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Isolation and Characterization of Mutants of the Plant Growth-Promoting Rhizobacterium *Pseudomonas putida* GR12-2 That Overproduce Indoleacetic Acid

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Abstract. Following transposon Tn5 mutagenesis of the plant growth-promoting rhizobacterium *Pseudomonas putida* GR12-2, mutants that were able to grow in the presence of the tryptophan analog 5-fluorotryptophan were selected. Seven of the 50 5-fluorotryptophan-resistant mutants overproduced the phytohormone indoleacetic acid (IAA). Of these seven mutants, the highest level of IAA was observed with strain *P. putida* GR12-2/*aux*1, which produced four times the amount of indoleacetic acid synthesized by the wild-type strain. Strain *P. putida* GR12-2/*aux*1, in contrast to the wild type, lost the ability to stimulate the elongation of the roots of canola seedlings under gnotobiotic conditions. The growth rate, siderophore production, and 1-aminocyclopropane-1-carboxylate deaminase activity of mutant strain *P. putida* GR12-2/*aux*1 were identical to those of the wild-type strain. The role of IAA in the mechanism of plant growth stimulation by *P. putida* GR12-2 and other plant growth-promoting rhizobacteria is discussed.

A beneficial free-living soil bacterium, often referred to as a plant growth-promoting rhizobacterium or PGPR [19], can affect plant growth either indirectly or directly [8]. Indirect promotion of plant growth occurs when a PGPR lessens or prevents the deleterious effects of one or more phytopathogenic organisms. The direct promotion of plant growth usually entails either providing the plant with a compound that is synthesized by the bacterium or facilitating the uptake of certain nutrients from the environment. For example, various PGPR strains can fix atmospheric nitrogen, synthesize siderophores that can solubilize iron from the soil and provide it to plant cells, synthesize phytohormones that can enhance plant growth, may have mechanisms for solubilization of minerals such as phosphorus, or may synthesize enzymes that can modulate plant growth and development [8]. A particular PGPR, such as *Pseudomonas putida* GR12-2 [20], may affect plant growth and development by any one or more of these mechanisms. Prominent among the mechanisms used by PGPR strains are bacterial synthesis of the phytohormone indoleacetic acid (IAA) and bacterial regulation of the production of ethylene in developing seedlings. In the latter case, the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC) is sequestered and then hydrolyzed from the plant through the action of the enzyme ACC deaminase [9, 10, 11, 16]. Furthermore, since IAA can stimulate the activity of the enzyme ACC synthase, thereby increasing the amount of ACC in the plant, bacterially synthesized IAA can not only stimulate cell elongation and cell division, but may also inhibit plant growth by promoting the synthesis of ethylene. As a first step toward developing a more detailed understanding of how bacterially synthesized IAA affects plant growth and development, a number of mutants of *P. putida* GR12-2 that overproduce IAA have been isolated and characterized.

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Materials and Methods

Pseudomonas putida GR12-2 was originally isolated from the rhizosphere of an Arctic plant [20] and was kindly provided by Gerry Brown, Agrium, Inc. (Saskatoon, Sask.). It is an effective root colonizer of canola (*Brassica campestris* cv. Tobin) and promotes canola root elongation under gnotobiotic conditions [21]. Growth in liquid medium was monitored by measuring the optical density of the cultures at 600 nm.

The transposon Tn5 was introduced into *P. putida* GR12-2 by conjugation with *E. coli* MM294A/pRK602 [4]. Transconjugants of *P. putida* GR12-2 with Tn5 inserted into the chromosomal DNA were selected on solid DF (Dworkin and Foster) salts minimal medium plus 2.0 g/L ammonium sulfate [6] containing 50 μg/ml kanamycin after growth for 5 to 7 days at room temperature (22°C ± 1°C). Under these conditions, only *P. putida* GR12-2 cells that contain an inserted Tn5 sequence will proliferate.

Kanamycin-resistant cells were grown in liquid DF salts minimal medium containing kanamycin and then plated onto solid DF salts minimal medium plus ammonium sulfate containing 0.01 mg/ml 5-fluorotryptophan, and incubated at 22 ± 1°C for 7–10 days. Auxotrophic mutants are unable to grow on DF salts minimal medium.

The stimulation of canola seedling root elongation under gnotobiotic conditions by *P. putida* GR12-2 and mutant strains was assessed according to the procedure described by Tang and associates [27] following growth on tryptic soybean broth (TSB) medium (Difco) plus 0.1 mg/ml tryptophan.

The amount of siderophore secreted into the medium was determined according to the "universal" siderophore assay [26]. The amount of siderophore was expressed as the μmoles of the siderophore rhizobactin per 10^9 bacterial cells.

IAA levels secreted into the growth medium were measured by the method of Tang and Bonner [28], with the formation of the IAA-$FeCl_3$ reagent complex being monitored at 535 nm. A plot of the absorbance of the complex at 535 nm versus IAA concentration was linear from 1 to 12 μg/ml of IAA.

The assay for ACC deaminase activity was based on the method of Honma and Shimomura [15]. Ninety-microliter aliquots of crude bacterial cell extract were incubated with 10 μl of 1.0 M ACC for 2 h at 30°C followed by the addition of 1.9 ml of 0.56 M HCl and 0.3 ml of 2,4-dinitrophenylhydrazine. The mixture was incubated for 15 min at 30°C; then 2 ml of 2 M NaOH was added and the absorbance read at 540 nm. A plot of the absorbance at 540 nm versus the α-ketobutyrate concentration was linear between 0.2 and 1.2 μmoles of α-ketobutyrate. ACC deaminase activity was expressed as units of enzyme activity where 1 unit was the amount of activity required to produce 1 μmole of α-ketobutyrate per min. Protein concentrations were determined by the procedure of Bradford [3] with bovine serum albumin as a standard.

Isolation of plasmid and chromosomal DNAs, restriction enzyme digestion, agarose gel electrophoresis, Southern blotting, primer labeling, DNA hybridization, and autoradiography were performed as described by Maniatis and colleagues [23].

Results

After transposon mutagenesis of *P. putida* GR12-2 with Tn5, 50 colonies that were resistant to both kanamycin and 5-fluorotryptophan were isolated. Seven of the 50 mutants produced more IAA than the wild-type *P. putida* GR12-2 (Table 1). These mutants were designated as *P. putida* GR12-2/*aux*1–7, reflecting the fact that these mutants are altered in some region of their DNA that affects the synthesis of indoleacetic acid (auxin). Of these seven, mutant *P. putida* GR12-2/*aux*1 secreted the highest level of IAA, approximately four times the amount of IAA secreted by the wild type, *P. putida* GR12-2.

Table 1. Quantitation of IAA secreted into the growth medium by wild-type and 5-FT-resistant mutants of *P. putida* GR12-2

Strain	IAA secreted (μg/ml) (n = 6, ±SEM)
P. putida GR12-2	2.01 ± 0.13
P. putida GR12-2/*aux*1	8.28 ± 0.50
P. putida GR12-2/*aux*2	5.23 ± 0.57
P. putida GR12-2/*aux*3	6.09 ± 0.30
P. putida GR12-2/*aux*4	3.84 ± 0.53
P. putida GR12-2/*aux*5	4.80 ± 0.25
P. putida GR12-2/*aux*6	4.41 ± 0.19
P. putida GR12-2/*aux*7	4.33 ± 0.14

Paired *t*-tests of comparisons between wild-type GR12-2 and each of the seven mutants showed that each mutant was significantly different from wild-type, *P. putida* GR12-2, in IAA production. Also, a paired *t*-test indicated that *P. putida* GR12-2/*aux*1 was significantly different from all of the other mutants.

Table 2. Effect of wild-type and IAA-overproducing mutants of *P. putida* GR12-2 on root elongation of canola seedlings

Seeds treated with	Root length ± SEM (mm)
A. *P. putida* GR12-2	69.0 ± 2.3
B. *P. putida* GR12-2/*aux*1	29.7 ± 2.6
C. *P. putida* GR12-2/*aux*2	77.2 ± 2.5
D. *P. putida* GR12-2/*aux*3	68.4 ± 3.2
E. *P. putida* GR12-2/*aux*4	79.1 ± 3.5
F. *P. putida* GR12-2/*aux*5	77.2 ± 3.3
G. *P. putida* GR12-2/*aux*6	73.6 ± 3.2
H. *P. putida* GR12-2/*aux*7	76.2 ± 3.0
I. $MgSO_4$	44.5 ± 1.7

Paired *t*-tests indicated that treatments A, C, D, E, F, G, and H were not significantly different ($P > 0.05$) from each other, but each of them was significantly different from treatments B and I ($P < 0.01$). Also, treatments B and I were significantly different from each other ($P < 0.01$). For each treatment, 30 seeds were tested.

With the exception of *P. putida* GR12-2/*aux*1, all of the selected mutants stimulated canola root elongation to the same extent as the wild type, *P. putida* GR12-2, and in each case to an extent significantly different from seeds treated with 0.1 M $MgSO_4$ (Table 2). Canola seeds that were treated with *P. putida* GR12-2/*aux*1 gave shorter, thicker, and more laterally branched roots than seeds treated with wild-type *P. putida* GR12-2, and a paired *t*-test showed that the difference in the root lengths was significant. All further studies focused on *P. putida* GR12-2/*aux*1.

The mutant *P. putida* GR12-2/*aux*1 was compared with the wild-type bacterium in several ways: its ability to grow in TSB medium at 25°C, synthesize siderophores, and produce the enzyme ACC deaminase (Table 3). In these tests, *P. putida* GR12-2/*aux*1

Table 3. Some biological activities of the mutant *P. putida* GR12-2/*aux*1 compared with wild-type *P. putida* GR12-2

Cells	Generation time at 22°C (h)[a]	Rhizobactin equivalents (μmol)[b]	ACC deaminase specific activity (units/mg)[c]
P. putida GR12-2	1.5 ± 0.2	12.48 ± 0.36	12.3 ± 1.5
P. putida GR12-2/*aux*1	1.5 ± 0.3	12.05 ± 0.36	13.8 ± 1.2

[a] Growth was monitored in TSB medium (n = 3, ±SEM).
[b] Siderophore secreted into the medium after growth in iron-depleted medium for 60 h (n = 4, ±SEM).
[c] Activity of cell-free extracts prepared after growth on DF salts minimal medium plus ACC (n = 4, ±SEM).

behaved identically to the wild-type bacterium (Table 3). This is in marked contrast to the behavior of a transformant of *P. putida* GR12-2 that carries the broad host range plasmid pGSS15 [13, 14]. In that case, the presence of the plasmid debilitated the bacterium in a number of metabolic functions, including growth rate and siderophore production as a consequence of a metabolic load imposed by the plasmid. By these criteria, the inability of the mutant *P. putida* GR12-2/*aux*1 to stimulate the elongation of canola roots can be attributed solely to the overproduction of IAA.

When *P. putida* GR12-2 and *P. putida* GR12-2/*aux*1 chromosomal DNA were digested with restriction enzyme *Sal*I and hybridized with a radiolabeled probe for Tn5 DNA, three bands were detected in the lane containing *P. putida* GR12-2/*aux*1, but none were detected in the lane with *P. putida* GR12-2 DNA (data not shown). The DNA bands observed were 8.0, 4.2, and 4.0 kb in length. Since there is one *Sal*I cleavage site in the middle of the Tn5 sequence [17, 25], hybridization of *Sal*I-digested chromosomal DNA with radiolabeled Tn5 should yield two bands if there is a single copy of Tn5 in the chromosomal DNA, and four bands if there are two copies of Tn5. The simplest explanation for the presence of three bands is that the 8.0-kb band resulted from the partial digestion of the chromosomal DNA and contained the entire Tn5 sequence with flanking chromosomal DNA; the 4.2 kb represents one portion of Tn5 with flanking chromosomal DNA; and the 4.0 kb represents the other piece of Tn5 with its flanking chromosomal DNA. The fact that the size of the larger band at 8.0 kb is about the same as the sum of the two smaller bands is consistent with the view that there is one copy of Tn5 inserted in *P. putida* GR12-2/*aux*1.

Discussion

A better understanding of the role played by bacterial IAA in plant root development [2, 22, 24] might be obtained by comparing the behavior of PGPR mutants that produce altered levels of IAA compared with the wild-type strain. To this end, Tn5 mutants of *P. putida* GR12-2 that were resistant to the toxic effects of 5-fluorotryptophan were selected.

Resistance to 5-fluorotryptophan can occur in several different ways. First, uptake of this tryptophan analog may be blocked by alteration of a membrane protein. Second, mutagenesis may have activated the synthesis or activity of an enzyme that degrades 5-fluorotryptophan. Third, altered regulation of the expression of one or more enzymes could lead to an increase in the biosynthesis of tryptophan, the precursor of IAA. In this case, overproduction of tryptophan or one of its metabolites, such as IAA, that is normally secreted into the growth medium could compete with the toxic analog for entry into the cell and/or integration into newly synthesized proteins [12]. In a study of IAA mutants in *Azospirillum*, large colonies on 5-fluorotryptophan-containing plates surrounded by small 5-fluorotryptophan-sensitive colonies were observed [12]; this arrangement of colonies on the plate suggested that the large central colony secreted tryptophan or tryptophan metabolites that afforded some measure of protection to the surrounding colonies by competing with the toxic 5-fluorotryptophan. In the present work, large colonies surrounded by small colonies in 5-fluorotryptophan-containing medium were not observed. Therefore, all 5-fluorotryptophan-resistant colonies were isolated and tested for the production of IAA.

When the seven mutants that overproduced IAA were tested for their ability to stimulate canola root elongation under gnotobiotic conditions, only the mutant that overproduced IAA to the greatest extent (*P. putida* GR12-2/*aux*1) inhibited root elongation. The others behaved just like the wild-type bacterium despite the fact that some of the other mutants produced two to three times the level of IAA synthesized by the wild type. This indicates that small changes in the level of bacterial IAA can dramatically alter the response of the plant to the bacterium (Tables 1 and 2) and is consistent with the existence of a threshold level of exogenous IAA that can be added to plants, above which the roots of the treated plant are unable to develop normally.

The inhibitory effect of *P. putida* GR12-2/*aux*1 on root growth may result from the increased level of IAA interacting with the enzyme ACC synthase in the

plant and stimulating the synthesis of ACC, the immediate precursor of the hormone ethylene. After seed germination, too much ethylene can inhibit root elongation [7]. This explanation is consistent with the hypothesis that the enzyme ACC deaminase in *P. putida* GR12-2 permits canola roots to elongate by lowering the amount of ethylene in the developing seedling [8–11, 16]. With *P. putida* GR12-2/*aux*1 the high level of IAA could act to increase the ACC level in the plant to the point where some, but not all, of the additional ACC is hydrolyzed by the bacterial ACC deaminase and the ethylene level becomes elevated.

When a PGPR such as *P. putida* GR12-2 produces too high a level of one phytohormone (for example, IAA), the hormonal balance critical to the growth and development of root tissues may be disrupted, and the bacterium switches from an organism that stimulates root elongation to one that inhibits it (that is, a phytopathogen). In this context it is noteworthy that after removal of cytokinin and IAA biosynthesis genes from the T-DNA of the Ti plasmid of *Agrobacterium tumefaciens*, transformation of susceptible plants no longer results in the formation of a crown gall [18]. In other words, following the removal of the ability to synthesize phytohormones, the Ti plasmid, while still infective, is no longer tumor-inducing. Furthermore, when sour cherry cuttings and tomato seedlings were treated with either wild-type or an IAA-overproducing mutant of *P. fluorescens* BSP53a, root weights of the plants that were inoculated with the mutant decreased significantly in comparison with treatment with the wild-type strain [5]. In another study, it was reported [12] that sugar beet seedlings that were inoculated with strains of the phytopathogen *Pseudomonas syringae pv. savastanoi* had much shorter roots than plants inoculated with mutant strains of this bacterium that produced less IAA [22].

The results obtained in this study viz-a-viz the growth rate of the IAA overproducing strains are consistent with those of others showing that IAA-overproducing mutants of the PGPR *Azospirillum brasilense* grew at a rate similar to that of the wild type [12] and that both IAA-underproducing and IAA-overproducing mutants of *Azospirillum lipoferum* exhibited normal growth rates when compared with that of the wild type [1]. These results indicate that differences in IAA production have no impact on the bacterial growth rate.

The results from this study indicate that Tn5 insertion into the genome of *P. putida* GR12-2 to generate *P. putida* GR12-2/*aux*1 did not impair any essential activities of this microorganism that might affect cell growth or its plant growth promotion properties other than IAA synthesis. If Tn5 had inserted within the structural genes for IAA synthesis, it is likely that the insertion would have destroyed gene expression and prevented IAA biosynthesis. It is, therefore, assumed that the Tn5 insertion occurred within a region of the DNA that regulates the expression of the IAA synthesis gene(s).

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