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Petroleum biodegradation capacity of bacteria and fungi isolated from petroleum-contaminated soil

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ABSTRACT

We investigated the potential for petroleum hydrocarbon biodegradation by 95 bacterial and 160 fungal strains isolated from a former petrochemical plant. We tested whether soil origin, culture media type, and strain taxonomy influenced the degradation of added petroleum hydrocarbon compounds. Preliminary screening was based on two colorimetric tests using 2,6-dichlorophenolindophenol and p-iodonitrotetrazolium indicators, to assess microbial strain tolerance to crude oil. Top-performing strains in these screening assays were then assessed for their ability to mineralize a mixture of four polycyclic aromatic hydrocarbons (PAH) for 49 days, using GC-MS quantification. The aerobic activity of these candidate strains was also assessed by respirometry over the first 24 days of incubation. On average, PAH degradation by microbial isolates from soil that was lightly, moderately, and highly contaminated with petroleum was equally efficient, and the type of culture medium used did not significantly impact mean biodegradation. Phylogenetic affiliation had a strong and significant effect on PAH biodegradation. Fungal isolates belonging to the group *Sordariomycetes,* and bacterial isolates belonging to the groups *Actinobacteria*, *Betaproteobacteria,* and *Gammaproteobacteria* showed high potential for PAH degradation. Three of the strains tested by GC-MS, *Rhodococcus* sp., *Trichoderma tomentosum*, and *Fusarium oxysporum,* significantly degraded all four PAH compounds in the mixture compared to the control.

1. Introduction

The widespread exploitation, transportation, and consumption of crude oil has attracted public attention to the fate of petroleum hydrocarbon (PHC) compounds in the environment. Crude oil contains a complex mixture of many thousands of chemicals, including aromatics such as polycyclic aromatic hydrocarbon (PAH) compounds. PAHs are of concern to human health and the environment, since they are highly persistent, can negatively impact soil functions, and pose mutagenic risks to microorganisms, plants, and animals (Williams et al., 2013; Cao et al., 2009). Consequently, avenues for rehabilitating PHC-contaminated soils are of wide general interest.

A number of technologies have been developed for the rehabilitation of PHC-contaminated sites, including physical, chemical, and biological approaches (Gan et al., 2009; Rayu et al., 2012; Li et al., 2010). Physico-chemical methods are expensive, may create further waste, and in many cases, simply transfer pollutants from one phase to another (Haritash and Kaushik, 2009; Khan et al., 2004). The use of living organisms for the rehabilitation of contaminated sites, also known as bioremediation, has attracted considerable research interest over the last decade, as a sustainable and cost-effective alternative to chemical treatment (Chikere et al., 2011; Kanaly and Harayama, 2010). Biodegradation of PHCs by natural populations of bacteria and fungi is well known (Megharaj et al., 2011; Palanisamy et al., 2014), as many microorganisms are able to use hydrocarbons as both energy and carbon sources (Montagnolli et al., 2015b). Through different extracellular and intracellular enzymatic activities reviewed by Fritsche and Hofrichter (2008), these microorganisms can mineralize PHCs (i.e. fully degrade them, with CO_2 as an end-product). The rate and extent of mineralization depend of the metabolic abilities of the microorganisms (Dobler et al., 2000). Numerous studies have demonstrated the potential for PAH bioremediation by bacteria

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(Palanisamy et al., 2014; Khan et al., 2013; Lu et al., 2011) and fungi (Sayara et al., 2011; Isola et al., 2013; Lee et al., 2015). However, determining the biodegradation potential of microorganisms remains challenging, since the most recalcitrant pollutants are degraded much more slowly than the more labile components. In addition to abiotic factors that limit complete degradation, indigenous microorganisms that can effectively tolerate and/or rapidly degrade PHCs may be present at very low abundance, or may only be able to degrade certain compounds (Tahhan et al., 2011; Sayara et al., 2009). To overcome slow innate PHC biodegradation in soil, adjustments to nutrient concentrations, oxygen, pH and/or temperature (biostimulation), along with microbial inoculation (bioaugmentation), may be used (Sayara et al., 2010; Betancur-Galvis et al., 2006; Tahhan et al., 2011).

For bioaugmentation to be efficient in practice, inoculated microorganisms must tolerate contaminants, efficiently degrade compounds of interest, and thrive in the target environment (Yao et al., 2015; Bisht et al., 2015). These microbial traits have been evaluated using culture-based assays, molecular methods, and analytical chemistry techniques like gas chromatography mass spectrometry (GC-MS) (Sayara et al., 2011). Since some of these techniques are costly, colorimetric methods have also been used to rapidly estimate the biodegradation capacity of microorganisms (Montagnolli et al., 2015b; Puškárová et al., 2013). Respirometry has also been used as a sensitive and effective method for quantifying PHC biodegradation (Coello Oviedo et al. 2009; Oyelami et al., 2013), by assessing CO_2 and O_2 production by organisms exposed to PHCs (Montagnolli et al., 2015a).

Although only a small portion of the microorganisms found in soil environments can be characterized through cultivation, a large number of microorganisms have been isolated from contaminated sites using different nutrient-rich (standard) and impoverished media, supplemented with various types and concentrations of PHCs (selective) (Stefani et al., 2015; Jacques et al., 2009). Polluted soils are of particular interest as sources for cultivation, since microbes in these soils are more likely to have developed multiple tolerance mechanisms, allowing them to survive and function effectively in the presence of PHCs (Caliz et al., 2012; Oriomah et al., 2014). Although bioremediation has become a crucial technology for *in situ* PHC removal, difficulties in identifying organisms that degrade high molecular weight compounds could limit our ability to enhance biodegradation. Current research in this area is limited by the identification of organisms that degrade complex aromatic structures.

This investigation aimed to evaluate the petroleum biodegradation efficiency of 95 bacterial and 160 fungal strains isolated from a former petrochemical plant that is highly petroleum-contaminated. We used screening tests based on colorimetric and quantitative analyses to determine the utility of these isolates in PHC biodegradation. Our specific objectives were to: (i) compare the potential of standard and selective media to isolate effective PHC-degrading microorganisms, (ii) evaluate the relationship between soil contaminant concentration and strain phylogeny with petroleum biodegradation efficiency, and (iii) identify a consortium of two to four microbial strains that could be useful for further *in vitro* assessments of hydrocarbon degradation pathways and potentially for *in situ* bioaugmentation.

2. Materials and methods

2.1. Experimental site and isolation of microorganisms

Bacterial and fungal strains used in this study were isolated from soil samples obtained at the site of a former petrochemical plant in Varennes, Quebec, Canada (45°41′56″N, 73°25′43″ W). This site has been contaminated for the last forty years by a variety of petroleum waste products related to the petro-chemical industry. Experimental design and sampling, DNA isolation, amplification, and sequencing of microbial isolates was previously described by Stefani et al. (2015). Briefly, the study area of approximately 2500 m^2 was divided in five plots of 300 m^2 each. The 24 soil samples from each plot were pooled to obtain representative composite soil samples and analysed for F1–F4 hydrocarbons (sum of all aromatic and aliphatic hydrocarbon compounds with chain lengths of C10–C50) by Maxxam Analytics (Montreal, Quebec, Canada). Results from hydrocarbon analyses (Table 1) revealed an increasing contamination gradient from plots 1 through 5, which led us to classify the blocks into three discrete contaminant levels: slightly contaminated (plots SC1, SC2), contaminated (plot C3), and highly contaminated (plots HC4, HC5). Bacterial strains were isolated from these three sections on standard media (tryptic soy agar, TSA, containing 30 g L^{-1} of tryptic soy broth (TSB)) and selective media (plates containing 2- to 30-fold diluted TSB (1–15 g L^{-1}) along with various concentrations of diesel engine oil or crude oil, or that were coated with crude oil) Table S1 (Supporting information). Fungal strains were isolated on standard media (potato dextrose agar, PDA, containing 24 g L^{-1} of potato dextrose broth (PDB)) and selective media (plates containing 3- to 39-fold diluted PDA (1–12 g L^{-1} of PDB) along with various concentrations of diesel engine oil or crude oil, or that were coated with crude oil) Table S1 (Supporting information). Sanger sequencing data have been deposited in GenBank under the accession numbers KP177318-KP177405 and KP177406-KP177454 for bacteria and fungi, respectively (Stefani et al., 2015).

2.2. Selection of microorganisms and inoculum preparation

A total of 781 bacterial strains and 279 fungal strains were isolated and sequenced from SC, C, and HC soils using standard and selective media. Among these isolates, 95 bacterial and 160 fungal strains were selected for this screening test. The criteria for selecting these microbial isolates were: (i) they were not classified as known pathogens of humans, animals, or plants; and (ii) they covered an array of major groups, allowing phylogenetic comparison. Selected fungal isolates belonged to three major taxonomic groups: 45 *Dothideomycetes*, 73 *Sordariomycetes*, and 42 *Mucoromycotina*. Selected bacterial isolates belonged to five major taxonomic groups: 30 *Actinobacteria*, 23 *Bacilli*, 9 *Alphaproteobacteria*, 10 *Betaproteobacteria,* and 23 *Gammaproteobacteria*.

Before performing screening tests, bacterial and fungal cultures were grown for two weeks in Tryptic Soy Broth (TSB) and Potato Dextrose Broth (PDB), respectively. Bacterial isolates were then transferred to 96-well plates containing 200 μL of TSB in each and incubated at 25 °C under aerobic conditions in a rotary shaker adjusted to 100 rpm. At the exponential growth phase (optical density at 600 nm of 0.5–0.9), plates were centrifuged at 3500 rpm for 10 min and the supernatant was removed. Bacterial pellets were rinsed with 200 μL of Phosphate-buffered saline (PBS) (Sigma-Aldrich, Montreal, Canada), and re-centrifuged at 3500 rpm for 10 min. The supernatant was removed and 200 μL of minimum culture medium Bushnell-Haas (BH) was added to each well. The density of bacteria in each well was estimated by counting cells using a hemocytometer and light microscopy at 100× magnification.

Fungal isolates were cultivated in 250 ml flasks filled with 100 ml of PDB and inoculated with a mycelium disk (5 mm diameter). Cultures were incubated at 22 °C for two weeks under agitation at 100 rpm. Fresh mycelia were harvested using 1 μL sterile inoculation loops (Sarstedt, Montreal, Canada), washed with sterile deionized water, centrifuged at 3500 rpm for 15 min, and