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r-strategist versus K-strategist for the application in bioremediation of hydrocarbon-contaminated soils

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ABSTRACT

A laboratory study was undertaken to determine the degradative potentials of the pure bacterial strains *Mycobacterium frederiksbergense* IN53 (*Actinobacteria*, a K-strategist) and *Acinetobacter* sp. IN47 (*Gammaproteobacteria*, an r-strategist) as well as a combination of these strains under harsh environmental conditions (high hydrocarbon load: 50,883 and 46,682 mg kg^{-1} dry weight of soil for non-sterile and sterile soil samples, respectively; moisture content lower than 20%). A decrease in total petroleum hydrocarbons (TPH) and the activity of soil enzymes (dehydrogenase and lipase) indicated that the effects of *M. frederiksbergense* IN53 and the consortium were equivalent. However, these two microbial treatments showed significantly better degradative actions than *Acinetobacter* sp. IN47. Similar biodegradation yields were observed in sterile and non-sterile soil samples. These results suggest that bioaugmentation with *M. frederiksbergense* IN53 (K-strategist) provides a better approach for the treatment of hydrocarbon-contaminated sites in which the optimum remediation bioprocess conditions are difficult to maintain.

1. Introduction

Contamination by petroleum hydrocarbons causes critical environmental damage and health problems. Therefore, there has been increased attention on developing and implementing innovative cleanup technologies (Chen et al., 1998; de la Huz et al., 2005). Bioremediation is a widely accepted and cost-effective solution for the removal of petroleum hydrocarbons from contaminated soils and sediments (Atlas, 1995). This approach is now globally recognized and often used as an alternative to other methods. Moreover, bioremediation technologies are believed to be environmentally friendly and efficient (Juwarkar et al., 2010). Bioremediation strategies include the application of competent living organisms to detoxify or eliminate xenobiotics from polluted ecosystems through the organisms' various metabolic capabilities (Gaskin and Bentham, 2010). Bioremediation generally can be divided into 2 main categories: (1) biostimulation – adjustment of nutrients and/or other supplements to enhance degradation by indigenous microbes and (2) bioaugmentation – introduction of microorganisms to polluted sites to accelerate the removal of undesired compounds (Tyagi et al., 2011). The latter approach has received much attention in recent years (Mrozik and Piotrowska-Seget, 2010). Bioaugmentation with specialized microbes that possess a high genetic potential to metabolize a wider spectrum of petroleum hydrocarbons is much more effective than biostimulation (Bento et al., 2005; Stallwood et al., 2005). There are numerous reports of successful laboratory studies of bioaugmentation, but there is less information concerning field trials (Łebkowska et al., 2011; Winquist et al., 2014). However, in certain cases, bioaugmentation does not produce satisfactory results. Poor results are mainly attributed to the poor bioavailability of pollutants (Moliterni et al., 2012), protozoan predation (Yu et al., 2011) or competition from native microbiota (Lladó et al., 2013). Therefore, choosing the best remediation technique is a complex problem and should be considered individually for each case of pollution (Steliga et al., 2012). The potential success of bioremediation depends on factors such as the activity of the introduced microorganisms and the physicochemical characteristics of the soil (Bopathy, 2000; El Fantroussi and Agathos, 2005). These factors can be particularly important when one deals with a large contaminated area where there is no possibility to sustain appropriate soil moisture and/or nutrient levels.

The question that remains unanswered is what would be the main criteria for selecting competent microorganisms. Most reports have demonstrated that the microbes should possess a

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degradative profile that is fitted to the chemical composition of the pollutants and that the microbes should also be adapted to the local environment (Lebeau, 2011). Microbial consortia, consisting of highly specialized hydrocarbon-degrading strains, have been used for the removal of pollutants in the laboratory and in field bioremediation studies (Gojgic-Cvijovic et al., 2012; Auffret et al., 2014; Kumar et al., 2014). Microorganisms forming consortia have shown better degradative potential than single isolated strains (Cerqueira et al., 2011; Kadali et al., 2012; Festa et al., 2013; Zhang et al., 2014) due to their mutual cooperation (Mikesková et al., 2012) and synergy. However, there has been limited research on the coexistence and competition within particular communities of hydrocarbon-degrading strains (Rivelli et al., 2013). Most known hydrocarbon degraders belong either to *Proteobacteria* or *Actinobacteria*, and the members of these large taxonomic groups are frequently used for bioremediation purposes (Tyagi et al., 2011). These phyla also represent two different life strategies: members of *Proteobacteria* are usually considered to be fast-growing bacteria (r-strategists), whereas *Actinobacteria* are mostly slow-growing organisms (K-strategists) (Lebeau, 2011). Thus, these different phyla should display different growth patterns and metabolic activity when they are introduced to contaminated soil.

The present study aimed to better understand hydrocarbon biodegradation by comparing the performance of individual strains with the performance of a constructed microbial consortium. The hypothesis was that microbes which can adapt better to harsh environmental conditions (high hydrocarbon load and soil moisture limitation) are the key-players in the successful removal of pollutants also when they are introduced as a consortium. The remediation effectiveness of the consortium strains determine the effectiveness of bioaugmentation. Two bacterial strains, *Mycobacterium frederiksbergense* IN53 (*Actinobacteria*, a K-strategist) and *Acinetobacter* sp. IN47 (*Gammaproteobacteria*, an r-strategist), which are capable of degrading a broad spectrum of petroleum hydrocarbons, were tested either separately or in a consortium. This report presents the results of monitoring hydrocarbon bioremediation through changes in soil lipase and dehydrogenase activity, determining the hydrocarbon content and analyzing microbial abundance with FISH (fluorescence *in situ* hybridization) techniques.

2. Materials and methods

2.1. Soil characteristics

The soil samples used in this study were collected from a G-24 waste pit area located in an oil and gas plant in Grabownica (southeast Poland). The contamination of this site has resulted from storing drilling wastes on the site for over 50 years. The sampling depth was between the surface layer and the 30 cm depth. This clayey soil mainly contained quartz (53%), clay minerals (35%), fledspar (6%) and traces of halite, calcite, pyrite and anhydrite. The main physical and chemical parameters of the soil were determined as described previously (Steliga et al., 2012) and are summarized in Table A.1. The total number of indigenous, heterotrophic, aerobic microorganisms was low (approx. 10^2 colony-forming units (CFU)$\cdot g^{-1}$ dry weight of soil).

2.2. Microorganisms

The *M. frederiksbergense* IN53 (*Actinobacteria*, the K-strategist) strain used in this study came from the hydrocarbon-degrading microbial collection of the Department of Microbiology (at the Oil and Gas Institute – National Research Institute, Poland) and was previously tested as a member of a hydrocarbon-degrading consortium (Steliga et al., 2012). The 16S rDNA sequence of this strain has been deposited in the NCBI database under accession number JN572675 (*M. frederiksbergense* IN53). *Acinetobacter* sp. IN47 (*Gammaproteobacteria*, the r-strategist) was isolated in this study from weathered, oil-polluted soil collected from a G-7 waste pit (at the same location as the G-24 waste pit). Strain identity was determined based on microscopic observations, morphology, growth on selective agar media, and biochemical profile (API tests, Biomerieux). In addition, the strain was phylogenetically identified by 16S rDNA sequencing analysis (CB DNA, Poznań, Poland) as described previously (Steliga et al., 2012). The 16S rDNA sequence of strain IN47 has been deposited in the NCBI database under accession number KF918751 (*Acinetobacter* sp. IN47). Both strains were tested for utilization of a wide range of hydrocarbons. Strain growth on a mineral medium was examined after the medium was supplemented with the following compounds: alkanes, BTEX (benzene, toluene, ethylobenzene, *o*-, *m*-, *p*-xylene) and PAHs (polycyclic aromatic hydrocarbons). Additionally, hydrocarbon-degradation capabilities were examined according to the methods of Wrenn and Venosa (1996) as well as Bučková et al. (2013). *M. frederiksbergense* IN53 degraded linear and branched alkanes as well as selected PAHs, whereas *Acinetobacter* sp. IN47 degraded linear and branched saturated aliphates (Table A.2). Both microbes possess lipolytic activities, which were indirectly tested by growth in mineral medium supplemented with vegetable oil (1% (v/v)).

Pure cultures of the individual strains were grown in nutrient broth (Difco, USA) supplemented with sodium acetate (0.2% (w/v); POCH, Poland), incubated at room temperature with shaking at 150 rpm for 24 and 72 h for *Acinetobacter* sp. IN47 and *M. frederiksbergense* IN53, respectively. A microbial consortium, consisting of *Acinetobacter* sp. IN47 and *M. frederiksbergense* IN53, was constructed by mixing equal proportions of both strains.

2.3. Bioremediation experiment

The experimental setup was designed to assess the TPH (total petroleum hydrocarbon) removal of each strain, acting independently or within a consortium, either in the absence or the presence of native microbiota. Each of the microcosms were prepared by filling a glass jar with 500 g of soil from the G-24 waste pit. Samples M0–M3 were autoclaved at 15 psi and 121 °C for 20 min. The proper C:N:P ratio was adjusted by addition of $(NH_4)_2SO_4$ (0.5 mg kg^{-1} dry weight of soil) and K_2HPO_4 (1.2 mg kg^{-1} dry weight of soil). Microcosms M1–M3 and M5–M7 were inoculated with 0.5 ml g^{-1} dry weight of soil of the appropriate inoculum (the final amount of the introduced inoculum was 10^5 cells g^{-1} dry weight of soil) as follows:

- microcosm M1 – sterile soil inoculated with a microbial consortium consisting of *Acinetobacter* sp. IN47 and *M. frederiksbergense* IN53
- microcosm M2 – sterile soil inoculated with *M. frederiksbergense* IN53
- microcosm M3 – sterile soil inoculated with *Acinetobacter* sp. IN47
- microcosm M4 – non-inoculated, non-sterile soil to investigate hydrocarbon degradation by indigenous microorganisms
- microcosm M5 – non-sterile soil inoculated with a microbial consortium consisting of *Acinetobacter* sp. IN47 and *M. frederiksbergense* IN53
- microcosm M6 – non-sterile soil inoculated with *M. frederiksbergense* IN53
- microcosm M7 – non-sterile soil inoculated with *Acinetobacter* sp. IN47.

The treatments included two controls: (1) microcosm M0, a non-inoculated sterile soil as a reference to assess abiotic hydrocarbon