ROLE OF *PSEUDOMONAS* RHAMNOLIPID SURFACTANTS IN BIODEGRADATION OF SLIGHTLY SOLUBLE HYDROCARBONS

By

Yimin Zhang

A Dissertation Submitted to the Faculty of the

DEPARTMENT OF SOIL AND WATER SCIENCE

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

1995

INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

A Bell & Howell Information Company
300 North Zeeb Road. Ann Arbor, MI 48106-1346 USA
313/761-4700 800/521-0600

ROLE OF *PSEUDOMONAS* RHAMNOLIPID SURFACTANTS IN BIODEGRADATION OF SLIGHTLY SOLUBLE HYDROCARBONS

By

Yimin Zhang

A Dissertation Submitted to the Faculty of the

DEPARTMENT OF SOIL AND WATER SCIENCE

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

1995

THE UNIVERSITY OF ARIZONA
GRADUATE COLLEGE

As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Yimin Zhang

entitled ROLE OF PSEUDOMONAS RHAMNOLIPID SURFACTANTS IN BIODEGRADATION OF SLIGHTLY SOLUBLE HYDROCARBONS

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy

Raina M. Miller	5/15/95 Date
Ian Pepper	5/15/95 Date
Mark Brusseau	15 May 95 Date
Norval Sinclair	5-15-95 Date
Chuck Gerba	5-15-95 Date

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Dissertation Director Raina M. Miller	5/15/95 Date

STATEMENT BY AUTHOR

This dissertation has been submitted in partial fulfillment of requirements for an advanced degree at the University of Arizona and is deposited in the University Library to be made available to borrowers under rules of the Library.

Brief quotations from this dissertation are allowable without special permission, provided that accurate acknowledge of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the Graduate College when in this or her judgement the proposed use of the material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author.

SIGNED: 

ACKNOWLEDGEMENTS

The research contained in this dissertation was motivated and strongly supported by my advisor Raina M. Miller. Her encouragement, insight, and scientific expertise aided me throughout this work. I am thankful for her professional and personal involvement in my life. I would also like to acknowledge the assistance of my committee members: Ian Pepper, Norval Sinclair, Mark Brusseau and Chuck Gerba. They provided advice on aspects of my research work which is acknowledged.

As with any graduate school experience much is learned from fellow students. This was certainly the case for me, as I enjoyed interaction with Xiaojiang Wang, Guiyun Bai, David Herman, Mary Huber, Jerry Miller and many others. I appreciate your friendship and help, especially during the slow months.

This research was made possible by financial support from the U.S. Department of Energy (DOE) Subsurface Science Program, Office of Health and Environmental Research (OHER) Grant No: DE-FG03-93ER61526.-

Finally, love and support from my wife Ming Li has made life easier and has kept me focused on aspects of life that are most important.

TABLE OF CONTENTS

TABLE OF CONTENTS - *Continued*

TABLE OF CONTENTS - *Continued*

LIST of TABLES

LIST OF ILLUSTRATIONS

LIST OF ILLUSTRATIONS - *Continued*

ABSTRACT

Biodegradation of hydrocarbons is often limited by the low water solubility. Surfactants can increase hydrocarbon solubility, however, the effects of surfactants on hydrocarbon biodegradation are not well known. Therefore, the purpose of this research is to investigate the fundamental interactions between surfactants, hydrocarbons and microorganisms. This three-way interaction was studied in the defined experimental system consisting of *Pseudomonas* rhamnolipid surfactants, hydrocarbons and *Pseudomonas* hydrocarbon degraders. A variety of system factors affecting biodegradation were examined. These factors included surfactant structure, surfactant concentration, hydrocarbon structure and cell hydrophobicity of microorganisms.

Pseudomonas sp. are able to produce several rhamnolipid types. Among three rhamnolipid types tested in this study, dirhamnolipid methyl ester had the greatest effect on alkane solubilization and biodegradation. Monorhamnolipid acid exhibited higher solubilization of alkanes than dirhamnolipid acid. Monorhamnolipid acid at high concentrations (> 0.1 mM) enhanced alkane biodegradation more effectively than dirhamnolipid acid. In contrast, dirhamnolipid acid enhanced degradation more effectively at low concentrations (< 0.1 mM).

The biodegradation of alkanes was affected by microbial cell surface hydrophobicity. Results showed that inherently slow alkane degraders had low cell hydrophobicity while the inherently fast alkane degraders had high cell hydrophobicity. Rhamnolipids enhanced cell hydrophobicity of the slow degraders but had no effect on the cell hydrophobicity of the fast degraders. The rate at which the cells became

hydrophobic depended on the rhamnolipid concentration and was directly related to the rate of alkane biodegradation.

Rhamnolipid mixtures had a different effect from single rhamnolipids on solubilization and biodegradation of alkanes. The effect can be synergistic or additive depending on the surfactant mixture. For instance, the mixture of dirhamnolipid acid and methyl ester had a synergistic effect on solubilization and additive effect on biodegradation.

The effect of rhamnolipids on the hydrocarbon biodegradation varied with hydrocarbon structure. For instance, enhanced biodegradation of model alkanes was determined by both rhamnolipid structure and concentration. In contrast, enhanced phenanthrene biodegradation seemed to depend only on rhamnolipid concentration.

The results of this research suggest that rhamnolipids have potential use for remediation of petroleum-contaminated sites. Further research is recommended to investigate the effect of rhamnolipids on hydrocarbon biodegradation in soil systems.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Nature of the problem

Limited availability of hydrocarbons to microorganisms is a major factor affecting biodegradation of hydrocarbons. The problem is caused by the low water solubility and the limited surface area of hydrocarbons due to the formation of separate phases in aqueous solution. Since microbial uptake of hydrocarbons occurs only from the aqueous phase (dissolved hydrocarbon) or at hydrocarbon-water interface, an increase in hydrocarbon solubility or in the interfacial area between the hydrocarbon and water may enhance hydrocarbon degradation. One potential approach to enhancing hydrocarbon biodegradation is to use surfactants because surfactants can increase dissolved hydrocarbon concentration by solubilization or increase the interfacial area of hydrocarbon between water and hydrocarbon by emulsification. However, the effects of surfactants on hydrocarbon biodegradation are not well understood. Therefore, the purpose of this dissertation was to investigate the fundamental interactions between one type of microbially produced surfactant (rhamnolipid), hydrocarbons and microorganisms.

Literature review

Microbial Uptake of Slightly Water Soluble Hydrocarbons

The low water solubility of hydrocarbons poses special problems for microorganisms capable of utilizing such substrates as sole sources of carbon and

energy. Since the first step in aromatic (Gibson, 1971) or aliphatic (Ratledge, 1978) hydrocarbon degradation is the introduction of molecular oxygen into the molecules by cell-associated enzymes, the immiscible solid or liquid hydrocarbons have to be transported into the cells before being degraded. The uptake involves two steps: the contact of cell surface with substrate and the translocation of substrate through cell membrane. The uptake of hydrocarbons by microorganisms in the absence of surfactants generally follows two modes: (i) uptake of hydrocarbons dissolved in the aqueous phase, and (ii) uptake of hydrocarbons by direct contact of cells with liquid droplets or solid particles. In the first case, microbial uptake of hydrocarbons is limited by substrate water solubility and mass transfer into the aqueous phase. In the second case, microbial uptake of hydrocarbons is limited by the availability of substrate surface area for cell attachment. Because of the difference in physicochemical properties between aromatic hydrocarbons and aliphatic hydrocarbons, microbial uptake of these two types of hydrocarbons may take place through different modes.

Uptake of Slightly Water Soluble Aromatic Hydrocarbons

Aromatic hydrocarbons containing two or more fused benzene rings also called polycyclic aromatic hydrocarbons (PAHs) exist in the solid phase and have low water solubility as shown in Table 1.1. The uptake rate of the hydrocarbon by microorganisms is directly related to the water solubility of the substrate. The greater the solubility of the hydrocarbon, the faster the growth rate. For example, the respective solubilities of naphthalene, phenanthrene, anthracene and naphthacene are 30, 1.6, 1.3 and 0.001 mg/l.

Table 1.1 Physical properties of selected aromatic and aliphatic hydrocarbons

Compound[1]	C atoms	Mol.wt.	m.p. (°C)[2]	b.p. (°C)[2]	Solubility (mg/l)
Naphthalene	10	128.2	80	218	30
Biphenyl	12	154.2	70	255	7
Fluorene	13	166.2	116	293	1.9
Phenanthrene	14	178.2	100	338	1.6
Anthracene	14	178.2	216	340	1.3
Pyrene	16	202.3	150	393	0.15
Naphthacene	18	228.3	257	450	0.001
n-hexane	6	86.2	-94.3	68.7	12.3
n-decane	10	128.3	-31.0	174.0	0.05
n-hexadecane	16	226.4	19.0	287.0	5.2×10^{-5}
n-octadecane	18	254.5	28.0	317.0	4.0×10^{-6}
n-eicosane	20	282.6	36.7	343.0	3.1×10^{-7}
n-hexacosane	26	366.7	56.4	412.2	1.3×10^{-10}

[1]Aromatic data selected from Mackay et al. (1992); aliphatic data from Eastcott et al. (1988).
[2]m.p. is the melting point and b.p.the boiling point at normal temperature and pressure.

The generation times of pure cultures of bacteria grown on naphthalene, phenanthrene, and anthracene measured were 1.5, 10.5 and 29 h respectively and no organism was isolated to utilize naphthacene, the most insoluble hydrocarbon studied (Wodzinski and Johnson, 1968).

Microbial utilization of solid phase hydrocarbons has been generally thought to be restricted to hydrocarbon in the dissolved state. Studies with phenanthrene (Wodzinski and Coyle, 1974), bibenzyl, and naphthalene (Wodzinski and Bertolini, 1972) showed that addition of solid substrate to a culture already saturated with substrate had no effect on the generation time of the culture used. Phase-contrast microscopy did not show significant numbers of cells on the surface of aromatic hydrocarbon as it underwent decomposition (Stucki and Alexander, 1987). However, some researchers have found that microorganisms can directly attach to and utilize aromatic hydrocarbons in the solid state. For instance, Guerin and Jones (1988) reported that *Mycobacterium* sp. colonized phenanthrene particles and proliferated while it was attached to the hydrocarbon particles.

The uptake of slightly soluble aromatic hydrocarbons is limited by the mass transfer of substrate from the solid phase to the aqueous phase. Mass transfer can be achieved by reducing the particle size of the substrate particles, thereby increasing the surface area and resulting in a higher dissolution rate. Studies with 4-chlorobiphenyl, naphthalene (Thomas et al., 1986) and phenanthrene (Köhler et al., 1994) showed that the substrate particles of smaller size were utilized faster than those of larger size. Comparisons of mass transfer and degradation rates suggest that spontaneous mass

transfer rates are a key factor governing the rates of aromatic hydrocarbon degradation (Thomas et al., 1986).

Passage of hydrophobic molecules through cell membrane generally occurs by simple diffusion (Nikaido and Vaara, 1985). This seems true for aromatic hydrocarbons. Bateman et al. (1986) compared the interaction of naphthalene with cells and membrane vesicles. They showed that movement of naphthalene into cells did not require a protein carrier, an activated membrane, or ATP and that uptake did not exhibit saturation or respond to competition by structurally similar compounds.

Uptake of Slightly Water Soluble Aliphatic Hydrocarbons

Aliphatic hydrocarbons exist as either liquids (≤ C16) or solid (> C16) and are are characterized by extremely low water solubility (Table 1.1). Uptake of aliphatic hydrocarbons often requires direct contact of cells with the hydrocarbon substrate. This is because the water solubility of many aliphatic hydrocarbons is too low to support microbial growth, and also because mass transfer of the hydrocarbon into aqueous solution is slow (Stucki and Alexander, 1987). Microbial utilization of liquid *n*-alkanes by direct contact of cells with the substrate has been extensively demonstrated. Nakahara et al. (1977) observed that *Candida lipolytican* grown on hexadecane mainly attached to large oil droplets and formed flocs consisting of clumps of cells, oil drops and air bubbles. More recently, Goswami and Singh (1991) demonstrated 75 to 80 % of *Pseudomonas* cells growing on hexadecane adhered to oil droplets even after centrifugation at $10^4 \times g$.

Less information is available about the uptake of solid *n*-alkanes and this has resulted in conflicting opinions about the uptake mode. One study (Zilber et al., 1980) with a marine pseudomonad grown on *n*-tetracosane showed that over 80% of the cells were bound to large pieces of *n*-tetracosane during the early exponential growth phase. This cell-associated *n*-tetracosane was preferentially utilized and could completely account for the observed growth of free cells. However, the other study (Cameotra, et al., 1983) with a different pseudomonad grown on same substrate showed that cells did not attach to the substrate. The uptake of *n*-tetracosane occurred through substrate solubilization by an extracellular solubilizing factor produced from cells. EDTA inhibited hydrocarbon solubilization by inactivating the factor, thereby inhibiting the growth of cells on alkane. Thus, adaptation to hydrocarbon uptake is strain specific and may involve either direct attachment to the substrate or may require the presence of a solubilizing factor, which can affect the physical state of solid alkane by solubilization and enhance uptake.

The uptake rate of aliphatic hydrocarbons by microorganisms by either mechanisms (attachment or solubilization) is limited by the availability of surface area of the substrate for cell attachment. The increase of surface area of the substrate generally leads to an enhanced biodegradation (Wang and Ochoa, 1972; Katinger, 1973). Two ways have been proposed to artificially increase the surface area of the hydrocarbon. One way is to increase the hydrocarbon volume by increasing substrate concentration or by addition of nonutilizable solvent. The other way is to increase the dispersion of the hydrocarbon by mechanical agitation-stirring or by addition of

surfactants. Liquid hydrocarbon is easily dispersed while the dispersion of solid hydrocarbon is harder to achieve since greater energy is required to disperse solid hydrocarbons than is required to disperse liquids (Zilber, et al., 1980).

The mechanism of translocation of hydrocarbons across the cell membrane is not yet clear. Käppeli and Fiechter (1981) considered that the process was passive in hexadecane transport by *Candida tropicalis* on the basis of the lack of influence of pH, temperature and metabolic inhibitors. However, other researchers believe an active process is required. Bassel and Mortimer (1985) showed that hexadecane uptake by *Yarrowia lipolytica* is inducible and inhibited by the metabolic inhibitors KCN and 2,4-dinitrophenol. They also isolated mutants defective in hexadecane transport. Other studies also suggest that hydrocarbons may be taken up against a concentration gradient. Studies on the ultrastructure of hydrocarbon-utilizing microorganisms have consistently reported the presence of intracytoplasmic inclusions, which have been positively identified as unchanged substrate (Scott and Finnerty, 1976a,b). The driving force for intracellular hydrocarbon accumulation is at present unknown.

Enhancement of Microbial Uptake of Slightly Water Soluble Hydrocarbons

While the low solubility and hydrophobicity of hydrocarbons often limits the availability of the substrate to microorganisms, in some cases the growth of microorganisms on hydrocarbons is faster than expected. Microorganisms have developed two strategies to facilitate substrate uptake. The first is the increased affinity of microorganisms for hydrocarbons, which is generally believed to result from

increased hydrophobicity of the cell surface. The second is the capacity of microorganisms to produce and release extracellular surface active compounds. The role of biosurfactants is believed to enhance the availability of hydrocarbons to microorganisms by solubilizing and emulsifying the substrate in aqueous solution. Microorganisms may use either one or two above strategies in hydrocarbon uptake.

Enhancement of microbial affinity for hydrocarbons

Microbial development of cell affinity for hydrocarbons seems to be induced and a direct result of growth on hydrocarbons. For example, Käppeli and Fiechter (1976) observed that *Candida tropicalis* demonstrated a 25% higher adsorption capacity for hydrocarbons when growing on hydrocarbons than when metabolizing glucose. Similarly, by contact angle measurements of an aqueous drop on a smooth lawn of cells, Neufeld et al. (1980) reported that *Acinetobacter calcoaceticus* cells grown on hexadecane had higher cell hydrophobicity than that of cells grown on sodium citrate, a water soluble substrate. Miura et al. (1977) were able to correlate the rate of hydrocarbon degradation to cell affinity for hydrocarbons. They demonstrated that yeast strains with low affinity for hydrocarbon had low degradation rates, while yeasts with high hydrocarbon affinity had high degradation rates. The importance of cell adherence in the microbial uptake of *n*-alkane was also reported by Rosenberg and Rosenberg (1981). They found that wild type *Acinetobacter calcoaceticus* was able to rapidly grow on hexadecane under conditions of limited agitation and at low initial cell densities. In contrast, a mutant defective in hydrocarbon adherence did not grow for at least 54 h

under the same conditions.

Microbial affinity for hydrocarbons can be quantitatively described by microbial cell surface hydrophobicity, a physical property resulting from the chemical nature of the outermost cell surface. There are a number of ways used to assess microbial cell surface hydrophobicity. It can be measured by contact angle measurements (Fletcher and Marshall, 1982), adhesion to liquid hydrocarbons (Rosenberg et al, 1980), two-phase partitioning (Gerson and Akit, 1980), hydrophobic interaction chromatography (Smyth et al., 1978), salting-out aggregation (Lindall et al., 1981), and adherence to solid surfaces (Rosenberg, 1981).

A variety of surface components that influence microbial cell hydrophobicity have been identified (Rosenberg and Kjelleberg, 1986) and listed in Table 1.2. However, there are relatively few studies concerning these components and their relation to hydrocarbon degradation. In one study, Rosenberg et al. (1982) demonstrated that thin fimbriae play a major role in adherence in hydrocarbon and enabling growth of *Acinetobacter calcoaceticus* on hexadecane. A mutant lacking these fimbriae was unable to adhere to hydrocarbon or grow on hexadecane. Käppeli et al. (1978) reported the mannan-fatty acid complex at the cell surface of *Candida tropicalis* was related to the alkane binding. The complex was induced during growth on alkane but not during growth on glucose. Masking of the mannan by complexation with concanavalin A reduced the affinity of the cells for alkane.

Table 1.2 Some proposed hydrophobic (hydrophilic) components on the cell surface of hydrocarbon-utilizing microorganisms

Component	Microorganisms	Reference
Thin fimbriae	*Acinetobacter calcoaceticus* RAG-1	Rosenberg and Rosenberg (1981)
Mycolic acids	*Corynebacterium* and related bacteria	Bendinger et al. (1993)
Mannan-fatty acid complexes	*Candida tropicalis*	Käppeli et al. (1978)
Lack of exopolysaccharides	*Pseudomonas fluorescens*	Pringle et al. (1983)

Biosurfactants

Surfactants are divided into two groups on the basis of their origin: chemically synthesized surfactants and biologically synthesized surfactants. Although both chemical surfactants and biosurfactants are now being used in the study of hydrocarbon degradation, there are at least three reasons for special interest in biosurfactants: (i) Biosurfactants produced by microorganisms may have natural interactions with microorganisms, which may affect hydrocarbon biodegradation. Furthermore, the study of this interaction will help to understand the physiological role of surfactants in nature and even modify surfactant structures to make them more effective in hydrocarbon degradation. (ii) Microorganisms are able to produce surfactants with a naturally wide diversity of structure, each of which may have different interaction with hydrocarbon and cells and consequently different effect on hydrocarbon degradation. Therefore, we may isolate highly effective surfactants by screening the structural pool of biosurfactants. (iii) Biosurfactants are considered natural products with low toxicity and biodegradability. Thus, the release of biosurfactants may be easier to justify to appropriate regulatory agencies than release of synthetic surfactants.

The topic of microbial surfactants has been reviewed within the broader scope of biosurfactants by Zajic and Panchal (1976) and Zajic and Seffens (1984). Hommel and Ratledge (1993) have reviewed metabolic pathways and regulation involved in the biosynthesis of surfactants. Rosenberg (1986) and Hommel (1990) in their reviews have discussed the possible physiological role of biosurfactants in nature. Recently, the reviews of Fiechter (1992) and van Dyke et al. (1991) have predicted the potential

application of biosurfactants to industrial and environmental problems. This section places particular emphasis on structural diversity and natural role of commonly isolated biosurfactants: glycolipid surfactants and polymeric surfactants, which are thought to be related to the microbial uptake of hydrocarbons.

The most important group of biosurfactants produced by hydrocarbon-utilizing microorganisms are glycolipids, in which carbohydrates are combined with long chain aliphatic acids or hydroxy-aliphatic acids. Glycolipids usually have low molecular weight ranging from 400 to 1000. There are diverse structures of glycolipids produced by different genera or species of microorganisms as showed in Figure 1.1. These include trehalose lipids of *Mycobacterium* sp. and related bacteria (Rapp et al., 1979), rhamnolipids of *Pseudomonas* sp.(Itoh et al., 1971), sophorose lipids of *Torulopsis* sp.(Tulloch, 1968), also mannosyl-erythritol lipids of *Candida* sp. and related yeasts (Kobayashi et al., 1987), and cellobiose lipids of *Ustilago* sp.(Bhattacharjee et al., 1970).

Species of microorganisms are able to produce glycolipid mixtures containing components that have minor structural differences. For example, *Pseudomonas* sp. produce rhamnolipids containing either mono- or dirhamnosyl unit combined with two units of β-hydroxydecanoic acids. Syldatk et al. (1985) isolated two rhamnolipids with only one unit of the fatty acid. Rhamnolipids in which an third decanoyl moiety was linked in 2' and 2" position with the mono- and dirhamnosyl unit, respectively, were described by Yamaguchi et al.(1976). The terminal carboxyl group of rhamnolipids can also be esterified to form nonionic mono- and dirhamnolipid methyl esters (Hirayama

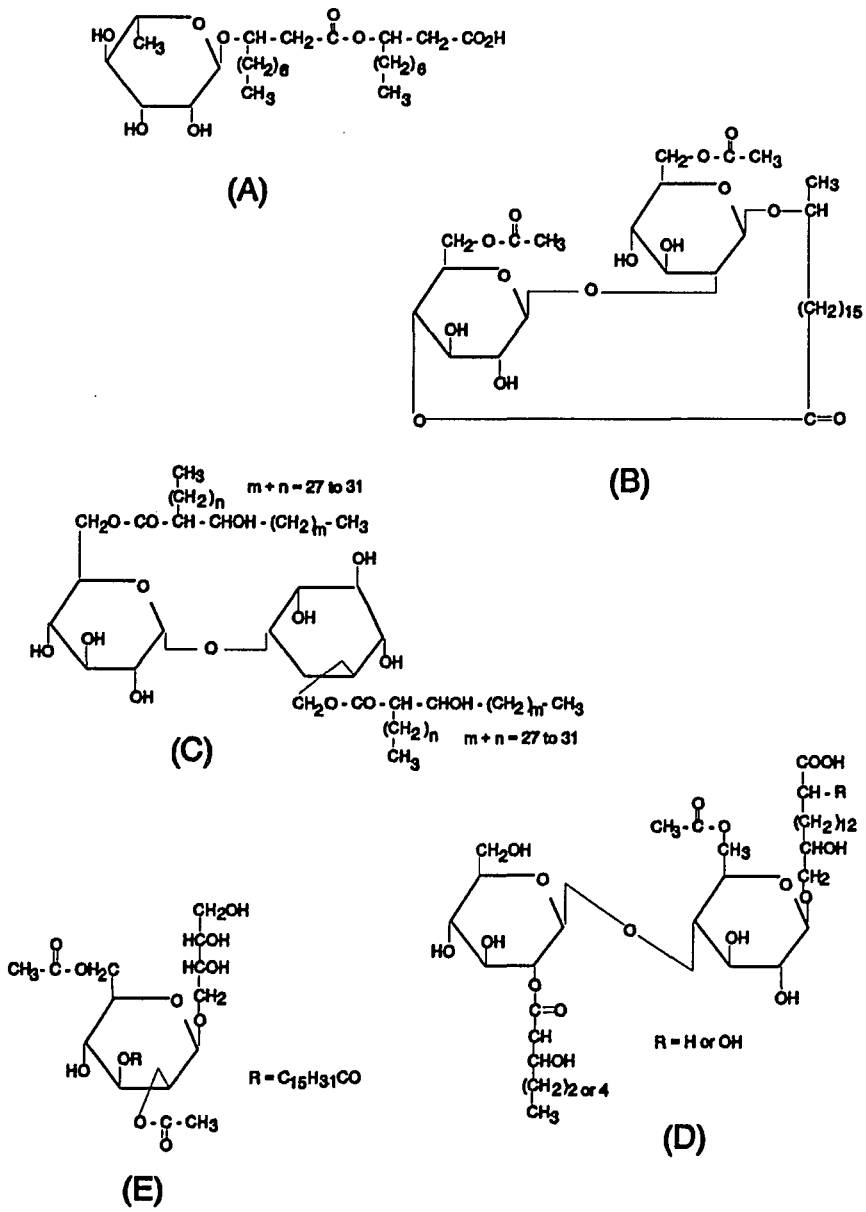

Figure 1.1 Structure of selected glycolipids from microorganisms
(A) Monorhamnolipid of *Pseudomonas aeruginosa.*
(B) Lactonic sophorose lipid of *Torulopsis bombicola.*
(C) Trehalose-6-monocorynomycolate of *Rhodococcus erythropolis.*
(D) Cellobioselipid of *Ustilago zeae.*
(E) Mannosylerythritol lipid of *Shizonella melanogramma.*

and Kato, 1982). Similar differences have been shown for sophorose lipids. *Torulopsis* sp. produce sophorose lipids containing a sophorosyl unit with a 17-hydroxyoctadecanoic acid either in the lactonic form or in the acid form (Tulloch et al., 1968). *Mycobacterium* and related bacteria produce trehalose lipids having the same general structure of two α-branched β-hydroxy fatty acids (corynomycolic acid) esterified to a trehalose unit. It is possible to obtain modified glycolipids by altering the substrate used to grow the bacteria. When species of *Arthrobacter*, *Corynebacterium*, and *Nocardia* were grown on sucrose (Suzuki et al., 1974) or fructose (Itoh and Suzuki, 1974), the trehalose portion of the lipids was substituted. When sucrose was the substrate, two sucrose glycolipids having one or two corynomycolic acids were isolated. Similarly, when fructose was the substrate, fructose 6-corynomycolate and fructose 1,6-dicorynomycolate were isolated.

The other important group of biosurfactants from hydrocarbon-utilizing microorganisms are polymeric surfactants, which usually have high molecular weight of more than 10,000 and capacity to emulsify hydrocarbons in water. A typical representative of this type of biosurfactants is Emulsan produced from hydrocarbon-degrading organism *Acinetobacter calcoaceticus* RAG-1 (Zuckerber at al., 1979). Emulsan contained a polyanionic D-galactosamine backbone with fatty acid esters and amides, as well as noncovalently associated protein. It effectively emulsifies hydrocarbons at low concentrations (0.001 to 0.01%). Goswami and Singh (1991) isolated hydrocarbon emulsifying and *n*-hexadecane pseudosolubilizing factors from *Pseudomonas* M1 grown on *n*-hexadecane, which were characterized as lipoprotein and

glycoprotein, respectively. Both factors act in a synergistic manner to provide enhanced hydrocarbon transport to cells through pseudosolubilization.

Now the question is what is the natural role of these biosurfactants. At present, there is no defined answer. Because biosurfactants are often produced under nutrient limiting conditions e.g. nitrogen limitation (Syldatk and Wanger, 1987), it is generally agreed that the production of biosurfactants is closely associated with the increased availability of nutrients by microorganisms. The enhancement of the uptake of slightly water soluble carbon sources such as hydrocarbons by microorganisms is believed to be one of major functions of biosurfactants in environment. This statement is supported by two pieces of evidence: (i) the growth of microorganisms on hydrocarbon is often accompanied by the production of surface active agents (Rambeloarisoa et al., 1984), (ii) addition of biosurfactants stimulates hydrocarbon degradation by microorganisms (Oberbremer et al., 1990). However, other work has shown that addition of biosurfactants may inhibit hydrocarbon degradation by microorganisms (Falatko and Novak, 1992). In addition, biosurfactants are also produced by some microorganisms during growth on water soluble carbon sources. For example, rhamnolipids are produced by *Pseudomonas* sp. on many different substrates, such as *n*-alkanes, sugars and vegetable oils (Robert et al., 1989). Therefore, microorganisms may use biosurfactants for other physiological purposes.

Rosenberg (1986) suggested three possible roles of surfactants in environment: adhesion of microorganisms to interfaces, emulsification of slightly soluble substrates in aqueous solution, and desorption of microorganisms from interfaces. His hypothesis was

based on the fact that microbial adhesion is an important physiological mechanism for growth and survival in the natural environment. A special case for adhesion is growth of bacteria on slightly soluble hydrocarbons. Microbial surfactants might be naturally adapted for this purpose to play a role in adhesion and in solubilization/emulsification of substrate. Following attachment growth, conditions at interfaces may become unfavorable. For example, toxins accumulate and crowded conditions may prevent transport of necessary nutrients. Surfactants may now play a role in desorption by accumulating and forming a hydrophilic film at the interface. This allows desorption of cells so they can find a new habitat. This hypothesis suggests that biosurfactants are involved in adhesion and desorption of microorganisms at interfaces. However, no evidence was presented to support the hypothesis.

As indicated in the previous paragraphs, Biodegradation of hydrocarbons in the presence of surfactants involves a complicated three-way interaction between substrate, microbial cell and surfactant molecule. Biodegradation rates in any system are determined by this three-way interaction, which is discussed in detail in the following sections.

Interaction between surfactant and hydrocarbon

Surfactants are amphiphilic compounds with both polar and nonpolar moieties in each molecule. The polar moiety of the molecule has an affinity for water and other polar substances, while the nonpolar moiety is hydrophobic and oriented away from the

water. At surfactant concentrations less than a compound-specific threshold value, surfactant molecules exist predominantly in monomeric form, with some fraction being sorbed at system interfaces. The surfactant concentration at which monomers begin to assemble in ordered, colloidal aggregates is termed the critical micelle concentration (cmc). At surfactant concentrations greater than the cmc, additional surfactant is incorporated into the bulk solution through micelle formation (Martin et al., 1969). Micelles may take the geometry of sphere, oblate and prolate ellipsoids with a size of 2-5 nm in diameter in solution (Robson and Dennis, 1977). Vesicles or liposomes can be considered specific types of micelles with more or less spherical structures consisting of lamellar or bilayer micelles arranged in one or more concentric spheres. A well-known example of such vesicles is phospholipid liposomes, artificially made lipid vesicles used extensively to study membrane and function (Huang, 1969).

Three types of interactions between surfactant and hydrocarbon in the aqueous solution may occur depending on surfactant structure, surfactant concentration, and hydrocarbon structure and its physical state. They are association of surfactant monomer with hydrocarbon, micelle-induced solubilization, and emulsification (Figure 1.2). These interactions are described in detail in the following paragraphs.

Association of surfactant monomers with hydrocarbon takes place when surfactant concentration is below the cmc (Fig. 1.2A). The solubility enhancement by the surfactant is generally so low that it cannot be measured. However, with highly hydrophobic compounds, e.g. DDT, a measurable increase in apparent solubility may occur (Kile and Chiou, 1989).

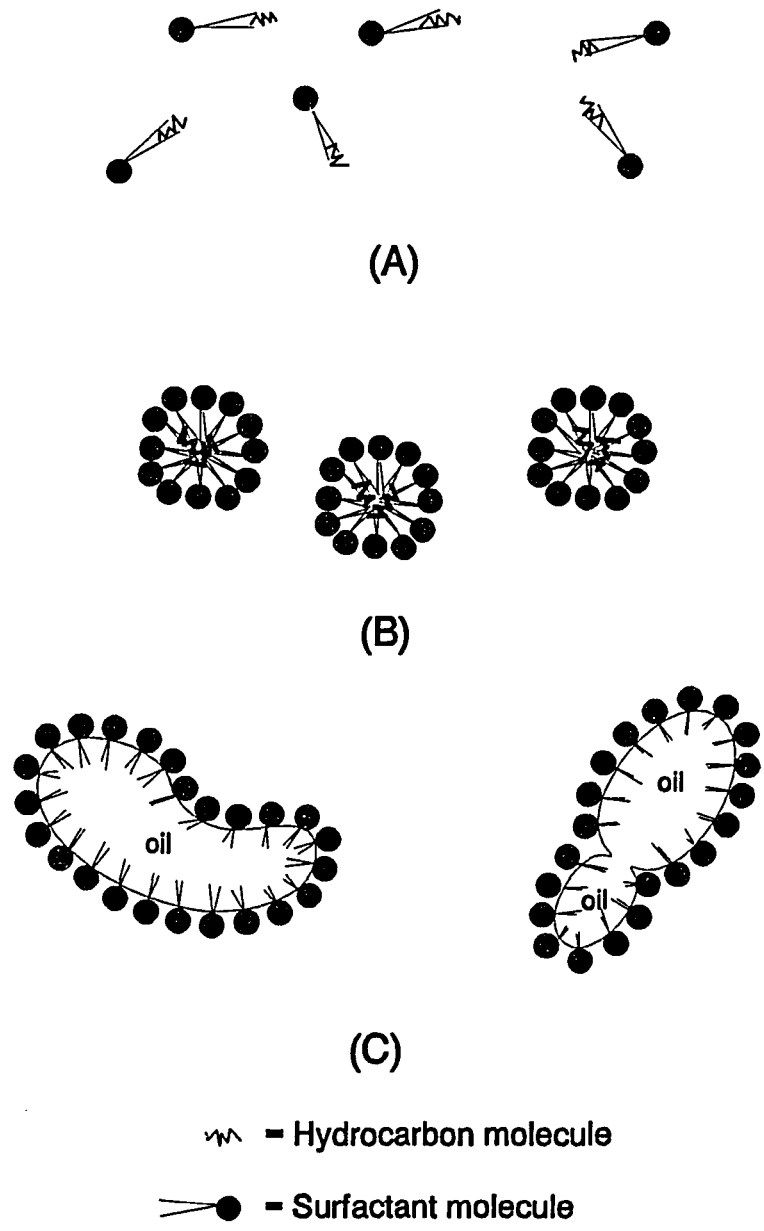

Figure 1.2 Three types of interaction between surfactant and hydrocarbon
(A) Association of surfactant monomers with hydrocarbon.
(B) Micelle-induced solubilization.
(C) Emulsification

Solubilization of hydrophobic compounds by surfactant generally commences at the cmc and is a linear function of surfactant concentration at surfactant concentrations greater than the cmc (Moroi, et al., 1982, 1983; Edwards at al., 1991). The enhancement effects are attributed to an incorporation of hydrophobic molecules into the hydrophobic core of each micelle in surfactant solution (Fig. 1.2B). The amount of organic compound that is solubilized depends on surfactant structure, ionic strength, temperature, solubilizate size and physical state (Attwood and Florence, 1983). For example, the solubilization of hydrophobic compounds by nonionic surfactants is closely proportional to the lipid tail content of the surfactant, whereas the solubilization by ionic surfactants is less accountable in terms of their lipid tails. (Kile and Chiou, 1989). Addition of a small amount of neutral electrolyte to solutions of ionic surfactants enhances the solubilization of hydrocarbons (Klevens, 1950). For ionic surfactants an increase in temperature generally results in an increase in the extent of solubilization of hydrocarbons (Kuieda and Shinoda, 1979). Hydrocarbon size is indirectly correlated to solubilization with increasing molecular weight causing decreasing solubilization. Crystalline solids generally show less solubility in micelles than do liquids of similar structure, the latent heat of fusion presumably opposing the change.

An emulsion is a significantly stable suspension of droplets of liquid of a certain size within a second, immiscible liquid. The term significantly stable means relative to the intended use and may range from a few minutes to a few years. Emulsification is distinguished from solubilization by (i) In emulsification the solubilizate is in a separate phase from the solution, whereas in solubilization the solubilizate is in the same phase

as the solution, (ii) Emulsions are thermodynamically unstable whereas solubility is thermodynamically stable. Emulsions are divided into three types based on the size of the dispersed droplets: (i) macroemulsions, opaque emulsions with droplets > 400 nm size, easily visible under a microscope; (ii) miniemulsions, blue-white emulsions with droplet sizes between 100 nm and 400 nm; (iii) microemulsions, transparent dispersions with droplets < 100 nm in size.

In the formation of emulsions, one of the two immiscible liquids is broken up into droplets that are dispersed in the second liquid (Fig. 1.2C). The emulsion produced without adding an emulsifying agent is thermodynamically unstable and quickly breaks by coalescence of the dispersed droplets. The function of the emulsifying agent is to stabilize this basically unstable system for a sufficient time so that it can perform some function. The emulsifying agent does this by adsorption at the liquid-liquid interface as an oriented interfacial film. This oriented film performs two functions: (i) It reduces the interfacial tension between the two liquids and consequently the thermodynamic instability of the system resulting from the increase in the interfacial area between the two phases; (ii) It decreases the rate of coalescence of the dispersed liquid particles by forming mechanical, steric, and electrical barriers around them. In the formation of macroemulsions, the reduction of interfacial tension reduces the amount of mechanical work required to break the inner phase into dispersed particles. In the case of microemulsions, the interfacial tension is reduced, at least temporarily, to such a low value that emulsification occurs spontaneously.

Emulsifying agents produced by microorganisms have substrate specificity. For

example, *Acinetobacter calcoaceticus* RAG-1 emulsan did not emulsify pure aliphatic (e.g., hexadecane), aromatic (e.g., methylnaphthalene), or cyclic (e.g., dicyclohexane) hydrocarbons (Rosenberg et al., 1979). In contrast, petroleum fractions which contain an appropriate mixture of aliphatic and aromatic compounds, such as Agha Jari crude oil or gas oil, were emulsified. Fractions such as kerosene and gasoline, which contain mainly aliphatics, were emulsified only if an aromatic compound was added. The emulsifying agent from *Pseudomonas cepacia* grown on 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) was found to only emulsify chlorinated aromatic compounds such as 2,4,5-T, 2,3,4,6-tetrachlorophenol and 2,4-dichlorophenoxyacetic acid (2,4-D); however, nonchlorinated compounds such as biphenyl, dibenzothiophene and hexadecane were not emulsified (Banerjee, et al., 1983).

Interaction between surfactant and microbial cells

In an aqueous system containing surfactant, hydrocarbon and microbial cells, surfactant molecules may interact or even sorb onto the cell surface and consequently change cell surface hydrophobicity, thus affecting the microbial uptake of hydrocarbon. However, little information is available about the effect of the surfactant-cell interaction on biodegradation largely because current research mostly focus on the effect of surfactant-hydrocarbon interaction on biodegradation. Any interaction between surfactant and microbial cells is determined by chemical structure and physical properties of microbial cell surface and surfactant. The microbial cell surface contains hydrophobic as well as hydrophilic sites. The former are composed mainly of lipids and lipid part

of lipopolysaccharide. The latter consist of charged groups such as the carboxyl, phosphate, amino, or guanidyl group and the noncharged hydroxyl group. Because the isoelectric point (pI) of microorganisms is between pH 2 and pH 5 (James, 1991), microorganisms are negatively charged at neutral pH. Similarly, surfactants contain hydrophobic and hydrophilic moieties. The hydrophilic moiety of a surfactant molecule may carry positive (cationic) or negative (anionic) or no charge (nonionic). Figure 1.3 shows four possible types of interactions between surfactants and cells. They are an electrostatic interaction, a hydrophobic interaction, hydrogen bonding and cationic bridging. The following sections discussing surfactant-cell interactions are speculative, where possible supporting evidence is cited.

An electrostatic interaction is referred to as the attachment of a surfactant molecule to a cell through neutralization of two oppositely charged groups carried by the respective surfactant and cell. In this way, cationic surfactants may sorb onto the cell surface with the positively charged hydrophilic head group oriented toward the negatively charged surface and its hydrophobic group oriented away from the surface, making the cell surface hydrophobic (Fig. 1.3A). For example, the cationic surfactant cetylpyridinium chloride (CPC) significantly enhanced microbial adhesion to the liquid hexadecane and the solid hydrophobic surface polystyrene (Goldberg et al., 1990b). The adhesion was inhibited by the presence of inorganic cations such as Na^+ and Mg^{2+} and low pH. This suggests that the observed "hydrophobicity" of the microbial cells in the presence of cationic surfactants was due to a loss of surface electronegativity (Goldberg et al, 1990a). The enhanced cell hydrophobicity by cationic surfactants should increase

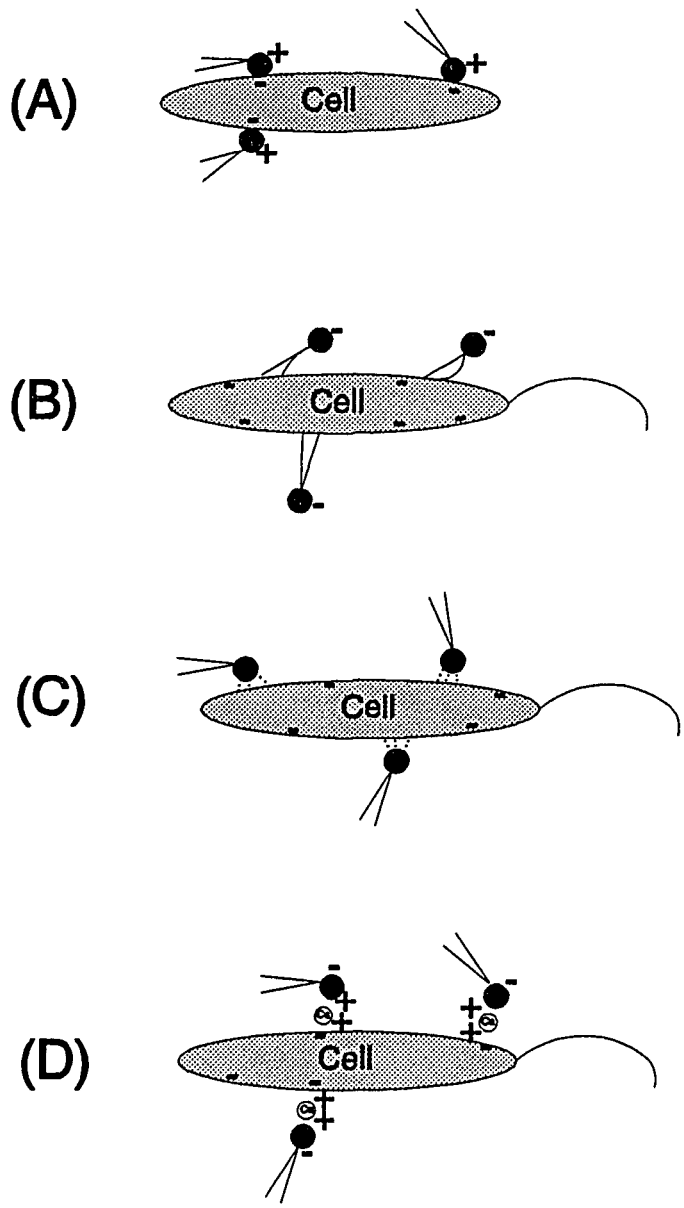

Figure 1.3 Proposed interactions between surfactant and cell
(A) Electrostatic interaction.
(B) Hydrophobic interaction.
(C) Hydrogen bonding
(D) Cation bridging

the microbial uptake of hydrocarbon. Unfortunately, cationic surfactants may inhibit microbial growth by their toxicity, which results from disruption of cell membrane integrity by surfactant interaction with lipid structural components(Swisher, 1970). In view of this fact it is not surprising that biosurfactants are either anionic or nonionic (Zajic and Panchal, 1976; Zajic and Seffens, 1984).

A second possible interaction is the hydrophobic interaction between surfactant and cell (Fig. 1.3B). Nonionic surfactants may sorb onto the cell surface with the hydrophobic moiety oriented toward the cell surface and with the hydrophilic moiety away from the surface. For example, the sorption of nonionic surfactant polethylene glycol mono-*n*-oleylether on the bacterial cell surface may involve the hydrophobic interaction between the alkyl group of the surfactant and the hydrophobic sites on the bacterial cell surface (Noda and Kanemasa, 1986). The extent of surfactant adsorption was found to be directly proportional to cell hydrophobicity. Similarly, anionic surfactants may sorb onto the cell surface through hydrophobic interaction. For example, the electrophoretic mobility of streptococcal cells was increased by the addition of the anionic surfactant sodium dodecyl sulphate (SDS) (Hill et al., 1963). This was probably due to the adhesion of the hydrocarbon chain of SDS to the lipid on the cell surface. This type of interaction should make cells more hydrophilic and thus reduce cell attachment to hydrophobic substances.

A third possible interaction between surfactant and cell is that the hydrophilic moiety of nonionic or anionic surfactants binds to the hydrophilic sites on the cell surface by hydrogen bonding, making cells more hydrophobic (Fig. 1.3C). In

particular, glycolipid bisurfactants may attach to the cell surface of their producing organisms in this way. The adhesion of glycolipids to the cell surface through sugar receptors has been reported (Krivan et al., 1989).

A fourth possibility is the association between an anionic surfactant and a cell through cation bridging (Fig. 1.3D). The formation of the cation bridge requires the presence of divalent metal ions such as Ca^{2+} and Mg^{2+} in solution. The function of metal ions is to link the negatively charged group of the surfactant to the negatively charged cell surface. However, no supporting evidence is available.

Effect of surfactants on the uptake of slightly water soluble hydrocarbons

Although surfactants can increase the amount of slightly water soluble hydrocarbons present in the aqueous phase, results of studies on the effects of surfactant additions on microbial degradation of hydrocarbons are diverse. Tables 1.3, 1.4 and 1.5 are summaries of recently reported effects of surfactants on biodegradation of aromatic hydrocarbons (Table 1.3) and aliphatic hydrocarbons (Table 1.4) as well as hydrocarbons in soil-water systems (Table 1.5). The presence of surfactants has been reported to be beneficial, detrimental or ineffective to the extent and rate of biodegradation. However, the mechanisms of enhancement or inhibition of biodegradation by addition of surfactants are not yet known.

In considering the effect of surfactant on the microbial uptake of hydrocarbon, one should keep in mind that there is a three-way interaction between surfactant, hydrocarbon and microbial cell. The rate of biodegradation in the system depends on

Table 1.3 Summary of reported effects of surfactants on degradation of aromatic hydrocarbons and related compounds

Overall Effect	Hydrocarbon	Surfactant	Microorganism	Reference
+	Phenanthrene, Fluoranthene	Brij 35, Triton X-102, et.	*Mycobacterium* sp.	Tiehm, (1994)
+	Phenanthrene	Tween 20, 40, 60, 80, 85	*Mycobacterium* sp.	Guerin and Jones, (1988)
+	Naphthalene in heptamethyl nonane	Triton X-100	*Arthrobacter* sp.	Efroymson and Alexander, (1991)
+	Naphthalene in emulsion	Mixture of Tergitol NP-14 and Neodol 25-3A	*Pseudomonas putida*	Cox and Williams, (1980)
+	PCBs in emulsion	Ligninsulfonate	*Pseudomonas* sp.	Liu, (1980)
+	4-Chlorobiphenyl in emulsion	β-Cyclodextrin	Mixture of *Arthrobacter* sp. and *Pseudomonas* sp.	Hiramoto, et al., (1989)
0	Naphthalene in micelle	Brij 30, Triton X-100	Mixed culture	Liu et al., (1995)
0	Phenanthrene	Emulsan	Pure culture	Foght et al., (1989)
-	Phenanthrene Anthracene Carbazole	Emulsan	Mixed culture	Foght et al., (1989)
-	Toluene, m-Xylene, Naphthalene, 1,2,4-Trimethylbezene	Unidentified biosurfactants	Mixed culture	Falatko and Novak, (1992)

+, enhancement, -, inhibition, 0, no effect, PCB, polychlorinated biphenyl

Table 1.4 Summary of reported effects of surfactant on degradation of aliphatic hydrocarbons and crude oil

Overall effect	Hydrocarbon	Surfactant	Microorganism	Reference
+	Decane, Tetradecane in micelle	Neodol 25-9, Neodol 25-7 Neodol 25-3	*Pseudomonas aeruginosa, Ochrobacterium anthropi*	Bury and Griffin, (1993)
+	Octadecane, Hexadecane	Rhamnolipid	*Pseudomonas oleovorans*	Churchill et al., (1995)
+	Octadecane	Rhamnolipid	*Pseudomonas aeruginosa*	Zhang and Miller, (1992)
+	Octadecane, Hexatriacontane in liposome	Phospholipid	*Pseudomonas* sp.	Miller and Bartha, (1989)
+	Crude oil in emulsion	Mixed anionic and nonionic surfactant	Mixed culture	Robichaux and Myrick, (1972)
+	Hexadecane in emulsion	Triton X-100, Brij 35, Fatty acids, Lipids, et.	*Acinetobacter lwoffii, Pseudomonas aeruginosa*	Breuil and Krushner, (1980)
-	Pristane, Hexadecane	Emulsan	Pure or mixed culture	Foght et al.,(1989)
-	Hexadecane in heptamethylno nane	Triton X-100	*Arthrobacter* sp.	Efroymson and Alexander, (1991)

+, enhancement, -, inhibition

Table 1.5 Summary of reported effects of surfactant on hydrocarbon degradation in soil-water systems

Overall effect	Hydrocarbon	Surfactant	Inoculum	Reference
+	Tetradecane, Hexadecane, Pristane	Rhamnolipid	No	Jain et al., (1992)
+	Lubricating oil	Corexit 7764	Mixed culture	Rittman and Johnson, (1989)
+	Mixed aliphatic hydrocarbons	Glycolipid biosurfactants	No	Oberbremer et al., (1990)
+	Phenanthrene	Afonic 810-60, Novel II 1412-56 (low concentration)	No	Aronstein et al., (1991)
0				
-		(high concentration)		
	Phenanthrene	Triton X-100, Brij30, Tergitol NP-10 (low concentration)	Mixed culture	Laha and Luthy, (1991)
0				
-		(high concentration)		

+, enhancement, -, inhibition, 0, no effect

the interaction between these components. It is generally accepted that solubilization of hydrocarbon by surfactants should have a positive effect on biodegradation rate. This effect may vary with hydrocarbon structures because of different uptake mechanisms between aromatic and aliphatic hydrocarbons as described above. For aromatic hydrocarbons, surfactants may enhance biodegradation by increasing the substrate solubility and mass transfer. For aliphatic hydrocarbons, surfactants may enhance biodegradation by increasing the substrate surface area for microbial attachment. This seems true in some cases, especially when emulsions of hydrocarbon are formed in the presence of surfactants (Tables 1.4 and 1.5). However, rate enhancement of hydrocarbon degradation is never greater than that of hydrocarbon solubilization by surfactants (Zhang and Miller, 1992). In some cases availability and uptake of hydrocarbon may be decreased even though solubilization increased (Falatko and Novak, 1992). Thus, it is obvious that consideration of solubilization alone is not enough to explain the effect of surfactants on hydrocarbon degradation. Therefore, it is important to understand the effect of surfactant-cell interaction on hydrocarbon degradation.

In a system containing surfactant, hydrocarbon and microbial cells, hydrocarbon is encapsulated into micelles or exists as surfactant-coated droplets or particles. Therefore, surfactants may reduce the hydrocarbon uptake by interference with contact between cell and the substrate. The inhibition of hydrocarbon degradation often occurs when the surfactant concentration is high. For example, Laha and Luthy (1991) reported that biodegradation of phenanthrene was not inhibited by nonionic surfactants until the surfactant concentration reached the cmc. However, some current studies with

hydrocarbons solubilized in micelles (Miller and Bartha, 1989; Bury and Miller, 1993; Liu et al., 1995) show that the hydrocarbon is readily bioavailable and degradable by microorganisms. For example, Liu et al. (1995) showed that solubilization of naphthalene by micelles of Brij 30 or Triton X-100 had the same degradation rates as in the absence of the surfactant. Bury and Miller (1993) reported that solubilization of decane and tetradecane in micelles of Neodol 25-9, 25-7 or 25-3 enhanced rates of hydrocarbon degradation relative to the control.

Why are results of effects of surfactants on microbial degradation so diverse that no generalization can be made? Clearly, each research study uses its own biodegradation system, which differs in hydrocarbon, surfactant and microorganism (Tables 1.3, 1.4, 1.5). Biodegradation rates depend on various factors of each system, which are described as followed:

(1) Chemical structure of hydrocarbon It is generally believed that the microbial uptake of aromatic hydrocarbons occurs in dissolved state and the uptake of aliphatic hydrocarbons by cell attachment (Thomas et al., 1986; Nakahara et al., 1977). It is possible that for aromatics, surfactants affect the uptake of the micellar hydrocarbon and for aliphatics, surfactants affect the attachment of cells to the hydrocarbon. However, no information is available.

(2) Physical state of hydrocarbon Dispersion of hydrocarbon by surfactants is greater in liquid state than in solid state. Therefore, surfactants may have greater effects on biodegradation of liquid hydrocarbons than on that of solids.

(3) Water solubility of hydrocarbon Enhanced solubilization of

polar and relative soluble hydrocarbons by surfactants is relatively smaller than that of nonpolar and slightly soluble hydrocarbons. Therefore, surfactants may have larger impacts on the biodegradation rate of very slightly soluble hydrocarbons than on that of more water soluble hydrocarbons. For example, Churchill et al. (1995) showed that the extent of rate enhancement of hydrocarbon mineralization resulting from rhamnolipid addition was significantly greater for the sparingly soluble alkanes, hexadecane and octadecane, than for the more soluble aromatics, toluene and 2-methylnaphthalene.

(4) Structure of surfactant Two surfactants with different structures may have different physical properties such as surface tension, interfacial tension, water solubility. Therefore, they may have different interactions with hydrocarbon and microbial cell and consequently different effects on biodegradation. For example, investigation of the effect of sophorose lipids on alkane degradation showed that lactonic form inhibited hexadecane biodegradation, while the acid form stimulated hexadecane biodegradation (Ito and Inoue, 1982; Ito et al., 1980).

(5) Concentration of surfactant Solubilization of hydrocarbons by surfactants is directly proportional to surfactant concentration. Considering solubilization alone, rates of enhancement of degradation by surfactants should be higher at high concentration than at low concentration. In contrast, surfactant at high concentration may form physical barriers between hydrocarbon and cells thus reducing biodegradation. For example, a study by Aronstein et al. (1991) showed that nonionic surfactants at low concentration stimulated biodegradation of sorbed phenanthrene in soil but the biodegradation was inhibited or ineffective when the concentration was high.

(6) Surface property of microbial cell Very important but less understood is the effect of cell surface property on hydrocarbon biodegradation in the presence of surfactant. Cell hydrophobicity has an important role in hydrocarbon uptake in the absence of surfactant. It may also be important in the presence of surfactant. Other cell surface properties e.g. surface charge may also affect hydrocarbon biodegradation in the presence of surfactants.

In summary, the effects of surfactants on hydrocarbon degradation depend on various factors of a system containing surfactant, hydrocarbon and microbial cell. Some of these factors have positive effects on biodegradation while others have negative effects on biodegradation. The overall effect of surfactants on biodegradation relies on the dominant factors among them. Current studies on the effects of surfactants on biodegradation only consider these factors affecting surfactant-hydrocarbon interaction but neglect the importance of surfactant-cell interaction. As a result, it is difficulty to interpret the current observations on the effects on surfactant addition on hydrocarbon biodegradation. In order to understand the mechanisms of effects of surfactants on hydrocarbon biodegradation, further studies are needed (i) to define cell surface properties affecting hydrocarbon degradation in the presence of surfactants, (ii) to study the effect of the surfactant-cell interaction on hydrocarbon degradation, (iii) to establish the relationship between surfactant structure and hydrocarbon biodegradation, and (vi) to investigate the physiological role of biosurfactants in nature.

Dissertation format

This dissertation comprises four chapters following by two appendices. Chapter 1 is the introduction to this dissertation. Chapters 2 and 3 are summaries of experimental results of the most current researches performed. Chapter 4 is the summary of this dissertation and recommendations for further work. Appendices A and B are two peer-reviewed papers describing initial research performed during graduate study.

The first paper attached to appendix A was published in the June issue of Applied and Environmental Microbiology in 1994. I designed and conducted all the experiments except for rhamnolipid structure identification by FAB mass spectra. Dr. Raina Miller, co-author, helped analyze the data and write the paper.

The second paper attached to appendix B will be published in Applied and Environmental Microbiology in this year (1995). I designed and executed all the experiments. Dr. Guigen Li in Chemistry Department of the University of Arizona helped perform ^{1}H NMR. Dr. Raina Miller, co-author, helped analyze the data and write the paper.

CHAPTER 2

SOLUBILITY AND BIODEGRADATION OF *N*-ALKANES IN THE PRESENCE OF *PSEUDOMONAS* RHAMNOLIPID SURFACTANT MIXTURES

Introduction

The biodegradation of hydrocarbons is often restricted because of the low water solubility. In previous work we have shown that the addition of rhamnolipid biosurfactants can enhance *n*-alkane biodegradation by increasing solubility of the substrate and by increasing cell hydrophobicity of the degrading microorganism (Zhang and Miller, 1992 and 1994). The rates of enhancement of biodegradation were found to depend on surfactant structure, surfactant concentration, physical state of the substrate and cell surface hydrophobicity of microorganism (Zhang and Miller, 1992, 1994 and 1995). However, all the work was done with individual biosurfactants. In reality, biosurfactants produced from most microorganisms are mixtures containing several types of surfactants that have minor structural differences. For instance, *Pseudomonas* sp. often produce a mixture of monorhamolipid acid and dirhamnolipid acid (Syldatk et al., 1985). Sophorose lipids from *Torulopsis* yeasts can consist of a mixture of up to six different structural components in the lactonic and acidic form of the lipids (Asmer et al., 1988). The growth of *Rhodococcus* sp. on *n*-alkanes produced trehalose lipids containing a homolog mixture of mycolic acids which were esterified to a disaccharide trehalose unit (Ramp et al., 1979). However, the role of these mixed surfactants in hydrocarbon biodegradation is not clear.

Mixed surfactant solutions usually have different surface active properties than single surfactant. For instance, mixtures of different synthetic surfactant often exhibit synergistic effects on the surface active properties of the systems, resulting in lower critical micelle concentrations (cmc) and interfacial tensions than would be expected based on the properties of the single surfactant alone (Rosen, 1989). Because of different surface active properties, surfactant mixtures would be expected to have a different effect from single surfactants on hydrocarbon solubility and biodegradation. Therefore, the purpose of this study was to evaluate some of the physicochemical properties of mixed rhamnolipids and to evaluate the effect of these mixtures on the solubility and biodegradation of *n*-alkanes. Two different mixed rhamnolipid systems were used in this study: dirhamnolipid acid (dR-A)/dirhamnolipid methyl ester (dR-Me) (anionic-nonionic mixture) and monorhamnolipid acid (mR-A)/dirhamnolipid acid (anionic-anionic mixture). These two rhamnolipid systems were chosen because dirhamnolipid acid and methyl ester have different physico-chemical properties while mono- and dirhamnolipid acids have similar physio-chemical properties (Table 2-1). Two model *n*-alkanes used were hexadecane (liquid) and octadecane (solid), which have similar chemical structures and physical properties except for their physical state at 25°C. Solubility and biodegradation of *n*-alkanes in the presence of mixed rhamnolipids in aqueous solution were determined and compared with the results from single rhamnolipids.

Materials and methods

Microorganisms

Pseudomonas aeruginosa ATCC 9027 was obtained from the American Type Culture Collection (Rockville, MD). The culture was maintained at 4°C on *Pseudomonas* agar P medium (Difco, Detroit, Mich.) by monthly transfers. Experiments by measuring the surface tension of culture supernatant showed that *P. aeruginosa* ATCC 9027 did not produce biosurfactants during growth in mineral salts medium containing *n*-alkanes. Also, the culture did not utilize rhamnolipids as a sole source of carbon.

Rhamnolipids

Monorhamnolipid acid (mR-A) was produced and purified from *Pseudomonas aeruginosa* ATCC 9027 as described by Zhang and Miller (1992, 1994). Dirhamnolipid acid (dR-A) was a gift from Kyowa Hakko Kogyo Co., Ltd (Tokyo, Japan). Dirhamnolipid methyl ester (dR-Me) was synthesized from dirhamnolipid acid by the diazomethane method as previously described (Zhang and Miller, 1995). Mixed rhamnolipid solutions were prepared by mixing two types of rhamnolipids at equal molar ratios. The surface tension and interfacial tension of rhamnolipids were determined using a Fisher (Pittsburgh, PA) surface tensiometer (Model 21), that employs the du Nouy ring method. The interfacial tension was measured between hexadecane and 0.1 M, pH 7.0 phosphate buffer. The critical micelle concentration (cmc) of rhamnolipids was determined from a semi-logarithmic plot of surface tension against the surfactant concentration (Magaritis, 1979).

Solubilization tests

The apparent solubility of hexadecane and octadecane in rhamnolipid solutions was determined by using [1-^{14}C]hexadecane (specific activity, 2.2 mCi/mmol, 98% pure) and [1-^{14}C]octadecane (specific activity, 3.6 mCi/mmol, 98% pure) obtained from Sigma, St. Louis, MO. A mixture of alkane and [^{14}C]alkane dissolved in chloroform was added to a series of test tubes (16 × 100 mm). After evaporation of solvent, 2 ml of rhamnolipid solution in 0.1 M, phosphate buffer (pH 7.0) was added. The final concentrations of alkane were 4 mM and the alkane specific activity was 0.5 mCi/mmol. For octadecane, the test tubes were then incubated at 37°C in a water bath for 30 sec, to melt the coated octadecane and then cooled at room temperature until the octadecane solidified on the surface of the solution. The test tubes were then incubated at 23°C with gyratory shaking (200rpm). After 24 h, the solutions were filtered through a Whatman GF/D filter (pore size, 10 μm) and 0.2 ml was added to 5 ml of Scintiverse BD (Fisher, Pittsburgh, Pa). Radioactivity was determined with a Packard (Meridem, Conn.) Tri-Carb liquid scintillation counter (model 1600 TR) and used to calculate hydrocarbon solubility.

Biodegradation tests

Microbial utilization of hexadecane and octadecane was determined by measurement of protein increases as an indication of cell growth on *n*-alkanes, which served as a sole source of carbon (Zhang and Miller, 1995). Ten ml of mineral salts medium containing rhamnolipids was added to 2 × 13 cm test tubes containing 4 mM

alkane. For octadecane, the solid alkane was melted and then cooled at room temperature. The tubes were inoculated with a 2.5% inoculum of ATCC 9027 (late log phase) grown in Kay's minimal medium (Warren, et al., 1960) at 37°C for 24 h and incubated with gyratory shaking (200 rpm) at 23°C. Periodically, 0.5-ml samples were taken from each test tube and heated for 10 min with 0.05 ml of 1 N NaOH, and the protein content was determined by the method of Lowry et al. (1951).

Results

Surface active properties of mixed rhamnolipids

Mixed rhamnolipids had different surface active properties from single rhamnolipids as shown in Table 2.1. For the dR-A:dR-Me mixture (1:1 ratio), the mixture had surface tension (30 dyn/cm) lower than both the dR-A (36 dyn/cm) and the dR-Me (31 dy/cm). This mixture also had interfacial tension (0.5 dyn/cm) lower than the dR-A (5 dyn/cm) but slightly higher than the dR-Me (< 0.1 dyn/cm). Interestingly, mixing of the slightly soluble dR-Me (solubility = 0.04 mM) with the high water soluble dR-A (solubility $>$ 15 mM) (Zhang and Miller, 1995) increased the solubilization of the dR-Me in aqueous phase. As a result, the solution of the mixture (1:1 ratio) was clear. For the mR-A:dR-A mixture (1:1 ratio), the mixture had surface tension (30 dyn/cm) and interfacial tension (2 dyn/cm) higher than the dR-A (36 and 5 dyn/cm) but lower than the mR-A (28 and 1 dyn/cm). The mixture had the same cmc value (0.1 mM) as the single rhamnolipids did.

Alkane solubilization by mixed rhamnolipids

The dR-A:dR-Me mixture (1:1 ratio) generally showed a better effect on the solubilization of hexadecane (Fig. 2.1A) and octadecane (Fig. 2.1B) than individual dirhamnolipids. As shown in Figure 2.1A, the mixture had the same enhancement of the solubility of hexadecane as the dR-Me did. However, the mixture enhanced the solubility much more than the dR-A did. At surfactant concentrations from 0 to 0.1 mM, there was a linear dependence of alkane solubilization on surfactant concentrations. The linear portion of each plot was used to calculate a molar solubilization ratio (MSR = moles hydrocarbon solubilized/mole of surfactant). The MSR for the mixture and the dR-Me was 5.2 while the MSR for the dR-A was 0.13, a difference of 41-fold. The mixture exhibited a synergistic effect on the solubilization of octadecane (Fig. 2.1B). The solubility of octadecane was increased by the mixture much more than by either the dR-Me or the dR-A. The MSR for the mixture was 2.2 while the MSR for the dR-Me and dR-A was 0.074 and 0.15, a respective difference of 29-fold and 14-fold respectively.

In contrast, the mR-A:dR-A mixture (1:1 ratio) demonstrated an additive effect on the solubilization of hexadecane as shown in Figure 2.2. The solubility of hexadecane was enhanced by the mixture (MSR = 0.2) more than by the dR-A (MSR = 0.0019) but less than by the mR-A (MSR = 0.44).

Effect of rhamnolipid mixtures on alkane biodegradation

Biodegradation of hexadecane (Fig. 2.3A) and octadecane (Fig. 2.3B) by *P.*

aeruginosa ATCC 9027 was examined in the presence of the dR-A:dR-Me mixture. In this set of experiments, rhamnolipid concentrations used were 0.05 mM and cell growth was measured by protein increase. As shown in Figure 2.3, the effect of the mixture on alkane biodegradation was additive. The rates (slope of each curve) of the biodegradation of hexadecane and octadecane were enhanced by the mixture more than by the dR-acid but less than by the dR-Me relative to the control.

Biodegradation of hexadecane by *P. aeruginosa* ATCC 9027 was also examined in the presence of varying concentrations of the mR-A:dR-A mixture (1:1 ratio). As shown in Figure 2.4, rates of enhancement of hexadecane biodegradation were dependent on rhamnolipid concentrations. At a surfactant concentration of 0.05 mM, the mixture had the same rate of enhancement of biodegradation as the mR-A, but the rate enhancement was slightly less than the dR-A relative to the control. At a surfactant concentration of 0.1 mM, both the mixed and the single rhamnolipids had almost the same rates of enhancement of biodegradation relative to the control. However, at surfactant concentration of 0.4 mM, the biodegradation was totally inhibited by the mixture and the dR-A but was enhanced by the mR-A relative to the control.

Discussion and suggestions for further study

In this study, the effect of mixing two single rhamnolipids on the solubilization and biodegradation of *n*-alkanes was investigated. In some cases, the effect was additive and in others synergistic depending on the mixture tested. For instance, the dR-A:dR-Me mixture generally had a synergistic effect on alkane solubilization (Fig. 2.1) while

the dR-A:mR-A mixture showed an additive effect on alkane solubilization (Fig. 2.2). The differential effects of surfactant mixtures on solubilization can be explained on the basis of the molecular interaction between single surfactants (Rosen, 1989). For synergistic effects, there is a strong interaction between surfactant constituents. For additive effects, there is a weak interaction or no interaction between surfactant constituents. For instance, in the presence of dR-A, the solubility of dR-Me was greatly increased, probably through association of dR-A and dR-Me monomers. As a result, the effective concentration of dR-Me was increased and thus, the dR-A:dR-Me mixture showed a synergistic effect on alkane solubilization.

The effect of rhamnolipid mixtures on alkane biodegradation was more complicated. Though the dR-A:dR-Me mixture generally showed a better effect on the alkane solubility than the dR-Me (Fig. 2.1), the alkane biodegradation was enhanced by the mixture less than by the dR-Me alone (Fig. 2.3). These results suggest that the alkane solubilized by the dR-A:dR-Me mixture is less available than that dissolved by the dR-Me alone. One possible explanation for this is that the dR-Me is a nonionic surfactant (no charge) while the dR-A:dR-Me mixture is an anionic surfactant (negative charge). It is possible that the electronic repulsion between the negatively-charged microbial cell surface and the negatively-charged mixture micelles may reduce the interaction of the micelles with the cells, thus decreasing the biodegradation rate.

We are interested in surfactant mixtures for two reasons: i) microorganisms produce surfactants as mixtures; and ii) mixed surfactants may have some unique

properties useful in contaminant remediation. For example, of the three rhamnolipids tested in this study, the dR-Me had the largest effect on both solubilization and biodegradation of alkanes. However, the low water solubility of the dR-Me limits its potential for use in remediation. By using this surfactant in a mixture, its solubility was greatly increased (Table 1) and its effects on solubilization and biodegradation were not impacted negatively (Figs. 2-1 and 2-3). Thus, the dR-A:dR-Me mixture has much improved potential for removal of hydrocarbons from soil either by solubilization (soil flushing) or enhanced biodegradation. The results in this study appear to be the first report for biosurfactant mixtures and are encouraging. Further work has to be done to find an optimal mixed surfactant system for use in remediation. Various ratios of mixed rhamnolipids need to be tested to determine the optimal mixture ratio. In addition, various surfactant structures (both biological and synthetic) should be mixed to find the optimal mixed surfactant system. Finally, mixed surfactants need to be tested with other hydrocarbons such as aromatic hydrocarbons. In complement to these optimization studies, efforts need to be made to understand the physiological basis for production of biosurfactant mixtures by microorganisms.

Table 2.1 Physico-chemical properties of rhamnolipids and their mixtures

Rhamnolipids/ mixtures	Property			
	Aqueous solubility (mM)	Surface tension (dyn/cm)	Interfacial tension (dyn/cm)[1]	cmc (mM)
mR-A	>15	28	1	0.1
dR-A	>15	36	5	0.1
dR-Me	0.04	31	<0.1	0.04
dR-A:dR-Me (1:1 molar ratio)	>15	30	0.5	0.10
mR-A:dR-A (1:1 molar ratio)	>15	30	2	0.1

[1]Interfacial tension was measured between hexadecane and 0.1 M, pH 7.0 phosphate buffer.

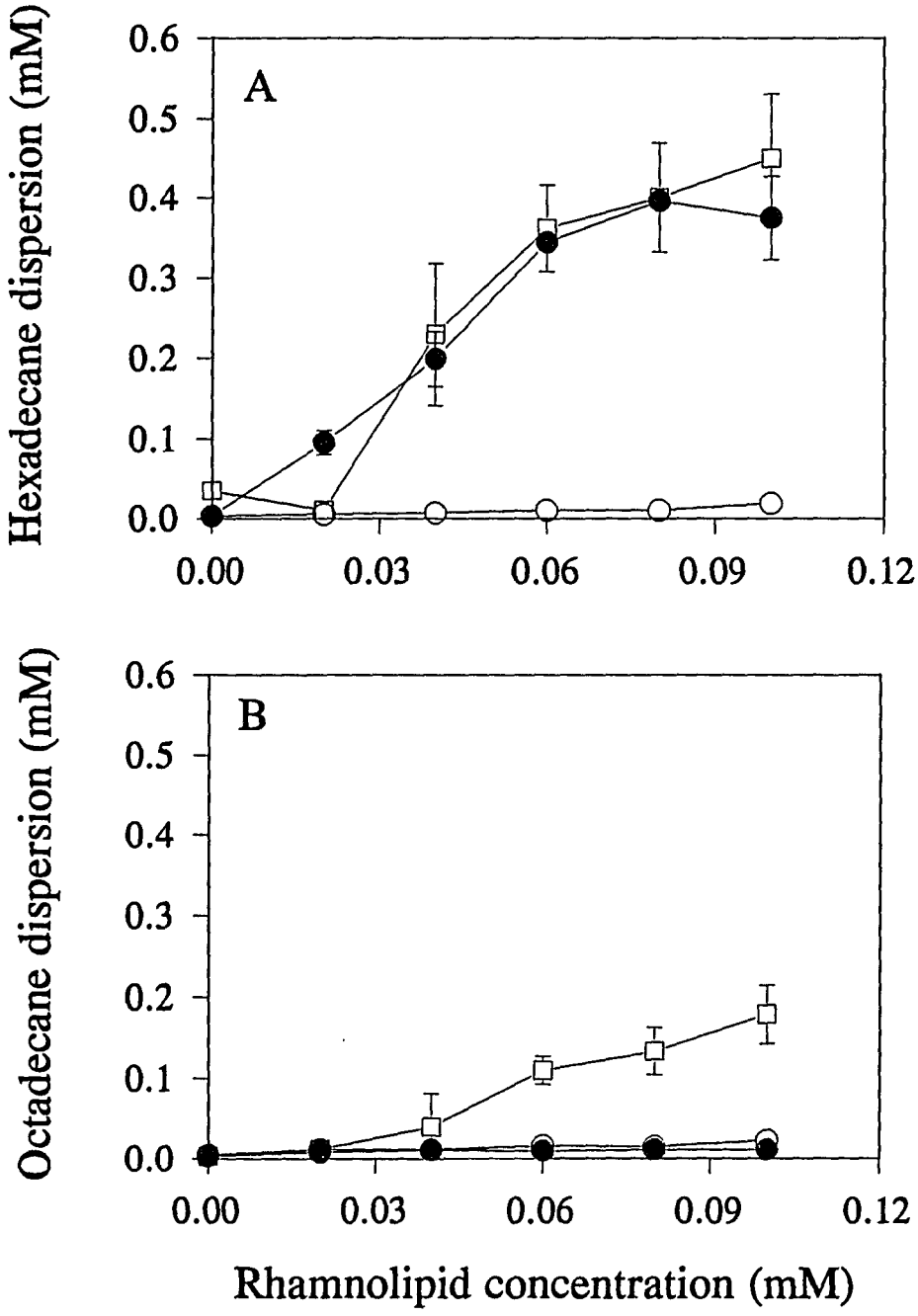

Figure 2.1 Effect of dirhamnolipid acid, dirhamnolipid methyl ester and a 1:1 mixture on the apparent aqueous solubility of hexadecane and octadecane. (A) hexadecane; (B) octadecane. Symbols: ○ dirhamnolipid acid, ● dirhamnolipid methyl ester, and □ the mixture (1:1 ratio).

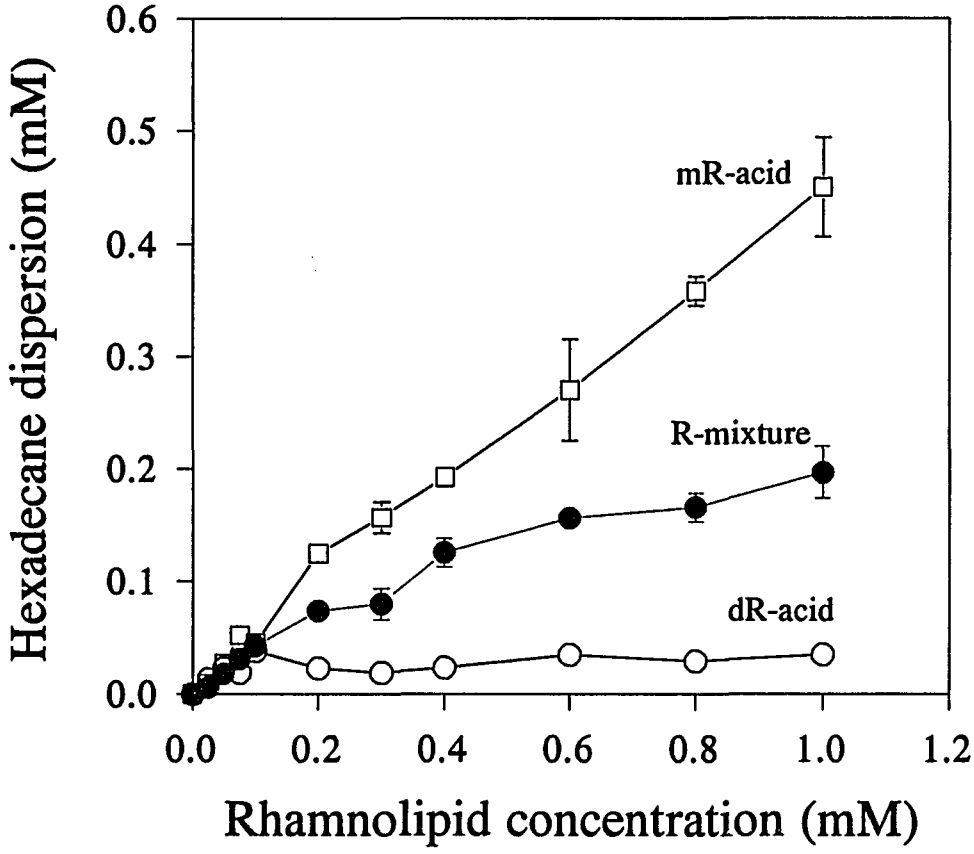

Figure 2.2 Effect of monorhamnolipid acid, dirhamnolipid acid and a 1:1 mixture on the apparent aqueous solubility of hexadecane. Symbols:□ monorhamnolipid acid, ○ dirhamnolipid acid, and ● the mixture (1:1 ratio).

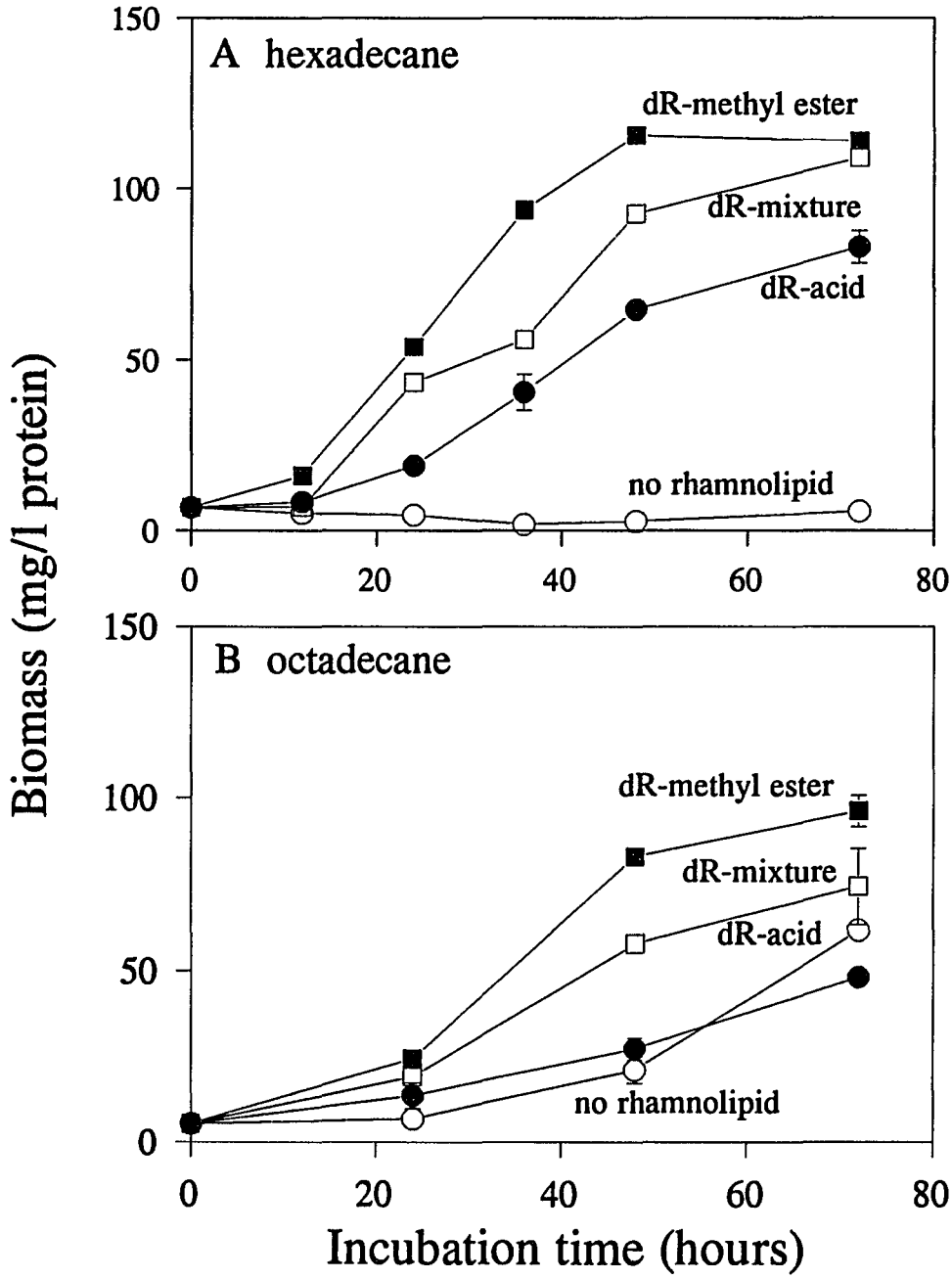

Figure 2.3 Effect of dirhamnolipid acid, dirhamnolipid methyl ester and a 1:1 mixture on the biodegradation of hexadecane (4 mM) and octadecane (4 mM) by *Pseudomonas aeruginosa* ATCC 9027. (A) hexadecane; (B) octadecane. Symbols: ○ no rhamnolipids, ● 0.05 mM dirhamnolipid acid, □ 0.05 mM rhamnolipid mixture (1:1 ratio) and ■ 0.05 mM dirhamnolipid methyl ester.

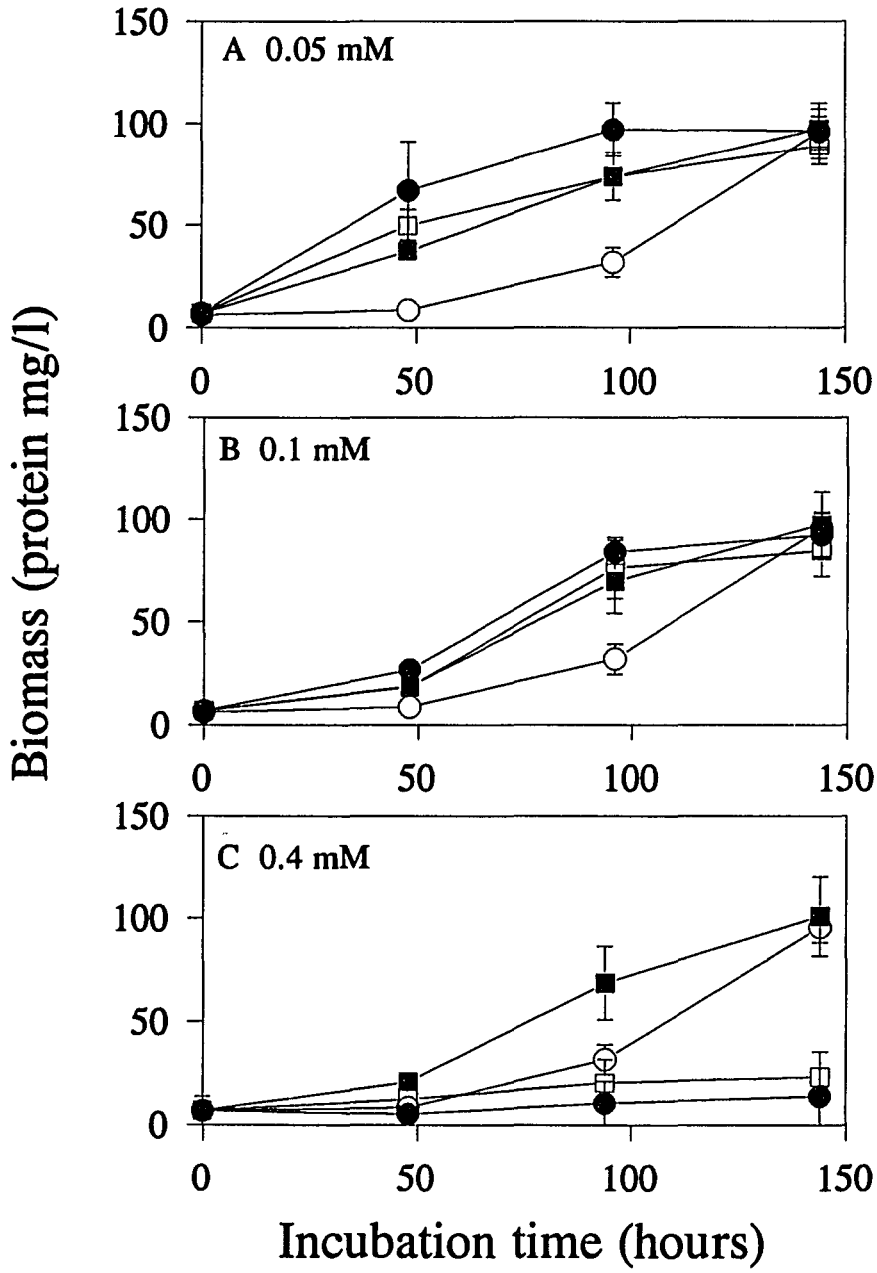

Figure 2.4 Effect of different concentrations of mono-, dirhamnolipid acids and their mixture on the biodegradation of hexadecane (4 mM) by *Pseudomonas aeruginosa* ATCC 9027. (A) 0.05 mM; (B) 0.1mM; (C) 0.4 mM Symbols: ○ no rhamnolipids, ■ monorhamnolipid acid, ● dirhamnolipid acid, and □ the mixture (1:1 ratio).

CHAPTER 3

ENHANCED PHENANTHRENE DEGRADATION BY *PSEUDOMONAS* RHAMNOLIPIDS REQUIRED FOR LIMITED INTERFACIAL AREA

Introduction

Polycyclic aromatic hydrocarbons (PAH) are hazardous compounds because they are toxic, carcinogenic and mutagenic (Amgren et al., 1979). Therefore, remediation of PAH contaminated sites is of general interest. High-molecular-weight PAH such as pyrene and naphthacene (≥ four rings) are highly resistant to biodegradation while low-molecular-weight PAH (≪ four rings) (especially naphthalene, anthracene, and phenanthrene) are considered biodegradable (Gibson and Subramanian, 1984; Weissenfels et al., 1990). However, rate of biodegradation of PAH compounds is often limited by their low water solubility and dissolution rates (Volkering et al., 1992). These limitations may be overcome by enhancing the mass transfer of PAH from the solid to the aqueous phase or by increasing the interfacial area between the PAH and water. For example, Thomas et al. (1986) showed that rates of degradation were higher when PAH was presented as small particles.

Surfactants may increase PAH biodegradation by increasing both the solubility of PAH and the dissolution rate from solid PAH to aqueous solution. Solubilization of PAH results from incorporation of the substrate into micelles (Edwards, et al., 1990), and it has been reported that PAH solubilized in surfactant micelles is readily degradable by microorganisms (Liu et al., 1995). The increase of PAH solubility and dissolution

rate by surfactants should enhance biodegradation. However, attempts to enhance the biodegradation rates of PAH by the use of surfactants have yielded contradictory results (Liu et al., 1995). Surfactants were found to either enhance or inhibit PAH degradation. Unfortunately, because different biodegradation systems were used it is difficult to compare and interpret these results. Thus, it is very important to evaluate the effect of surfactants on PAH degradation in a defined system.

In previous work we have investigated the effect of surfactants on the biodegradation of *n*-alkanes in such a well-defined system (Zhang and Miller, 1992, 1994, 1995). The system consisted of *Pseudomonas* rhamnolipids, hydrocarbon and *Pseudomonas* hydrocarbon degraders. In this system, the degraders are unable to produce and utilize rhamnolipids during growth on hydrocarbons, and rhamnolipids are not toxic to the degraders. The purpose of this study was to investigate the biodegradation of PAH in this degradation system. The PAH used in this study was phenanthrene, a solid compound with the water solubility of 1.6 mg/l (Mackay et al., 1992). The microorganisms used were four environmental isolates that degrade phenanthrene. Because the interfacial area between the substrate and water controls the mass transfer rate of PAH in aqueous solution and is directly related to the rate of PAH degradation (Thomas et al., 1986), biodegradation was investigated under the conditions of both limited and unlimited substrate interfacial area. In this study, interfacial area of phenanthrene was controlled by the size of surface area coated with the substrate.

Materials and methods

Microorganisms

Pseudomonas putida CRE7 was obtained from Mike Montgomery, Geo-Centers, Inc., Naval Research Lab., Washington, DC. Three species-unidentified phenanthrene degraders SW1, SW2 and SW3 were isolated from Tucson area soils. These cultures were maintained at 4°C on mineral salts medium (MSM) (Zhang and Miller, 1992) agar plates using phenanthrene as sole carbon source and transferred monthly. None of cultures produced biosurfactants during growth in mineral salts medium containing phenanthrene.

Enrichment culture

Microorganisms that were capable of growth on phenanthrene as sole source of carbon and energy were isolated by enrichment from several local Tucson soils. A 2-g sample of soil was added to 50 ml of MSM (Zhang and Miller, 1992) containing phenanthrene (2.8 mM) in 250-ml Erlenmeyer flasks and incubated with gyratory shaking (200 rpm) at 23°C for four weeks. The mixed cultures were then transferred with a 5% inoculum to the fresh liquid medium every other week for three times and finally streak plated on MSM agar plates. The plates were then sprayed with phenanthrene in ethyl ether until the agar surface was covered with thin film of phenanthrene. After incubation at 23°C for two weeks, single colonies were picked and restreaked onto new plates for final isolation.

Rhamnolipids

Monorhamnolipid acid was produced and purified from *Pseudomonas aeruginosa* ATCC 9027 as described by Zhang and Miller (1992, 1994). Dirhamnolipid acid in a crystalline solid was a gift from Kyowa Hakko Kogyo Co., Ltd (Tokyo, Japan).

Solubilization tests

The solubility of phenanthrene in rhamnolipid solutions was determined by using [9-^{14}C]phenanthrene (specific activity, 13.1 mCi/mmol, >98% pure) obtained from Sigma, St. Loius, MO.. A mixture of phenanthrene and [^{14}C]phenanthrene dissolved in chloroform was added to test tubes (16 × 100 mm). After evaporation of solvent for 24 h under hood, 2 ml of rhamnolipid solution in 0.1 M, pH 7.0, phosphate buffer was added. The final concentration of phenanthrene was 2.8 mM and the phenanthrene specific activity was 8 μCi/mmol. The test tubes were incubated at 23°C with gyratory shaking (200rpm). After 24 h, the solutions were filtered through a Corning syringe filter (pore size, 0.2 μm) and 0.2 ml was added to 5 ml of Scintiverse BD (Fisher, Pittsburgh, Pa). Radioactivity was determined with a Packard (Meridem, Conn.) Tri-Carb liquid scintillation counter (model 1600 TR).

Rhamnolipid biodegradation

Utilization of rhamnolipids by phenanthrene degraders was determined by measurement of microbial growth on rhamnolipids as a sole source of carbon. Ten ml of MSM containing rhamnolipids (1.0 mM) was added to a series of 2 × 13 cm test tubes. The tubes were inoculated with a 2.5% inoculum of degraders grown in mineral

salts medium containing phenanthrene (2.8 mM) at 23°C for 3 days and incubated with gyratory shaking (200 rpm) at 23°C. Periodically, 0.5-ml samples were taken from each test tube and heated for 10 min with 0.05 ml of 1 N NaOH, and the protein content was determined by the method of Lowry et al. (1951).

Phenanthrene mineralization tests

For experiments where interfacial area between substrate and water was not limiting, a mixture of phenanthrene and [^{14}C]phenanthrene dissolved in chloroform was used to coat the bottom (50 cm^2) of modified 125-ml micro-Fernbach flasks (Wheaton, Millville, N.J.), designed for the collection of $^{14}CO_2$ and ^{14}C-volatile compounds. For experiments where interfacial area between substrate and water was limited, 50 μl of chloroform-phenanthrene mixture was carefully added to the bottom of a tilted flask (30°) and coated onto a 1.5 cm^2 area. After evaporation of solvent, 20 ml of MSM containing rhamnolipid was added to each flask. The final concentration of phenanthrene was 1.0 mM, and phenanthrene specific activity was 2.3 μCi/mmol. The flasks were inoculated with a 2.5 % inoculum of isolates grown on MSM containing phenanthrene at 23°C for 3 days. The flasks were incubated with gyratory shaking (200 rpm) at 23°C and were flushed periodically as described Marinucci and Bartha (1979) to collect $^{14}CO_2$ and ^{14}C-volatile organic compounds.

Results

Phenanthrene solubilization by rhamnolipids

Phenanthrene solubilization was directly proportional to rhamnolipid concentration (Fig. 3.1). The data in Figure 3.1 could be fitted by two linear functions which are described by the equation $y = ax + b$, where for monorhamnolipid, $a = 0.16$ mol/mol and $b = 0.011$ mM; for dirhamnolipid, $a = 0.029$ mol/mol and $b = 0.017$. The slope of each plot is also the molar solubilization ratio (MSR = moles hydrocarbon solubilized/mole surfactant), a constant often used to describe surfactant solubilizing capacity. The MSR for monorhamnolipid was 0.16 and for dirhamnolipid was 0.029, a 5.5-fold difference.

Microbial utilization of rhamnolipids

The biodegradation of rhamnolipids was examined by growth of four phenanthrene degraders on rhamnolipids as sole carbon and energy sources. Neither *P. putida* CRE7 or (Fig. 3.2A) nor isolate SW2 (Fig. 3.2C) grew on mono- and dirhamnolipids. Isolate SW1 did not grow on dirhamnolipid (Fig. 3.2B) and isolate SW3 did not grow on monorhamnolipid (Fig. 3.2D). Isolate SW1 did grow on monorhamnolipid after 72 h incubation and isolate SW3 grew on dirhamnolipid after 48 h incubation.

Effect of rhamnolipids on phenanthrene mineralization under conditions of limited substrate surface area

The effect of rhamnolipid concentration on the phenanthrene mineralization by *P. putida* CRE7 under conditions of limited substrate surface area (1.5 cm^2 area coated

with phenanthrene) was examined in the presence of both mono- and dirhamnolipid. As shown in Figure 3.3, both mono- and dirhamnolipid stimulated the rate of phenanthrene mineralization at the higher rhamnolipid concentration tested (3.5 mM). There was little effect of the lower rhamnolipid concentration (0.35 mM). In the absence of rhamnolipids, phenanthrene mineralization was linear and the calculated mineralization rate (slope of the plot) calculated was 0.33 % phenanthrene evolved as $^{14}CO_2$ per hour. Total mineralization in 72 hr for *P. putida* CRE7 was 24 % in the absence of rhamnolipids, 28 % in the presence of 0.35 mM, and 40 % in the presence of 3.5 mM.

Effect of rhamnolipid on phenanthrene degradation under conditions of unlimited substrate surface area

The effect of rhamnolipids on the phenanthrene mineralization by *P. putida* CRE7 was also examined using a large surface area (50 cm^2 area coated with phenanthrene). As shown in Figure 3.4, neither rhamnolipid structure (mono- or dirhamnolipids) nor concentration (0.35 and 3.5 mM) influenced phenanthrene biodegradation. However, the rates of phenanthrene mineralization in this experiment were much faster and were nonlinear in comparison to substrate surface area limited conditions.

Effect of rhamnolipid on phenanthrene degradation by various phenanthrene degraders

Figure 3.5 shows the effect of rhamnolipids on phenanthrene mineralization under

limited substrate surface area by the other three phenanthrene degraders SW1, SW2 and SW3 used in this study. Dirhamnolipid at a concentration of 3.5 mM was used. Similar to *P. putida* CRE7 under substrate surface area limited condition, there was a linear dependence of phenanthrene mineralization on incubation time in the absence of rhamnolipids. The rates (% phenanthrene evolved as $^{14}CO_2$ per hour) of mineralization were 0.31 %/h for the SW1, 0.29 %/h for the SW2 and 0.29 %/h for the SW3. Rhamnolipids enhanced phenanthrene biodegradation by all three isolates, however, the rate of the enhancement varied with each isolate. Thus, phenanthrene mineralization in 72 h was 27.2 % by SW1, 31.6 % by SW2 and 34.3 % by SW3. In comparison, only 21 % of the substrate was mineralized in the absence of rhamnolipid.

Discussion and suggestion for further study

In this study, the effect of rhamnolipids on the phenanthrene biodegradation was investigated under conditions of both limited (1.5 cm^2) and unlimited (50 cm^2) surface area of the substrate. Phenanthrene biodegradation was enhanced by rhamnolipid only under substrate surface area limited conditions. These results indicate that the enhancement did not result from the increased solubility of phenanthrene. At first, the solubility of phenanthrene was enhanced by monorhamnolipid more than by dirhamnolipid (Fig. 3.1), but both rhamnolipids had the same effect on phenanthrene biodegradation. Secondly, under substrate surface area unlimited condition the rates of phenanthrene biodegradation in the absence of surfactants were the same as those in the presence of surfactants. The reasons for this enhancement are not yet known. It may

result from the enhanced mass transfer of phenanthrene by rhamnolipids under substrate surface area limited condition or from the enhanced cell hydrophobicity of microorganisms by rhamnolipids (Zhang and Miller, 1994). Therefore, in further study we should determine the dissolution rates of phenanthrene under both substrate surface area limited and unlimited conditions and should compare the cell surface hydrophobicities of test isolates both in the presence and in the absence of rhamnolipids.

Phenanthrene biodegradation rates in the absence of surfactants may be controlled only by the rates of mass transfer of the substrate to solution and may not be related to the cell surface properties of test isolates. This is supported by the fact that all four isolates had almost the same phenanthrene mineralization rates (~ 0.30 % phenanthrene evolved as $^{14}CO_2$ per hour) under substrate surface area limited conditions. However, the rates of phenanthrene mineralization in the presence of surfactants varied with each individual isolate (Figs. 3.3 and 3.5). This may result from differential availability of rhamnolipid-solubilized phenanthrene to each isolate, which may be related to the cell surface properties of the isolates. Therefore, cell surface properties of these organisms should be examined.

One concern for the use of rhamnolipids in hydrocarbon biodegradation is that hydrocarbon degraders may utilize rhamnolipids as preferential carbon source thus inhibiting biodegradation. Rhamnolipids in this study were found to be more resistant to biodegradation than phenanthrene. This was also demonstrated by Oberbremer et al. (1990), who found that the glycolipid biosurfactant used in their study were degraded only after they had facilitated degradation of hydrocarbons in a soil-slurry system.

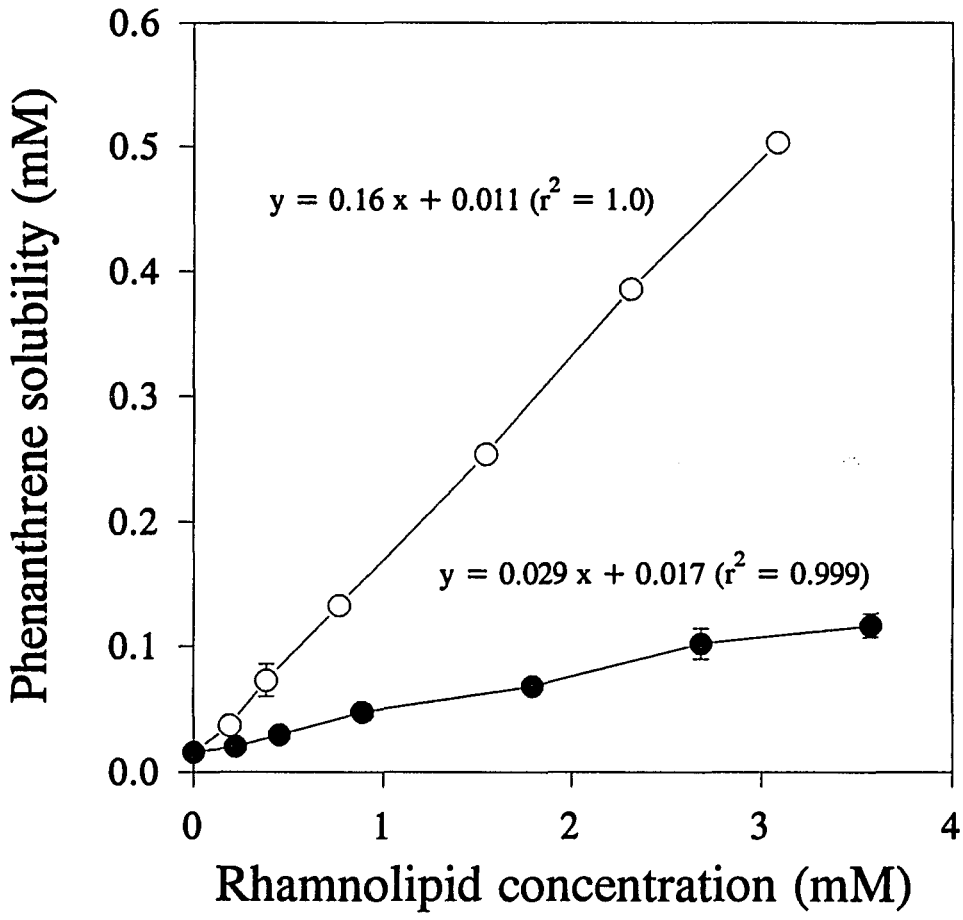

Figure 3.1 Effect of monorhamnolipid acid and dirhamnolipid acid on the aqueous solubility of phenanthrene. Symbols: ○ monorhamnolipid acid, and ● dirhamnolipid acid.

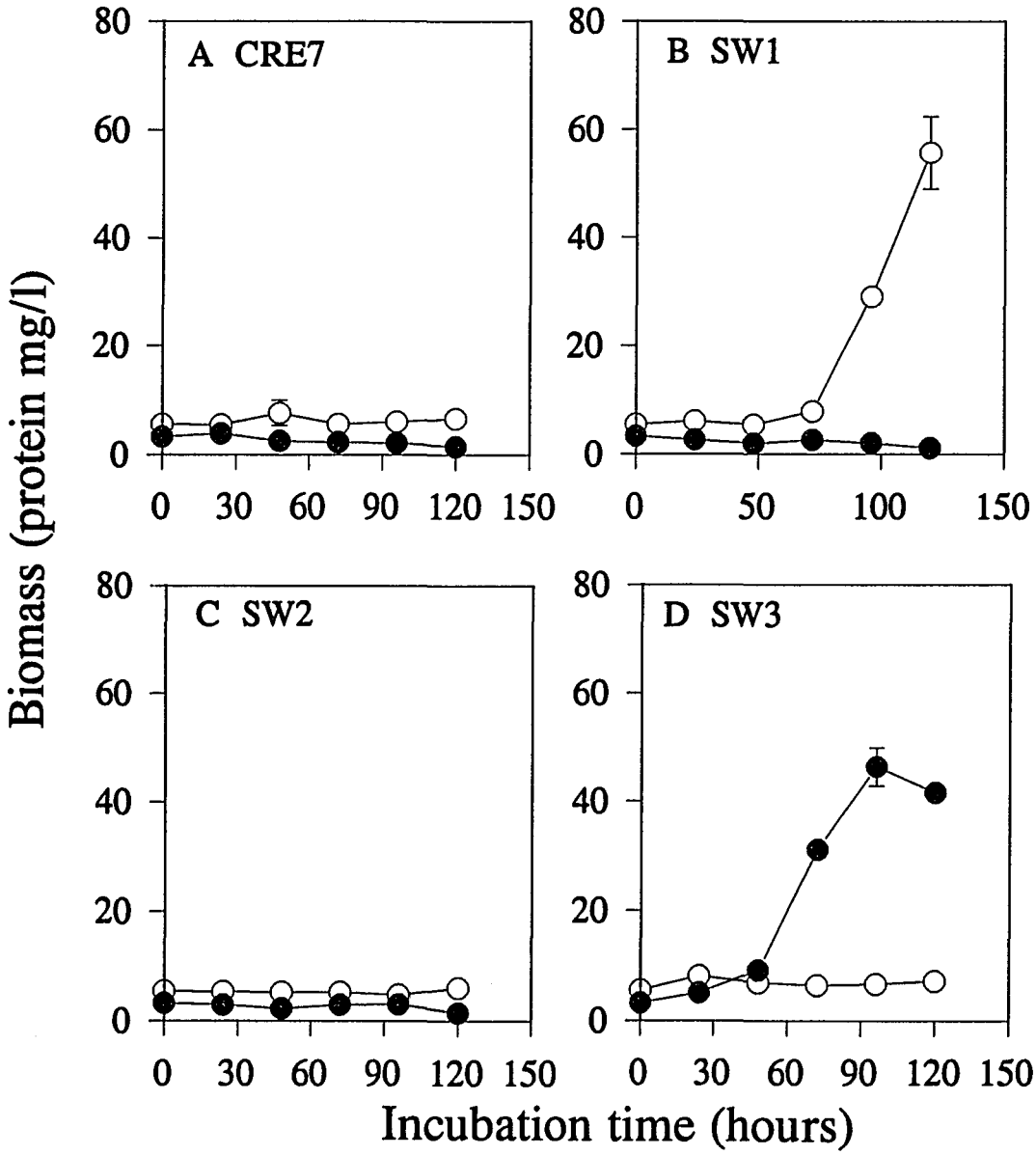

Figure 3.2 Biodegradation of rhamnolipids (1.0 mM) by four phenanthrene degraders. (A) *P. putida* CRE7; (B) SW1; (C) SW2; (D) SW3. Symbols: ○ monorhamnolipid, and ● dirhamnolipid.

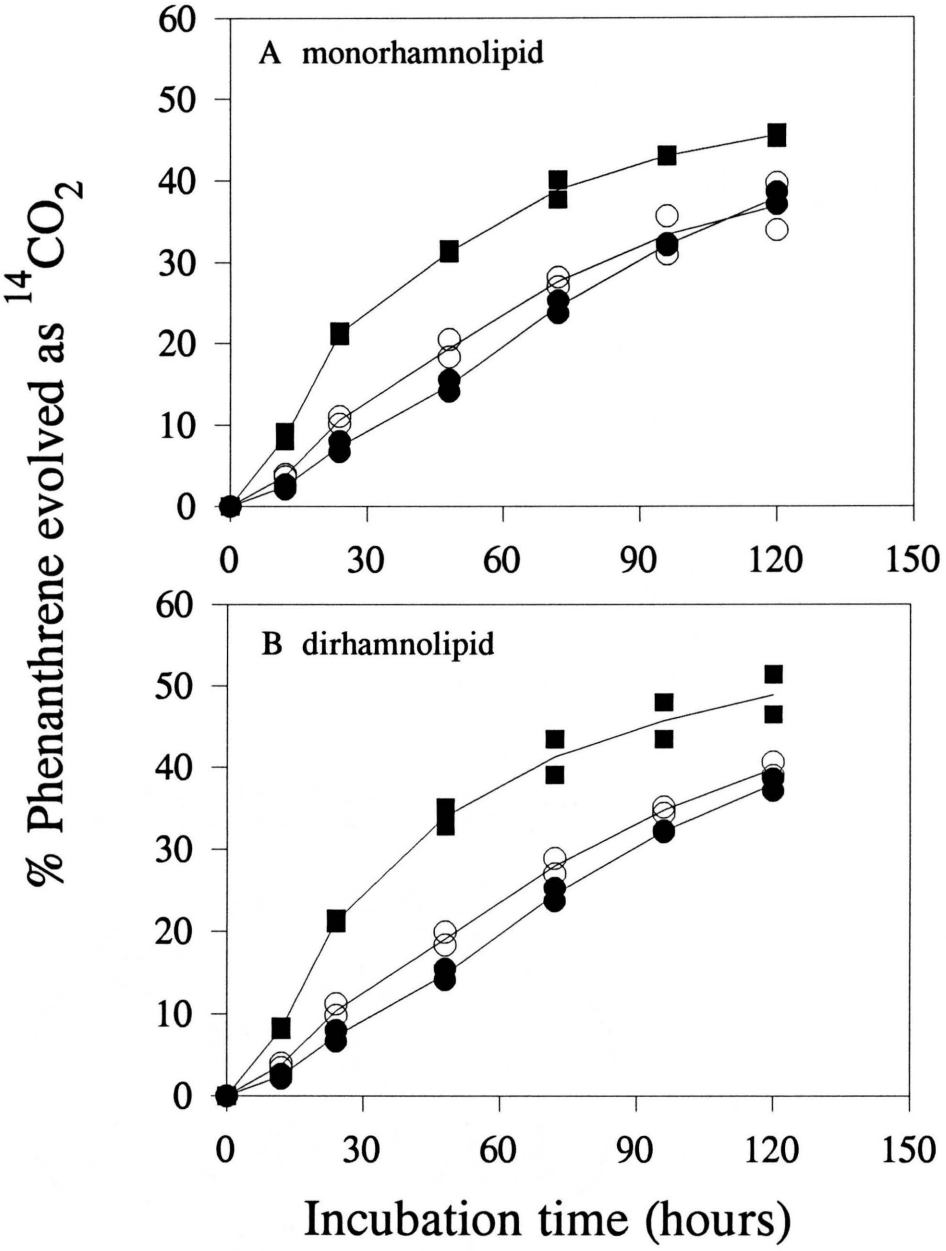

Figure 3.3 Effect of mono- and dirhamnolipid concentrations on the mineralization of phenanthrene (1.0 mM) by *P. pudita* CRE7 under substrate surface-area-limited conditions. (A) monorhamnolipid acid, (B) dirhamnolipid acid. Symbols: ● no rhamnolipids, ○ 0.35 mM rhamnolipids, and ■ 3.5 mM rhamnolipids.

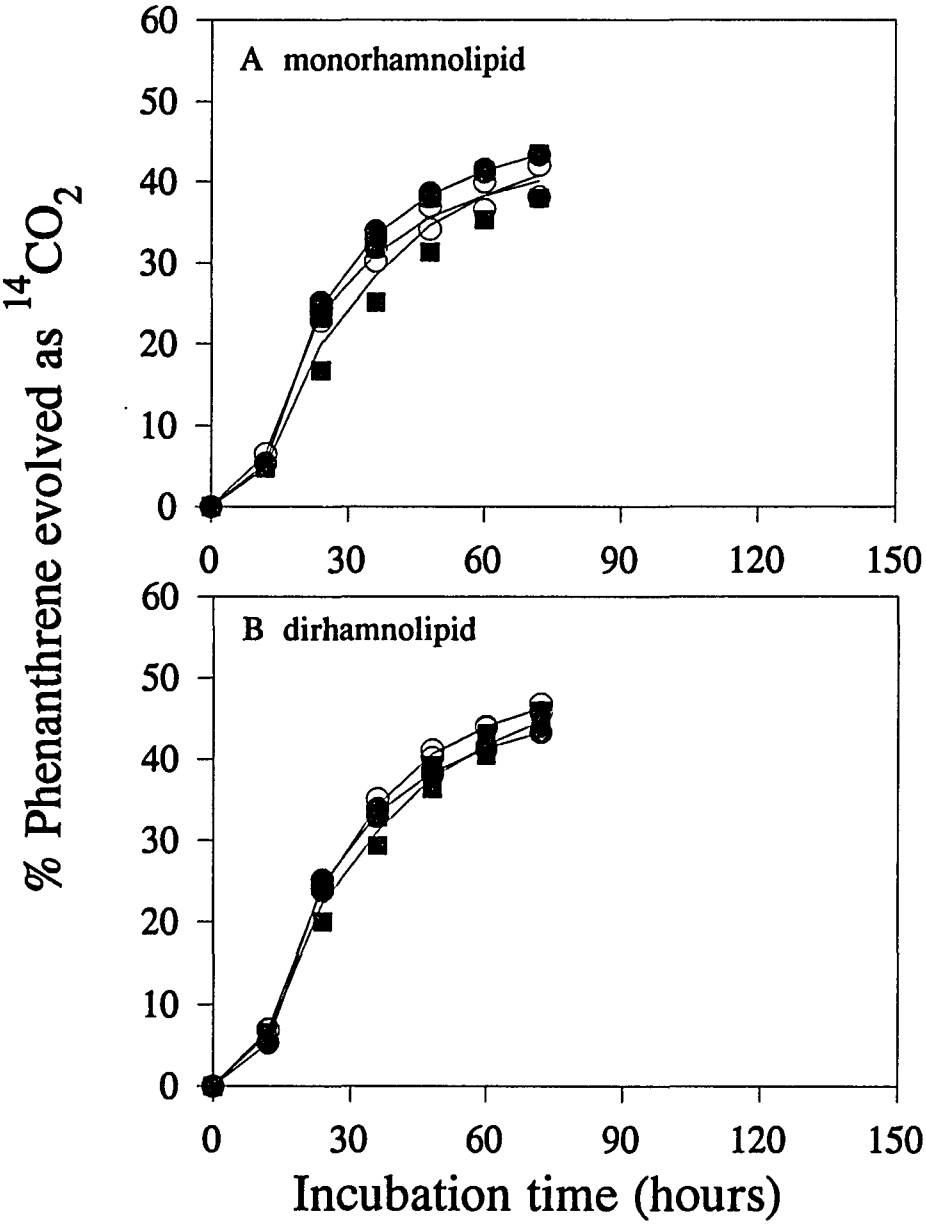

Figure 3.4 Effect of mono- and dirhamnolipid concentrations on the mineralization of phenanthrene (1.0 mM) by *P. pudita* CRE7 under substrate surface-area-unlimited conditions. (A) monorhamnolipid acid, (B) dirhamnolipid acid. Symbols: ● no rhamnolipids, ○ 0.35 mM rhamnolipids, and ■ 3.5 mM rhamnolipids.

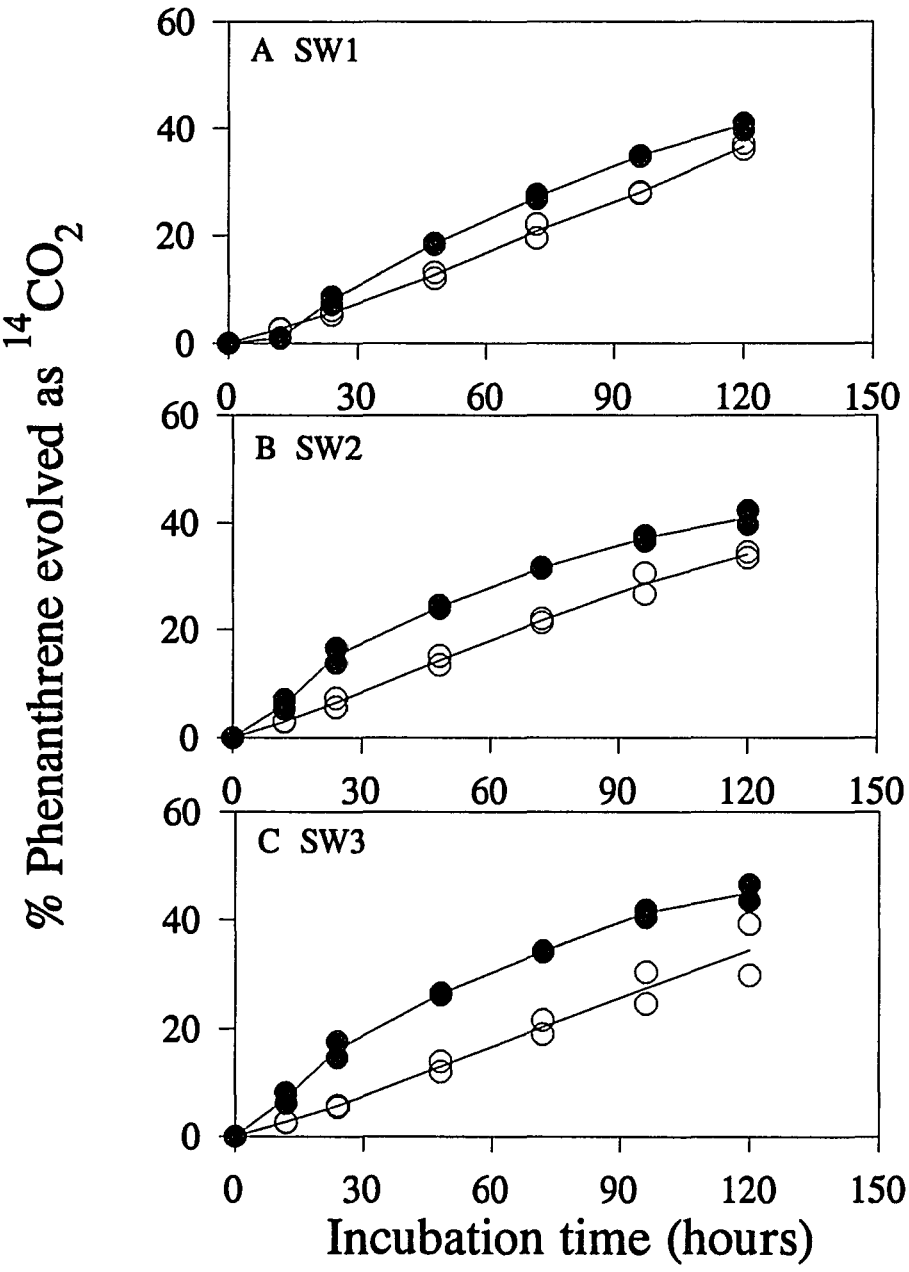

Figure 3.5 Mineralization of phenanthrene (1.0 mM) under substrate surface area limited conditions by a variety of phenanthrene degraders in the presence of dirhamnolipid (3.5 mM). (A) SW1; (B) SW2; and (C) SW3. Symbols: ○ without rhamnolipids, ● rhamnolipid.

CHAPTER 4

SUMMARY AND RECOMMEDATIONS

Summary

The effect of biosurfactants on the biodegradation of hydrocarbons by pure cultures has been investigated. The system consisted of defined biosurfactants, hydrocarbons and microorganisms. A variety of system factors that affect biodegradation were examined in the system. These factors included surfactant structure, surfactant concentration, hydrocarbon structure, physical state of hydrocarbons, and cell hydrophobicity of microorganisms. The major findings from this research are:

1) A new experimental system consisting of *Pseudomonas* rhamnolipids, hydrocarbons and *Pseudomonas* hydrocarbon degraders has been developed to determine the effect of biosurfactants on hydrocarbon degradation rates. The advantages of the system are: i) *Pseudomonas* sp. and rhamnolipids are respectively, commonly isolated environmental hydrocarbon degraders and biosurfactants; ii) Rhamnolipids are not toxic to *Pseudomonas* sp.; iii) *Pseudomonas* sp. are unable to use rhamnolipids as sole carbon and energy source so that hydrocarbon degradation in the system can be simply determined by measuring biomass increase. The results derived from the system are highly comparable.

2) Solubilization and biodegradation of *n*-alkanes by rhamnolipids depended on surfactant structure. Among three rhamnolipids tested in this study, dirhamnolipid methyl ester was found to have the greatest effect on solubilization and alkane biodegradation. For the other two rhamnolipids tested, monorhamnolipid acid exhibited higher solubilization of alkanes than dirhamnolipid acid. Monorhamnolipid acid at high concentration ($\rangle$ 0.1 mM) enhanced alkane biodegradation more effectively than dirhamnolipid acid. In contrast, dirhamnolipid acid enhanced degradation more effectively at low concentration ($\langle$ 0.1 mM).

3) Mixed rhamnolipids had a different effect from single rhamnolipids on solubilization and biodegradation of *n*-alkanes. The effect can be synergistic or additive depending on the surfactant mixture. The mixture of dirhamnolipid acid and methyl ester generally had a synergistic effect on solubilization and additive effect on biodegradation. The mixture of mono- and dirhamnolipid acid showed an additive effect on solubilization and acted as similarly to one or the other individual rhamnolipids in biodegradation, depending on concentration tested. Both mixed and single rhamnolipids at low concentration was found to effectively enhance hydrocarbon biodegradation.

4) The effect of rhamnolipids on the hydrocarbon biodegradation varied with hydrocarbon structure. For instance, the rate of enhancement of hexadecane and octadecane was determined by both rhamnolipid structure and concentrations. In contrast, the rate of enhancement of phenanthrene biodegradation only depended on

rhamnolipid concentrations. This may be due to the different mechanism used by microorganisms for the uptake of aliphatic hydrocarbons and aromatic hydrocarbons in the presence of surfactants. However, no information is available in this dissertation.

5) The physical state of hydrocarbons serving as substrate affected hydrocarbon biodegradation rates. Hexadecane (liquid) and octadecane (solid) had similar chemical structure and physical properties except for physical state at room temperature. In the absence of surfactants, octadecane was degraded at a faster rate than hexadecane. However, in the presence of surfactant, the degradation of hexadecane was more rapid than that of octadecane. This was due to a greater increase in dispersion of the liquid alkane than the solid alkane hexadecane in the presence of surfactants.

6) The biodegradation of *n*-alkanes was affected by microbial cell surface hydrophobicity. The inherently slow alkane degraders had low cell hydrophobicity while the inherently fast alkane degraders had high cell hydrophobicity. Rhamnolipids enhanced cell hydrophobicity of the slow degraders but had no effect on the cell hydrophobicity of the fast degraders. The rate at which the cells became hydrophobic depended on the rhamnolipid concentration and was directed related to the rate of alkane biodegradation.

These findings suggest that rhamnolipids have potential use for remediation of petroleum-contaminated sites. Before application of rhamnolipids to environments,

some further work has to be done and is discussed in the following section.

Recommendations for further work

This research has focused on the effect of *Pseudomonas* rhamnolipid biosurfactants on the biodegradation of hydrocarbons in aqueous solution. Through this investigation, an improved understanding of the interaction between surfactant, cell and hydrocarbon and the effect of this interaction on hydrocarbon biodegradation was obtained. However, this study is limited for two reasons: i) Rhamnolipids were found to enhance or inhibit hydrocarbon biodegradation. We had a good understanding of positive effect of surfactants such as solubilization. However, we had little knowledge about the mechanisms of inhibitory effect of surfactants; and ii) This research showed that the interaction between surfactant and cell could affect hydrocarbon biodegradation. For instance, the enhanced cell hydrophobicity by rhamnolipids increased alkane biodegradation. However, we still lack an understanding of this interaction at the molecular level. Therefore, one aspect of further research is to deeply understand the mechanisms of hydrocarbon transport into cells at the molecular level. The other aspect of further research is to investigate the effect of surfactants on hydrocarbon biodegradation in real environments especially in soil systems.

The increase of the aqueous concentration of hydrocarbons by micelle-induced solubilization is generally considered a positive effect on biodegradation. However, the question concerning the availability of dissolved hydrocarbons to microorganisms remains to be answered. Microorganisms may take up dissolved hydrocarbon by fusing

micelles with microbial cell membrane or by partitioning micellar hydrocarbon into cells. In order to understand the uptake process, we must investigate the interaction between surfactant and cells. Some new techniques will be required. For instance, electron microscopy technique may be useful to visualize the interaction between micelle and cells. The preparation of [^{14}C]labeled rhamnolipids and a monoclonal antibody to rhamnolipid may help investigate the interaction between rhamnolipid and cell.

The study in this dissertation was confined to investigating the effect of surfactants on hydrocarbon biodegradation in aqueous solution. The results from this study may be applicable to aqueous environments such as river, lake and ocean. However, in nature most hydrophobic contaminants tend to sorb to solid matrices such as soil. Therefore, an important aspect of further study is to investigate the effect of surfactants on hydrocarbon biodegradation in soil systems. It would be expected that the effect of surfactants on hydrocarbon biodegradation in soil system is quite different from that in aqueous system. In soil system, surfactants may not only increase the solubility of hydrocarbon but also increase the desorption of hydrocarbon from soil. Surfactants themselves may sorb to soil so that the effective concentration of surfactants used in soil may be higher than in aqueous solution. In addition, surfactants may affect the sorption or desorption of microorganisms to soil thus affecting biodegradation. Because of complexity of real soil, initial tests may be performed in a simple model system consisting of surfactant, microorganisms, hydrocarbon and a sandy soil. Later, soil organic matter and clay may be added to the system. Finally, the effect of surfactants on hydrocarbon biodegradation may be determined in a variety of real soils.

Pseudomonas sp. were used in this research as model hydrocarbon degraders. In reality, other hydrocarbon degraders also exist in environments. Therefore, a further study will be required to investigate the effect of rhamnolipids on the hydrocarbon degradation by other degraders before rhamnolipids are applied to contaminated sites. This study is very important because rhamnolipids may be toxic to other microorganisms thus inhibiting hydrocarbon biodegradation. Other microorganisms may utilize rhamnolipids as preferential carbon and energy source thus also inhibiting hydrocarbon biodegradation. In order to conduct this study, a variety of hydrocarbon degraders may be isolated from soils or obtained from other labs. These hydrocarbon degraders should represent major groups of hydrocarbon degraders in soils.

Hydrocarbons often exist in environments as a mixture. The biodegradation of one structural hydrocarbon may be affected by the presence of other structural hydrocarbon in the mixture. This is because that mixed hydrocarbons may have different properties from unmixed hydrocarbons in respect to water solubility, toxicity and physical state. For instance, the mixture of liquid alkane and solid PAH may yield liquid or solid mixture depending on their ratio. Therefore, it would be very interesting to examine the effect of surfactants on the biodegradation of mixed hydrocarbons. Because we know little about the biodegradation of mixed hydrocarbons, the research on the effect of surfactants on the biodegradation of mixed hydrocarbon may be done at two stages. In the first stage, tests may be performed to study the effect of mixed components on the biodegradation of hydrocarbons without the presence of surfactants. In the second stage, this effect may be investigated in the presence of surfactants.

APPENDIX A

EFFECT OF A *PSEUDOMONAS* RHAMNOLIPID BIOSURFACTANT ON CELL HYDROPHOBICITY AND BIODEGRADATION OF OCTADECANE

Yimin Zhang and Raina M. Miller*

Department of Soil and Water Science
University of Arizona
Tucson, AZ 85721

Published:

Applied and Environmental Microbiology

Volume 60, pp. 2101-2106, 1994

*Corresponding author

Abstract

In this study, the effect of a purified rhamnolipid biosurfactant on the hydrophobicity of octadecane-degrading cells was investigated to determine whether difference in rates of octadecane biodegradation resulting from the addition of rhamnolipid to four strains of *Pseudomonas aeruginosa* could be related to measured differences in hydrophobicity. Cell hydrophobicity was determined by a modified bacterial adherence to hydrocarbon (BATH) assay. Bacterial adherence to hydrocarbon quantitates the preference of cell surfaces for the aqueous phase or the aqueous-hexadecane interface in a two-phase system of water and hexadecane. On the basis of octadecane biodegradation in the absence of rhamnolipid, the four bacterial strains were divided into two groups: the fast degraders (ATCC 15442 and ATCC 27853), which had high cell hydrophobicities (74 and 55% adherence to hexadecane, respectively), and the slow degraders (ATCC 9027 and NRRL 3198), which had low cell hydrophobicities (27 and 40%, respectively). Although in all cases rhamnolipid increased the aqueous dispersion of octadecane at least 10^4-fold, at low rhamnolipid concentrations (0.6 mM), biodegradation by all four strains was initially inhibited for at least 100 h relative to controls. At high rhamnolipid concentrations (6 mM), biodegradation by the fast degraders was slightly inhibited relative to controls, but the biodegradation by the slow degraders was enhanced relative to controls. Measurement of cell hydrophobicity showed that rhamnolipids increased the cell hydrophobicity of the slow degraders but had no effect on the cell hydrophobicity of the fast degraders. The rate at which the cells became hydrophobic was found to depend on the rhamnolipid concentration and

was directly related to the rate of octadecane biodegradation. These results suggest that the bioavailability of octadecane in the presence of rhamnolipid is controlled by both aqueous dispersion of octadecane and cell hydrophobicity.

Introduction

Biodegradation of organic compounds with limited water solubility is slow because of the low availability of these compounds to microbial cells. As shown in previous work, the availability of slightly soluble organic compounds can be enhanced by microbially produced surfactants (biosurfactants), which can increase aqueous dispersion by many orders of magnitude (Zhang and Miller, 1992). In many instances, biosurfactants also stimulate the biodegradation of organic compounds. For example, alkane degradation is stimulated by rhamnolipids (Zhang and Miller, 1992), sophorose lipids (Ito and Inoue, 1982; Oberbremer et al., 1990), and phospholipids (Käppeli and Finnerty, 1979). However, biosurfactants can also inhibit biodegradation. Inhibition can be genus specific or substrate specific. For example, sophorose lipids were found to stimulate hydrocarbon degradation by a *Torulopsis* sp. but to inhibit degradation by other yeast genera (Ito and Inoue, 1982). Substrate-specific inhibition was demonstrated by Falatko and Novak (1992), who showed that biosurfactants produced from growth on glucose or vegetable oil inhibited biodegradation of gasoline hydrocarbons, while biosurfactants produced from growth on gasoline did not inhibit degradation.

The reason for variable biosurfactant enhancement of biodegradation is not yet known. Clearly, though, biodegradation requires uptake of the substrate by the cell,

which in turn requires contact between the substrate and the cell. Contact is determined by two factors: (i) available substrate surface area and (ii) affinity of microbial cells for the substrate. Biosurfactants increase dispersion or surface area for microbial attachment, which should increase biodegradation. However, there is evidence that biosurfactants may also interfere with the interaction between biosurfactant-dispersed substrates and microbial cells (Humphries, et al., 1986; Rosenberg, et al., 1983).

The objective of this research was to investigate the effect of different rhamnolipid concentrations on cell hydrophobicity and the resultant impact on biodegradation of octadecane. It has been suggested previously that the hydrophobicity of the cell surface is an important factor in predicting adhesion to surfaces (van Loosdrecht, et al. 1987). Thus, cell hydrophobicity was used as a measure of potential cell affinity for hydrophobic substrates and was determined by the bacterial adherence to hydrocarbon (BATH) assay. In this study, four *Pseudomonas aeruginosa* strains which differed in octadecane biodegradation rates were used to determine the effect of rhamnolipid on cell hydrophobicity and octadecane biodegradation.

Materials and methods

Microorganisms

P. aeruginosa ATCC 9027, ATCC 15442, ATCC 27853, and NRRL 3198 were obtained from the American Type Culture Collection (Rockville, Md) or from the University of Arizona Undergraduate Program in Microbiology Culture Collection. The cultures were stored at 4°C on nutrient agar plates or *Pseudomonas* agar P plates (Difco,

Detroit, Mich.) and transferred monthly. All four strains produced rhamnolipids during growth in phosphate-limited proteose peptone-glucose-ammonium salts (PPGAS) medium (Cheng, et al., 1970). None of these strains produced rhamnolipid during growth in mineral salts medium (Zhang and Miller, 1992) containing substrates used in this study, e.g., octadecane and glucose. Also, none of these strains can utilize rhamnolipid as a sole carbon source.

Rhamnolipid production

P. aeruginosa ATCC 9027 rhamnolipid was produced and partially purified as described by Zhang and Miller (1992). The partially purified yellow rhamnolipid residue was then dissolved in 1 ml of chloroform and applied to a Silica Gel 60 (Aldrich, Milwaukee, Wis.) chromatography column (17 by 1.5 cm). The column was eluted with chloroform to remove the yellow pigment associated with the partially purified rhamnolipid and was then eluted with chloroform-methanol (10:1). The eluant fractions contained one anthrone-positive component,which was further analyzed by fast atom bombardment (FAB) mass spectrometry. Purified rhamnolipid was quantified by determinations of weights and amounts of rhamnose.

FAB mass spectra were obtained by using mass analyzer 1 of a custom-built four-sector instrument (AMD intdectra, Harpstedt, Germany) of BEBE (B, magnetic; E, electric fields) configuration equipped with two KWS MC 68000 computer systems for instrument control and data acquisition. The ion source consisted of a 20-kV Cs-ion gun operating at a thermoionic emission current of 2 A at 12 kV. The samples were

dissolved in high-performance liquid chromatography grade methanol, and an equal volume of thioglycerol was added. Four microliters of the resulting solution was placed on the target of the inlet probe held at 10°C with chilled methanol-water (1:1 [vol/vol]) at a source temperature of 34°C. The mass range 100 to 2,000 Da was scanned at 60-s/decade at a resolution of 2,000 (10% valley definition). The mass spectra are reported as the average of 10 accumulated scans.

[^{14}C] rhamnolipid

Some experiments in this study required the use of [^{14}C]rhamnolipid. This was synthesized by adding D-[U-^{14}C]glucose (specific activity, 251 mCi/mmol), which was obtained from Sigma(St. Louis, Mo.) into the PPGAS growth medium. The final concentration of glucose in the medium was 7.5 g/liter and was a mixture of unlabeled and ^{14}C-labeled glucose with a specific activity of 0.43 μCi/mmol. [^{14}C]rhamnolipid was purified and quantified as described above. The specific activity of the [^{14}C]rhamnolipid was 1.1 μCi/mmol, which indicated a 2.9% yield of [^{14}C]rhamnolipid from [^{14}C]glucose on the basis of recovery of radioactivity.

Biodegradation tests

Octadecane biodegradation of octadecane was measured either by protein determination or by detection of $^{14}CO^2$ evolved during growth on [^{14}C]octadecane (Zhang and Miller, 1992). For protein determination experiments, octadecane dissolved in chloroform was coated onto the bottom of a 250-ml flask. The solvent was then

evaporated. Mineral salts medium (50 ml) containing rhamnolipid (0, 0.6, or 6 mM) was added and the flask was placed briefly (30 s) into a 37°C water bath to melt the coated octadecane. The flask was then maintained at room temperature until the octadecane resolidified on the surface of the medium. The final octadecane concentration was 3.9 mM. Each flask was then inoculated with a 5% *P. aeruginosa* inoculum which had been grown in Kay's minimal medium (Warren, et al. 1960) at 37°C for 24 h. The flasks were incubated at 23°C in a gyratory shaker (200 rpm). For protein measurements, 1-ml samples were removed periodically and heated for 1 min with 0.1 ml of 1 N NaOH (to lyse cells), and the protein content was determined (Lowry, et al. 1951).

Mineralization experiments were performed with [1-^{14}C]octadecane purchase from Sigma (specific activity, 3.6 mCi/mmol; 98% purity). A mixture of octadecane and [^{14}C]octadecane from Sigma was used to coat the bottoms of modified 125-ml micro-Fernbach flasks (Wheaton, Millville, N.J.), which were designed for the collection of $^{14}CO_2$ and ^{14}C-labeled volatile compounds. The solvent was evaporated, and 20 ml of mineral salts medium containing rhamnolipid (0, 1.2 or 7.6 mM) was added to each flask. The final concentration of octadecane was 1.6 mM, and octadecane specific activity was 1.4 μCi/mmol. The octadecane was melted and the flasks were inoculated and incubated as described above. The micro-Fernbach flasks were periodically flushed through a series of six traps to collect $^{14}CO_2$ and ^{14}C-volatile organic compounds. Radioactivity was determined with a Packard (Meriden, Conn.) Tri-Cab liquid scintillation counter (model 1600 TR).

BATH assay

The relative hydrophobicity of bacterial cells was measured by BATH assay (Rosenberg, et al., 1980). Bacterial cells to be assayed were prepared by the following procedure. The cells were first washed twice to remove any interfering solutes, particularly rhamnolipid that was added in some of the experiments. The cells were then resuspended in buffer salts solution (pH 7.0) containing 16.9 g K_2HPO_4, 7.3 g KH_2PO_4, 1.8 g urea, and 0.2 g $MgSO_4.7H_2O$ to give an optical density (OD) at 400 nm of 1.0. Cells (4.0 ml) and hexadecane (1.0 ml) were mixed in a screw-top test tube (1.5 by 8 cm), the test tube was vortexed for 60 s. After vortexing, the hexadecane and aqueous phases were allowed to separate for 30 min. The aqueous phase was then carefully removed with a Pasteur pipette, and the turbidity of the aqueous phase at 400 nm was measured. Hydrophobicity is expressed as the percentage of adherence, which is calculated as follows: 100 × (1 - OD of aqueous phase/OD of the cell suspension).

The effect of rhamnolipid on the cell adherence to hexadecane was also determined. For these experiments, cells were harvested, washed twice, and resuspended in buffer slats solution as described above. Then, various concentrations of rhamnolipid (0 to 0.008 mM) were added to the cell solution, and BATH assay was performed.

Measurement of cell association with rhamnolipid

Bacterial cells grown in Kay's minimal medium at 37°C for 24 h were washed twice with mineral salts medium. Washed cells were added to 10 ml mineral salts medium (OD at 400 nm of 2.0) containing [^{14}C]rhamnolipid (6 mM; specific activity,

0.75 nCi/mmol). This solution was incubated with and without octadecane (3.9 mM) in 125 ml flasks at 23°C with 200 rpm of gyratory shaking. Periodically, 1-ml samples were withdrawn and centrifuged. The cell pellet was washed twice, and radioactivity was determined.

Results

Rhamnolipid purification and structure

The purified rhamnolipid was analyzed by thin-layer chromatography using a chloroform-methanol-water (65:25:4) solvent system. One anthrone-positive spot with an R_f value of 0.70, which corresponds to a monorhamnolipid, was identified (Parra, et al., 1989). The sample was further analyzed by FAB mass spectroscopy. Mass spectra of the purified rhamnolipid were compared with known C_{18}-, C_{20}-, and C_{22}-purified monorhamnolipid samples generously provided by Graham W. Tayler (Rendell, et al., 1990). An intense signal molecular ion was found at *m/z* 643 (I_{rel} = 100.00%) which corresponds to $(M + K)^+$, where M is the C_{20} monorhamnolipid (Fig.1.). A series of signals at *m/z* 515 (I_{rel} = 31.88%), and 599 (I_{rel} = 2.87%) indicated a mixture of four monorhamnolipids, C_{18}, C_{20}, C_{22}, and C_{24}, although the signals for the C_{18} and C_{24} monorhamnolipids were very weak.

An average molecular weight of 504, which represents the C_{20} monorhamnolipid, was used in this study to calculate rhamnolipid concentrations when concentrations were determined by weight. We found that calculated rhamnolipid concentration values determined by weight were within 6 % of the concentration values calculated by the

rhamnose assay described in our previous work (Zhang and Miller, 1992).

Effect of rhamnolipid concentration on biodegradation rates

As shown in Fig. 2, in the absence of rhamnolipid, cumulative CO_2 production showed a short lag phase and then rapid production of CO_2, which then tapered off after 100 h. In the presence of high rhamnolipid concentration (7.6 mM), mineralization was enhanced relative to that of the control. Interestingly, low rhamnolipid concentrations initially inhibited the rate of mineralization relative to that of the control; however, the presence of either high or low rhamnolipid concentrations resulted in a higher cumulative amount of CO_2 produced after 300 h than the amount produced in the absence of rhamnolipid.

A comparison of cumulative protein productions by four different rhamnolipid-producing strains of *P. aeruginosa* during growth on octadecane is shown in Fig. 3. The rate of protein production (see slope of each line), a measure of octadecane biodegradation, varied among the four bacterial strains. In the absence of rhamnolipid, ATCC 15442 and ATCC 27853 were found to degrade octadecane rapidly (fast degraders), which ATCC 9027 and NRRL 3198, the slow degraders. The same addition of rhamnolipid to ATCC 15442 and ATCC 27853, the fast degraders, did not seem to affect biodegradation. Interestingly, the addition of a lower concentration of rhamnolipid (0.6 mM) inhibited octadecane biodegradation by all four strains for at least 100 h. After this initial inhibition, the biodegradation rates for ATCC 9027 (a slow degrader) and ATCC 15442 (a fast degrader) increased until they were comparable with controls.

Effect of rhamnolipid on cell hydrophobicity

Table 1 compares the effects of rhamnolipid on the cell hydrophobicities of the four *Pseudomonas* strains when they were grown on octadecane in the presence or absence of rhamnolipid. When they were grown on octadecane alone, the fast degraders, ATCC 15442 and 27853, had higher cell hydrophobicities (75 and 55%, respectively) than the slow degraders, ATCC 9027 and NRRL 3198 (27 and 40%, respectively). When grown on octadecane in the presence of rhamnolipid, the hydrophobicities of the slow degraders increased dramatically, while the hydrophobicities of the fast degraders remained unchanged. Additional experiments were carried out to investigate the effect of different carbon sources on the cell hydrophobicities of slow degraders. These carbon sources ranged from very water soluble (acetate, citrate, glucose, and succinate) to slightly water soluble (hexadecane [0.0063 mg/liter] and octadecane [0.006 mg/liter])(Singer and Finnerty, 1984). As shown in Table 2, rhamnolipid increased the cell hydrophobicities of both ATCC 9027 and NRRL 3198 only when they were incubated in the presence of the slightly soluble carbon sources, e.g., hexadecane and octadecane.

Effect of cell hydrophobicity on biodegradation rates

Figure 4 compares the change in cell hydrophobicity (Fig. 4B) with cumulative protein production (Fig. 4A) during growth of *P. aeruginosa* ATCC 9072 on octadecane. As shown in Fig. 4B, the development of hydrophobicity in ATCC 9027 cells was dependent on rhamnolipid concentration. In the absence of rhamnolipid, there

was a slight increase in cell hydrophobicity during growth to a final value of approximately 20% at 300 h. In contrast, the high rhamnolipid concentration (7.6 mM) stimulated an increase in hydrophobicity of ATCC 9027 cells to 80% in 100 h. At the lower rhamnolipid concentration (1.2 mM), there was a 100-h lag period before development of cell hydrophobicity started. After this lag period, the rate of increase in hydrophobicity was similar to the rate of increase at high rhamnolipid concentrations that was seen from 0 to 100 h. Development of high hydrophobicity appears to correlate with the rate of biodegradation (Fig. 4A). At the high rhamnolipid concentration, hydrophobicity increased immediately, and the initial rate of biodegradation was high. At the low rhamnolipid concentration, both development of hydrophobicity and octadecane biodegradation were inhibited for the first 100 h. After development of hydrophobicity started at 100 h, the rate of octadecane biodegradation became comparable to the rate seen at high rhamnolipid concentration from 0 to 100 h (Fig. 4A).

Rhamnolipid reduction of cell attachment to hydrocarbon

The results already presented have shown that biodegradation of octadecane by fast degraders was initially inhibited by rhamnolipid. Similarly, slow degraders were initially inhibited by the low rhamnolipid concentration (Fig. 3). To investigate the initial inhibition of octadecane degradation in the presence of rhamnolipid, cell adherence to hexadecane in the presence of rhamnolipid was determined for each of the test strains. For this experiment, cells were pelleted, washed, and then resuspended in

buffer salts solution. The rhamnolipid was then added to the resuspended cells, and the BATH assay was performed. In all cases, the BATH test showed that extremely low rhamnolipid concentrations (0.008 mM) dramatically decreased the adhesion of cells to hexadecane. It should be emphasized that this BATH test did not measure true cell surface hydrophobicity but rather the apparent hydrophobicity in the presence of low concentrations of rhamnolipid. (Table 1 shows the actual hydrophobicity of each strain in the absence of rhamnolipid.) As shown in Fig. 5, the apparent hydrophobicity of each test strain was reduced with increasing concentrations of rhamnolipid of up to 0.008 mM. This experiment was also run at higher rhamnolipid concentrations (≥0.01 mM rhamnolipid); however, analysis of the BATH results was not possible because of the formation of rhamnolipid-hexadecane emulsions.

To investigate whether there was a strong interaction between the cells and the rhamnolipid, the cells were incubated with [^{14}C]rhamnolipid in the presence and absence of octadecane. After incubation periods of up to 48 h, no measurable [^{14}C]rhamnolipid was incorporated into the cells in either test.

Discussion

Results of an earlier study from this laboratory showed that the aqueous dispersion of octadecane could be increased over 10^4-fold by the addition of rhamnolipid (Zhang and Miller, 1992). The increase in aqueous dispersion was correlated with an increase in the rate of octadecane biodegradation. The results of the present study imply that the rate of octadecane biodegradation is dependent on both the aqueous dispersion

of octadecane and on the surface properties of the degrading cells. As shown in Tables 1 and 2, cell hydrophobicity can be induced to change in the presence of a combination of both rhamnolipid and slightly soluble substrate, e.g., octadecane. The importance of cell surface properties for the biodegradation of slightly soluble organic compounds has been indicated by previously by Rosenberg and Rosenberg (1981), who showed that the rate of hydrocarbon degradation by bacterial cells was dependent on cell affinity. Bacteria with high affinity for hydrocarbons utilized hexadecane more effectively than those with low affinity. Similarly, in this study, cells with high hydrophobicity, a measurement which can be used to indicate the affinity of cells for a nonpolar substrate, showed increased utilization of octadecane.

In examining the interaction among rhamnolipid, substrate, and degrading cells, the effects of rhamnolipid can be categorized as positive or negative. Positive effects can be summarized as (i) the increased dispersion of substrate and (ii) the enhancement of cell hydrophobicity. The negative effect of rhamnolipid is an apparent interference in the interaction between the microbial cells and octadecane.

Although the effect of rhamnolipid on octadecane dispersion was concentration dependent, both the low and high rhamnolipid concentrations used in this study significantly increased octadecane dispersion ($>10^4$-fold). Therefore, rhamnolipid-induced enhancement of cell hydrophobicity, which is also an effect of rhamnolipid addition, seems to be an important factor in determining the biodegradation rate of slightly soluble organic compounds. Rhamnolipid was particularly effective for cells with low initial hydrophobicity (slow degraders). For these cells, high rhamnolipid

concentrations induced an immediate rapid increase in cell hydrophobicity (and biodegradation), whereas in the presence of low rhamnolipid concentrations, there was a 100-h lag period before cell hydrophobicity (and biodegradation) began in increase. These results imply that there is a concentration-dependent interaction of rhamnolipid with the degrading cells which seems to stimulate the development of cell hydrophobicity. In contrast, octadecane biodegradation by fast degraders, which had a high initial cell hydrophobicity, was either unaffected by the addition of rhamnolipid (high rhamnolipid concentrations). In this case, rhamnolipid addition did not have any effect on the measured hydrophobicity of the degrading cells.

The negative effect of rhamnolipid addition is an apparent interference in the interaction between the microbial cells and octadecane. This may be due to rhamnolipid-induced interference in the contact between the degrading cells and the substrate (octadecane). This hypothesis is supported by the data in Fig. 5, which showed that extremely small amounts of rhamnolipid severely reduced the adhesion of cells to hexadecane in the BATH assay (Fig. 5). One possible explanation for this interference is that at pH 7.0, both the bacterial cell walls and the rhamnolipid-octadecane complexes carry a net negative charge. Therefore, electrostatic repulsion between the similarly charged cells and complexes may prevent their interaction. To begin investigation of the interaction among the rhamnolipid, the octadecane, and the degrading cells, we performed some initial experiments which showed that filtration of rhamnolipid-octadecane solutions through a 300,000-molecular-weight membrane to remove particulate octadecane did not affect the octadecane biodegradation rate (data

not shown). This suggests that cells can obtain octadecane directly from the rhamnolipid-octadecane micellar structures described by Champion et al. (1995). However, after incubation in filtrated rhamnolipid-octadecane solutions, rinsed cells showed no accumulation of [^{14}C]rhamnolipid, suggesting that rhamnolipid is not taken up by the cell. Thus, there is only a weak association between the cell surface and the rhamnolipid-octadecane structures in comparison with the stronger associations which have been suggested for other systems (e.g., phospholipids), such as fusion between biosurfactant structures and the cell membrane (Jones and Osborn, 1977; Singer and Finnerty, 1984).

In summary, the results of the study present show that the enhancement of biodegradation of slightly soluble organic compounds, e.g., by biosurfactants is not simply a matter of increased dispersion of the organic compounds. The rhamnolipid used in this study also affected the surface properties of the degrading cells, in some cases leading to enhanced biodegradation (ATCC 9027 and NRRL 3198) and in other cases inhibiting biodegradation (ATCC 15442 and TACC 27853). Thus, there is a complex interaction among the rhamnolipid, substrate, and the degrading cells which needs to be explored more carefully before the full potential of this biosurfactant can be realized in practical applications, e.g., bioremediation of petroleum-contaminated sites. This observation can be extended to include the many different biosurfactants produced in nature. There are a variety of chemical structures which are produced; however, presently very little is known about the relationship between the chemical structures of these biosurfactants and their ability to either increase dispersion of slightly soluble

organic compounds or to change cell surface properties. Successful use of biosurfactants in remediation will require knowledge of such structure-function relationships.

References

Champion, J.T., J.C. Gilkey, H. Lamparski, J. Retterer, and R.M. Miller. Submitted for pubilication.

Cheng, K. J., J. D. Ingram, and J. W. Costerton. 1970. Release of alkaline phosphatase from cells of *Pseudomonas aeruginosa* by manipulation of cation concentration and of pH. J. Bacteriol. 104:748-753

Falatko, D.F., and J.T. Novak. 1992. Effects of biologically produced sufactants on the mobility and biodegradation of petroleum hydrocarbons. Water Environ. Res. 64:163-169

Humphries, M., F. Jaworzyn, and J.B. Cantwell. 1986. The effect of a range of biological polymers and synthetic surfactants on the adhesion of a marine *Pseudomonas* sp strain NCMB 2021 to hydrophilic and hydrophobic surfaces. FEMS Microbiol. Lett. 38:299-308

Itoh, S., and T. Suzuki. 1972. Effect of rhamnolipids on growth of *Pseudomonsa aeruginosa* mutant deficient in *n*-paraffin-utilizing ability . Agrc. Biol. Chem. 36:1233-1235

Jones, N.C., and M.J. Osborn. 1977. Interaction of *Salmonella typhimurium* with phospholipid vesicles. J. Biol. Chem. 252:7398-7404

Käppeli, O., and W.R. Finnerty. 1979. Partition of alkane by an extracellular vesicle derived from hexadecane-grown *Acinetobacter.* J. Bacteriol. 140:707-712

Lowry, O. H., N. J. Rosebrough, A.L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275

Miller, R.M., and R. Bartha. 1989. Evidence from liposome encapsulation for transport-limited microbial metabolism of solid alkanes. Appl. Environ. Microbiol. 55:269-274

Oberbremer, A., R. Müller-Hurtig, and F. Wagner. 1990. Effect of the addition of microbial surfactants on hydrocarbon degradation in a soil population in a stirred reactor. Appl. Microbiol. Biotechnol. 32:485-489

Parra, J.L., J. Guinea, M.A. Manresa, M. Robert, M.E. Mercade, F. Comelles, and M.P. Bosch. 1989. Chemical Characterization and physicochemical behavior of biosurfactants. J. Am. Chem. Soc. 66:141-145

Rendell, N. B., G.W. Taylor, M. Somerville, H. Todd, T. Wilson, and P.J. Cole. 1990. Characterization of *Pseudomonas* rhamnolipids. Biochim. Biophys. Acta. 1045:189-193

Rosenberg, E., A. Gottlieb, and M. Rosenberg. 1983. Inhibition of bacterial adherence to hydrocarbons and epithelial cells by emulsan. Infect. Immun. 39:1024-1028

Rosenberg, M., Gutnick, D., and E. Rosenberg. 1980. Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. FEMS Microbiol. Lett. 9:29-33

Singer, M.E., and W.R. Finnerty. 1984. Microbial metabolism of straight-chain and branched alkanes, p. 1-59. *In* R.M. Atlas (ed.), Petroleum microbiology. Macmillan publishing Co., New York

van Loosdrecht, M.C.M., J. Lyklema, W. Norde, G. Schraa, and A.J.B. Zehnder. 1987. The role of bacterial cell wall hydrophobicity in adhesion. Appl. Environ. Microbiol. 53:1893-1897

Warren, A. J., A. F. Ellis, and J. J. R. Campbell. 1960. Endogenous respiration of *Pseudomonas aeruginosa*. J. Bacteriol. 79:875-880

Zhang, Y., and R. M. Miller. 1992. Enhanced octadecane dispersion and biodegradation by a *Pseudomonas* rhamnolipid surfactant (biosurfactant). Appl. Environ. Microbiol. 58:3276-3282

Table 1 Hydrophobicity of *P. aeruginosa* cells (% adhered) grown on octadecane in the presence or absence of rhamnolipid

P. aeruginosa strain	% Adhered to hexadecane (mean ± SD)	
	Octadecane[a]	Octadecane + rhamnolipid[b]
ATCC 9027	27 ± 3	79 ± 6
NRRL 3198	40 ± 3	77 ± 6
ATCC 15442	74 ± 2	71 ± 5
ATCC 27853	55 ± 4	51 ± 4

[a]Bacteria were grown in mineral salts medium with octadecane at 23°C for 6 days, washed, and assayed.
[b]Bacteria were grown in mineral salts medium with octadecane ad 6 mM rhamnolipid at 23°C for 6 days, washed to remove rhamnolipid, and assayed.

Table 2 Effect of carbon source on cell hydrophobicity of slow degraders

Carbon source	% adhered to hexadecane (mean± SD)			
	ATCC 9027		NRRL 3198	
	- Rhamnolipid	+ Rhmanolipid (6 mM)	- Rhamnolipid	+ Rhamnolipid (6 mM)
Hexadecane[a]	24 ± 3	83 ± 2	40 ± 7	53 ± 2
Octadecane[a]	27 ± 3	79 ± 6	40 ± 3	77 ± 6
Acetate[b]	36 ± 7	30 ± 9	8 ± 2	29 ± 1
Citrate[b]	19 ± 3	24 ± 6	11 ± 2	23 ± 8
Glucose[b]	20 ± 5	23 ± 4	61 ± 2	35 ± 10
Succinate[b]	22 ± 5	25 ± 7	49 ± 2	28 ± 1

[a]Bacteria were grown in mineral salts medium with octadecane at 23°C for 6 days, washed, and assayed.
[b]Bacteria were grown in mineral salts medium with octadecane at 23°C for 1 days, washed, and assayed.

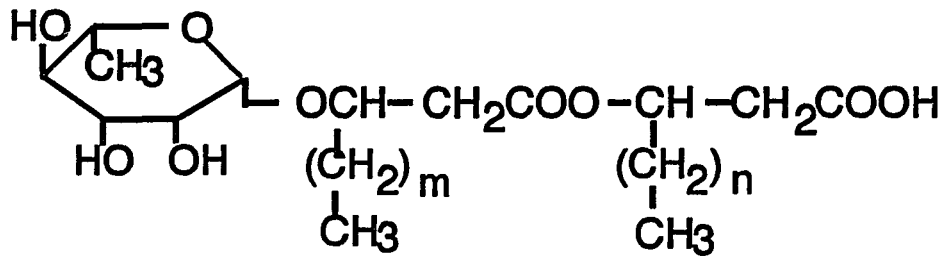

Fig. 1 *P. aeruginosa* ATCC 9027 monorhamnolipid structure. For C_{18} monorhamnolipid, m +n = 10; for C_{20}, m + n = 12; for C_{22}, m + n = 14; for C_{24}, m + n = 16.

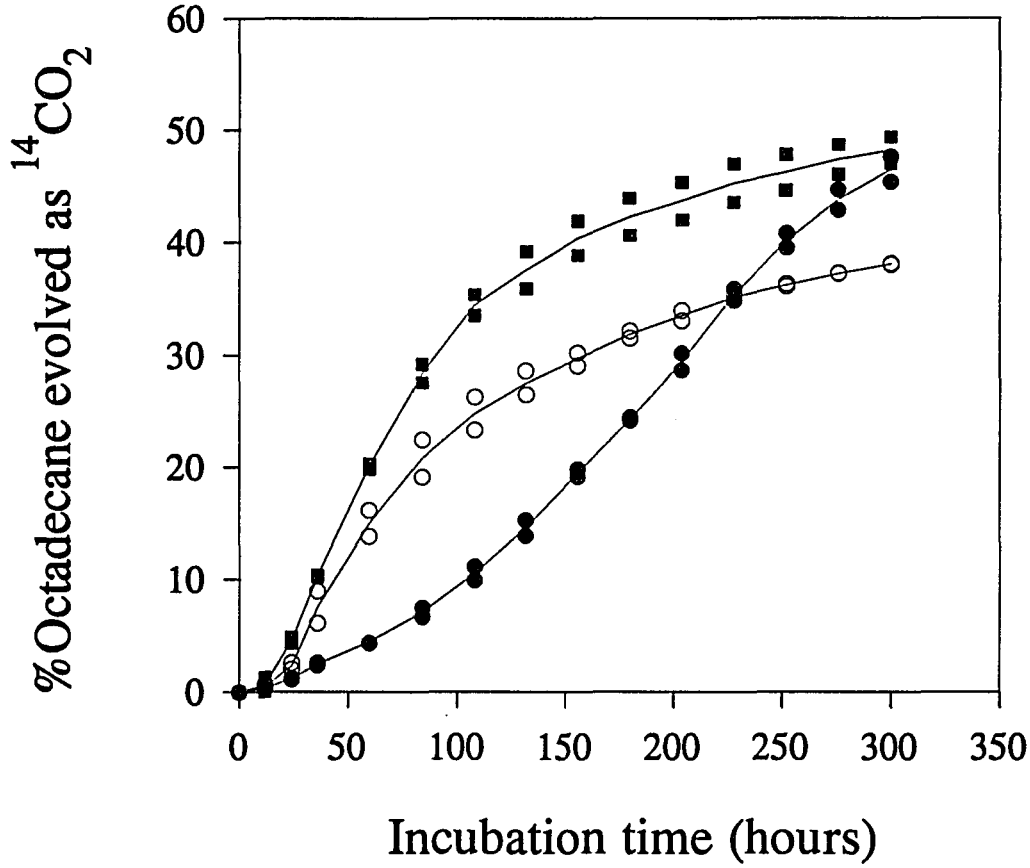

Fig. 2 Effect of rhamnolipid concentration on the mineralization of octadecane by *P. aeruginosa* ATCC 9027. A mixture of [^{14}C]octadecane (1.6 mM) and rhamnolipid was incubated in mineral salts medium with *P. aeruginosa* with gyratory shaking (200 rpm) at 23°C. Mineralization was measured described in Materials and Methods. ○, no rhamnolipid; ●, 1.2 mM rhamnolipid; ■, 7.6 mM rhamnolipid. This experiment was done in duplicate, and each point represents a duplicate sample.

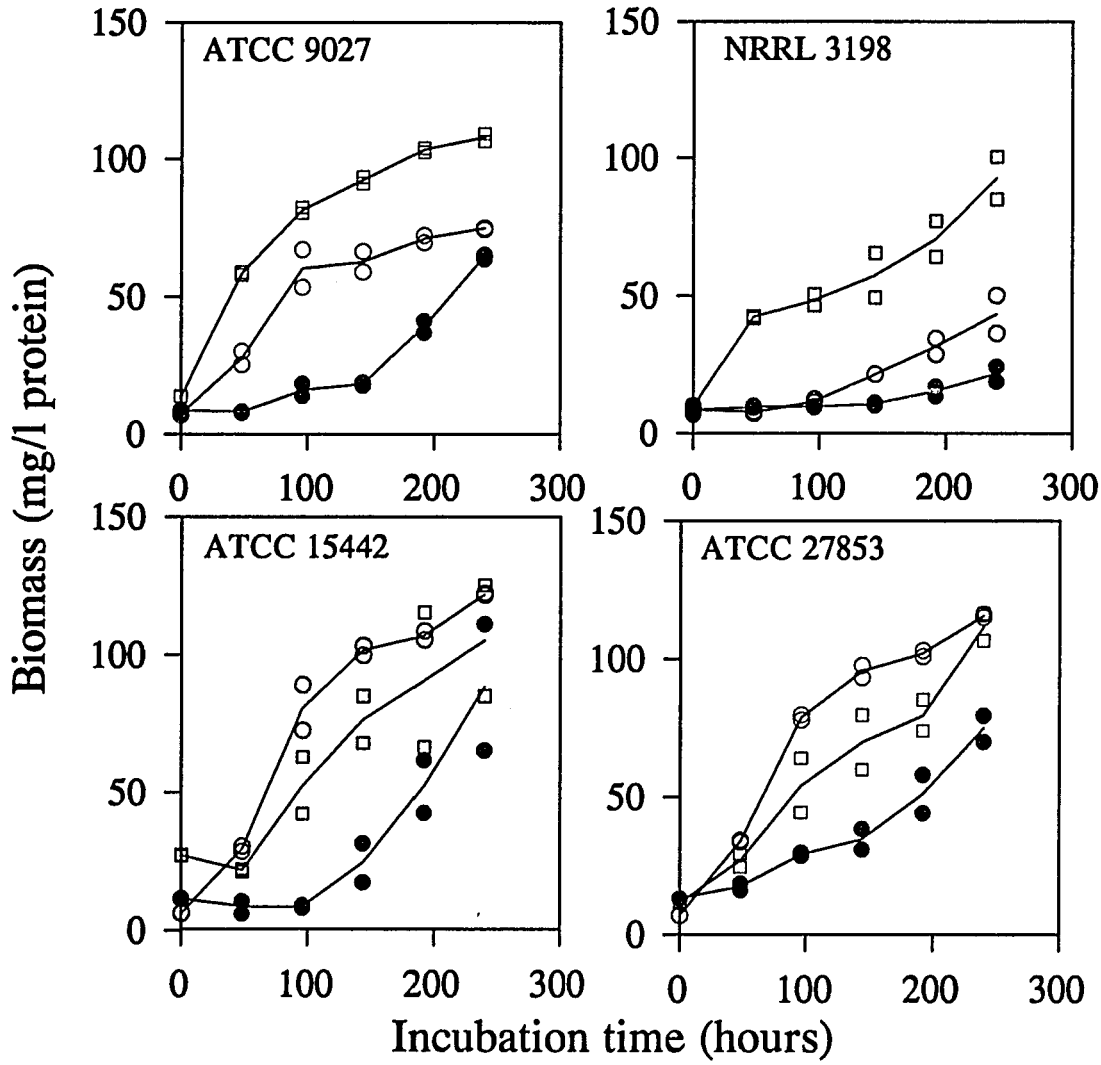

Fig. 3 Effect of rhamnolipid on octadecane biodegradation by four *P. aeruginosa* strains. A mixture of octadecane (3.9 mM) and rhamnolipid in mineral salts medium was inoculated with *P. aeruginosa* and the resulting mixture was incubated with gyratory shaking (200 rpm) at 23°C. Biomass was measured as described in Materials and Methods. ○, no rhamnolipid; ●, 0.6 mM rhamnolipid; □, 6 mM rhamnolipid.

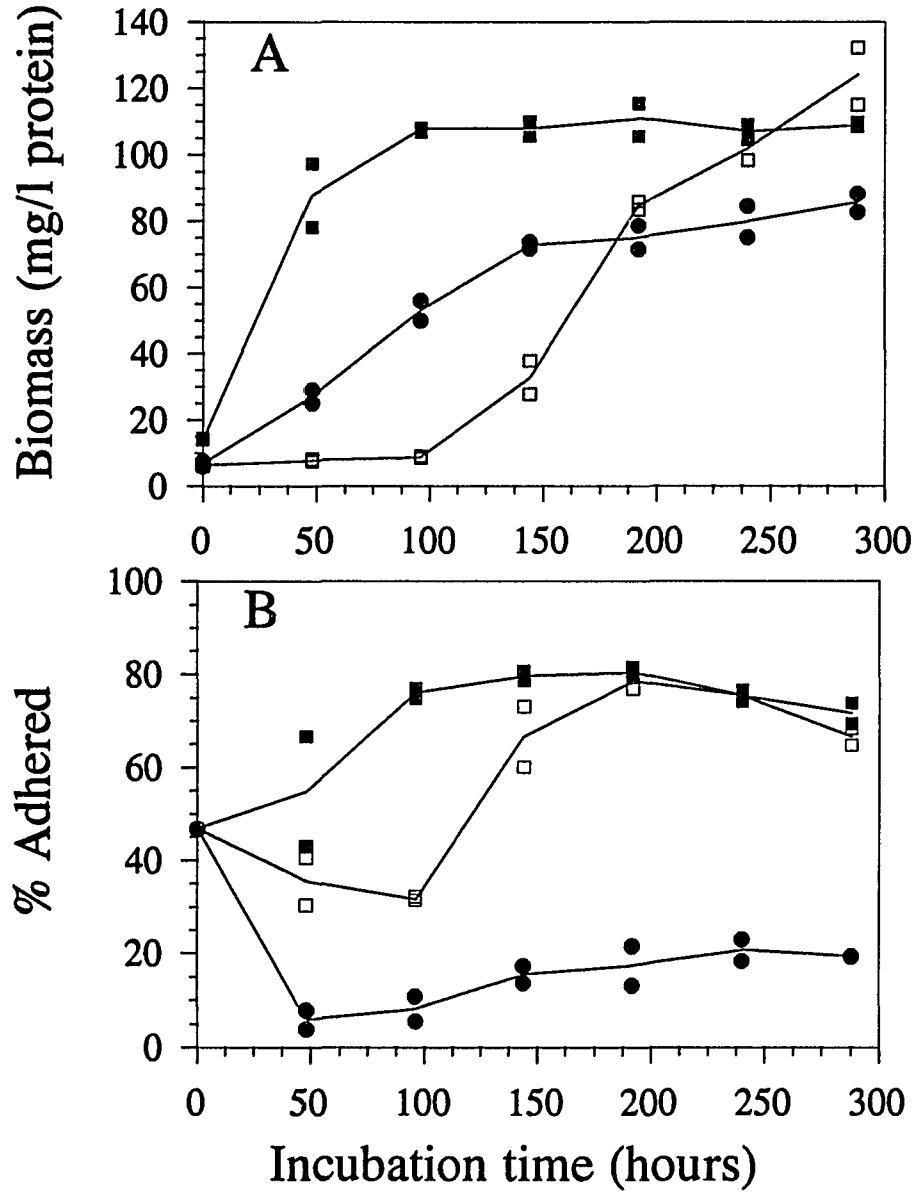

Fig. 4 Effect of rhamnolipid on the development of cell hydrophobicity and biodegradation of octadecane by *P. aeruginosa* ATCC 9027. A mixture of octadecane (3.9 mM) and rhamnolipid in mineral salts medium was inoculated with *P. aeruginosa* ATCC 9027, and the resulting mixture was incubated with gyratory shaking (200 rpm) at 23°C. (A) Amount of protein were determined as measure of biodegradation; (B) cells were assayed by the BATH test to determine hydrophobicity as described in Materials and Methods. ●, no rhamnolipid; □, 1.2 mM rhamnolipid; ◆, 7.6 mM rhamnolipid.

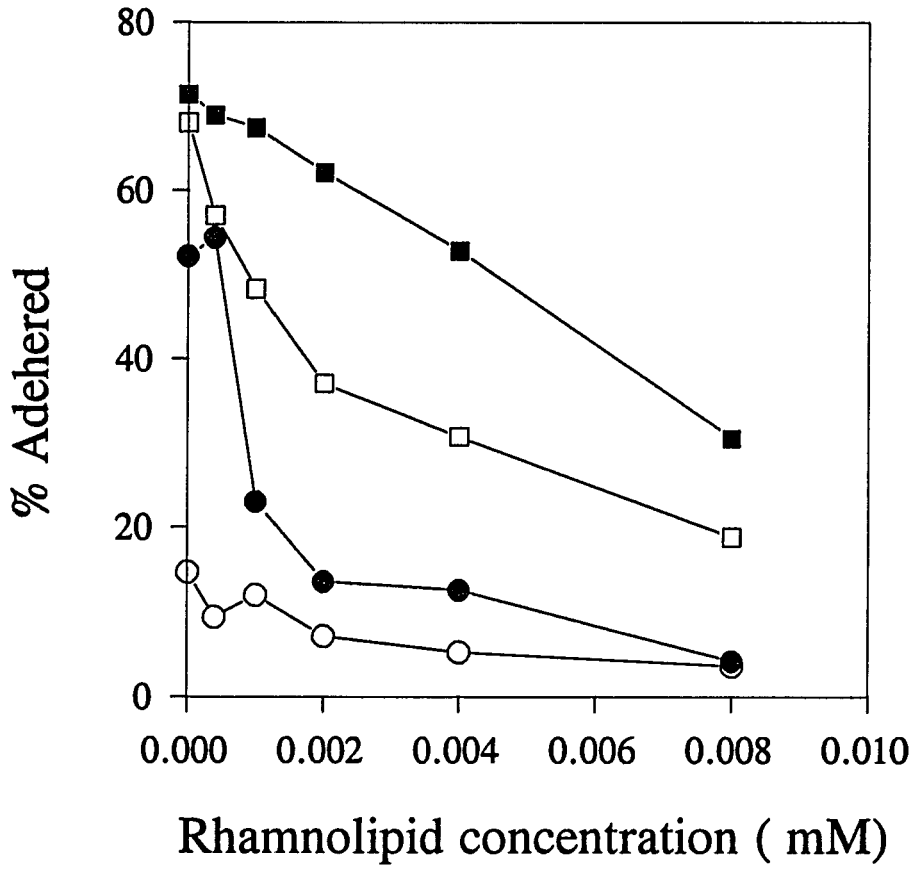

Fig. 5 Effect of rhamnolipid concentration on adherence of *P. aeruginosa* cells to hexadecane. *P. aeruginosa* cells were grown in Kay's minimal medium with gyratory shaking (200 rpm) at 37°C for 24 h. Cells were harvested and washed twice, and various concentrations of rhamnolipid were added. The BATH was performed as described in Materials and Methods. ■, ATCC 15442; □, ATCC 27853; ●, ATCC 9027; ○, NRRL 3198.

APPENDIX B

EFFECT OF RHAMNOLIPID (BIOSURFACTANT) STRUCTURE ON SOLUBILIZATION AND BIODEGRADATION OF *N*-ALKANES

Yimin Zhang and Raina M. Miller*

Department of Soil and Water Science
University of Arizona
Tucson, AZ 85721

Published in

Applied and Environmental Microbiology

Volume 61, pp. 2247-2251, 1995

*Corresponding author

Abstract

A study was conducted to quantify the effect of rhamnolipid biosurfactant structure on degradation of alkanes by a variety of *Pseudomonas* isolates. Two dirhamnolipids were studied, a methyl ester (dR-Me) and an acid form (dR-A). These rhamnolipids have different properties with respect to interfacial tension, solubility, and charge. For example, the interfacial tension between hexadecane and water was decreased to < 0.1 dyn/cm by the dR-Me but was only decreased to 5 dyn/cm by the dR-A. Solubilization and biodegradation of two alkanes in different physical states, liquid and solid, was determined at dirhamnolipid concentrations ranging from 0.01 to 0.1 mM (7 to 70 mg/l). The dR-Me markedly enhanced hexadecane (liquid) and octadecane (solid) degradation by eight different *Pseudomonas* strains except for one strain which exhibited extremely high cell surface hydrophobicity, the growth of which was inhibited on octadecane. The dR-A also enhanced hexadecane degradation by all degraders but more modestly than the dR-Me. For octadecane, the dR-A only enhanced degradation by strains with low cell surface hydrophobicity.

Introduction

One promising approach to increasing biodegradation rates of organic compounds with limited water solubility is the addition of biosurfactants (Francy, et al., 1991; Jain, et al., 1992; Miller, 1995). In previous work we have shown that biosurfactants affect the rate of hydrocarbon biodegradation in two ways: by increasing solubilization/dispersion of the hydrocarbon, and by changing the affinity between

microbial cells and hydrocarbons by inducing increases in cell surface hydrophobicity (Zhang and Miller, 1992 and 1994). These studies were performed using a purified monorhamnolipid biosurfactant produced by *Pseudomonas aeruginosa* ATCC 9027. However, most microorganisms produce biosurfactant mixture that are structurally similar, but which may have quite different physico-chemical properties. For example, rhamnolipids produced by *P. aeruginosa* strains have four main structural types: monorhamnolipid acid, monorhamnolipid methyl ester, dirhamnolipid acid (dR-A), and dirhamnolipid methyl ester (dR-Me). These rhamnolipids can be produced in mixtures of varying composition (Hirayama and Kato, 1982; Itoh, et al., 1971). Similarly, *Torulopsis* sp. produce sophorolipids in an acidic and a lactonic form(Tulloch, et al., 1968), and *Arthrobacter paraffineus* can produce either trehalose lipids or sucrose lipids depending on the carbon source used in medium (Suzuki, 1974).

Since biosurfactant structure is characteristic of the producing species and the available carbon source during growth, biosurfactant structures may play different roles in hydrocarbon metabolism. For example, investigation of the effect of sophorolipid types on alkane degradation showed that lactonic form inhibited hexadecane biodegradation, while the acid form stimulated hexadecane biodegradation (Ito and Inoue, 1982; Ito, et al., 1980). This example illustrates the importance of biosurfactant structure in determining hydrocarbon degradation rates. But it is not yet clear how structure impacts degradation rates. It has been established that surfactant solubilization/dispersion of organic compounds, which is related to interfacial tension, is dependent on surfactant structure (Lang, et al., 1984). However, increased dispersion

does not always lead to increased biodegradation (Miller, 1995). Therefore, it must be concluded that it is the three-way interaction between biosurfactant, substrate and cell that is crucial to achieve enhanced biodegradation rates. There have been few studies to date concerning the effect of surfactant structure on the interaction of surfactants with hydrocarbons and microbial cells. In this study two structurally different dirhamnolipids were investigated for their effect on both substrate dispersion and cell aggregation and the resulting impact on biodegradation rates of n-alkanes. The dirhamnolipids used in this study were an anionic dirhamnolipid acid (dR-A), and a nonionic dirhamnolipid methyl ester (dR-Me) both of which are shown in Fig. 1. Model substrates used were two n-alkanes that exist in different physical states at room temperature: hexadecane (liquid) and octadecane (solid). The degrading organisms used were a variety of laboratory and environmental *Pseudomonas* isolates, with different inherent alkane biodegradation rates.

Materials and methods

Microorganisms

Pseudomonas aeruginosa ATCC 9027, ATCC 15442, ATCC 27853, and NRRL 3198 were obtained from the American Type Culture Collection (Rockville, MD) or from the University of Arizona Undergraduate Program in Microbiology Culture Collection. The cultures were stored at 4°C on *Pseudomonas* agar P medium (Difco, Detroit, Mich.) and transferred monthly. *Pseudomonas fluorescens* isolates were obtained from the U.S. Department of Energy Subsurface Microbiological Culture

Collection (Fliermans and Balkwill, 1989). The isolates were stored at 4°C on PTYG medium and transferred monthly. None of these strains produced biosurfactants during growth in mineral salts medium (Zhang and Miller, 1992) containing n-alkanes. Also, none of these strains utilize dirhamnolipid as sole source of carbon.

Dirhamnolipid acid and methyl ester

Crystalline dR-A was gift from Kyowa Hakko Kogyo Co., Ltd (Tokyo, Japan). The dR-Me was synthesized from dR-A by the diazomethane method (Tulloch, et al., 1968). Diazomethane was prepared from Diazald® using the procedure described by Aldrich Technical Information Bulletin No. AL-180. The dR-A (2 g) was dissolved in methanol (50 ml) and a solution of diazomethane in ethyl ester was added until the diazomethane yellow color persisted. The excess diazomethane and solvent were evaporated. The dR-Me product was purified by elution with chloroform-methanol (35:1) from a Silica Gel 60 (Aldrich, Milwaukee, WI.) chromatography column (14 × 2.5 cm). The dR-Me was analyzed by thin layer chromatography (TLC) using a chloroform methanol:water (65:25:1) solvent and was identified by ^{1}H NMR ($CDCl_3$) (Model AM 250, Bruker Co., Germany). The unique chemical shift (δ) at 3.68 in the ^{1}H NMR spectra indicated the methyl group ($COOCH_3$) of the dR-Me.

The surface tension and interfacial tension of dR-Me and dR-A were determined in 0.1 M, pH 7.0, phosphate buffer using a Fisher (Pittsburgh, Pa) surface tensiometer (Model 21), that employs the du Nouy ring method. All reported interfacial tension values were measured between hexadecane and water. The critical micelle concentration

(cmc) of dR-Me and dR-A was determined from a semi-logarithmic plot of surface tension against the surfactant concentration (Magaritis, et al., 1979).

Dispersion tests

The of hexadecane and octadecane in dirhamnolipid solution was determined using [1-^{14}C]hexadecane (specific activity, 2.2 mCi/mmol, 98% pure) and [1-^{14}C]octadecane (specific activity, 3.6 mCi/mmol, 98% pure), Sigma, St. Louis, MO. A mixture of alkane and [^{14}C]alkane dissolved in chloroform were added to test tubes (16 × 100 mm). After evaporation of solvent, 2 ml of rhamnolipid solution in 0.1 M, pH 7.0, phosphate buffer was added. The final concentration of alkanes was 4 mM, and the alkane specific activity was 0.5 mCi/mmol. For octadecane, the test tubes were incubated at 37°C in a water bath for 30 sec, to melt the coated octadecane and the cooled at room temperature until the octadecane solidified on the surface of solution. The test tubes were then incubated at 23°C with gyratory shaking (200 rpm). After 24 h, the solutions were filtered through a Whatman GF/D filter (pore size, 10 μm) and 0.2 ml was added to 5 ml of Scintiverse BD (Fisher, Pittsburgh, Pa). Radioactivity was determined with a Packard (Meriden, Conn.) Tri-Carb liquid scintillation counter (model 1600 TR).

Biodegradation tests

Alkane biodegradation was determined both by measurement of alkane mineralization and by measurement of protein increase as an indication of cell growth.

For mineralization experiments, a mixture of alkane and [^{14}C]alkane dissolved in chloroform was used to coat the bottom of modified 125-ml micro-Fernbach flasks (Wheaton, Melville, N.J.), designed for the collection of $^{14}CO_2$ and ^{14}C-volatile compounds. The solvent was evaporated, and 20 ml of mineral salts medium containing rhamnolipid was added to each flask. The final concentration of alkane was 4 mM, and alkane specific activity was 0.6 μCi/mmol. For octadecane, the solid alkane was melted and then cooled at room temperature. The flasks were inoculated with a 2.5% inoculum of *Pseudomonas* sp. grown in Kay's minimal medium (Warren, et al., 1960) at 37°C for 24 h. The flasks were incubated with gyratory shaking (200 rpm) at 23°C and were flushed periodically as described by Marinucci and Bartha (1979) to collect $^{14}CO_2$ and ^{14}C-volatile organic compounds.

For protein measurement, 10 ml of mineral salts medium containing dirhamnolipid was added to test tubes containing 4 mM alkane. The tubes were inoculated and incubated as described above. Periodically, 0.5-ml samples were taken from each test tube and heated for 10 min with 0.05 ml of 1 N NaOH, and the protein content was determined by the method of Lowry et al. (1951).

Results

Physical properties of dirhamnolipid acid and methyl ester

High performance liquid chromatography of the dR-A showed four components that differed slightly in fatty acid structure. These have been identified by Kyowa Hakko Kogyo Co. as the C-18, C-20, and C-22 saturated, and C-18 monounsaturated

dirhamnolipids. The structure of the major C-20 component (70 %) is shown in Fig.1. Our analysis of the dR-A by thin layer chromatography (TLC) showed only one anthrone positive spot with a R_f value of 0.46 using a chloroform-methanol-water (65:25:1) solvent system. In comparison, the dR-Me synthesized from the dR-A displayed two anthrone positive spots on a TLC plate with R_f values of 0.75 and 0.81 using the same solvent system.

As expected, the water solubility of the two dirhamnolipids varied greatly. The dR-A had an aqueous solubility of more than 15 mM while the water solubility of the dR-Me was several orders of magnitude lower, 0.04 mM (Table 1). Surface tension, interfacial tension, and cmc values were measured and compared for the two dirhamnolipids (Table 1). These data showed that the dR-Me had a lower cmc and produced lower surface and interfacial tension in solution than the dR-A.

Alkane dispersion by dirhamnolipids

Aqueous dispersion tests measured the concentration of alkane-rhamnolipid complexes less than 10 μm in diameter. As shown in Fig. 2A, the dispersion of hexadecane was enhanced by the dR-Me much more than by the dR-A. The increase in hexadecane dispersion was linear until 0.1 mM dirhamnolipid and linear portion of each plot was used to calculate a molar solubilization ratio (MSR = moles organic compound solubilized/mole of surfactant). The MSR for the dR-Me was 5.2 and for the dR-A was 0.13, a difference of 40-fold. The difference was apparent visually as well. An emulsion formed in the presence of the dR-Me while in the presence of the dR-A,

some hexadecane was dispersed and some hexadecane still floated on the surface of the water.

In contrast, the dispersion of octadecane was increased only slightly by both the dR-Me and the dR-A (Fig. 2B). The calculated MSR values, similar for both the dR-Me and dR-A, were 0.074 mM and 0.15 mM octadecane/mM rhamnolipid. Visually, both dirhamnolipid forms distributed octadecane into solution as small particles, but the particles produced by dR-Me were smaller resulting in a more extensive alkane surface area.

Effect of dirhamnolipids on alkane biodegradation

Figures 3 and 4 show the effect of 0.05 mM dR-A and dR-Me on the degradation of hexadecane (Fig. 3) and octadecane (Fig. 4) by *P. aeruginosa* ATCC 9027. As shown in these figures, the increase in biodegradation was determined by both measurement of alkane mineralization and protein was similar. Examination of the slopes of each curve showed that the dR-Me was most effective in stimulating the rate of biodegradation for both hexadecane (Fig. 3) and octadecane (Fig.4). For hexadecane, rate of mineralization was stimulated 8-fold by the dR-Me and 1.6-fold by the dR-A in comparison to the control. For octadecane, the rate of mineralization was stimulated 1.6-fold by the dR-Me in comparison to the control, but the dR-A had a slightly slower maximum rate of biodegradation than the control after an initial lag period for the control. Interestingly, in the presence of dirhamnolipids, hexadecane was always mineralized more quickly than octadecane (Figure 3B and 4B), which in the absence of

dirhamnolipids, octadecane was mineralized (9.7 %) faster than hexadecane (4.9 %).

Effect of dirhamnolipid concentration on alkane biodegradation

The biodegradation of alkanes by *P. aeruginosa* ATCC 9027 was also examined at varying concentrations of the dirhamnolipids. In this set of tests, cell growth was measured by protein increase after 24 h and 48 h incubation. For hexadecane (Fig. 5), biodegradation was enhanced at the lowest rhamnolipid concentration (0.01 mM) and an increase in surfactant concentration to 0.07 mM had no effect on biodegradation. Similar to hexadecane, the biodegradation of octadecane was enhanced at the lowest dR-Me concentration tested (0.02 mM) and a further increase in surfactant concentration to 0.1 mM had no effect on biodegradation (data not shown). There was little effect of the dR-A at any concentration tested (0.02 to 0.1 mM) on octadecane degradation (data not shown).

Effect of dirhamnolipid on biodegradation of hexadecane and octadecane by different Pseudomonas isolates

The effect of the dR-A and dR-Me on alkane biodegradation by eight *Pseudomonas* isolates is summarized in Table 2. The eight strains were divided into two groups based on their growth rates on alkanes in the absence of dirhamnolipids :ATCC 27853, ATCC 15442, and OB 316 utilize alkanes rapidly (fast degraders); while ATCC 9027, NRRL 3198, BO 267, BO 307, and BO 138 degrade alkanes more slowly (slow degraders).

Addition of the dR-Me enhanced degradation of hexadecane by all strains with the exception of ATCC 15442, the growth of which was inhibited on octadecane. Total growth (in terms of protein increase) on hexadecane in the presence of the dR-Me was similar for both slow and fast degraders. These data and the data in Fig. 3A suggest that the substrate was completely utilized within the 48-h timeframe of the experiment. The increase in total growth in 48-h in the presence of the dR-Me can be quantified from the data in Table 2. For hexadecane, growth of the slow isolates was increased 6.2 to 38.7-fold, while growth of the fast isolates was increased 2.9 to 3.2-fold. Although none of the isolates tested degraded octadecane completely in 48-h, the pattern of degradation of octadecane in the presence of dR-Me was similar to that of hexadecane. Degradation by the slow degraders (1.8 to 4.9-fold) was stimulated more than degradation by the fast degraders (ATCC 27853 and BO 310 were stimulated 1.2-fold, ATCC 15442 was inhibited).

Behavior of the dR-A was more complex than the dR-Me. Similar to the dR-Me, degradation of hexadecane and octadecane by slow degraders was enhanced, but much more modestly. Degradation of hexadecane by the fast degraders was also enhanced slightly for two of the three strains tested. However, octadecane degradation by the fast degraders was inhibited relative to the control for all three strains.

Discussion

We have previously reported that a monorhamnolipid acid biosurfactant enhanced dispersion and biodegradation of octadecane (Zhang and Miller, 1994). The results of

this study show that dirhamnolipids can achieve comparable levels of dispersion and biodegradation but at extremely low concentrations, as low as 0.01 mM, while monorhamnolipid concentrations required to stimulate biodegradation were 6 to 7 mM. The environmental significance of these results is two-fold. First, it may be feasible to stimulate in situ production of surfactants in this concentration range. In situ production has already been suggested by results of a study by Oberbremer et al. (1989) which showed that a reduction in surface tension of the fluid phase in a stirred soil bioreactor was correlated to the onset of biodegradation of petroleum hydrocarbons with low water solubility in the bioreactor. Second, the cost of ex situ production and the environmental impact resulting from exogenous addition of surfactants in such low concentration is reduced.

Results of this study demonstrate that surfactant effects on hydrocarbon biodegradation depend to some degree on: surfactant structure, physical state of the alkane, amount alkane dispersion/emulsification, degrading isolate, and the degree of cell aggregation. Although these factors are interdependent, each can be considered separately in order to help interpret the system as a whole.

Surfactant structure

Modification of the dirhamnolipid carboxyl group to a methyl ester caused a large difference in surfactant effectiveness. As shown in Table 1, the dR-A was much less effective in reducing interfacial tension between hexadecane and water than the dR-Me. The difference can be attributed to the carboxylic acid group (pK_a 5.6) which confers

a negative charge on the dR-A at neutral pH. This charge caused an enhanced interaction of the rhamnolipid with water and a weaker interaction between the rhamnolipid and hexadecane in comparison to the dR-Me. Thus, the dR-A was less effective at reducing interfacial tension between hexadecane and water. A second result of modification of the dR-A to the dR-Me was a dramatic decrease in surfactant water solubility. It should be noted that the reported value of the dR-Me cmc (0.04 mM) in table 1 was equal to its maximum water solubility. Thus it is possible that the measured minimum surface tension of dR-Me was due to limited water solubility and the true cmc was not reached.

Physical state

The physical state of the n-alkane serving as substrate also affected alkane biodegradation rates. In the absence of surfactants, octadecane was consistently degraded at a faster rate than hexadecane (Table 2). This was surprising since thermodynamically the uptake of a solid compound should require more energy than uptake of a liquid, suggesting the biodegradation of octadecane would be slower than that of hexadecane. In contrast, in the presence of surfactants, the degradation of hexadecane was always more rapid than degradation of octadecane. This was correlated with a greater increase in dispersion of the liquid alkane than the solid alkane by the surfactants studied.

Alkane dispersion

Alkane dispersion seems to be one of the most important factors in determining alkane degradation rates. The dispersing ability of dirhamnolipids was related to their ability to reduce interfacial tension. The low interfacial tension produced by the dR-Me (< 0.1 dyn/cm) caused emulsification of hexadecane, the liquid alkane. In contrast, the dR-A acted as a weak dispersant. The solid physical state of octadecane prevented emulsification, however, the size of the octadecane particles in solution with the dR-Me were visually much smaller than those produced by the dR-A. This resulted in a much more extensive available surface area in the presence of the dR-Me. Alkane biodegradation was directly related to alkane dispersion. Thus, growth was greatest on hexadecane in the presence of the dR-Me which caused an increase of 10^5-fold in hexadecane dispersion to 0.4 mM or ~90 mg/l (Fig.2). For hexadecane, the data in Table 2 and Fig. 3A show that with the addition of the dR-Me all isolates showed an increase in growth and achieved complete substrate utilization in 48 h. In contrast to the dR-Me, the dR-A caused an increase of only 10^3-fold in hexadecane dispersion to 0.01 mM (~2 mg/l). The resulting effect on alkane degradation was an enhancement, but this enhancement was 2 to 3-fold smaller than the enhancement by the dR-Me (Table 2).

While dispersion of octadecane was increased to similar levels (~0.01 mM) by both dirhamnolipid forms (Fig. 2), it was visually apparent that the octadecane particle size distribution was very different in the presence of the two rhamnolipids. The particles formed in the presence of the dR-Me were much smaller and more numerous that for the dR-A. The resulting effects on biodegradation of octadecane were several:

1) in comparison to hexadecane, increases in octadecane biodegradation were much smaller, 2) in contrast to hexadecane, only biodegradation of slow degraders was enhanced, and 3) for the slow degraders there was a 1.5 to 2-fold greater enhancement of octadecane biodegradation by the dR-Me than by the dR-A.

Degrading isolate

This study investigated biodegradation by two types of bacteria: slow degraders that exhibit relative low cell surface hydrophobicity and a relative low inherent rate of alkane biodegradation, and fast degraders that have higher cell surface hydrophobicity and a higher rate of alkane biodegradation. Considering all alkane/surfactant combinations examined, alkane degradation was greatest for both types of degraders in the dR-Me/hexadecane combination. For this combination, the relative enhancement in growth by fast degraders was less than that for slow degraders but all isolates achieved complete substrate utilization within 48 h (table 2). This was the general pattern in all cases; alkane degradation by the slow degraders was stimulated by dirhamnolipid addition more than degradation by the fast degraders.

In all cases, both dirhamnolipids stimulated alkane degradation by the slow degraders. However, for the fast degraders there were two alkane/surfactant combinations where the presence of the surfactant inhibited degradation of the alkane relative to the control. These combinations were octadecane/dR-A and octadecane/dR-Me (Table 2). A common factor observed in all experiments that showed inhibition of degradation was the appearance of large aggregates (1 to 2 mm in diameter) in culture

solution. These aggregates suggest that there is a specific surfactant-hydrocarbon-cell interaction which causes inhibition of uptake and degradation of the hydrocarbon. Investigation of aggregate formation is the focus of on-going work in our laboratory.

In summary, dirhamnolipids have exciting potential for remediation of petroleum contaminated sites because of the low rhamnolipid concentration required for effective stimulation of biodegradation. The dR-Me was particularly effective in stimulating alkane biodegradation, however, the low water solubility of the dR-Me may limit its usefulness in environmental settings. This shortcoming can potentially be overcome by using rhamnolipid mixtures containing both the dR-A and the dR-Me forms. Initial work in our laboratory on mixtures has shown that a 1:1 mixture of dR-A and dR-Me markedly increased the solubility of the dR-Me and the mixed dirhamnolipids were also more effective in alkane biodegradation than the dR-A alone (data not shown). Although most *Pseudomonas* strains produce mixed rhamnolipids, it is not known how many of these strains are able to produce rhamnolipid methyl ester. The current method for the isolation of rhamnolipids (acid precipitation) selects for the dirhamnolipid acid forms but is not suitable for the isolation of rhamnolipid esters. Thus, new methods must be developed to improve the isolation and detection of rhamnolipid methyl esters.

References

Fliermans, C.B.and D.L. Balkwill. 1989. Life in the terrestrial deep surface. BioScience. 39:370-377

Francy, D.S., J.M. Thomas, R.L. Raymond, and C.H. Ward. 1991. Emulsification of hydrocarbons by subsurface bacteria. J. Ind. Microbiol. 8:237-246

Hirayama, T., and I. Kato. 1982. Novel methyl rhamnolipids from *Pseudomonas aeruginosa*. FEBS Lett. 139:81-85

Itoh, S., H. Honda, F. Tomita, and T. Suzuki. 1971. Rhamnolipids produced by *Pseudomonas aeruginosa* grown on *n*-paraffin. J. Antibiot. 12:855-859

Ito, S., and S. Inoue. 1982. Sophorolipids from *Torulopsis bombicola*: possible relation to alkane uptake. Appl. Environ. Microbiol. 43:1278-1283

Ito, S., M. Kinta, and S. Inoue. 1980. Growth of yeasts on *n*-alkanes: inhibition by a lactonic sophorolipid produced by *Torulopsis bombicola*. Agri. Biol. Chem. 44:2221-2223

Jain, D.K., H. Lee, and J.T. Trevors. 1992. Effect of addition of *Pseudomonas aeruginosa* UG2 inocula or biosurfactants on biodegradation of selected hydrocarbons in soil. J. Ind. Microbiol. 10:87-93

Lang., S., A. Gibon, C. Syldatk and F. Wanger. 1984. Comparison of interfacial active properties of glycolipid from microorganisms. In *surfactants in solution*. Mittal, K.L., and B. Lindman, (eds), Plenum Press, New York. p.1365-1376

Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall. 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. 193:265-275

Magaritis, A., K. Kennedy, J.E. Zajic, and D.F. Gerson. 1979. Bisurfactant production by *Nocardia erythropolis*. Develop. Ind. Microbiol. 20:623-630

Marinucci, A.C. and R. Bartha. 1979. Apparatus for monitoring the mineralization of volatile ^{14}C-labeled compounds. Appl.Environ. Microbiol. 38:1020-1022

Miller, R.M. 1995. Surfactant-enhanced bioavailability of slightly soluble organic compounds. In Bioremediation - Science & Applications. (H.Skipper, ed.) Soil Science Society of America special publication, Madison, WI, in press.

Oberbremer, A., and R. Müller-hurtig. 1989. Aerobic stepwise hydrocarbon degradation and formation of biosurfactants by an original soil population in a stirred reactor. Appl. Microbiol. Biotechnol. 31:582-586

Singer, M.E., and W.R. Finnerty. 1984. Microbial metabolism of straight-chain and branched alkanes, p.1-59. *In* R.M. Atlas (ed.), Petroleum microbiology. Macmillan Publishing Co., New York.

Suzuki, T., H. Tanaka, and S. Itoh. 1974. Sucrose lipids of *Arthrobacter, Corynebacteria and Nocardia* grown on sucrose. Agr. Biol. Chem. 38:557-563

Tulloch, A.P., A. Hill, and J.F.T. Spencer. 1968. Structure and reactions of lactonic and acididc sophorosides of 17-hydroxyoctadecanoic acid. Can J. Chem. 38:557-563

Warren, A.J., A.F. Ellis, and J.J.R. Campbell. 1960. Endogenous respiration of *Pseudomonas aeruginosa.* J. Bacteriol. 79:875-880

Zhang, Y. and R.M. Miller. 1992. Enhanced octadecane dispersion and biodegradation by a *Pseudomonas* rhamnolipid surfactant (biosurfactant). Appl. Environ. Microbiol. 58:3276-3282

Zhang, Y., R.M. Miller. 1994. Effect of a *Pseudomonas* rhamnolipid biosurfactant on cell hydrophobicity and biodegradation of octadecane. Appl. Environ. Microbiol. 60:210-2106

Table 1 Physico-chemical properties of dirhamnolipis and *n*-alkanes used in this study

Property	C_{16}	C_{18}	Dirhamnolipid acid	Dirhamnolipid methyl ester
Aqueous solubility (mM)	[1]2.8×10^{-5}	[1]2.3×10^{-5}	>15	0.04
Density[1](g/l)	773	777	-	-
Surface tension (dyn/cm)	-	-	36	31
Interfacial tension [2](dyn/cm)	-	-	5	< 0.1
cmc (mM)	-	-	0.1	0.04

[1]From Singer and Finnerty (1984).
[2]Interfacial tension was measured between hexadecane and water

Table 2 Effect of dR-A and dR-Me on biodegradation of hexadecane and octadecane

Bacterial strain[b]	Growth in 48 h[a]					
	Hexadecane[c]			Octadecane[c]		
	no dR	dR-A[d]	dR-Me[d]	no dR	dR-A[d]	dR-Me[d]
slow degraders						
ATCC 9027	3±1	65±1 (21.7)	116±2 (38.7)	39±2	44±6 (1.1)	49±4 (1.8)
NRRL 3198	11±2	38±4 (3.5)	118±15 (10.7)	34±1	46±4 (1.4)	92±4 (2.7)
BO 267	12±3	21±0 (1.8)	116±3 (9.7)	15±2	40±1 (2.7)	74±2 (4.9)
BO 307	10±2	33±2 (3.3)	110±19 (11)	25±4	43±9 (1.7)	61±4 (2.4)
BO 138	19±5	36±1 (1.9)	117±15 (6.2)	31±3	42±2 (1.4)	73±11 (2.4)
fast degraders						
ATCC 27853	32±9	51±4 (1.6)	94±2 (2.9)	68±7	35±3[e] (0.5)	83±3 (1.2)
ATCC 15442	37±4	46±4 (1.2)	118±15 (3.2)	59±5	40±6[e] (0.7)	12±1[e] (0.2)
BO 310	41±7	42±8 (1.0)	126±4 (3.1)	65±4	51±4[e] (0.8)	79±2 (1.2)

[a] Growth is expressed in mg/l protein (mean ± SD). Numbers in parentheses with follow are the increase in growth due to dirhamnolipid, calculated from: growth in presence of dirhamnolipid/growth in absence of dirhamnolipid.

[b] Strains with the prefix BO were supplied by Dr, David Balkwill from the U.S. Department of Energy Subsurface Microbiological Culture Collection. A total 11 strains were tested. Seven of these strains did not degrade either alkane and were not affected by rhamnolipid addition.

[c] Hexadecane and octadecane concentration used was 4 mM.

[d] dR-Me and dR-A used was 0.05 mM.

[e] Biodegradation was inhibited by dirhamnolipid addition.

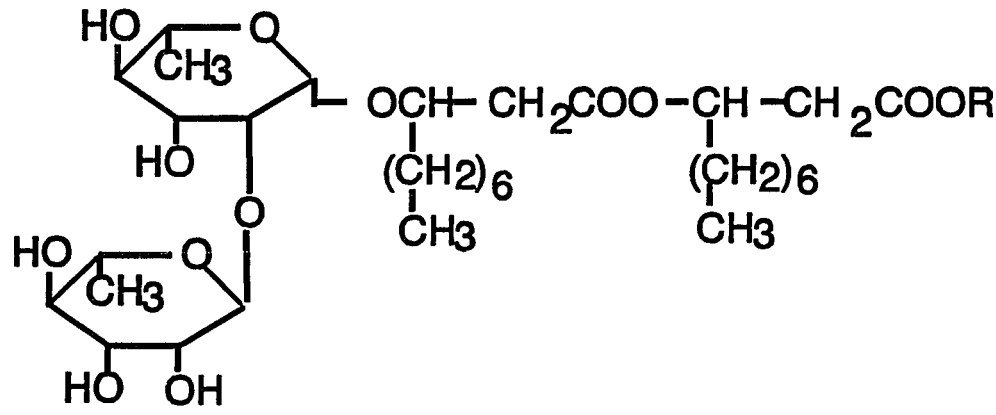

Figure 1 Structure of a *Pseudomonas* dirhamnolipid. For dR-A, R = H; for dR-Me, R = CH_3.

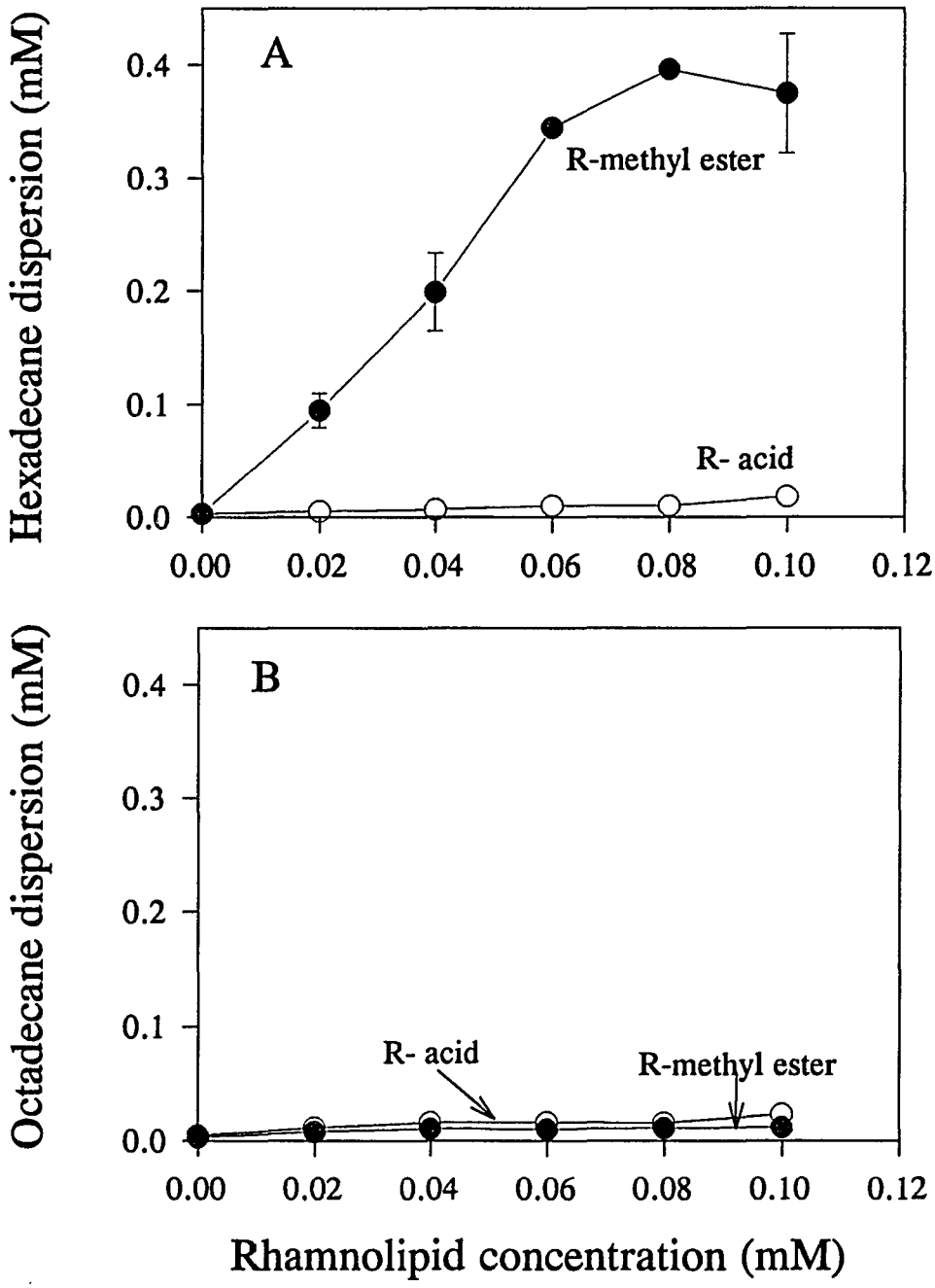

Figure 2 Effect of dirhamnolipid on the apparent aqueous solubility of hexadecane and octadecane. Solutions containing dirhamnolipid and [^{14}C]hexadecane or [^{14}C]octadecane (4 mM) were incubated with gyratory shaking at 200 rpm and 23°C for 24 h. Solubility was measured as descried in Materials and Methods. (A) hexadecane; (B) octadecane. Symbols: ● dR-Me , ○ dR-A.

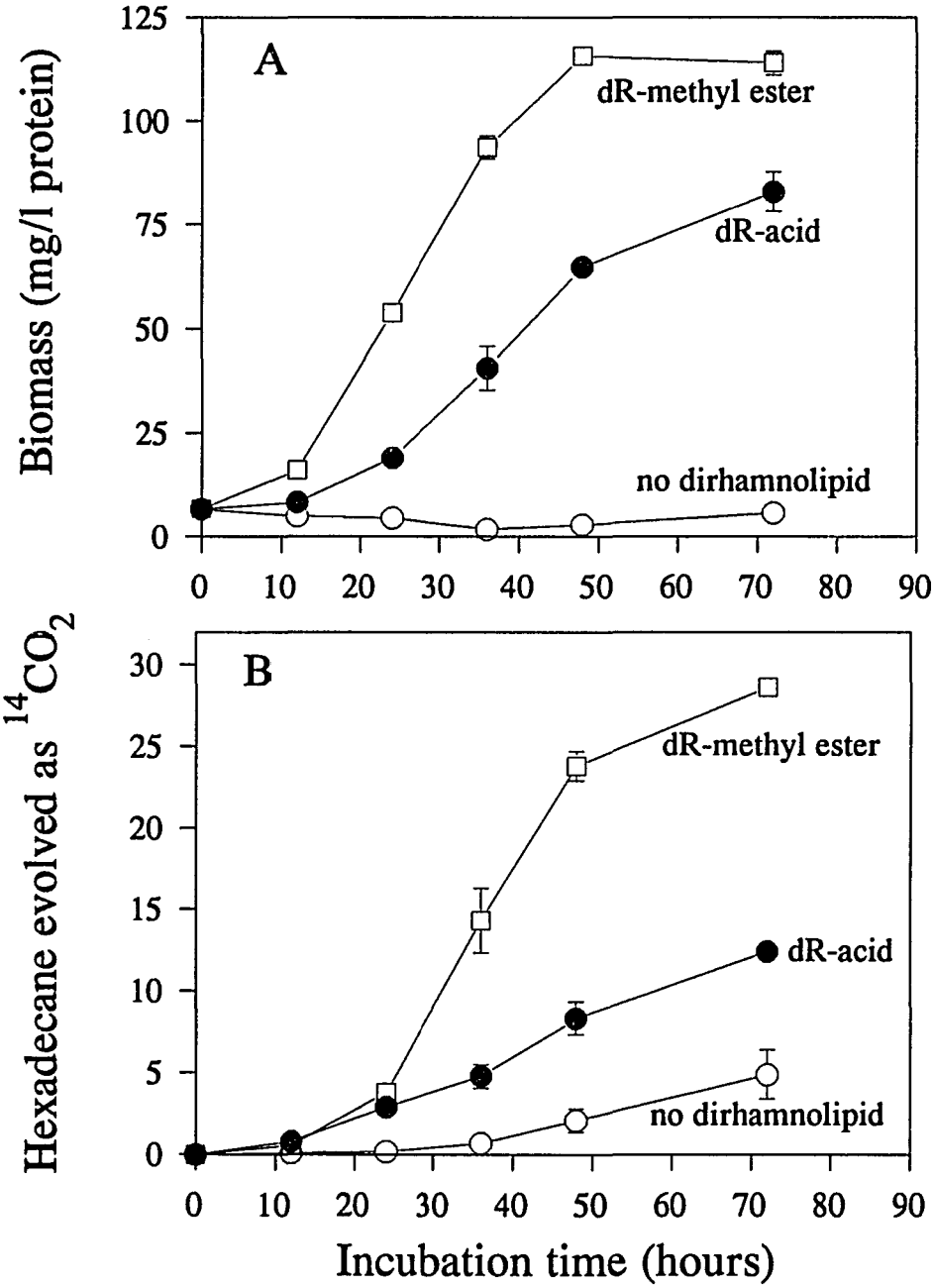

Figure 3 Effect of dirhamnolipid on the biodegradation of hexadecane (4 mM) by *Pseudomonas aeruginosa* ATCC 9027. (A) protein increase was used to measure biodegradation; (B) alkane mineralization was used to measure biodegradation. Symbols: ○ no dirhamnolipid, ● 0.05 mM dR-A, □ 0.05 mM dR-Me.

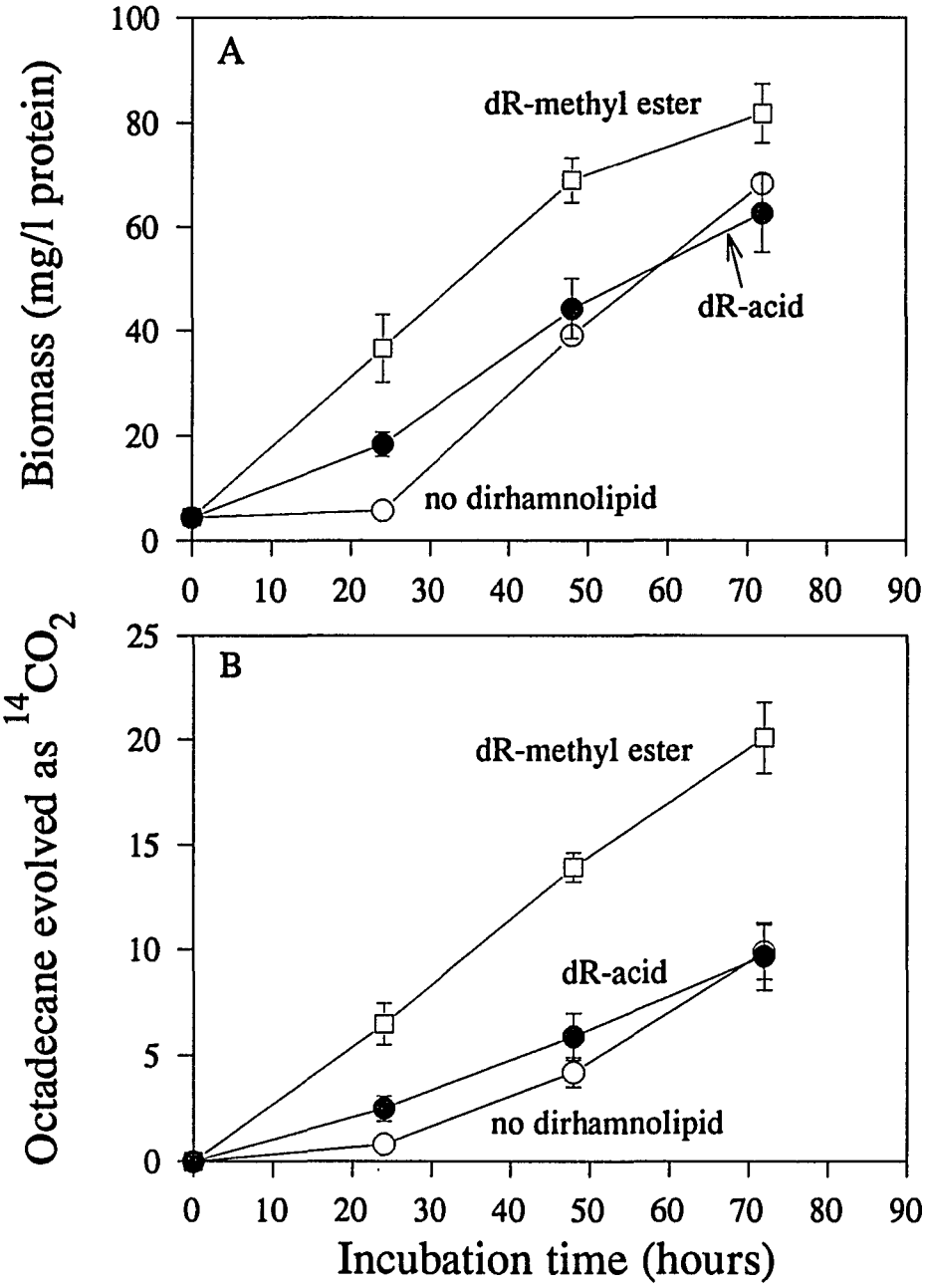

Figure 4 Effect of dirhamnolipid on the biodegradation of octadecane (4 mM) by *Pseudomonas aeruginosa* ATCC 9027. (A) protein increase was used to measure biodegradation; (B) alkane mineralization was used to measure biodegradation. Symbols: ○ no dirhamnolipid, ● 0.05 mM dR-A, □ 0.05 mM dR-Me.

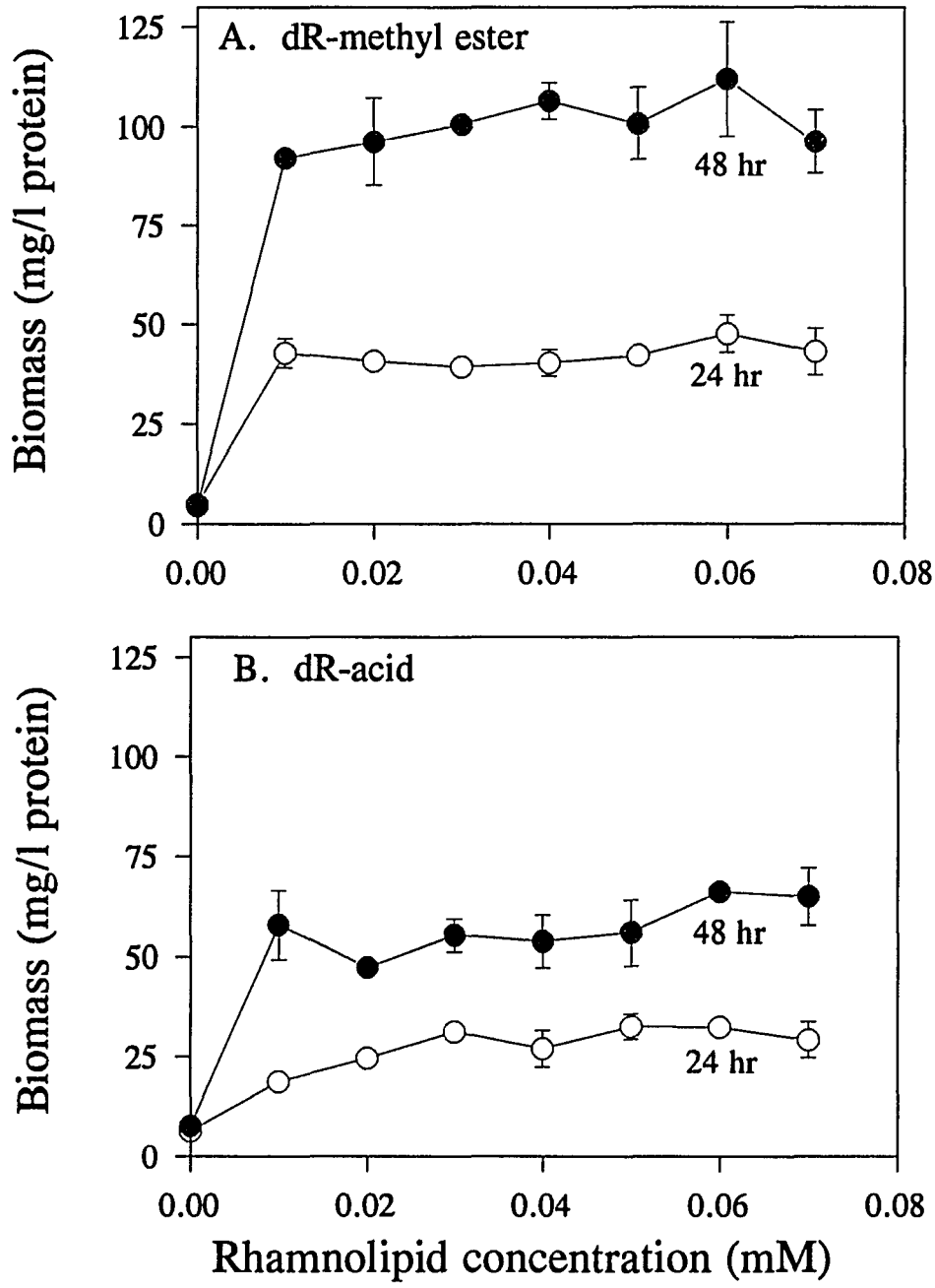

Figure 5 Effect of dirhamnolipid concentration on the biodegradation of hexadecane (4 mM) by *Pseudomonas aeruginosa* ATCC 9027. Protein was determined as described in Materials and Methods. (A) dR-Me; (B) dR-A. Symbols: ○ 24 h incubation, ● 48 h incubation.

REFERENCES

Amgren, M., F. Grieser, and J.K. Thomas. 1979. Dynamic and static aspects of solubilization of neutral arenes in ionic micellar solutions. J. Am. Chem. Soc. 101:279-292

Aronstein, B.N. , Y.M. Calvillo, and M. Alexander. 1991. Effect of surfactants at low concentrations on the desorption and biodegradation of sorbed aromatic compounds in soil. Environ. Sci. Technol. 25:1728-1731

Asmer, H-J, S. Lang, F. Wanger and V. Wray. 1988. Microbial production, structure elucidation and bioconversion of sophorose lipids. J. Am. Chem. Soc. 65:1460-1466

Attwood, D., and A. Florence. 1983. Surfactant systems: their chemistry, pharmacy and biology. Chapman and Hall. New York.

Banerjee, S., S. Duttagupta, and A.M. Chakrabarty. 1983. Production of emulsifying agent during growth of *Pseudomonas cepacia* with 2,4,5-trichlorophenoxyacetic acid. Arch. Microbiol. 135:110-114

Bateman, J.N., B. Speer, L. Feduik, and R. Hartline. 1986. Naphthalene association and uptake in *Pseudomonas putida*. J. Bacteriol. 166:155-161

Bassel, J.B., and R.K. Mortimer. 1985. Identification of mutations preventing *n*-hexadecane uptake among 26 *n*-alkane non-utilizing mutants of *Yarrowia* (*Saccharomycopsis*) *lipolytica*. Curr. Genetics 9:579-586

Bendinger, B., H.H.M. Rijnaarts, K.Altendorf, and A.J.B. Zehnder. 1993. Physicochemical cell surface and adhesive properties of coryneform bacteria related to the presence and chain length of mycolic acid. Appl. Environ. Microbiol. 59:3973-3977

Bhattacharjee, S.S., R.H. Haskins, and P.A.J. Gorin. 1970. Location of acyl groups on two partly acylated glycolipids from strains of *Ustilago* (smut fungi). Carbohydrate Res. 13:235-246

Breuil, C., and D.J. Kushner. 1980. Effects of lipid, fatty acid, and other detergents on bacterial utilization of hexadecane. Can J. Microbiol. 26:223-231

Bury, S.J., and C.A. Miller. 1993. Effect of micellar solubilization on biodegradation rates of hydrocarbons. Environ. Sci. Technol. 27:104-110

Cameotra, S.S., H.D. Singh, A.K. Hazarika and J.N. Baruah. 1983. Mode of uptake of insoluble solid substrates by microorganisms. II: uptake of solid n-alkanes by yeast and bacterial species. Biotechnol. Bioeng. 25:2945-2956

Churchill, S.A., R.A. Griffin, L.P. Jones, and P.F. Churchill. 1995. Biodegradation rate enhancement of hydrocarbons by an oleophilic fertilizer and a rhamnolipid biosurfactant. J. Environ. Qual. 24:19-28

Cox, D.P., and A.L. Willianms. 1980. Biological process for converting naphthalene to cis-1,2-dihydroxy-1,2-dihydronaphthalene. Appl. Environ. Microbiol. 39:320-326

Eastcott, L, W.Y. Shiu, and D. Mackay. 1988. Environmentally relevant physical chemical properties of hydrocarbons: a review of data and development of simple correlations. Oil Chem. Pollut. 4:191-216

Edwards,D.A., R.G.Luthy, and Z.Liu. 1991. Solubilization of polycyclic aromatic hydrocarbons in micellar nonionic surfactant solutions. Environ. Sci. Technol. 25:127-133

Efroymson, R.A., and M. Alexander. 1991. Biodegradation by an *Arthrobacter* species of hydrocarbons partitioned into an organic solvent. Appl. Environ. Microbiol. 57:1441-1447

Falatko, D. F., and J.T. Novak. 1992. Effects of biologically produced surfactants on the mobility and biodegradation of petroleum hydrocarbons. Water Environ. Res. 64:163-169

Fiechter, A. 1992. Biosurfactants:moving towards industrial application. Tibtech 10:208-216

Fletcher, M., and K.C. Marshall. 1982. Bubble contact angle method for evaluating substratum interfacial characteristics and its relevance to bacterial attachment. Appl. Environ. Microbiol. 44:184-192

Foght, J.M., D.L. Gutnick, and D.W.S. Westlake. 1989. Effect of emulsan on biodegradation of crude oil by pure and mixed bacterial cultures. Appl. Environ. Microbiol. 55:36-42

Gerson, D.F., and J. Akit. 1980. Cell surface energy, contact angles and phase partition II. Bacterial cells in biphasic aqueous mixtures. Biochim. Biophys. Act. 602:281-284

Gibson, D.T. 1971. The microbial oxidation of aromatic hydrocarbons. Crit. Rev. Microbiol. 1:199-223

Gibson, D.T. and V. Subramanian. 1984. Microbial degradation of aromatic hydrocarbons, P. 181-252. *In* D.T. Gibson (ed.), Microbial degradation of organic compounds. Marcel Dekker, Inc., New York.

Goldberg, S., R.J. Doyle and M. Rosenberg. 1990. Mechanism of enhancement of microbial cell hydrophobicity by cationic polymers. J. Microbiol. 172:5650-5654

Goldberg, S., Y. Konis, and M.Rosenberg. 1990. Effect of cetylpyridinium chloride on microbial adhesion to hexadecane and polystyrene. Appl. Environ. Microbiol. 56:1678-1682

Goswami, P. and H.D. Singh. 1991. Different modes of hydrocarbon uptake by two *Pseudomonas* species. Biotechnol. Bioeng. 37:1-11

Guerin, W.F., and G.E. Jones. 1988. Mineralization of phenanthrene by a *Mycobacterium* sp.. Appl. Environ. Microbiol. 54:937-944

Hill, M.J., A.M. James, and W. E. Maxted. 1963. Some physical investigations of the behavior of bacterial surfaces. X. The occurrence of lipid in the streptococcal cell wall. Biochim. Biophys. Acta. 75:414-424

Hiramoto, M., H. Ohtake, and K. Toda. 1989. A kinetic study of total degradation of 4-chlorobiphenyl by a two-step culture of *Arthrobacter* and *Pseudomonas* strains. J. Ferm. Bioeng. 68:68-70

Hirayama, T., and I. Kato. 1982. Novel methyl rhamnolipids from *Pseudomonas aeruginosa.* FEBS letters 139:81-85

Hommel, R.K. 1990. Formation and physiological role of biosurfactants produced by hydrocarbon-utilizing microorganisms. Biodegradation 1:107-119

Hommel, R. F. and C. Ratledge. 1993. Biosynthetic mechanisms of low molecular weight surfactants and their precursor molecules. In N. Kosaric (ed.) Biosurfactants. Vol. 48. Marcel Dekker, Inc. New York.

Huang, C. 1969. Studies on phosphatidycholine vesicles, formation and physical characteristics. Biochemistry 8:344-351

Ito, S., M. Kinta, and S. Inoue. 1980. Growth of yeasts on n-alkanes: inhibition by a lactonic sophorolipid produced by *Torulopsis bombicola.* Agri. Biol. Chem. 44:2221-2223

Ito, S., and S. Inoue. 1982. Sophorolipids from *Torulopsis bombicola*: possible relation

to alkane uptake. Appl. Environ. Microbiol. 43:1278-1283

Itoh, S., H. Honda, F. Tomita, and T. Suzuki. 1971. Rhamnolipids produced by *Pseudomonas aeruginosa* grown on n-paraffin. J. Antibiot. 12:855-859

Itoh, S. and T, Suzuki. 1974. Fructose lipids of *Arthrobacter*, *Corynrbacteria*, *Nocardia* and *Mycobacteria* growth on fructose. Agric. Biol. Chem. 38:1443-1449

Jain, D.K., H, Lee, and J.T.Trevors. 1992. Effect of addition of *Pseudomonas aeruginosa* UG2 inocula or biosurfactants on biodegradation of selected hydrocarbons in soil. J. Ind. Microbiol. 10:82-93

James, A.M. 1991. Charge properties of microbial cell surfaces. *In* N. Mozes, P.S. Handley, H.J. Busscher, and P.G. Rouxhet (ed.) Microbial cell surface analysis. VCH Publishers, Inc. New York

Katinger, H.W.D. 1973. Influence of interfacial area and nonutilizable hydrocarbons on growth kinetics of *Candida* sp. in hydrocarbon fermentations. Biotechnol. Bioeng. Symp. 4:485-505

Käppeli, O., and A. Fiechter. 1976. The mode of interaction between the substrate and cell surface of hydrocarbon-utilizing yeast *Candida tropicalis*. Biotechnol. Bioeng. 18:967-974

Käppeli, O., and A. Fiechter. 1981. Properties of hexadecane uptake by *Candida tropicalis*. Curr. Microbiol. 6:21-26

Käppeli, O., M. Muller, and A. Fiechter. 1978. Chemical and structural alterations at the cell surface of *Candida tropicalis* induced by hydrocarbon substrate. J. Bacteriol. 133:952-958

Klevens, H.B. 1950. Effect of electrolytes upon the solubilization of hydrocarbon and polar compounds. J.Am.Chem.Soc. 72:3780-3785

Kile, D.E., and C.T. Chiou. 1989. Water solubility enhancement of DDT and Trichlorobenzene by some surfactants below and above the critical micelle concentration. Environ. Sci. Technol. 23:832-838

Kobayashi, T., S. Ito, and K. Okamoto. 1987. Production of mannsylerythritol by *Candida* sp. KSM-1529. Agric. Biol. Chem. 51:1715-1716

Köhler, A., M. Schüttoff, D. Bryniok, and H-J Knackmuβ. 1994. Enhanced

biodegradation of phenanthrene in a biphasic culture system. Biodegradation 5:93-104

Krivan, H.C., L.D. Olson, M.F. Barile, V. Ginsburg, and D.D. Roberts. 1989. Adhesion of *Mycoplasma pneumoniae* to sulfated glycolipids and inhibition by dextran sulfate. J. Biol. Chem. 264:9283-9288

Kuieda,H., and K. Shinoda. 1979. Solution behavior of aerosol OT/water/oil system. J. Colloid Interface Sci. 70:577-583

Laha, S., and R.G. Luthy. 1991. Inhibition of phenanthrene mineralization by nonionic surfactants in soil-water systems. Environ. Sci. Technol. 25:1920-1930

Lindall, M., A. Faris, T. Wadstrom and S. Hjerten. 1981. A new test based on 'salting out' to measure relative surface hydrophobicity of bacterial cells. Biochim. Biophs. Acta. 677:471-676

Liu, D. 1980. Enhancement of PCBs biodegradation by sodium ligninsulfonate. Water Res. 14:1467-1475

Liu, Z. A.M. Jacobson, and R.G. Luthy. 1995. Biodegradation of naphthalene in aqueous nonionic surfactant systems. Appl. Environ. Microbiol. 61:145-151

Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275

Mackay, D, W.Y. Shiu, and K.C. Ma. 1992. Illustrated handbook of physical-chemical properties and environmental fate for organic chemical. Vol.2. Lewis Publishers, Chelsea, Michigan.

Magaritis, A., K. Kennedy, J.E. Zajic, and D.F. Gerson. 1979. Biosurfactant production by *Nocardia erythropolis*. Develop. Ind. Microbiol. 20:623-630

Marinucci, A.C. and R. Bartha. 1979. Apparatus for monitoring the mineralization of volatile ^{14}C-labeled compounds. Appl. Environ. Microbiol. 38:1020-1022.

Martin, A.N., J. Swarbrick, A. Cammarata. 1969. Physical pharmacy. Lea and Febiger. Philadephia, PA.

Miller, R.M., and R. Bartha. 1989. Evidence for liposome encapsulation for transport-limited microbial metabolisms of solid alkanes. Appl. Environ. Microbiol. 55:269-274

Miura, Y., M. Okazaki, S-I. Hamada, S. Murakawa, and R. Yugen. 1977. Assimilation of liquid hydrocarbon by microorganisms. I. mechanisms of hydrocarbon uptake.

Biotechnol. Bioeng. 19:701-714

Moroi, Y., H. Noma, and R. Matuura. 1983. Solubilization of N-alkylpenothiazine in aqueous anionic surfactant micelles. J.Phys. Chem. 87:872-876

Moroi, Y., K.Sato, H. Noma, and R. Matuura. 1982. Solubilization of phenothiazine in aqueous surfactant micelles. J.Phys. Chem. 86:2463-2468

Nakahara, T., L.E. Erickson, and J.R. Guiterrez. 1977. Characteristics of hydrocarbon uptake in culture with two liquid phases. Biotechnol. Bioeng. 19:9-25

Neufeld, R.J., J.E. Zajic, and D.F. Gerson. 1980. Cell surface measurements in hydrocarbon and carbohydrate fermentations. Appl. Environ. Microbiol. 39:511-517

Nikaido, H., and M. Vaara. 1985. Molecular basis of bacterial outer membrane permeability. Microbiol Rev. 49:1-32

Noda, Y., and Y. Kanemasa. 1986. Determination of hydrophobicity on bacterial surfaces by nonionic surfactants. J. Bacteriol. 167:1016-1019

Oberbremer, A., R. Müller-hurting, and H. Wanger. 1990. Effect of addition of microbial surfactants on hydrocarbon degradation in a soil population in a stirred reactor. Appl. Microbiol. Biotechnol. 32:485-489

Pringle, J.H., M. Fletcher, and D.C. Ellwood. 1983. Selection of attachment mutants during the continuous culture of *Pseudomonas flurescens* and relationship between attachment ability and surface composition. J.Gen. Microbiol. 129:2557-2559

Rambeloarisoa, E., J.F. Rontani, G, Giusti, Z. Duvnjak, and J.C. Bertrand. 1984. Degradation of crude oil by a mixed population of bacteria isolated from sea-surface foams. Marine Biol. 83:69-81

Rapp, P., H. Bock, V. Wray, and F. Wanger. 1979. Formation, isolation and characterization of trehalose dimycolates from *Rodococcus erythropolis* grown on n-alkanes. J. Gen. Microbiol. 115:491-503

Ratledge, C. 1978. Degradation of aliphatic hydrocarbons, p. 1-46. *In* R.J. Watkison (ed.) Developments in biodegradation of hydrocarbons, Vol. I. Applied Science Publishers, London.

Rittman, B.E., and N.M. Johnson. 1989. Rapid biological clean-up of soils contaminated with lubricating oil. Water Sci. Technol. 121:209-219

Robert, M., Mercadé, M.P. Bosch, J.L. Parra. M.J. Espuny, M.A. Manresa, and J. Guinea. 1989. Effect of the carbon source on biosurfactant production by *Pseudomonas aeruginosa* 44T1. Biotechnol. Lett. 12:871-874

Robichaux, T.J., and H.N. Myrick. 1972. Chemical enhancement of biodegradation of crude-oil pollutants. J. Petr. Technol. 24:16-20

Robson, R.J., and E.A. Dennis. 1977. The size, shape, and hydration of nonionic surfactant micelles. Triton X-100. J. Phys. Chem. 81:1075-1078

Rosen, M.J. 1989. Surfactants and interfacial phenomena, 2nd ed.. John Wiley and Sons. New York.

Rosenberg, E. 1986. Microbial surfactants. CRC Crit. Rev. Biotechnol. 3:109-132

Rosenberg, E., A. Perry, D.T. Gibson, and D.L. Gutnick. 1979. Emulsifier of *Arthrobacter* RAG-1: specificity of hydrocarbon substrate. Appl. Envrion. Microbiol. 37:409-413

Rosenberg, M. 1981. Bacterial adherence to polystyrene: a replica method of screening for bacterial hydrophobicity. Appl. Environ. Microbiol. 42:375-377

Rosenberg, M., E. A. Bayler, J. Dellarea, and E. Rosenberg. 1982. Role of thin fimbriae in adherence and growth of *Acinetobacter calcoaceticus* RAG-1 on hexadecane. Appl. Environ Microbiol. 44:929-937

Rosenberg, M., D. Gutnick, and E. Rosenberg. 1980. Adherence of bacteria to hydrocarbons: a simple method for measuring cell surface hydrophobicity. FEMS Microbiol. Lett. 9:29-33

Rosenberg, M., and E. Rosenberg. 1981. Role of adherence in growth of *Acinetobacter calcoaceticus* RAG-1 on hexadecane. J. Bacteriol. 148:51-57

Rosenberg, M., and S. Kjelleberg. 1986. Hydrophobic interactions: role in bacterial adhesion. Adv. Microbiol. Ecol. 9:353-393

Scott, C.C.L., and W.R. Finnerty. 1976a. A comparative analysis of the ultrastructure of hydrocarbon-oxidizing microorganisms. J. Gen. Microbiol. 94:342-350

Scott, C.C.L., and W.R. Finnerty. 1976b. Characterization of intracytoplasmic hydrocarbon inclusions for the hydrocarbon-oxidizing *Acinetobacter* sp HO1-N. J. Bacteriol. 127:481-489

Smyth, C.J., P. Jonsson, E. Olsson, O. Soderlind, J. Rosengren, S. Hjerten, and T. Watstrom. 1978. Difference in hydrophobic surface characteristics of porcine enteropathogenic *Escherichia coli* with or without K88 antigen as revealed by hydrophobic interaction. Infec. Immu. 22:462-472

Stucki, G., and M. Alexander. 1987. Role of dissolution rate and solubility in biodegradation of aromatic compounds. Appl. Environ. Microbiol. 53:292-297

Suzuki, T., H. Tanaka, and S. Itoh. 1974. Sucrose lipids of *Arthrbacter*, *Corynebacteria* and *Nocardia* grown on sucrose. Agr. Biol. Chem. 38:557-563

Swisher, R.D. 1970. Surfactant biodegradation. Surfactant Science Series No.3. Marcel Dekker, Inc. New York.

Syldatk, C., S. Long, F. Wagner, V. Wray, and L. Witte. 1984. Chemical and physical characterization of four interfacial-active rhamnolipids from *Pseudomonas* spec. DSM 2874 grown on n-alkanes. Z. Naturfrch. 40:52-67

Syldatk, C., and F. Wagner. 1987. Production of biosurfactants. *In* N. Kosaric (ed). Biosurfactants: production, properties, applications. M. Dekker, New York

Thomas, J.M., J.R. Yordy, J.A. Amador, and M. Alexander. 1986. Rates of dissolution and biodegradation of water-insoluble organic compounds. Appl. Environ. Microbiol. 52:290-296

Tiehm, A. 1994. Degradation of polycyclic aromatic hydrocarbons in the presence of synthetic surfactants. Appl. Environ. Microbiol. 60:258-263

Tulloch, A.P.Hill, and L.F.T. Spencer. 1968. Structure and reactions of lactonic and acidic sophoresides of 17-hydroxyoctadecanoic acid. Can J. Chem. 46:3337-3351

van Dyke, M.I., H. Lee and J.T.Trevors. 1991. Applications of microbial surfactants. Biotechnol. Adv. 9:241-252

Volkering, F., A.M. Breure, A. Sterkenburg, and J.G.van Andel. 1992. Microbial degradation of polycyclic aromatic hydrocarbons: effect of substrate availability on bacterial growth kinetics. Appl. Microbiol. Biotechnol. 36:548-552

Wang, D.I.C., and A. Ochoa. 1972. Measurement on the interfacial area of hydrocarbon in yeast fermentations and relationships to specific growth rates. Biotechnol. Bioeng. 14:345-360

Warren, A.J., A.F.Ellis, and J.J.R.Campbell. 1960. Endogenous respiration of

Pseudomonas aeruginosa. J. Bacteriol. 53:1893-1897

Weissenfels, W.D., M. Beyer, and J. Klein. 1990. Degradation of phenanthrene, fluorene and fluoranthene by pure bacterial cultures. Appl. Microbiol. Biotechnol. 32:479-484

Wodzinski, R.S., and D. Bertolini. 1972. Physical state in which naphthalene and bibenzyl are utilized by bacteria. Appl. Microbiol. 23:1077-1081

Wodzinski, R.S., and J. E. Coyle. 1974. Physical state of phenanthrene for utilization by bacteria. Appl. Environ. Microbiol. 27:1077-1081

Wodzinski, R.S., and M.J. Johnson. 1968. Yields of bacterial cells from hydrocarbons [sic]. Appl. Microbiol. 16:1886-1891

Yamaguchi, M., M. Sato, and K. Yamada. 1976. Microbial production of sugar lipids. Chem. Ind. 17:741-742

Zajic, J.E. and C.J. Panchal. 1976. Bioemusifiers. CRC Crit. Rev. Microbiol. 5:39-66

Zajic, J.E. and W. Seffens. 1984. Biosurfactants. CRC Crit. Rev. Biotechnol. 1:87-107

Zhang, Y., and R.M. Miller. 1992. Enhanced octadecane dispersion and biodegradation by a *Pseudomonas* rhamnolipid surfactant (biosurfactant). Appl. Environ. Microbiol. 58:3276-3282

Zhang, Y., and R. M. Miller. 1994. Effect of a *Pseudomonas* rhamnolipid biosurfactant on cell hydrophobicity and biodegradtion of octadecane. Appl. Environ. Microbiol. 60:2110-2106

Zhang, Y., and R. M. Miller. 1995. Effect of rhamnolipid (biosurfactant) structure on solubilization and biodegradation of n-alkanes. Appl. Environ. Microbiol. 61:2247-2251

Zilber, I.K., E. Rosenberg, and D. Gutnick. 1980. Incorporation of ^{32}P and growth of pseudomonad UP-2 on n-tetracosane. Appl. Environ. Microbiol. 40:1086-1093

Zuckerberg, A., A. Diver, Z.,Peeri, D.L. Gutnick, and E. Rosenberg. 1979. Emulsifier of *Arthrobacter* RAG-1: chemical and physical properties. Appl. Environ. Microbiol. 37:414-420