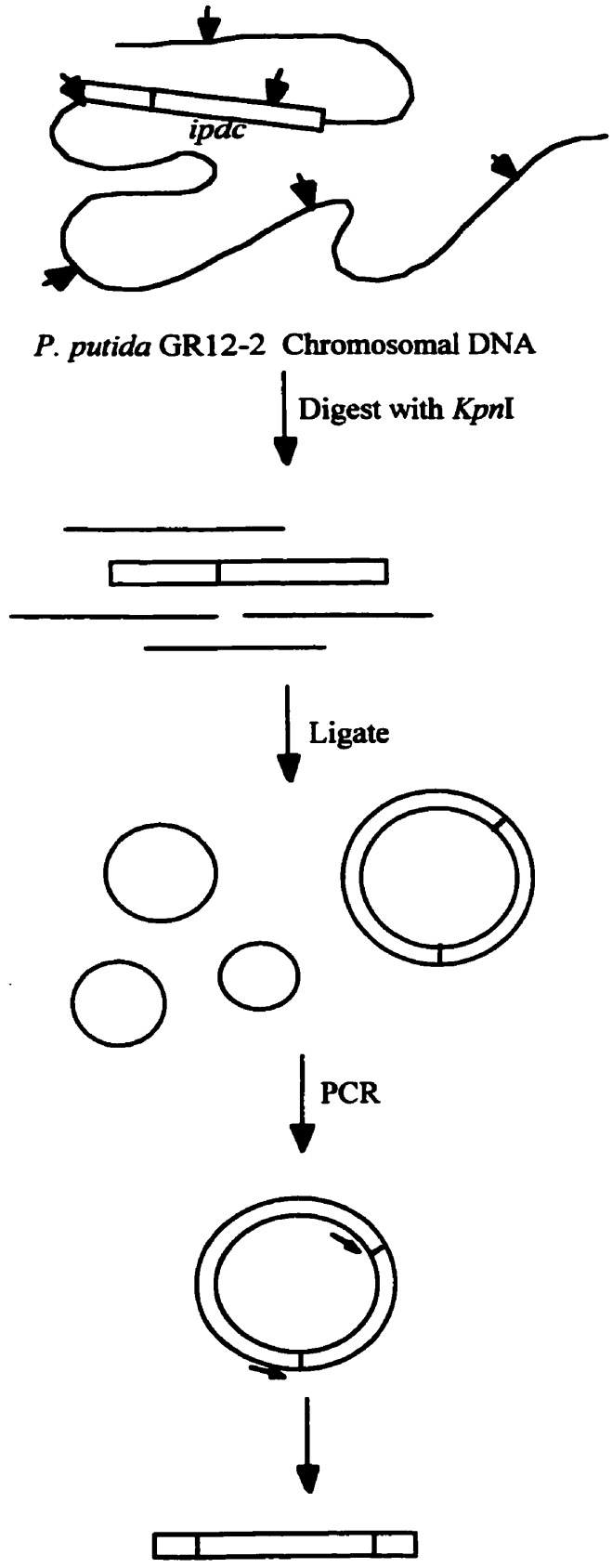

ipdc
P. putida GR12-2 Chromosomal DNA
Digest with KpnI
Ligate
PCR

Figure 16. Nucleotide sequence of the *P. putida* GR12-2 *ipdc* gene showing positions within the gene for binding of PCR primers used to amplify the region upstream of the *ipdc* gene. The binding position for each primer and the direction of amplification are indicated by the arrows. The unique *Kpn*I site within the *ipdc* gene is underlined.

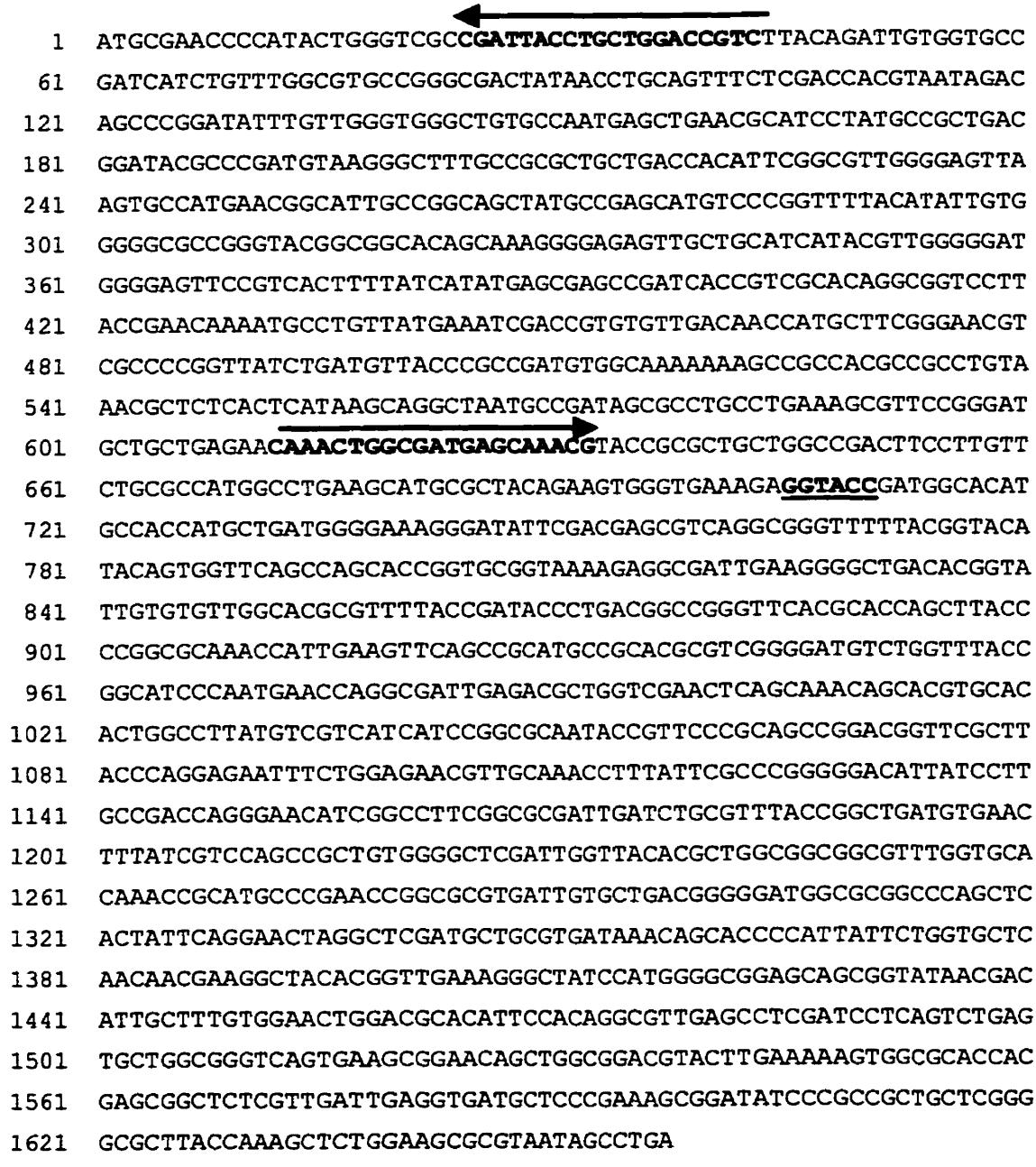
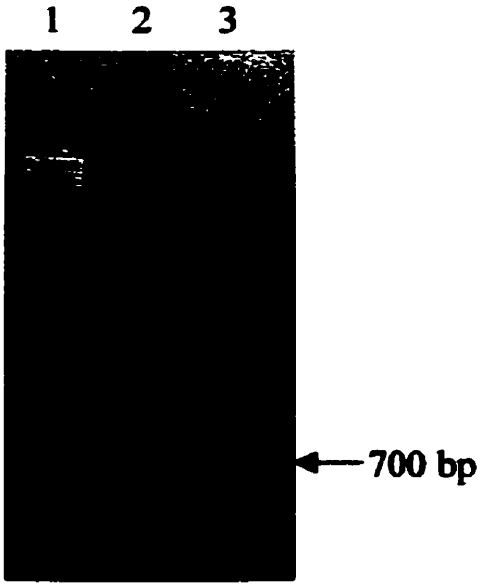

1 ATGCGAACCCCATACTGGGTCGCCGATTACCTGCTGGACCGTCTTACAGATTGTGGTGCC
61 GATCATCTGTTTGGCGTGCCGGGCGACTATAACCTGCAGTTTCTCGACCACGTAATAGAC
121 AGCCCGGATATTTGTTGGGTGGGCTGTGCCAATGAGCTGAACGCATCCTATGCCGCTGAC
181 GGATACGCCCGATGTAAGGGCTTTGCCGCGCTGCTGACCACATTCGGCGTTGGGGAGTTA
241 AGTGCCATGAACGGCATTGCCGGCAGCTATGCCGAGCATGTCCCGGTTTTACATATTGTG
301 GGGGCGCCGGGTACGGCGGCACAGCAAAGGGGAGAGTTGCTGCATCATACGTTGGGGGAT
361 GGGGAGTTCCGTCACTTTTATCATATGAGCGAGCCGATCACCGTCGCACAGGCGGTCCTT
421 ACCGAACAAAATGCCTGTTATGAAATCGACCGTGTGTTGACAACCATGCTTCGGGAACGT
481 CGCCCCGGTTATCTGATGTTACCCGCCGATGTGGCAAAAAAAGCCGCCACGCCGCCTGTA
541 AACGCTCTCACTCATAAGCAGGCTAATGCCGATAGCGCCTGCCTGAAAGCGTTCCGGGAT
601 GCTGCTGAGAACAAACTGGCGATGAGCAAACGTACCGCGCTGCTGGCCGACTTCCTTGTT
661 CTGCGCCATGGCCTGAAGCATGCGCTACAGAAGTGGGTGAAAGAGGTACCGATGGCACAT
721 GCCACCATGCTGATGGGGAAAGGGATATTCGACGAGCGTCAGGCGGGTTTTTACGGTACA
781 TACAGTGGTTCAGCCAGCACCGGTGCGGTAAAAGAGGCGATTGAAGGGGCTGACACGGTA
841 TTGTGTGTTGGCACGCGTTTTACCGATACCCTGACGGCCGGGTTCACGCACCAGCTTACC
901 CCGGCGCAAACCATTGAAGTTCAGCCGCATGCCGCACGCGTCGGGGATGTCTGGTTTACC
961 GGCATCCCAATGAACCAGGCGATTGAGACGCTGGTCGAACTCAGCAAACAGCACGTGCAC
1021 ACTGGCCTTATGTCGTCATCATCCGGCGCAATACCGTTCCCGCAGCCGGACGGTTCGCTT
1081 ACCCAGGAGAATTTCTGGAGAACGTTGCAAACCTTTATTCGCCCGGGGGACATTATCCTT
1141 GCCGACCAGGGAACATCGGCCTTCGGCGCGATTGATCTGCGTTTACCGGCTGATGTGAAC
1201 TTTATCGTCCAGCCGCTGTGGGGCTCGATTGGTTACACGCTGGCGGCGGCGTTTGGTGCA
1261 CAAACCGCATGCCCGAACCGGCGCGTGATTGTGCTGACGGGGGATGGCGCGGCCCAGCTC
1321 ACTATTCAGGAACTAGGCTCGATGCTGCGTGATAAACAGCACCCCATTATTCTGGTGCTC
1381 AACAACGAAGGCTACACGGTTGAAAGGGCTATCCATGGGGCGGAGCAGCGGTATAACGAC
1441 ATTGCTTTGTGGAACTGGACGCACATTCCACAGGCGTTGAGCCTCGATCCTCAGTCTGAG
1501 TGCTGGCGGGTCAGTGAAGCGGAACAGCTGGCGGACGTACTTGAAAAAGTGGCGCACCAC
1561 GAGCGGCTCTCGTTGATTGAGGTGATGCTCCCGAAAGCGGATATCCCGCCGCTGCTCGGG
1621 GCGCTTACCAAAGCTCTGGAAGCGCGTAATAGCCTGA

Figure 17. The region upstream of the *P. putida* GR12-2 *ipdc* gene. A, Inverse PCR products following agarose gel electrophoresis (Lane 3). The 700 bp fragment carrying the upstream region is indicated by the arrow. Lane 1, DNA size markers; Lane 2, PCR products from water as a template. B, Nucleotide sequence of the upstream region showing possible binding sites for sigma factor RpoS (overlined) and ribosomes (underlined), and the translation start codon for indolepyruvate decarboxylase (in bold). The position of the start codon and the direction of translation for a putative potassium ion channel protein is indicated by an arrow.

**A**




**B**

```
  1  ATCCACATATTCCAGCCCCATACGCTTCAGGCTTTGATCCAGGCTGGCAACCAGATATTT
       V  Y  E  L  G  M  R  K  L  S  Q  D  L  S  A  V  L  Y  K  R
 61  GCGTGACCCCCAGTCGCCGTAGGGGCCATCCCACATGGTATAACCCGCTTTGGTCGAGAT
       S  G  W  D  G  Y  P  G  D  Q  M  T  Y  G  A  K  T  S  I  I
121  GATCAGCTCGTCGCGCCATGGCAGGAAATCTTCCTGCAAAATGCGACCGAAATTACGTTC
       L  E  D  R  W  P  L  F  D  E  Q  L  I  R  G  F  N  R  E  A
181  GGCTGAGCCGGGAGGAGGACCGTAATTATTGGCAAGGTCGAAATGCGTAATACCCAGATC
       S  G  P  P  P  G  Y  N  N  A  L  D  F  H  T  I  G  L  D  F
241  GAACGCGCGTTGTAAAAGTTGACGGCTGTTTTCGACAAGCGTGGCGTCGCCAAAATTGTG
       A  R  Q  L  L  Q  R  S  S  N  E  V  L  T  A  D  G  F  N  H
301  CCACAACCCGAGTGAGATGGCAGGCAACCTGAGTCCGCTTTGCCCGCAGCGGCGATACTG
       W  L  G  L  S  I  A  P  L  R  L  G  S  Q  G  C  R  R  Y  Q
361  CATTGTCTGATAACGATTTTTGTCCGGCTGGTAACCCATTCTGATGCCCTCTGGCGCTGA
       M
421  AAGGAAAAATCAGTGTATACGTTTACATTTACATGAAAAAAAAGAGCATAGCGCAGCCTT
481  TTTTGTAAAGCATTCTTTCCATGCCCTTCTTACGACCAATTCTGGACAGCCATCACGCTT
541  CTTTAATACTCAAAGTGAGGTCAACGTCAGAAGGACACCTGTTATGCGAACCCCATACTG
                                                  M  R  T  P  Y  W
601  GGTCGCCCATTACCTGCTGGACCGTCTTACAGATTGTGGTGCCGATCATCTGTTTGGCGT
       V  A  H  Y  L  L  D  R  L  T  D  C  G  A  D  H  L  F  G  V
```

transcriptional machinery is significantly similar (63% identical over approximately 400 bp) to the corresponding nucleotide sequence upstream of the *ipdc* gene from *Erwinia herbicola* (Brandl and Lindow, 1996). This region may encode a potassium ion channel protein subunit similar to that found in many organisms including microorganisms, animals and plants (Fig. 18). The protein is transcribed from the strand opposite to that carrying the *ipdc* gene, beginning from the translation start codon 220 bases upstream of the start codon for indolepyruvate decarboxylase (Fig. 17B).

That the isolated sequence indeed corresponded to the region upstream from the *ipdc* gene was confirmed by several means. As expected, the sequence of the products from inverse PCR revealed that the 5' end was identical to approximately 60 bases just upstream from the *Kpn*I site in the *ipdc* gene, and similarly, the 3' end of the PCR products was identical to the first 23 bases of the *ipdc* gene. In addition, PCR primers designed from the 5' end of the isolated upstream sequence and from the 3' end of the *ipdc* structural gene, amplified a region of *P. putida* GR12-2 genomic DNA of expected size (2.2 kb, Fig. 19A) and with expected restriction enzyme recognition sites (Fig. 19B).

The proximity of the promoter to the *ipdc* gene, and the presence of a putative transcription termination sequence and a putative gene transcribed from the opposite strand in the region downstream of the *ipdc* gene, indicate that the genes involved in IAA biosynthesis in this bacterium are not organized in an operon.

**Characterization of *ipdc* Promoter Activity**

The 700 bp PCR fragment was initially inserted into vector pGEM®-T by ligating the single deoxyadenosine on the 5' ends of the PCR products, generated by *Taq* polymerase,

Figure 18. Alignment of the open reading frame upstream of the *P. putida* GR12-2 *ipdc* gene with the sequences for a potassium ion channel protein from *E. coli* (E.c), *Arabidopsis thaliana* (A.t) and *Homo sapiens* (H.s). The numbers shown to the right of the *P. putida* GR12-2 sequence (P.p) indicate the nucleotide position upstream relative to the indolepyruvate decarboxylase start codon.

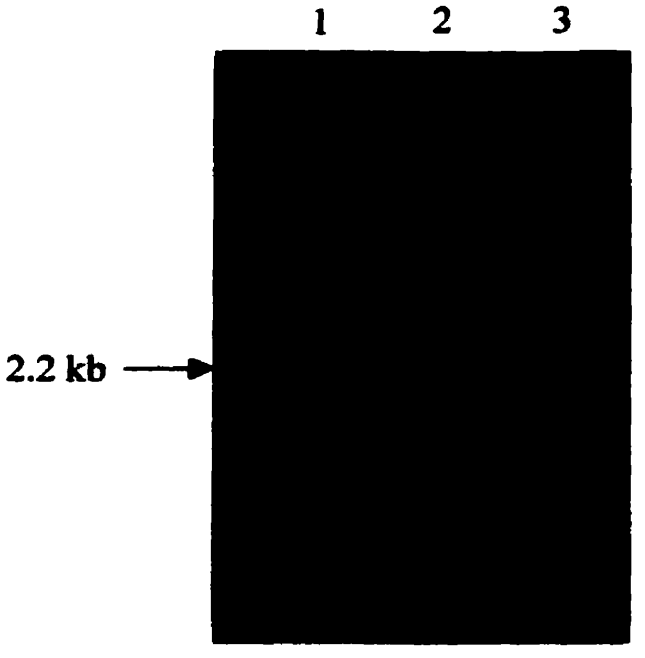
```
P.p   ----------------------MQYRRCGQSGLRLPAISLGLWHNFGDATLVENSSRQLL 334
E.c   ----------MVWLANPERYGQMQYRYCGKSGLRLPALSLGLWHNFGHVNALE-SQRAIL 49
A.t   ----------------------MQYKNLGKSGLKVSTLSFGAWVTFGNQLDVK-EAKSIL 37
H.s   MYPESTTGSPARLSLRQTGSPGMIYRNLGKSGLRVSCLGLGTWVTFGGQITDE-MAEQLM 59

P.p   QRAFDLGITHFDLANNYGPPPGSAERNFGRILQEDFLPWRDELIISTKAGYTMQDGPYGD 514
E.c   RKAFDLGITHFDLANNYGPPPGSAEENFGRLLREDFAAYRDELIISTKAGYDMWPGPYGS 109
A.t   QCCRDHGVNFFDNAEVYAN--GRAEEIMGQAIRE--LGWRRSDIVISTKIFWGGPGPNDK 93
H.s   TLAYDNGINLFDTAEVYAA--GKAEVVLGNIIKK--KGWRRSSLVITTKIFWGGKAETER 115

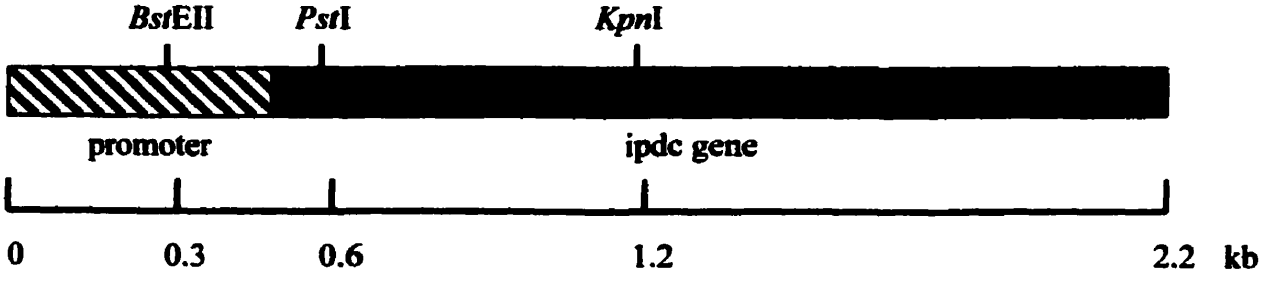
P.p   WGSRKYLVASLDQSLKRMGLEYVD---------------------------------- 586
E.c   GGSRKYLLASLDQSLKRMGLEYVDIFYSHRVDENTPMEETASALAHAVQSGKALYVGISS 169
A.t   GLSRKHIVEGTKASLKRLDMDYVDVLYCHRPDASTPIEEAVRAMNYVIDKGWAFYWGISE 153
H.s   GLSRKHIIEGLKASLERLQLEYVDVVFANRPDPNTPMEETVRAMTHVINQGMAMYWGTSR 175
```

Figure 19. The 2.2 kb genomic fragment carrying the *P. putida* GR12-2 *ipdc* gene and its upstream flanking region. A, Agarose gel electrophoresis of PCR products using primers designed to anneal to the 5' end of the upstream region and the 3' end of the *ipdc* gene from *P. putida* GR12-2 (Lane 1) and water (Lane 2) as a template. Lane 3, 1 kb ladder DNA size markers. B, Map of restriction enzyme recognition sites.

A




B



with compatible thymidine overhangs in the cloning site of the vector. The promoter fragment was subsequently transferred from pGEM®-T as an *Nco*I-*Sal*I fragment to *Nco*I/*Hin*dIII-digested pQF70 carrying the *luxAB* reporter gene to generate pQFPROM (the *Sal*I site was abolished but the *Hin*dIII site was regenerated following treatment with Klenow polymerase to fill in sticky ends) (Fig. 20).

Light production (LuxAB activity) in transformed *E.coli* DH5α cells, driven by the *ipdc* promoter, was quantified in a luminometer before isolating plasmids for further manipulation. *E. coli* DH5α cells carrying plasmids with the promoter fragment inserted upstream of *luxAB* in pQFPROM produced about one hundred times more light in rich medium than cells carrying plasmids without the promoter inserted (pQF70) (Table 4). Because *P. putida* GR12-2 is naturally resistant to ampicillin (100 μg/mL), cells transformed with pQFPROM could not be selected using the ampicillin resistance marker on the plasmid. Therefore, a kanamycin resistance gene was inserted into the *Pvu*II site of pQFPROM to generate pQFPROM-Kan (Fig. 20). Colonies of both *E. coli* DH5α/pQFPROM and *P. putida* GR12-2/pQFPROM-Kan growing on agar plates produced light in the presence of n-decyl aldehyde, whereas corresponding control cells, *E. coli* DH5α/pQF70 and *P. putida* GR12-2/pQF70-Kan, did not (Fig. 21).

Effect of tryptophan and RpoS on promoter activity

Preliminary experiments indicated that *ipdc* promoter activity in *P. putida* GR12-2/pQFPROM-Kan, assayed by measuring light production by LuxAB, increased sharply in the presence of tryptophan around the onset of stationary phase. To determine whether the stationary phase sigma factor RpoS regulates the *ipdc* promoter, *P. putida* GR12-2/pQFPROM-Kan, and *P. putida* GR12-2/pQF70-Kan as a negative control, were

Figure 20. Construction of promoter reporter plasmids pQFPROM and pQFPROM-Kan. The 0.6 kb *Nco*I-*Sal*I *ipdc* promoter fragment was excised from the pGEM-T vector and inserted into the *Nco*I-*Hin*dIII site upstream of the *luxAB* reporter gene in pQF70 to create pQFPROM. The orientation of the promoter was preserved by ligation into the *Nco*I site; the *Sal*I site was abolished but the *Hin*dIII site was regenerated following treatment with Klenow polymerase to fill in sticky ends and subsequent blunt end ligation. The kanamycin resistance gene, carried on a 2.3 kb *Eco*RI fragment, was removed from pHP45Ω-Kn and inserted into the *Pvu*II site in pQFPROM to generate pQFPROM-Kan.

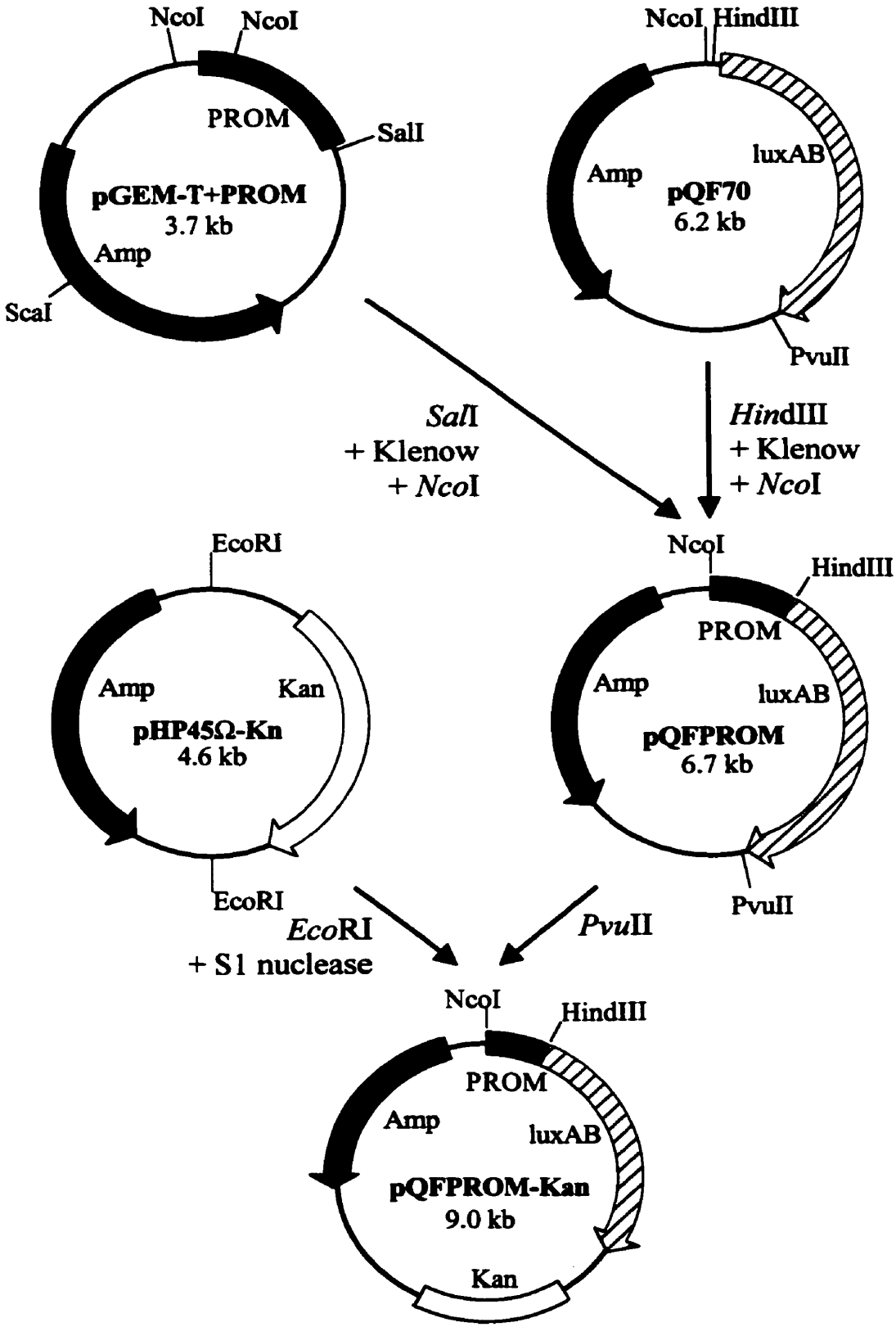
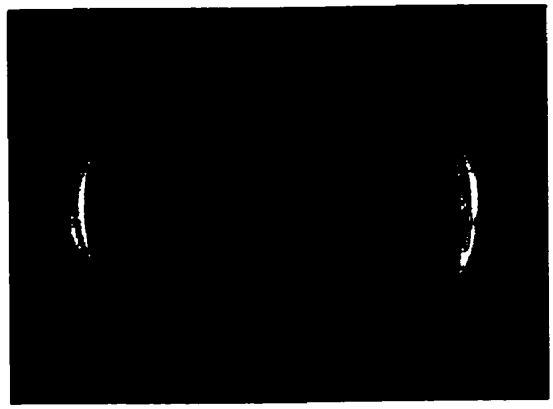

NcoI
NcoI
PROM
SalI
pGEM-T+PROM
3.7 kb
Amp
ScaI
NcoI HindIII
luxAB
Amp
pQF70
6.2 kb
PvuII
SalI
+ Klenow
+ NcoI
HindIII
+ Klenow
+ NcoI
EcoRI
Amp
Kan
pHP45Ω-Kn
4.6 kb
EcoRI
NcoI
HindIII
PROM
Amp
luxAB
pQFPROM
6.7 kb
PvuII
EcoRI
+ S1 nuclease
PvuII
NcoI
HindIII
PROM
Amp
luxAB
pQFPROM-Kan
9.0 kb
Kan

Table 4. Light production (Lux activity) in *E. coli* cells carrying reporter plasmids with and without the *P. putida* GR12-2 *ipdc* promoter inserted upstream of *luxAB* (pQF70 and pQFPROM, respectively).

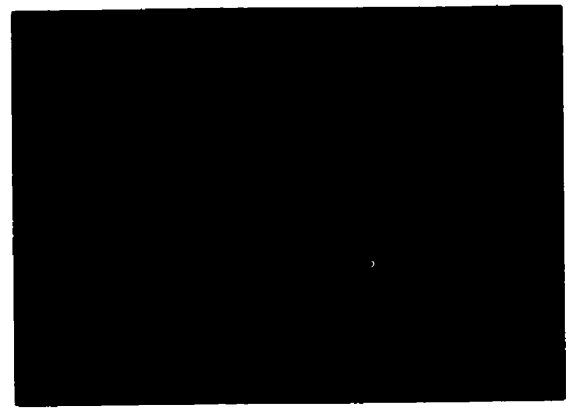
| Strain | Lux Activity (RLU) | |
|---|---|---|
| | Trial 1 | Trial 2 |
| *E. coli*/pQF70 | 68 | 63 |
| *E. coli*/pQFPROM | 6676 | 6830 |

Figure 21. *E. coli* DH5α/pQFPROM and *P. putida* GR12-2/pQFPROM-Kan, carrying the *ipdc* promoter region upstream of the *luxAB* reporter gene, produce light in the presence of n-decyl aldehyde whereas control cells without the promoter region, *E. coli* DH5α/pQF70 and *P. putida* GR12-2/pQF70-Kan, do not. A and B, *E. coli* DH5α/pQF70 (top half of plates) and *E. coli* DH5α/pQFPROM (bottom half of plates) in the light (A) and in the dark (B). C and D, *P. putida* GR12-2/pQF70-Kan (top half of plates) and *P. putida* GR12-2/pQFPROM-Kan (bottom half of plates) in the light (C) and in the dark (D).

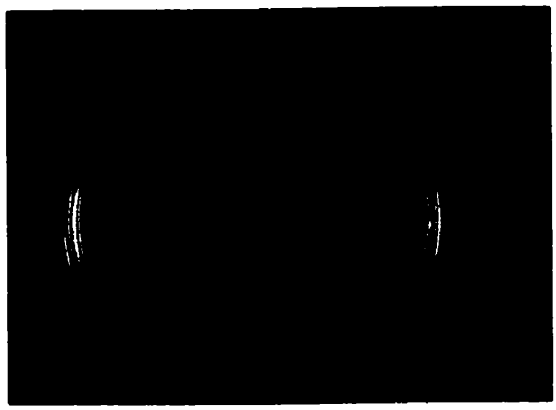
A



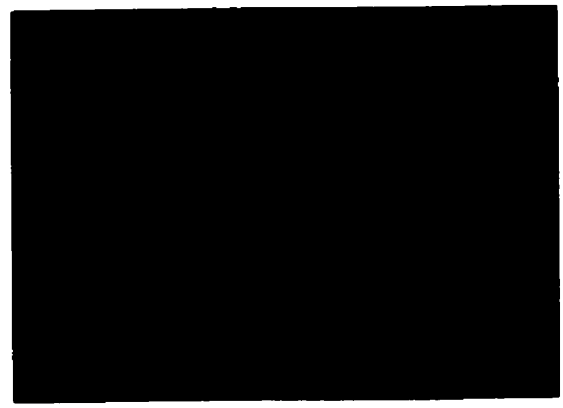
B



C



D



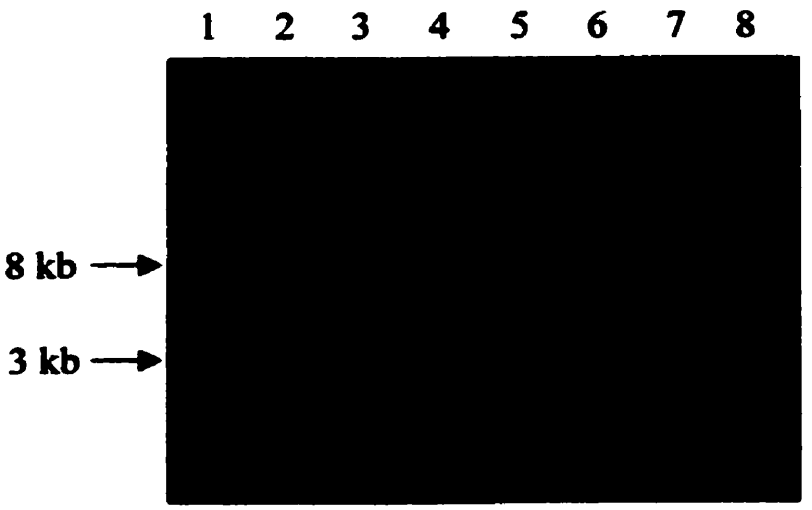
transformed with pJEL5649 (pRpoS) which carries the gene encoding RpoS from *Pseudomonas fluorescens* Pf-5 (Sarniguet et al., 1995). Resistance to both kanamycin (encoded on pQF70-Kan and pQFPROM-Kan) and tetracycline (encoded on pRpoS), and the pattern of bands for uncut and *Eco*RI-digested plasmids isolated from *P. putida* GR12-2/pQF70-Kan/pRpoS (Fig. 22A, lane 3) and from *P. putida* GR12-2/pQFPROM-Kan/pRpoS (Fig. 22B, lane 3) in an agarose gel, confirmed that these strains carry both pRpoS (Figs. 22A, lanes 2 and 3; Fig. 22B, lane 2) and the *luxAB* reporter plasmid (Figs. 22A, lanes 6 and 7; Fig. 22B, lane 4).

The growth of all four strains, that is, *P. putida* GR12-2/pQF70-Kan, *P. putida* GR12-2/pQFPROM-Kan, *P. putida* GR12-2/pQF70-Kan/pRpoS, and *P. putida* GR12-2/pQFPROM-Kan/pRpoS, was similar whether cultured in the presence or absence of tryptophan (Fig. 23A and 23B). The rate of cell proliferation was maximal from about 8 hours until the onset of stationary phase at around 20 hours.

Production of IAA by all strains in minimal medium without tryptophan was very low throughout the 48 hour period of the experiment, never reaching more than 1 μg/mL. In media supplemented with tryptophan (200 μg/mL), IAA was first apparent in cultures of *P. putida* GR12-2 strains carrying extra copies of *rpoS* (on pRpoS) at 16 hours (Fig. 24A and 24B). At this time, IAA production in cells without pRpoS was still very low. These latter cells without pRpoS began to produce IAA in the presence of tryptophan four hours later, and at levels well below those of cells overexpressing RpoS. Strains carrying extra copies of the *ipdc* promoter, on pQFPROM-Kan, always lagged behind corresponding strains carrying pQF70-Kan, which does not carry the promoter fragment, in IAA production, although this was alleviated somewhat by RpoS overproduction in the early stages of the

Figure 22. Agarose gel electrophoresis of plasmids isolated from *P. putida* GR12-2/pQF70-Kan/pRpoS (A; Lane 3, *Eco*RI digest) and *P. putida* GR12-2/pQFPROM-Kan/pRpoS (B; Lane 4, uncut; Lane 5, *Eco*RI digest) to confirm the presence of pRpoS (A; Lane 2, uncut; Lane 3, *Eco*RI digest; B; Lane 2, *Eco*RI digest), and pQF70-Kan (A; Lane 6, uncut; Lane 7, *Eco*RI digest) or pQFPROM-Kan (B; Lane 4, *Eco*RI digest). (A; Lanes 1 and 8, and B; Lanes 1 and 5, 1 kb ladder DNA size markers)

A




B

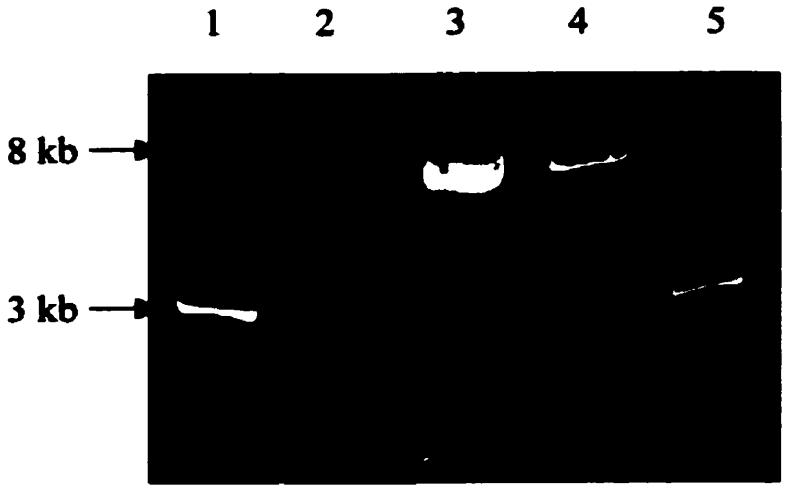
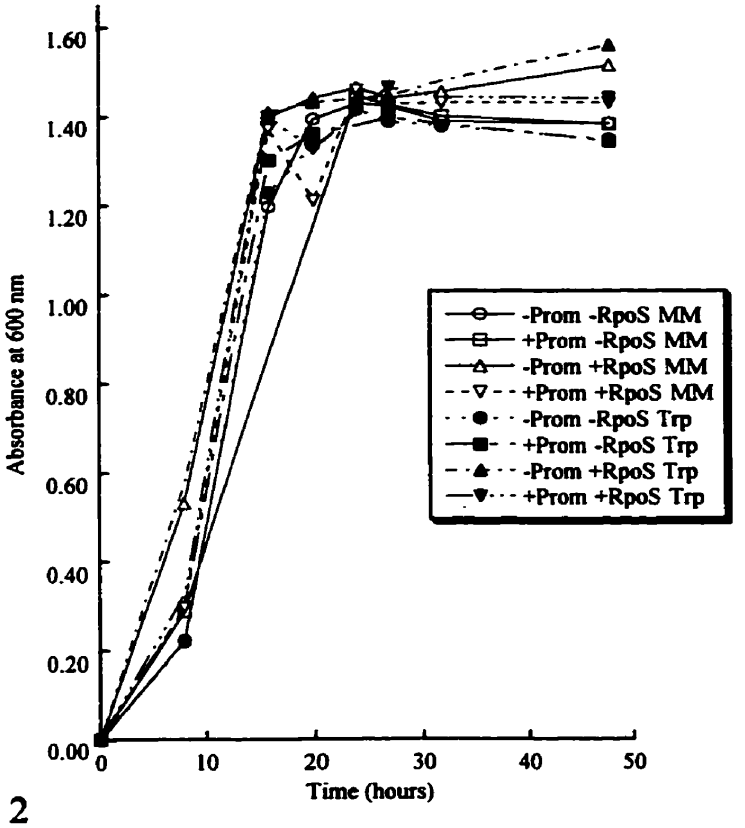


Figure 23. Growth of *P. putida* GR12-2/pQF70-Kan, *P. putida* GR12-2/pQFPROM-Kan, *P. putida* GR12-2/pQF70-Kan/pRpoS, and *P. putida* GR12-2/pQFPROM-Kan/pRpoS in the presence (Trp) and absence (MM) of tryptophan (200 μg/mL) over a 48 hour period. Growth was determined spectrophotometrically by the absorbance of the cultures at 600 nm. (+Prom indicates the presence of pQFPROM-Kan; -Prom indicates the presence of pQF70-Kan and ±RpoS indicates the presence or absence of pRpoS in cells; n=2 for each strain at each time point.)

Exp. 1


Absorbance at 600 nm
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0
10
20
30
40
50
Time (hours)
-Prom -RpoS MM
+Prom -RpoS MM
-Prom +RpoS MM
+Prom +RpoS MM
-Prom -RpoS Trp
+Prom -RpoS Trp
-Prom +RpoS Trp
+Prom +RpoS Trp

Exp. 2

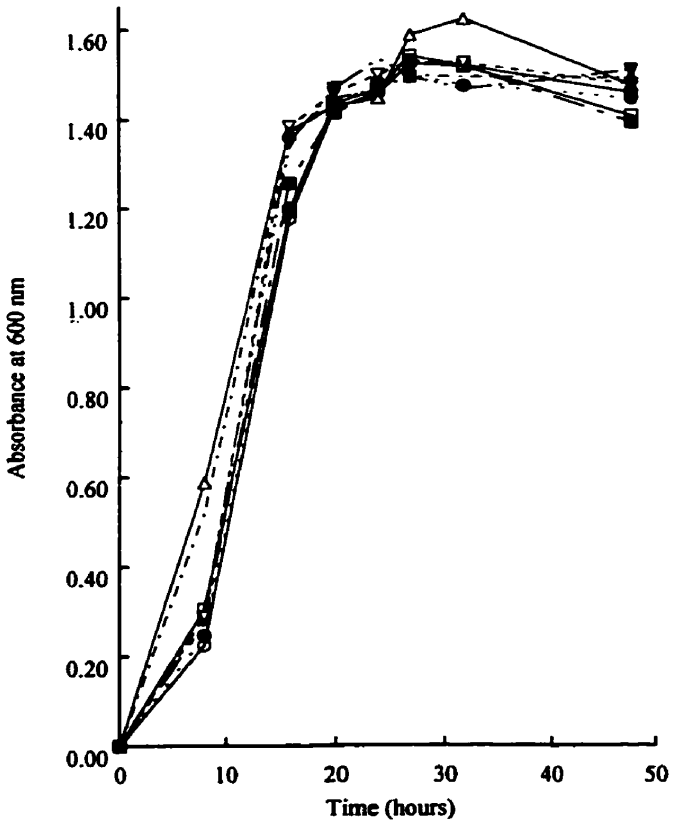
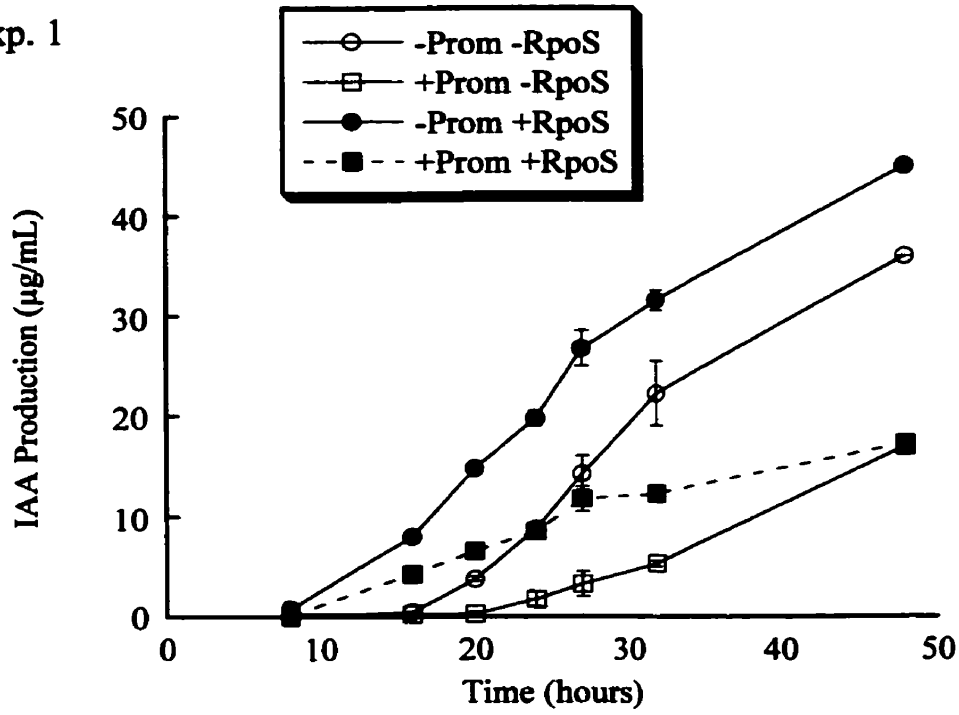
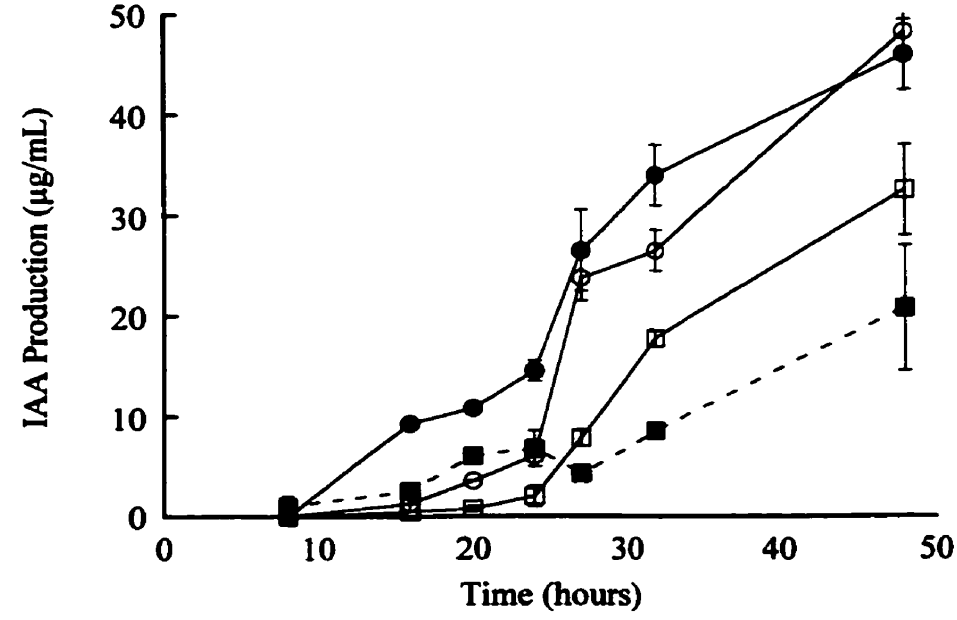

Absorbance at 600 nm
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0
10
20
30
40
50
Time (hours)

Figure 24. IAA production by *P. putida* GR12-2/pQF70-Kan (-Prom) and *P. putida* GR12-2/pQFPROM-Kan (+Prom) in the presence of tryptophan (200 μg/mL) and in the presence or absence of pRpoS (±RpoS) over a 48 hour period. In each experiment, n=2 for each strain at each time point; error bars indicate standard error of the mean (SE). Where error bars are not visible, they are smaller than the marker.

Exp. 1


-Prom -RpoS
+Prom -RpoS
-Prom +RpoS
+Prom +RpoS
IAA Production (µg/mL)
Time (hours)
0
10
20
30
40
50

Exp. 2


IAA Production (µg/mL)
Time (hours)
0
10
20
30
40
50

growth cycle. These trends continued as IAA accumulated in the culture media, at least to 48 hours; however, in the later stationary phase, overproduction of RpoS did not influence IAA production.

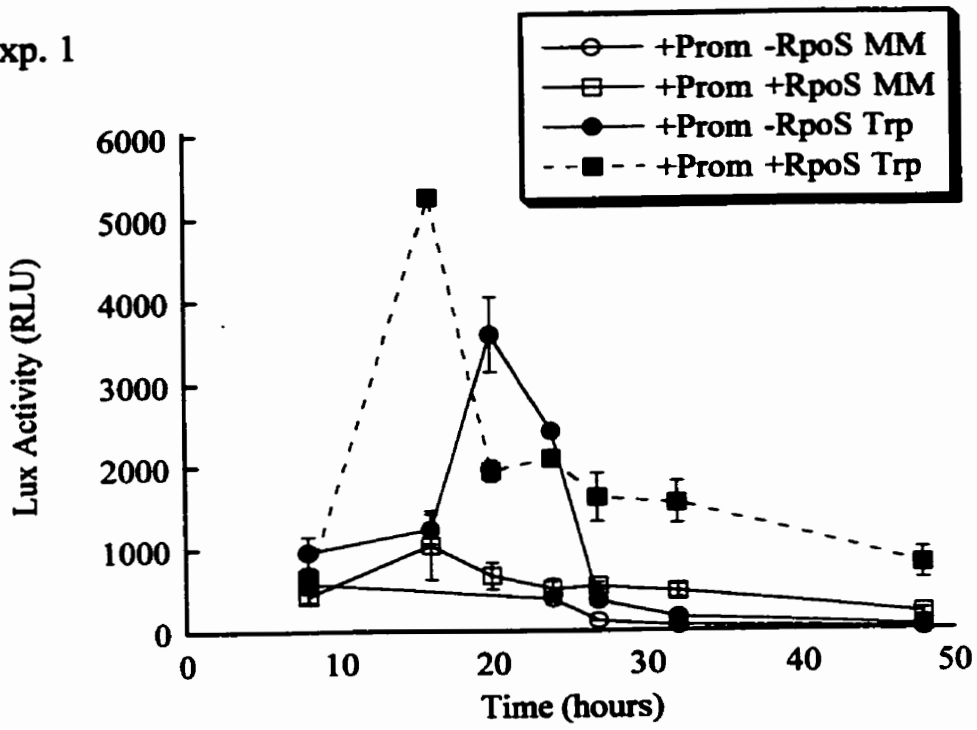
When the promoter was absent, as in control cells *P. putida* GR12-2/pQF70-Kan and *P. putida* GR12-2/pQF70-Kan/pRpoS, cells did not produce light (always less than 1 RLU; for comparison, light producing cells reached values ranging from several hundred to several thousand RLU). In cells carrying the promoter fragment (on pQFPROM-Kan), LuxAB activity increased at between 16 to 24 hours, and then decreased rapidly (Fig. 25). In the presence of tryptophan, this increase in promoter activity was approximately 5 times greater than in cells grown without tryptophan. Light was emitted earlier, and was maintained at a higher level later in the growth cycle, in cells carrying extra copies of *rpoS*, compared to corresponding cells without pRpoS. However, at the onset of stationary phase, at around 20 hours, overproduction of RpoS did not result in a further increase in promoter/LuxAB activity.

Effect of canola seed exudate on promoter activity

Exudate collected from canola (Hyola 401) seeds 3, 6.5 and 10 hours after imbibition in 0.03 M $MgSO_4$, did not influence IAA production (Fig. 26A) in *P. putida* GR12-2/pQFPROM-Kan (+Prom) or *P. putida* GR12-2/pQF70-Kan (-Prom). IAA levels in the culture medium of cells treated with exudate were very low, less than 1 μg/mL, and were not significantly different from cells grown in minimal medium without added exudate. However, light emission decreased slightly in *P. putida* GR12-2/pQFPROM-Kan cultures supplemented with exudate compared to light production in the absence of exudate.

Figure 25. Activity of the *ipdc* promoter as determined by LuxAB activity (measured in relative light units, RLU) in *P. putida* GR12-2/pQFPROM-Kan (+Prom) in the presence (Trp) and absence (MM) of tryptophan (200 μg/mL), and in the presence or absence of pRpoS (±RpoS) over a 48 hour period. In each experiment, n=2 for each strain at each time point; error bars indicate SE. Where error bars are not visible, they are smaller than the marker.

Exp. 1


+Prom -RpoS MM
+Prom +RpoS MM
+Prom -RpoS Trp
+Prom +RpoS Trp
Lux Activity (RLU)
6000
5000
4000
3000
2000
1000
0
0
10
20
30
40
50
Time (hours)

Exp. 2

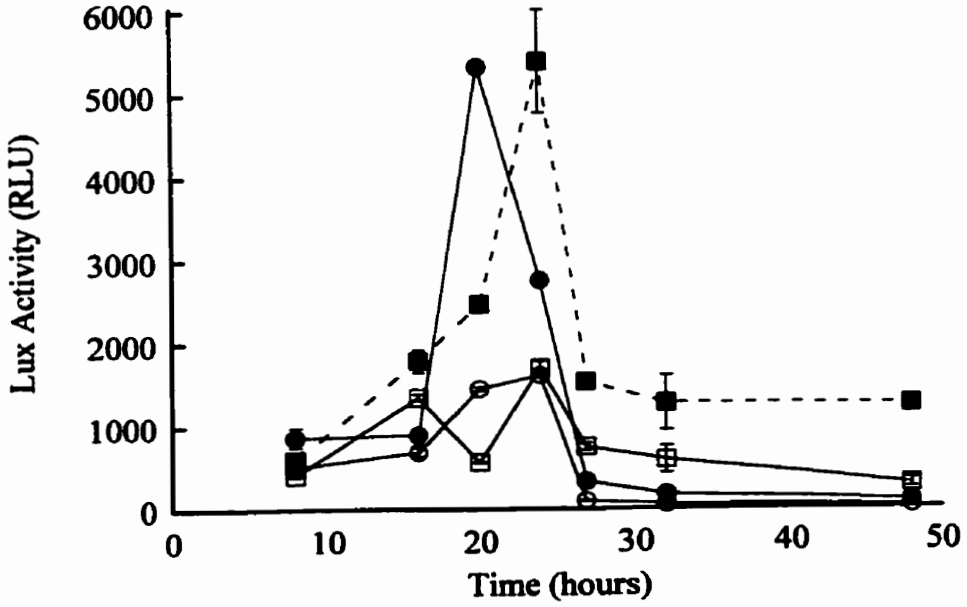
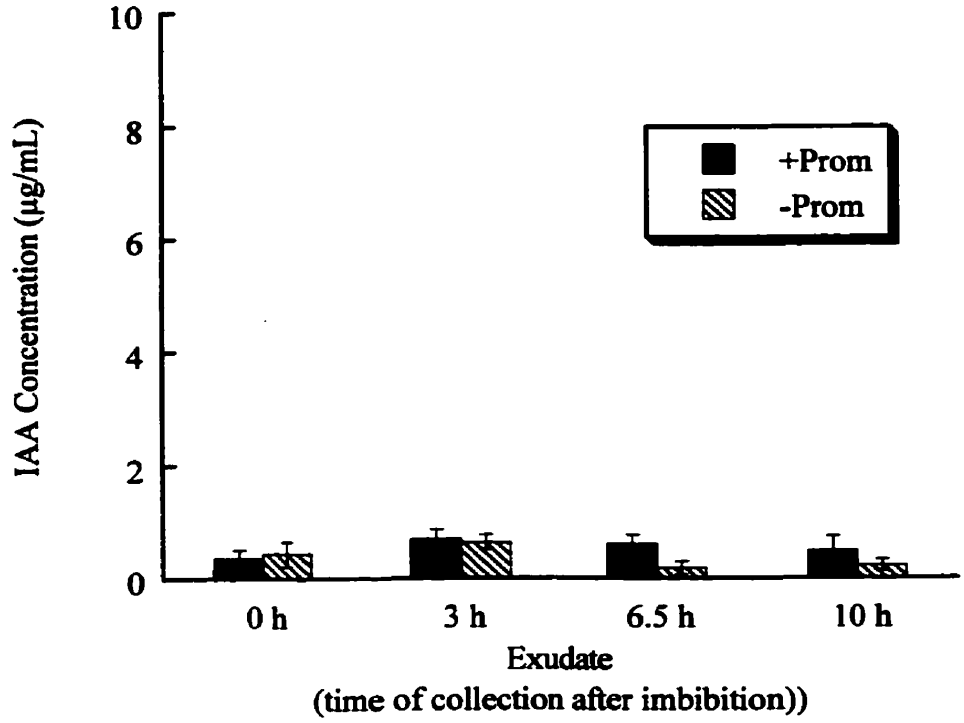
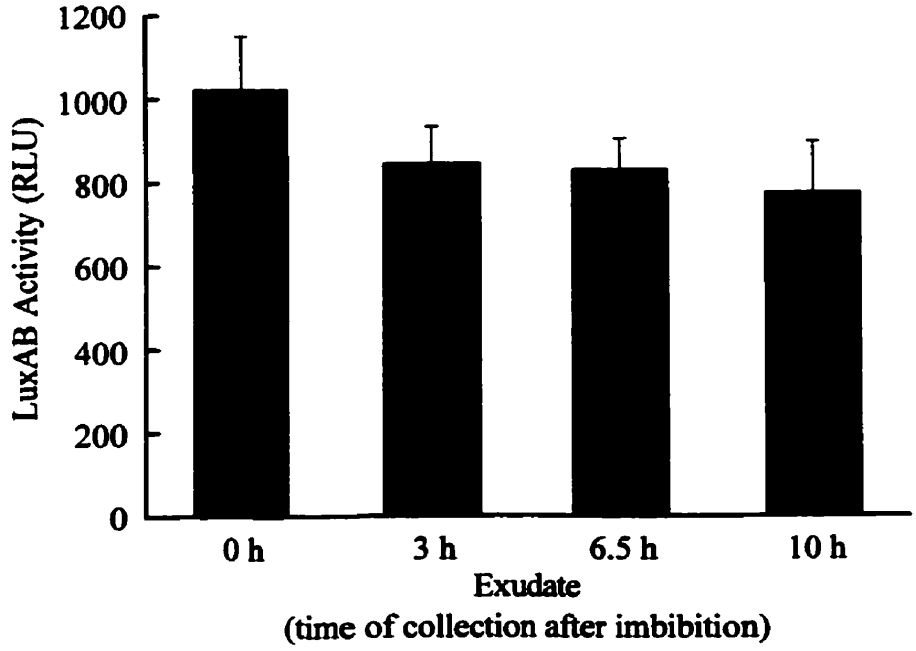

Lux Activity (RLU)
6000
5000
4000
3000
2000
1000
0
0
10
20
30
40
50
Time (hours)

Figure 26. IAA production (A) and *ipdc* promoter activity (LuxAB activity; measured in relative light units, RLU) (B) in *P. putida* GR12-2/pQF70-Kan (-Prom) and *P. putida* GR12-2/pQFPROM-Kan (+Prom) in media supplemented with exudate collected from canola seeds 3, 6.5 and 10 h following imbibition in 0.03 M $MgSO_4$. Note that 0 indicates cultures where sterile distilled water was added in place of exudate, and that, for clarity, values for LuxAB activity (B) in *P. putida* GR12-2/pQF70-Kan were omitted because they were negligible (< 1 RLU) compared with values for *P. putida* GR12-2/pQFPROM-Kan. In each experiment, n=4 for each treatment and error bars indicate standard error of the mean (SE). Where error bars are not visible, they are smaller than the marker. The analysis of variance showed that Lux activity in *P. putida* GR12-2/pQFPROM-Kan cells treated with exudate (B) was significantly lower (at P value < 0.05) than in cells grown in the absence of exudate ($F_{1/3} = 12.2$).

A


10
8
6
4
2
0
IAA Concentration (µg/mL)
+Prom
-Prom
0 h
3 h
6.5 h
10 h
Exudate
(time of collection after imbibition))

B

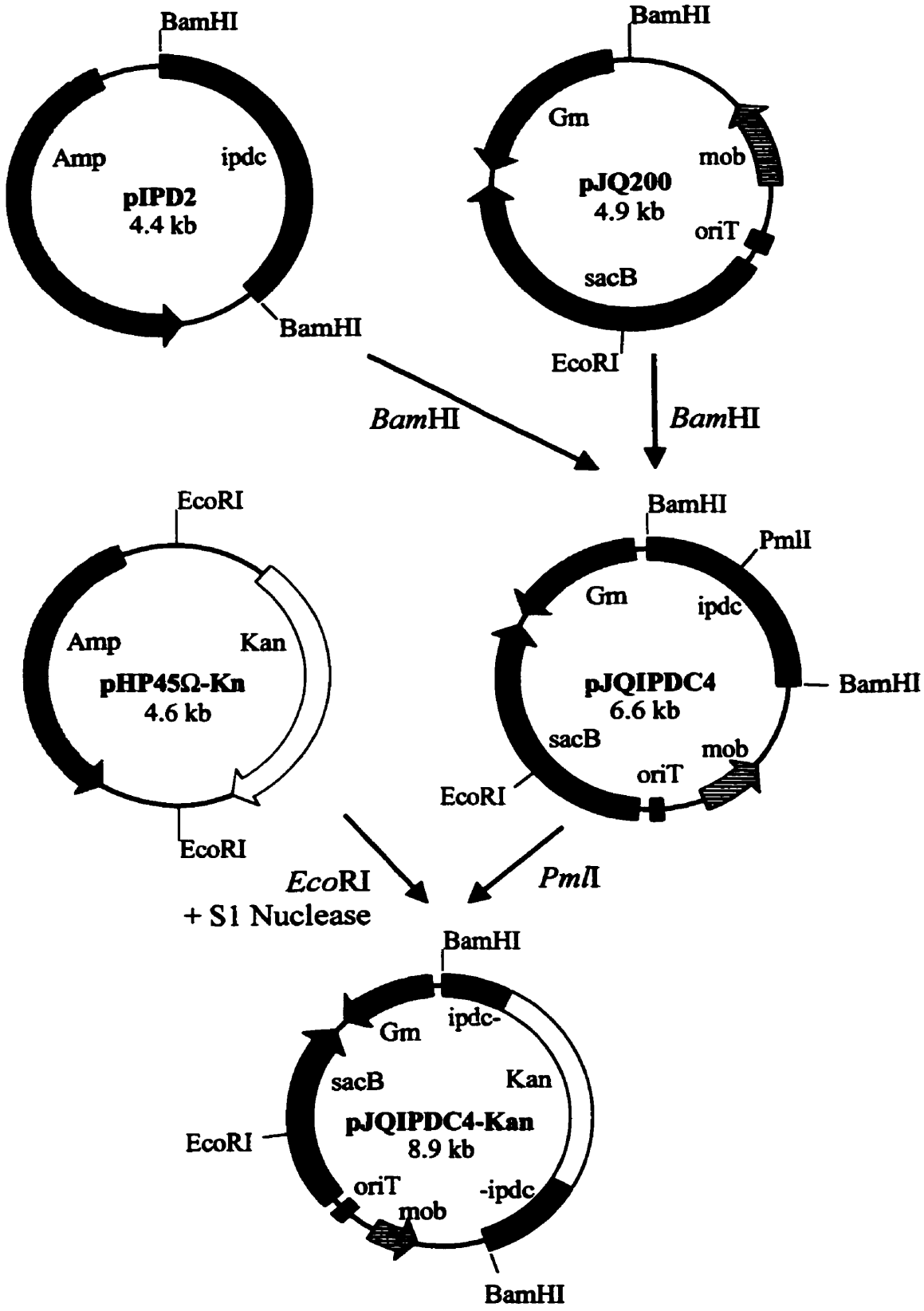

1200
1000
800
600
400
200
0
LuxAB Activity (RLU)
0 h
3 h
6.5 h
10 h
Exudate
(time of collection after imbibition)

**Construction of an IAA-Deficient Mutant of *P. putida* GR12-2**

Vector pJQ200 (Quandt and Hynes, 1993) was chosen as a vehicle for delivery of the *ipdc* sequence, disrupted by insertion of a gene for kanamycin resistance, into the genome of *P. putida* GR12-2 because it has an origin of transfer (*oriT*) and *mob* genes from plasmid RP4 enabling transfer of the vector from *E. coli* S17.1 (Simon et al., 1983) into *P. putida* GR12-2 via conjugation. However, once in *P. putida* GR12-2 the plasmid cannot replicate because it has an origin of replication derived from pACYC184 that is functional only in enterobacteria (Quandt and Hynes, 1993). Thus, following transfer of the vector to *P. putida* GR12-2, kanamycin resistant cells can only arise if the kanamycin resistance gene has been inserted into the *ipdc* gene in the genome by a double crossover between homologous *ipdc* sequences on the plasmid and in the chromosome. In addition, because gentamicin acetyltransferase and SacB are encoded on the vector, selection for the absence of these traits, that is, selection for sensitivity to gentamicin and resistance to the lethal effects of SacB in the presence of sucrose, selects against the incorporation of the entire plasmid into the genome that would result from a single crossover event.

The 1.7 kb *Bam*HI fragment carrying the *ipdc* gene from pIPD2 was subcloned into the *Bam*HI site in the multiple cloning site of pJQ200 to yield pJQIPDC4 (Fig. 27). A kanamycin resistance gene, carried on a 2.3 kb *Eco*RI fragment from pHP45Ω-Kn (Fellay et al., 1987; Prentki and Krisch, 1984), was inserted into the unique *PmlI* site of pJQIPDC4, roughly in the middle of the *ipdc* gene. The orientation of the kanamycin resistance gene in the resulting plasmid (pJQIPDC4-Kan) was determined by restriction enzyme mapping of plasmids isolated from transformed cells of *E. coli* S17.1 that were initially selected on the basis of kanamycin and gentamicin resistance. In addition to confirming the expected size

Figure 27. Construction of plasmid pJQIPDC4-Kan used for insertional mutagenesis of the *ipdc* gene in the genome of *P. putida* GR12-2. The *ipdc* gene was transferred from pIPD2 to vector pJQ200 as a 1.7 kb *BamHI* fragment to generate pJQIPDC4. The 2.3 kb *Eco*RI fragment from pHP45Ω-Kn, carrying a kanamycin resistance gene, was inserted into the *Pml*I site in the *ipdc* gene in pJQIPDC4 by blunt end ligation following S1 nuclease treatment of the *Eco*RI sticky ends. The resulting plasmid was designated pJQIPDC4-Kan.


BamHI
Amp
ipdc
pIPD2
4.4 kb
BamHI
BamHI
Gm
mob
pJQ200
4.9 kb
oriT
sacB
EcoRI
BamHI
BamHI
EcoRI
Amp
Kan
pHP45Ω-Kn
4.6 kb
EcoRI
BamHI
PmlI
Gm
ipdc
pJQIPDC4
6.6 kb
BamHI
sacB
mob
oriT
EcoRI
EcoRI
+ S1 Nuclease
PmlI
BamHI
Gm
ipdc-
sacB
Kan
pJQIPDC4-Kan
8.9 kb
EcoRI
oriT
mob
-ipdc
BamHI

(8.9 kb) of the plasmids from positive transformants by agarose gel electrophoresis of digested plasmids (Fig. 28), insertion of the kanamycin resistance gene into the *ipdc* gene on pJQIPDC4-Kan was verified by an increase of 2.3 kb in the size of PCR products compared to those from pJQIPDC4 using primers designed to amplify the *ipdc* gene (Fig. 29).

Following the transfer of pJQIPDC4-Kan from *E. coli* S17.1 to *P. putida* GR12-2 by conjugation, transconjugants were initially selected on Simmon's Citrate agar (on which *E. coli* donor cells cannot grow) containing kanamycin (on which non-transformed *P. putida* GR12-2 cells cannot grow). After three days, the surface of the Simmon's Citrate plates was almost covered with small colonies and, in addition, 20 larger colonies were visible. Cells from eight large colonies were carefully picked and subcultured onto Simmon's Citrate agar plus kanamycin in order to isolate single colonies, and then onto DF salts minimal agar (on which *E. coli* cannot grow) containing kanamycin to confirm that they were indeed derived from *P. putida* GR12-2; four transconjugants were selected for further analysis. Growth on TSB agar containing kanamycin and 5% sucrose, and lack of growth on TSB agar containing gentamicin, indicated that the kanamycin resistance gene, but not the remainder of the plasmid, had been inserted into the chromosome of all four selected transconjugants. Replacement of the functional *ipdc* gene in the chromosome of *P. putida* GR12-2 with the *ipdc* gene disrupted by the kanamycin resistance gene from pJQIPDC4-Kan was confirmed by PCR using primers designed to amplify the *ipdc* gene, and whole cell lysates of transconjugants and wild-type *P. putida* GR12-2 as templates. As can be seen in Fig. 30, PCR products from transconjugants (Lanes 2, 3, and 5) are 2.3 kb larger, corresponding to the size of the kanamycin resistance gene fragment, than PCR products from the wild-type strain (Lane 6); PCR products were confirmed to contain the *ipdc* sequence by Southern

Figure 28. *Sal*I digestion products of pJQIPDC4-Kan following agarose gel electrophoresis. Lanes 1 and 7, 1 kb ladder DNA size marker; Lanes 2-6, plasmids pJQIPDC4-Kan isolated from positive transformants. The plasmid shown in lane 5 was chosen for further manipulation.

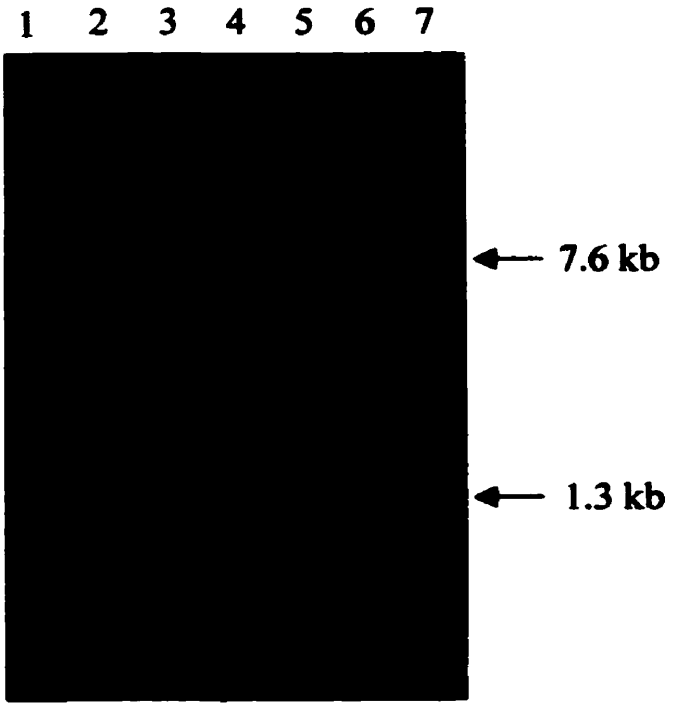

1 2 3 4 5 6 7
7.6 kb
1.3 kb

Figure 29. Products of PCR amplification of the *P. putida* GR12-2 *ipdc* gene in pJQIPDC4-Kan (Lane 2) and pJQIPDC4 (Lane 3) following agarose gel electrophoresis. Lane 1, PCR products from water as a template; Lane 4, 1 kb ladder DNA size markers.

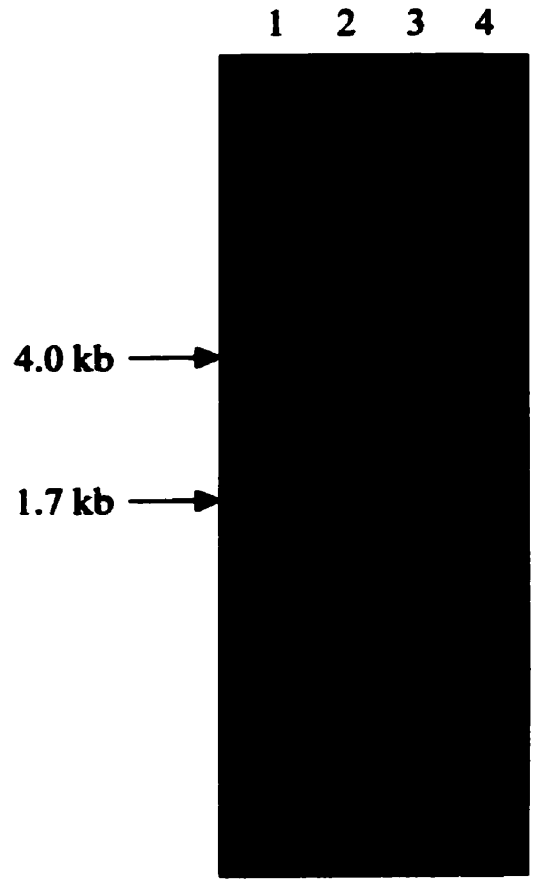
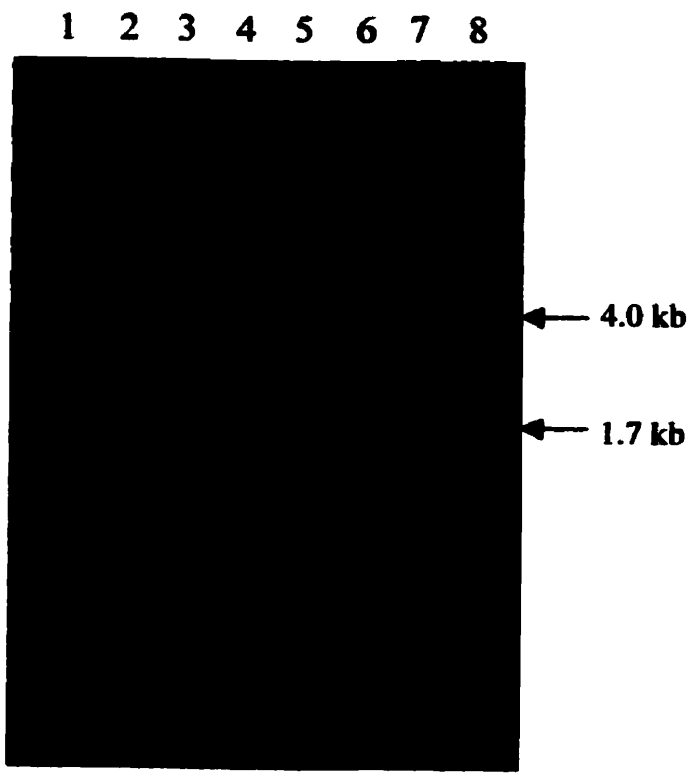

1 2 3 4
4.0 kb
1.7 kb

Figure 30. Products of PCR amplification of the *ipdc* gene from whole cell lysates of transconjugants (Lanes, 2-5) and wild-type (Lane 6) *P. putida* GR12-2. The 2.3 kb increase in the size of the PCR products from the transconjugants compared to those from the wild-type strain confirms that the kanamycin resistance gene was inserted into the *ipdc* gene in the *P. putida* GR12-2 genome. Lanes 1 and 7, 1 kb ladder DNA size markers; Lane 8, PCR products from water as a template.


1 2 3 4 5 6 7 8
4.0 kb
1.7 kb

hybridization (Fig. 31, lanes 3 and 4). In addition, Southern hybridization confirmed the presence of a larger *Eco*RI fragment carrying the *ipdc* gene in the chromosome of the mutant strain compared to the wild-type bacterium (Fig. 31, lanes 1 and 2).

**Characterization of the IAA-Deficient Mutant of *P. putida* GR12-2**

IAA production

In the absence of tryptophan supplements, both the IAA-deficient mutant and wild-type *P. putida* GR12-2 produced very low levels of IAA (Fig. 32). However, when both strains were grown in the presence of 50 μg/mL (or approx. 0.25 mM) tryptophan for approximately 42 hours, wild-type *P. putida* GR12-2 responded by producing substantial levels of IAA, while the mutant was not capable of producing significant amounts of IAA (Fig. 32). As the concentration of tryptophan in the growth medium was increased, so did IAA production by the wild-type strain. In contrast, IAA production by the mutant strain remained low. The apparent slight increase in IAA concentration in the medium of mutant cultures supplemented with high levels of tryptophan (500 μg/mL) is most likely due to accumulation of indolepyruvic acid, which can also react with Salkowski's reagent used to estimate IAA concentration colorimetrically, albeit to a lesser extent than IAA (Fig. 33). Indolepyruvic acid is the product of catalysis of tryptophan by tryptophan transaminase, the first step in the IAA biosynthetic pathway, and the substrate for indolepyruvate decarboxylase, which is no longer functional in the mutant. The growth of the mutant and wild-type strains of *P. putida* GR12-2 was not affected by the addition of high levels of tryptophan to the medium (Fig. 34).

Figure 31. Southern hybridization of *Eco*RI-digested genomic DNA from an IAA-deficient mutant (Lane 1) and a wild-type strain (Lane 2) of *P. putida* GR12-2, and the products of PCR amplification of the *ipdc* gene from whole cell lysates of the IAA-deficient mutant (Lane 3) and the wild-type strain (Lane 4), with a DIG-labeled *ipdc* gene probe.

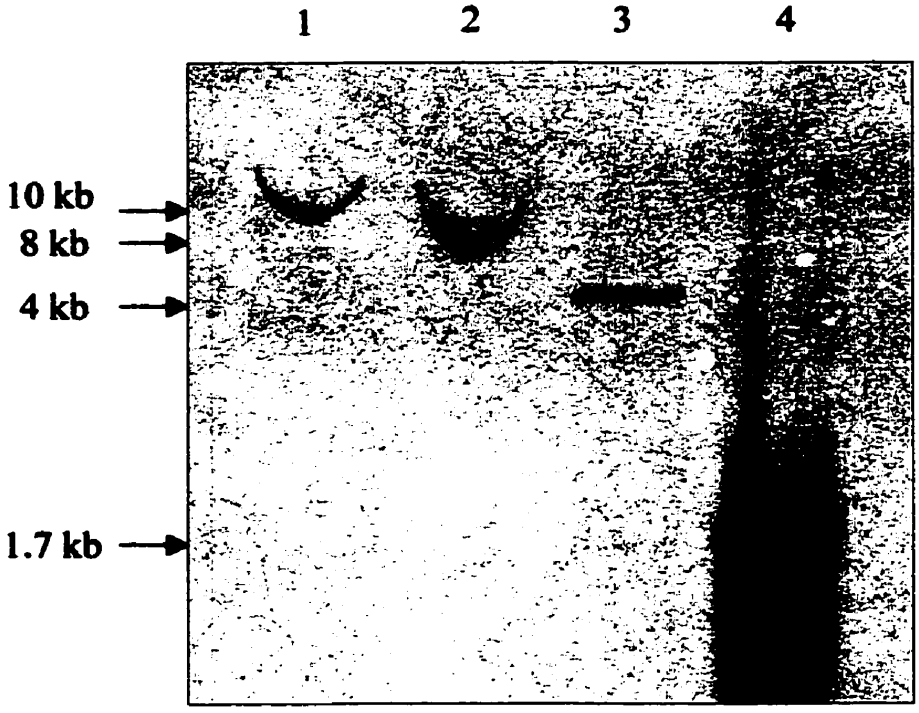

1
2
3
4
10 kb
8 kb
4 kb
1.7 kb

Figure 32. Production of IAA by wild-type and an IAA-deficient mutant of *P. putida* GR12-2 in the presence of various concentrations of tryptophan. The concentration of IAA in the cell-free growth media was measured by reaction with Salkowski's reagent after 42 hours of growth and adjusted to account for small differences in cell numbers among the various cultures (absorbance of the cultures at 600 nm). Error bars indicate standard error of the mean (SE); n=3.

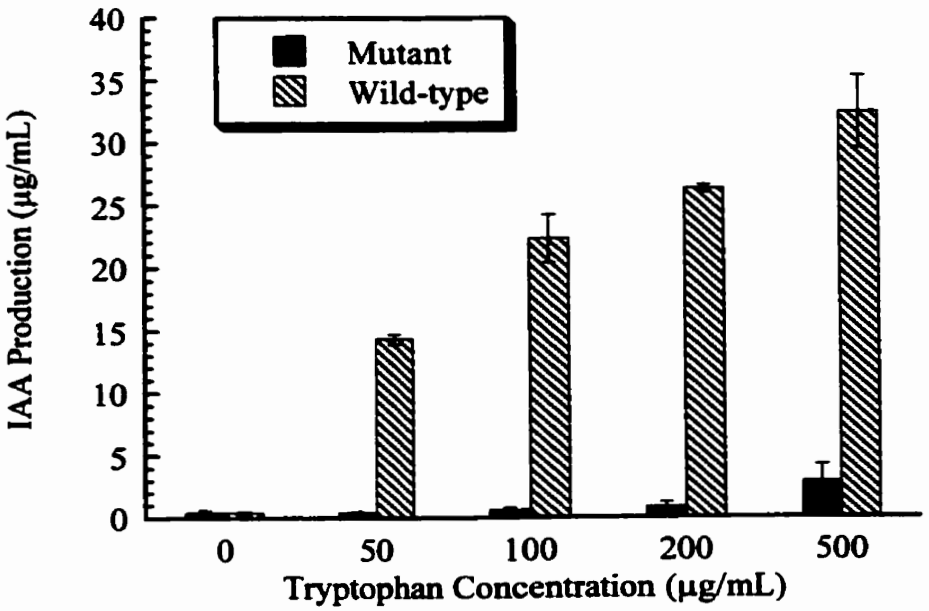

Mutant
Wild-type
IAA Production (µg/mL)
40
35
30
25
20
15
10
5
0
0
50
100
200
500
Tryptophan Concentration (µg/mL)

Figure 33. Reaction of indoleacetic acid (IAA) and indolepyruvic acid (IPyA) with Salkowski's reagent used for the colorimetric estimation of IAA concentration (absorbance of the reaction products at 535 nm). Although the concentration of IAA is expressed elsewhere in μg/mL (see Fig. 32), the concentration here is expressed in mM to account for differences in molecular weight between IAA (175 g/mole) and indolepyruvic acid (203 g/mole); however, for the purposes of conversion, 0.5 mM of these compounds corresponds roughly to 100 μg/mL. Error bars indicate standard error of the mean (SE); n=2.

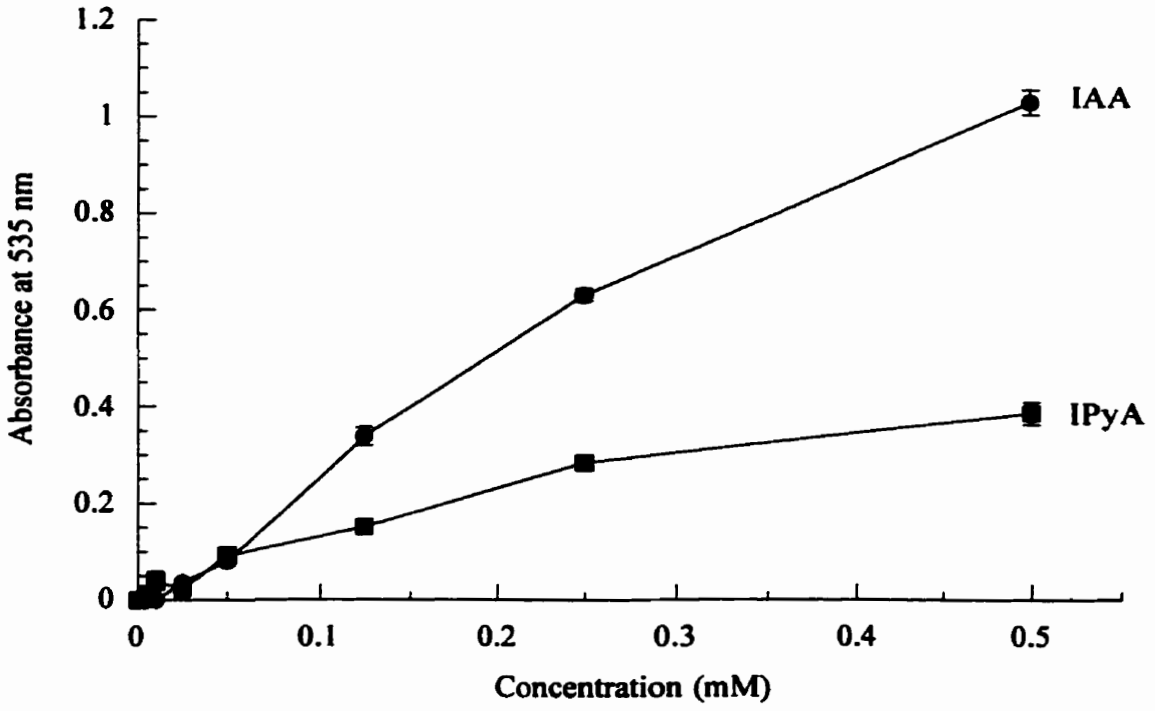
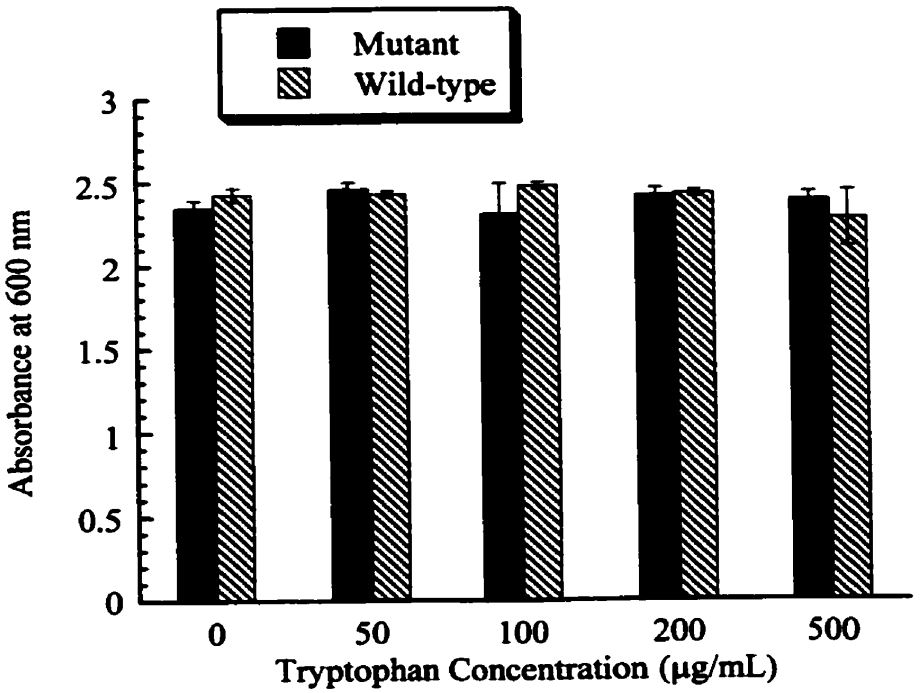

1.2
1
0.8
0.6
0.4
0.2
0
Absorbance at 535 nm
0
0.1
0.2
0.3
0.4
0.5
Concentration (mM)
IAA
IPyA

Figure 34. Growth of wild-type and an IAA-deficient mutant of *P. putida* GR12-2 in the presence of various concentrations of tryptophan as measured by the optical density (turbidity) of 42-hour cultures at 600 nm. Error bars indicate standard error of the mean (SE); n=3.


Mutant
Wild-type
Absorbance at 600 nm
3
2.5
2
1.5
1
0.5
0
0
50
100
200
500
Tryptophan Concentration (µg/mL)

Root elongation assays

IAA produced by *P. putida* GR12-2 has a significant impact on the ability of this bacterium to stimulate the growth of primary roots of canola seedlings. Whereas roots from seeds treated with wild-type *P. putida* GR12-2 were on average 35% longer than those from uninoculated control seeds after five days, the lengths of roots from seeds treated with the IAA-deficient mutant were not significantly different from those from uninoculated control seeds (Fig. 35, Table 5). IAA produced by the wild-type strain had no effect on shoot length, as shoots from seeds inoculated with wild-type *P. putida* GR12-2 were not different in length from shoots from uninoculated seeds (Fig. 36). Similarly, wild-type *P. putida* GR12-2 did not have an effect on the fresh or dry weight of shoots and roots (Table 6). As expected then, abolishing IAA production also did not affect these parameters.

Rooting assays

Mung bean cuttings, excised from above the roots after seven days of growth in vermiculite and placed in either water or a bacterial suspension, showed visible roots at the base of the stem after five days. After eight days, the cuttings in water had a few long roots, on average about six 3.7 mm long roots (Table 7) growing from just above the base (Fig. 37). More than three times as many adventitious roots developed in a suspension of wild-type *P. putida* GR12-2 (Table 7). Most of these were very small, less than 1 mm long (Fig. 38), distributed over several centimeters up from the base of the stem, sometimes with a few longer roots right at the base (Fig. 37). Roots that developed in the suspension of IAA-deficient mutant of *P. putida* GR12-2 were both abundant and long (Fig. 37), likely the best situation for propagation of the cutting in the long term. Twice as many roots were present on these cuttings compared to those growing in water, and these were generally longer than

Figure 35. Lengths of roots from canola seeds treated with wild-type or an IAA-deficient mutant of *P. putida* GR12-2 and from uninoculated control seeds after five days of growth. The average lengths of roots from five separate experiments are presented; n=300 roots/treatment, error bars indicate standard error of the mean (SE). The analysis of variance indicated that roots from uninoculated control seeds were significantly shorter (at P value < 0.01) than those from seeds treated with wild-type *P. putida* GR12-2 ($F_{1/8}$ = 30.1), but were not different from roots from seeds treated with the IAA-deficient mutant ($F_{1/8}$ = 4.3).

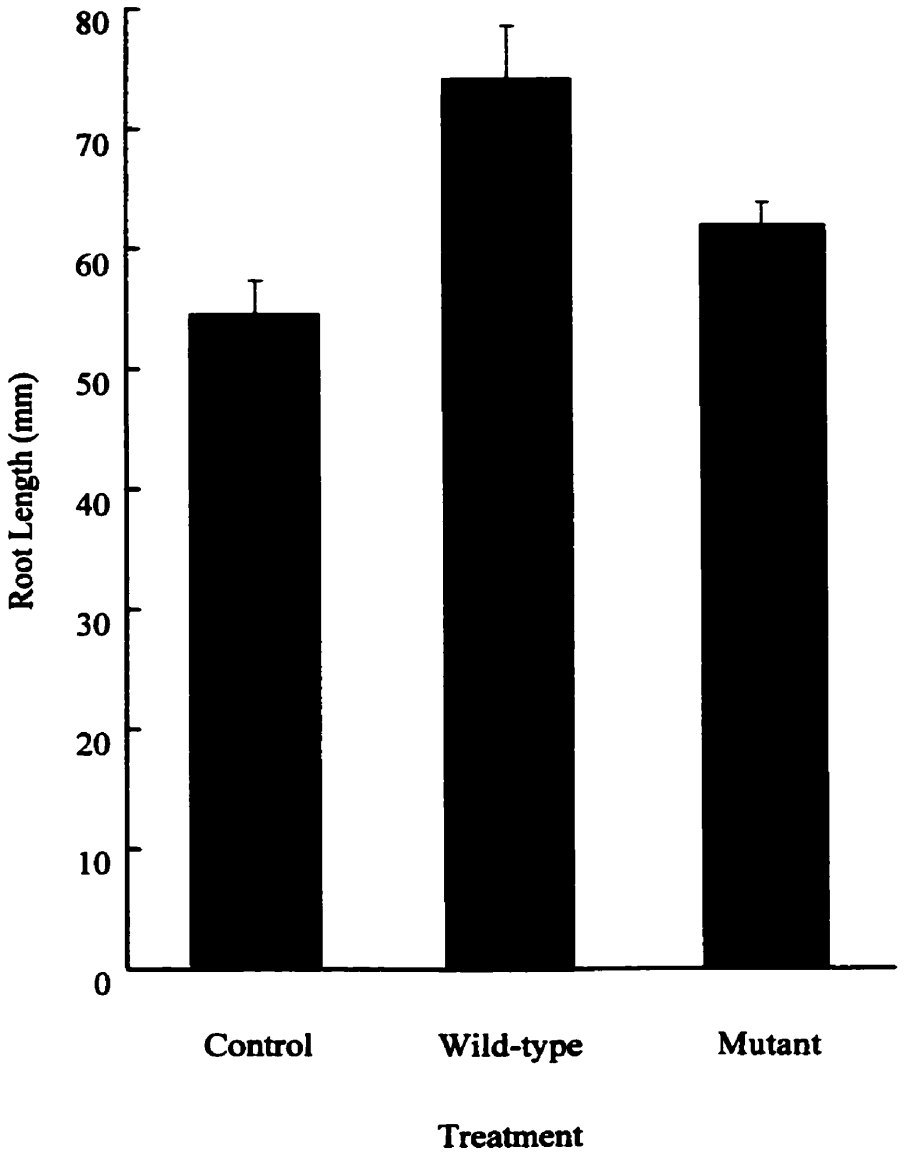

80
70
60
50
40
30
20
10
0
Root Length (mm)
Control
Wild-type
Mutant
Treatment

Table 5. Length of five day old canola seedling roots from seeds treated with either wild-type or an IAA-deficient mutant of *P. putida* GR12-2 or $MgSO_4$ as a negative control. The root lengths from five separate root elongation assays are presented as the mean value of approximately 60 roots ± SE (standard error of the mean).

| Assay No. | Average Root Length (mm ± SE) | | |
|---|---|---|---|
| | Control | IAA- Mutant | Wild-type |
| 1 | 59.6 ± 1.6 | 56.3 ± 2.3 | 87.1 ± 2.1 |
| 2 | 45.9 ± 1.3 | 60.6 ± 1.7 | 63.9 ± 1.8 |
| 3 | 61.2 ± 2.0 | 68.1 ± 2.2 | 81.6 ± 2.5 |
| 4 | 53.2 ± 1.4 | 60.8 ± 1.7 | 66.4 ± 1.7 |
| 5 | 52.4 ± 1.1 | 63.6 ± 2.1 | 71.9 ± 2.2 |

Figure 36. Lengths of shoots from canola seeds treated with wild-type or an IAA-deficient mutant of *P. putida* GR12-2 and from uninoculated control seeds after five days of growth. In each assay, 60 seeds were used for each treatment, error bars indicate standard error of the mean (SE). The analysis of variance indicated that shoots from uninoculated control seeds were not significantly different from those from seeds treated with wild-type *P. putida* GR12-2 ($F_{1/4} = 5.1$) or from shoots that developed from seeds treated with the IAA-deficient mutant ($F_{1/4} = 3.9$).

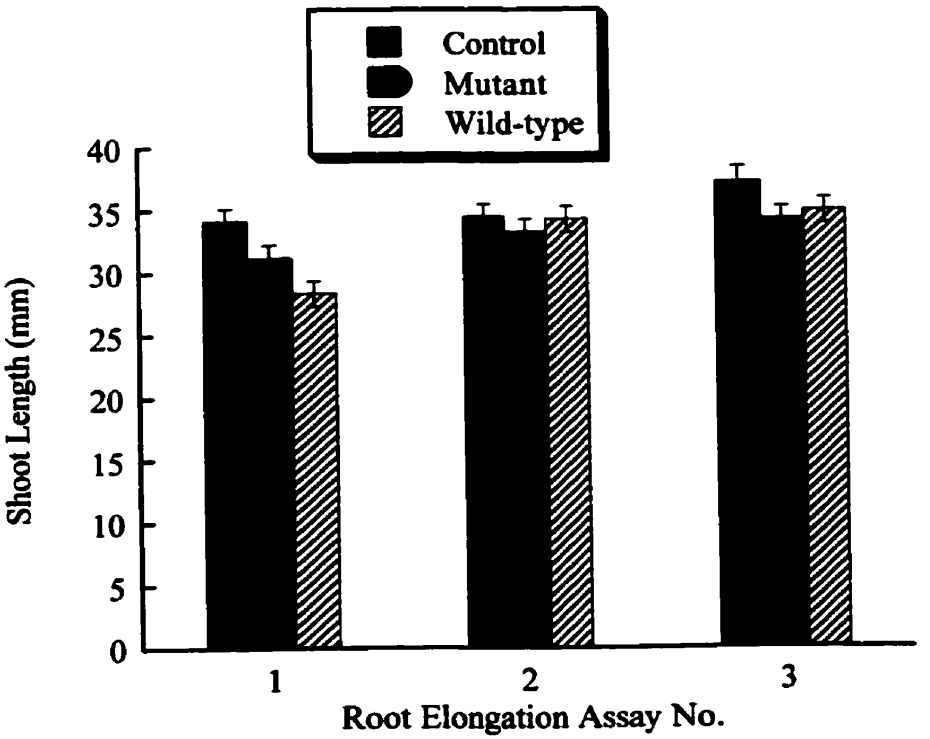

Control
Mutant
Wild-type
Shoot Length (mm)
40
35
30
25
20
15
10
5
0
1
2
3
Root Elongation Assay No.

Table 6. Fresh and dry weight of five day old canola seedling roots and shoots from seeds treated with wild-type or IAA-deficient *P. putida* GR12-2 or $MgSO_4$ as a control (n =60).

| Treatment | Root Fresh Weight (mg ± SE) | Shoot Fresh Weight (mg ± SE) | Root Dry Weight (mg ± SE) | Shoot Dry Weight (mg ± SE) |
|---|---|---|---|---|
| Control | 10.5 ± 0.4 | 34.6 ± 1.0 | 0.66 ± 0.02 | 3.5 ± 0.1 |
| IAA-Mutant | 12.0 ± 0.4 | 35.8 ± 0.9 | 0.66 ± 0.03 | 3.6 ± 0.1 |
| Wild-type | 11.5 ± 0.4 | 36.0 ± 0.7 | 0.68 ± 0.02 | 3.7 ± 0.1 |

Figure 37. Adventitious roots on mung bean cuttings after eight days of incubation in water, or in a suspension of wild-type or an IAA-deficient mutant of *P. putida* GR12-2.

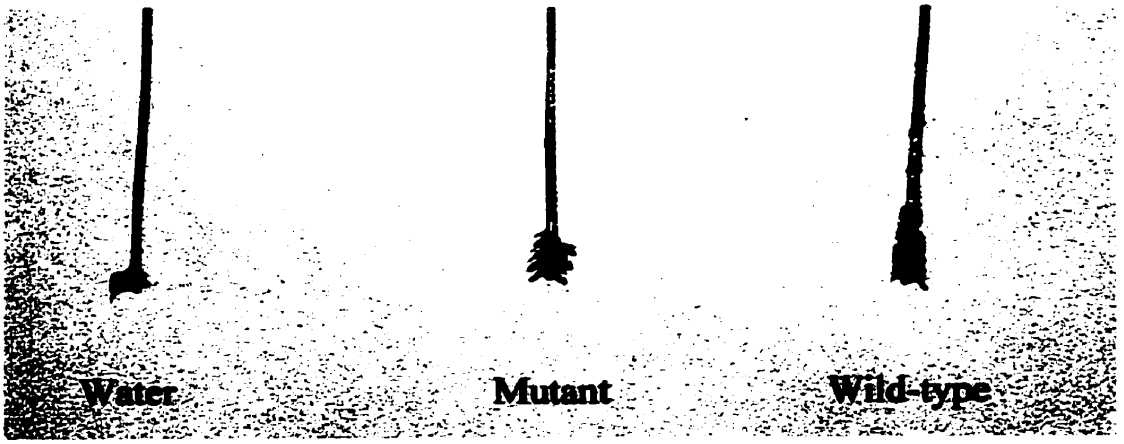
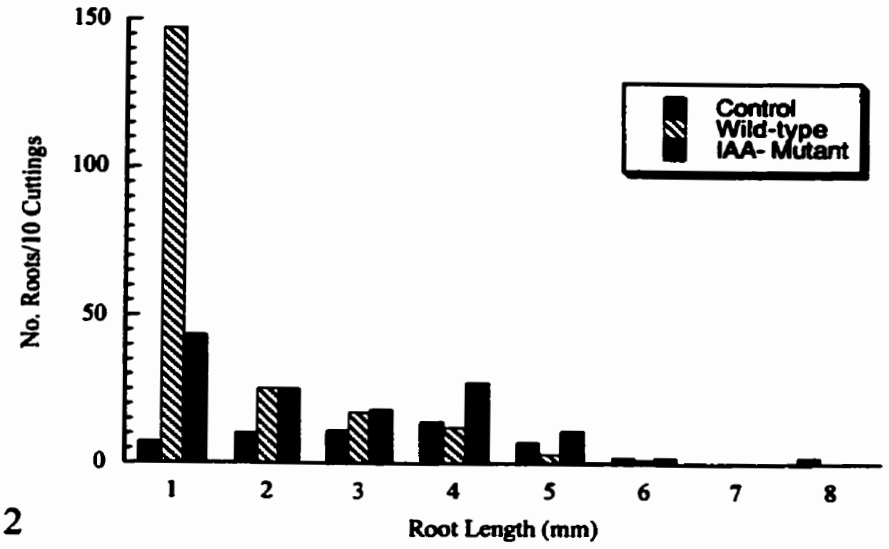

Water
Mutant
Wild-type

Table 7. Effect of wild-type and an IAA-deficient mutant of *P. putida* GR12-2 on the number and length of adventitious roots on mung bean cuttings. Roots were measured eight days after treatment with the bacteria. In each experiment, ten cuttings were measured for each treatment; SE indicates standard error of the mean. The analysis of variance indicated that significantly (at P value < 0.05) more ($F_{1/4}$ = 19.2) and shorter ($F_{1/4}$ = 17.8) adventitious roots developed on cuttings treated with wild-type *P. putida* GR12-2 than in water, and that the number and length of adventitious roots that developed on cuttings treated with the IAA-deficient mutant were intermediate between these two ($F_{1/4}$ = 0.4 for abundance; $F_{1/4}$ = 0.3 for length).

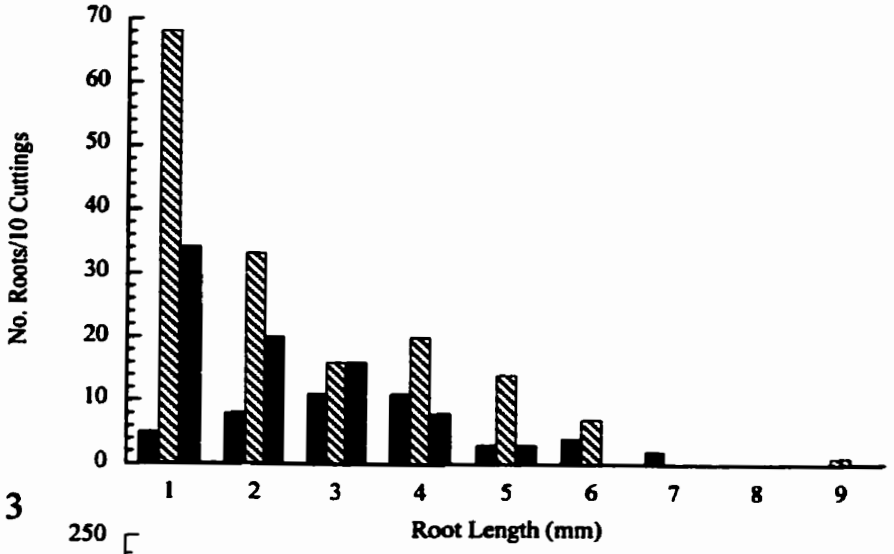
| Treatment | Roots/Cutting (No. ± SE) | | | Avg. Root Length (mm ± SE) | | |
|---|---|---|---|---|---|---|
| | Exp. 1 | Exp. 2 | Exp. 3 | Exp. 1 | Exp. 2 | Exp. 3 |
| Water | 6.8 ± 1.0 | 5.5 ± 1.3 | 6.3 ± 0.6 | 3.4 ± 0.2 | 3.4 ± 0.2 | 4.2 ± 0.2 |
| IAA-Mutant | 13.8 ± 1.9 | 10.1 ± 1.8 | 14.2 ± 1.6 | 2.6 ± 0.1 | 2.1 ± 0.1 | 2.9 ± 0.2 |
| Wild-type | 20.3 ± 2.2 | 16.1 ± 3.8 | 33.4 ± 3.4 | 1.6 ± 0.1 | 2.4 ± 0.1 | 1.4 ± 0.1 |

Figure 38. Number of adventitious roots of each length (1-9 mm) that developed on ten mung bean cuttings after eight days of incubation in water (control), or in a suspension of wild-type or an IAA-deficient mutant of *P. putida* GR12-2, in three separate experiments.

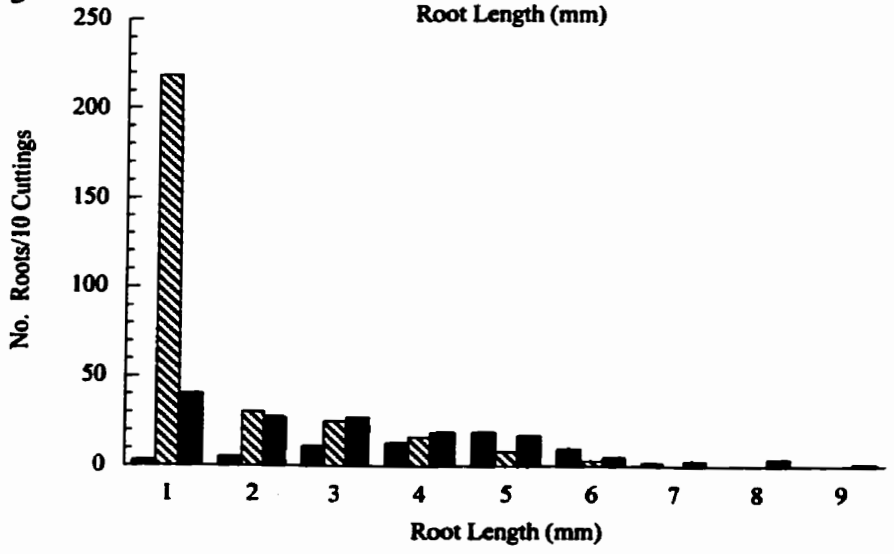
Exp. 1




Exp. 2




Exp. 3



those that developed in the wild-type bacterial suspension (Table 7, Fig. 38).

# DISCUSSION

## Indolepyruvate Decarboxylase

The amino acid sequence determined from the *ipdc* gene isolated from *P. putida* GR12-2 reveals a protein of 552 amino acids with a predicted molecular weight of approximately 60 kDa. It is not encoded in an operon containing the other genes involved in the biosynthesis of IAA by the indolepyruvic acid pathway as it is transcribed from its own promoter and has a transcription termination sequence just downstream of the translation stop codon. Also, the *ipdc* gene is flanked by two genes, transcribed from the opposite strand on the chromosome, one encoding a putative potassium ion channel protein in the 5' flanking region, and the other, a protein similar to an *E. coli* protein of unknown function in the 3' flanking region; rarely are two genes transcribed from complementary DNA strands in the same region of DNA. It is reasonable that the enzymes involved in the indolepyruvic acid pathway are not expressed from an operon because the genes for the first enzyme in the pathway, an aromatic aminotransferase, are often present in multiple copies in a single bacterium, and can utilize substrates other than tryptophan (Kuo and Kosuge, 1970; Liu et al., 1982; Ruckäschel et al., 1988; Soto-Urzua et al., 1996; Kittel et al., 1989; Koga et al., 1994; Brandl et al., 1996). Not only does this enzyme lack specificity for L-tryptophan, but it prefers to utilize other amino acids as substrates. For example, the $K_m$ values for the aromatic aminotransferase from *Azospirillum brasilense* for L-tyrosine, L-histidine, and L-phenylalanine were 0.19, 0.35, and 0.43 mM, respectively, compared to 1.05 mM for L-tryptophan (Soto-Urzua et al., 1996). Thus, this aromatic aminotransferase is not solely an IAA biosynthesis enzyme.

Indolepyruvate decarboxylase from *P. putida* GR12-2 is similar in sequence to

indolepyruvate decarboxylase from *Enterobacter cloacae* FERM BP-1529, which was isolated from the rhizosphere of cucumber (Koga et al., 1991), and to the same protein from *Erwinia herbicola* 299R, an epiphytic bacterium isolated from pear (Brandl and Lindow, 1996). The *E. cloacae* indolepyruvate decarboxylase is a 552 amino acid protein with a molecular weight of 60 kDa and the protein from *E. herbicola* consists of 550 amino acids. In contrast, indolepyruvate decarboxylase from two *Azospirillum brasilense* strains, Sp245 and Sp7 (Costacurta et al., 1994; Zimmer et al., 1998), is somewhat different in sequence from those from the aforementioned bacteria including *P. putida* GR12-2, although identified conserved regions are present. These *Azosprillum* strains are capable of producing high levels of IAA in the presence of tryptophan, compared to *P. putida* GR12-2 which is a moderate IAA producer. This could be due to differences in the regulation of IAA synthesis, or alternatively, to differences in indolepyruvate decarboxylase activity.

Attempts to isolate the *ipdc* gene from *Enterobacter cloacae* CAL3, another bacterium capable of producing levels of IAA much higher than *P. putida* GR12-2 (Patten, 1996), using homology-based methods such as colony hybridization and PCR, were unsuccessful, possibly because this gene from *E. cloacae* CAL3 is also different from that from the other bacterial strains. Support for this can be found in Southern blots of *P. putida* GR12-2 and *E. cloacae* CAL3 genomic DNA in which the probe, the *ipdc* gene from *E. cloacae* FERM BP-1529, hybridized strongly to a fragment of the *P. putida* GR12-2 genome but gave only a faint signal from the *E. cloacae* CAL3 genome. Perhaps there is some correlation between the indolepyruvate decarboxylase sequence present in a bacterium and the levels of IAA produced. Alternatively, the differences among the sequences may simply manifest evolutionary relationships among these strains, with *P. putida* GR12-2 being

more closely related to *E. cloacae* FERM BP-1529 and *E. herbicola* 299R than to the *Azospirillum brasilense* strains. Interestingly, the putative potassium ion channel protein upstream of *ipdc* in *P. putida* GR12-2 is also present upstream of *ipdc* in *E. herbicola* 299R (GenBank Accession No. L80006) suggesting that not only the *ipdc* gene sequences, but also the organization of genes in this region, are conserved in these two organisms. Whether or not the organization of these genes is conserved because it is important for their function is not clear. There is some evidence that a gene similar to *ipdc* is present in other Enterobacteriaceae such as *Klebsiella aerogenes* and *Enterobacter agglomerans*, although only partial sequences have been obtained as PCR products from primers designed to anneal to conserved regions within the gene (Zimmer et al., 1994); function has not been confirmed.

Koga (1995) has extensively characterized indolepyruvate decarboxylase from *E. cloacae* FERM BP-1529 as a homotetramer that requires thiamine diphosphate and $Mg^{2+}$ as cofactors and has a high specificity and high affinity ($K_m$=15 μM) for indolepyruvic acid (Koga et al., 1992). Thiamine diphosphate and $Mg^{2+}$ are involved in the formation and stability of the tetramer. Pyruvate decarboxylases from the bacterium *Zymomonas mobilis* (Dobritzsch et al., 1998) and from the yeast *Saccharomyces cerevisiae* (Kellermann et al., 1986) share extensive similarity to indolepyruvate decarboxylase from *E. cloacae* FERM BP-1529 and from *P. putida* GR12-2, especially in many of the residues that, from the crystal structure and site-directed mutagenesis of pyruvate decarboxylase, are believed to be involved in cofactor and substrate binding (Candy et al., 1996; Lobell and Crout, 1996; Schenk et al., 1997; Dobritzsch et al., 1998). Pyruvate decarboxylase from *Z. mobilis* is also a homotetramer; each subunit is composed of 568 amino acids with a molecular mass of 60 kDa. This enzyme catalyzes the decarboxylation of pyruvic acid to acetaldehyde and

$CO_2$, a key step in the fermentation of glucose to alcohol.

The similarity among the sequences for pyruvate decarboxylases and indolepyruvate decarboxylases, and the ability of indolepyruvate decarboxylase to utilize pyruvate as a substrate, albeit with a relatively low binding affinity ($K_m$=2.5 mM) (Koga et al., 1992), suggests that indolepyruvate decarboxylase may have evolved from pyruvate decarboxylase. Alterations in some of the substrate binding residues may have changed the substrate preference of an ancestral pyruvate decarboxylase molecule for indolepyruvate rather than pyruvate. That pyruvate decarboxylase is the ancestral protein rather than indolepyruvate decarboxylase, is suggested by some of the characteristics of pyruvate decarboxylase: 1) It is the key enzyme in energy metabolism under anaerobic conditions that were believed to be present on ancient earth; 2) It is more ubiquitous than indolepyruvate decarboxylase, being present in a wide range of organisms including bacteria, fungi, plants (Kelley et al., 1991; Hossain et al., 1996) and animals (Zhang et al., 1993). This suggests that pyruvate decarboxylase was conserved through evolution, although more recent lateral transfer of the gene among these organisms cannot be ruled out.

Indolepyruvate decarboxylase is also similar to the acetolactate synthase large subunit, involved in the biosynthesis of branched chain amino acids in bacteria, fungi, plants, and archaea (Bowen et al., 1997; Chipman et al., 1998; Pang and Duggleby, 1999). Acetolactate synthase, also a thiamine-diphosphate- and $Mg^{2+}$-requiring enzyme, catalyzes two different reactions, both involving the decarboxylation of pyruvate. In one reaction acetolactate synthase converts two molecules of pyruvate to acetolactate, the precursor of valine and leucine, while in the other, acetolactate synthase converts one molecule of pyruvate and one molecule of 2-ketobutyrate to acetohydroxybutyrate, a key step in the

synthesis of isoleucine (Gollop et al., 1990). Although the overall identity between indolepyruvate decarboxylase and acetolactate synthase is moderate (approximately 25%), conserved residues are concentrated in several regions of the proteins.

It is possible that the gene for acetolactate synthase gave rise to *ipdc*, or vice versa. The reaction that results in the biosynthesis of IAA from tryptophan via the indolepyruvic acid pathway is, in reverse, an amino acid biosynthesis reaction. In fact, tryptophan aminotransferase, the enzyme that catalyzes the first step in the indolepyruvic acid pathway, preferentially catalyzes the conversion of indolepyruvic acid to tryptophan (Koga et al., 1994). The $K_m$ value of tryptophan aminotransferase for indolepyruvic acid is 24 μM, whereas the $K_m$ value for L-tryptophan is 3.3 mM. Only when indolepyruvate decarboxylase is present, and indolepyruvate levels are therefore low, does the reaction favor IAA synthesis.

The sequence similarity among genes encoding acetolactate synthase in archaea and bacteria, and the ubiquity of the enzyme among organisms, suggest that it is an ancient molecule, arising before the divergence of bacteria and archaea (Bowen et al, 1997). However, others argue that on ancient earth, amino acids were abundant and therefore proteins that catalyze amino acid biosynthesis would be a late development (Chang and Cronan, 1988). Rather, they suggest that pyruvate oxidase, another thiamine diphosphate-dependent enzyme similar in sequence to both indolepyruvate decarboxylase and acetolactate synthase, was the ancestral molecule. Pyruvate oxidase catalyzes the oxidative decarboxylation of pyruvate to acetate but also has acetolactate synthase activity (Chang and Cronan, 1988). Thus, while there is an evolutionary relationship among indolepyruvate decarboxylase, acetolactate synthase, pyruvate oxidase and pyruvate decarboxylase, the precise phylogenetic linkages are not clear.

Indulging in some speculation, if acetolactate synthase or pyruvate decarboxylase gave rise to indolepyruvate decarboxylase in bacteria, then it is equally possible that one of these genes in plants gave rise to the elusive plant indolepyruvate decarboxylase. Careful phylogenetic analysis of acetolactate synthase genes from a variety of organisms, suggests that the eukaryotic, nuclear-encoded acetolactate synthase genes are of bacterial origin (Funke et al., 1999). Acetolactate sythase is also encoded on some plastid genomes such as the chloroplast chromosome of the red alga, *Porphyra umbilicalis* (Reith and Munhulland, 1993). This, and the presence of a similar acetolactate synthase gene in cyanobacteria (Maestri and Joset, 2000), suggests that the gene was transferred to eukaryotes from bacteria during the endosymbiosis that gave rise to the chloroplast. Perhaps, after transfer to plants and a gene duplication event, an acetolactate synthase paralogue diverged to generate plant indolepyruvate decarboxylase. A similar duplication, and divergence, in bacteria would yield bacterial indolepyruvate decarboxylase with a sequence different from that from plants. Such a scenario would explain why, despite biochemical evidence for the presence of indolepyruvate decarboxylase in plants (McQueen-Mason and Hamilton, 1989; Cooney and Nonhebel, 1991; Nonhebel et al., 1993), a gene similar to bacterial *ipdc* has not been found.

**Regulation of Indolepyruvate Decarboxylase Expression**

IAA accumulates in the culture medium of *P. putida* GR12-2 and many other bacterial cells grown in the presence of exogenous tryptophan (Koga et al., 1991b; Ernsten et al., 1987; Kaneshiro et al., 1983; Patten, 1996; Brandl and Lindow, 1996; Barbieri et al., 1986; Omay et al., 1993). When tryptophan is not added, only very low levels of IAA are produced. From this, it is concluded that exogenous tryptophan is required for IAA

synthesis. But is it required to regulate the expression or activity of the enzymes involved in IAA biosynthesis, or is it simply required as a substrate, to drive the reaction in favor of IAA synthesis? As noted above, Koga et al. (1994) suggest that tryptophan aminotransferase, the first enzyme in the indolepyruvic acid pathway, preferentially catalyzes the synthesis of tryptophan from indolepyruvic acid rather than the reverse reaction which leads to IAA production. However, when the levels of indolepyruvic acid are low, or possibly when the levels of tryptophan are high, the synthesis of IAA is favored. Because appreciable levels of IAA are not produced unless an external source of tryptophan is supplied to the bacterial cells, endogenous levels of tryptophan are not sufficient for IAA production. There is likely a high demand for tryptophan by bacterial cells as it is used to produce many essential compounds such as proteins and vitamins (Martens and Frankenberger, 1993). Perhaps IAA, a secondary metabolite that is not required for cell proliferation, is not important enough for the cell to use up limited amounts of available endogenous tryptophan for its production.

To determine if exogenous tryptophan is an activator of transcription of IAA biosynthesis genes, transcriptional fusions can be constructed in which the promoter region of an IAA gene is inserted upstream of a reporter gene, and then expression of the reporter gene compared in the presence and absence of tryptophan. To date, the promoter regions from only a few indolepyruvate decarboxylase genes have been studied. Only in one case was tryptophan found to increase the expression of indolepyruvate decarboxylase. Zimmer et al. (1998) inserted the *lacZ* reporter gene in the genome of *Azospirillum brasilense* Sp7 such that it was expressed by the native *ipdc* promoter and found that β-galactosidase activity increased three- to four-fold in the presence of 50 μg/mL tryptophan. In contrast, the *ipdc* promoter in *A. brasilense* Sp245 (Vande Broek et al., 1999) and in *Erwinia herbicola* 299R

(Brandl and Lindow, 1997), fused to the *gus* reporter gene and to an ice nucleation gene, respectively, in the bacterial chromosome, was not influenced by exogenous tryptophan, although in both bacteria the levels of IAA were substantially elevated when the culture medium was supplemented with tryptophan.

Both *ipdc* promoter activity and IAA production in *P. putida* GR12-2 are increased in response to exogenous tryptophan. By late-log/early stationary phase, LuxAB (luciferase from the bacterium *Vibrio harveyi*) activity driven by the *ipdc* promoter in pQFPROM-Kan, was about five-fold higher in cells grown in the presence of 200 μg/mL L-tryptophan than in medium without tryptophan. At this time, tryptophan-induced IAA was also beginning to accumulate in the culture medium. The rapid decline in LuxAB activity after this stage may be due to the instability of luciferase and/or to reduced transcription, especially as the inducer, tryptophan, is being depleted as a consequence of IAA production. Luciferase may be recognized as foreign by *P. putida* GR12-2 and degraded rapidly by proteases, a common problem for recombinant proteins that is often a function of the protein sequence and the host cell (Murby et al., 1996). Terminal sequencing of the products of limited proteolysis of the alpha subunit of bacterial luciferase (LuxA) indicated a protease-labile C-terminal region (Noland et al., 1999). In contrast to luciferase, indolepyruvate decarboxylase may be more stable, although the turnover rate for this enzyme has not been determined. If transcription of *ipdc* is induced only for a short period of time, as is indicated by the sharp peak in LuxAB activity in the presence of tryptophan and the lack of continuous transcription, then indolepyruvate decarboxylase would have to be a reasonably stable protein in order to have continued production of IAA for at least an additional 28 hours.

Several tryptophan-regulated promoters have been identified in bacteria; these usually

control the transcription of genes involved in tryptophan metabolism. Expression of most of these genes, such as those involved in the biosynthesis of tryptophan (e.g., the *E. coli trp* operon) and other amino acids (e.g., the *aroH* and *aroL* operons) are repressed by tryptophan (Pittard, 1996; Caligiuri and Bauerle, 1991; Heatwole and Sommerville, 1992; Lawley and Pittard, 1994). In *E. coli*, repression is mediated by the tryptophan repressor TrpR, which is also negatively regulated by tryptophan at the transcriptional level, and by transcription attenuation (Pittard, 1996; Santillán and Mackey, 2001; Khodursky et al., 2000). Mironov et al. (1999) established a recognition rule for tryptophan-regulated promoters in *E. coli* and *Haemophilus influenzae* by identifying sequences common to the upstream regions of genes known to be regulated by tryptophan. This approach assumes that the cognate regulatory molecules are also conserved. The consensus sequence T(T/C)GTACT(A/C)GT(T/G)AAC-TAGTACA was found in the promoters of *trpR*, the *trp* operon, *aroH*, and *mtr* (a tryptophan-specific permease), all of which are negatively regulated by TrpR. They then used this recognition rule to identify putative tryptophan-regulated genes in unannotated bacterial genomes.

Very few genes are known to be positively regulated by tryptophan. In contrast to the *E. coli trp* operon, the *trpBA* operon for tryptophan biosynthesis in fluorescent pseudomonads is positively regulated by its cognate regulatory molecule TrpI, however, tryptophan has not been implicated in the mechanism (Auerbach et al., 1993). Rather, indoleglycerol phosphate, an intermediate in tryptophan synthesis, mediates the binding of TrpI to the *trpBA* promoter, causing the DNA to bend such that transcription by RNA polymerase is facilitated (Piñeiro et al., 1997).

Genes likely to be up-regulated by tryptophan are those involved in tryptophan

catabolism; however, the known mechanisms of activation do not seem to apply to tryptophan-induced transcription of *ipdc* in *P. putida* GR12-2. For example, tryptophan oxygenase, which catalyzes the first step in the conversion of tryptophan to niacin via the kynurenine pathway, is subject to allosteric activation by tryptophan; this of course does not explain the increased transcription of the *luxAB* reporter gene from the *ipdc* promoter. Using DNA microarrays to measure changes in transcript levels in response to exogenous tryptophan in *E. coli*, Khodursky et al. (2000) found that only transcription from the *tnaAB* operon was increased by tryptophan. This operon encodes tryptophanase (TnaA), which converts tryptophan to indole and pyruvate, and tryptophan permease (TnaB). In the absence of tryptophan, transcription of *tnaAB*, which is regulated by catabolite repression, is terminated in the leader region (*tnaC*) of the operon (Stewart and Yanofsky, 1985). When tryptophan is present, termination is inhibited and expression of *tnaAB* proceeds. A leader peptide similar to TnaC (GenBank Accession No. I54862) is not apparent in the region upstream of *ipdc* in *P. putida* GR12-2, therefore it is unlikely that indolepyruvate decarboxylase expression is regulated in a similar manner.

Given that only a small number of promoters known to be positively-regulated by tryptophan are available, identification of a tryptophan-responsive element using the method of Mironov et al. (1999) is not possible at this time. However, the reduced production of IAA in cells carrying pQFPROM-Kan compared to negative control cells carrying pQF70-Kan supports the hypothesis that a transcription factor is involved in the expression of *ipdc*. Many extra copies of the *ipdc* promoter on the multicopy plasmid pQFPROM-Kan would compete with the chromosomal *ipdc* promoter for binding of such a factor. It is not likely that a high level of expression of LuxAB on the plasmid is placing a metabolic load on the

cells because growth of these cells was similar to that of *P. putida* GR12-2/pQF70-Kan cells, in which LuxAB was not expressed. Also, despite the fact that tryptophan was continuously present in the culture medium, LuxAB activity did not increase for approximately 20 hours, perhaps awaiting the production/activation of a transcription factor. Although not likely to be applicable to bacterial cells, an element required for induction of transcription in response to tryptophan was present in the upstream region of both an aromatic aminotransferase and an indolepyruvate decarboxylase homologue in *Saccharomyces cerevisiae*; the cognate transcriptional activator was also identified (Iraqui et al., 1999).

It is possible that tryptophan only indirectly induces expression of indolepyruvate decarboxylase. *Azospirillum irakense*, a low IAA producer, accumulates more anthranilate than *Azospirillum* strains that synthesize higher levels of IAA (Zimmer et al., 1991). When *A. irakense* was transformed with genes for enzymes that metabolize anthranilate, for example, those from the tryptophan biosynthesis operon, anthranilate levels were reduced and IAA levels were increased (Zimmer et al., 1991). Exogenous or high levels of endogenous tryptophan can reverse suppression of IAA production by anthranilate by preventing anthranilate synthesis through feedback inhibition of anthranilate synthase, the enzyme that catalyzes the synthesis of anthranilate from chorismic acid (Denenu and Demain, 1981; Hartmann et al., 1983). Alternatively, tryptophan may indirectly induce indolepyruvate decarboxylase expression by increasing expression of tryptophan aminotransferase. This enzyme converts tryptophan to indolepyruvic acid which may be responsible for increased expression of indolepyruvate decarboxylase. The tryptophan aminotransferase from *E. herbicola* was shown to be regulated by tryptophan (Clark et al., 1992; Jaeger et al., 1999). It would be very difficult to test the hypothesis that indolepyruvic

acid activates the *ipdc* promoter by adding indolepyruvic acid to the culture medium because indolepyruvic acid is extremely unstable *in vitro*.

IAA itself has been shown to regulate expression of indolepyruvate decarboxylase in *A. brasilense* Sp245 (Vande Broek et al., 1999). A sequence similar to the auxin-responsive element found in the promoter of this gene is not present in the *P. putida* GR12-2 *ipdc* promoter. Lambrecht et al. (1999) believe that this element was derived from plants because it is similar in sequence to the auxin-responsive element found in promoter of some plant auxin-regulated genes (Ulmasov et al., 1999), and because its proximity to the RNA polymerase $\sigma^{54}$ recognition sequence in the *A. brasilense ipdc* promoter is suggestive of the modular arrangement of elements conserved in the hormone-inducible promoters of plants.

Since exogenous tryptophan is required for bacterial IAA production, it must be present in the rhizosphere for bacteria to synthesize IAA when associated with their host plants. One possible source of tryptophan is in the exudates of seeds and roots of plants. Seed and root exudates are known to contain many different compounds, mainly sugars, amino acids (including tryptophan (Martens and Frankenberger, 1994)), and organic acids, some of which can increase protein expression in bacteria (Van Bastelaere et al., 1993; Overbeek and van Elsas, 1995; Jaeger et al., 1999). It was hypothesized that a compound, possibly tryptophan, present in exudates collected from imbibed canola seeds, could activate the *ipdc* promoter and induce production of IAA in *P. putida* GR12-2. Seed exudates were expected to increase IAA expression to a greater extent than root exudates because the concentration of many amino acids is higher in seed exudates than in root exudates (Penrose, 2000), and because PGPR are believed to exert their effect early, before roots have developed, and therefore bacterial IAA, if involved in plant growth promotion, must also be

present early.

Contrary to expectation, exudates collected from canola seeds at 3, 6.5 and 10 hours after imbibition slightly inhibited transcription from the *ipdc* promoter in *P. putida* GR12-2/pQFPROM-Kan, although the exudates had no effect on IAA production. This does not necessarily preclude that tryptophan, or some other inducing factor, is not present in seed exudates. Levels of such a factor in the volume of exudates added to the cultures may be outside the range required to stimulate promoter activity. One explanation for lack of induction of *ipdc* is that the exudate was collected from uninoculated seeds and therefore sufficient levels of an appropriate inducer were not available. It is well known that bacteria stimulate exudation from seeds and roots (Lee and Gaskins, 1982; Heulin et al., 1987; Laheurte and Berthelin, 1988; Mehag and Killham, 1995), possibly by alteration of plant cell membrane permeability, a process that may be influenced by bacterial IAA (Rausch et al., 1984; Brummel and Hall, 1987; Bashan and Levanony, 1991). It is also possible that bacteria may stimulate exudation of specific compounds, such as tryptophan. For example, bacteria that synthesize ACC deaminase are a sink for that enzyme's substrate, the amino acid 1-aminocyclopropane-1-carboxylic acid (ACC) (Penrose and Glick, 2001).

Certainly, levels of tryptophan available in exudates from roots of *Avena barbata* treated with *E. herbicola* 299R were adequate to increase the activity of a tryptophan-responsive promoter in *E. herbicola* 299R (Jaeger et al., 1999). IAA production has been shown to be induced in the presence of leaf extracts in *Xanthomonas axonopodis* pv. *citri* (Costacurta et al., 1998), and *ipdc* promoter activity, assessed by fusing the promoter to a gene for ice nucleation protein or for green fluorescent protein, increased dramatically when *E. herbicola* 299R was associated with the leaves and flowers of various plants, in some

cases, more so than levels induced under the best conditions *in vitro* (Brandl and Lindow, 1997; Brandl et al., 2001).

During the logarithmic phase of bacterial cell growth, expression of many of the genes required for cell proliferation is controlled by the sigma factor, RpoD ($\sigma^{70}$) (Jishage and Ishihama, 1995). RpoD is a component of bacterial RNA polymerase and is responsible for promoter recognition. When available nutrients begin to be depleted, cells respond by increasing production of another sigma factor known as RpoS ($\sigma^{38}$) (Jishage and Ishihama, 1995). This stationary phase sigma factor competes with RpoD for association with RNA polymerase and directs RNA polymerase to the promoters of genes whose products enable the bacterial cells to survive under starvation or stress conditions (McCann et al., 1991; O'Neal et al., 1994; Lazazzera, 2000). Examples of genes shown to be regulated by RpoS include those involved in DNA repair, in the determination of cell morphology (cells become more compact under nutrient stress conditions), in thermotolerance, and in virulence (Loewen and Hengee-Aronis, 1994; Schellhorn et al., 1998). It is likely that, outside of laboratory conditions, bacterial cells spend most of their time in such a survival mode, during which stationary phase responsive genes are expressed. IAA biosynthesis genes, and specifically *ipdc*, in *P. putida* GR12-2 seem to belong in this group of stationary phase genes for the following reasons: 1) IAA production normally begins in the late-log/early stationary phase, at the same time as RpoS levels are known to increase (Zhou and Gottesman, 1998; Jishage and Ishihama, 1995), and accumulates into the later stationary phase of cell growth, at least to 48 hours. Recombinant cells carrying constitutively produced RpoS produce IAA earlier, and continue to do so at consistently elevated levels, compared to cells that produce natural levels of RpoS; 2) The *ipdc* promoter of *P. putida* GR12-2 contains a sequence

similar to the consensus sequence CTATACT recognized by RpoS (Espinosa-Urgel et al., 1996). Brandl et al. (2001) also indicate that indolepyruvate decarboxylase is regulated by RpoS in *Erwinia herbicola* 299R; 3) Although constitutive production of RpoS does not increase transcription of *ipdc* at its peak, suggesting that RpoS is not normally limiting at this stage of cell growth, it does cause the promoter to be activated earlier, when RpoS is not naturally produced. In addition, constitutively produced RpoS prolongs a significantly higher level of transcription at the later stages of the cell cycle, when nutrients in the culture medium are severely depleted and RpoS is likely to be in high demand. Elevated levels of *ipdc* transcription are seen both in cultures grown in minimal medium supplemented with tryptophan and in those grown without tryptophan, although in the latter case the increase in transcription compared to cells lacking extra RpoS is not as dramatic.

**Role of Indoleacetic Acid in Plant Growth Promotion**

To understand how microbial IAA influences plant growth, mutants can be created that overproduce or underproduce IAA, and any alterations from the plant's response to the wild-type strains can be observed. Despite the inability to obtain bacterial mutants completely deficient in IAA synthesis, some mutants with significantly reduced levels have been generated by several means. Where IAA biosynthesis genes are plasmid-encoded, IAA underproducing mutants have been isolated following curing of plasmids from the phytopathogens *Pseudomonas syringae* pv. *savastanoi* and *Agrobacterium tumefaciens*, using acridine orange (Comai and Kosuge, 1980) and heat and ethidium bromide treatment (Liu et al., 1982), respectively, and by transposon mutagenesis of the Ti plasmid of *A. tumefaciens* (Liu et al., 1982) and a large plasmid from *Erwinia herbicola* pv. *gypsophilae*

(Clark et al., 1993). Spontaneous mutants have also been identified in *P. syringae* pv. *savastanoi* (Smidt and Kosuge, 1978; Surico et al., 1984; Soby et al., 1994). Where IAA genes are located in the chromosome, chemical mutagenesis has been used to isolate an aromatic aminotransferase deficient mutant of *A. tumefaciens* (Liu et al., 1982) and transposon insertion has interrupted IAA biosynthesis genes in *P. syringae* pv. *syringae* (Mazzola and White, 1994) and in *Azosprillum lipoferum* (Abdel-Salam and Klingmüller, 1987).

Replacement of a wild-type IAA biosynthesis gene in the chromosome of a bacterial cell with a non-functional version will also abolish IAA production. This technique, known as marker exchange, necessitates a knowledge of the gene sequence that is to be disrupted. The target gene sequence, subcloned onto a suicide plasmid that cannot replicate in the host bacterium, is interrupted with a marker gene, for example, a gene encoding antibiotic resistance. Following transformation of the host bacterium with this construct, one can select for double cross-over recombination events between homologous regions of the target sequence on the plasmid and on the chromosome by growing cells in the presence of an appropriate antibiotic. Resistant cells will have integrated the antibiotic resistant marker into the genomic sequence. The plasmid, which would now contain the wild-type IAA gene sequence, would be lost as it cannot replicate in these cells. This technique has been used successfully to generate indolepyruvate decarboxylase deficient mutants of *E. herbicola* (Brandl and Lindow, 1996) and *A. brasilense* (Costacurta et al., 1994).

Marker exchange was also used here to successfully generate an IAA-deficient mutant of *P. putida* GR12-2. Mutant cells are resistant to kanamycin indicating that the kanamycin resistant gene was transferred from pJQIPDC4-Kan to the chromosome of

*P. putida* GR12-2 by homologous recombination, and the cells were also resistant to sucrose but not to gentamicin, evidence that the entire plasmid carrying the genes for SacB and gentamicin acetyltransferase (Quandt and Hynes, 1993) had not been incorporated into the genome by a single cross-over event. The most conclusive evidence for the creation of an IAA-deficient mutant was the lack of IAA production in the presence of tryptophan, conditions under which the wild-type strain produces copious amounts of IAA. As mentioned above, mutants with reduced ability to synthesize IAA have been generated for some other bacteria, however, these strains still produce low levels of IAA in the presence of tryptophan (Abdel-Salam and Klingmüller, 1987; Clark et al., 1993; Costacurta et al., 1994; Brandl and Lindow, 1996). It was concluded that an alternate pathway for IAA biosynthesis is present in these bacteria; many bacteria are known to possess genes for at least two different IAA biosynthesis pathways (Kuo and Kosuge, 1970; Liu et al., 1982; Manulis et al., 1991b; Clark et al., 1993).

The low level of IAA apparently present in the culture medium of the IAA-mutant of *P. putida* GR12-2 supplemented with high levels of tryptophan is more likely a consequence of the accumulation of indolepyruvic acid which can react with Salkowski's reagent, used in the colorimetric determination of IAA concentration, than to the synthesis of IAA by an alternate pathway. That the Salkowski's reactive product is not IAA is further evidenced by the color of the reaction product which was purple-red rather than pink-red indicative of IAA. The Salkowski's reactive product was present only at very low, barely detectable, levels in cultures supplemented with lower levels of tryptophan (e.g., 50 μg/mL), levels of tryptophan sufficient to induce IAA production in the wild-type strain. The contribution of an alternative, tryptophan-inducible pathway to the production of IAA would be expected to

be higher than that observed in cultures containing 50 µg/mL of tryptophan. Southern hybridization and PCR indicated that only a single copy of *ipdc* is present in the *P. putida* GR12-2 genome. Southern blots containing *P. putida* GR12-2 genomic DNA failed to hybridize to the *iaaM/iaaH* genes from indoleacetamide pathway used as a probe, even under low stringency conditions (Patten, 1996), however, lack of significant homology necessary for hybridization cannot be ruled out. The presence of a Salkowski's reactive product, rather than an alternate IAA biosynthesis pathway, may also explain the apparent low levels of IAA production in *A. lipoferum* mutants (Abdel-Salam and Klingmüller, 1987). High performance liquid chromatography (HPLC) could be used to differentiate between IAA and indolepyruvic acid, and any other indole compounds secreted into the culture medium by the IAA mutant strains.

Loss of the ability to produce IAA following disruption of the *ipdc* gene confirms that *P. putida* GR12-2 produces IAA via the indolepyruvic acid pathway. This provides more support for the hypothesis that plant growth-promoting bacteria such as *Azospirillum* spp. (Costacurta et al., 1994) and *Enterobacter cloacae* (Koga et al., 1991b) produce IAA via the indolepyruvic acid pathway in contrast to plant pathogens which seem to preferentially synthesize IAA via the indoleacetamide pathway (Kuo and Kosuge, 1970; Schroeder et al., 1984; White and Ziegler, 1991; Mazzola and White, 1994). Indeed, rendering the *ipdc* gene inactive by insertional mutagenesis, and thereby abolishing IAA production by this pathway, significantly reduces the ability of *P. putida* GR12-2 to promote primary root growth in canola seedlings. It is known from application of exogenous IAA (Thimann, 1938; Evans et al., 1994), or application of diluted culture extracts or low density inocula of bacteria that produce high levels of IAA (Harari et al., 1988; Selvadurai et al., 1991; Beyerler et al.,

1997), that low concentrations of IAA can stimulate primary root elongation; however, this is the first report to demonstrate directly that bacterial IAA plays a major role in promotion of root elongation when the bacterium is associated with its host plant.

IAA secreted by a bacterium may promote root growth directly. Application of exogenous IAA has been shown to regulate the expression of many plant genes and to influence a variety of physiological responses in plants including cell elongation and cell division. It is generally believed that cell elongation, which occurs within minutes of exposure to exogenous IAA, is explained by cell wall acidification, whereas IAA-induced cell division is a later effect involving specific changes in gene expression (Theologis, 1986; Hagen, 1987). In the acid growth theory, acidification of the plant cell apoplast leads to loosening of the cell wall and subsequently, to cell extension as the uptake of water increases (Brummel and Hall, 1987; Rayle and Cleland, 1992). IAA acts on the external surface of the cell to activate a plasma membrane-bound $H^+$-ATPase which pumps hydrogen ions into the apoplast. The decrease in apoplastic pH may activate enzymes that can cleave bonds between cell wall components.

Exogenous IAA has both a rapid effect on mRNA levels, within minutes of application, as well as a delayed effect, hours after exposure (Theologis, 1986; O'Neill and Scott, 1987). Hagen (1987) has identified over forty mRNAs that are either up- or down-regulated in response to IAA. The early mRNAs encode small, short-lived, nuclear proteins with DNA-binding domains (Goldsmith, 1993; Garbers and Simmons, 1994; Abel and Theologis, 1996) and may encode transcription factors that control auxin-regulated gene expression. Products of the later RNAs have been identified as ribosomal RNAs, RNA polymerase I, ribosomal proteins, peroxidases, cellulases, glucanases, and pectin enzymes

(Theologis, 1986; O'Neill and Scott, 1987; Garbers and Simmons, 1994).

Although roots from seeds inoculated with wild-type *P. putida* GR12-2 were consistently longer than those from uninoculated seeds, there was some variability in the response of plants to the IAA-deficient mutant. The roots from seeds treated with the mutant were generally shorter than those from seeds treated with the wild-type strain, and were usually, but not always, as short as those from uninoculated seeds, suggesting that IAA production, or lack thereof, is not solely responsible for influencing primary root growth. Bacteria can use one or more of several mechanisms to promote plant growth. ACC deaminase, produced by many plant growth-promoting bacteria (Shah et al., 1997; Glick et al., 1998; Belimov et al., 1998; Shah et al., 1998), including *P. putida* GR12-2 (Jacobson et al., 1994), is also involved in the stimulation of root elongation in seedlings (Glick et al., 1994; Li et al., 2000). ACC deaminase hydrolyzes plant ACC, the immediate precursor to ethylene, and thereby prevents the production of plant growth-inhibiting levels of ethylene (Penrose et al., 2001). Mutants of plant growth-promoting bacteria that no longer produce ACC deaminase, have lost the ability to stimulate root elongation (Glick et al., 1994; Li et al., 2000). Thus, it is possible that both IAA and ACC deaminase work in concert to stimulate root elongation. IAA produced by *P. putida* GR12-2 may indirectly promote root growth by influencing ACC deaminase activity. Exogenous IAA is known to increase the transcription and activity of ACC synthase (Peck and Kende, 1995), which catalyzes the production of ACC from S-adenosylmethionine. Because ACC stimulates ACC deaminase activity in bacteria (Honma and Shimomura, 1978; Jacobson et al., 1994; Li and Glick, 2001), perhaps when bacterial IAA is not available to induce sufficient ACC production via ACC synthase, ACC deaminase activity is low and therefore plant ethylene levels remain

high enough to inhibit root growth. In addition, bacterial IAA may indirectly increase ACC deaminase activity by stimulating exudation of metabolites, including ACC, from plant roots.

Although, great effort was made to exactly replicate each root elongation assay, seed formation and storage conditions prior to inoculation with the bacteria may have varied, especially humidity levels and temperature. During the development and maturation drying of seeds, the content of free IAA and other hormones such as abscisic acid decreases (Buchanan et al., 2000; Mayer and Poljakoff-Mayber, 1989). Upon rehydration, the hormone levels within the seeds increases (Mayer and Poljakoff-Mayber, 1989), and thus their responsiveness to additional input of these hormones from associated bacteria may diminish. In plants, endogenous levels of IAA may be suboptimal or optimal for growth (Pilet and Saugy, 1987). If IAA levels within seeds stored under humid conditions increased to levels optimal for root development, then IAA contributed by a bacterium would be ineffective; that is, the seeds would no longer be sensitive to exogenous IAA, or a lack thereof.

The inability of the IAA-deficient mutant to stimulate root growth may be less a consequence of loss of IAA directly than a consequence of a reduced ability to colonize plant surfaces. Recently, Brandl and Lindow (1998) have shown that an *E. herbicola* 299R mutant strain in which IAA production was abolished, could no longer compete with the wild-type strain for colonization of bean leaves and pear flowers. *In vitro*, the mutant strain grew at the same rate and reached the same cell density as the wild-type strain indicating that growth of the mutant was not debilitated.

IAA does not seem to influence shoot growth, at least within the first five days of seedling growth. Shoots are generally less sensitive to auxin than are roots and require higher levels of exogenous IAA for growth stimulation (Thimann, 1952).

While low levels of IAA stimulate root elongation, high levels of bacterial IAA, whether from IAA-overproducing mutants or strains that naturally secrete high levels, or from high density inocula, stimulate the formation of lateral and adventitious roots (Barbieri et al., 1986; Loper and Schroth, 1986; Barbieri and Galli, 1993; Sawar and Kremmer, 1995; Xie et al., 1996; Mayak et al., 1997; Beyerler et al., 1997). *P. putida* GR12-2 cells that produce wild-type levels of IAA stimulated the formation of many short adventitious roots on mung bean cuttings, and an IAA-overproducing mutant induced even more adventitious roots than the wild-type strain (Mayak et al., 1997). In contrast, the IAA-deficient mutant of *P. putida* GR12-2 stimulated fewer roots than the wild-type bacterium and these were generally longer than those induced by the wild-type strain.

Several lines of evidence suggest a role for IAA in lateral and adventitious root development (reviewed by Malamy and Benfey, 1997). Exogenous IAA increases root initiation. *Arabidopsis* mutants with higher levels of endogenous IAA have more lateral roots, while auxin-resistant mutants have fewer. IAA initiates rooting by establishing a population of rapidly dividing pericycle cells (Laskowski et al., 1995; Casimiro et al., 2001). Recent work is attempting to elucidate the mechanism by which the IAA signal is transduced to stimulate cell division for root formation. Nakazawa et al. (2001) have identified a gene (*dfl1*) that is induced by IAA and is involved in the initiation and development of lateral roots in *Arabidopsis*. Rooting in a *dfl1*-overproducing mutant was significantly reduced and *dfl1* antisense transgenic plants had significantly more lateral roots suggesting that DFL1 negatively regulates root formation. Rogg et al. (2001) also describe an *Arabidopsis* gene (*iaa28*) whose product represses IAA-induced genes involved in lateral root initiation.

Initiation of adventitious and lateral roots may be mediated by IAA-induced ethylene.

An ACC deaminase-negative mutant of *P. putida* GR12-2 which is no longer capable of reducing ethylene levels in plants, stimulated more small adventitious roots than the wild-type strain (Mayak et al., 1997). The increase in the number of roots on the cuttings correlated with an increase in ethylene production. Mung bean cuttings treated with 1-methylcyclopropene, a cyclic ethylene analogue which binds ethylene receptors in plants and thereby prevents perception of ethylene (Sisler and Serek, 1997), also prevents the initiation of adventitious roots (Saleh et al., in preparation). The IAA-deficient mutant of *P. putida* GR12-2 would no longer be able to stimulate ACC synthase, and therefore ethylene synthesis in plants, thus fewer adventitious roots are initiated on the cuttings. An attempt was made to measure ethylene levels stimulated in cuttings by the IAA-deficient mutant in the first 4.5 hours after treatment using the method described by Mayak et al. (1997), however, levels were outside the lower limit of detection. That more roots are still stimulated by the mutant compared to treatment with water, suggests that the presence of the bacteria alone is sufficient to induce root initiation. Again, this may be related to ethylene; inoculation with a bacterium imposes a certain amount of stress on the plants and ethylene production is a well-known plant stress response (Deikman, 1997).

High levels of exogenous or bacterial IAA, and therefore high levels of ethylene, have also been shown to inhibit elongation growth in roots (Rahman, 2001; Xie et al., 1996; Beyerler et al., 1997; Sawar and Kremmer, 1995; Loper and Schroth, 1986). Thus, while adventitious roots that formed on mung bean cuttings inoculated with wild-type *P. putida* GR12-2 were very short, most less than 1mm long, the roots stimulated by the IAA-deficient mutant strain were longer. Because cuttings were suspended in a bacterial suspension for eight days, they were exposed to a high inoculum density continuously for an extended

period of time. Cuttings treated with the wild-type bacterium would therefore be exposed to a high level of IAA throughout this period.

From a practical point of view, treatment of cuttings with an IAA-deficient mutant may be a beneficial method for propagation of plants. Certainly while many adventitious roots are desirable, longer roots with more surface area through which the plants can absorb nutrients and water from the soil would be advantageous. Treatment with the IAA-deficient mutant of *P. putida* GR12-2 provides just such a compromise between the many short roots stimulated by the wild-type strain and the few long roots produced by treatment with water. Before such a treatment would be viable commercially, it would have to be demonstrated that it stimulates root development in cuttings better than, or at least as well as, current treatments, that it has the desired effect on plants that are horticulturally more valuable than mung beans, and that, in the long term, cuttings with more and longer roots are propagated more successfully than current treatments.

**Conclusion**

Finally, we are left with the question as to why bacteria have evolved to produce IAA, a plant hormone which does not apparently function as such in bacterial cells. Others have suggested that IAA production is a mechanism by which bacterial cells can detoxify high levels of tryptophan or tryptophan analogues that are deleterious to bacterial cells. Some IAA biosynthetic enzymes can convert methylated and halogenated substrates to less toxic compounds (Hutcheson and Kosuge, 1985; Yamada et al., 1985; Bar and Okon, 1992), however, these enzymes are from the indoleacetamide pathway which does not explain the presence of enzymes involved in the indolepyruvic acid pathway. It is also doubtful that high

levels of tryptophan would often be encountered by bacterial cells in nature. Rather, it is expected that the converse would usually be the case, as levels of tryptophan in the soil and even in the rhizosphere are low (Weibull et al., 1990; Kravchenko et al., 1991).

IAA production may have evolved in bacteria because it is important in the bacterial-plant relationship. In this research and that of others, it has been shown that bacterial IAA plays a role in the development of the host plant root system, and in phytopathogens such as *Agrobacterium tumefaciens*, IAA stimulates the formation of gall tumors; in both situations the advantage for the bacterium is an increased food supply. As much as 30% of the metabolic products of the carbon fixed by plants may be lost from roots into the rhizosphere as exudates, lysates and mucilage (Martens and Frankenberger, 1994) providing a rich source of carbon and energy for root-associated bacteria; bacterial IAA may enhance the release of plant metabolites from roots. Brandl and Lindow (1998) postulate that IAA-deficient mutants are less competitive on plant surfaces because decreased levels of IAA result in decreased exudation of nutrients from plants. It may be significant that nutrient-deprived *Azospirillum* sp. (Yahalom et al., 1990; Omay et al., 1993), and *Erwinia herbicola* (Brandl and Lindow, 1997) increase IAA production, thereby providing a mechanism to promote root exudation and obtain nutrients essential for their growth. Here it has been shown that production of indolepyruvate decarboxylase and IAA in *P. putida* GR12-2 are regulated by tryptophan, a component of many plant exudates, and by RpoS, a stationary phase sigma factor that regulates gene expression in response to nutrient stress.

If it is true that bacteria have evolved to produce IAA because it is involved in their relationship with the host plant, then one would expect only plant-associated bacteria to possess genes for IAA synthesis. However, this is not the case. A search of the sequenced

bacterial genomes suggests that a protein highly similar to indolepyruvate decarboxylase is widespread among bacteria, even in those that are not normally associated with plants. For example, a protein 66% identical (77% similar) to indolepyruvate decarboxylase was found in *Salmonella typhimurium*. Several species of *Mycobacterium*, including *M. leprae* and *M. tuberculosis*, also carry a sequence with a high degree of similarity (47% identity, 61% similarity) to indolepyruvate decarboxylase. The extent of similarity is much higher than that between indolepyruvate decarboxylase and closely related proteins such as pyruvate decarboxylase and acetolactate synthase which are 35% and 25% identical (50% and 40% similar), respectively, suggesting that the similar proteins found in the genome databases are indolepyruvate decarboxylase rather than another related protein.

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