Rapid communication

Expression of alkane monooxygenase (alkB) genes by plant-associated bacteria in the rhizosphere and endosphere of Italian ryegrass (Lolium multiflorum L.) grown in diesel contaminated soil

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Bacterial alkane degradation genes are expressed in the rhizosphere and in the plant interior.

\textbf{A R T I C L E   I N F O}

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\textbf{A B S T R A C T}

For phytoremediation of organic contaminants, plants have to host an efficiently degrading microflora.
To assess the role of endophytes in alkane degradation, Italian ryegrass was grown in sterile soil with 0, 1 or 2% diesel and inoculated either with an alkane degrading bacterial strain originally derived from the rhizosphere of Italian ryegrass or with an endophyte. We studied plant colonization of these strains as well as the abundance and expression of alkane monooxygenase (alkB) genes in the rhizosphere, shoot and root interior. Results showed that the endophyte strain better colonized the plant, particularly the plant interior, and also showed higher expression of alkB genes suggesting a more efficient degradation of the pollutant. Furthermore, plants inoculated with the endophyte were better able to grow in the presence of diesel. The rhizosphere strain colonized primarily the rhizosphere and showed low alkB gene expression in the plant interior.

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1. Introduction

Phytoremediation combines the use of plants and their associated microorganisms to degrade toxic organic contaminants (Pilon-Smits, 2005). The efficiency of a phytoremediation process depends largely on the presence and activity of the plant-associated microflora carrying degradation genes required for enzymatic break-down of organic pollutants. The rhizosphere as well as the plant apoplast have been reported to host degrading bacteria (Newman and Reynolds, 2005). Endophytes are defined as bacteria that reside within plant tissue without conferring pathogenicity and frequently show plant growth-promoting activities (Sessitsch et al., 2004; Ryan et al., 2008). There is increasing interest in the role of endophytes in phytoremediation applications as they can serve as vector delivering biodegradative capacities inside the plant (Barac et al., 2004). However, it is not fully understood, whether potentially degrading endophytes are active once inside the plant and thereby contribute to the detoxification of the pollutant and better survival of the plant under toxic conditions.

Highly diverse alkane degraders containing alkane monooxygenase genes (alkB) have been isolated from the plant environment (van Belien et al., 2002; Hamamura et al., 2005; Kaimi et al., 2007). The abundance of alkB genes has been assessed in the rhizosphere and in the root interior (Siciliano et al., 2001; Whyte et al., 2002), but alkB gene expression under natural conditions has been rarely addressed (Powell et al., 2006).

The aim of this study was to analyze plant colonization and activities of two strains, the endophyte \textit{Pseudomonas} sp. strain ITRI53 and the rhizosphere strain \textit{Rhodococcus} sp. ITRH43, isolated from Italian ryegrass (Andria et al., submitted for publication). This study shows efficient plant colonization by the endophyte and high alkB gene expression inside the plant, particularly in the presence of high diesel oil concentrations.

2. Materials and methods

2.1. Bacterial strains and plant experiment

\textit{Pseudomonas} sp. strain ITRI53 and \textit{Rhodococcus} sp. strain ITRH43 were isolated from the root interior and the rhizosphere of Italian ryegrass, respectively. Both strains have the capacity to degrade alkanes and contain identical or highly similar alkane monooxygenase (alkB) gene sequences (Andria et al., submitted for publication). Strains were cultivated in 10% Luria Bertani broth amended with 1% (v/v) filter-sterilized diesel at 30 °C. Cells were harvested by centrifugation and resuspended in sterile 0.9% NaCl solution.
For the plant experiment Magenta boxes were filled with 72.5 g air-dried soil mixed with 40% (v/v) sand and sterilized by 30 kGy γ-radiation. Before sowing, the soil was amended with either 0%, 1% or 2% (v/v) filter-sterilized diesel and then mixed with 25 ml inoculant suspension (app. 10^5 CFU/ml) containing either strain ITR53, strain ITRH43 or sterile 0.9% NaCl solution. Control treatments with the amendment of diesel but without bacterial inoculation were included. Seeds of Italian ryegrass were surface-sterilized in a 20% (v/v) NaOCl solution for 10 min, washed 5 times with sterile water and were germinated on sterile filters at 25 °C. Twenty plantlets were placed in each box and each treatment was replicated. Plants were grown at 25 °C in a sterile environment and subjected to a cycle of 16 h light and 8 h dark for 2 months. Plants were watered with equal amounts of sterile water.

2.2. Sampling and extraction of DNA and RNA

After 2 months, shoots were cut from 2 cm above soil and the remaining plants were harvested to obtain root and rhizosphere samples. Rhizosphere soil was collected by gently sampling the soil closely attached to the root surface. Subsequently, the roots were washed several times in sterile water. Roots and shoots (1 g) were surface-sterilized as described by Reiter et al. (2003), replacing distilled sterile water by DEPC-treated water. Sterility was checked by plating on tryptic Soy Agar plates (TSA, Merck).

DNA from rhizosphere was extracted using FastDNA Spin Kit for soil (QBiogene), whereas RNA was isolated with the FastRNA Pro Soil-Direct Kit (MP Biomedicals). Roots and shoots were briefly ground in liquid N2 and microbial cells were disrupted by bead-beating (Reiter et al., 2003). For isolation of DNA and RNA, the DNase I enzyme (Ambion) digestion products were examined on 2% agarose gels. No primer-dimers were detected. Serial dilutions were plated on 10% TSA in duplicates and amplified (Whyte and Greer, 1996), digested with AluI (Invitrogen), electrophoresed on 1.5% agarose gels and compared with the profiles obtained from the inoculant strains. Isolates and inoculant strains had identical restriction patterns.

2.3. Abundance of inoculant strains

Surface-sterilized roots and shoots (1 g) were cut into small pieces. Rhizosphere soil as well as root and shoot pieces were suspended in 2 ml of 0.9% (w/v) NaCl solution and shaken at 180 rpm for 30 min. After plant and soil particles settled, the soil as well as shoot and root pieces were suspended in 2 ml of 0.9% (w/v) NaCl solution and the abundance of inoculant strains was determined using qRT-PCR. The amplification efficiency was calculated by serial dilution of stocks containing purified PpalkB PCR products from strain sp. ITRH43 in association with diesel amendment 0%, 1% and 2%. The standard error of the distribution is presented in parentheses.

<table>
<thead>
<tr>
<th>Bacterial inoculant</th>
<th>Pseudomonas sp. ITR53</th>
<th>Pseudomonas sp. ITR53</th>
<th>Rhodococcus sp. ITR53</th>
<th>Rhodococcus sp. ITR53</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diesel (v/v CFU/g dry weight)</td>
<td>1%</td>
<td>2%</td>
<td>3%</td>
<td></td>
</tr>
<tr>
<td>Bacterial inoculant: Pseudomonas sp. ITR53</td>
<td>0%</td>
<td>0.7 A</td>
<td>0.8 A</td>
<td>1.1 B</td>
</tr>
<tr>
<td>Bacterial inoculant: Rhodococcus sp. ITR53</td>
<td>0%</td>
<td>1.0 A</td>
<td>1.6 A</td>
<td>2.0 A</td>
</tr>
<tr>
<td>Diesel (v/v CFU/g dry weight)</td>
<td>1%</td>
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<td>3%</td>
<td></td>
</tr>
<tr>
<td>Bacterial inoculant: Pseudomonas sp. ITR53</td>
<td>0%</td>
<td>1.6 A</td>
<td>2.0 A</td>
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</tr>
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<td>1.6 A</td>
<td>2.0 A</td>
</tr>
</tbody>
</table>

2.4. Quantitative analysis of the abundance and expression of alkB genes

Reverse transcription was performed with 10–20 ng RNA, the specific primer PpalkB (Bustin, 2000) and Superscript II Reverse Transcriptase (Invitrogen) according to the manufacturer’s instructions. Abundance and expression of alkB genes were quantified by real-time PCR using a cycler IQ (BioRad). Specific qRT-PCR primers were designed based on the conserved region within the alkB of the inoculant and closely related strains (Genbank accession number FJ014898-FJ014915, AJ332397, AJ344083, AJ320569 and AY034587). The specificity of the designed primers RTalkB-f (5’-ATCCCGCTGACGAAGTAATC-3’) and RTalkB-r (5’-CGCGCAACTCTTCTTATTGACG-3’) was checked by using Primer 3 and BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Standards for qRT-PCR were generated by serial dilution of stocks containing purified PpalkB PCR products from strain ITR53 DNA. Analyses were performed using purified genomic DNA from ITR53 (ITR53 gDNA). Gene copy numbers were calculated as described by Powell et al. (2006). Reaction mixtures (25 μl) contained 12.5 μl of Q Mix (Biobonf), 2 μl 10 mg/ml BSA, 0.8 μl DMSO, 0.5 μl 2 μM of each primer, 25–50 ng of DNA/cDNA template and RNase-free water. Thermal cycling conditions were: 4 min at 94 °C followed by 40 cycles of 94 °C for 20 s, 60 °C for 30 s, 72 °C for 45 s followed by a melt curve from 50 to 100 °C. Besides melt curve analysis, PCR products were examined on 2% agarose gels. No primers-dimers were detected. Serial dilutions of DNA and cDNA were spiked with 10^6 copies of amplified alkB genes to check for real-time PCR inhibition (López-Gutiérrez et al., 2004). Highly linear standard curves (r^2 values > 0.95, PCR efficiency > 98%) over the dilution range and a detection limit of 10^3 copies were obtained indicating no PCR inhibition. The alkB gene copy numbers were quantified relative to a standard curve of positive control and were normalized to the copy number of control plants. Statistical analysis was based on Levene’s Significant Difference Tests using SAS software (SAS Institute Inc., Cary, NC, USA) and regression analysis using Microsoft Excel.

3. Results and discussion

Colonization of alkane degrading inoculant strains was determined by cultivation and a cultivation-independent approach. Both approaches clearly showed that strain ITR53, originally isolated from...
We observed that plants inoculated with strain ITRI53 showed better growth and survival in the presence of diesel. All (20) plants were able to grow in unpolluted soil, whereas less plants were able to grow in the presence of diesel (Table 2). Only 15 and 9 plants inoculated with strain ITRI53 survived in the presence of 1% and 2% diesel, respectively, but 20 and 15 plants, respectively, survived when inoculated with strain ITRH43. Diesel oil is toxic to plants even at low levels (Adam and Duncan, 2003) and strain ITRI53 protected plants more efficiently than strain ITRH43. It might be that strain ITRI53, which belongs to the genus *Pseudomonas*, increased stress tolerance in the plant as many *Pseudomonas* have been identified so far with plant growth-promoting properties (Compant et al., 2005). Furthermore, strain ITRI53 colonized plants better and might have degraded degraded more efficiently and thereby protected the plants.

Both strains principally expressed *alkB* genes indicating an active role in the degradation of the pollutant and *alkB* gene expression was found in all compartments (Table 1, Fig. 1). The differences between strains ITRI53 and ITRH43 in regard to *alkB* gene expression followed essentially the same patterns as *alkB* abundance data. However, both strains showed higher *alkB* expression in relation to *alkB* abundance in the shoot interior than in the root interior indicating that root and shoot metabolites influenced *alkB* gene expression. It appears that the endophyte strain maintained its degrading activity in the rhizosphere and in the plant interior independently from the diesel concentration, whereas the rhizosphere strain needed a higher diesel concentration as selective pressure to maintain its degradation activity within plant tissues, but not in the rhizosphere. However, for both inoculants, *alkB* gene abundance and expression were the highest in rhizosphere (Table 1) indicating that root exudates provide nutrients for bacterial growth and co-metabolites for alkane degradation (Olson et al., 2003). In conclusion, we showed that bacterial *alkB* genes can be expressed not only in the rhizosphere but also in planta. The endophyte strain tested was superior than the rhizosphere strain regarding colonization and *alkB* gene expression. Furthermore, inoculation of the endophyte strain resulted in better survival of plants which might be due to plant growth-promoting and/or alkane degrading activities. Such differences in endophytic populations might be an important factor that could help to explain the huge differences among plant species and even cultivars in sensitivity/tolerance to hydrocarbon contaminants in soils. Further studies will address the application potential of endophytes in phytoremediation applications.

**Fig. 1.** Mean values of Colony Forming Unit (CFU), *alkB* abundance and *alkB* expression of endophyte *Pseudomonas* sp. ITRI53 and rhizosphere bacteria *Rhodococcus* sp. ITRH43 at rhizosphere, root and shoot interior of Italian ryegrass, in presence of 0%, 1% and 2% of diesel. Error bars represent standard error. The asterisk (*) shows significant difference between means for every parameter (CFU, *alkB* abundance and *alkB* expression) in different diesel concentration for the same inoculant. Means followed by the same letters are not significantly different according to Least Significant Difference test (*P < 0.05*).


