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Characterization of the bacterial archaeal diversity in hydrocarbon-contaminated soil

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ABSTRACT

A polyphasic approach combining culture-based methods with molecular methods is useful to expand knowledge on microbial diversity in contaminated soil.

Microbial diversity was examined in soil samples from a former industrial site in the European Alps (mainly used for aluminum production and heavily contaminated with petroleum hydrocarbons) by culture-dependent and culture-independent methods. The physiologically active eubacterial community, as revealed by fluorescence-in-situ-hybridization (FISH), accounted for 6.7% of the total (DAPI-stained) bacterial community. 4.4% and 2.0% of the DAPI-stained cells could be attributed to culturable, heterotrophic bacteria able to grow at 20 °C and 10 °C, respectively. The majority of culturable bacterial isolates (34/48) belonged to the *Proteobacteria* (with a predominance of *Alphaproteobacteria* and *Gammaproteobacteria*), while the remaining isolates were affiliated with the *Actinobacteria*, *Cytophaga–Flavobacterium–Bacteroides* and *Firmicutes*. A high fraction of the culturable, heterotrophic bacterial population was able to utilize hydrocarbons. *Actinobacteria* were the most versatile and efficient degraders of diesel oil, n-alkanes, phenol and PAHs. The bacterial 16S rRNA gene clone library contained 390 clones that grouped into 68 phylotypes related to the *Proteobacteria*, *Bacteroidetes*, *Actinobacteria* and *Spirochaetes*. The archaeal 16S rRNA gene library contained 202 clones and 15 phylotypes belonging to the phylum *Euryarchaeota*; sequences were closely related to those of methanogenic archaea of the orders *Methanomicrobiales*, *Methanosarcinales*, *Methanobacteriales* and *Thermoplasmatales*. A number of bacterial and archaeal phylotypes in the clone libraries shared high similarities with strains previously described to be involved in hydrocarbon biodegradation.

Knowledge of the bacterial and archaeal diversity in the studied soil is important in order to get a better insight into the microbial structure of contaminated environments and to better exploit the bioremediation potential by identifying potential hydrocarbon degraders and consequently developing appropriate bioremediation strategies.

1. Introduction

Petroleum hydrocarbons are the most widespread contaminants in the environment. The contamination of soil with high levels of hydrocarbons results in an increased soil organic carbon content, which – depending on composition and concentration – may be utilized for microbial growth or may be toxic to microorganisms (Bossert and Bartha, 1984; Alexander, 1999; Maier et al., 2000). The impact of low and high doses of environmental pollutants such as hydrocarbons can range from stimulation to total inhibition of microorganisms (Ramakrishnan et al., 2010). The capacity of a broad spectrum of microorganisms to utilize hydrocarbons as the sole source of carbon and energy was the basis for the development of biological remediation methods. The ability to degrade hydrocarbons is widespread among soil microorganisms. They may adapt rapidly to the contamination, as demonstrated by significantly increased numbers of hydrocarbon degraders after a pollution event (Margesin and Schinner, 2001; Greer et al., 2010).

Microbial community structures in hydrocarbon-contaminated soils are influenced by a number of factors, such as soil type, concentration and bioavailability of the contaminants, nutrient contents, temperature, oxygen content and pH (Margesin and Schinner, 2001; Greer et al., 2010). To evaluate soil microbial community composition in contaminated soils, culture-dependent and culture-independent methods have been used (Margesin and Schinner, 2005; Alonso-Gutierrez et al., 2009; Fabiani et al., 2009). Microbial abundance is often based on culture-dependent methods. However, culturable cells may only represent less than 1% of the total microbial community in an environment (Amann et al., 1995; Rappé and Giovannoni, 2003) and numerous bacteria enter a viable but non-culturable (VBNC) state in response to environmental stress (McDougald et al., 2009). Therefore, culture-independent, molecular assays, such as profiling soil DNA, rRNA, or phospholipid fatty acids, are increasingly used in environmental microbiology. Culture-independent approaches have been claimed to be more reliable for diversity analyses given the established cultivation methods favor the isolation of fast-growing microorganisms (Felske et al., 1999). Direct recovery of bacterial 16S rDNA from soil theoretically represents the entire microbial population from environmental samples (Spiegelman et al., 2005). However,

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molecular methods also have their limitations, such as variable efficiency of lysis and DNA extraction and differential amplification of target genes (Kirk et al., 2004).

Studies on microbial community composition in contaminated alpine soils have focused so far on the bacterial population (Margesin et al., 2003b; Labbé et al., 2007; Margesin et al., 2007), whereas information on the impact of *Archaea* is missing. In this study, we used a combination of culture-dependent and culture-independent methods (analysis of *Bacteria* and *Archaea* 16S rRNA gene clone libraries and fluorescence-in-situ-hybridization (FISH)) to investigate the microbial diversity in soil samples from an Alpine hydrocarbon-contaminated industrial site. Knowledge of the bacterial and archaeal diversity in the studied soil is important in order to get a better insight into the microbial structure of contaminated environments and to better exploit the bioremediation potential by identifying potential hydrocarbon degraders and consequently developing appropriate bioremediation strategies. Since both traditional, culture-based, and molecular methods have their limitations (Kirk et al., 2004), a multi-technique (polyphasic) approach combining these methods is advantageous.

2. Materials and methods

2.1. Sampling site and soil analysis

Soil samples were collected from an industrial site in March 2008. The study site was located in the European Alpine region in Bozen/Bolzano, South Tyrol, Italy. It used to be a former industrial district, built in 1930 and mainly used for aluminum production. In the 70s the production was reduced, and in 1990 the area was closed down and expropriated. Currently the area is not anymore used as industrial site and unused. Storage tanks for heavy oil (formerly used for cheap energy supply) were located at a depth of 1–3 m below ground surface. In 2008, the upper surface (0–4 m) was removed in the course of a remediation treatment in order to lay open an area of approximately 1000 m^2 that was contaminated with hydrocarbons due to pregressive leakage of heavy oil storage tanks. Our study area within this area had a size of approx. 100 m^2.

Five composite soil samples (15 kg each; each obtained from 5 subsamples) were collected with the help of a bucket from the study area and immediately transported to the laboratory. All soil samples were gently crumbled and sieved through an 8-mm screen in order to eliminate rough materials, thoroughly mixed, and stored at field humidity in polyethylene bags at 4 °C until processing.

Each sample was examined (2–3 replicates) for physico-chemical and microbiological (see Section 2.2) parameters. Physico-chemical parameters (soil dry mass, soil carbonate content, soil pH in $CaCl_2$, nutrient content) were measured as described (Schinner et al., 1996). Hydrocarbon content in the range of C_{10} to C_{40} was determined by gas chromatography after extraction with heptane (DIN ISO 16703, modified according to ÖNORM EN 14309).

2.2. Enumeration of culturable aerobic soil bacteria

Numbers of culturable soil bacteria were determined by the plate-count method for viable cells on R2A agar containing cycloheximide (100 mg L^{-1}) (Margesin et al., 2011). Hydrocarbon-utilizers were quantified on oil-agar containing diesel oil (Margesin and Schinner, 1997). Colony-forming units (CFU) were counted after 14, 21 and 28 days at 10 °C and 20 °C, respectively. All enumerations were performed with three replicates and CFUs were calculated on an oven-dry mass (105 °C) basis.

2.3. Phylogenetic analysis of culturable bacteria

Genomic DNA of 73 culturable bacterial strains, differing in phenotypic characteristics (colony morphology, pigmentation, growth characteristics) was extracted using the UltraClean Microbial DNA isolation kit (Mo Bio Laboratories). The 16S rRNA genes were amplified as described earlier (Zhang et al., 2010a). Strains having >98% 16S rRNA gene sequence similarity and matching the same GenBank sequence were assigned to the same phylotype (see also Section 2.10.).

2.4. Characterization of culturable bacteria

The 48 strains that were identified as potentially unique (see Section 3.5) were characterized with regard to their growth temperature range, degradation abilities and enzyme activities.

2.4.1. Growth temperature range

Suspensions of bacterial cells (pre-grown on R2A agar plates at 15 °C) in 0.9% NaCl were used to inoculate R2A agar plates that were incubated at 1, 5, 10, 15, 20, 25, 30 and 37 °C, using two replicates per strain and temperature. Growth was monitored up to an incubation time of 7–21 days.

2.4.2. Utilization of aliphatic and aromatic hydrocarbons for growth

Suspensions of bacterial cells in 0.9% NaCl were used to inoculate mineral medium (without yeast extract) agar plates (Margesin and Schinner, 1997) that contained one of the following hydrocarbons as the sole source of carbon: diesel oil (30 μL per plate), phenol (2.5 mM), naphthalene, anthracene, pyrene (2 and 10 mg of each compound per plate, dissolved in acetone). Inoculated plates without hydrocarbons as well as sterile hydrocarbon-containing medium served as negative controls. Two replicates were used for all experiments. Plates were incubated up to 14–21 days at 15 °C and growth was monitored regularly.

Diesel-oil utilizing strains were further tested in liquid culture (mineral medium) on their ability to degrade n-alkanes (C_{16}, C_{20} and C_{28}; each 800 mg L^{-1}) and high amounts of diesel oil (3800 mg L^{-1}). After 14–21 days at 15 °C, the residual hydrocarbon concentration was measured by gas chromatography after extraction with heptane (DIN EN ISO 9377-2, modified). Strains able to utilize phenol on agar plates were grown in liquid culture at 15 °C in the same medium with increasing phenol concentrations in order to determine the highest amount of phenol that could be degraded by these strains (Margesin et al., 2003a).

2.4.3. Screening for enzyme activities

Amylase, protease, cellulase and esterase–lipase activities were tested as described (Margesin et al., 2003a; Gratia et al., 2009) on R2A agar supplemented with starch, skim milk (each compound 0.4% w/v), carboxymethylcellulose and trypan blue (0.4% and 0.01% w/v, respectively) or Tween 80 and $CaCl_2$ (0.4% v/v and 0.01% w/v, respectively). Plates were evaluated after 7 days at 15 °C.

2.5. Total bacterial counts

Total counts of bacteria were determined in the filtrates of soil suspensions by non-selective DAPI (4′,6′-diamino-2-phenylindole; 1.5 μg mL^{-1}) staining using CitiDAPI (2.5 μg DAPI mL^{-1}, Citifluor™ AF1 antfading; Electron Microscopy Sciences) and epifluorescence microscopy as described below (see Section 2.6.).

2.6. Fluorescence-in-situ-hybridization (FISH)

FISH analysis was done as described by Margesin et al. (2011) according to a procedure after Bertaux et al. (2007), using Nycodenz-based cell extraction (Barra Caracciolo et al., 2005). The oligonucleotide probe EUB338 (*Eubacteria*; Stahl and Amann, 1991) was used to quantify *Eubacteria* (Loy et al., 2007). The probe NONEUB (Wallner et al., 1993) was used as a negative control. The probes EUB338 and NONEUB were Cy-3 labeled. Since soil particles interfered due to autofluorescence with counting by automated methods (Kobabe et al.,