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Genetic diversity of culturable bacteria in oil-contaminated rhizosphere of *Galega orientalis*

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Bacterial diversity during rhizoremediation in oil-contaminated soil is characterized by a combination of molecular methods.

Abstract

A collection of 50 indigenous *meta*-toluate tolerating bacteria isolated from oil-contaminated rhizosphere of *Galega orientalis* on selective medium was characterized and identified by classical and molecular methods. 16S rDNA partial sequencing showed the presence of five major lineages of the Bacteria domain. Gram-positive *Rhodococcus*, *Bacillus* and *Arthrobacter* and gram-negative *Pseudomonas* were the most abundant genera. Only one-fifth of the strains that tolerated *m*-toluate also degraded *m*-toluate. The inoculum *Pseudomonas putida* PaW85 was not found in the rhizosphere samples. The ability to degrade *m*-toluate by the TOL plasmid was detected only in species of the genus *Pseudomonas*. However, a few *Rhodococcus erythropolis* strains were found which were able to degrade *m*-toluate. A new finding was that *Pseudomonas migulae* strains and a few *P. oryzihabitans* strains were able to grow on *m*-toluate and most likely contained the TOL plasmid. Because strain specific differences in degradation abilities were found for *P. oryzihabitans*, separation at the strain level was important. For strain specific separation $(GTG)_5$ fingerprinting was the best method. A combination of the single locus ribotyping and the whole genomic fingerprinting techniques with the selective partial sequencing formed a practical molecular toolbox for studying genetic diversity of culturable bacteria in oil-contaminated rhizosphere.

Keywords: Oil contamination; Rhizosphere; Bacterial diversity; Molecular identification; TOL plasmid

1. Introduction

The perennial forage legume of *Galega orientalis* (goat's rue), with its nitrogen-fixing symbiont *Rhizobium galegae* H1174, and various other rhizosphere bacteria have good potential for rhizoremediation of oil-contaminated soil (Suominen et al., 2000), since plants enhance the bioremediation of oil-contaminated soils by stimulating microbial degradation activity in the rhizosphere (Radwan et al., 1995). Not only do the plant roots supply nutrients such as amino acids, carbohydrates and organic acids for rhizosphere bacteria (Anderson et al., 1993), but they may also help bacteria degrade toxic organic chemicals by releasing phospholipid surfactants that modify the physical and chemical properties of the rhizosphere and thus the bioavailability of organic pollutants (Read et al., 2003). Root exudates may create favourable conditions also for co-metabolism. In addition to plant surfactants, Nielsen and Sørensen (2003) have shown that many *Pseudomonas fluorescens* strains produce different cyclic lipopeptides

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with antifungal and biosurfactant properties in specific habitats like the rhizosphere.

Pseudomonas putida PaW85 contains an archetypal and conjugative TOL plasmid called pWW0 (Bayley et al., 1977; Williams and Murray, 1974). Self-transmissible TOL plasmids have the potential to spread to other bacteria in the soil. The *xyl* genes located on TOL plasmids code for enzymes responsible for the degradation of monoaromatic oil compounds, BTEX (benzene, toluene, ethyl benzene and xylenes) (Assinder and Williams, 1990). Petrol stations and garages are typical sources of these highly soluble oil compounds. The *meta*-cleavage pathway on TOL plasmids differs significantly from the chromosomal β-ketoadipate or *ortho*-cleavage pathway in being able to tolerate alkyl substituents on the catechol. This enables TOL-harbouring bacteria to utilize also e.g. *meta*-toluate (3-methylbenzoate), our model compound. One of the key enzymes in the *meta*-cleavage pathway is the *xylE* gene product called catechol 2,3-dioxygenase, the substrate of which is 3-methylcatechol. The breakdown of catechol can be detected as a yellow product called 2-hydroxymuconic semialdehyde.

PCR-based DNA-typing methods of culturable bacteria are nowadays universally applicable, simple and rapid. However, oil-degrading bacteria have not yet been studied systematically with these methods. Besides the knowledge of total bacterial communities, we still need information on individual culturable bacteria in order to combine different metabolic functions to relevant bacterial species. Restriction fragment length polymorphism analysis (RFLP) of amplified 16S rRNA genes provides an estimate of the phylogenetic relationships between bacteria at species and higher taxonomic levels (Gurtler et al., 1991; Laguerre et al., 1994), while repetitive sequence-based polymerase chain reaction (rep-PCR) genomic fingerprinting is used to produce species and strain specific fingerprints of different bacterial genomes (Laguerre et al., 1996; Nick et al., 1999; Versalovic et al., 1991, 1994). DNA primers corresponding to repetitive extragenic palindromic (REP), enterobacterial repetitive intergenic consensus (ERIC) and a subunit of the BOX element (BOXA) sequences as well as to a repetitive trinucleotide ($(GTG)_5$) are available for this purpose. REP and ERIC have been the most popular primers thus far (de Bruijn, 1992; Judd et al., 1993; Louws et al., 1994). Most of the studies using these DNA-fingerprinting methods have focused on one species or genus at a time. We, however, wanted to know, how molecular identification methods could be used for parallel identification of several bacterial genera from the complex environment of oil-contaminated rhizosphere.

In the present study we applied molecular techniques to settle a compact set of methods suitable for monitoring the diversity of culturable bacteria during in situ bioremediation, and to gain genetic information about potential oil degraders in the contaminated rhizosphere. The aims of the present study were (1) to establish a collection of bacteria from oil-contaminated rhizosphere with the capacity to grow on a medium containing *m*-toluate, (2) to phenotypically characterize the culture collection by determining the tolerance of the isolates to *m*-toluate and their capacity to degrade *m*-toluate, (3) to taxonomically characterize the collection of indigenous *m*-toluate-tolerating isolates by using 16S rDNA PCR-RFLP, rep-PCR genomic fingerprinting and partial 16S rRNA gene sequencing.

2. Materials and methods

2.1. Microcosms and the harvest of rhizosphere soil samples

Microcosms with oil- (OS) or *m*-toluate-contaminated soil (MS) were set up. Soil types were classified according to Elonen (1971) and basic soil characteristics were analysed by Viljavuuspalvelu Ltd (Mikkeli, Finland). Oil-contaminated, dry and humous sandy soil (4% organic C; pH 7.3; conductance 10×mS/cm 10.0; levels of exchangeable nutrients mg/l: Ca, 9350; P, 17; K, 122; Mg, 574; S, 1820; total N, 0.17) was from a 20-year-old land-farming field for oil refinery wastes in southern Finland. This oil soil contained 10% total hydrocarbons (THC) as measured gravimetrically by carbon tetrachloride extraction (SFS 3009, 1980). The original oil soil was diluted with fine sand to a 2% final THC concentration. The concentrations of calcium and sulphur were very high in the OS. Metal concentrations (mg/kg DW; As, 53; Hg, 0.67; Cd, 0.45; Cr, 94; Cu, 123; Pb, 40; Ni, 145; Zn, 312; V, 233) were determined from the oil soil by Neste, Corporate Technology, Analytical Research (Porvoo, Finland). Due to the high metal concentration, the OS had high conductance. The concentrations of arsenic, copper, mercury, nickel, zinc and vanadium exceeded maximum values for agricultural use. MS was prepared from agricultural soil by adding 10% *m*-toluate (pH 13) to get the final *m*-toluate concentration of 3000 mg/l of soil. Agricultural soil was from the Partala Research Station for Ecological Agriculture (MTT, Juva, Finland) (4% organic C; pH 6.3; conductance 10×mS/cm 2.5; levels of exchangeable nutrients mg/l: Ca, 1920; P, 2.6; K, 133; Mg, 195; S, 42.3; total N, 0.18). This soil was moist and humous fine sand. It contained no indigenous *Rhizobium galegae* bacteria.

Plants in OS or MS were treated in two different ways and grown in an open greenhouse at an average summer temperature of 16 °C in Helsinki: *Galega orientalis* seeds inoculated with *R. galegae* HI174 were grown with or without the presence of a peat layer inoculated with *Pseudomonas putida* PaW85. Peat-based *P. putida* PaW85 inoculants were prepared as described by Suominen et al.