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Degradation of oil by fungi isolated from Gulf of Mexico beaches

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ABSTRACT

Fungi of the Ascomycota phylum were isolated from oil-soaked sand patties collected from beaches following the Deepwater Horizon oil spill. To examine their ability to degrade oil, fungal isolates were grown on oiled quartz at 20 °C, 30 °C and 40 °C. Consistent trends in oil degradation were not related to fungal species or temperature and all isolates degraded variable quantities of oil (32–65%). Fungal isolates preferentially degraded short ($<C_{18}$; 90–99%) as opposed to long (C_{19}–C_{36}; 7–87%) chain *n*-alkanes and straight chain C_{17}- and C_{18}-*n*-alkanes (91–99%) compared to their branched counterparts, pristane and phytane (70–98%). Polycyclic aromatic hydrocarbon (PAH) compounds were also degraded by the fungal isolates (42–84% total degraded), with a preference for low molecular weight over high molecular weight PAHs. Overall, these findings contribute to our understanding of the capacity of fungi to degrade oil in the coastal marine environment.

1. Introduction

Oil degradation by fungi is well established, but the diversity of species and the associated mechanisms and degradation pathways are yet to be fully explored (Marco-Urrea et al., 2015). Estimates of fungal diversity range from 1.5 million species (Hawksworth, 2001) to 3.5–5.1 million species (O'Brien et al., 2005) encompassing a wide range of fungi that possess diverse metabolisms adapted for life in heterogeneous environments (Stajich et al., 2009). In the environment, fungi may play an important role in the cycling of organic carbon (Hedges and Oades, 1997; Read and Perez-Moreno, 2003), nitrogen and phosphorus (Dighton et al., 2014; Hodge and Fitter, 2010; Read and Perez-Moreno, 2003) as well as the degradation of xenobiotic compounds including those derived from oil (Harms et al., 2011). Fungi capable of degrading oil have been identified in both terrestrial (April et al., 1998, 1999; Atlas, 1981; Atlas and Bartha, 1992; Capotorti et al., 2004; Covino et al., 2015; Marco-Urrea et al., 2015; Mineki et al., 2015; Reyes-César et al., 2014; Harms et al., 2011 and references therein) and marine environments (Al-Nasrawi, 2012; Bartha and Atlas, 1977; Cerniglia and Sutherland, 2010; Da Silva et al., 2003; Fedorak et al., 1984; Hassanshahian et al., 2012; Passarini et al., 2011; Walker et al., 1975) although they are relatively understudied in the latter (Richards et al., 2012). The major fungal phyla/subphyla involved in the biodegradation of oil include the Ascomycota, Basidiomycota, and Mucoromycotina, with specific fungal genera including, Aspergillus, Cephalosporium, Penicillium, Candida, Torulopsis Saccharomyces, Paecilomyces, Gliocladium, Yarrowia, Pichia, Pleurotus, Geotrichum, Talaromyces, Cladosporium, Fusarium, Alternaria, Mucor, Polyporus, Rhizopus, and Rhodotolura (Harms et al., 2011 and references therein).

Owing to the ubiquity and persistence of oil in the coastal marine environment (e.g. Farrington and McDowell, 2004; White et al., 2013), the degradation of petroleum by marine fungi is of particular interest. Prior studies have examined the biodegradation of oil by marine fungi by quantifying changes in the total mass of oil over time (e.g. Al-Nasrawi, 2012) or by examining changes in the quantity of oil-derived compounds including straight chain *n*-alkanes, branched alkanes (e.g. pristane and phytane) as well as polycyclic aromatic hydrocarbons (PAHs; April et al., 1999; Argumedo-Delira et al., 2012; Fedorak et al., 1984; Haritash and Kaushik, 2009; Harms et al., 2011; Johnsen et al., 2005; Marco-Urrea et al., 2015; Passarini et al., 2011; Reyes-César et al., 2014; Walker et al., 1975; Wu et al., 2010). Fungi are capable of degrading this structurally diverse range of oil-derived compounds by employing a variety of mechanisms including intracellular enzymes such as cytochrome P450 monooxygenases, nitroreductases and transferases as well as extracellular enzymes such as laccases and fungal peroxidases (Harms et al., 2011).

To explore the ability of fungi present on beaches in the Gulf of Mexico to degrade oil, we have characterized three species of marine fungi isolated from oil-soaked sand patties collected from Gulf coast beaches impacted by the 2010 Deepwater Horizon (DWH) oil spill. These oil-soaked sand patties first described by Aeppli et al. (2012), are also known as surface residual balls (SRB's; Plant et al., 2013), tar balls and tar patties (Milkov et al., 2011; Wang and Roberts, 2013) persist on Gulf coast beaches today and contain a mixture of weathered oil, sand, water (Elango et al., 2014) and traces of the anionic surfactant dioctyl sodium sulfosuccinate (DOSS), which is a component of the dispersant mixtures Corexit 9527 and Corexit 9500A (Nalco Co.) used at the time of the DWH spill (White et al., 2014). This study examines

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the ability of the isolated marine fungi to degrade oil at 20, 30 and 40 °C. Considering that marine fungi are still a relatively unexplored group (Damare et al., 2012; Richards et al., 2012), we seek to contribute to a better understanding of both their taxonomic and functional diversity.

2. Materials and methods

2.1. Sample collection

During fieldwork to collect oil-soaked sand patty samples that originated from the DWH oil spill, filamentous fungi were found growing on oil-soaked sand patties (Supplementary Fig. 1). These patties were collected in June 2012, from Gulf Shores (Alabama, USA; Lat/Long 30°24′N, −87°74′W) and Dauphin Island (Alabama, USA; Lat/Long 30°25′N, −88°12′W) approximately 24 months after the DWH oil spill. Samples were composed of either a single patty or a composite of several patties collected in close proximity (~10 m) to each other.

2.2. Fungal isolation and culture techniques

To screen for fungal isolates that were able to degrade oil, portions of fungal hyphae were aseptically transferred from sand patties to the center of agar plates made from an artificial seawater (AFSW) minimal medium (final concentration in 1 L: 450 mM NaCl, 10 mM KCl, 9 mM $CaCl_2$, 30 mM $MgCl_2 \cdot 6H_2O$, 16 mM $MgSO_4 \cdot 7H_2O$ at pH 7.8, with 1.5% agarose) supplemented with crude oil (0.5 g/L) after sterilization. If radial growth from the initial inoculation point occurred, the fungi isolates were transferred and maintained in petri dishes containing agar-solidified (2% agar) potato dextrose medium (Supplementary Fig. 2E, 3E and 4D).

2.3. Fungal characterization

Fungal mycelia were dissected from PDA (Potato Dextrose Agar) using sterile tweezers, for morphological identification (as in Alexopoulos and Mims, 1996; Bessey, 1950) and genomic DNA extraction. DNA was extracted using the PowerSoil® DNA Isolation Kit (MoBio Laboratories, Inc., Solana Beach, CA) according to the manufacturer's protocol. Plugs of PDA containing fungal hyphae were taken using a sterile coring device and tweezers and placed into a bead tube for extraction. The remaining steps were performed as per the manufacturer's instruction. Purity and quantity of DNA were assessed using a NanoDrop 1000 spectrophotometer (Thermo Scientific) and gel electrophoresis of a 5 μL aliquot on a 1% agarose gel containing 0.5 μg mL^{-1} ethidium bromide. Fungal internal transcribed spacer (ITS) region was PCR-amplified using primers, which target a wide range of fungal species (ITS-1F, 5′-CTTGGTCATTTAGAGGAAGTAA-3′, and ITS-4 5′-TCCTCCGCTTATTGATATGC-3′) (White et al., 1990). Primers LR0R 5′-ACCCGCTGAACTTAAGC-3′ and LR5 5′-TCCTGAGGGAAACTTCG-3′ (Vilgalys and Hester, 1990), which amplify the 28S large ribosomal subunit were also used to aid in identification of fungal isolates. PCR amplifications were carried out in a 25 μL mixture containing 2.5 μL total genomic DNA as a template, 400 nM of each primer, 2.5 mM of each dNTP, 2.0 μL of TaKaRa Ex-*Taq*™ buffer, and 1.25 U of TaKaRa Ex-*Taq*™ (TaKaRa). Cycling conditions for the 28S rRNA primer pair were as follows: initial denaturing at 94 °C for 3 min, 35 cycles of 94 °C for 60 s, 52 °C for 50 s and 72 °C for 60 s, followed by a final elongation step at 72 °C for 10 min. Cycling conditions for ITS1F/ITS4R initial denaturing at 95 °C for 2 min, 35 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 60 s, followed by a final elongation step at 72 °C for 10 min. 28 rRNA and ITS sequences were analyzed using the Sequencher® software package (version 5.2, Gene Codes Corporation, Ann Arbor, MI USA http://www.genecodes.com) to remove low-quality sequence data. No chimeras were identified using the UCHIME algorithm implemented in Mothur (Schloss et al., 2009). ITS and 28S rRNA sequences were aligned to related sequences retrieved from BLAST searches and from described strains where full sequences were available prior to manual edits as necessary using the package software ClustalX 2.1 (Larkin et al., 2007). Phylogenetic trees were constructed with the Molecular Evolutionary Genetics Analysis (MEGA) Software Version 6.06 (Tamura et al., 2007) using the neighbor-joining method with 10,000 bootstrap repetitions. All sequences were submitted under the GenBank accession numbers KR260888–KR260899.

2.4. Light- and electron microscopy

Wet mount and slide cultures of fungal isolates were visualized with the lactophenol cotton blue (LPCB) stain and Light microscopy using a Nikon SMZ1500 Stereo Microscope fitted with a Nikon Digital Sight DS-Ri1 digital camera. For scanning electron microscopy (SEM) fungal mycelia were fixed in 2.5% glutaraldehyde and subjected to a series of ethanol/acetone/Tetramethylsilane (TMS) dehydrations. After the fixation process, desiccated samples were mounted on double-sided carbon tape and sputter-coated with gold prior to imaging. SEM was performed using a High Resolution JEOL JSM-6510LV, with a standard conventional SE detector.

2.5. Oil degradation experiments

To determine if the fungi isolates could utilize crude oil as a sole carbon source, a petri dish was filled with sterile quartz sand mixed with crude oil (10 mg/g) with a sterile wire mesh and 0.22 μM filter (Whatman) placed on top to provide a support for fungal growth. One milliliter of sterile MilliQ water was added to each petri dish to maintain moisture. Each petri dish was then capped to minimize evaporative losses of water and volatilization of oil. After approximately 2–3 days when the growth of fungal isolates on PDA had proceeded radially outward from the inoculation point, the fungal mycelia were sampled with a sterile coring device. Mycelia-containing agar plugs obtained with the sterile coring device were then placed on top of the filter paper. Care was taken to ensure that all agar plugs were of similar size. Triplicates were conducted for each species and incubated for 6 weeks at 20, 30 or 40 °C. Sterile MiliQ water (1 mL) was added every 1–2 weeks to maintain moisture. Identical incubations without agar plugs and fungi were set up in triplicate as abiotic controls.

2.6. Oil extraction and analysis

After 6 weeks, fungal agar plugs, filter paper and wire mesh were removed and the petri dishes containing the sand and oil mixture and were dried in an oven at 45 °C overnight. These samples were then extracted by sonication with dichloromethane (3 × 10 mL for 30 min). Extracts were, dried over $NaSO_4$ and reduced in volume by rotary evaporation before splitting the extract in two and spiking one-half with 9,10-dihydrophenanthrene (0.25 μg), and the other half with stearyl palmitate (25 μg), both as injection standards. Half of the extracts were analyzed on an Agilent 7890 series gas chromatograph interfaced to a flame ionization detector (FID). Compounds were separated on a J&W DB-XLB capillary column (30 m, 0.25 mm internal diameter (I.D.), 0.25-μm film) with helium carrier gas at a constant flow of 1 mL min^{-1}. The GC oven had an initial temperature of 40 °C (1 min hold) and was ramped at 5 °C min^{-1} until 320 °C (15 min hold). Total oil was quantified by integrating the total FID area and using response factors determined from crude oil standards. The total FID area for all chromatograms included resolvable branched and straight chain alkanes and a small unresolvable complex mixture (UCM). Quantities of *n*-alkanes, pristane and phytane were calculated using response factors determined from pure standards. The other half of the extracts were analyzed on the aforementioned GC coupled to an Agilent 5975 mass selective detector (MSD) for the quantification of PAHs. The MSD was operated in the selected-ion monitoring mode for quantitation of target PAHs, which included: naphthalene, C_1-, C_2-,

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