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Isolation and characterization of new plant growth-promoting bacterial endophytes

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ABSTRACT

Those bacterial endophytes that also provide some benefit to plants may be considered to be plant growth-promoting bacteria (PGPB) and can facilitate plant growth by a number of different mechanisms. In the work that is reported here, soil samples from several locales around the world were used as a starting point for the isolation of new endophytes. Subsequently, those newly isolated endophytes that were able to utilize the plant compound 1-aminocyclopropane-1-carboxylate (ACC) as a sole source of nitrogen, as a consequence of possessing the enzyme ACC deaminase, were selected for additional characterization. More specifically, ACC deaminase-expressing strains were tested for IAA synthesis, siderophore production, phosphate solubilization activity, optimal growth temperature, salt tolerance, and antibiotic sensitivity. In addition, the partial DNA sequences of the16S rRNA genes of the characterized strains were determined so that the taxonomic identity of each strain could be assessed, and the ability of some of these strains to facilitate the growth of canola plant roots under controlled gnotobiotic conditions was measured.

1. Introduction

Plant growth-promoting bacterial endophytes have the ability to colonize a plant's interior and to establish a special type of relationship where both partners may derive benefits from this interaction (Hallmann et al., 1997; Reiter and Sessitsch, 2006). Bacterial endophytes have been reported to promote plant growth by a number of different mechanisms. These mechanisms include phosphate solubilization activity (Verma et al., 2001; Wakelin et al., 2004), production of phytohormones (Lee et al., 2004), nitrogen fixation (Compant et al., 2005b; Watanabe et al., 1979), siderophore biosynthesis (Lodewyckx et al., 2002; Wang et al., 1993), and supplying essential nutrients to the host plant (Costa and Loper, 1994; Puente et al., 2009b). Bacterial endophytes may also promote plant growth as a consequence of the bacterium expressing the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase which cleaves ACC to α-ketobutyrate and ammonia and thereby decreases ethylene levels in host plants (Sessitsch et al., 2005; Sun et al., 2009). A model describing the role of bacterial ACC deaminase in decreasing the level of ethylene in plants was developed (Glick et al., 1998) and recently refined (Glick et al., 2007a,b). In this model, it is argued that a plant growth-promoting bacterium with ACC deaminase activity can sequester and cleave the ethylene precursor, ACC, and thereby lower potentially deleterious ethylene levels in the plant host, altering its physiology (Glick, 2004, 2005; Glick et al., 2007a,b).

Previous studies have indicated that endophytic bacteria can promote plant growth by altering plant physiology including osmotic pressure regulation, changes in stomatal responses, adjustment in root size and morphology, modification of nitrogen accumulation and metabolism, and increased uptake of certain minerals (Compant et al., 2005a,b). Bacterial endophytes can also be used as biocontrol agents. Antibiotic production (Aria, 1976; Demain, 1981; Ezra et al., 2004; Goodfellow et al., 1988) and lytic enzyme production, e.g. hydrolases (Chernin and Chet, 2002), chitinases (Frankowski et al., 2001), laminarinases (Lim et al., 1991), and glucanases (Singh et al., 1999), are two common modes of endophyte-based biocontrol. Moreover, endophytes have also been reported to trigger induced systemic resistance (ISR)-based plant growth promotion (Ait Barka et al., 2000, 2002; Benhamou et al., 1996a,b; Brooks et al., 1994; Conn et al., 1997; M'Piga et al., 1997; Sharma and Nowak, 1998; Viswanathan and Samiyappan, 1999). In addition, several endophytic bacteria have been shown to facilitate various phytoremediation strategies (Barac et al., 2004; Van Aken et al., 2004; Puente et al., 2009b).

Most studies, both in the laboratory and in the field, that have employed endophytes in the past have not selected or tested for the presence of the enzyme ACC deaminase. However, based on extensive studies with rhizospheric plant growth-promoting bacteria, it is assumed that endophytic plant growth-promoting bacteria that

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contain this activity will also be significantly superior to endophytic strains without this activity in promoting plant growth under a wide range of environmental conditions. Therefore, the purpose of the work reported here was to isolate new ACC deaminase-containing bacterial endophytes from a number of geographically different soil samples, to screen them for their plant growth-promoting capacities, and finally to use them as environmentally friendly adjuncts to agricultural practice.

2. Materials and methods

2.1. Isolation of bacterial endophytes

A total 15 different soil samples were collected from various soils in Canada (collected on the campus of the University of Waterloo and from the Kitchener, Ontario), Lyon, France (kindly provided by Dr. Yvan Moënne-Loccoz), Haifa, Israel (kindly provided by Dr. Shimon Gepstein), Evora, Portugal (kindly provided by Dr. Solange Oliveira), and the Blacksburg Virginia, United States of America (kindly provided by Dr. Jerzy Nowak). Surface sterilized tomato seeds (*Solanum lycopersicum* Heinz 722) were sown in the above mentioned soils in green plastic pots (11 cm × 9 cm). Roughly 10–15 seeds were sown per pot, and pots were incubated at room temperature on the lab bench top; following 4–5 weeks of growth the tomato plants were harvested. Bacterial endophytes were then isolated based on the method described by the Sturz et al. (1998) and Surette et al. (2003). Each plant was separated into roots, stems, and leaves and then thoroughly washed with tap water to remove any adhering soil. The plant tissues were then surface disinfected by a 3 min treatment with commercial bleach (5.25% available chlorine), transferred to a 3% hydrogen peroxide solution for 3 min, and finally rinsed three times with sterile milli-Q water. A 10% solution of Tween 20 was added to the first rinse solution. Surface-sterilized tissues were used to inoculate tryptic soy agar (TSA) (Bacto™ Becton, Dickinson and company Sparks, MD, USA) plates to ascertain that, subsequent to the above mentioned washing procedure (McInroy and Kloepper, 1995a; Sturz et al., 1998; Surette et al., 2003), plant tissue surfaces did not contain any culturable microorganisms. In fact, in all cases when this was done, no bacterial growth was found following a 48 h incubation at 30 °C.

Tomato plant tissues (root, stem, and leaf) were then homogenized in 10 ml 3× Ringer's solution (Surette et al., 2003) using an ethanol-sterilized mortal and pestle, incubated at room temperature (21–23 °C) in an orbital shaker for 1 h, and serially diluted to 10^{-3} with 3× Ringer's solution. Aliquots of 100 μl of each dilution were plated out onto tryptic soy agar (TSA), Luria agar (LA) (Fisher Scientific, New Jersey, USA), and King's B agar (KBA) (20 g proteose peptone 3, 10 ml glycerol, 1.5 g K_2HPO_4, 1.5 g $MgSO_4 \cdot 7H_2O$ and 15 g agar in 1 l of Milli-Q water) plates in duplicate, then the plates were incubated at 25 °C for 72 h. Morphologically different colonies (based on size, shape, and color) were selected; not more than two colonies were selected per plate. Individual colonies were subcultured on respective growth medium plates for further screening and to make −80 °C glycerol culture stocks.

2.2. ACC deaminase activity measurement

ACC deaminase activity was determined for all of the newly isolated strains according to the protocol described by Penrose and Glick (2003) with a standard curve of α-ketobutyrate between 0.05 and 0.5 μmoles.

2.3. IAA production assay

The ability of bacterial endophytes to produce IAA was measured based on the colorimetric method described by Glickmann and Dessaux (1995) and Patten and Glick (2002) with some modifications. Aliquots of 20 μl of an overnight grown bacterial culture were used to inoculate 5 ml TSB without and with tryptophan (200 μg ml^{-1} or 500 μg ml^{-1}) and incubated at 30 °C for 24 h. Overnight cultures were centrifuged and 1 ml supernatant was mixed with 4 ml Salkowski's reagent (Gordon and Weber, 1951), incubated for 20 min at room temperature before the absorbance was measured at 535 nm. The concentration of each sample was calculated from a standard plot ranging from 0.01 to 0.4 μg ml^{-1} pure IAA (Sigma).

2.4. Siderophore production assay

This assay was done qualitatively and is based on competition for iron between a ferric complex of chrome azurol S (CAS), an indicator dye, and a siderophore produced by the microorganism. The iron is removed from CAS by the siderophore (which binds iron more tightly) and a positive reaction is indicated by a color change of the CAS reagent from blue to orange (Schwyn and Neilands, 1987). A 5 μl aliquot of an overnight bacterial culture in KB medium was spotted onto a CAS agar plate (Alexander and Zuberer, 1991) in triplicate and incubated at 30 °C for 3–4 days.

2.5. Salt tolerance

A 20 μl aliquot of an overnight test culture was inoculated into 1% proteose peptone plus 0.0%, 0.5%, 1%, 2%, 3%, 4%, 5%, 7%, or 10% salt. After 24–48 h, the absorbance of the culture was determined at 600 nm, with uninoculated medium serving as a blank.

2.6. Antibiotic sensitivity/resistance

Five different antibiotics were selected for testing according to their modes of action. Ampicillin (50 μg ml^{-1}), erythromycin (50 μg ml^{-1}), kanamycin (50 μg ml^{-1}), novobiocin (50 μg ml^{-1}), and tetracycline (15 μg ml^{-1}) were added separately to 5 ml TSB, before 20 μl of an overnight test culture was added and then incubated at 30 °C in a shaking water bath for 24–48 h. The absorbance at 600 nm of all samples was measured using uninoculated TSB as a blank.

2.7. Optimal growth temperature

Optimal growth temperatures were investigated by growing the test strains at a range of temperatures from 15 °C to 50 °C in a growth curve machine (Lab Systems Thermo Electron Growth Curves Bioscreen C Microplate Reader, Diversified Equipment Company Inc. Lorton, VA) following the protocol suggested by the manufacturer. Each growth curve experiment was done in triplicate in TSB medium and run for 3 days.

2.8. Production of ammonia

The ability of bacterial strains to produce ammonia was assessed as described by Marques et al. (2010). In this method 20 μl of an overnight grown test culture was inoculated into 5 ml of 1% proteose peptone broth and incubated at 30 °C in a shaking water bath. After 24–48 h, 0.5 ml Nesseler's reagent was added to the culture and the color change was noted, a yellow coloration indicates the positive result while the intensity of color is indicative of the amount of ammonia produced by the test strain.

2.9. Gnotobiotic root elongation assay

The ability of newly isolated bacterial endophytes to promote the growth of canola roots was carried out and monitored as