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Diversity of endophytic bacteria in *Lolium perenne* and their potential to degrade petroleum hydrocarbons and promote plant growth

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HIGHLIGHTS

- Most of the isolates belonged to *Pseudomonas*, *Bacillus* and *Curtobacterium* genus.
- Selected endophytic bacteria showed multiple PGP abilities.
- Strains *Rhodococcus* sp. seem to be good candidate for enhancement phytodegradation.
- The taxonomic diversity of endophytic bacteria is tissue specific.

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ABSTRACT

The aim of this study was to assess the ability of twenty-nine endophytic bacteria isolated from the tissues of ryegrass (*Lolium perenne* L.) to promote plant growth and the degradation of hydrocarbon. Most of the isolates belonged to the genus *Pseudomonas* and showed multiple plant growth-promoting abilities. All of the bacteria that were tested exhibited the ability to produce indole-3-acetic acid and were sensitive to streptomycin. These strains were capable of phosphate solubilization (62%), cellulolytic enzyme production (62%), a capacity for motility (55%) as well as for the production of siderophore (45%), ammonium (41%) and hydrogen cyanide (38%). Only five endophytes had the emulsification ability that results from the production of biosurfactants. The 1-aminocyclopropane-1-carboxylate deaminase (ACCD) gene (*acdS*) was found in ten strains. These bacteria exhibited ACCD activities in the range from 1.8 to 56.6 μmol of α-ketobutyrate mg^{-1} h^{-1}, which suggests that these strains may be able to modulate ethylene levels and enhance plant growth. The potential for hydrocarbon degradation was assessed by PCR amplification on the following genes: *alkH*, *alkB*, *C23O*, *P450* and *pah*. The thirteen strains that were tested had the *P450* gene but the *alkH* and *pah* genes were found only in the *Rhodococcus fascians* strain (L11). Four endophytic bacteria belonging to *Microbacterium* sp. and *Rhodococcus* sp. (L7, S12, S23, S25) showed positive results for the *alkB* gene.

1. Introduction

The contamination of soil environments by petroleum hydrocarbons has become a serious problem. Due to their negative biological effects (carcinogenic, neurotoxic) and tendency to spread into ground and surface waters, there is an urgent need to remove these compounds from soil. The most promising method that has been proposed for hydrocarbon degradation is phytoremediation because it is low in cost, friendly to the environment and has a positive impact on soil fertility and structure. The effectiveness of this technique largely depends on the presence and activity of plant-associated microorganisms, especially endophytic bacteria (Hardoim et al., 2008; Li et al., 2012), which live inside plant tissues without causing any apparent symptoms of disease. Because they probably live inside most, if not all, plant species, the possibility of finding new beneficial endophytic microorganisms is considerable (Ryan et al., 2008).

Recent attention has been paid to the partnership between plants and plant growth-promoting endophytes (Luo et al., 2012; Afzal et al., 2013), which are able to assist plant growth and development through various plant growth-promoting (PGP) capabilities such as nitrogen fixation, the solubilization of minerals; the production of phytohormones, ammonia, hydrogen cyanide, siderophores, 1-aminocyclopropane-1-carboxylate deaminase (ACCD) enzyme; the synthesis of surface-active compounds that are called biosurfactants; their antagonistic activity against plant pathogens (Hardoim et al., 2008; Pacwa-Płociniczak et al., 2011; Rashid

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et al., 2012). It is well documented that ACCD activity in plant-associated bacteria significantly enhances the production of plant biomass and petroleum degradation as compared to bacteria that only have the ability to degrade hydrocarbons (Afzal et al., 2011; Yousaf et al., 2011; Khan et al., 2013). Moreover, endophytic bacteria may produce various hydrolytic enzymes that are involved in the decomposition of plant compounds. Many of them have a similar chemical structure to organic toxic pollutants such as petroleum hydrocarbons. Therefore, endophytes have a great biochemical potential and exhibit a natural competence for xenobiotic degradation during the phytoremediation (Wang and Dai, 2011).

The genes that encode the enzymes that are involved in hydrocarbon degradation have been described in a wide range of endophytic bacteria that belong to *Arthrobacter*, *Bacillus*, *Enterobacter*, *Pantoea* and *Pseudomonas* genus (Porteous-Moore et al., 2006; Phillips et al., 2008; Andria et al., 2009; Yousaf et al., 2010). Furthermore, it has been observed that plants growing on petroleum contaminated soils recruited endophytes that possessed hydrocarbon-degrading genes (Khan et al., 2013).

Many plants have been tested for their ability to tolerate and remove petroleum hydrocarbons. Among others, ryegrass has been shown to be more suitable for the cleanup of petroleum contaminated soils (Kirk et al., 2005; Rezek et al., 2008; Tang et al., 2010; Barrutia et al., 2011). Because of the potential role of ryegrass in phytoremediation, the characterization of the bacteria that are associated with ryegrass tissues may provide valuable information about the potential economic and environmental benefits of using endophytic bacteria in phytoremediation. To the best of our knowledge, this is the first experiment that describes endophytic strains, their PGP traits, hydrocarbon degradation potential and other important abilities that are necessary for the interior colonization of plant tissues. Thus, the objectives of this study were (1) the isolation of endophytic bacteria from the different tissues of ryegrass; (2) the assessment of the PGP mechanisms such as phosphate solubilization, the production of indole-3-acetic acid (IAA), ammonia, siderophores, the activity of ACCD and the synthesis of HCN; (3) the evaluation a strain's ability for motility, the synthesis of cellulolytic enzymes, the production of biosurfactants and sensitivity/resistance to antibiotics (streptomycin and ampicillin); (4) the detection of the gene encoding ACCD (*acdS*) and (5) the determination of the degradation potential of endophytic isolates using the PCR method and primers that are specific for the following genes: *alkH* (alkane hydroxylase), *alkB* (alkane monooxygenase), *C23O* (catechol-2,3-dioxygenase), *P450* (cytochrome P450-type alkane hydroxylase, CYP153) and *pah* (alpha subunit of the PAH-ring hydroxylating dioxygenases).

2. Materials and methods

2.1. Isolation and characterization of endophytic bacteria

Endophytic bacteria were isolated from ryegrass which grew in industrial soil in Zabrze, Southern Poland. Healthy plants were collected at the flowering stage. Stems and leaves were surface sterilized with 70% ethanol, 5% sodium hypochlorite, and 10% hydrogen peroxide. The samples were rinsed three times in sterile distilled water to remove the disinfectant. The successful sterilization process was verified by a plated final wash onto TSA medium and the plates were incubated at 28 °C for 4 d. If no microbial growth was found, the plant tissues were used for further analysis. Finally, the stems and leaves were separately macerated in 0.9% NaCl using a mortar and pestle and a 100 μL suspension was plated onto a TSA medium and incubated at 28 °C for 7 d. The morphologically different bacterial colonies were selected, sub-cultured and stored at −20 °C for further analysis.

Endophytic bacterial strains were identified based on whole-cell fatty acid profiles using the MIDI-MIS system (MIDI, Sherlock TSBA Library version 6.1, Microbial ID, Newark, DE, USA) as described in Piotrowska-Seget et al. (2005). In addition, the identification was confirmed using the standard physiological method (Holt et al., 1994).

2.2. Detection of catabolic genes

Genomic DNA was extracted from liquid cultures of the strains that were tested using a GeneMatrix Bacterial & Yeast Genomic DNA Purification Kit (EURx DNA Gdansk, Poland) according to the protocol. Total DNA extracts were used for PCR amplification using the primers that are specific for the following genes: *alkH*, *alkB*, *C23O*, *P450* and *pah*. The primer sequences are listed in Table 1. PCR was performed in a 25 μL final volume reaction mixture containing: 50 ng of DNA, 0.5 mM of each primer, 200 mM of each dNTP, 1 × Dream Taq reaction buffer (Thermo Scientific) and 1 U of Dream Taq DNA Polymerase (Thermo Scientific). The parameters for the reaction cycle were as follows: initial denaturation for 5 min at 95 °C; 30 cycles of 1 min denaturation at 95 °C; 45 s annealing at the correct temperature for the gene (Table 1) and elongation for 1 min at 72 °C; final elongation for 5 min at 72 °C and at 4 °C until further use. Amplification was confirmed in a 2% agarose gel stained with ethidium bromide.

2.3. ACCD activity

Endophytic bacteria were screened for the presence of gene *acdS* encoding ACCD enzyme. The DNA isolation and PCR reaction were prepared in the same manner as above-described for the analysis of the hydrocarbon degradation genes. Only strains with a positive reaction to specific *acdS* starters (Table 1) were chosen for the measurement of ACCD activity. ACCD activity was determined according to a modified Honma and Shimomura (1978) method as described by Saleh and Glick (2001). The activity was expressed in μmol of α-ketobutyrate $mg^{-1}\,h^{-1}$. The protein concentration of the microbial cell suspensions was determined using the Bradford (1976) method.

2.4. PGP activities

The phosphate solubilization ability of the isolates was determined on a Pikovskaya agar medium. The presence of a clear zone around the bacterial colonies indicated the solubilization of phosphate. The halo size was calculated by subtracting the colony diameter from the total diameter. IAA, ammonia, and HCN production was examined using a method described by Porteous-Moore et al. (2006), Phillips et al. (2008) and Płociniczak et al. (2013). Siderophore production was screened on a Chrome Azurol S (CAS) assay according to the method of Schwyn and Neilands (1987). A change in the blue color of the CAS medium surrounding the bacterial colony to orange or yellow indicated the production of siderophores.

2.5. Evaluation of other abilities of endophytic bacteria

Extracellular cellulase activity was tested on indicator plates with a carboxymethyl cellulose (CMC) medium. The production of the enzyme was detected by flooding the surface of the medium with Lugol's iodine. It was considered to be a positive result when the colonies were surrounded by a yellow halo zone. The motility of the cultured bacteria was examined on a nutrient broth containing 0.2% agar. After a 24–48 h incubation, the ability for bacterial