



Ultrastructural Insights into Microbial Life at the Hydrocarbon: Aqueous Environment Interface 27

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Contents

1	Introduction	410
2	Fossil Fuel Production and Spills	410
3	Oil Well Souring	411
4	Sulfate Reduction Using <i>Desulfovibrio vulgaris</i>	411
5	Biofuel Production	412
6	The Role of Imaging in Microbe-Hydrocarbon Research	413
7	Research Needs	415
8	Future Studies	416
	References	416

Abstract

Despite the harmful effects observed when bacteria grow in a hydrocarbon-rich environment, some have been able to overcome the potential toxicity; however, specific interactions that operate at the hydrocarbon/aqueous interface remain unknown due to the difficulty of studying these interactions. Fortunately, there have been vast improvements in sample preparation such as the introduction of high-pressure freezing/freeze substitution (HPF/FS) which are able to preserve the ultrastructure while imaging. This process has been a gateway to a greater understanding of the ultrastructure of these interactions which could present deeper insight into the many processes that involve hydrocarbons. These processes include events such as catastrophic oil spills that give the opportunity to study the hydrocarbon/aqueous interface for the potential of utilizing new mechanisms in future disasters. This follows the possibility of reducing industrial oil souring by studying

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sulfate-producing bacterium, as well as furthering our understanding in biofuel production, where engineered microbes are used to produce hydrocarbon fuels.

1 Introduction

Microbial life at the hydrocarbon/aqueous environment interface is one of the most fascinating yet ill-understood phenomena in microbiology, in part, due to the challenges faced by microbes when interacting with a hydrocarbon surface. Furthermore understanding of the hydrocarbon/aqueous environment interface is of high economic significance, be it in the context of oil spills, oil well souring, or the production of biofuels.

2 Fossil Fuel Production and Spills

As a result of fossil fuel drilling, hydrocarbons can be released incrementally from natural leaks in the ocean floor or by catastrophes and can cause havoc on microbial communities in freshwater and oceanic bodies of water which are known to influence the biogeochemical cycles and food webs of the entire planet (Zehr 2010).

The Deepwater Horizon oil spill was the second largest accidental marine oil spill in the history of the oil industry and is an example of how hydrocarbons can devastate bacterial communities. This spill resulted in a huge influx of hydrocarbons (around 780,000 cubic meters or 210 million US gallons) from the Macondo well (MC252) over 3 months of being released into the Gulf of Mexico (Baelum et al. 2012). This tragic accident provided an opportunity to enrich and isolate indigenous hydrocarbon-degrading bacteria and to identify the hydrocarbon concentrations and microbial community composition (Baelum et al. 2012; Hazen et al. 2010). There was also the opportunity to study the effect of the oil dispersant COREXIT and the iron source FeCl_2 on floc formation and the microbial degradation of oil (Macondo MC252), *since these reagents are often used in oil spills, yet their effects on indigenous microbes in the environment and their potential influence oil degradation rates had been previously unknown* (Baelum et al. 2012).

When isolated and enriched with Macondo MC252, the indigenous microbial communities demonstrated approximately 60% to 25% degradation of the oil with or without COREXIT, with no negative effects of high amounts of COREXIT on the growth of indigenous microorganisms from the isolation site. Additionally, FeCl_2 was shown to increase respiration rates, but not the total amount of hydrocarbons degraded (Baelum et al. 2012). Enrichment with MC252 and/or COREXIT in the absence of FeCl_2 also leads to floc formation, with sequences representative of *Colwellia* becoming dominant. This species specificity was confirmed to rapidly degrade high amounts of MC252, demonstrating the evolution of flocs during exposure to high concentrations of MC252 (Baelum et al. 2012).

The ecological significance of high hydrocarbon amounts on microbial composition was further shown by studies done with the oil on the shore of Grand Isle,

Louisiana, that caused a shift in the community structure toward hydrocarbonoclastic consortia. Gene sequencing demonstrated a diverse array of known petroleum hydrocarbon-degrading microorganisms, including *Marinobacter* strains (Lamendella et al. 2014).

Petroleum hydrocarbon degradation/catabolism is an energetically favorable process that occurs under both aerobic and anaerobic conditions utilizing pathways that involve terminal oxidation (subterminal oxidation, ω -oxidation, and β -oxidation), reduction, hydroxylation, and dehydrogenation reactions (Varjani 2017; Abbasian et al. 2015). In aerobic conditions, hydrophobic hydrocarbon methyl groups undergo oxidation to form an alcohol, which is then dehydrogenated via an aldehyde into a corresponding carboxylic acid, which can then be metabolized by the β -oxidation pathway of fatty acids (Varjani 2017; Das and Chandran 2011). Corresponding oxygenases and peroxidases to this degradation mechanism have also been identified for the purpose of microbial degradation of hydrocarbon pollutants; examples include *alkB* gene for alkane monooxygenase, *xylE* gene for catechol dioxygenase, and *nahAc* gene for naphthalene dioxygenase (Varjani 2017). We have made progress in elucidating a number of variables that affect biodegradation of hydrocarbons, including oxygen availability, optimal temperature range, and adequate pH (Ratcliffe 2017). However for microbe-, host-, environment-, and location-specific mechanisms of catabolism, the details of the hydrocarbon-microbe interaction remain largely unknown.

3 Oil Well Souring

Apart from oil spills, there is also economic value in preventing the souring of oil wells. Sulfate-reducing bacteria can metabolize hydrocarbons present in the aqueous environment of an oil well, using sulfate as a terminal electron acceptor, thus reducing sulfate to hydrogen sulfide gas, with a foul odor and corrosive properties. Oil is considered “sour” if it contains more than 0.5% sulfur and accelerates corrosion of the metal infrastructure used for oil production and processing (Leffler 2008). Similarly, sulfur would damage engine components if it were not removed when oil is refined in fuels. High-sulfur oil is more costly to refine and as a result is less valuable than low-sulfur oil (Leffler 2008). One mechanism for the souring of oil is sulfate-reducing bacteria present in the seawater, which oxidizes hydrocarbons in the aqueous interface and produces sulfides in the process. The *Desulfobulbaceae* family and its notable member *Desulfovibrio vulgaris* (*D. vulgaris*) are known to contribute to oil well souring (Muyzer and Stams 2008).

4 Sulfate Reduction Using *Desulfovibrio vulgaris*

Sulfate reducers like *D. vulgaris* are able to survive in a variety of toxic conditions, including hydrocarbon environments by adjusting their metabolism to the point that they can use sulfate as the terminal electron acceptor to generate sulfide, as long as they have hydrogen, formate, lactate, ethanol, or other organic compounds, such as

crude oil, as a carbon source (Liamleam and Annachhatre 2007). The US Department of Energy has sponsored detailed research of *D. vulgaris* as a sulfate reducer; as a result, it is a well-studied model system for sulfate reducers. Genome sequence analysis revealed a complex, periplasmic cytochrome network, along with details about its transmembrane electron transport and cytoplasmic sulfate reduction capabilities (Heidelberg et al. 2004).

This research identified cytoplasmic isozymes as a critical component in hydrogen cycling and an unexpectedly high number of formate dehydrogenases. The latter suggests a system of chemiosmotic energy conservation by the diffusion of an uncharged metabolic intermediate, i.e., formate, from the cytosol with subsequent periplasmic oxidation (Heidelberg et al. 2004).

5 Biofuel Production

Microbes have a significant role to play in the production of biogenic transportation fuels (Lee et al. 2008). Microbial metabolic pathways are reengineered to produce hydrophobic precursors to biofuels or final products, which when produced in high quantity are either stored inside the cells (due to the hydrophobic effect) or continuously secreted. Hydrocarbons are relatively simple organic substances that, while being comprised of only carbon and hydrogen, constitute a large variety of compounds, each with their own unique chemical properties. For example, hydrocarbons with aliphatic structure will behave differently to aromatic compounds. Thus biodegradability of hydrocarbons can be ranked from highest to lowest as follows: linear alkanes > branched alkanes > low-molecular-weight alkyl aromatics > monoaromatics > cyclic alkanes > polyaromatics \gg asphaltenes (Atlas 1981; Leahy and Colwell 1990). However, in most cases, hydrocarbons negatively interfere with membrane function: The Douglas fir terpene α -pinene and toluene led to massive leakage of the cytoplasm of *S. cerevisiae* (Andrews et al. 1980) and *E. coli* (de Smet et al. 1978). *Candida* grown in increasing concentration of n-alkanes reduces glucose utilization (Gill and Ratledge 1972). Phospholipid bilayer integrity is compromised, resulting in the breakdown of membrane ion gradients (Sikkema et al. 1994) and thus cell death.

In our own biofuel-related research, we studied *E. coli* and yeast producing various titers of hydrocarbons, and at higher titer, we find microbes to be ultrastructurally highly disturbed (see Fig. 1), with microbes trying to compensate for the toxicity by compartmentalization. While it seems clear that hydrocarbons have a devastating effect on microbes, the exact reason for its toxicity is poorly understood. At the core of the mystery lies how microbes deal with the challenge put forward by hydrophobic, lipophilic surfaces on the microbial cellular membranes and macromolecular bacterial organization. It would seem from a chemical point of view that hydrocarbons could act like an organic solvent, thus posing a real threat to the integrity of lipid bilayer-based membranes and with it to the ion gradients across such membranes needed to allow proper physiological function. It seems almost ironic that microbial cells may literally sit on an infinite carbon source, yet struggling to keep alive and metabolically productive. And while many bacteria are not up for

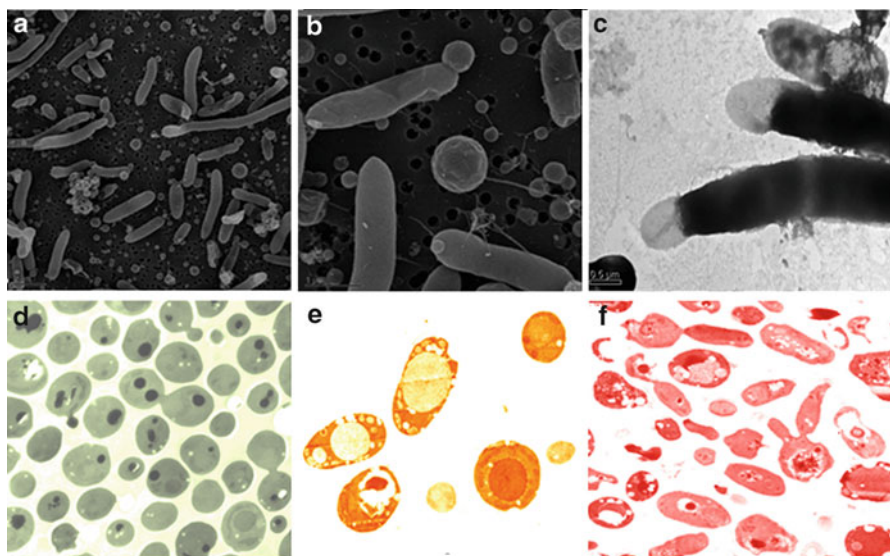


Fig. 1 Ultrastructural analysis of *E. coli* (top row) and *S. cerevisiae* (bottom row) producing biofuels. Low magnification (a) and higher magnification (b) scanning electron micrographs of *E. coli* producing biofuels, resulting in the formation of globular structures pinching off from the bacterial membrane surface. Whole-mount TEM imaging of *E. coli* (c) confirms that compartmentalization of biofuel into droplets, marked by asterisks; (d–e) *S. cerevisiae* producing increased titers of biofuels. Note that under low titer conditions (d), cells show a healthy uniform shape (note normal-looking uniform dark, electron dense vacuoles), whereas medium (e) and high (f) titers resulting in cells with increasingly distorted ultrastructural morphology, including distressed overall shape as well as irregular internal features, including partially solvent-extracted vacuoles, a likely storage place in plant cells

the task, there are some that not only will be able to cope with such hostile conditions but also thrive in such hydrophobic hydrocarbon-rich environments. Interestingly, gram-negative bacteria exhibited higher tolerance against lipophilic compound when compared to gram-positive bacteria (Harrop et al. 1989). The higher tolerance of gram-negative bacteria presumably is due to the presence of lipopolysaccharides in its outer membrane, which may constitute a protective layer. Another strategy to increase tolerance against hydrocarbons is the expression of bacterial membrane protein efflux pumps, which can result in rapid removal of toxic hydrocarbon from the inside of microbes. Engineering *E. coli* to express an efflux pump from *A. borkumensis* allowed increased limonene production yield (Dunlop et al. 2011).

6 The Role of Imaging in Microbe-Hydrocarbon Research

The mysteries of how exactly microbes have adapted to the challenges of hydrophobicity are complicated by the fact that bacteria rarely exist as individual, independent cells but mostly are found in biofilms. When forming biofilms, a large

number of microbes display completely different expression profiles, compared to their planktonic state (Sauer et al. 2002). Therefore it would be expected that hydrocarbon-challenged microbes will form complex microbial communities that are difficult to study.

Imaging may provide unique insight in the hydrocarbon/aqueous environment interface. For instance, synchrotron radiation-based Fourier transform infrared (SR-FTIR) spectromicroscopy studied bacterial floc groups, including their low-resolution structure and chemical composition. Bacterial floc groups are formed by flocculation or the aggregation of oil, biomass, and carbohydrates (Baelum et al. 2012). Strongest peaks indicate the intensity of radiation measured by spectromicroscopy and are presented as absorbance units (a.u.). With higher concentrations of a particular element of a particular chemical bond, the intensity of the emitted IR radiation is higher. This is a measurement of spatial distribution MC252 oil and proteins as compared to measurements taken elsewhere in the sample. Thus the relative location of the non-degraded oil, the oil degradation products, and the bacteria can be determined from such FTIR spectra, revealing that the pollutant oil initially accumulates in the floc groups and then is degraded over time.

When it comes to trying to understand the hydrocarbon/aqueous environment interface, we need to consider a high-resolution imaging mode like electron microscopy. Whole-mount negative stain and cryo-imaging of microbes (2D or 3D via electron tomography) are options but face the challenge that the entire sample thickness (microbe and environment) needs to be thinner than $\sim 0.5\text{--}1\ \mu\text{m}$, which can be difficult to accomplish for individual bacteria and is clearly not achievable for biofilms without sectioning (McDonald and Auer 2006). While cryo-sectioning of frozen-hydrated samples followed by cryo-EM 2D projection or 3D electron tomography is a possibility, this approach suffers from a low success rate and low throughput, with less than ten labs in the world having the necessary background to do it. However, such a sample preparation approach (cryo-sectioning) may be viewed as the gold standard for obtaining unstained, vitrified samples that upon TEM imaging do not suffer from sample preparation artifacts. Fortunately, there is an alternative approach that is technically much less challenging and yields excellent results in terms of contrast and preservation: high-pressure freezing/freeze substitution (HPF/FS), which cryogenically freezes and thus physically immobilizes the biological system within milliseconds at liquid nitrogen temperature, with the high pressure discouraging ice crystal formation (McDonald and Auer 2006; Matias et al. 2003). Not only does this physical fixation allow us to capture the cellular scenery without disturbing the ultrastructure, but it also allows for the cells be viable once thawed (Hunter and Beveridge 2005). The process of high-pressure freezing/freeze substitution is initiated by the immersion of the specimens on Isopore membrane filters in 10% glycerol or 20% bovine serum albumin in CYE medium (Palsdottir et al. 2009). After immersion, the filters are sandwiched between two aluminum planchettes (Palsdottir et al. 2009). Next specimens are cryofixed in a high-pressure freezer such as the Lecia EM PACT2. The Lecia EM PACT2 is the most advanced high-pressure freezer which includes a rapid transfer system (RTS) that allows one to do correlative light and electron microscopy with high time resolution (McDonald

et al. 2007). Once cyrofixed specimens are in anhydrous acetone containing 1% osmium tetroxide and 0.1% uranyl acetate and infiltrated with Epon-Araldite, they can be used in a freeze substitution system (McDonald et al. 2007). During the freeze substitution process, samples are kept at low (dry ice) temperature (-90°C), where the cellular water is gradually replaced by an organic solvent by letting the sample sit for 48 h at this temperature (Jhamb and Auer 2015). After the allotted time, the samples should slowly be warmed up until they reach -30°C , and then they should maintain this temperature for 3 h before they are warmed to 0°C (Jhamb and Auer 2015). During the warm-up period, the samples can undergo heavy metal staining. Such samples can then be resin embedded and ultrathin sectioned, before being examined by room temperature TEM. HPF/FS in combination with electron tomography has been successfully applied to a variety of biofilms, including the well-studied soil bacterium, *Myxococcus xanthus* (Palsdottir et al. 2009), and *Desulfovibrio vulgaris* (manuscript in preparation) with unprecedented insight into microbial community organization and microbial interaction with its extracellular environment. It is expected that with a concerted funding effort, the secrets of microbial interaction with its hydrocarbon environment could be revealed, including the presence of lipopolysaccharides (LPS) on the outer membrane surface or even layers of exopolysaccharides (EPS) that may protect the microbes from external hydrocarbon or the presence of specialized compartments or other strategies that protect from hydrocarbon toxicity from the inside, e.g., in the context of biofuel production.

For transmission electron microscopy, sample preparation is critical to obtain reproducible, reliable results. Despite new techniques, the greatest source of error in thin sections for TEM still lies in sample preparation (Cheville and Stasko 2014). Sample preparation treats specimens of interest in a series of reactions, beginning with primary fixation, postfixation, dehydration, resin infiltration, and embedding.

7 Research Needs

Fixation is done with 2% glutaraldehyde at pH 7.2 (McDonald and Zalpuri 1997), postfixation with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer (Cheville and Stasko 2014). Postfixation is followed by rinses with buffer (McDonald and Zalpuri 1997). Dehydration with ethanol series (35% increased to 100%) is critical for removing remaining water in the sample in order for embedding media to effectively infiltrate the specimen during the subsequent treatment (Cheville and Stasko 2014). Resin infiltration (with accelerator BDMA) for samples is done stepwise by increasing the resin-to-ethanol ratio. Samples are then put in three pure resin exchanges (McDonald and Zalpuri 1997). Samples are embedded in epoxy resin. Epoxide resin is intolerant of water and will not polymerize if the sample is not thoroughly dehydrated, resulting in rubbery blocks that cannot be cut into ultrathin sections (Cheville and Stasko 2014). Samples are embedded into molds and put into the oven at 60°C for 2 days (McDonald and Zalpuri 1997). Sample preparation is followed by ultrathin sectioning of the polymerized blocks in order to

allow electrons from the microscope beam to pass through thin cross sections of the sample. Ultramicrotomes are used to cut section into 100 nm or less, optimally 70 nm thick. These sections are then collected on coated grids, followed by positive staining to increase contrast of the images (Cheville and Stasko 2014).

8 Future Studies

Research on studying the microbe-hydrocarbon environment would benefit from the subcellular localization of candidate proteins that are suspected to be involved. Such proteins are ideally tagged using green fluorescent protein (GFP and related)-tagged fusion proteins, which work well under aerobic conditions, or SNAP-tagged fusion proteins that also work under anaerobic conditions (Chhabra et al. 2011). In any case, such signals are used as the starting point for photoconversion, which allows subcellular protein localization at higher (TEM) resolution. While this approach works well in some circumstances, in others – e.g., where internal metal deposits can interfere with a clean interpretation of the TEM imaging data (manuscript in preparation) – more work is needed to solve this challenging problem.

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