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A novel procedure for rapid isolation of plant growth promoting pseudomonads

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Abstract: A rapid and novel procedure for the isolation of plant growth promoting rhizobacteria (PGPR) is described. This method entails screening soil bacteria for the ability to utilize the compound 1-aminocyclopropane-1-carboxylate (ACC) as a sole N source, a trait that is a consequence of the presence of the activity of the enzyme ACC deaminase. This trait appears to be limited to soil bacteria that are also capable of stimulating plant growth. Seven different soil samples from two geographically disparate locations were found to contain pseudomonads that were able to to utilize ACC as a N source. Each of the seven strains was shown, by the ability of the bacterium to promote canola seedling root elongation under gnotobiotic conditions, to be a PGPR. The method described here may be used to replace the otherwise slow and tedious process of testing individual bacterial strains for their ability to promote plant growth, thereby significantly speeding up the process of finding new PGPR.

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Key words: plant growth promoting rhizobacteria, PGPR, 1-aminocyclopropane-1-carboxylate, ACC, ACC deaminase, bacterial fertilizer, soil bacteria.

Résumé : Une méthode nouvelle et rapide d'isolement de rhizobactéries qui favorisent la croissance des plantes (PGPR) est décrite. Cette méthode consiste à sélectionner des bactéries du sol capables d'utiliser le composé 1-aminocyclopropane-1-carboxylate (ACC) comme unique source d'azote, un caractère relié à la présence de l'activité d'une enzyme déaminase de l'ACC. Ce trait caractéristique semble limité aux bactéries du sol qui sont aptes à stimuler la croissance des plantes. Sept échantillons de sols différents provenant de deux sites disparates géographiquement contenaient des pseudomonades capables d'utiliser l'ACC comme source d'azote. Par leur aptitude à promouvoir l'élongation des racines de plantules de canola dans des conditions gnotobiotiques, chacun des sept souches obtenues s'est révélée être une PGPR. La méthode ici décrite peut être utilisée en remplacement d'autres méthodes longues et fastidieuses pour tester l'aptitude des souches bactériennes individuelles à promouvoir la croissance des plantes et, ainsi, accélérer significativement la découverte de nouvelles PGPR.

Mots clés : rhizobactéries promotrices de la croissance des plantes, PGPR, 1-aminocyclopropane-1-carboxylate, ACC, déaminase de l'ACC, engrais bactérien, bactéries du sol.
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Plant growth promoting rhizobacteria (PGPR) include a diverse group of free-living soil bacteria that can stimulate the growth of plants by one or more of a number of different mechanisms (Davison 1988; Kloepper et al. 1989; Glick 1995). These bacteria may stimulate plant growth either indirectly or directly. Indirect stimulation of plant proliferation includes preventing phytopathogens from inhibiting plant growth and development (O'Sullivan and O'Gara 1992; Sivan and Chet 1992; Cook 1993; Glick 1995); direct stimulation provides plants with compounds such as fixed nitrogen, phytohormones, or solubilized iron from the soil (Brown 1974; Kloepper et al. 1988, 1989; Glick 1995).

We recently discovered that, in addition to its more well characterized and documented PGPR attributes, the bacterium *Pseudomonas putida* GR12-2 (Lifshitz et al. 1986) contains the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Jacobson et al. 1994; Glick et al. 1994*a*, 1994*b*). This enzyme functions to hydrolyze ACC, the immediate precursor of ethylene that is synthesized in plant tissues (Yung et al. 1982), although, paradoxically, in bacteria ethylene is probably synthesized by a pathway that does not include ACC (Arshad and Frankenberger 1992). It was postulated that, following the binding of *P. putida* GR12-2 to the plant seed coat (Hong et al. 1991), this enzyme might act to stimulate plant growth, root elongation in particular, by sequestering and then

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Table 1. PGPR activity of *Pseudomonas* spp. isolated from soil samples.

Experiment	Strain	Length	*n*
1	None	65.9±3.7	30
	GR12-2	100.3±3.9	27
	UW1	93.6±4.1	25
	UW2	96.2±4.0	26
	UW3	86.7±4.1	24
	UW4	81.1±3.9	27
2	None	67.5±1.3	96
	GR12-2	96.7±1.4	85
	UW1	84.4±1.5	76
	UW2	76.3±1.5	73
	UW3	71.2±1.4	81
	UW4	76.1±1.4	83
3	None	46.3±2.2	37
	GR12-2	77.8±2.2	38
	UW1	73.5±2.3	34
	UW2	68.7±2.1	42
	UW3	56.3±2.3	35
	UW4	60.4±2.4	32
4	None	31.4±1.4	83
	GR12-2	43.0±1.5	65
	CAL1	37.5±1.5	65
	CAL2	44.0±1.5	66
	CAL3	39.0±1.5	66
5	None	32.9±1.2	90
	GR12-2	42.9±1.2	85
	CAL1	44.6±1.3	67
	CAL2	45.1±1.1	93
	CAL3	45.7±1.2	79
6	None	44.8±2.1	58
	GR12-2	59.5±2.1	58
	CAL1	59.8±2.1	57
	CAL2	58.1±2.3	50
	CAL3	54.2±2.2	56

NOTE: The data are reported as the mean length of canola roots in millimetres ± 1 SE. *n* is the number of seeds treated.

hydrolyzing ACC from germinating seeds thereby lowering the level of ACC, and hence the level of ethylene in seeds (Glick et al. 1994*a*, 1994*b*). This hypothesis was examined by selecting and subsequently testing three separate mutants of *P. putida* GR12-2 that were devoid of any ACC deaminase activity and therefore do not hydrolyze ACC (Glick et al. 1994*a*, 1994*b*). Unlike wild-type *P. putida* GR12-2, and consistent with the above-mentioned hypothesis, these three mutants were unable to promote the elongation of canola roots in growth pouches under gnotobiotic conditions (Lifshitz et al. 1987; Glick et al. 1994*a*, 1994*b*). These results suggest that the plants that are treated with these mutants of *P. putida* GR12-2 synthesize ethylene to the same extent as when the seeds are not treated with any bacterium.

A prediction of our model of plant growth stimulation by bacterial ACC deaminase is that any bacterium that contains this enzyme and is capable of binding to plant seeds or roots in the soil should also be able to promote root elongation. In other words, any soil bacterium with ACC deaminase activity should also be a PGPR. Following this line of thought, *Pseudomonas* spp. that were able to utilize ACC as a sole source of nitrogen were isolated from several different soil samples. These bacteria were then tested for the ability to promote the proliferation of canola roots in sterile growth pouches. Consistent with the hypothesis of the role of ACC deaminase in soil bacteria, in each case examined, a *Pseudomonas* spp. that was able to utilize ACC as a sole source of nitrogen also displayed PGPR activity.

The PGPR *P. putida* GR12-2 was originally isolated from the rhizosphere of grasses in the High Canadian Arctic (Lifshitz et al. 1986). It was routinely grown at room temperature (22 ± 1°C) on typtone-soy broth (TSB) medium (Difco Laboratories, Detroit, Mich.), or on DF salts minimal medium (Dworkin and Foster 1958) supplemented with either 2.0 g of ammonium sulfate or 3.0 mM ACC as a nitrogen source.

Plant growth promoting activity was quantified by measuring the effect of a bacterium on the root length of developing canola seedlings in polyester growth pouches (Northrup King Co., Minneapolis, Minn.) under gnotobiotic conditions (Lifshitz et al. 1987). This assay overcomes any confounding effects that may arise as a result of the soil composition or the presence of other soil microorganisms when the PGPR activity of a bacterium is assessed under more natural conditions (Tang et al. 1994). Canola (*Brassica campestris* cv. Tobin) seeds, provided by Dr. G. Brown, Cominco Fertilizers (Saskatoon, Sask.), were surface sterilized by being soaked for 5 min in a 10% solution of liquid soap and then rinsed several times with sterile distilled water before they were resuspended in either 100 mM $MgSO_4$, or suspensions of bacterial strains in 100 mM $MgSO_4$, for 1 h at room temperature. Six canola seeds were germinated in each growth pouch. The pouches were covered with plastic wrap and kept at 25 ± 2°C for 3.5–5 days with a light level of about 18 μmol photons·m^{-2}·s^{-1}. The root lengths obtained with newly isolated strains were compared with those obtained from the cases in which no bacterium was added to the germinating seeds and in which *P. putida* GR12-2, a well-established PGPR, was added. The data from each experiment were analyzed by analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) simultaneous pairwise mean comparisons.

Rhizosphere soil samples from Waterloo, Ontario, Canada, and from several locations in California, U.S.A., were screened for new PGPR as follows. One gram of soil was added to 50 mL of sterile medium (10 g proteose peptone, 10 g casein hydrolysate, 1.5 g anhydrous $MgSO_4$, 1.5 g K_2HPO_4, and 10 mL glycerol; PAF medium) in a 300-mL flask and incubated with constant shaking, at 22°C for the Waterloo samples or at 30°C for the Californian samples. After 24 h, a 1-mL aliquot was removed from the growing culture and transferred to 50 mL of sterile PAF medium in a 300-mL flask. The flask was incubated with constant shaking for 24 h, at 22°C for the Waterloo samples or at 30°C for the Californian samples. The first two incubations enriched for pseudomonads and reduced the number of fungi in the culture. A 1-mL aliquot was removed from the second growing culture and transferred to 50 mL of sterile DF salts minimal medium with 2 g/L $(NH_4)_2SO_4$ as a N source, contained in a 300-mL flask. The flask was incubated

as previously described. A 1-mL aliquot was removed from the third growing culture and transferred to 50 mL of sterile DF salts minimal medium with 0.3033 g of ACC · 0.5 H_2O/L as a N source contained in a 300-mL flask. The flask was incubated as previously described. The heat-labile ACC was filter sterilized through a 0.2-μm membrane and the filtrate was added to the DF salts minimal medium after autoclaving. Dilutions of the final culture were plated onto solid DF salts minimal medium containing 3 mM ACC and incubated at room temperature for 48 h. Replicate colonies, grown on solid PAF medium, were tested for the ability to synthesize fluorescent siderophores. Because all of the colonies that had been isolated from a particular sample displayed a similar colony morphology and rate of growth, only a single fluorescent colony from each soil sample was selected for further testing to avoid isolating multiple copies of the same bacterium. The Waterloo strains were designated *Pseudomonas* sp. UW1–UW4 (and were originally associated with the roots of beans, clover, corn, and reeds, respectively) while the California strains were designated *Pseudomonas* sp. strains CAL1–CAL3 (and were from fields in San Benito, King City, and Fresno, where oats, tomato, and cotton, respectively, were grown). The designation of these bacteria as pseudomonads was made primarily on the basis of growth on selective media and fluorescent siderophore production. A more complete characterization of these organisms is currently underway. All of the soil samples were collected in late summer.

ACC deaminase activity is an essential component of the PGPR activity of at least one soil pseudomonad, *P. putida* GR12-2 (Glick et al. 1994*a*, 1994*b*). Reasoning that this enzyme activity might also be present in other PGPR, we used seven different rhizosphere soil samples as starting material as part of an effort to isolate novel PGPR strains. Starting with soil samples from diverse and geographically separated environments allows us to ask whether the association of pseudomonad ACC deaminase activity (as inferred from the ability of a bacterium to utilize ACC as a sole source of nitrogen) with PGPR activity is a relatively ubiquitous phenomenon.

Each of the *Pseudomonas* strains that utilized ACC as its sole N source significantly stimulated canola root elongation ($p < 0.02$; Table 1). ANOVA analysis and Tukey's HSD simultaneous pairwise mean comparisons of the data summarized in Table 1, experiment 1, showed a significant difference between the blank (no treatment) and all treatments except for strain UW4; in experiment 2 there was a significant difference between the blank and all treatments except for strain UW3; in experiment 3 there was a significant difference between the blank and all treatments. In experiments 1–3 *P. putida* GR12-2 stimulated canola root elongation to the greatest extent followed by strains UW1 and UW2, with UW3 and UW4 always stimulating root elongation the least. While none of these four newly isolated strains stimulated root elongation to the same extent as *P. putida* GR12-2, all of these strains were nonetheless active as PGPR. In addition, with strains CAL1–CAL3, all experiments showed a significant difference between the blank and all other treatments, without exception. These three newly isolated strains stimulated root elongation to approximately the same extent as *P. putida* GR12-2.

PGPR are typically isolated from soil samples by a laborious and time-consuming process that generally includes the use of biological assays to assess either the ability of the isolated bacteria to stimulate plant growth directly or to act as a biological control agent that can prevent, or at least diminish, phytopathogen-induced damage to plants (Chambel et al. 1994; van Tran et al. 1994). Initial screens to enrich for PGPR often include the ability of the bacteria to fix N or to produce antibiotics. However, neither of these traits nor, until now, any other trait, has been found to be strictly associated with PGPR activity. On the other hand, we have found that following enrichment for fluorescent pseudomonads, bacteria that are able to proliferate on a minimal medium containing ACC as a N source all show statistically significant PGPR activity. This is consistent with the model which suggests that the enzyme ACC deaminase is an important component of the mechanism used by PGPR to stimulate root elongation (Glick et al. 1994*a*, 1994*b*).

Whether soil bacteria other than fluorescent pseudomonads contain ACC deaminase activity remains to be determined. If they do, then bacteria such as bacilli and azospirilla may be screened by a variation of the protocol described in this paper and novel PGPR may be readily selected. Experiments to this end are currently in progress in our laboratory.

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Hydrogen production from continuous fermentation of xylose during growth of *Clostridium* sp. strain No. 2

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Abstract: Experimental conditions are presented for continuous fermentation of xylose and glucose to hydrogen (H_2), using *Clostridium* sp. strain No. 2 growing in 300 mL of culture containing 0.3% substrate, either without pH control or with the pH controlled at 6.0. The H_2 production rate increased in proportion to increasing dilution rate within the range of about 0.4 to 1.0 h^{-1}. The maximal H_2 production rates of 21.03 and 20.40 mmol $\cdot h^{-1} \cdot L^{-1}$ were obtained from xylose and glucose with dilution rates of 0.96 and 1.16 h^{-1}, respectively, at pH 6.0. About 2.06 mol H_2 with a dilution rate of 0.21 h^{-1} at pH 6.0 and 2.36 mol H_2 with a dilution rate of 0.18 h^{-1} were formed per mole of xylose and glucose consumed, respectively, at uncontrolled pH.

Key words: hydrogen production, continuous fermentation, xylose, *Clostridium* sp. strain no. 2.

Résumé : Nous présentons ici les conditions expérimentales pour la production d'hydrogène (H_2) à partir du xylose et du glucose en fermentation continue à l'aide de *Clostridium* sp. souche n° 2 cultivé dans 300 mL d'un milieu contenant 0,3% de substrat où le pH est soit non-contrôlé ou contrôlé à 6,0. Le taux de production de H_2 augmentait en proportion de l'augmentation du taux de dilution à l'intérieur d'une limite de 0.4 à 1.0 h^{-1}. Des valeurs de production maximale de H_2 de 21,03 et de 20.40 mmol $\cdot h^{-1} \cdot L^{-1}$ ont été obtenues à

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