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Isolation and characterization of indigenous thermophilic bacteria active in natural attenuation of bio-hazardous petrochemical pollutants

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Abstract

Common petrochemical compounds, such as homocyclic polyaromatic hydrocarbons and heterocyclic NOS-polyaromatics (NOS-compounds), were used as the sole carbon and energy source to enrich indigenous bacteria harboring the catabolic ability to degrade these compounds from petroleum-contaminated soils from Kuwait. Chemical analysis of the extracted soil materials revealed residual amounts of oil (<5% w/w), presumably of heavy oil fractions with elevated S-content. Aerobic culturable mesophilic polyaromatic hydrocarbon- and NOS-degraders were abundant in these soils, whereas their moderately thermophilic counterparts constituted only a minor fraction. Glucose stimulated the growth of mesophiles and drastically suppressed the number of thermophiles. 16S rDNA was amplified by PCR from nine of the purified thermophilic strains, using primers specific for eubacteria. Sequencing of 900 bp of the 16S rDNA and database homology search tentatively aligned these isolates to low G+C Gram positive bacteria of the family *Bacillaceae*. Electron microscopy characterization revealed endospore-forming bacilli varying in size, with well-structured cell walls. Gas chromatography and mass spectrometry (GC/MS) analysis revealed a versatile catabolic ability of the pure and mixed cultures to degrade all tested compounds. The metabolism of the offered substrates does not involve co-metabolism, since all pure cultures consumed the offered substrates completely.

Keywords: Thermophilic bacteria; PAHs; NOS-compounds; Natural attenuation; Biodegradation

1. Introduction

Environmental pollution problems have gained increasing public and governmental awareness during recent decades. In spite of the many regulations that have been made and the safety measures that have been taken, environmental pollution of aquatic and terrestrial ecosystems caused by crude oil is still among the serious problems facing us. In this context, the dramatic increase in the production, refining, and distribution of petroleum, as well as oil spillage catastrophes, have brought with them an ever-increasing pollution problem in oil-producing countries of the Gulf region.

Among the common petrochemical compounds are polyaromatic hydrocarbons (PAHs) and heterocyclic NOS-containing compounds (NOS-compounds). The physico-chemical properties of PAH and NOS-compounds, such as low water solubility, high adsorption coefficient, and high stability of the aromatic ring displayed by the resonance energy of the aromatic ring and the inertness of C–H and C–C bonds, combine to make contaminated sites sinks for these compounds (Kropp and Fedorak, 1998; Kanaly and Harayama, 2000; Dean-Ross et al., 2002; Van Hamme et al., 2003). Accordingly, many of these compounds have been considered as priority pollutants which exert biohazardous effects on both human and other living organisms in the environment (Kramer and Van der Heijden, 1990; Rosmarie, 1991; Richardson, 1996). Fortunately, the microbial flora inhabiting polluted sites have developed fascinating metabolic machineries for detoxification and degradation of a wide range of PAH and

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NOS-compounds (Alexander, 1981; Atlas, 1981; Bressler and Fedorak, 2000; VanBriesen, 2001; Van Hamme et al., 2003; Abed and Köster, 2005). Isolation, identification, and genetic manipulation of a vast number of indigenous bacterial species for the bioremediation of pollutants have been the focus of many investigations worldwide (Gibson and Subramanian, 1984; Alexander, 1994; Atlas, 1995; Atlas and Bartha, 1998; Nojiri et al., 1999; Van Hamme et al., 2003; Ghazali et al., 2004). However, there is still a need to isolate more microbial species with novel enzymatic activities that are crucial for environmental as well as biotechnological applications, such as those that work in aquatic as well as organic media, have catalytic stability over a wide pH range and enhanced activities at high temperature and salinity.

The present investigation aimed to isolate and characterize indigenous thermophilic bacteria active in natural attenuation of biohazardous petrochemicals. The strains showing promising biodegradation potential will be further characterized to be used in some biotechnological applications.

2. Materials and methods

2.1. Chemicals

All chemicals and biochemicals were of analytical grade. PAHs, namely phenanthrene (PH), anthracene (AN), pyrene (PY), naphthalene (NA), fluorine (FL), and NOS-compounds benzothiophene (BT), dibenzothiophene (DBT), dibenzofuran (DBF), and carbazole (CA), were purchased from Fluka (Switzerland) and Acrose (USA). Other chemicals, biochemicals, and culture media were purchased from Difco (France), Fluka (Switzerland), and Sigma (USA). Molecular biology reagents and enzymes were purchased from Promega (USA) and Qiagen (Germany).

2.2. Culture media

Chemically defined media (CDM) were prepared according to Mohamed et al. (1999) and supplemented with trace elements (Van Hamme et al., 2000) and vitamins (VL7, Pfennig, 1978). Carbon sources, glucose (10 mM), PAH or NOS-compounds (1 mM) were added from filter-sterilized stock solutions. PAH and NOS-compound stock solutions were prepared in dimethyl sulfoxide (DMSO) except for AN, which was prepared in acetone.

2.3. Crude-oil contaminated soil samples

Oil-contaminated soil samples were collected from seven different sites with an oil-pollution history (oil lakes at Burqan oil fields) in the state of Kuwait. The soil samples (2 kg each) were collected randomly from the top soil layer (15 cm in depth).

2.4. Soil elemental analysis for total C, H, S and N

Ten grams of each oil-contaminated soil was extracted in three successive steps with a total of 60 mL ethylacetate. The residues obtained after evaporation were analyzed by a thermal conductivity analyzer to determine the total carbon, hydrogen, sulfur, and nitrogen contents. The elemental analysis was performed in the Kuwait Institute for Scientific Research (KISR), Kuwait.

2.5. Enrichment and enumeration of culturable bacterial communities inhabiting oil-contaminated soils

The total count and the abundance of the culturable bacterial communities enriched with PAHs or NOS-compounds (1 mM of each individual compound) at 37, 60, and 75 °C were tested in CDM. For each soil site, 12 flasks containing 100 mL CDM were prepared: three flasks without exogenously added C-source, three flasks supplemented with glucose, three flasks supplemented with a mixture of PAHs, and the last three flasks supplemented with a mixture of NOS-compounds. Each flask was inoculated with 10 g soil and incubated on a rotary shaker (150 rpm). A set of four flasks was incubated at each temperature. During the incubation period, the flasks were supplemented every 3 days with PAHs, NOS-compounds, or glucose. After 2 weeks of incubation, 10 mL was taken from each flask and 10-fold dilutions prepared and plated (100 and 200 μL) on the same media. The number of colonies was counted every 24 h for 4 days and the total number of cells in the original undiluted samples calculated and expressed as CFU g^{-1} soil (colony forming units per g soil). This experiment was run with separate duplicates and the mean values presented.

2.6. Purification of indigenous thermophilic bacteria utilizing PAHs and NOS-compounds

Ten milliliters of cultures growing at 60 °C with either a mixture of PAHs or NOS-compounds were transferred to fresh media and incubated at 60 °C on a rotary shaker (150 rpm). After 3 days of incubation, each flask was supplemented again with either PAHs or NOS-compounds. After 7 days incubation, 10 mL from each flask was transferred to fresh medium and incubated under the same conditions. These procedures were repeated for three successive transfers within 28 days. The bacterial cultures growing after the last transfer were collected by centrifugation (9000 rpm). Cells were suspended in 10 mL of the same medium containing 20% glycerol (w/v), stored at −70 °C and referred to as the original enrichment. To isolate and purify PAHs and NOS thermophilic degraders growing with individual compounds, the original enrichments were used to inoculate flasks containing CDM with 1 mM of each individual compound (CA, BT, DBT, DBF, NA, AN, PH, FL, PY). The cultures were transferred in three successive passages (every 4 days) to fresh medium containing the same substrate.

The bacteria growing with each individual compound were collected by centrifugation and suspended in the same medium. A portion of each culture was serially diluted, plated on the same medium, and incubated for 3 days. The most frequent colonies on the plates, based on their distinct morphology, were selected. Each colony was picked and streaked several times on Luria-Bertani (LB) agar supplemented with the same C-source to ascertain purity of the culture. The purity of the selected colonies was also checked by microscopic examination.

2.7. Utilization of individual PAHs and NOS-compounds by pure thermophilic bacterial cultures

Fourteen isolates of the purified thermophilic bacteria (enriched at 60 °C), designated as 4a, 4b, 6a, 6b, 6c, 9a, 9b, 9b′, 12a, 12b, 13a, 13b, 14a, and 14b, were tested for their capability to degrade individual PAHs and NOS-compounds at 1 mM concentration. Tubes containing 8 mL of CDM with the respective compound were inoculated with 300 μL of each pure culture (optical density at 580 nm of around 5). To test for synergetic or antagonistic interactions between cultures isolated from the same plate, equal volumes of different cultures enriched with the same substrate were mixed in a total volume of 300 μL and used to inoculate similar tubes. The tubes were incubated with shaking (200 rpm) at 60 °C and samples (1.5 mL) were withdrawn at 12, 24, 48, and 96 h to determine the concentration of C source in the cell-free supernatant. Control tubes containing only DMSO were also tested. The supernatant was acidified with 30 μL HCl (6 M) and extracted in total volume of 9 mL ethyl acetate