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Functional Roles of Biosurfactants in Bacterial and Environmental Processes

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Environmental Toxicology

by

Richard Wilson Belcher

December 2012

Dissertation Committee:
Dr. David Crowley, Chairperson
Dr. Jay Gan
Dr. Sharon Walker

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The Dissertation of Richard Wilson Belcher is approved:

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Committee Chairperson

University of California, Riverside

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There is not enough room in a paragraph to express my deepest appreciation for the friendship I have developed with my mentor, Dr. David Crowley. He has always been in my corner when I needed backup and has been constantly understanding of the difficulties that are inherent to scientific progress. We have shared many enduring memories both inside and outside the lab. Laughs have been had, tears have been shed, and our sweat and blood have been put into this dissertation. My writing style and research-oriented mind has evolved under his tutelage and I will deeply miss sharing minds as we did on these dissertation chapters. Always be looking for that power spot in your travels, David.

I want to thank Dr.s Jay Gan and Sharon Walker for their invaluable contributions as members of my committee. I would also like to thank UCR and the many colleagues I have been graced to know during my stay here. The ladies in the AEE office have been a joy to chat with and have devoted their time to conducting the business and financial end in a timely and professional manner. I have been truly blessed with amazing friends all over campus. They have been critical to my sanity; having someone to share my triumphs and tragedies as well as having someone to critically analyze my research protocols and writings.

As the first member of my extended family to pursue a doctorate degree, one of my motivations was to make my family proud. Despite being two thousand miles away, they were inspiration during discouragement, guidance when I was lost, and throughout it

all they were that familiar voice over the phone when I needed it most. Lastly, I would thank my Lord Jesus Christ for guiding me through the trials and tribulations that are endured during graduate school.

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This work is dedicated to my dearly departed sister, Joanna.

May the dreams of the fallen dwell in the hearts of the loved ones they touched.

ABSTRACT OF THE DISSERTATION

Functional Roles of Biosurfactants in Bacterial and Environmental Processes

by

Richard Wilson Belcher

Doctor of Philosophy, Graduate Program in Environmental Toxicology
University of California, Riverside, December 2012
Dr. David Crowley, Chairperson

Biosurfactants are amphipathic molecules exuded by bacteria that play critical roles in a variety of bacterial and environmental processes due to their interfacial interactions. The involvement of biosurfactants in these processes has vast potential to enhance bioremediation and expedite swarming motility, to name a few, and research into this arena is pivotal. Surface tension reduction by surface active agents can induce swarming motility lending competence of plant growth-promoting soil inocula. Bioavailability of inaccessible petroleum hydrocarbons can be promoted by biosurfactant presence by a myriad of mechanisms. Many new biosurfactants are still being found from microbes living in petroleum concentrated soils and novel habitats such as natural asphalt seeps should be considered.

The Crowley lab first revealed bacteria in the Rancho La Brea tar pits where they have adapted degradative enzymes to grow on aromatic hydrocarbons and produce biosurfactants to enhance the bioavailability of these petrochemicals. Extracted bacterial cells were enriched on monoaromatics and a polycyclic aromatic hydrocarbon (phenanthrene). Isolates were screened for biosurfactant production with a variety of

techniques using a novel medium. Thirty percent of the isolated bacteria were able to produce biosurfactants and were exclusively enriched on BTEX compounds. *Stenotrophomonas maltophilia* RB91B, *Pseudomonas* sp. RB91F, and *Shewanella* sp. RB91G were shown to produce biosurfactants that could lower surface tension down to 52 ± 0.6, 35 ± 3.1, and 54 ± 0.7 mN/m, respectively.

Leading off from Chapter Two, the most potent biosurfactant was used along with a phenanthrene-degrading bacterium, to assess if mineralization of ^{14}C-phenanthrene was affected and what possible mechanisms are involved. *Pseudomonas* sp. RB91F was shown to produce a polymeric surface active agent comprised of carbohydrate, protein, and lipid that could lower surface tension down to 36 ± 0.4 mN/m at a CMC of 925 mg L^{-1} and could emulsify kerosene, *n*-hexane, toluene, xylene, and gasoline. Degradation by *Delftia tsuruhatensis* RB91H was enhanced by the polymeric bioemulsifier produced by *Pseudomonas* sp. RB91F depending on amended CMC levels. At each CMC level, unique mechanisms appear to be dominant based on cell surface hydrophobicity, growth on bioemulsifier, as well as micellar transport and sequestration.

The Orwin lab discovered a *Variovorax paradoxus* strain capable of swarming motility that may produce a biosurfactant within its wetting agent. Using specially designed extraction and purification techniques, the wetting agent was assayed for surface activities and any biosurfactant present was characterized. The results strongly suggest that *V. paradoxus* EPS produces a lipopeptide biosurfactant that can lower surface tensions to 30 ± 0.5 mN/m at a CMC of 215 mg L^{-1}. The biosurfactant has a

molar mass of 679.3 m/z and is currently being structurally characterized to determine if it's a novel surface active agent. The data seems to suggest that variowettin is related to the serrawettin class of lipopeptide wetting agents produced by members of the *Serratia* genera. This dissertation, therefore, identifies environmental isolates with biosurfactant-producing capabilities that have a range of critical functions to enhancing bacterial and environmental processes.

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Chapter One: Introduction

Biological surface active agents, or biosurfactants, are structurally diverse amphiphiles produced by microorganisms. They contain hydrophobic and hydrophilic components enabling them to attach to a multitude of surfaces and interact with interfaces between different phases. This can result in biosurfactants lowering surface and interfacial tensions at air-water and water-oil interfaces, formation of stable emulsions, and solubilization and desorption of hydrophobic compounds from particle surfaces (Georgiou et al. 1992). These functions can shape the ecological niches for bacteria within microbial communities by altering the availability of substrates for growth, and by allowing bacteria to enter into liquid phases having different levels of hydrophobicity. This phenomenon is particularly important with respect to biodegradation of petroleum chemicals in which biosurfactants increase the bioavailability of petroleum compounds by increasing their mobility and diffusion in aqueous media, and by enabling bacteria to attack oil droplets and particles that are covered with oil films (Rouse et al. 1994). Swarming bacteria can also colonize novel surfaces rapidly by enabling biofilm formation, and the development of highly coordinated rafts of bacterial cells that move across particle surfaces (Daniels et al. 2004). Given their fundamental role in microbial ecology and biotechnology, research on biosurfactants extends back through studies that have been carried out since the 1950s. However, there is still relatively little understanding of the diversity and properties of these compounds, and new analytical methods enable new insight into how these compounds function. The research reported in this dissertation focuses in particular on the critical roles of biosurfactants with respect to

remediation of soils polluted with polycyclic aromatic hydrocarbons and their role in the motility of bacteria that are able to use a mechanism known as “swarming” in order to rapidly move over the surfaces of materials that they colonize.

Role of Surfactant in Remediation of Environmental Pollution

Technologies combining the ability of certain bacteria to degrade hydrophobic organic pollutants along with other bacteria that produce biosurfactants that can mobilize these substances are now being considered as remediation strategies to help clean up hazardous waste sites. Whether speaking of industrialized or developing countries, all civilizations generate hazardous wastes that pollute the environment. A waste can be classified as hazardous if it has characters that can lead to illness, death or some other harm to humans and other biotic organisms when mishandled or released into the environment (EPA 2007). Hazardous waste produced from industrial, agricultural, medical, municipal or household activities can pollute the air, water, or soil and is often transported among these media over varying distances that can sometimes end up far from the original source. Contamination of soils is a common occurrence due to precipitation of atmospheric chemicals, runoff or drift from chemical applications, rupture of a storage site, or industrial discharge. Soil pollutants are a concern when they become transportable through leaching into groundwater and as runoff into bodies of water and contaminate our drinking water and fisheries. Risk of exposure also exists with city and rural life when contaminants migrate in the air as smog or during contact with polluted agricultural soil. For these reasons, the federal government passed the

Comprehensive Environmental Response, Compensation, and Liability Act in 1980 allowing the U.S. Environmental Protection Agency (EPA) to identify and clean up contaminated terrestrial sites. Those sites that meet the highest priority for cleanup are called Superfund sites (EPA 2007). Containment of the most concentrated areas of pollution generally involve physical isolation and burial or incineration, after which residual contaminants are treated using remediation strategies involving microorganisms that can degrade the remaining materials to safe concentrations.

Many techniques are used to remove waste from soil such as hauling contaminated soil to a disposal site or the *ex situ* (off site) or *in situ* (on site) remediation of organic contaminants (Fig. 1). Intensive remediation strategies involving mechanical, physical, thermal, and chemical strategies to remove or reduce pollutants (Khan et al. 2004) tend to be the most expensive methods and can result in invasive procedures and treatments that disrupt the contaminated site. Thus it is often desirable only to use these methods to treat highly contaminated areas, after which adjacent areas with lower levels of contamination are treated using bioremediation. With bioremediation, there are two strategies in which microorganisms may be directly introduced, termed "bioaugmentation" or by another process termed "biostimulation" (Iwamoto and Nasu 2001). The latter involves altering the environmental conditions to stimulate the activity of indigenous microorganisms that can break down the hazardous compounds into less toxic byproducts or that can completely mineralize the contaminants to carbon dioxide, water, and harmless minerals (Singleton 1994). Since the process is based on selective enrichment of degrader organisms, many factors can be manipulated to enhance the

process such as altering the soil pH, optimizing soil moisture and aeration, and by addition of nutrients or substances that stimulate the growth of the degrader population and bioavailability of the contaminants (Boopathy 2000). In general, most contaminants are degraded more rapidly under aerobic conditions due to the greater ability to generate energy and produce an effective biomass. However, some substances, particularly halogenated aromatic pollutants require a two step process in which the chlorine atoms are removed by reductive dehalogenation using the chemical as an electron acceptor for respiration, followed by an aerobic process to degrade the dechlorinated metabolites. Addition of oxygen and rate-limiting essential nutrients like nitrogen are often useful biostimulation strategies to stimulate aerobic microbial degradation by the native populations. The process of bioaugmentation may use degrader organisms, or addition of biosurfactant-producing bacteria. (El Fantroussi and Agathos 2005). In some cases, it may be possible to stimulate *in situ* production of biosurfactants through the addition of nontoxic organic materials or culture of plants that enhance the growth and activity of biosurfactant-producing bacteria. For example, the plant-produced fatty acid, linoleic acid, appears to support the growth and activity of Mycobacteria that degrade polycyclic aromatic hydrocarbons (Yi and Crowley 2007). The use of bioremediation is still only a small component among the various technologies that are used to treat contaminated sites (Fig. 1), but is becoming increasingly important for final cleanup of contaminant residues.

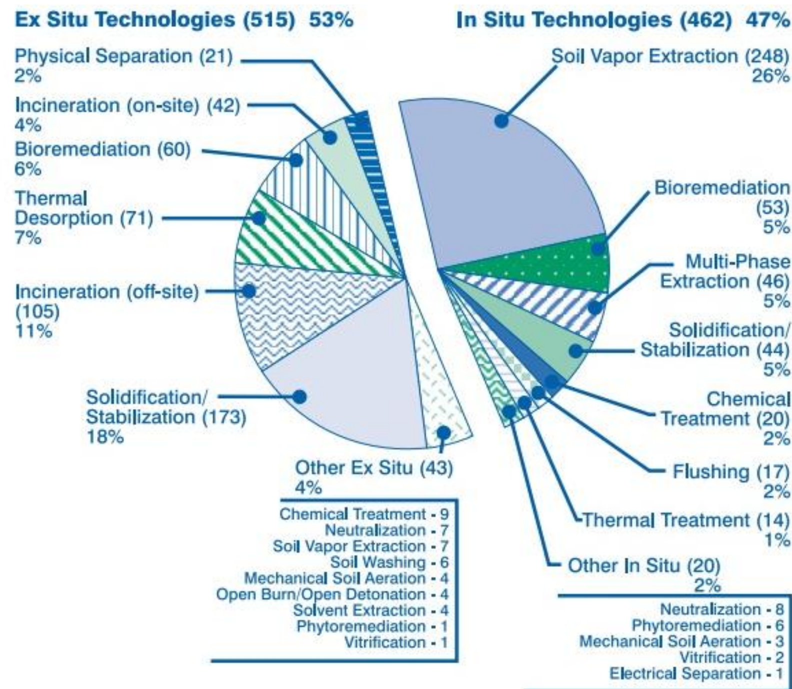

Fig. 1 *Ex situ* and *in situ* technologies employed by the EPA (fiscal year 1982 – 2005; total projects: 977) (EPA 2007)

Polycyclic Aromatic Hydrocarbons

Among the most common organic pollutants associated with Superfund sites are the group of compounds known as polycyclic aromatic hydrocarbons (PAHs). These chemicals are produced during incomplete combustion, and are also components of oil and asphalt materials from which petroleum chemicals are produced. Due to the wide use of petroleum as an energy source, PAHs are now ubiquitous in the environment. There are many different PAHs, ranging from the most simple compound, naphthalene, which consists of two fused benzene rings, to largely insoluble high molecular weight substances containing five or more fused rings. The PAHs with intermediate molecular weights are the most problematic as contaminants as they have partial solubility in water and are highly toxic to most living organisms. Human exposure to PAHs can be

detrimental as many are potentially mutagenic (White and Claxton 2004; Lemieux et al. 2008; Durant et al. 2006), carcinogenic (Smith et al. 2000; Boffetta et al. 1997; Li et al. 2002), and toxic (Romero et al. 1997; Miller and Ramos 2001; Mounho et al. 1997). The EPA has therefore designated 16 PAHs as priority pollutants for remediation. Unfortunately, PAHs are highly resistant to degradation as they are hydrophobic, have a high solid-water distribution ratio, and contain dense π-electron clouds that protect them from nucleophilic attack (Harms and Bosma 1997; Johnsen et al. 2005). The relative recalcitrance of different PAHs with respect to biodegradation is based partially on their structural arrangement; linear being less stable than angular, for instance (Wilson and Jones 1993). Aside from biodegradation, other methods by which PAHs may be eliminated or transported to other media include volatilization and abiotic losses such as leaching or transport on particle surfaces. Disappearance by volatilization and abiotic losses is more common in two to three ring PAHs, but less likely for those with more than three rings. Despite these limitations, many bacteria are still able to degrade PAHs, but their low solubility and concomitant low bioavailability leads to a slow degradation rate and limits the growth of high cell densities of degrader bacteria that are needed to achieve rapid degradation.

While low molecular weight PAHs are slightly soluble in water, in soils they often partition into nonaqueous-phase liquids or are sorbed on to clay and organic matter or become physically trapped by diffusing into nanopores within solid particles. This greatly limits the ability of microorganisms or their enzymes to access these molecules for use as a growth substrate or degradation by extracellular enzymes (Alexander 1999).

As the enzymes required for degradation are largely intracellular or are associated with the cell envelope, energy-dependent transport pathways or passive diffusion followed by partitioning into the cell membrane are the primary processes by which the hydrocarbon molecules enter bacterial cells (Van Hamme 2004; Whitman et al. 1998; Bateman et al. 1986). The initial degradation begins under aerobic reactions via dioxygenase enzymes that incorporate two oxygen atoms into one of the aromatic rings to form a *cis* dihydrodiol (Haritash and Kaushik 2009). Subsequent oxidations prepare the aromatic rings to be cleaved by meta or ortho fission, which is then followed by central degradation pathways that proceed to complete mineralization or production of aliphatic compounds or pericondensed PAHs with aliphatic chains that accumulate as particularly recalcitrant compounds (Chung and Violi 2011). Assimilation of the carbon derived from a PAH yields energy when PAH metabolites enter the citric acid cycle, producing reducing agents necessary for NADH and ATP production. Mineralization of PAHs can occur when utilizing the contaminant as a growth substrate and degrading them into harmless byproducts like CO_2.

Due to the low solubility of high molecular weight PAHs, which are often too insoluble to support growth, high molecular weight PAHs tend to be degraded mainly by a process involing cometabolism in which growth on low molecular PAHs induce low specificity enzymes able to also attack the high molecular weight PAHs. Cometabolism occurs in the presence of two compounds that have structural similarities and results in degradation of a compound that would otherwise not be able to produce usable energy (Hwang and Cutright 2002). In addition to cometabolism, the release of biosurfactants

plays a key role in the mobilization of PAHs by facilitating their transport to the cell surface where they can be attacked by enzymes located on the surface of the cell envelope or in pores in the cell wall where dioxygenases are tethered to the cell wall. The surfactants can also help to modify the interface between the cell envelope and the PAHs by modifying the hydrophobicity of the bacterial cell wall.

Biosurfactants and Degradation of Petroleum Hydrocarbons

The first biosurfactant to be chemically characterized was a rhamnolipid (branched glycolipid) that is produced by *Pseudomonas aeruginosa*. At the time, this lipid was studied only with respect to its toxicity (Jarvis and Johnson 1949). It was not until later that it was shown to dramatically improve the growth of *P. aeruginosa* in *n*-hexadecane media and its surface active properties came to light (Hisatsuka et al. 1971), and the ability of biosurfactants to lower surface tension and increase emulsification was conclusively demonstrated. Biosurfactant research in the 80s focused on growth promotion increases responding to enhanced bioavailability of *n*-alkanes (Pareilleux 1979; Gerson and Zajic 1978). Production of many biosurfactants was also shown to be stimulated by the presence of aliphatic hydrocarbons, indicating that the compounds were being actively induced as a means to increase access to hydrophobic molecules (Suzuki et al. 1969). This was established mainly by comparing the relative amounts of biosurfactants that are produced when bacteria were grown on *n*-alkanes as compared to water-soluble substrates such as glucose (Rapp et al. 1979; Ramsay et al. 1988; Neufield

et al. 1983). By the 1990s, it was widely accepted that biosurfactants can play important roles in petroleum hydrocarbon degradation.

Surfactant molecules have an amphiphilic structure in which a hydrophilic portion on one end of the molecule allows biosurfactants to be soluble in water and a hydrophobic part of the molecule allows intraction with hydrophobic substances. This property also leads to the concentration of surfactant molecules at interfaces, including the air-water interface (Volkering et al. 1998). When the ordered structure of water molecules at the air-water interfaces is disrupted, surface tension decreases. Likewise, the association of the nonpolar region of biosurfactants with nonpolar PAH molecules reduces the formation of strong interfaces on hydrophobic surfaces where PAHs tend to accumulate in microdroplets or to associate with hydrophobic surfaces of clay and organic matter. At high concentrations, stable aggregates of 10 to 200 biosurfactant molecules will form a spherical structure called a "micelle". The concentration at which this occurs is called the critical micelle concentration (CMC) (Cameotra et al. 2010). At the CMC, hydrophobic substances are partitioned into the interior of the micelle that is oriented with polar regions of the micelle interfacing with adjacent water molecules. The CMC is thus often used to measure the efficiency of a biosurfactant, although biosurfactants may still affect the molecular interactions between water and the hydrophobic substance at concentrations below the CMC. In measurements of the CMC, the surface tension reduction is generally measured with respect to that of the air-water interface for pure water, which has a surface tension of 72 mN/m. This is quantified by instruments that physically measure the force needed to disrupt the stable surface of

water molecules that form a surface film in which the polar side of the water molecules (oxygen atoms) orient toward the air interface. Through the addition of a biosurfactant, the surface tension can be reduced to as low as 30 ± 5 mN/m. Another useful method for screening for biosurfactant activity is the ability of a substance to stabilize emulsions of hydrocarbon and aqueous phases.

PAHs can be transported to the surface of bacterial cells only if they are mobilized into the aqueous phase by association with a surfactant (Wodzinski and Bertolini 1972; Wodzinski and Coyle 1974). When micelles form, solid PAHs may partition into the interior of the micelle, which increases the hydrocarbon's apparent solubility (Noordman et al. 2000). This is considered to be one of the major mechanisms in which biosurfactants enhance the bioavailability of PAHs (Schippers et al. 2000). Once PAH molecules are associated with the surfactant, they may remain entrapped in the micellar core (also known as the micellar pseudophase), or directly released to the bacterial cell if the cell wall is sufficiently hydrophobic to interact with the surfactant (Fig. 2). Non-micellar transport is possible through cell surface modifications by surfactant molecules (Al-Tahhan et al. 2000), and interactions between pollutants and micelle-like aggregates or single surfactant molecules (Barkay et al. 1999). These modes of action are expounded upon in biosurfactant-based bioremediation studies. For instance, most solubilization studies suggest that the percentage of PAH solubilized is dependent on biosurfactant concentration (Das et al. 2008; Gu and Chang 2001). Solubilization of petroleum hydrocarbons by biosurfactants generally leads to an increase in biodegradation in liquid media (Zhang et al. 1997; Jacques et al. 2005) and soil

(Cameotra and Singh 2008; Whang et al. 2008). However, there are also studies that

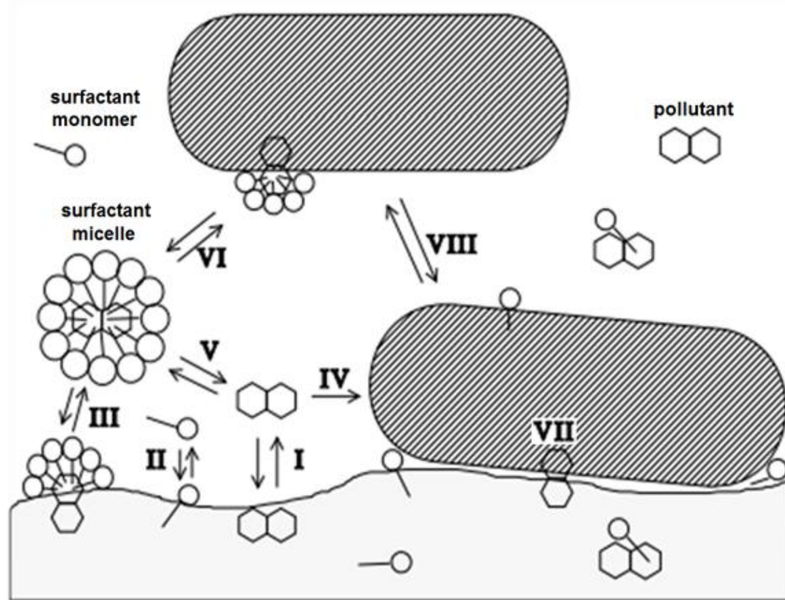

Fig. 2 Schematic overview of the interaction between bacteria, soil, PAH (shown here as naphthalene), and biosurfactants: **I)** solid/aqueous phase partitioning of PAH, **II)** sorption of biosurfactant onto soil, **III)** micellar solubilization of PAH, **IV)** microbial uptake of PAH from aqueous phase, **V)** partitioning of PAH from micellar pseudophase to aqueous phase,**VI)** direct microbial uptake from micellar core, **VII)** direct microbial uptake from solid phase, **VIII)** sorption of bacteria onto soil (Volkering et al. 1997)

show biosurfactants can inhibit PAH degradation (Shin et al. 2005; Guha and Jaffe 1996). This may occur when bacteria are unable to access PAHs that are contained in the center of the micelles and the cell envelope is not compatible to interact with the surfactant in a manner that allows the release and diffusion into the cell membrane for uptake by the bacteria.

When microorganisms are able to interact with the surfactant, the presence of the biosurfactant will frequently lead to enhanced growth of the microorganism, which accesses the carbon contained in the PAH, or possibly by use of the surfactant as a

substrate for growth (Whang et al. 2008; Rahman et al. 2003). Synthetic surfactants are considered to have greater potential for toxicity (Tiehm 1994), possibly by causing delipidation of the cell membrane of bacteria that are exposed to certain surface active agents. Thus, biosurfactants can also have antimicrobial activities aside from serving as a carrier for hydrophobic substance mobilization (Benincasa et al. 2004; Haba et al. 2003). During micellar-dependent mobilization, growth reduction may be concentration-dependent especially at supra-CMC levels where the PAH is sequestered in the micellar pseudophase (Guha and Jaffe 1996). Furthermore, biodegradability of biosurfactants should be considered as many can be used as a sole carbon source (Zajic et al. 1977). Many opponents therefore argue against biosurfactants and prefer to use synthetic surfactants in remediation. On the other hand, synthetic surfactants that are poorly degradable can potentially lead to greater dispersal of the contaminant through leaching of contaminant laden micelles into deeper zones of the soil profile or into ground water.

Some synthetic surfactants share the degradation-enhancing properties that occur with biosurfactants and due to their low production costs dominate the $23 billion global surfactant industry. Commercially, synthetic surfactants have proved useful for inclusion in cleaning products, for delivery of pharmaceuticals, and for applications in biological sciences, personal hygiene, oil drilling, and many other markets. However, with respect to environmental applications, biosurfactants are increasing in popularity for several reasons. Chemical surfactants are created from petroleum feedstocks; whereas, biosurfactants can be generated from microorganisms, provided that inexpensive feedstocks for microbial culture can be used (Mukherjee et al. 2006). Other advantages

include higher biodegradability, lower toxicity, higher selectivity and specific activity at extreme temperatures, pH, and salinity. The diversity of biosurfactants that may reside in nature thus offers molecules having many interesting and potentially useful chemical properties for a wide range of applications (Table 1). Current research is developing biotechnologies that can increase yields and lower costs associated with biosurfactant production to compete with the synthetic market.

As with most secondary metabolites, the quantities of biosurfactant that can be produced are strongly influenced by the types of carbon and nitrogen sources that are used to culture the biosurfactant-producing microorganisms. One of the major challenges is optimizing the composition of growth media for high yields that are needed for commercialization (Mukherjee et al. 2006). The biosynthesis of biosurfactants is a complex process that can involve shifting microbial pathways toward *de novo* and substrate-dependent reactions that will yield biosurfactant (Georgiou et al. 1992). Carbon sources that are commonly used for inducing bacteria to produce surfactants include

Table 1 Biosurfactants, producing organisms, and their applications in recent years (Makkar et al. 2011)

Organism	Type of biosurfactant	Potential Applications	Reference
Rhodococcus erythropolis 3C-9	Glucolipid and a trehalose lipid	Oil spill cleanup operations	(Peng et al. 2007)
Pseudomonas aeruginosa S2	Rhamnolipid	Bioremediation of oil contaminated sites	(Chen et al. 2007)
Pseudozyma siamensis CBS 9960	Mannosylerythritol lipid	Promising yeast biosurfactant	(Morita et al. 2008a)
Pseudozyma graminicola CBS 10092	Mannosylerythritol Lipid	washing detergents	(Morita et al. 2008b)
Pseudomonas libanensis M9-3	Lipopeptide	Environmental and biomedical applications	(Saini et al. 2008)
Bacillus subtilis strain ZW-3	Lipopeptide	Potential in pharmaceutics, environmental protection, cosmetic, oil recovery	(Wang et al. 2008b)
Rhodococcus sp. TW53	Lipopeptide	Bioremediation of marine oil pollution.	(Peng et al. 2008)
Pseudozyma hubeiensis	Glycolipid	Bioremediation of marine oil pollution	(Fukuoka et al. 2008)
R. wratislaviensis BN38	Glycolipid	Bioremediation applications	(Tuleva et al. 2008)
Bacillus subtilis BS5	Lipopeptide	Bioremediation of hydrocarbon-contaminated sites	(Abdel-Mawgoud et al. 2008)
Azotobacter chroococcum	Lipopeptide	Environmental applications.	(Thavasi et al. 2008b)
Pseudomonas aeruginosa BS20	Rhamnolipid	Bioremediation of hydrocarbon-contaminated sites.	(Abdel-Mawgoud et al. 2009)
Micrococcus luteus BN56	Trehalose tetraester	Bioremediation of oil-contaminated environments.	(Tuleva et al. 2009)
Bacillus subtilis HOB2	Lipopeptide	Enhanced oil recovery, bioremediation of soil and marine environments, and food industries.	(Haddad et al. 2009)
Pseudomonas aeruginosa UFPEDA 614	Rhamnolipid	Bioremediation.	(Neto et al. 2009)
Nocardiopsis alba MSA10	Lipopeptide	Bioremediation	(Gandhimathi et al. 2009)
Pseudoxanthomonas sp. PNK-04	Rhamnolipid	Environmental applications.	(Nayak et al. 2009)
Pseudozyma parantarctica	Mannosylmannitol lipid,	Emulsifiers and/or washing detergents	(Morita et al. 2009)
Pseudomonas alcaligenes	Rhamnolipid	Environmental applications.	(Oliveira, et al. 2009)
Pseudomonas koreensis	Lipopeptide	Biocontrol Agent	(Hultberg et al. 2010)
Pseudomonas fluorescens BD5	Lipopeptide	Bioremediation and biomedicine.	(Janek et al. 2010)
Candida bombicola	Sophorolipids	Environmental applications.	(Daverey and Pakshirajan 2010a, b)
Brevibacterium aureumMSA13	Lipopeptide	MEOR	(Kiran et al. 2010b)
Nocardiopsis lucentensis MSA04	Glycolipid	Bioremediation in the marine environment.	(Kiran, et al. 2010a)
Bacillus velezensis H3	Lipopeptide	Industrial strain for the Lipopeptide production.	(Liu et al. 2010)
Calyptogena soyoae	Mannosylerythritol lipid	Bioremediation processes in the marine environment.	(Konishi et al. 2010)
Burkholderia plantari DSM 9509	Rhamnolipid	Detergents and pharmaceutical industry	(Hörmann et al. 2010)

hydrocarbons, carbohydrates, alcohols, or vegetable oils. Solubility of the carbon substrate may also have a role in enhancing surface activities, yield, and emulsification properties. Robert et al. (1989) and Abouseoud et al. (2008) suggest that hydrophobic, water immiscible carbon sources promote in the production of biosurfactants; whereas, others Illori et al. (2005) and Das et al. (2009) claim that water soluble carbon substrates

can also be used. Along with choice of which carbon source to use, the choice of nitrogen source also may be important, although evidence seems to support the idea that nitrogen source is not as critical as carbon source, but rather it is the ratio of carbon to nitrogen (Davis et al. 1999; Mulligan and Gibbs 1989). Production of many biosurfactants is growth-linked, such that the substances are released into the culture medium toward the end of active growth , and accumulate after the cells have reached the stationary phase (Hommel 1990). Nitrogen limitation (or a high carbon:nitrogen ratio) has been shown to enhance biosurfactant production. This is speculated to occur when nitrogen-limited cells convert remaining carbon to biosurfactant which serves as a means to store otherwise labile carbon in the growth medium (Fig. 3). Regardless of the reason, the ratio of carbon:nitrogen, should be considered when designing biosurfactant-inducing media.

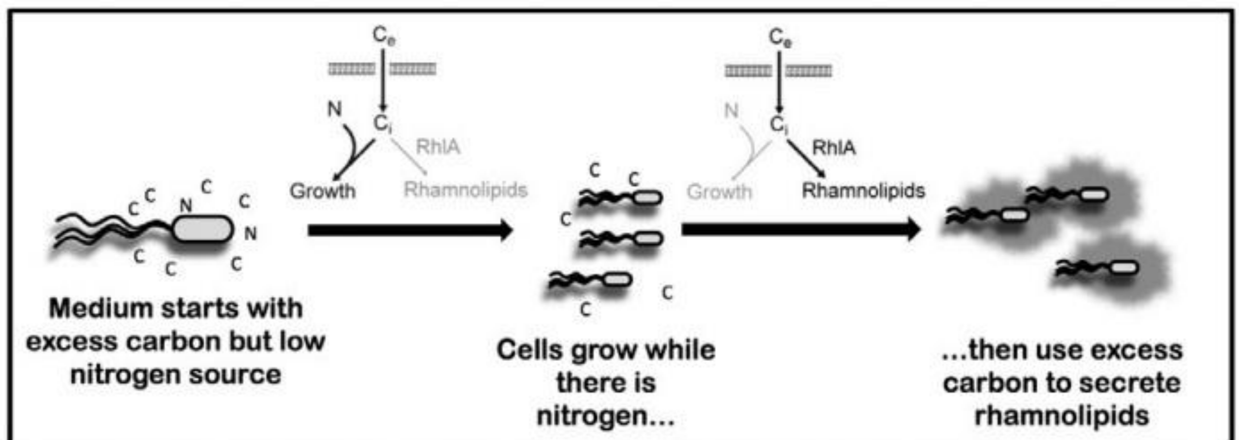

Fig. 3 *Pseudomonas aeruginosa* like many bacteria are induced to produce their biosurfactants in nitrogen-limiting conditions (Xavier et al. 2011)

After biosurfactant production, any downstream processing often requires removal of cells from the culture medium by centrifugation and filtration to prevent the macromolecular composition (lipid, carbohydrate, and protein) from contaminating

biosurfactant extracts. After removing the cells, the cell free supernatant can be assayed for surfactant content using a tensiometer. One such instrument uses the du Nouy ring method to determine surface tension, which is measured in mN/m (Lecomte du Nouy 1919). The presence of a biosurfactant is evident when the surface tension of pure water is reduced from 72 mN/m down to approximately 40 – 50 mN/m (Willumsen and Karlson 1997; Cooper 1986). To accomplish further analysis, a partially purified compound is often required, which is traditionally derived by precipitation and solvent extraction (Banat et al. 2010). At this level of purification, there are a wide array of methods that can be used to screen for the presence of the surfactant, including the drop collapse method (Jain et al. 1991; Burch et al. 2010), oil spreading (Morikawa et al. 2000), and hemolysis (Carrillo et al. 1996). Of these methods, the drop collapse assay is considered one of the most reliable, simple, and rapid (Yin et al. 2009; Youssef et al. 2004). This method has been employed for screening for surfactant-producing microorganisms in oil-contaminated water and soil (Bodour et al. 2003; Batista et al. 2006; Plaza et al. 2006). In another assay, the emulsification activity can be assessed by vortexing biosurfactant in aqueous solution with a hydrophobic liquid and measuring any stable emulsion that forms 24 hours later (Cooper and Goldenberg 1987). Compositional identification can be discerned with thin layer chromatography and colorimetric methods. Full structural identification requires chromatographic purification followed by mass spectrometry and spectroscopy (infrared or nuclear magnet resonance) (Satpute et al. 2010). These procedures are effective strategies to describe the chemical properties of the biosurfactant and identify the class or type of biosurfactant that is produced.

The Rancho La Brea Tar Pits

Petroleum microbiology has led to the discovery of many bacterial species that are capable of degrading a wide range of contaminants including PAHs (Zhao et al. 2008; Zhao et al. 2009; Farias et al. 2008), nitrogen and sulfur-containing compounds (Kaiser et al. 1996; Kropp and Fedorak 1998), and aromatic hydrocarbons, alkanes, and other substances contained in petroleum. In addition to aerobic degradation, some microorganisms also are able to degrade hydrocarbons under anaerobic conditions (Grishchenkov et al. 2000; Wilkes et al. 2000; Boopathy 2004). When petroleum contaminants enter into an ecosystem, the microorganisms respond by changing their metabolic pathways, communicating and interacting intercellularly, and horizontal transfer of critical genes (Brito et al. 2006). It appears that all soils contain bacteria that can degrade petroleum compounds, but the extent to which populations adapt and new species evolve over long periods of exposure to petroleum is still an open question. Oil-degrading populations in "pristine" soils will be in the range of a few hundred to several thousands cells per gram of soil, which can increase in population densities ranging into hundreds of millions of cells per gram of soil (Johnsen and Karlson 2007). Most studies focus on bacteria isolated from recently petroleum-contaminated sites, while few have examined the species of bacteria that occur in sites that have had a long term history of exposure, i.e. natural asphalts contained in pitch lakes and tar pits, or oil seeps. During the seepage process leading to the accumulation of asphalt, the lighter portions evaporate or volatilize under high pressure leaving behind an extreme environment containing a mixture of very recalcitrant hydrocarbons. Asphalts contain high concentrations of heavy

oil components such as asphaltenes, as well aromatic alkanes, monoaromatics, and PAHs. Microorganisms living in the asphalt have adapted to the limited access to water or air, the use of highly recalcitrant carbon sources, and exposure to high concentrations of potentially toxic metals and chemicals (Table 2). Microbial life in the largest natural asphalt deposit at Pitch Lake located in the town of La Brea in southern Trinidad have further adapted within particular niches in the petroleum matrix to become unique and diverse island-like populations capable of anaerobic hydrocarbon degradation (Schulze-Makuch et al. 2011). The Rancho La Brea tar pits is another asphalt seep, located in downtown Los Angeles, and through the efforts of our lab, new bacterial taxa as well as novel dioxygenases have been identified from this extreme environment (Kim and Crowley 2007). Since the vast majority of these are so far unculturable on laboratory media, it is of particular interest to focus first on the culturable bacteria in the asphalt that may produce biosurfactants. Such bacteria may likely include species that produce novel biosurfactants that can be used to remediate harsh sites contaminated with PAHs.

pH of suspension		EC (μS/cm)	% C	% N	% S	C/N ratio	Concn (μg/ml) of:												
1:1 ($CaCl_2$)	1:5 (H_2O)						Na	Ca	Mg	K	Al	Cd	Cr	Fe	Zn	Cu	Mn	Pb	Ni
6.3	5.36	46	22.04	0.3	0.97	74.16	13	2,902	265	86.1	356	2.2	2.65	484	33.9	23.4	13	ND	89.9

Table 2. Chemical properties of tar pit site (Kim and Crowley, 2007)

The Rancho La Brea tar pits has had several fossil excavation sites over the years, but currently they are working on only one dig site, Pit 91. In the research reported in this dissertation, asphalt samples from Pit 91 were collected and placed into liquid culture media to enrich for biosurfactant producers and PAH degraders. Kim and Crowley (2007) completed a comprehensive survey of Pit 91, which included a characterization of its

chemical properties (Table 2) and phylogenetic analyses of culturable and nonculturable bacteria, and a list of gene sequences that may encode novel aromatic ring hydroxylating dioxygenases. Their findings suggest that Pit 91 may contain candidate species that could be used to produce biosurfactants. For instance, they were able to culture several species and strains of *Pseudomonas* spp.; this genus is well known bacteria that produce biosurfactants. The presence of genes encoding both known and potentially novel dioxygenases further suggest that this natural seep may contain many PAH-degrading bacteria. To address these hypotheses, the research in Chapter Two reports the results of studies that screened for bacteria that produce biosurfactants when grown on different substrates. Chapter Three follows with a report on studies that were conducted to examine the effect of biosurfactant obtained by a microorganism that inhabits the tarpits for its effects on PAH degradation by a nonsurfactant-producing PAH-degrading bacterium, also from the tar pits.

Natural Roles of Biosurfactants Outside the World of Bioremediation

Biosurfactants assist their bacterial producers in other ways than just enhancing bioavailability of recalcitrant petroleum contaminants. Many bacteria, for instance, are dependent on biosurfactants to reduce the friction between their cells and the surfaces they colonize to facilitate rapid movement through a process known as swarming. This is the most rapid form of motility in microorganisms; which allows those strains that can swarm to rapidly occupy and colonize biotic and abiotic surfaces. The swarming behavior also showcases one of the most interesting examples of multicellular behavior among

unicellular microorganisms as they communicate intracellularly, differentiate into new hyperflagellated morphotypes, and then form groups or rafts of cells that colonize a surface environment as a collective (Harshey 1994). This specialized surface translocation makes swarming bacteria highly competitive for dominating new surfaces as compared to other bacteria that only spread by cell division along the edges of their colonies (Butler et al. 2010; Kaiser 2007). Aside from benefits associated with swarming, biosurfactants that are involved in this process can have effects that contribute to fitness and competition. Many biosurfactants are antibiotics, providing added protection against competitors from colonizing neighboring surfaces (Raaijmakers et al. 2010). Since some potential growth substrates such as hydrophobic compounds, including PAHs, are adsorbed to surfaces, swarming bacteria may be able to use their biosurfactants to enhance the bioavailability of hydrophobic substances for use as growth substrates (Arino et al. 1998). Biosurfactants and swarming behavior are therefore important to general studies of bacteriology, biomedicine, and environmental microbiology.

Swarming, much like the biosurfactants that facilitate this behavior, requires specific conditions to be induced. Since it typically occurs at high cell densities and results in hyperflaggelation, nutrient conditions must be rich to promote cell growth and enough ATP that is used as chemical energy to propel cells via the rotation of their multiple flagella (Kearns 2010). These processes take time to occur resulting in a swarming lag during which time planktonic cells transition to become swarm cells. Differentiation will occur during this transitional period and often leads to the creation of more flagella responsible for fast movement and cell elongation to increase surface area

contact with neighboring cells aiding in intracellular communication and raft (side-by-side cell groups) formation (Kearns 2010). When induced on agar media, the surface conditions must also be sufficiently soft (0.4 – 0.7 % agar), but not aqueous. If the medium is too soft (< 0.4 % agar) swimming – a similar flagellar-driven motility pattern that occurs within liquid – will be preferentially induced (Harshey 2003). Swarming is often regulated by quorum sensing which is triggered at high cell densities activating a regulatory system. Initially, N-acyl-homoserine lactones (AHLs) which are low molecular weight signals (also called quorom sensors) are released and increase alongside population size (Daniels et al. 2004). When AHLs reach a threshold concentration, they bind and activate transcriptional regulators that are often upstream of genes responsible for biosurfactant production. In certain strains, biosurfactant will be released into a pool of wetting agent that surrounds an advancing colony to promote swarming.

Wetting agent is comprised of liquid released extracellularly when bacteria live on water-repellent surfaces (Matsuyama and Nakagawa 1996) or taken from the substrate which is why low viscosity media is typically used (0.4 – 0.7 % agar) when assaying swarming in the lab (Bees et al. 2000; Mezanges et al. 2012). Wetting agent composition is variable depending on species and habitat type, but in many microorganisms a biosurfactant is required for swarming to occur. Surfactant advances the wetting agent edge forward and also reduces surface tension of the liquid. Bacteria are so small they are susceptible to intermolecular forces within water, which has a high surface tension (72 mN/m) (Matsuyama and Nakagawa 1996). However, surfactant presence in the wetting

agent can lower surface tension as low as 30 mN/m, reducing this intermolecular force (Fauvart et al. 2012). The formation of hyperflagellated swarmer cells in coordinated rafts overcomes any other forces present allowing for rapid travel across the substrate. To better understand swarming, the identity of biosurfactants involved need to be determined by extracting the amphiphiles from the wetting agent.

Biosurfactants are classified based on the types of microorganism from which they are produced, and by their chemical composition. Perhaps due to the highly regulated systems that are involved, swarming is conserved among three major classes: Firmicutes, Alphaproteobacteria, and Gammaproteobacteria (Fig. 4). Low molecular weight biosurfactants mainly have the traditional polar head and nonpolar tail; whereas, high molecular weight biosurfactants are polymeric (Neu 1996). The latter are known primarily for their emulsification properties and are often referred to as bioemulsifiers (Franzetti et al. 2011). As of yet, no bioemulsifier has been extracted from wetting agents. Although low molecular weight biosurfactants can have emulsification properties,

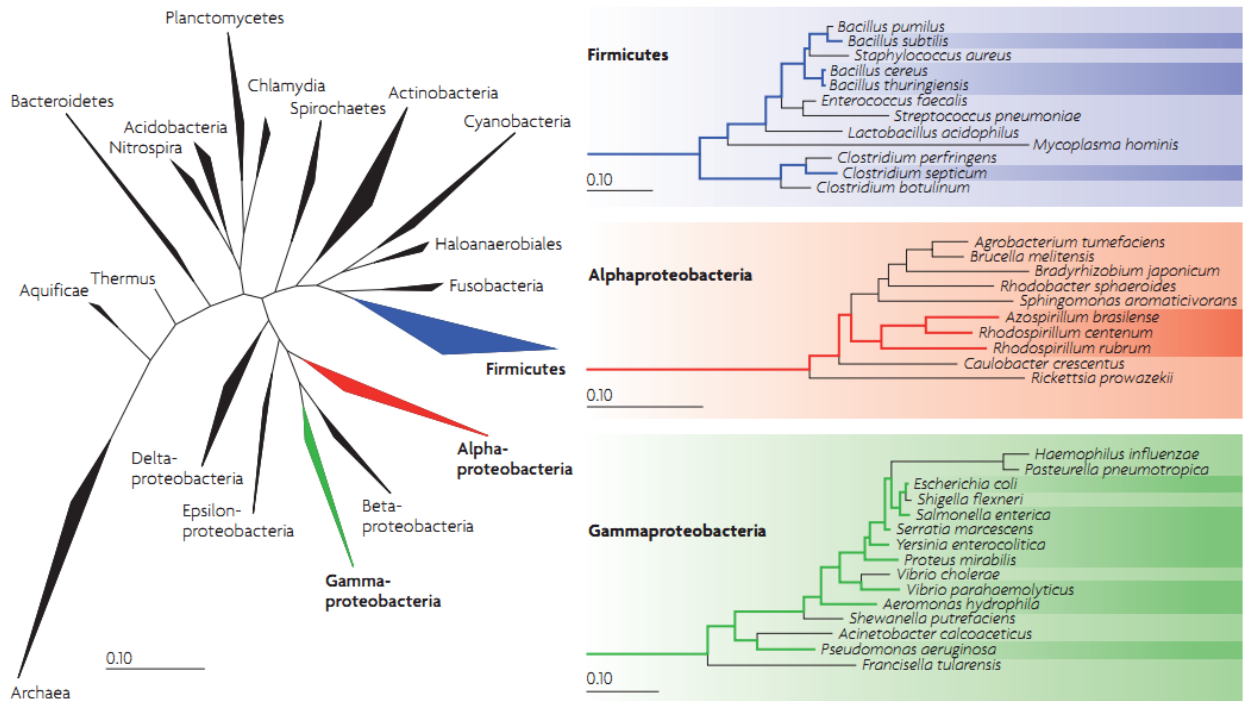

Fig. 4 Phylogenetic distribution of swarming motility. Trees were generated from 1,547 aligned positions using the neighbor-joining algorithm on distances determined under the HKY8F+I+G substitution model in PAUP* v4.0b10. The scale bar represents a distance of 0.1 substitutions per site (Kearns 2010)

they are commonly known for their reduction of surface tensions. Since fatty acids are universal components in all biosurfactants, low molecular weight biosurfactants can primarily be distinguished by their head group. Wetting agents will contain either a glycolipid or lipopeptide which have a carbohydrate or cyclic peptide head group, respectively. Cyclic lipopeptides are of particular interest to human and environmental microbiology since they have antiviral (Vollenbroich et al. 1997; Huang et al. 2006), antibacterial (Huang et al. 2007; Bais et al. 2004), and antifungal properties (Tendulkar et al. 2007). Attributed to peptide charge (Bonmatin et al. 2003) and fatty acid tail length (Kracht et al. 1999), lipopeptides can disrupt lipid membranes and envelopes. When

coupled with the capacity of cells to rapidly colonize surfaces, lipopeptide-producing swarmers may be considered beneficial biocontrol agents or harmful pathogens (Raaijmakers et al. 2010).

Surfactin, produced by *Bacillus subtilis*, is the most potent surfactant produced by swarming bacteria, and has the ability to reduce water surface tension from 72 mN/m to 27 mN/m at a CMC as low as 20 μm (Peypoux et al. 1999). Peptidic construction of the head group is controlled by a non-ribosomal peptide synthetase (NRPS) called surfactin synthetase (encoded by the *srfA* codon), a multimodular enzyme complex that sequentially adds amino acids to a 13 – 15C β-hydroxy fatty acid tail (Fig. 5, A). Lactonization of the terminal amino acid by a thioesterase (TE) to a hydroxyl on the fatty acid results in the cyclic nature of the peptide (Fig. 5, A). Homologs of the *srfA* operon is responsible for production of surfactin variants amongst other members of the *Bacillus* genera (Peypoux et al. 1999) such as pumilacidin in *B. pumilus* (Naruse et al. 1990) and lichenysin in *B. licheniformis* (Yakimov et al. 1998) (Fig. 5A). The amino acid sequence is highly conserved with these cyclic lipopeptides and variations appear to be due to substitutions in residues 2, 4, and 7 (Fig. 5B). However, these other *Bacillus* spp. and their surfactin variants have yet to be associated with swarming motility.

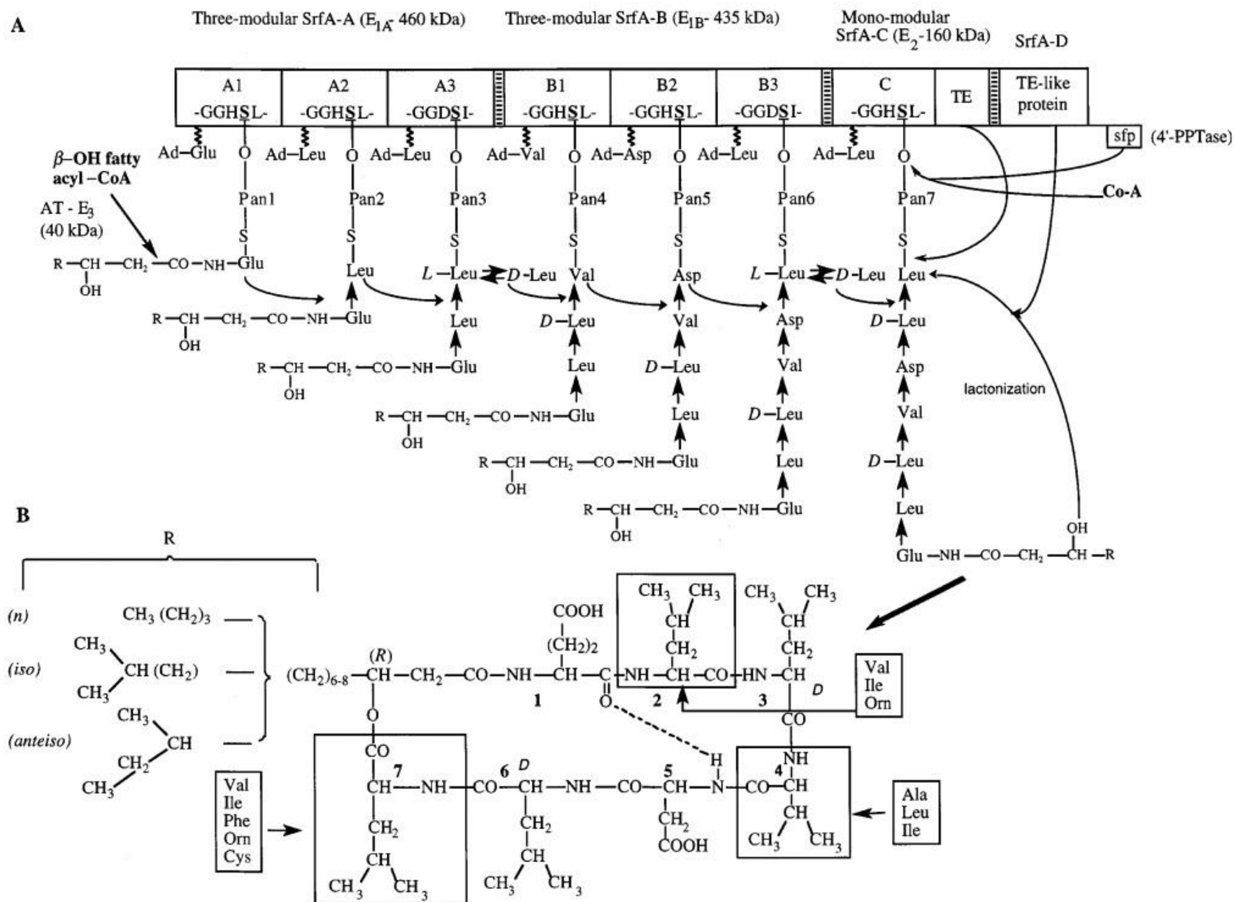

Fig. 5 A) Schematic representation of the surfactin synthetase and of biosynthesis steps. **B)** Primary structure of surfactin (amino acids 2, 4, and 7 are variable according to genetic methods and/or directed biosynthesis) (Peypoux et al. 1999)

Conservation in the chemical structures of lipopeptides has also been observed with *Serratia marcescens* strains. Serratamolide (later reclassified as serrawettin W1), an antibiotic originating from the synthetase encoded by the *swrW* gene was found in pigmented strains of *S. marcescens* NS 38, 274, and ATCC 13380 and is a cyclotetradepsipeptide comprised of alternating serine and β-hydroxydecanoic acid residues (Li et al. 2005; Kiryushkin et al. 1967; Wasserman et al. 1961, 1962; Matsuyama et al. 1985). Nonpigmented strains, however, generate lipopeptides with five amino acid

residues that have differing sequences. Serrawettin W2, for instance, exuded by NS 25 and MG1 strains has the sequence: Leu-Ser-Thr-Phe-Ile (Matsuyama et al. 1992; Eberl et al. 1999). Meanwhile, serrawettin W3, released by strain NS 45 has the sequence: Thr-Ser-Val-Leu-Ile (Matsuyama et al. 1986). An unidentified marine bacterium that can swarm also produces four similar lipopeptides, kailuin A-D, with the conserved sequence: Leu-Ser-Thr-Leu-Ile (Harrigan et al. 1997). Genetic regulation and tertiary structure has yet to be determined for serrawettin W3, but serrawettin W3 is traced back to the *swrA* gene. Swarming-inducible surface conditions are sensed by a flagellar operon *flhDC* that triggers cell differentiation while a density-dependent quorum sensing operon *swrIR* induces *swrA* (Fig. 6) (Eberl et al. 1999; Givskov et al. 1997). This *luxIR*-type quorum sensing system has only been found in proteobacteria, and the biosurfactant-producing *S. marcescens* strain SS-1 keeps its *spnIR* codon on a transposon, suggesting some proteobacteria may share quorum sensing abilities via horizontal gene transfer (Wei et al. 2004; Wei et al. 2006). The betaproteobacterium, *Variovorax paradoxus* EPS has recently been shown to have surfactant-dependent swarming and it may also have an NRPS sharing homology to *swrW* (Jamieson et al. 2009; Han et al. 2011). This may be another example of how structurally similar lipopeptides can be conserved amongst different bacteria.

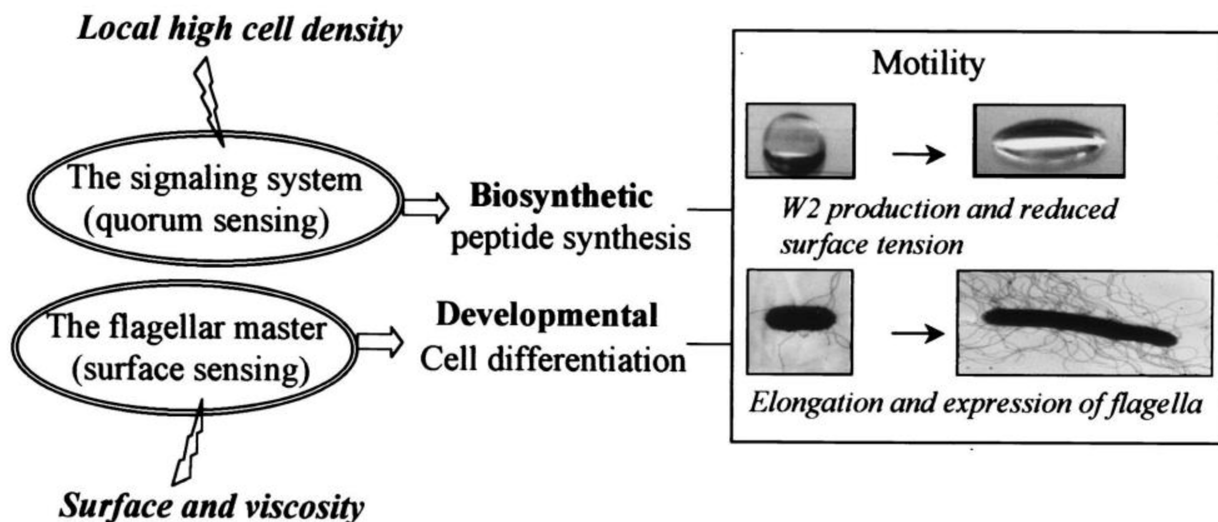

Fig. 6 Quorum sensing schematic of swarming motility within *S. marcescens* (Eberl et al. 1999)

*Variovorax paradoxu*s is a gram-negative soil bacterium known for a variety of traits such as plant growth promotion (Belimov et al. 2001), metal tolerance (Abou-Shanab et al. 2007), and contaminant biodegradation (Snellinx et al. 2003). Although quorum sensing has yet to be observed in *V. paradoxus*, it has the ability to degrade AHLs as a carbon and nitrogen source (Leadbetter and Greenberg 2000). Aside from swarming motility and biosurfactant production, quorum sensing can regulate virulence factors contributing to plant pathogenesis. When living in plant tissues as endophytes,*V. paradoxus* with the capacity to degrade specific AHL targets could behave as quorum quenchers and be employed as biocontrol agents against plant pathogens (Uroz 2003). Recently, *V. paradoxus* has been observed with swarming motility that appears to be dependent on a biosurfactant (Jamieson et al. 2009). As stated earlier, NRPS homology implies the biosurfactant is a cyclic lipopeptide suggesting another resource this endophyte could use in preventing plant pathogenesis. In Chapter Four, I report the

purification and chemical characterization of a biosurfactant that is produced by *V. paradoxus* EPS.

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Chapter Two:

Isolation of Biosurfactant-Producing Bacteria from the Rancho La Brea Tar Pits

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Abstract

This research was conducted to identify culturable surfactant-producing bacterial species that inhabit the 40,000-year-old natural asphalt seep at the Rancho La Brea tar pits in Los Angeles, CA. Using phenanthrene, monocyclic aromatic hydrocarbons, and tryptic soy broth as growth substrates, culturable bacteria from the tar pits yielded ten isolates, of which three species of gamma-proteobacteria produced biosurfactants that accumulated in spent culture medium. Partially purified biosurfactants produced by these strains lowered the surface tension of water from 70 to 35–55 mN/m and two of the biosurfactants produced 'dark' halos with the atomized oil assay, a phenomenon previously observed only with synthetic surfactants. Key findings include the isolation of culturable biosurfactant-producing bacteria that comprise a relatively small fraction of the petroleum-degrading community in the asphalt.

Keywords Asphalt – Biodegradation – Bioremediation – Biosurfactant – Heavy oil – Petroleum – Surfactant

Introduction

Biosurfactants are structurally diverse amphiphiles that are secreted into the environment to facilitate the diffusion and uptake by microorganisms of hydrophobic substances from aqueous media. Currently there is interest in the identification of novel biosurfactants or biosurfactant-producing microorganisms for environmental cleanup and bioremediation (Muthusamy et al. 2008). Biosurfactants serve to isolate individual petroleum molecules in micelles and can differentially affect the bioavailability of petroleum to microorganisms, depending on how the surfactant interacts with the cell envelopes of the broad suite of bacterial species that are present in the oil-degrading community. The frequency of occurrence of biosurfactant producers in petroleum-degrading communities has not been well examined, but such organisms could potentially play a keystone role in shaping the species composition and activity of petroleum-degrading consortia by favoring growth of those bacteria that can access hydrocarbons that are mobilized by the predominant surfactants.

Biosurfactant production is highly variable, depending on the composition of the growth medium that is used to culture the microorganisms. Glucose and glycerol are commonly used to induce surfactant production, which can be detected by measuring changes in the surface tension of the spent medium (Amaral et al. 2009; Batista et al. 2006). Another method for detecting surfactant synthesis uses an assay in which compounds in the spent medium are screened for their ability to collapse water droplets (Bodour and Miller-Maier 1998). This latter assay, while rapid and easy, is limited by the

need for high concentrations of surfactant to generate a positive response (Satpute et al. 2010; Walter et al. 2008). To improve the sensitivity of this assay, Burch et al. (2010) developed a novel approach that relies on the interaction of atomized oil with the amphipathic molecules to generate observable halos. Using these methods, culturable bacteria can readily be screened for production of compounds with surfactant activity.

Biosurfactants play a pivotal role in the biodegradation of hydrophobic aromatic compounds contained in petroleum and heavy oil (Banat et al. 2010), but the extent to which various petroleum-degrading microorganisms produce these substances is still unknown. The frequency of surfactant-producing microorganisms in petroleum degradation has mostly been studied for soils and sediments with recent oil contamination, where 30 % of culturable bacteria typically screen as positive for biosurfactant production (Bayoumi et al. 2010; Yu and Huang 2011). Since long term exposure to petroleum hydrocarbons would be expected to select for evolution of biosurfactant-producing bacteria via horizontal gene transfer and metabolic switching (Brito et al. 2006), natural oil seeps should contain bacteria that produce effective surfactants that can be used by many different petroleum-degrading species that are indigenous to petroleum-dominated habitats.

The research reported here surveyed culturable surfactant-producing bacteria that are present in the Rancho La Brea tar pits in Los Angeles, CA. This natural asphalt seep is approximately 40,000 years old. Prior characterization of this seep showed that the bacterial species inhabiting the site are highly diverse and are comprised of several

hundred species, only a few of which are culturable on agar media (Kim and Crowley 2007). In the present study, we examined surfactant production using a range of media, and the general characteristics of the biosurfactants that could be isolated in spent culture media. This study lays the foundation for future research aimed at understanding how these biosurfactants may influence competition between different species within petroleum-degrading bacterial consortia.

Materials and Methods

Sample Collection

Samples of asphalt-permeated soil from a natural heavy oil seep were collected from Pit 91 of the Rancho La Brea tar pits in downtown Los Angeles. The samples were obtained by scraping away the upper surface with a sterile knife to expose fresh asphalt, after which samples were scooped from the upper 10 cm into sterile 50 ml plastic tubes. A 1-l sample was collected, from which 50 g subsamples were used for experimentation. The samples were then transported to the laboratory and stored at 4 °C until use for screening of biosurfactant production by culturable petroleum degraders.

Extraction of Bacterial Cells from Natural Asphalt

Asphalt (10 g) was added to a 250-ml Erlenmeyer flask containing 90 ml of a modified potassium citrate buffer (Williamson et al. 2011) substituted with sodium citrate and amended with 0.5 % (v/v) Tween 20 and 0.05 M sodium pyrophosphate (Amalfitano and Fazi 2008) and adjusted to a pH of 8. The slurry was shaken for 18 h at 300 rev/min

at 4 °C, after which bacterial cells were separated out from the asphalt-soil mixture using a 60 % (w/v) Nycodenz solution to float the cells above a Nycodenz cushion. The mixtures were centrifuged at 10,000 rev/min for 25 min at 4 °C (Accurate Chemical and Scientific Corp., Westbury, NY, USA). The cells were stored as stock cell suspensions at 4 °C for subsequent culture on different media.

Enrichment Culture of Biosurfactant-Producing Bacteria

Unless otherwise noted, all media were adjusted to a pH of 7.2 and solidified with 1.5 % (w/v) agar (Difco). Heat-tolerant gram-positive species were selected by heating the Nycodenz-prepared cells at 70 °C for 10 min, after which cells were grown in liquid tryptic soy broth (TSB) (Bacto) and were spread on to agar-solidified TSB to isolate single colonies.

Microorganisms capable of growing on monoaromatic hydrocarbons were enriched on Bushnell-Haas agar (Atlas 2005) as previously described (Kleinheinz and Bagley 1997) using benzene, toluene, ethylbenzene, m-xylene, o-xylene, and p-xylene (BTEX) as carbon sources (Sigma-Aldrich, St. Louis, MO, USA; Fisher Scientific, Fair Lawn, NJ, USA). Serial dilutions of Nycodenz-prepared cells (100 μl) were spread onto agar solidified Bushnell-Haas medium in glass Petri plates. BTEX compounds were pipetted on to filter paper discs placed on the inside of the glass lid, after which the plates were inverted to allow growth of cells on the BTEX vapors. Isolates were obtained after incubation at 28 °C for 3–10 days.

Enrichment cultures of bacteria that grew on phenanthrene (PHE) (Eastman Kodak Company, Rochester, NY, USA) were produced in 250-ml Erlenmeyer flasks containing 100 ml of mineral salts medium (MSM) that contained 50 mg l^{-1} PHE as a sole carbon source in MSM medium containing (per liter): KH_2PO_4, 1.36 g; $(NH_4)_2SO_4$, 0.66 g; Na_2HPO_4, 0.42 g; $MgSO_4 \cdot 7H_2O$; and a trace mineral solution, 10 ml. The trace mineral solution contained (mg l^{-1}) $CaSO_4 \cdot 2H_2O$, 250; $FeSO_4 \cdot 7H_2O$, 200; $MnSO_4 \cdot H_2O$, 20; $Na_2MoO_4 \cdot 2H_2O$, 10; $CuSO_4 \cdot 5H_2O$, 31.3; $CoSO_4 \cdot 7H_2O$, 10; and H_3BO_3, 5. Cultures were grown for 10 days with continuous shaking on a horizontal shaker at 150 rev/min at 28 °C. Other enrichment cultures were produced using 5 mg of Pit 91 asphalt-soil that was placed into fresh MSM medium that contained 100 mg l^{-1} PHE and incubated in the same manner. After repeating the process for a third subculture in fresh MSM medium that contained 100 mg l^{-1} PHE, cells were extracted and plated on solidified MSM medium containing a PHE film produced using sublimation (Alley and Brown 2000). Isolates were screened for PHE utilization by production of clearing zones.

Screening for Biosurfactant-Producing Bacteria

Initial screening for biosurfactant production was performed using liquid cultures produced on medium containing (per liter): Na_2HPO_4, 1 g; K_2HPO_4, 1 g; NH_4NO_3, 1 g; $MgSO_4 \cdot 7H_2O$, 1 mM; $CaCl_2 \cdot 2H_2O$, 0.1 mM; glucose, 15 g; and glycerol, 15 ml. For comparison, two surfactant-producing strains and one non-producing bacterial strain were included as controls. The positive controls were *Pseudomonas aeruginosa* PAO1, which produces a rhamnolipid biosurfactant, and *Bacillus subtilis* ATCC 21332, which produces

the biosurfactant, surfactin. Both strains were obtained as gifts from Paul Orwin (California State University, San Bernadino, CA, USA) and have been recently described in the literature (Zheng et al. 2012). *Escherichia coli* DH5α, which does not produce biosurfactant, was used as a negative control. Biosurfactant production by these and the test strains was evaluated using an atomized oil assay (Burch et al. 2010). In brief, plates that contained solidified medium were inoculated with bacterial isolates and sprayed uniformly with a light mineral oil (Fisher Scientific; Catalogue No. BP2629) using a Paasche H airbrush (Paasche Airbrush Company, Chicago, IL, USA) at 10 ψ. After growth at 22 °C for 8 days, observable halos were measured in indirect light from the leading edge of the colony to the edge of the halo.

To obtain larger quantities of biosurfactants, strains that screened positive on agar medium were transferred to liquid cultures where the surfactants could be assayed from the spent medium. Cultures were produced in 250-ml Erlenmeyer flasks containing 30 ml of liquid medium and were grown aerobically in a shaker-incubator at 150 rev/min at 28 °C for 8 days. After incubation, the cells were removed by centrifugation at 10,000 rev/min for 20 min, after which the medium was passed through a 0.2 µM polycarbonate filter. The presence of biosurfactant was detected using the drop collapse method as modified from (Bodour and Miller-Maier 1998) using 6 µl of cell-free supernatant dropped onto 4 µl of 10W40 motor oil (Ace Hardware Corporation, Oakbrook, IL, USA). Droplet formation was compared to that produced using sterile fresh medium. Surface tension was measured for cell-free supernatants by the Du Nouy ring method on a Model 10 tensiomat (Fisher Scientific).

Samples from cultures that had displayed 'dark' halos during the atomized oil assay were re-tested using their cell-free supernatants to confirm that the halos were not produced by diffuse spreading of bacterial cells. For this purpose, 5 µl aliquots of crude cell-free extract from 'dark' halo-producing bacteria and *P. aeruginosa* PAO1, as a representative 'bright' halo-producing bacterium, were dropped onto solidified surfactant screening medium and sprayed with atomized oil to confirm halo type.

Identification of Bacterial Isolates

Colony-PCR (50 µl) of 16S rRNA genes was performed on cell lysis products (PCR-Lyse Solution, Epicentre) from individual cultures using a reaction mixture of nuclease-free water, 1X GoTaq Green Master Mix (Promega, Madison, WI, USA), and 0.2 µM of each primer, 63F and 1387R (Invitrogen) (Marchesi et al. 1998). Amplification was carried out using a MJ Research PTC-200 Thermal Cycler. After a hot start at 94 °C for 1 min, PCR amplification was carried out for 30 cycles at 94 °C for 1 min, 55 °C for min, and 72 °C for 3 min. A final extension step was carried out at 72 °C for 10 min. The PCR products were purified using a MinElute PCR Purification Kit (Qiagen), ligated into pGEM-T vectors (Promega), and transformed into *Escherichia coli* TOP10 competent cells (Invitrogen). Plasmids from clones were purified with Plasmid MiniKit I (Omega Bio-Tek) and 16S rRNA genes were sequenced by the Institute for Integrative Genome Biology (University of California Riverside, Riverside, CA, USA). Sequence similarities to known bacteria were determined using the Greengenes database (DeSantis et al. 2006) and submitted to GenBank for accession numbers (JQ764829–JQ764838). A neighbor-

joining phylogenetic tree was constructed with aligned 16S rRNA gene sequences using 1000 bootstrap replications on Mega5 (Tamura et al. 2011).

Results

Enrichment of Culturable Bacteria

Enrichment cultures produced on BTEX compounds and PHE generated nine isolates. Bacteria isolated using volatile BTEX compounds as carbon sources yielded isolated RB91B, RB91E, RB91F, RB91G, RB91H, RB91J, and RB91K (Table 1). These strains were identified as bacteria similar to *Stenotrophomonas maltophilia* d109, *Stenotrophomonas maltophilia* RBE1CD-58, *Pseudomonas* sp. Z27-2, *Shewanella* sp. MFC_2, *Delftia tsuruhatensis* H1, *Cellulosimicrobium cellulans* NCIMB, and *Sphingobacterium spiritivorum* ATCC 33861. Among these strains, RB91C, RB91H, RB91I and RB91K, produced clearing zones on PHE (Table 1). These strains were closely related to *Stenotrophomonas maltophilia* JCM 1976, *Delftia tsuruhatensis* H1, *Microbacterium* sp. 0702P1-2, and *Sphingobacterium spiritivorum* ATCC 33861, respectively. Among the 4 isolates that grew on PHE, RB91H and RB91K also grew on BTEX compounds. Among the isolates produced by direct culture on TSB, only one strain, RB91D (*Stenotrophomonas maltophilia* PSM-1) produced biosurfactant (Table 1). With respect to their broader phylogeny, 6 strains

Table 1 Culturable strains and their mode of enrichment and capacity to produce surfactants

Strain (GenBank accession number)	Enrichment source	Surfactant production	Closest relative (GenBank accession number)	Similarity (%)
RB91B (JQ764829)	BTEX	+	*Stenotrophomonas maltophilia* d109 (FJ950652.1)	99.87
RB91C (JQ764830)	PHE	–	*Stenotrophomonas maltophilia* JCM 1976 (AB294554.1)	99.86
RB91D (JQ764831)	TSB	–	*Stenotrophomonas maltophilia* PSM-1 (FJ888386.1)	100
RB91E (JQ764832)	BTEX	–	*Stenotrophomonas maltophilia* RBE1CD-58 (EF111112.1)	99.63
RB91F (JQ764833)	BTEX	+	*Pseudomonas* sp. Z27-2 (HM584486.1)	99.73
RB91G (JQ764834)	BTEX	+	*Shewanella sp.* MFC_2 (HM589853.1)	100
RB91H (JQ764835)	PHE; BTEX	–	*Delftia tsuruhatensis* H1 (GQ868495.1)	99.82
RB91I (JQ764836)	PHE	–	*Microbacterium sp.* 0702P1-2 (HM222654.1)	100
RB91J (JQ764837)	BTEX	–	*Cellulosimicrobium cellulans* NCIMB (X79453.1)	100
RB91K (JQ764838)	PHE; BTEX	–	*Sphingobacterium spiritivorum* ATCC 33861 (NZ_ACHA01000008.1)	99.38

(RB91B–RB91G) were gamma-proteobacteria, one (RB91H) was a beta-proteobacterium, 2 were gram-positive actinomycetes (RB91I and RB91J), and one strain (RB91K) was *Sphingobacterium* (Fig. 1).

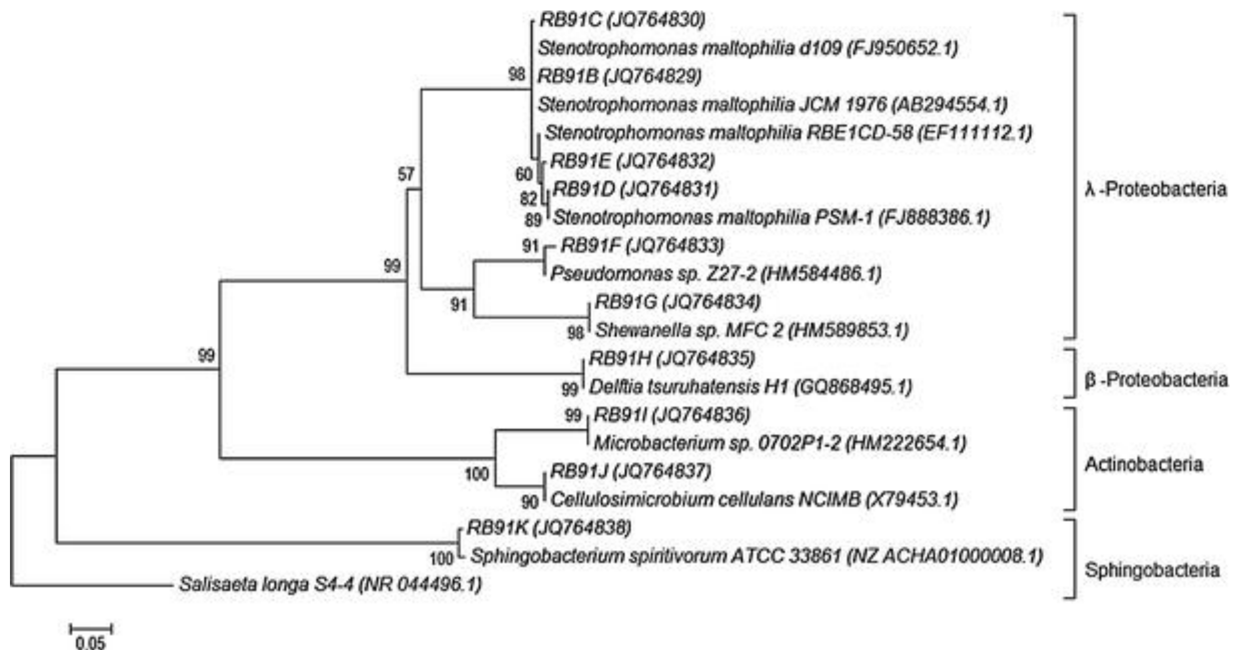

Fig. 1 Phylogenetic tree of ten strains based on their 16S rRNA gene sequences and closest relatives. *Bar* represents 0.05 nucleotide substitutions. Values in the *parentheses* are the GenBank accession numbers

Screening of Biosurfactant-Producing Isolates

Of the ten strains cultured from Pit 91, only three produced biosurfactant in liquid culture (Table 1). In relation to biosurfactant efficacy based on surface tension reduction, the strains ranked as follows: DH5α < RB91G < RB91B < RB91F ≤ ATCC 21332 < PAO1 (Table 2). The surfactants contained in the cell-free supernatants from these cultures were also assayed using the drop collapse method, which confirmed surfactant production by the three isolates from Pit 91, and the two positive control strains (Table 2). Measurements using the du Nouy ring method mirrored those using the atomized oil assay (Table 2, Fig. 2). Biosurfactant produced by RB91B and RB91G both produced 'dark' halos with the atomized oil assay and produced halos ≤0.5 cm (Fig. 3, Table 2).

Table 2 Surfactant presence after 8 days growth on solid agar or in liquid media as assayed by atomized oil spray or drop collapse assay, respectively

Bacterial strain	Atomized oil spray assay (Avg halo (cm) ± SD)	Drop collapse assay	Surface tension (mN/m)
RB91B	+(0.5 ± 0.1)	+	52.7 ± 0.6
RB91F	+(1.1 ± 0.1)	+	35 ± 3.1
RB91G	+(0.3 ± 0.1)	+	54 ± 0.7
Escherichia coli DH5α	–	–	69.8 ± 1.8
Pseudomonas aeruginosa PAO1	+(1.0 ± 0.2)	+	30.3 ± 0.8
Bacillus subtilis ATCC 21332	+(1.1 ± 0.1)	+	34.5 ± 2.1

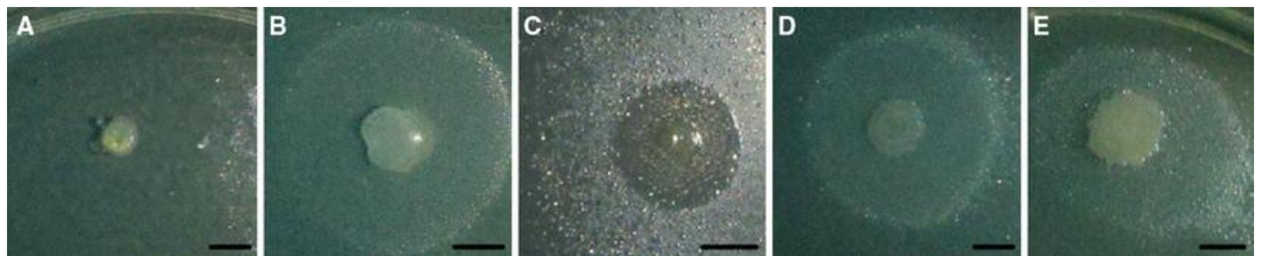

Fig. 2 Biosurfactant production after 8 days growth as indicated by a halo surrounding a colony. **A)** *E. coli* DH5α, **B)** RB91F, **C)** RB91B, **D)** *B. subtilis* ATCC 21332, and **E)** *P. aeruginosa* PAO1. *Bars* represent 0.5 cm

Fig. 3 Atomized oil assay of cell-free extract from **A)** *P. aeruginosa* PAO1, **B)** RB91G, and **C)** RB91B

Discussion

Previous research on the bacterial diversity of the Rancho La Brea tar pits has shown that 16S rRNA gene sequences from this habitat represent at least 300 species of bacteria (Kim and Crowley 2007), of which only a few are culturable on defined media. Here we used a wider range of media than in our original work in an attempt to isolate a broad range of bacteria that could be screened for biosurfactant production. Nonetheless, the vast majority detected using molecular methods remained unculturable and our research yielded only ten distinct strains, of which 3 produced biosurfactants. This is about the same frequency of biosurfactant-producing bacteria reported for soils that have been more recently contaminated by anthropogenic activities and suggest that even in matrices with a long exposure to petroleum hydrocarbons, only a relatively small fraction of the community produces biosurfactants (Bayoumi et al. 2010; Yu and Huang 2011).

Many of the culturable surfactant-producing bacterial species that were identified in the tar pits were similar to those that are commonly identified in petroleum-contaminated soils. This suggests that these bacteria are ubiquitous and/or may reflect the enrichment culture methods that were used to isolate them. Crude oil, which contains BTEX and PHE, is commonly used to isolate petroleum-degrading bacterial strains that may be further screened for biosurfactant production (Bayoumi et al. 2010; Yu and Huang 2011). The culturable petroleum hydrocarbon-degrading strains obtained here represent several taxa, including Sphingobacteria, Actinobacteria and beta-proteobacteria (Fig. 1, Table 1). However, all 3 of the biosurfactant producers identified here belonged

to the gamma-proteobacteria (Table 1), which suggest limited distribution of these genes among bacteria. This is consistent with the observation that biosurfactant production involves operons that encode biosurfactant synthesis via nonribosomal multienzyme peptide synthase complexes (Das et al. 2008), primarily found in pseudomonads, or as indicated here in various species of gamma-proteobacteria that encompasses pseudomonads. It has recently been shown that surfactant-mediated increases in hydrocarbon bioavailability can lead to increased biomass and biodiversity in oil-contaminated sites (Baek et al. 2007; Taccari et al. 2012). This suggests that certain taxa of surfactant-producing species may play unique roles in determining the composition and population sizes of petroleum-degrading species in oil-degrading communities as well as the rate of petroleum degradation.

Among the biosurfactants that were partially purified and characterized in this research, the surfactant produced by RB91F had the highest surface activity as determined by measurements of surface tension. Both measurements of surface tension and results from the atomized oil assay revealed similar activity of the surfactant produced by this bacteria as measured for surfactin produced by *B. subtilis* and rhamnolipid from *P. aeruginosa* (Fig. 2, Table 2). *Pseudomonas* spp. are well known for their potent biosurfactants, especially rhamnolipids (Lang and Wullbrandt 1999). The other two strains, RB91B and RB91G, were tentatively identified as *S. maltophilia* and *Shewanella* sp., respectively. These two strains produced biosurfactants that displayed unique results with the atomized oil assay. This assay relies on the interaction of atomized oil with the amphiphiles, which generates observable ‘light’ or

‘dark’ halos depending on the height of the oil droplets (Burch et al. 2010). The biosurfactants made by these two species produced ‘dark’ halos (Fig. 3) which have never been observed with biosurfactants despite a comprehensive assessment of bacteria from aquatic, soil and plant environments (Burch et al. 2011). Surfactants that produce a ‘dark’ halo may have a large polar head, a feature that could be uniquely characterized with the water–oil interface based atomized oil assay as opposed to the water–air interface based drop collapse assay (Burch et al. 2011). Our data supports the versatility of this relatively new assay and suggests it may be useful for screening for different types of surfactants that segregate similarly according to their efficacy in reducing surface tension: DH5α < RB91G < RB91B < RB91F ≤ ATCC 21332 < PAO1 and their halo characteristics using the atomized oil assay (Table 1).

As bioreactors and other microbial technologies emerge for use in petroleum processing, there is still much to learn about the interrelationships between biosurfactants and petroleum-degrading microbial communities. Biosurfactants may alter the growth, community structure, and degradation efficacy of species within petroleum-degrading consortia. This study lays the foundation for future research employing these strains from the Rancho La Brea tar pits to investigate the extent to which biosurfactants and biosurfactant-producing bacteria influence PAH and petroleum-degrading consortia.

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Chapter Three:

Mineralization of ^{14}C-Phenanthrene Enhanced by Natural Asphalt Microorganisms

Abstract

Contamination of soils by used motor oil introduces low molecular weight polycyclic aromatic hydrocarbons (PAHs) into the environment where these chemicals pose a long term hazard to human and environmental health. While many bacteria are able to degrade PAHs, the the hydrophobic nature of these chemicals results in low water solubility and partitioning on mineral and organic matter surfaces where they are inaccessible for use as growth substrates. In previous work, we isolated a bioemulsifier-producing bacterium, *Pseudomonas* sp. st. RB91F from a natural asphalt seep, which is capable of emulsifying petroleum compounds. In this research, we examined the mechanisms by which this bioemulsifier affected PAH degradation by the PAH-degrading bacterium *Delftia tsuruhatensis* st. RB91H. Research characterizing the bioemulsifier showed that it was a polymeric surface active agent comprised of lipid, protein, and carbohydrate, and that at the critical micelle concentration (CMC), it could reduce the surface tension of pure water to 36 ± 0.4 mN/m. Microcosm experiments were conducted in sterile soil that was artificially contaminated with used motor oil spiked with 100 mg l^{-1} ^{14}C phenanthrene. The microcosms were then amended with bioemulsifier at concentrations above, at, and below the CMC and inoculated with *Delftia tsuruhatensis* RB91H. The results showed that mineralization of ^{14}C-phenanthrene was dependent on the bioemulsifier concentration, with the greatest amount of disappearance

at the CMC. A faster rate of disappearance after onset of PAH disappearance further suggested growth-linked degradation occurs at sub-CMC concentrations. In other experiments with pure cultures, cell surface hydrophobicity was shown to be decreased by the biosurfactant, which suggested that direct micellar uptake via hydrophilic interactions. Various other mechanisms were also suggested from data obtained at concentrations above the CMC. The results demonstrate complex interactions between a bioemulsifier and PAH-degrading microorganism that are dependent on the bioemulsifier concentration.

Keywords Bioemulsifier – Mineralization – Phenanthrene – Natural asphalt

Introduction

Production of biosurfactants greatly increases the desorption of hydrophobic petroleum hydrocarbons from soil particle surfaces, but thereafter leads to differential availability of the mobilized hydrocarbons to microorganisms that vary in their abilities to acquire hydrocarbons from the surfactant. One of the most important mechanisms appears to involve modification of the cell surface when amphiphilic biosurfactant molecule adhere to the cell envelope and facilitate transport of the substance into the cell membrane. However, bioavailability is also affected by the surfactant concentration. As biosurfactant concentrations increase in solution, the biosurfactant molecule form micelles that can result in encapsulation of the aromatic hydrocarbons in the interior of the micelles. This can result in both increased or decreased bioavailability to different species of PAH-degrading bacteria. In some cases, biosurfactants can be used as carbon substrates for growth, resulting in growth-linked biodegradation. For these reasons, a better understanding of the interactions between biosurfactants and PAH-degrading bacteria is important for optimizing the use of these compounds or inoculation with biosurfactant-producing microorganisms for bioremediation of contaminated soils.

As bioremediation technologies are being developed that incorporate the use of biosurfactants, novel habitats are being explored for bioprospecting microorganisms that produce these compounds. There is also interest in discovery of novel bacterial strains that are especially efficient in degrading recalcitrant petroleum hydrocarbons such as PAHs that are of concern as environmental contaminants (Shete et al. 2006; Cameotra

and Bollag 2003). To this end, natural asphalt seeps represent some of the most promising habitats for isolating new microorganisms that are capable of biosurfactant production and degrading PAHs (Schulze-Makuch et al. 2011; Kim and Crowley 2007). Microorganisms that have adapted to asphalt-permeated soil may require biosurfactants to gain access to recalcitrant petroleum substrates. Although it might initially be expected that most petroleum-degrading bacteria would produce biosurfactants, prior research has shown that only about 30% of culturable petroleum-degrading bacteria produce biosurfactants. This function also appears to be further limited to discrete taxonomic groups that use a nonribosomal process for synthesis of the enzymes that produce biosurfactants. Recently, we have identified culturable bacteria from the Rancho La Brea tarpits that have been shown to express both biosurfactant production and enzymes facilitating aromatic hydrocarbon degradation (Belcher et al. 2012). In this research, we were interested in the interactions of this biosurfactant with other bacteria that do not produce these compounds. The overarching hypothesis is that biosurfactant-producing bacteria may function as keystone organisms in petroleum-degrading bacteria consortia, and that the structure and composition of these communities may be influenced by the types of surfactants that are produced and their concentrations in the soil solution. There are two major types of biosurfactants produced by microorganisms classified as low molecular weights typically comprising a head and tail moiety or high molecular weights called “bioemulsifiers” that are polymeric. Here, we examined the interactions between the bioemulsifier that is produced by *Pseudomonas* sp. RB91F and the petroleum-degrading bacterium *Delftia tsuruhatensis* RB91H, both of which were isolated from the

Rancho La Brea tarpit. The research specifically focused on the effects of bioemulsifier concentration on the rate of mineralization of the PAH, phenanthrene, as a contaminant spiked into soil contaminated with used motor oil. Additional *in vitro* experiments examined changes in the cell surface hydrophobicity of the PAH-degrading bacterium to discern possible mechanisms by which the bioemulsifier affected uptake and use of phenanthrene.

Materials and Methods

Bacteria

Two bacterial strains that had been previously cultured from the tarpits and identified (Belcher et al. 2012) were used in this study. These included *Pseudomonas* sp. RB91F (JQ764833) which had been shown to produce a bioemulsifier; and *Delftia tsuruhatensis* RB91H (JQ764835), which had been shown to grow on phenanthrene (PHE) as a sole carbon source.

Partial Purification and Surface Activity Measurements of Bioemulsifier

All media were adjusted to pH 7.2 unless otherwise noted. Bioemulsifier production by *Pseudomonas* sp. RB91F was induced by growth in a medium previously described (Belcher et al. 2012). After 2 days incubation in a shaker (150 rev/min; 28 °C), partial purification was carried out by spinning down the cells at 10,000 rev/min for 20 min and filtering through a 0.2 μm polycarbonate filter. Cell-free supernatant was lowered to a pH of 2 and acid precipitated overnight at 4 °C. Solvent extraction with

chloroform:methanol (2:1, v/v) was carried out three times, using centrifugation at 10,000 rev/min for 20 min at 4 °C to separate and partially purify the bioemulsifier. Rotary evaporation (Brinkmann Instruments) removed the organic solvents and after resuspension of sample, lyophilization (Labconco) created a partially purified product. Samples were stored at -80 °C until further use.

Triplicate samples were assayed to measure the ability of the bioemulsifier to reduce the surface tension of pure water and to determine the critical micelle concentration (CMC). Surface tension measurements were conducted sued the du Nouy ring method on a Model 10 tensiomat (Fisher Scientific). Emulsification was determined using kerosene, *n*-hexane, toluene, gasoline, and xylene according to a method described previously (Cooper and Goldenberg 1987). After vortexing samples for 2 min and let rest for 24 h, the emulsification index (E_{24}) was calculated by dividing the height of the emulsion layer by the total volume.

Determination of Macromolecular Composition of Bioemulsifier

The orcinol assay (Koch et al. 1991) was used to measure carbohydrate content using glucose as a standard. Measurement of free fatty acids elucidated lipid content with oleic acid as a standard (Sadasivam and Manickam 1996). Protein content was measured using the Lowry method with bovine serum albumin as the standard (Lowry et al. 1951).

Thin layer chromatography (TLC) also analysed the sample for macromolecule composition. For protein determination, TLC was developed in *n*-butanol:acetic acid:water (60:30:10, v/v/v), sprayed with ninhydrin (0.1 % w/v in 95 % ethanol), and

heated at 110 °C for 5 min. Fatty acid composition was determined by developing TLC in chloroform:methanol:water (65:25:4, v/v/v), spraying with 2’7’-dichlorofluorescein (0.1 % w/v in 95 % ethanol), and heating at 110 °C for 20 min. TLC was developed in chloroform:acetic acid:water (60:30:10, v/v/v) for carbohydrate analysis and then sprayed with 90 % phenol:sulfuric acid:95 % ethanol (3:5:95, v/v/v) and heated at 110 °C for 15 min.

Partially purified product (1-2 mg) was mixed with KBr (200 mg) and analysed by fourier transform infrared spectroscopy (FTIR) on an Equinox 55 FTIR (Analytical Chemistry Instrumentation Facility at University of California, Riverside, CA, USA).

Mineralization of ^{14}C-PHE

A respirometer developed from Reid et al. (2001) was used to monitor mineralization of ^{14}C-PHE. To capture $^{14}CO_2$ released during mineralization of ^{14}C-PHE, 1 ml of 1 M NaOH was added to a 7 ml scintillation vial and suspended by an alligator clip hanging above the slurry attached to a Teflon plug. The experimental setup consisted of a 250-ml Erlenmeyer flask containing 1 ml of used motor oil to which 0.15 µCi of ^{14}C-PHE and 100 mg l^{-1} of ^{12}C-PHE dissolved in 500 µl of acetone was added. After evaporation of acetone, 9 g of a sterile loam soil (pH 7.7, 1% organic carbon) taken adjacent to Pit 9 at the Rancho La Brea tarpits was added. M9 media (Atlas 2004) containing bioemulsifier partially purified from *Pseudomonas* sp. RB91F created three treatments ranging from sub-CMC, at CMC, and supra-CMC. A fourth treatment containing no surfactant acted as a control. 30 ml of the M9 treatments were added to

each flask. *D. tsuruhatensis* RB91H was suspended in PBS to measure OD_{600} = 0.1 and diluted 1:100. 900 µl of the dilution was inoculated into the flasks and incubated in a shaker at 150 rev/min at 28 °C. The experimental treatments were performed with five replicates. During sample collection, 5 ml of Ultima Gold scintillation cocktail (PerkinElmer, Waltham, MA, USA) was mixed with the NaOH solution and fluorescence measured on a scintillation counter. Fresh NaOH solution was immediately replaced with a new vial during sampling.

Assessment of Bioemulsifier Production by D. tsuruhatensis RB91H

Bioemulsifier production was measured for cell cultures grown in soil microcosms consisting of a 250-ml Erlenmeyer flask, 10 g of sterile loam soil and 27 ml of M9 medium that contained 100 mg l^{-1} ^{12}C-PHE. After overnight growth of *D. tsuruhatensis* RB91H in tryptic soy broth, cells were spun down at 10,000 rpm for 20 min, washed twice with PBS, equilibrated to an OD_{600} = 0.1 (late log phase), and inoculated (3 ml) in triplicate to the slurries. Controls were created in triplicate by inoculation of soil slurries with 3 ml of sterile PBS. During 6 days incubation in a rotary shaker, 10-ml samples were taken every 2 days and centrifugation followed by filtration removed cells and sediment. Measurements of the ability of surface tension reduction in the supernatant were conducted with a tensiometer, and were compared to control microcosms that had not been inoculated to determined if growth on PHE induced biosurfactant production by *D. tsuruhatensis* RB91H.

Growth of D. tsuruhatensis RB91H on Bioemulsifier Produced by Pseudomonas sp. RB91F

The ability of the PAH-degrading bacterium, *D. tsuruhatensis* RB91H to grow on the bioemulsifier was examined using microcosms consisting of 50-ml Erlenmeyer flasks containing 30 ml of M9 medium and four concentrations of partially purified bioemulsifier produced by *Pseudomonas* sp. RB91F (no surfactant, sub-CMC, at CMC, and supra-CMC). The measurements were performed for triplicate microcosms per treatment. *D. tsuruhatensis* RB91H was inoculated (500 µl, $OD_{600} = 0.1$) and the samples were incubated for 3 days in the rotary shaker. Every day, 3 ml of sample was removed and growth was monitored at OD_{600} to determine if *D. tsuruhatensis* RB91H can grow on bioemulsifier as a sole carbon source.

Microbial Adhesion to Hydrocarbons (MATH) Assay

The mineralization experimental setup in 250-ml Erlenmeyer flasks was repeated with the exception of soil, used motor oil, and ^{14}C-PHE amendments. *D. tsuruhatensis* RB91H was grown overnight in M9 medium containing 100 mg l^{-1} ^{12}C-PHE and the four levels of bioemulsifier (no surfactant, sub-CMC, at CMC, and supra-CMC). Cells were centrifuged down, washed twice, and resuspended in PUM buffer (Pijanowska et al. 2006) at an $OD_{600} = 1$. MATH assay was then performed as previously described (Rosenberg et al. 1980) to determine the hydrophobicity of the cell surface of *D. tsuruhatensis* RB91H as affected by PHE and/or bioemulsifier presence.

Results and Discussion

Recently, *Pseudomonas* sp. RB91F was shown to produce a bioemulsifier that was partially characterized in the current study. Colorimetry showed the surface active agent to contain 8.5 % carbohydrate, 5.4 % protein, and 23.9 % lipid. TLC developed with ninhydrin revealed four spots with Rf values of 0.1, 0.46, 0.6, and 0.7. Fatty acid analysis on TLC showed two spots with Rf values of 0.7 and 0.9. We were unable to detect carbohydrate on TLC, but previous TLC analysis with bioemulsifier comprised of the same macromolecules had the same result (Suthar et al. 2008). Altogether, the analyses suggested the surfactant may be a high molecular weight bioemulsifier that is a complex of carbohydrate, lipid and protein. FTIR analysis (Fig. 1) also confirmed the

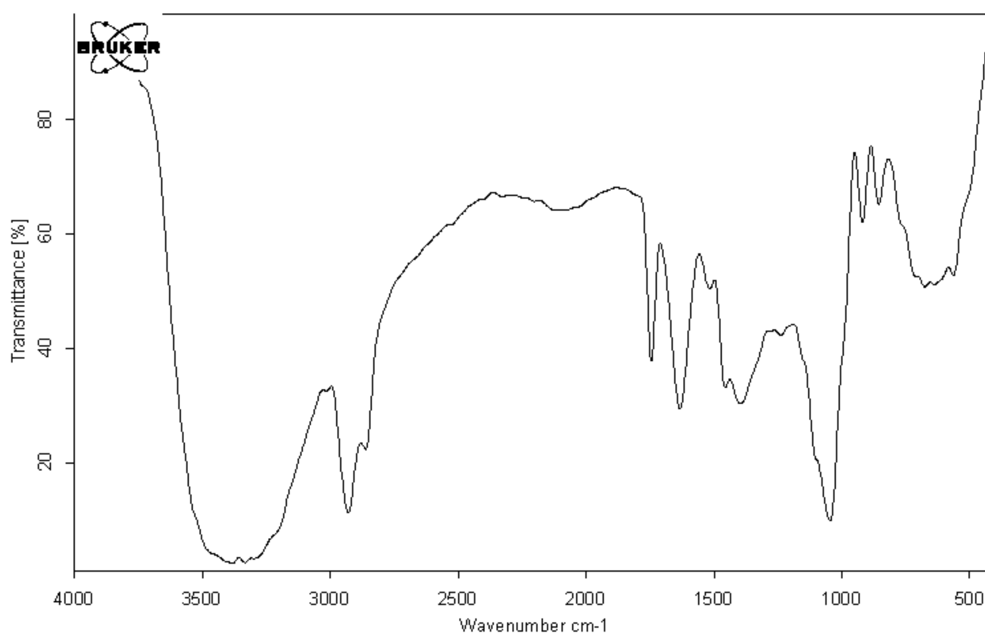

Fig. 1 FTIR spectrum of bioemulsifier from *Pseudomonas* sp. RB91F

presence of lipid, carbohydrate, and protein. A strong, broad peak between 3550-3200 cm^{-1} suggested an H-bonded –OH representative of the hydroxyls found on carbohydrates. Two peaks between 3000-2850 cm^{-1} were indicative of alkanes associated with the lipid component. The C=O (1740-1720 cm^{-1}) and –CO (1100-1040 cm^{-1}) peaks may represent carboxylic acid found at the terminus of fatty acid moieties. Amine presence was evident with the C=O amide I band (1695-1630 cm^{-1}). It appeared that *Pseudomonas* sp. RB91F produced a protein-carbohydrate-lipid complex. *Pseudomonas* spp. are capable of producing low molecular weight biosurfactants such as glycolipids (Lang and Wullbrandt 1999; Wu et al. 2008; Abouseoud et al. 2008), or lipopeptides (Nielsen et al. 1999; Saini et al. 2008) as well as the high molecular weight biosurfactants often referred to as bioemulsifiers (Bonilla et al. 2005; Freitas et al. 2009). Bioemulsifiers are complex polymers well known for emulsification properties useful in mobilization of hydrophobic compounds into the aqueous phase (Ron and Rosenberg 2001). The polymeric bioemulsifier was able to emulsify a variety of petroleum hydrocarbons such as kerosene (E_{24} = 40 ± 5 %), *n*-hexane (E_{24} = 49.3 ± 4.3), gasoline (E_{24} = 55.9 ± 2.4), toluene (E_{24} = 39.5 ± 3.5), and xylene (E_{24} = 59.0 ± 1.8). Emulsification was not apparent with other nonaqueous liquids that are not petroleum based implying the bioemulsifier is selective for emulsifying only petroleum compounds. The emulsifier was also able to reduce water's surface tension from 72 mN/m to 36 ± 0.4 mN/m at a CMC of 925 mg/L (Fig. 2). Reduction in surface tension and emulsification could be critical in the enhancement of PHE mineralization by a degrading bacterium also isolated from the tarpits.

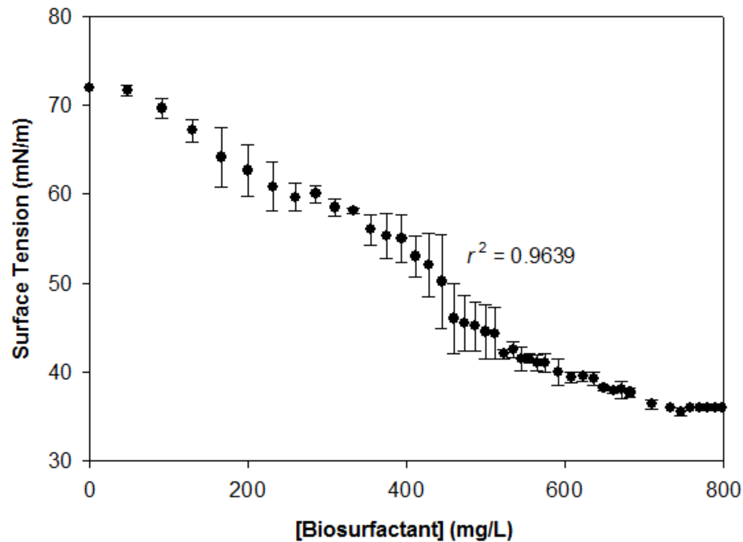

Fig. 2 Surface tension reduction of bioemulsifier produced by *Pseudomonas* sp. RB91F

In addition to their ability to degrade phenanthrene (Vacca et al. 2005; Chen and Hickey 2011), *Delftia* spp. are well known for their degradation of anilines (Zhang et al. 2010; Liu et al. 2002) and herbicides (Westendorf et al. 2003; Gonzalez et al. 2012). Although much has been discussed about the degradative properties of *Delftia* spp., few studies have examined the mechanisms by which enhanced degradation occurs in the presence of bioemulsifier. In the research reported here, we observed that when *Delftia tsuruhatensis* RB91H was provided with bioemulsifier, the rate of mineralization of ^{14}C-PHE appeared to be dependent on the concentration of the bioemulsifier (Fig. 3). Compared to a sterile control, *D. tsuruhatensis* RB91H was only able to reduce surface tension by 4 ± 0.5 mN/m when growing on PHE so the only biosurfactant present should be the bioemulsifier produced by *Pseudomonas* sp. RB91F. When bioemulsifier is present, mineralization began at 46 h, but was delayed until 65 h in its absence, suggesting the

bioemulsifier promotes more rapid degradation. Initially, up until 19 h, there was no disappearance of ^{14}C-PHE in all treatments (data not shown), suggesting a lag period. This is in agreement with prior observations in which bacteria acclimate to the presence of phenanthrene, which is dispersed in the hydrophobic phase presented by the used motor oil (Alexander 1999). In relation to total mineralization based on biosurfactant concentration, the treatments ranked as follows: no surfactant < supra-CMC < sub-CMC < at CMC (Fig. 3). The mass transfer of ^{14}C-PHE out of the used motor oil appears to be optimal at the CMC level of bioemulsifier when micelles enhance bioavailability for microbial uptake (Brown 2007; Guha and Jaffe 1996). Findings, however, suggest that there are unique mechanisms at play at the various levels of bioemulsifier concentration.

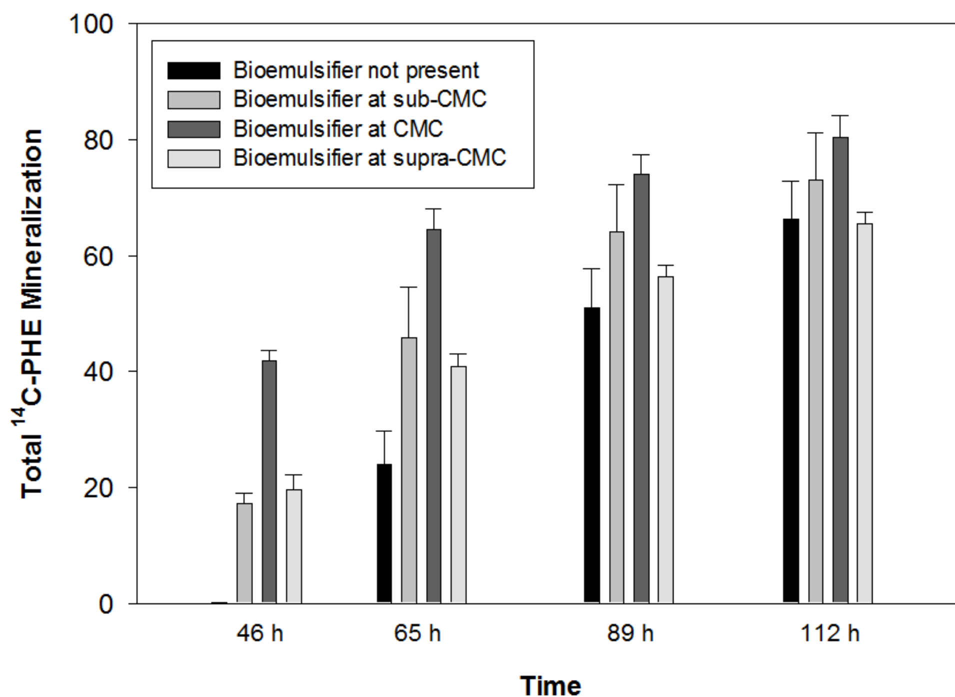

Fig. 3 Total mineralization of ^{14}C-PHE into $^{14}CO_2$ by *D. tsuruhatensis* RB91H in the presence of different levels of bioemulsifier (no surfactant, sub-CMC, at CMC, and supra-CMC). The four mineralization treatments were significantly different ($P < 0.001$; ANOVA)

After initial onset of degradation in all treatments, mineralization increased by one-fold or less except for the treatment at sub-CMC which had a two-fold increase (Fig. 3). Assimilation of ^{14}C-PHE and bioemulsifier as carbon substrates may elevate cell density resulting in a more rapid rate of ^{14}C-PHE mineralization, suggesting growth-linked metabolism may be the major degradation route at sub-CMC (Alexander 1999). The cell surface hydrophobicity of *D. tsuruhatensis* RB91H may also play a role at the varying levels of bioemulsifier. In the absence of bioemulsifier the cell wall was hydrophobic (47 %), and in the presence of bioemulsifier it was hydrophilic (sub-CMC: 13 %; at-CMC: 5 %; and supra-CMC: 16 %). When only in the presence of PHE, *D.*

tsuruhatensis RB91H has a relatively hydrophobic surface as it may be responding to the presence of the hydrophobic contaminant (Prabhu and Phale 2003). Addition of bioemulsifier into the system lowers the cell surface hydrophobicity which may be due to hydrophobic moieties coating the cell surface and exposing hydrophilic moieties (Neu 1996; Zhao et al. 2011). Attachment of bioemulsifiers leading to reduction of cell surface hydrophobicity can hinder biodegradation of hydrophobic substrates (Rosenberg et al. 1983), but in this study cell surface hydrophobicity of *D. tsuruhatensis* RB91H appears to be inversely related to PHE mineralization. Micelles with a hydrophilic exterior are most likely attracted to the reduced cell surface hydrophobicity allowing for direct attachment to the cell envelope and release of entrapped PAHs into the microorganism (Bouchez-Naitali et al. 1999). This may be the primary mode of ^{14}C-PHE uptake at CMC when the cell surface is at the highest hydrophilicity. Growth on the bioemulsifier as a sole carbon substrate was shown to be significantly different to the control, but surfactant-containing treatments were not significantly different from one another (data not shown). Since growth was not inhibited it appears evident that the bioemulsifier is not toxic to the bacterium. A decreased mineralization rate at supra-CMC compared to at CMC and sub-CMC may be due to elevated concentration of bioemulsifier being used as a preferred substrate or PHE reaching toxic levels as a result of micellar solubilization (Mougin 2002; Bramwell and Laha 2000; Rouse et al. 1994). Other possible explanations is that ^{14}C-PHE is sorbed onto admicelles bound to used motor oil (Noordman et al. 2000; Kord and Khaledi 1992) or irreversibly locked up within the micellar core (Wong et al. 2004; Whang et al. 2008).

Future research seems warranted with bioemulsifier-enhanced degradation of PAHs with *D. tsuruhatensis* RB91H. Studies should further investigate mechanistic effects and implementation of the degrader/bioemulsifier combination in more complex soil systems to determine impact of competition and physicochemical interactions with soil particles.

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Chapter Four:

Characterization of a Lipopeptide Biosurfactant Produced by *Variovorax paradoxus* EPS

Abstract

Swarming motility in gram-negative bacteria such as *Serratia marcescens* is mechanism for rapid translocation of bacterial cells across surfaces that is facilitated by a lipopeptide biosurfactant based wetting agent that is secreted by bacterial cells. The biosurfactants that mediate this process in *S. marcescens* are classified as serrawettins. Here we examined a similar compound that is produced by swarming colonies of *Variovorax paradoxus* EPS. Using tensiometry, the surfactant produced by *V. paradoxus* was shown to lower surface tension reduction of pure water to 30 ± 0.5 mN/m, and to have a critical micelle concentration of 215 mg l^{-1}. The biosurfactant, termed variowettin, yielded a positive reaction using the drop collapse assay and produced a 'bright' halo for the atomized oil assay. Akin to serrawettins, emulsification was absent with variowettin solutions. Based on results of colorimetric assays, Fourier transform infrared spectroscopy, and thin layer chromatography, variowettin was further characterized as a lipopeptide. According to tandem mass spectrometry, variowettin has a peak of *m/z* 679.3 which appears to be different than the known serrawettins. Altogether, the results suggest that variowettin may be classified within the serrawettin class of lipopeptide wetting agents.

Keywords Wetting agent – Lipopeptide biosurfactant – Surface translocation

Introduction

Biosurfactants are amphipathic molecules that are produced by bacteria to facilitate the interaction of the cell surface with hydrophobic environments or interfaces. A particular function of some surfactants is their ability to facilitate the rapid movement of cells across surfaces via a process called swarm motility. Among some of the most studied classes of biosurfactants are those based on cyclic lipopeptide molecules, which have a hydrophobic moiety comprised of non-polar amino acids and hydroxy fatty acids and a polar region that consists of a short chain of amino acids that are hydrophilic. Serrawettins are a group of lipopeptide biosurfactants released in the wetting agent that are produced by the swarm cells of *Serratia marcescens* that under morphological differentiation in order to move rapidly across air water interfaces associated with solid or semi-solid surfaces. This strategy is thought to serve as a means for the bacteria to rapidly dominate a potential resource that is then colonized by slower growing planktonic cells that fill in the regions behind the swarm front (Matsuyama et al. 2011). The surface activities of lipopeptides increase expansion of the wetting agent and allow bacteria to move rapidly along substrates in highly coordinated rafts (Bees et al. 2000; Eberl et al. 1999). This specialized surface translocation, termed swarming, allows for colonization of water-repelling surface habitats (Matsuyama and Nakagawa 1996). Lipopeptide production is also widespread in other bacteria where it is frequently associated with this type of multicellular surface behavior.

The first lipopeptide biosurfactant to be characterized was surfactin, which is still considered to be one of the most potent surface active agents in this class of molecule. As with other lipopeptides that have been described for strains of *Bacillus* spp., *Pseudomonas* spp., and *Serratia* spp., their synthesis is unique in that the enzymes that produce these compounds are proteins that are not constructed via normal processes involving ribosomes. These enzymes are known as nonribosomal peptide synthetases (NRPSs) (Raaijmakers et al. 2010), and so far have been shown to occur in relatively few bacterial taxa. The synthesis of NRPS is further thought to be regulated by cell density-dependent signals involving quorum sensing, which precede cell differentiation and swarming motility (Daniels et al. 2004). Signal molecules involved in quorum sensing may also stimulate biofilm formation in which immobile assemblages of cells are cocooned in a protective mucoid extracellular polysaccharide (EPS) that forms a matrix around clusters of swarm cells known as rafts (Frederick et al. 2011).

The recent discovery of gene sequences encoding putative NRPSs in the bacterium *V. paradoxus* that share homology with the serrawettin NRPSs has suggested that this bacterium may also produce a lipopeptide surfactant (Han et al. 2011). *V. paradoxus* EPS was named for its ability to produce an EPS that results in mucoid colonies. The EPS provides a porous matrix that enables passive diffusion of signal molecules and further functions for bacterial attachment to surfaces (Pehl et al. 2012). As with *Serratia*, swarming motility is observed for colonies of *Variovorax paradoxus* EPS when grown on agar media, which is accompanied by the production of a wetting agent production that appears to contain a biosurfactant (Jamieson et al. 2009). Our aim was to

confirm the presence of a biosurfactant within the wetting agent secreted by *V. paradoxus* EPS. We hypothesized that the biosurfactant produced by *V. paradoxus* EPS is chemically similar to the serrawettin class of cyclic lipopeptides. To assess the wetting agent with respect to surface activity, the wetting agent was characterized with respect to its ability to lower the surface tension of water, emulsification of oil, and by drop collapse and atomized oil assays. The chemical composition was further described using colorimetry, thin layer chromatography, Fourier transform infrared spectroscopy, and tandem mass spectrometry.

Materials and Methods

Microorganisms and their Growth Conditions

Variovorax paradoxus EPS (NC_014931.1) is a soil bacterium collected by Paul Orwin and has been described in a previous publication (Jamieson et al. 2009), *Serratia marcescens* MG1 (AY498856.1) was a gift from Michael Givskov (University of Copenhagen) and *S. marcescens* NS 45 was a gift from Jun-ichi Wakita (Chuo University).

Optimal production of the wetting agent produced by *V. paradoxus* EPS was observed on agar medium containing 0.5 % (w/v) yeast extract (YE) (Bacto) solidified with 0.5 % (w/v) agar (Fisher Scientific). The medium was allowed to dry open-faced for at least 20 minutes and spot inoculated from freshly grown bacterial isolates. Wetting agent was produced by *S. marcescens* MG1 on AB minimal medium as described by Lindum et al. (1998). The medium was supplemented with 0.5 % (w/v) peptone (Bacto)

and solidified with 0.5 % (w/v) agar (Bacto). Wetting agent produced by *S. marcescens* NS 45 was collected from cells grown on medium containing 0.5 % (w/v) peptone (Bacto), 1 % (w/v) glycerol (Fisher Scientific) and solidified with 0.5 % (w/v) agar (Bacto). All media were adjusted to pH 7.2. Samples collected from *V. paradoxus* EPS, *S. marcescens* MG1, and *S. marcescens* NS 45 were designated variowettin, serrawettin W2, and serrawettin W3, respectively. Controls in which wetting agent production was inhibited were generated by culture of the bacteria on identical media, but solidified with 1.5 % (w/v) agar to produce a harder, drier surface. Samples were collected from plates that were grown for 1-4 days in incubators maintained at 28 °C and 30 % RH. As observed previously with the serrawettins (Matsuyama et al. 2011), wetting agent production was observed by growing *V. paradoxus* EPS samples at 37 °C.

Partial Purification of Wetting Agent

Samples were combined from 60 plates recovered in 10 ml of Milli-Q water per plate. Cells were removed by centrifugation at 13,000 rev/min for 20 min, after which the supernatant was sterilized by filtration through a 0.2 µm pore filter (Millipore). Surfactant was extracted from the cell-free supernatant by lowering the pH to 2 using HCl, which causes the surfactant to precipitate from solution. After overnight incubation at 4 °C, the precipitate was extracted three times by centrifugation (13,000 rev/min, 4 °C, 20 min) with chloroform:methanol (2:1, v/v). The partially purified product was concentrated by rotary evaporation (Brinkmann Instruments) and dried by lyophilization (Labconco). Samples were stored at -80 °C until further use.

Screening Assays for Biosurfactant Presence in Wetting Agent

Surface Activity and Critical Micelle Concentration (CMC) Determinations

Surfactant properties of the partially purified products were determined by measuring the ability of different amounts of the substances to lower the surface tension of pure water using the du Nouy ring method with a Model 21 tensiomat (Fisher Scientific). CMC values were calculated by plotting surface tension against the logarithmic scale of the biosurfactant concentration. The intersections at which best fit lines representing the values below and above the lowest observed change in surface tension were used to determine the CMC values.

Drop Collapse Assay

The drop collapse method was used to confirm biosurfactant activity. The assays were carried out using the polystyrene lid of a 96-microwell plate (Sterilin Limited, Newport, UK). The qualitative assay was modified from Bodour et al. (2003) using 6 µl of sample dropped onto 4 µl of motor oil. MilliQ water was used as a negative control.

Atomized Oil Assay

The atomized oil assay was developed from the method described by Burch et al. (2010) to detect biosurfactant production. *V. paradoxus* was freshly inoculated (5 µl) onto 0.5 % and 1.5 % (w/v) agar containing 5 g l^{-1} YE. After 24 h growth, the plates were sprayed uniformly with a light mineral oil (Fisher Scientific) with a Paasche H airbrush

(Paasche Airbrush Company, Chicago, IL) at 10 ψ. Halos were observed in indirect light, and measured from the leading colony edge to the halo edge.

Emulsification Assay

Emulsification assays were performed according to Cooper and Goldenberg (1987) with kerosene, n-hexadecane, and olive oil. Measurements were taken with biosurfactant containing solutions over a range of concentrations and pH values (6 – 8).

Characterization of Biosurfactant

Colorimetric Analysis

Colorimetric analyses were used to determine the chemical composition of the partially purified products. Lipid content was based on detection of free fatty acids with oleic acid as a standard (Sadasivam and Manickam 1996). Protein content was measured using the Lowry method with bovine serum albumin as the standard (Lowry et al. 1951). Carbohydrate content was determined with a phenol-sulfuric acid assay using glucose as the standard (Dubois et al. 1956).

Thin Layer Chromatography

Samples were prepared for thin layer chromatography (TLC) by acid hydrolysis in 6N HCl at 100 °C for 20 h. The hydrolysed samples were then spotted onto silica gel TLC plates (Scientific Adsorbents Inc, Atlanta, GA) and eluted with n-butanol:acetic acid:H_2O (4:1:3, v/v/v). Plates were sprayed with ninhydrin (0.1% w/v in ethanol) to detect amino acids; 2',7'-dichlorofuorescein (0.1% w/v in ethanol) to detect fatty acids;

and anthrone (0.1% w/v in ethanol) to detect carbohydrates. After heating at 110 °C for 10 min, ninhydrin positive spots appeared pink, 2',7'-dichlorofluorescein positive spots appeared orange, and anthrone positive spots appeared yellow.

Structural Characterization of Biosurfactant

Partially purified products were desalted with C18 ZipTips (Millipore) and the molar mass of the most predominant compound was determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) at the Institute for Integrated Research on Materials Environment and Society at California State University, Long Beach, CA (iirmes.org). All samples were run on an Applied Biosystems 4800 MALDI-TOF/TOF mass spectrometer in the reflectron mode with α-cyano-4-hydroxycinnamic acid as the matrix. The six highest peaks were subjected to MS/MS analysis.

Partially purified product (1-2 mg) was mixed with KBr (200 mg) and analysed by fourier transform infrared spectroscopy (FTIR) on an Equinox 55 FTIR. FTIR analyses were carried out at the Analytical Chemistry Instrumentation Facility at University of California, Riverside, CA.

Results and Discussion

Swarming motility in *V. paradoxus* EPS and many other gram-negatives is associated with the production of an extracellular wetting agent (Jamieson et al. 2009). The wetting agents contain biosurfactants that lower surface tensions to assist in rapid

translocation of cells across surfaces (Kearns 2010). Since many factors stimulate a variety of swarming patterns in *V. paradoxus* EPS (Jamieson et al. 2009), a nutrient-specific medium was designed by Pehl et al. (2012) to exclude swarming and either induce or inhibit wetting agent production dependent on viscosity (Fig. 1). Low viscosity may allow *V. paradoxus* EPS to extract fluids

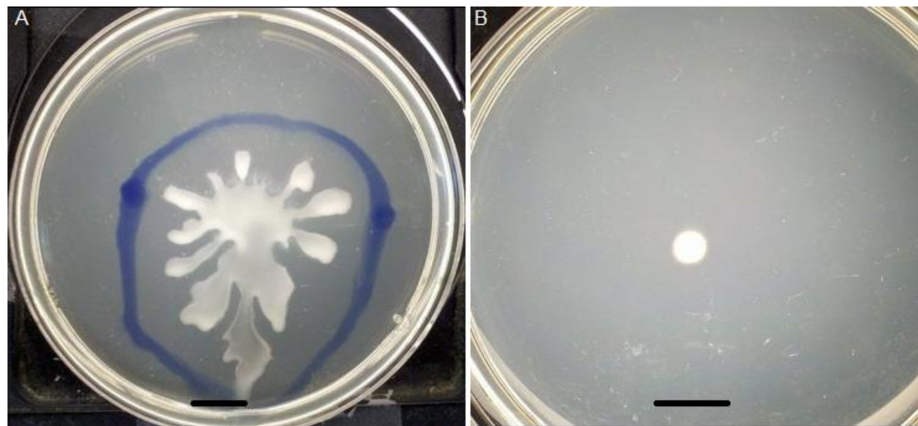

Fig. 1 *V. paradoxus* EPS grown on **A)** semi-solid YE resulting in a wetting agent zone as indicated with methylene blue or on **B)** solid YE resulting in no wetting agent. Bar = 1 cm

from semi-solid medium to generate the wetting agent (Bees et al. 2000). Here, the partially purified wetting agent was shown to have biosurfactant properties as determined by several independent assays. This included a postive response using the drop collapse assay and direct measurements in which the wetting agent was shown to lower surface tension of water from 70 to 30 ± 0.5 mN/m, and to have a CMC of 215 mg L^{-1} (Fig. 2). The control was negative for the drop collapse assay and only lowered surface tension from 70 to 66 ± 0.7 mN/m (Fig. 2).

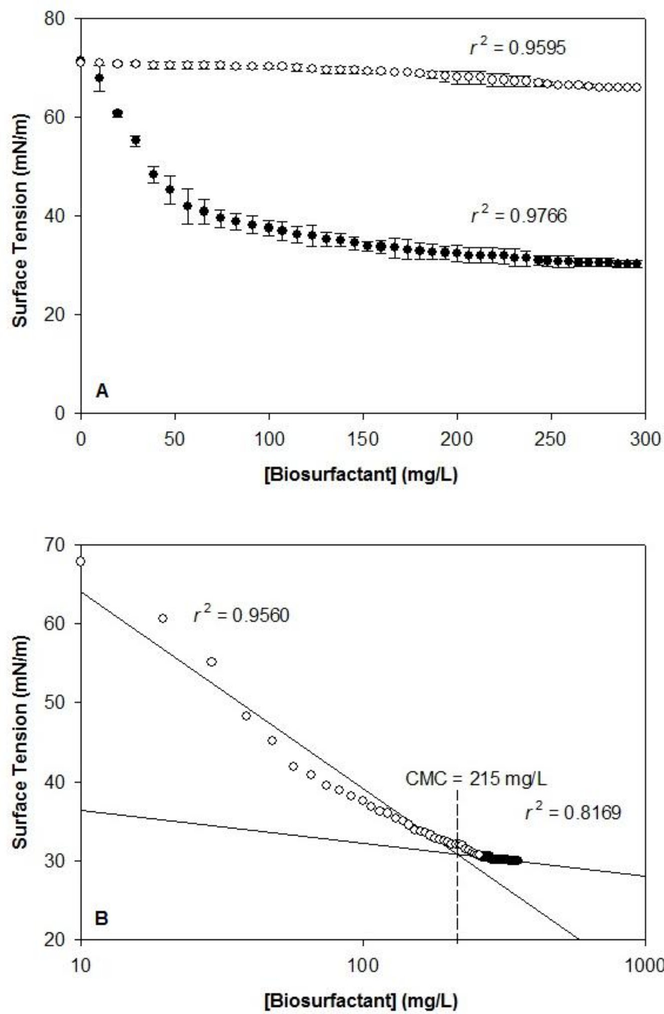

Fig. 2 A) Surface tension reductions of wetting agent (filled dots) compared to control (unfilled dots). **B)** Determing the CMC was the intersection of best fitted lines representing data below (filled dots) and above (unfilled dots) the lowest surface tension, 30 ± 0.5 mN/m

The atomized oil assay has never been used for wetting agents, but revealed a 'bright' halo for variowettin and no detectable halo for the control (Fig. 3). This data strongly supports our hypothesis that the wetting agent produced by *V. paradoxus* EPS contains a biosurfactant, which we call variowettin. Based on its ability to lower surface tension,

this wetting agent likely would help to facilitate swarming motility of this bacterium under conditions where cells are induced to initiate swarm motility.

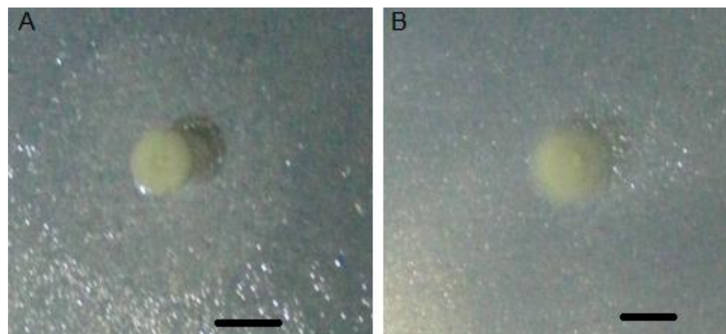

Fig. 3 Atomized oil assay results for **A)** variowettin with a 'bright' halo of 1.12 ± 0.15 cm and **B)** control with no halo present. Bar represents 0.5 cm

Along with their ability to lower surface tension, many biosurfactants are able to form stable emulsions with nonaqueous liquids to enhance carbon sequestration (Ron and Rosenberg 2001). Although low molecular weight biosurfactants such as lipopeptides can have emulsifying properties (Ramani et al. 2012), many high molecular weight polymers such as EPS are well known for this property (Ron and Rosenberg 2001). No such emulsions appeared to develop when variowettin solutions were tested with hydrophobic liquids. Emulsification activity was also absent with serrawettin W2 and serrawettin W3. However, within liquid cultures both *V. paradoxus* 7bcT5S and *S. marcescens* SmSA were able to exude bioemulsifiers (Franzetti et al. 2012; Roldan-Carrillo et al. 2011). If variowettin is related to the serrawettins, the limited capacity of only being able to reduce surface tensions may further define this class of surfactants. Colorimetric analyses identified variowettin as having 20.4 % protein content, 17.7 % fatty acid content, and 0.9 % carbohydrate content. Previous analyses of biosurfactants produced by *P.*

aeruginosa and *B. subilis* strains that produce lipopeptide type surfactant molecule,

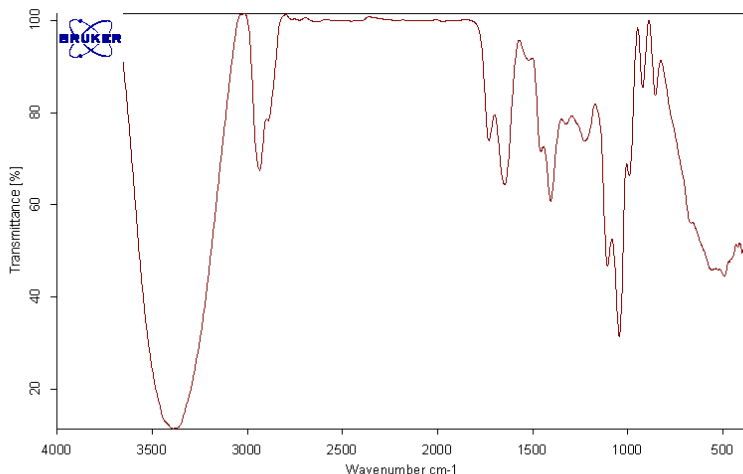

Fig. 4 FTIR spectrum of variowettin

which using similar methods, yielded comparable results such that it can be concluded that the biosurfactant produced *V. paradoxus* was a lipopeptide biosurfactant (Bordoloi and Konwar 2009; Das et al. 2009). FTIR results for variowettin are shown in Fig. 4. A strong, broad peak at 3390.42 cm^{-1} suggested a H-bonded –OH (3550-3200 cm^{-1}) which may belong to serine and/or threonine, both of which are amino acids found in serrawettins. Two peaks at 2935.35 and 2889.38 cm^{-1} are indicative of alkanes (3000-2850 cm^{-1}) that are likely associated with the lipid tail of the biosurfactant. The band at 1647.56 cm^{-1} may represent the C=O amide I band (1695-1630 cm^{-1}) and the small band at 1522.21 cm^{-1} likely represents an –NH amide II (1560-1500 cm^{-1}) band. A C=O (1740-1720 cm^{-1}) bond was observed at 1729.77 cm^{-1} and a –CO (1300-1000 cm^{-1}) group was represented by the two peaks at 1106.78 and 1044.37 cm^{-1}. These three peaks may describe the lactone present where the cyclic peptide binds to lipid. As with colorimetry, the FTIR results obtained with variowettin suggest that this compound is similar to the

lipopeptide produced by *P. aeruginosa* MTCC7815 (Bordoloi and Konwar 2009). The identity of variowettin as a lipopeptide was further confirmed with TLC based on positive reactions with ninhydrin and 2’,7’-dichlorofuorescein for spots separating with Rf values of .45 and 51, respectively. Similarily, serrawettin W2 had spots with Rf values of .45 and .53 while serrawettin W3 spots were measured at .47 and .50 for ninhydrin and 2’,7’-dichlorofuorescein, respectively. Also, just as with the serrawettins, wetting agent production in *V. paradoxus* EPS is inhibited by growth at 37 °C (Matsuyama et al. 2011). This evidence supports the identification of variowettin as a member of the lipopeptide class of serrawettins.

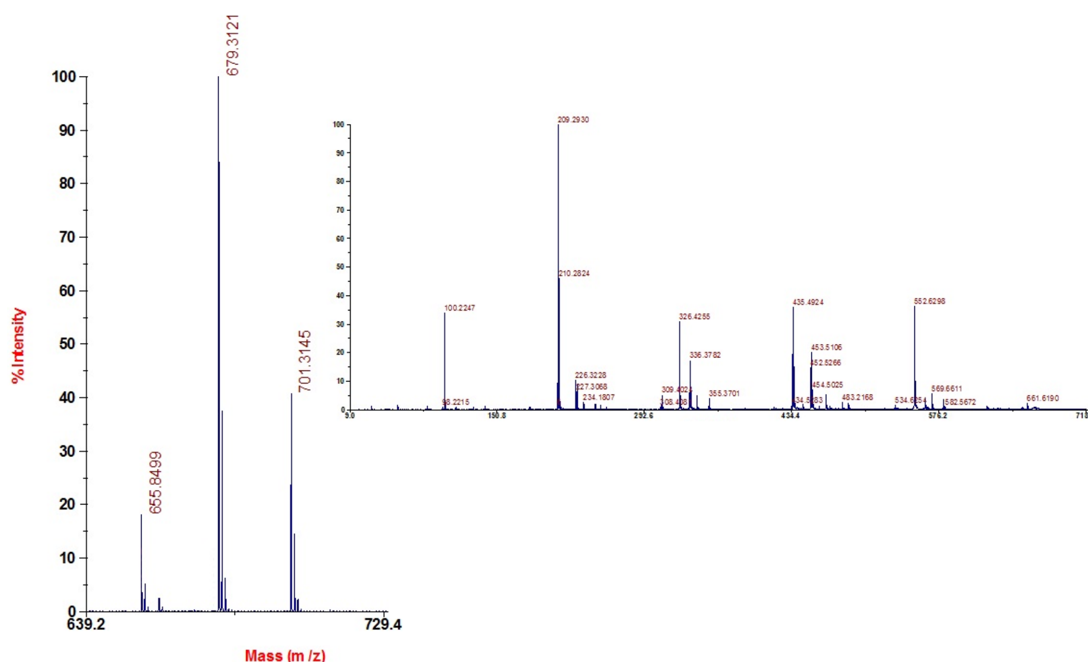

Fig. 5 MALDI-TOF mass spectrum of predominant peak found in variowettin. Inset shows the MS/MS of the compound found at *m/z* 679.3

To determine further structural relationships amongst the biosurfactants, MALDI-TOF-MS/MS was used to determine their predominant *m/z* peaks. Analysis of variowettin

composition indicated a predominance of a compound having a peak of *m/z* 679.3 (Fig. 5). Serrawettin W3 had a strong peak of *m/z* 690.2 which differs from the peak at *m/z* 683.0 previously reported (Matsuyama et al. 2011). This may be due to a different strain being used from the previous report. Serrawettin W2 had a predominant peak of *m/z* 754.3 which correlates to the pseudomolecular ion $[M + Na]^+$ at *m/z* 754.5 previously described by Lindum et al. (1998). Although not reported here, serrawettin W1 produced by *S. marcescens* 274 has a peak of *m/z* 658.0 (Matsuyama et al. 2011). Since variowettin has a peak differing to those of the serrawettins, tandem spectra may suggest that this is a novel lipopeptide. The tandem spectra were inconclusive in sequencing the nonribosomal peptides, but dereplication from the Norine database (http://bioinfo.lifl.fr/norine) further confirms the identity of serrawettin W2. Serrawettin W3 was not present in the database. Dereplication of variowettin suggested the presence of four fragmentation ions and a homology to montanastatin with a -62.13 Da modification. Although montanastatin is a cyclooctadepsipeptide sharing structural similarities to serrawettin W1, it lacks fatty acids which appear present in variowettin (Doi and Asano 2002; Matsuyama et al. 2011). Tertiary structure analysis of variowettin and serrawettin W3 is currently being conducted by Dr. Raina Maier at University of Arizona.

Swarming motility within *V. paradoxus* EPS may require the cyclic lipopeptide biosurfactant variowettin to induce rapid translocation within its wetting agent (Fig. 6). Variowettin shares similar properties to the serrawettin class of wetting agents, but appears to be novel. There are some serrawettin homolog NRPSs in *V. paradoxus* EPS that are suspected to be involved in the production of variowettin. Future research should

establish relationships between quorum sensing, swarming motility, variowettin production, and biofilm formation. Similarities with *S. marcescens* strains and their serrawettins should be considered further with respect to the class of cyclic lipopeptide swarming-associated wetting agents.

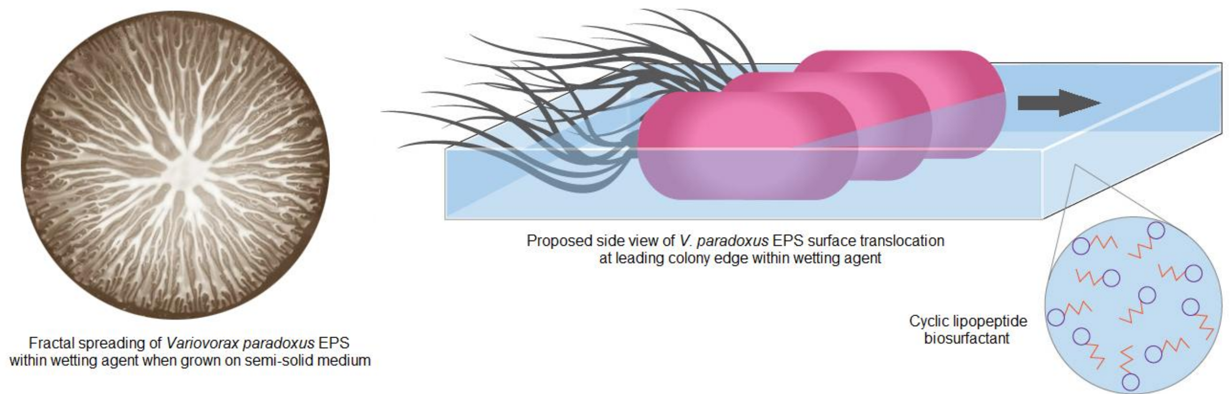

Fig. 6 Proposed model of variowettin's role in promoting swarming motility

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Chapter Five:

General Conclusions

Soil bacteria use biosurfactants to stimulate a variety of important biological and environmental processes ranging from PAH biodegradation to swarming motility. A small fraction of culturable isolates were selectively enriched on monoaromatic hydrocarbons to screen for biosurfactant producers. The phenanthrene-degrading capabilities of *Delftia tsuruhatensis* RB91H appears to be enhanced by the presence of a glycolipid released by *Pseudomonas* sp. RB91F, and is dependent on biosurfactant concentration. These results indicate selective enrichment may be an ideal way to screen for biosurfactant production and that degraders and biosurfactant producers from the tarpits are viable candidates for bioremediation technologies. The gram-negative betaproteobacterium, *Variovorax paradoxus* EPS was shown to produce a lipopeptide biosurfactant as a wetting agent. Presence of tensioactive wetting agent in swarming colonies of *V. paradoxus* strongly suggests that this lipopeptide is required for rapid motility which further elucidates cell physiology in this species.

My dissertation work is fortunate enough to be pioneering in various respects. It is the first to isolate culturable bacteria with biosurfactant production capabilities from the Rancho La Brea tar pits. To my knowledge, this is the first publication to identify biosurfactant production from the *Shewanella* genera. I also created an adaptable and novel medium that successfully stimulated biosurfactant production in liquid and solidified states across a wide variety of bacterial species. Although a relatively new

technique, my use of the atomized oil assay showed for the first time that bacteria from a natural asphalt seep can be successfully employed with the assay and that two of my cultured isolates produced ‘dark’ halos, a phenomenon previously only associated with synthetic surfactants. There is currently no biodegradation studies on bacterial isolates cultured from a terrestial natural asphalt environment aside from tar sands and underground petroleum reservoirs. My research is the first to showcase that these habitats, although rare, can provide more culturables that are relevant to the ever-growing bioremediation industry. Furthermore, I was able to discover a potentially novel lipopeptide biosurfactant produced by a member of a genus that has not been associated with biosurfactant production.

Partial determination of biosurfactants according to their surface activities and chemical composition is important for classification of potentially novel biosurfactants and elucidating mechanisms involved in the processes. *Pseudomonas* sp. RB91F produces what appears to be a high molecular weight polymeric biosurfactant comprising carbohydrate, protein, and lipid moieties that increases ^{14}C-PHE mineralization by *Delftia tsuruhatensis* RB91H dependant on the CMC level of the bioemulsifier. Cell surface interactions lead to reduced hydrophobicity of *D. tsuruhatensis* RB91H that may promote micellar uptake via hydrophilic-hydrophilic attraction when bioemulsifier concentration is at CMC. Since bioemulsifier can be a growth substrate for *D. tsuruhatensis* RB91H, and two-fold increase in total mineralization after degradation onset at sub-CMC suggests growth-linked biodegradation may be a major mechanism. When bioemulsifier is at supra-CMC, an array of mechanisms seem possible, but due to inhibition of

mineralization the sequestration of PAH within the micellar core seems most likely. Chapter Three highlights the probable mechanisms in play at the varying CMC levels of bioemulsifer in a one member soil slurry contaminated with used motor oil. In Chapter Four, *Variovorax paradoxus* EPS was shown to produce a cyclic lipopeptide as a wetting agent to promote swarming motility. By reduction of surface tensions, variowettin may allow the soil bacterium *V. paradoxus* to swarm rapidly along hydrated soil substrates and plant roots. Similarities with the surface activites, *m/z* values, and chemical compositions of the serrawettins suggest variowettin is a member of this class. Identification of a new lipopeptide-producing proteobacterium further displays the conservancy of swarming in that group.

Much of the work from this dissertation can be applied to soil studies. Phenanthrene degradation has been shown to be enhanced by biosurfactant presence in a motor oil-containing soil slurry. Currently, I am running a phytoremediation treatability study where motor oil contaminated soil has been amended with biochar inoculated with *Pseudomonas* sp. RB91F and *D. tsuruhatensis* RB91H. The biochar will hypothetically accumulate petroleum molecules into the proximity of the tarpit isolates and biosurfactant-enhanced biodegradation may reduce total petroleum hydrocarbon levels. The Orwin group has observed that *V. paradoxus* EPS inoculation onto roots of *Helianthus annuus* can stimulate plant growth promotion. If swarming can occur on the root surface, the lipopeptide production may be beneficial as a biocontrol agent against pathogenic bacteria. Future research with the bacteria studied in my dissertation may provide new avenues in improving growth conditions for soil micro- and macrobiota.