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Phylogenetic characterization of culturable bacteria and fungi associated with tarballs from Betul beach, Goa, India

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ABSTRACT

Tarballs are semisolid blobs of crude oil, normally formed due to weathering of crude-oil in the sea after any kind of oil spills. Microorganisms are believed to thrive on hydrocarbon-rich tarballs and possibly assist in biodegradation. The taxonomy of ecologically and economically important tarball-associated microbes, however, needs improvement as DNA-based identification and phylogenetic characterization have been scarcely incorporated into it. In this study, bacteria and fungi associated with tarballs from touristic Betul beach in Goa, India were isolated, followed by phylogenetic analyses of 16S rRNA gene and the ITS sequence-data to decipher their clustering patterns with closely-related taxa. The gene-sequence analyses identified phylogenetically diverse 20 bacterial genera belonging to the phyla *Proteobacteria* (14), *Actinobacteria* (3), *Firmicutes* (2) and *Bacteroidetes* (1), and 8 fungal genera belonging to the classes *Eurotiomycetes* (6), *Sordariomycetes* (1) and *Leotiomycetes* (1) associated with the Betul tarball samples. Future studies employing a polyphasic approach, including multigene sequence-data, are needed for species-level identification of culturable tarball-associated microbes. This paper also discusses potentials of tarball-associated microbes to degrade hydrocarbons.

1. Introduction

Tarballs are semisolid blobs of crude oil, normally formed due to weathering of crude oil in the sea after any kind of oil spills. They get advected long distance by ocean currents and waves to reach beaches, and cause pollution in coastal marine environment. Microbes originating either from sea-water or beach-sand get attached to tarballs and possibly affect the biodegradation of hydrocarbon-rich tarballs. Tarballs act as a substratum for physical attachment of various microbes and the byproducts formed during their degradation possibly support microbial growth (Shinde et al., 2017).

The taxonomy of tarball-associated microbes, however, needs improvement as DNA-based identification and phylogenetic characterization have been scarcely incorporated into it (Bacosa et al., 2016; Nkem et al., 2016; Sanyal et al., 2016; Shinde et al., 2017; Barnes et al., 2018). To the best of our knowledge, Nkem et al. (2016) were the first to report 16S rRNA gene-sequencing of culturable tarball-associated bacteria. They characterized two bacterial species, *Acinetobacter baumannii* and *Cellulosimicrobium cellulans* associated with tarballs from Malaysia based on a polyphasic approach. Recently, tarball-associated bacteria were studied using a culture-independent method from Texas beaches following the 2014 Texas city "Y" spill (Bacosa et al., 2016) which provided insights into their unculturable diversity in the context of tarball chemistry.

In case of tarball-associated fungi, Sanyal et al. (2016) were the first to report phylogenetically diverse filamentous-fungi and yeasts, based on ITS sequence-data, from touristically important Candolim beach in Goa. Recently, Barnes et al. (2018) reported two tarball-associated fungal species, *Fusarium equiseti* and *F. circinatum* from Betul beach, Goa and provided their multigene sequence-data. Nevertheless, information available on molecular diversity and DNA sequence-based identification of tarball-associated bacteria and fungi is scarce and patchy (Bacosa et al., 2016; Nkem et al., 2016; Sanyal et al., 2016; Shinde et al., 2017; Barnes et al., 2018), especially from tourist beaches of Goa state which has been facing tarball pollution since the 1970s (Nair et al., 1972; Suneel et al., 2013, 2016). This study, therefore, aimed to isolate both bacteria and fungi from tarballs collected from touristic Betul beach in south Goa and characterize their phylogenetic relationships based on 16S rRNA gene (for bacteria) and the ITS sequence data (for fungi) analyses.

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2. Material and methods

2.1. Sampling of tarballs

Betul is a coastal town located in the south Goa, India. The GPS coordinates for Betul are 15.144837°N 73.958244°E. The Betul beach was surveyed twice for tarball sampling, initially in October 2014 and subsequently in July 2016. Tarballs were handpicked randomly along a stretch on the beach wearing sterile nitrile gloves and collected in zip-lock bags. The bags were kept in ice box and carried to the laboratory. Samples were stored at 4 °C until further processing.

2.2. Isolation of bacteria

Tarball samples collected during October 2014 were processed for bacterial isolation using an isolation protocol, which was standardized in the lab after improving the procedure described by Tao et al. (2011). In the modified protocol, 1 g of tarball was weighed aseptically and washed with sterile sea water. The washed tarball samples were then added into a test tube containing 9 ml of sterile seawater. The sterile cotton swab was used to break tarballs and detach bacteria. The tubes were vortexed vigorously for 5–10 min. 1 ml of this was serially diluted further up to 10^{-5} dilution. 100 µl of each dilution was spread plated on Zobell Marine Agar (ZMA). The plates were then incubated at room temperature. After 48 h of incubation, the colony forming units (CFUs) were calculated.

Tarball samples collected during July 2016 were also processed for isolation of bacteria using the afore-mentioned procedure. To increase the recovery of diverse groups of bacteria, one more bacterial isolation protocol was standardized in the lab, after improving the isolation procedure described in Bayat et al. (2015). In this method, 1 g of the freshly collected tarball (from the July 2016 sample collection) was washed with sterile seawater to remove the particles adhered to it as much as possible and suspended it immediately in 100 ml of Zobell Marine Broth (ZMB) containing tarball (1 g/100 ml) as a source of hydrocarbon to promote the growth of hydrocarbon-utilizing bacteria. As tarballs were insoluble in water, sterile cotton swab was used to break them, so that maximum possible number of bacteria could get into the culture medium. The medium was then incubated on a rotary shaker at 120 rpm and 35 °C for 48 h. 1 ml of enriched medium was then transferred to ZMB containing tarball (1 g/100 ml) and further incubated on a rotary shaker at 120 rpm and 35 °C for 48 h. Final transfer was made in 100 ml of Bushnell Hass medium containing tarball as only carbon source. After the incubation, 1 ml from the final medium was serially diluted in sterile sea-water and spread plated on ZMA. Morphologically diverse bacterial colonies were further isolated by streak plate method.

The bacterial cultures were selected based on their colony morphology and purified for further studies (Table 1). Colony morphology characters were noted down, and Gram staining was performed for each isolate to confirm its purity. Purified bacterial cultures were maintained as 80% glycerol stocks and stored at −80 °C.

2.3. Isolation of fungi

Fungi were isolated from tarballs collected during July 2016 using the method described by Sanyal et al. (2016). Fungal colonies with unique culture morphology were selected for further characterization (Table 2) and their purified cultures were maintained at 4 °C.

2.4. DNA extraction and PCR amplification

DNA from both bacterial and fungal cultures was extracted using the Fungal and Bacterial DNA isolation kit (Zymo Research, USA) according to manufacturer's protocol. From fungal DNA, the ITS region was amplified by PCR using the primer-pair ITS1 and ITS4 (White et al., 1990), following the method detailed in Sanyal et al. (2016).

16S rRNA gene of bacteria was amplified by PCR using primer-pair 27F and 1492R (Piterina et al., 2010). The reaction for bacterial 16S rRNA gene amplification was performed in a 50 µl of volume, comprising 3 µl of template DNA (~50 ng/µl), 1 µl of each primer (20 pM) (Bioserve, India), 1 µl of dNTPs (10 mM) (Genaxy, India), 5 µl of Taq buffer A (10× with 15 mM $MgCl_2$) (GeNei, Bangalore), 1.5 µl of Taq polymerase (1 U/µl) (Chromous Biotech, India), 37.5 µl of nuclease-free water (HiMedia, India). The reaction was carried out at 95 °C for 5 min (initial denaturation), followed by 35 cycles at 95 °C for 1 min (denaturation), 50 °C for 1 min (primer annealing), 72 °C for 2 min (elongation), and a final extension at 72 °C for 10 min.

PCR products were purified using QIAquick PCR purification kit (QIAGEN) following manufacturer's protocol. After purification, PCR products were sequenced by ABI 3130 XL DNA sequencer, using primers mentioned above, at the Biological Oceanography Division, CSIR-NIO, Goa.

2.5. Phylogenetic analysis

The raw sequences obtained from the forward and reverse primers were checked for quality in DNA Dragon (SequentiX, Germany). Consensus sequences were prepared in the same software using both forward and reverse sequences, wherever possible. Two different DNA sequence datasets were analyzed, one for 16S rRNA gene sequence data-set (for bacteria) and the ITS sequence data-set (for fungi), in MEGA 7 (Kumar et al., 2016) using the newly-generated sequences and reference sequences retrieved from NCBI-GenBank.

3. Results

3.1. Phylogeny of tarball-associated bacteria

A list of bacterial cultures isolated in this study, along with information on their Gram nature and GenBank accession numbers for 16S rRNA gene sequences is presented in Table 1. The CFUs of bacteria were found to be 8.3×10^6 per gram of tarball for the samples collected during October 2014, while the samples collected during July 2016 yielded 5.07×10^5 CFUs per gram of tarball. Eight bacterial isolates were retrieved by the dilution plate method from the October 2014 tarball samples (Table 1, prefixed with “MOB-”). Twenty-five and sixteen bacterial isolates were retrieved from the July 2016 tarball samples by the dilution plate method and the enrichment culture technique, respectively. Numbers and alphabets are used to differentiate between the cultures isolated by serial dilution method and those isolated by enrichment culture technique, respectively (Table 1). Bacteria belonging to *Alcanivorax, Bhargavaea*, *Nitratireductor*, *Oceanimonas* and *Tistrella* were exclusively recovered from the enrichment culture technique (Fig. 1).

A Maximum Likelihood tree generated using the 16S rRNA gene sequence data-set in MEGA is shown in Fig. 1. *Aquifex pyrophilus* (M83548) was the designated outgroup. The system output details generated by MEGA for the bacterial tree construction are presented in Supplementary Document 1. In the bacterial phylogenetic tree shown in Fig. 1, 49 newly-generated sequences of tarball-associated bacteria from Betul beach clustered within four bacterial phyla [*Proteobacteria*: 38 (*Gammaproteobacteria*: 31, *Alphaproteobacteria*: 7), *Actinobacteria*: 6, *Firmicutes*: 4 and *Bacteroidetes*: 1].

In the *Gammaproteobacteria* clade (Fig. 1), 11 bacterial isolates clustered within *Pseudomonas* clade. B_BETUL_19 and B_BETUL_7 clustered with *Pseudomonas* sp. (MF948939, a pyrene degrader), coralline algae-associated bacterium (MG551789) and uncultured bacterium clone isolated from a polluted aquatic environment (KC208430). B_BETUL_4 exhibited proximity with the type of *P. sihuiensis* (NR 148251). MOB_V2 clustered with *Pseudomonas* sp. from the soil of Liangshui river of Beijing (KT380612). B_BETUL_G and B_BETUL_11 grouped with uncultured bacterium clone (JX047093), *P. stutzeri*