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Assimilation of hydrocarbons and lipids by means of biofilm formation

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ABSTRACT: Hydrophobic organic compounds (HOCs) that are used as substrates by bacteria, encompass a great variety of molecules, including contaminants such as hydrocarbons and natural components of the organic matter such as lipids. It is now well known that many bacterial strains use HOCs as carbon and energy sources for growth and form biofilms at the HOCs-water interface that are referred to as oleolytic biofilms. The formation of these biofilms appears to be a strategy to overcome the low accessibility of nearly water-insoluble substrates and is therefore a critical process in the biodegradation of hydrocarbons and lipids. Because oleolytic biofilms develop on a nutritive interface serving as both substratum and substrate, they represent an original facet of biofilm biology.

Introduction

Early studies on hydrocarbon biodegradation led to the observation that hydrocarbon-degrading bacteria had high affinity for oil droplets. Phase contrast and electron microscopy examination of *Acinetobacter sp.* growing on *n*-hexadecane revealed hydrocarbon spheres densely covered with bacterial cells and suggested close contact between the cells and oil droplets (Kennedy *et al.*, 1975). Since then, similar observations have been reiterated with various alkane-degrading strains, such as *Rhodococcus sp.* Q15 (Whyte *et al.*, 1999), *Acinetobacter venetianus* RAG-1 (Baldi *et al.*, 1999), *Oleiphilus messinensis* (Golyshin *et al.*, 2002), *Pseudomonas* UP-2 (Zilber Kirschner *et al.*, 1980) and *Marinobacter hydrocarbonoclasticus* SP17 (figure 1) (Klein *et al.*, 2008). Bacterial attachment to polycyclic aromatic hydrocarbons (PAHs) has also been described for *Pseudomonas* spp. (Eriksson *et*

28 *al.*, 2002; Mulder *et al.*, 1998), *Sphingomonas sp.* CHY-1 (Willison, 2004) and
29 *Mycobacterium frederiksbergense* LB501T (Bastiaens *et al.*, 2000). In biphasic culture
30 medium containing a poorly water-soluble substrate and the aqueous phase, substrate-bound
31 cells often coexist with cells floating freely in the aqueous phase. Although the presence of
32 hydrocarbon-bound cells at the interface assumes interfacial growth, demonstration of actual
33 substrate degradation and growth of the attached cells has been provided in only a few cases
34 (Efroymson and Alexander, 1991; Wick *et al.*, 2003; Zilber Kirschner *et al.*, 1980).

35 The sessile mode of life and the multilayered structure of cell populations growing at the
36 interface between hydrophobic organic compounds (HOCs) and water are reminiscent of
37 biofilms. During the last two decades, biofilms have been the subject of extensive
38 investigations. Most of our knowledge about the molecular biology of biofilms has been
39 derived from model strains such as *Pseudomonas aeruginosa* and *Escherichia coli*. This
40 research has revealed that biofilms are much more than the simple accretion of cells attached
41 to an interface. Biofilms are heterogeneous, highly organized structures possessing an
42 architecture that is essential to their functioning. Biofilm growth follows a stepwise pattern
43 involving differentiation and collective behavior of cells (Stewart and Franklin, 2008; Webb
44 *et al.*, 2003). Molecular studies of oleolytic biofilms growing at hydrophobic interfaces have
45 not yet gone far enough to conclude whether they share all the characteristics of extensively
46 studied model biofilms. However, properties characteristic of the biofilm lifestyle have been
47 identified. CSLM (Confocal Scanning Laser Microscopy) observation of a biofilm community
48 developing at polychlorinated biphenyl-water interface provided evidence of a stepwise
49 development pattern of the biofilm (Macedo *et al.*, 2005). Transcriptomic and proteomic
50 studies indicated that cells growing at the alkane-water interface experienced a profound
51 reshaping of their gene expression profile (Mounier *et al.*, 2014; Vaysse *et al.*, 2009; Vaysse
52 *et al.*, 2010). Extracellular polymeric substances (EPS), polysaccharides, DNA and proteins

have been detected during growth at the alkane-water interface, indicating production of an extracellular matrix, which is a typical trait of biofilms (Ennouri *et al.*, 2017; Whyte *et al.*, 1999).

In this chapter, biofilms on HOCs refer to multilayered, matrix-embedded bacteria or bacterial communities growing at the HOCs–water interface and using these compounds as a substrate. In such biofilms, the energy and the carbon, which fuel bacterial growth, are provided by the degradation of the substrate, which thus constitutes a nutritive interface serving as both substrate and substratum.

Multispecies biofilms on hydrophobic interface

During enrichment procedures on hydrocarbons, microbiologists have very often observed multispecies biofilms developing at the hydrocarbon–water interface. For example, Deppe *et al.* (2005) have observed by phase contrast microscopy oil droplets covered by a biofilm during isolation of a consortium enriched on crude oil from Arctic sea ice and seawater from Spitzbergen. Unfortunately, such observations received little attention and in consequence they are not always mentioned in the literature and are rarely fully documented, making it difficult to measure the occurrence of these biofilms among bacterial species. Stach *et al.* (2002) carried out a study devoted to the diversity of biofilm communities developing on PAHs. Biofilms developing on naphthalene- and phenanthrene-coated flow cells were isolated and their diversity compared with planktonic cultures enriched on the same hydrocarbons. The biofilm system showed a three times higher diversity of cultivable bacteria as compared to the enrichment culture. Molecular approaches revealed that the biofilm community contained a greater diversity of active species and of PAH-degradation genes than the planktonic enrichment community. The diversity of active species found in the biofilm closely matched the diversity found in the PAH-contaminated soil used as inoculum. This study

demonstrates that biofilm cultures represent a means to obtain PAH-degrading communities closely related to environmental situations suggesting that biofilm formation on hydrocarbons is a likely lifestyle in natural ecosystems. The existence of biofilms at oil-water interfaces in natural environments has been reported during the Deepwater Horizon oil spill where bacterial flocs were observed in the oil plume. Synchrotron radiation-based Fourier-transform infrared-spectra of these flocs indicated that their formation occurred on the surface of oil droplets and revealed the presence of oil-degradation products, polysaccharides and proteins (Baelum *et al.*, 2012; Hazen *et al.*, 2010).

Cell adhesion to hydrophobic compounds

In order to either develop a biofilm or to grow as single cell layers at the interface, bacteria must first approach and then adhere to the interface. Bacteria can reach surfaces by passive diffusion, random swimming, or taxis that is a directed motility in response to gradients of chemical and physical stimuli. Chemotaxis has been observed in response to single ring aromatic hydrocarbons, naphthalene, and hexadecane. Regrettably, no experiment designed to determine whether this behavior led to biofilm formation at the interface between water and hydrocarbons has been conducted (Lanfranconi *et al.*, 2003; Pandey and Jain, 2002). To date, random mobility like swimming has never been shown to play a role in adhesion to HOCs. Once cells have reached the interface, the initial adhesion step is a purely physicochemical process described by the traditional and extended DLVO theories of colloidal stability, which describes contact of cells to surfaces as the result of van der Waals interactions, Lewis acid–base interactions, and electrostatic interactions (Hermansson, 1999). The intensity of these interactions and hence the effectiveness of the binding depend on the cell surface properties (hydrophobicity, charge, roughness, etc...) as well as interface properties. This means that only bacteria exhibiting the proper surface properties will adhere on hydrophobic surfaces (for

a detailed review of bacterial adhesion to hydrocarbon, see Abbasnezhad *et al.*, 2011). In contrast to adhesion to biotic surfaces or insoluble polysaccharides like chitin or cellulose, no specific adhesins or receptors recognizing hydrocarbons or lipids have been identified so far. In many cases, adhered cells exhibit surface properties that are different from their soluble substrate-grown counterparts. These alterations of the cell surface are thought to strengthen adhesion after the initial interaction with the interface. For example, anthracene-grown cells of *Mycobacterium sp.* LB501T are more hydrophobic and more negatively charged than glucose-grown cells (Wick *et al.*, 2002). Changes in cell surface can be achieved by modification, production, or removal of their surface molecules. In Gram-positive bacteria, the presence and the chain length of mycolic acids are correlated with hydrophobicity and adherence (Bendinger *et al.*, 1993). Lipopolysaccharides are important determinants of cell surface properties in Gram-negative bacteria. Their chain length variation or their removal from the cell surface has been shown to be important for interacting with hydrocarbons (Al-Tahhan *et al.*, 2000). Capsular polysaccharides are another class of surface molecules playing a role in adhesion to hydrophobic compounds (Baldi *et al.*, 1999).

Adhesion to hydrophobic surfaces is also mediated by extracellular appendages such as fimbriae and pili. The importance of fimbriae in adherence to *n*-hexadecane was demonstrated by the isolation of a non-adherent mutant of *Acinetobacter calcoaceticus* RAG-1, which was devoid of fimbriae and defective for growth on hydrocarbons. The reappearance of fimbriae in adherent revertants was a strong argument in favor of the involvement of fimbriae in adhesion to hexadecane (Rosenberg *et al.*, 1982). The strains *Acinetobacter haemolyticus* AR-46 and *Acinetobacter sp.* Tol 5 produce pili at their cell surfaces when grown on *n*-hexadecane or triglycerides, respectively. Although the function of these organelles has not been elucidated it was presumed that they play a role during the adhesion to the hydrophobic substrate (Bihari *et al.*, 2007; Katsutoshi *et al.*, 2011). Changes in surface properties of cells

grown on hydrophobic substrates and inhibition of adhesion in the presence of soluble substrates indicate that the process of adhesion is regulated and that cells are able to respond to contact with hydrophobic interfaces.

Regulation and determinism of biofilm formation at the HOC-water interfaces

In biofilms growing on an inert substratum, all nutrients are supplied through the aqueous phase. In biofilms on HOCs, the situation is very different. These biofilms develop in a biphasic medium where the electron donor is provided by the non-aqueous phase and the electron acceptor (*e.g.*, oxygen) is available from the aqueous phase. It results in a geometry in which one side of the biofilm is in close association with the electron source, while the other contacts the source of the electron acceptor. Thus, cells within the biofilm experience two opposite gradients of acceptor and donor of electron generated by their simultaneous diffusion and consumption. These microscale chemical gradients presumably contribute to the physiological heterogeneity in the biofilm and exert a control on its development. The experiment carried out by Joannis-Cassan *et al.* (2005) demonstrated that biofilm growth on hydrocarbons can be limited either by carbon or by oxygen depletion. The authors studied biofilm growth in a liquid–liquid system consisting of an emulsion obtained by stirring dodecane in mineral medium. Biofilm growth occurred at the surface of a dodecane droplet. During growth, the droplet diameter was reduced from 200 μm to 160 μm . Biofilm growth ceased when it reached a maximum thickness of about 80 μm . A series of experiments demonstrated that inhibition of growth was caused by the diffusion limitation of both dodecane and oxygen within the biofilm but not by others factors such as nutrient exhaustion or product inhibition (Joannis-Cassan *et al.*, 2005).

Although adhesion to hydrocarbons does not seem to depend on the recognition of molecular structures, many HOC-degrading bacteria show a preference or specificity to the surface of

153 metabolizable hydrocarbons or lipids for biofilm formation (Johnsen and Karlson, 2004;
154 Klein *et al.*, 2008; Rodrigues *et al.*, 2005). The substrate/substratum specificity of biofilm on
155 hydrocarbons is certainly one remarkable feature that distinguishes them from other biofilms.
156 Biofilm formation tends to occur preferentially on less-soluble substrates and seems to be
157 regulated in function of substrate availability. Screening for biofilm formation capacity by
158 isolated PAHs-degrading strains showed that the majority of the tested strains formed biofilm
159 in microtiter wells coated with PAH crystals. For strains capable of growing on different
160 PAHs, it was observed that the percentage of adhering cells decreased with the solubility of
161 the PAHs, indicating that aqueous solubility of the substrate exerts a regulation on biofilm
162 development (Johnsen and Karlson, 2004). *Pseudomonas putida* ATCC 17514 exhibits
163 different growth patterns depending on the PAH properties on which it is feeding. CSLM
164 observation of a gfp-labeled derivative of this strain showed that growth on phenanthrene
165 occurred by forming a biofilm at the crystal surface, while on fluorene, which is more soluble
166 than phenanthrene, *P. putida* grew randomly between the crystals feeding on dissolved PAH
167 (Rodrigues *et al.*, 2005). Insoluble substrate preference for biofilm formation has also been
168 observed in *M. hydrocarbonoclasticus* SP17. This bacterium forms biofilms on a variety of
169 HOCs, including *n*-alkanes, wax esters, and triglycerides, but is unable to form biofilm (in
170 presence of acetate as substrate) on non-metabolizable alkanes (branched alkanes and *n*-
171 alkanes with more than 28 carbon atoms) and forms only weak biofilms on polystyrene with
172 10 times less biomass than on paraffin (Ennouri *et al.*, 2017; Klein *et al.*, 2008). The
173 preference for insoluble substrates suggests that bacteria forming biofilms on HOCs are able
174 to detect and recognize nutritive interfaces. It is reasonable to anticipate that control of
175 biofilm formation by substrate/substratum is exerted through a signal transduction pathway
176 and genetic regulatory mechanisms. Indeed, induction of genes at an interface was
177 demonstrated for the *pra* gene encoding the PA protein, an alkane inducible extracellular

protein exhibiting an emulsifying activity involved in hexadecane assimilation, and the *rhlR* gene coding for the transcriptional activator of rhamnolipids biosynthesis. Studies with liquid cultures on hexadecane of *P. aeruginosa* harboring a *pra::gfp* or *rhlR::gfp* fusion revealed specific transcriptional activity at the *n*-hexadecane–water interface (Holden *et al.*, 2002).

Biofilm formation as an adaptive response to optimize acquisition of insoluble HOCs

The first intuitive indications that biofilms could favor access to poorly soluble HOCs came from the observations that biofilm formation occurs in function of substrate solubility since it was shown that the more insoluble the substrate is, the more growth occurs at the water–HOC interface. Moreover, it was observed that several strains growing at the interface between nearly insoluble hydrocarbons and water do not release emulsifier or surface-active compounds into the bulk medium (Bouchez *et al.*, 1997; Bouchez-Naïtali *et al.*, 1999; Bouchez-Naïtali *et al.*, 2001; Klein *et al.*, 2008; Wick *et al.*, 2002). In these cases, cells do not access the substrate by surfactant-mediated transfer, during which cells contact emulsified, solubilized or pseudo solubilized hydrocarbons. Access to the insoluble substrate rather occurs by direct contact of the cells or extracellular structures with the hydrocarbon–water interface. Rosenberg demonstrated the importance of adhesion to hydrocarbons in the growth of *Acinetobacter calcoaceticus* RAG-1 on hexadecane in absence of any emulsifier (Rosenberg and Rosenberg, 1981). Thus, biofilm formation and adhesion to hydrocarbons would promote growth on hydrocarbons by facilitating interfacial access. The strongest evidence of an increase in access to HOCs by adhesion or biofilm formation arose from kinetic studies showing that growth at the interface occurs faster than the mass transfer rate of HOCs in the absence of bacteria would suggest (Bouchez-Naïtali *et al.*, 2001; Calvillo and Alexander, 1996; Harms and Zehnder, 1995; Wick *et al.*, 2002).

Mechanisms employed in biofilms for accessing HOCs are still poorly understood. On the one hand, it is not difficult to imagine that biofilms offer a way to optimize the effect of known mechanisms of acquisition of poorly soluble hydrophobic substrates. Surfactant production within a biofilm would limit its dilution in the bulk phase, facilitating the formation of micelles by keeping the concentration of the surfactant close to the critical micelle concentration (CMC). Biofilms also offer the advantage of holding the cell population in the vicinity of the HOC–water interface thus stimulating the mass transfer of HOCs by shortening the diffusive pathway (Wick *et al.*, 2002). On the other hand, biofilm lifestyle might offer possibilities of biofilm-specific mechanisms for HOCs accession. Biofilms are typically characterized by dense cell clusters embedded in extracellular polymeric substances. The formation of such structures involves profound changes in cell physiology and behavior requiring regulation of the expression of hundreds of genes. Such changes in cellular physiology has been indeed revealed by transcriptomic and proteomic studies on biofilms of *M. hydrocarbonoclasticus* growing at HOCs-water interfaces (Mounier *et al.*, 2014; Vaysse *et al.*, 2009; Vaysse *et al.*, 2010). The transition from the planktonic to the biofilm mode on HOCs entails change in the expression level of more than one thousand genes . Although most of these genes are of unknown function some of them are involved in cellular processes like lipid, alkanesand central metabolisms, chemotaxis, motility, transport and protein secretion. In view of such a reshaping of cellular functions and structural organization, the existence of biofilm-specific mechanisms for HOCs accession is conceivable.

For instance, biofilms can improve the assimilation of HOCs through their extracellular matrix. Many functions currently attributed to the biofilm matrix, *e.g.* adhesion to surfaces and retention of enzymes and metabolites, can have implications in the assimilation of HOCs (Flemming and Wingender, 2010). The retention properties of the matrix could maintain exo-products in the vicinity of cells and prevent their loss in the bulk medium. The action of

227 biosurfactants, which has been demonstrated to improve assimilation of hydrocarbons in some
228 cases (Perfumo *et al.*, 2010), could be greatly increased within a matrix by enabling their
229 accumulation up to the CMC and thus allow micellar transport of hydrocarbons (Guha and
230 Jaffé, 1996). In addition, EPS of the biofilm matrix may act as sorbents or emulsifiers that
231 could stimulate the mass transfer rate of HOCs (Harms *et al.*, 2010). Various strains of
232 *Acinetobacter sp.* produce extracellular complexes of polysaccharides or lipo-polysaccharides
233 and proteins called bio-emulsans that have the capacity to emulsify and increase the solubility
234 of hydrocarbons (Barkay *et al.*, 1999; for reviews see Ron and Rosenberg, 2002; Ron and
235 Rosenberg, 2010). Although the emulsifying activities were not localized within a biofilm
236 matrix, these results indicate that biopolymers could increase mass transfer rates of
237 hydrocarbons and hence stimulate their biodegradation. Direct interactions between EPS and
238 hydrocarbons have been evidenced in *A. venetianus* VE-C cells growing on diesel fuel where
239 nano-droplets incorporated in an extracellular matrix containing glyco-conjugates were
240 observed (Baldi *et al.*, 1999; Baldi *et al.*, 2003). Similarly, oil droplets were completely
241 covered with cells and EPS in a culture of *Rhodococcus sp.* strain Q15 on diesel fuel (Whyte
242 *et al.*, 1999). In both cases, EPS mediated the adhesion of cells to hydrocarbons and are
243 thought to participate in the uptake of hydrocarbons, although the mechanisms involved
244 remain unknown. Biofilm EPS can also serve as an adsorbent to store HOCs and allow their
245 subsequent utilization by the biofilm community (Wolfaardt *et al.*, 1995).

246 Biofilm matrices comprise extracellular proteins with various functions such as hydrolytic
247 enzymes or adhesion (Flemming and Wingender, 2010). The involvement of extracellular
248 proteins in alkane utilization was first evidenced in *A. calcoaceticus* ADP1 as it was shown
249 that a Type 2 Secretion System (T2SS) mutant showed reduced growth on alkanes (Parche *et*
250 *al.*, 1997). The OmpA-like AlnA protein of *A. radioresistens* KA53 and the PA protein from
251 *P. aeruginosa* PG201 and S7B1 are two extracellular proteins that have been shown to play a

role in alkane utilization in planktonic culture (Hardegger *et al.*, 1994; Kenichi *et al.*, 1977). These two proteins exhibit emulsifying properties that have been claimed to be the basis of their function in alkane assimilation although no clear cause-to-effect relationship has been established. During biofilm development on alkanes or triglycerides *M. hydrocarbonoclasticus* SP17 uses cytoplasmic proteins released by cell lysis and proteins secreted through the T2SS to form a proteinaceous matrix (Ennouri *et al.*, 2017). It was hypothesized that the surface activity of proteins could be exploited in oleolytic biofilm matrices to form a conditioning film at the HOC-water interface that could promote cell adhesion and colonization. Matrix proteins could also participate in the mass transfer of HOCs to the biofilm cells by forming micelles or acting as mobile sorbents.

Research Needs

The most exciting aspect of biofilms on HOCs is certainly to identify the features that distinguish them from other biofilms, that is to say, their substrate/substratum specificity and their capacity to overcome the low accessibility of a hydrophobic substrate. These two properties make biofilm formation a very efficient adaptive strategy to assimilate HOCs, which can provide a serious advantage in environments where carbon sources are scarce. The processes by which biofilms stimulate interfacial accession to nearly insoluble substrates remain to be elucidated. Substrate specificity of biofilm formation for HOCs surfaces presumably involves surface sensing and signal transduction pathways, which have not been revealed yet. Biofilm development during the assimilation of HOCs most likely requires coordination of fundamental processes such as architectural biofilm organization, physico-chemical interactions between biofilm and substrate, and the control of gene expression. Investigation of these processes will require multidisciplinary approaches aimed at (1) identifying the genes/proteins involved in biofilm formation, (2) deciphering the architecture

of biofilms, and (3) characterizing at the physico-chemical level the interactions between biofilm components (cells and extracellular matrix) with hydrophobic substrates. So far, investigations on biofilms on HOCs have been conducted on different strains growing on various substrates in diverse experimental setups. It was therefore not possible to correlate these results in order to draw a picture of the functioning of these biofilms. The study of additional?? model bacteria, chosen for their ability to form readily and reproducibly biofilms on HOCs, their genetic amenability, and the availability of their genome sequence would ensure the complementarity of the data obtained from multidisciplinary approaches. In addition to studies examining model single species biofilms on HOCs, investigations of the activities and biodiversity of multispecies biofilms isolated from samples collected from various environments are critical to gain full understanding of the ecological significance of these biofilms.

Due to their wide distribution in the environment, their recalcitrance, and their deleterious effect on human health, hydrocarbons have been the main molecules used in studies of biofilm formation on HOCs. However, other classes of HOCs should also be taken into consideration. In the natural environment, lipids represent a very abundant class of HOCs. For example in sea water they represent up to about 15 % of the organic carbon and its biodegradation is relevant to the global carbon cycle (Lee *et al.*, 2004). Consistent with this, some bacterial strains isolated for their hydrocarbon-degrading capacities also form biofilms on a larger panel of HOCs. This suggests that HOCs-degrading bacteria may have the ability to form oleolytic biofilms that can cope with several types of hydrophobic organic carbon they may encounter in the environment by adapting their physiology according to the chemical nature of the HOC.

Figure 1

M. hydrocarbonoclasticus SP17 biofilms growing on alkanes.

A and B, confocal scanning laser microscopy images of a biofilm covered *n*-hexadecane droplet. Hydrophobic regions including bacteria as well as hydrocarbon were stained with Nile Nile (red signal), glycoconjugates were stained with PSA lectin (green signal). In A, the data are presented as an isosurface projection where the two signals have been split. In B, the dataset is presented as an XYZ projection. The two signals were not separated, colocalized signals of the green and red channel appear in yellow indicating the colocalization of Nile red and lectin stain. Images courtesy by Pierre-Jo Vaysse and Thomas R. Neu (Helmholtz Centre for Environmental Research - UFZ, Magdeburg, Germany). C, picture of *M. hydrocarbonoclasticus* SP17 biofilm growing at the surface of solid eicosane.

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