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Hydrocarbon-degradation and MOS-formation capabilities of the dominant bacteria enriched in sea surface oil slicks during the *Deepwater Horizon* oil spill

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ABSTRACT

A distinctive feature of the Deepwater Horizon (DwH) oil spill was the formation of significant quantities of marine oil snow (MOS), for which the mechanism(s) underlying its formation remain unresolved. Here, we show that *Alteromonas* strain TK-46(2), *Pseudoalteromonas* strain TK-105 and *Cycloclasticus* TK-8 – organisms that became enriched in sea surface oil slicks during the spill – contributed to the formation of MOS and/or dispersion of the oil. In roller-bottle incubations, *Alteromonas* cells and their produced EPS yielded MOS, whereas *Pseudoalteromonas* and *Cycloclasticus* did not. Interestingly, the *Cycloclasticus* strain was able to degrade *n*-alkanes concomitantly with aromatics within the complex oil mixture, which is atypical for members of this genus. Our findings, for the first time, provide direct evidence on the hydrocarbon-degrading capabilities for these bacteria enriched during the DwH spill, and that bacterial cells of certain species and their produced EPS played a direct role in MOS formation.

1. Introduction

The Deepwater Horizon (DwH) blowout of April 20, 2010 is recorded as the worst oil spill in US history. Estimates of the overall magnitude of the release vary, with recent figures reporting approximately 3.19 million barrels (134 million gallons) of oil (U.S. v. BP et al., 2015), and at least 250,000 metric tonnes of natural gas, largely methane, released into the Gulf of Mexico over a period of 87 days (Valentine et al., 2010; Joye et al., 2011). Based on its magnitude, difficulty and complexity of the clean-up response, the spill was marked as one of the worst in the history of the oil and gas industry (Lubchenco et al., 2012). Two distinctive features set the DwH spill apart from other oil spills at sea. The first was the formation of a hydrocarbon-enriched plume (Du and Kessler, 2012; Ryerson et al., 2012) that became entrained as a lens at a depth of 1000–1300 m depth within the water column (Camilli et al., 2010; Diercks et al., 2010). Whilst this deepwater plume had, from the outset of the spill, attracted intense interest from the scientific community in tracking its movement, analysing its physicochemical properties and evolving microbial community, the formation of unprecedented quantities of marine oil snow (MOS) – the other distinctive feature of the DwH spill – gradually gained the interest of the scientific community, with the first reports to emerge on MOS by 2012 (Passow et al., 2012; Ziervogel et al., 2012). MOS is defined as mucilaginous floating organic matter with a “fluffy” off-white appearance, and which distinctively contains associated oil droplets. MOS formation and its impact to the Gulf, and during other spills where it was observed to have formed (i.e. *Ixtoc-I* and *Tsesis* oil spills), has received considerable attention, with > 50 of studies that consider MOS formation following the DwH spill (Vonk et al., 2015; Daly et al., 2016; Passow, 2016).

The large quantities of MOS observed during the DwH spill were observed during the first research cruise on *R/V Pelican* to the spill site in early May 2010, and were frequently encountered around the vicinity of surface oil slicks (Niu et al., 2011; Passow et al., 2012) and

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within deep water oil plumes (Niu et al., 2011). By June 2010, a little over a month after the onset of the spill, MOS was no longer visible on surface waters in the Gulf of Mexico, as it had subsequently sedimented to the ocean floor (Hollander et al., 2012; Joye et al., 2014). MOS sedimentation has also been suggested to have originated from the deepwater plume (Valentine et al., 2014). Significant hydrocarbon deposition to the seafloor was observed within 20 km of the spill site (Brooks et al., 2015; Romero et al., 2015; Romero et al., 2017; Stout et al., 2017) and including to the northern Gulf of Mexico (White et al., 2012; Montagna et al., 2013; Valentine et al., 2014; Chanton et al., 2015) as a product of MOS formation (Brooks et al., 2015). Estimates for the amount of weathered oil residues that were transported to the seafloor is comparable between studies, from 1.8–14% (Valentine et al., 2014) to 0.5–9% (Chanton et al., 2015), though still uncertain. Now, just over seven years on, the full environmental impact of this MOS-mediated oil sedimentation remains unresolved.

Although conjecture still surrounds what triggered MOS formation during the DwH spill, the prevailing evidence indicates that it was directly associated with the massive influx of crude oil into the Gulf of Mexico. In roller-bottle experiments performed under conditions simulating the Gulf spill by adding weathered oil collected from the sea surface near the Macondo wellhead, Ziervogel et al. (2012) elegantly demonstrated the importance of the oil in MOS formation, and that these amorphous aggregations could act as hotspots of microbial oil-degrading activity that significantly influenced carbon flux in surface oil slicks at DwH. Passow et al. (2012) provided further insight on the complexity of biological interactions that contributed to the formation of MOS, and Bælum et al. (2012) described the formation of flocs (synonymous with MOS) in incubations with seawater and oil from DwH, and showed *Colwellia* was a dominant member of the flocs formed. These studies in the Gulf of Mexico and elsewhere have explored the genesis of MOS formation, revealing the involvement of bacteria, oil, dispersants, mucilaginous polymers (e.g. TEP, EPS) and possibly also eukaryotic phytoplankton (Gutierrez et al., 2013a; Arnosti et al., 2016; Duran Suja et al., 2017; Passow et al., 2012; Fu et al., 2014; Passow, 2016). Whether any one of these biological contributors plays a protagonist role in triggering MOS formation remains unsubstantiated. During the DwH spill, their ubiquity in the Gulf water column suggests they had at least contributed in concert in the formation of MOS.

Marine snow particles are held together by carbohydrate-based polymers, such as transparent extracellular particles (TEP) and/or extracellular polymeric substances (EPS), that can be produced in large quantities by phytoplankton and bacteria. Certain groups of bacteria in the ocean are recognised for producing significant quantities of EPS that contribute to the total dissolved organic matter (DOM) pool in the ocean (Azam, 1998). A large fraction of this bacterial-derived EPS consists of glycoprotein (Long and Azam, 1996; Verdugo et al., 2004), which coincidentally reflects the chemical composition found in MOS that formed in surface and plume waters at DwH (Bælum et al., 2012; Passow et al., 2012; Ziervogel et al., 2012). It can be hypothesized that indigenous groups of EPS-producing bacteria that became enriched during the DwH spill had contributed, via an as yet unknown mechanism(s), to the formation of significant quantities of MOS observed during the spill. Since no methods currently exist that can match any type of EPS in an environmental sample to its biological source, one method to ascertain whether EPS produced by a bacterial group enriched during the DwH spill may had contributed to the formation of MOS is to study them in pure culture. Using roller-bottle incubations under conditions simulating oil-contaminated sea surface water, we previously showed that EPS produced by *Halomonas* sp. TGOS-10 – a hydrocarbon-degrading bacterium that had become enriched in sea surface oil slicks during the DwH spill – could trigger the formation of MOS in the presence of crude oil (Gutierrez et al., 2013a). Other studies also described MOS formation in similar laboratory-based incubations with seawater (Bælum et al., 2012; Passow et al., 2012; Ziervogel et al., 2012), but the role of EPS in this respect was not investigated. In this study, we test the hypothesis that other hydrocarbon-degrading bacteria that became enriched in surface oil slicks during the spill were involved in inducing the formation of MOS, as well as participating in the emulsification and degradation of the Macondo oil. MOS formation was evaluated in oil-amended roller bottle incubations in the laboratory over a period of 14 days using constant gentle turbulence to simulate conditions near the sea surface. We also determined the range of hydrocarbons in Macondo oil that these strains are capable of degrading, and we isolated the EPS produced by one of the strains in order to analyse its chemical characteristics and infer on its role in MOS formation and emulsification of the oil.

2. Materials and methods

2.1. Microorganisms used in this study

Alteromonas sp. strain TK-46(2) was originally isolated from a sea surface oil slick sample collected during a research cruise on RV *Pelican* on May 5th of 2010, ca. 0.86 miles from the site of the DwH blowout (28° 44.175′ N, 88° 22.335′ W). *Pseudoalteromonas* sp. strain TK-105 had been isolated from a deepwater plume sample collected at 1170 m depth (28° 41.686′ N, 88° 26.081′ W) during a subsequent cruise on the RV *Walton Smith* on 31st May 2010. The strains were selected for use in this study based on their ability to produce EPS, to degrade hydrocarbons, and because they were found enriched (based on 16S rRNA gene sequence identity) in sea surface oil slick samples collected during the DwH oil spill (Gutierrez et al., 2013a, 2013b); the TK-105 strain *Cycloclasticus* sp. strain TK-8 was isolated from the sea surface oil slick and included in this study because, whilst it too was found heavily enriched in sea surface oil slicks and the deepwater plume (Gutierrez et al., 2013b), this strain does not produce EPS and therefore served as a useful reference organism to compare with the EPS-producing strains TK-46(2) and TK-105. In addition, it had not previously been determined, by empirical investigation, the range of hydrocarbons in the Macondo crude oil that each of these strains is able to degrade, so was assessed here.

2.2. Hydrocarbon analysis

To determine the hydrocarbon species in Macondo crude oil that strains TK-8, TK-46(2) and TK-105 can degrade, a synthetic seawater medium, ONR7a (Dyksterhouse et al., 1995) was used and amended with surrogate Macondo crude oil (from the Marlin platform) as the sole carbon and energy source. For this, 250-ml pre-autoclaved glass Schott bottles were prepared containing 45 ml ONR7a, Macondo oil to ca. 100 mg/l final concentration, and inoculated with 5 ml of washed cells. For preparation of inocula, the strains were grown in ONR7a amended with Na-pyruvate (for TK-46(2) and TK-105) or phenanthrene (for TK-8); the cell biomass was washed three times, resuspended in sterile ONR7a to 5 ml and used for inoculation. Additional incubations were set up in the same way with the exception that 85% phosphoric acid (3% final concentration) was added, or the bottles were not inoculated; these controls served to analyse for any loss of hydrocarbons due to abiotic factors. All incubations were carried out in triplicate and incubated in parallel in the dark with gentle shaking (100 rpm) and at 21 °C, which is a temperature similar to that at the sea surface in the Gulf of Mexico during the time of the DwH oil spill. At the termination of the experiment (day 20), all the bottles were extracted for total petroleum hydrocarbons (TPH) and subsequent analysis for individual hydrocarbon constituents by gas chromatography/mass spectrometry (GC–MS), as detailed below.

For extraction of TPH, dichloromethane (DCM) was used at an oil/water mix to DCM ratio of 2:1. The DCM fraction was removed and the oil/water mix re-extracted an additional 3 times. The extracted oil sample was then diluted with DCM to ca. 5 ml and dried using anhydrous sodium sulphate. An aliquot of known volume was removed,