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Effect of Rhamnolipid (Biosurfactant) Structure on Solubilization and Biodegradation of *n*-Alkanes

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A study to quantify the effect of rhamnolipid biosurfactant structure on the degradation of alkanes by a variety of *Pseudomonas* isolates was conducted. Two dirhamnolipids were studied, a methyl ester form (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 dyne/cm by the dR-Me but was only decreased to 5 dyne/cm by the dR-A. Solubilization and biodegradation of two alkanes in different physical states, liquid and solid, were determined at dirhamnolipid concentrations ranging from 0.01 to 0.1 mM (7 to 70 mg/liter). The dR-Me markedly enhanced hexadecane (liquid) and octadecane (solid) degradation by seven different *Pseudomonas* strains. For an eighth strain tested, which exhibited extremely high cell surface hydrophobicity, hexadecane degradation was enhanced but octadecane degradation was inhibited. The dR-A also enhanced hexadecane degradation by all degraders but did so more modestly than the dR-Me. For octadecane, the dR-A only enhanced degradation by strains with low cell surface hydrophobicity.

One promising approach to increasing the biodegradation rates of organic compounds with limited water solubility is the addition of biosurfactants (2, 7, 12). In previous work, we have shown that biosurfactants affect the rate of hydrocarbon biodegradation in two ways: by increasing solubilization and dispersion of the hydrocarbon and by changing the affinity between microbial cells and hydrocarbons by inducing increases in cell surface hydrophobicity (18, 19). These studies were performed with a purified monorhamnolipid biosurfactant produced by *Pseudomonas aeruginosa* ATCC 9027. However, most microorganisms produce biosurfactant mixtures that are structurally similar but which may have quite different physicochemical 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 that vary in composition (3, 6). Similarly, *Torulopsis* spp. produce sophorolipids in acidic and lactonic forms (16), and *Arthrobacter paraffineus* can produce either trehalose lipids or sucrose lipids depending on the carbon source used in the medium (15).

Since biosurfactant structure is a 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 the lactonic form inhibited hexadecane biodegradation while the acid form stimulated hexadecane biodegradation (4, 5). This example illustrates the importance of biosurfactant structure in determining hydrocarbon degradation rates. But it is not yet clear how structure affects degradation rates. It has been established that surfactant solubilization and dispersion of organic compounds, which are related to interfacial tension, are dependent on surfactant structure (8). However, increased dispersion does not always lead to increased biodegradation (12). Therefore, it must be concluded that it is the three-way interaction among the biosurfactant, substrate, and cell that is crucial to achieving 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 effects 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 dR-A and a nonionic dR-Me, both of which are shown in Fig. 1. The 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. *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 *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 (1a). The isolates were stored at 4°C on PTYG (peptone, yeast extract, glucose) medium and transferred monthly. None of these strains produced biosurfactants during growth in mineral salts medium (18) containing *n*-alkanes. Also, none of these strains utilized dirhamnolipid as a sole source of carbon.

dR-A and dR-Me. Crystalline dR-A was a gift from Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan). The dR-Me was synthesized from dR-A by the diazomethane method (16). Diazomethane was prepared from Diazald by a procedure described elsewhere (1). The dR-A (2 g) was dissolved in methanol (50 ml), and a solution of diazomethane in ethyl ether 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, Wis.) chromatography column (14 by 2.5 cm). The dR-Me was analyzed by thin-layer chromatography with a chloroform-methanol-water (65:25:1) solvent and was identified by ^{1}H nuclear magnetic resonance ($CDCl_3$) (model AM 250, Bruker Co., Karlsrouhe, Germany). The unique chemical shift (δ) at 3.68 in the ^{1}H nuclear magnetic resonance spectra indicated the methyl group ($COOCH_3$) of the dR-Me.

The surface tensions of dR-Me and dR-A solutions in 0.1 M phosphate buffer (pH 7.0) were determined with a Fisher (Pittsburgh, Pa.) surface tensiometer (model 21) that employs the du Nouy ring method. All reported interfacial

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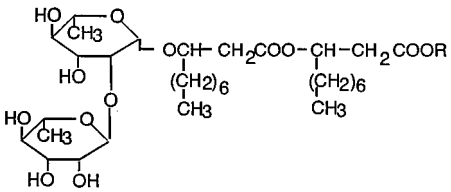

FIG. 1. Structure of a *Pseudomonas* dirhamnolipid. For dR-A, R = H; for dR-Me, R = CH_3.

tension values were measured between hexadecane and 0.1 M phosphate buffer solution (pH 7.0). The critical micelle concentration (cmc) of dR-Me and dR-A was determined from a semilogarithmic plot of surface tension against surfactant concentration (10).

Dispersion tests. The dispersion of hexadecane and octadecane in dirhamnolipid solution was determined with [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 was added to test tubes (16 by 100 mm). After the evaporation of the solvent, 2 ml of rhamnolipid solution in 0.1 M phosphate buffer (pH 7.0) was added. The final concentration of the 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 s to melt the coated octadecane and then were 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 (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). 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, Millville, N.J.) 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 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 (17) 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 (11) to collect $^{14}CO_2$ and ^{14}C-labeled 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. (9).

RESULTS

Physical properties of dR-A and dR-Me. 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 the 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 showed only one anthrone-positive spot with an R_f value of 0.46 by the 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 thin-layer chromatography plate with R_f values of 0.75 and 0.81 by 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.

TABLE 1. Physicochemical properties of dirhamnolipids and *n*-alkanes used in this study

Dirhamnolipid or *n*-alkane	Aqueous solubility (mM)	Density (g/liter)[a]	Surface tension (dyne/cm)	Interfacial tension (dyne/cm)[b]	cmc (mM)
C_{16}	2.8×10^{-5}[a]	773			
C_{18}	2.3×10^{-5}[a]	777			
dR-A	>15		36	5	0.1
dR-Me	0.04		31	<0.1	0.04

[a] From Singer and Finnerty (14).
[b] Interfacial tension between hexadecane and 0.1 M buffer (pH 7.0) was measured.

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 the linear portion of each plot was used to calculate a molar solubilization ratio (moles of organic compound solubilized/mole of surfactant). The molar solubilization ratio for the dR-Me was 5.2 and that 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 molar solubilization ratios, similar for both the dR-Me and dR-A, were 0.074 and 0.15, respectively. 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 increases in biodegradation determined by measurement of both alkane mineralization and protein were similar. An examination of the slopes of each curve show that the dR-Me was most effective in stimulating the rate of biodegradation for both hexadecane (Fig. 3) and octadecane (Fig. 4). For hexadecane, the rate of mineralization was stimulated 8-fold by the dR-Me

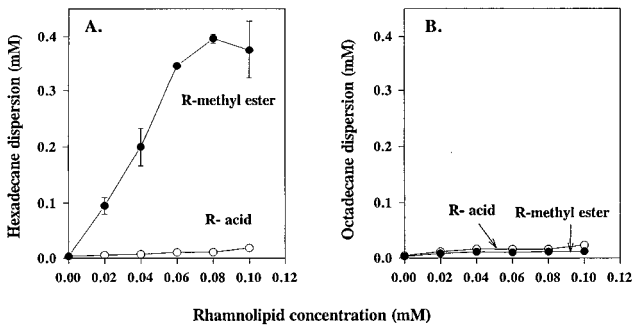

FIG. 2. Effect of dirhamnolipid on the apparent aqueous solubilities of hexadecane (A) and octadecane (B). Solutions containing dirhamnolipid and [^{14}C]hexadecane or [^{14}C]octadecane (4 mM) were incubated with gyratory shaking at 200 rpm and 23°C for 24 h. Solubility was measured as described in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

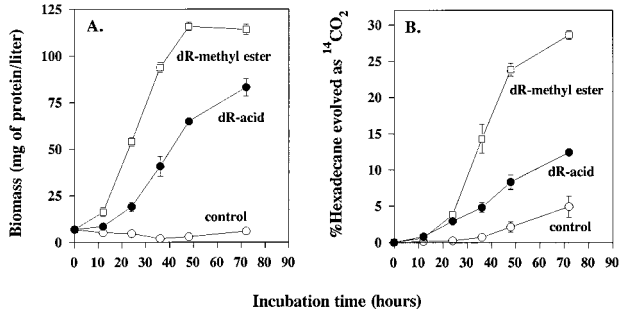

FIG. 3. Effect of dirhamnolipid on the biodegradation of hexadecane (4 mM) by *P. aeruginosa* ATCC 9027. (A) The protein increase was used to measure biodegradation (biomass is measured in milligrams of protein per liter). (B) Alkane mineralization was used to measure biodegradation. Error bars indicate standard deviations. Symbols: ○, no dirhamnolipid; ●, 0.05 mM dR-A; □, 0.05 mM dR-Me.

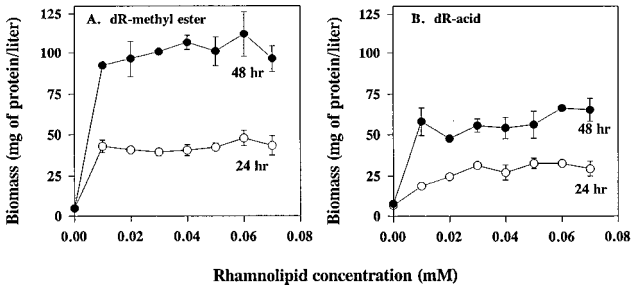

FIG. 5. Effect of dirhamnolipid concentration on the biodegradation of hexadecane (4 mM) by *P. aeruginosa* ATCC 9027. Protein was determined as described in Materials and Methods (biomass is measured in milligrams of protein per liter). Error bars indicate standard deviations. (A) dR-Me. (B) dR-A. Symbols: ○, 24-h incubation, ●, 48-h incubation.

and 1.6-fold by the dR-A by comparison with the control. For octadecane, the rate of mineralization was stimulated 1.6-fold by the dR-Me by comparison with the control, but the dR-A had a slightly slower maximum rate of biodegradation than that of the control after an initial lag period for the control. Interestingly, in the presence of dirhamnolipids, hexadecane was always mineralized more quickly than octadecane (Fig. 3B and 4B), while in the absence of dirhamnolipids, octadecane (9.7%) was mineralized 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 various concentrations of the dirhamnolipids. In this set of tests, cell growth was measured by protein increases after 24-h and 48-h incubations. 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 that of 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 dirhamnolipids on biodegradation of hexadecane and octadecane by different *Pseudomonas* isolates. The effect of 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 on the basis of their growth rates on alkanes in the absence of dirhamnolipids: ATCC 2785, ATCC 15442, and BO 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 the degradation of hexadecane and octadecane by all the strains, with the exception of ATCC 15442, the growth of which was inhibited on octadecane. This strain has been previously reported to have very high cell surface hydrophobicity (19). Total growth rates (in terms of protein increase) on hexadecane in the presence of the dR-Me were 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 time frame 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, the growth of the slow isolates was increased 6.2- to 38.7-fold, while the 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 the 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, and ATCC 15442 was inhibited).

The behavior of the dR-A was more complex than that of the dR-Me. In a manner similar to that of the dR-Me, dR-A enhanced the degradation of hexadecane and octadecane by slow degraders, 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 that by the control for all three strains.

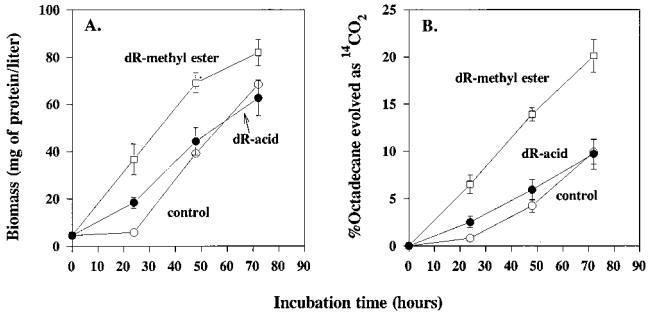

FIG. 4. Effect of dirhamnolipid on the biodegradation of octadecane (4 mM) by *P. aeruginosa* ATCC 9027. (A) The protein increase was used to measure biodegradation (biomass is measured in milligrams of protein per liter). (B) Alkane mineralization was used to measure biodegradation. Error bars indicate standard deviations. Symbols: ○, no dirhamnolipid; ●, 0.05 mM dR-A; □, 0.05 mM dR-Me.

DISCUSSION

We have previously reported that a monorhamnolipid acid biosurfactant enhanced dispersion and biodegradation of octadecane (19). 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 twofold. First, it may be feasible to stimulate in situ production of surfactants in this concentration range. In situ production has already been suggested by the results of a study by Oberbremer and Müller-Hurtig (13)

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

Bacterial strain[a]	Growth in 48 h[b]					
	Hexadecane[c]			Octadecane[c]		
	No dirhamnolipid	dR-A[d]	dR-Me[d]	No dirhamnolipid	d-RA[d]	dR-Me[d]
Slow degraders						
ATCC 9027	3 ± 1	65 ± 1 (21.7)	116 ± 2 (38.7)	39 ± 2	44 ± 6 (1.1)	69 ± 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 (0.5)[e]	83 ± 3 (1.2)
ATCC 15442	37 ± 4	46 ± 4 (1.2)	118 ± 15 (3.2)	59 ± 5	40 ± 6 (0.7)[e]	12 ± 1 (0.2)[e]
BO 310	41 ± 7	42 ± 8 (1.0)	126 ± 4 (3.1)	65 ± 4	51 ± 4 (0.8)[e]	79 ± 2 (1.2)

[a] Strains with the prefix BO were supplied by David Balkwill from the U.S. Department of Energy Subsurface Microbiological Culture Collection. A total of 11 strains were tested. Seven of these strains did not degrade either alkane and were not affected by rhamnolipid addition.
[b] Growth is expressed in milligrams of protein per liter (means ± standard deviations). The numbers in parentheses are the increases in growth due to dirhamnolipid and were calculated from growth in presence of dirhamnolipid/growth in absence of dirhamnolipid.
[c] The hexadecane and octadecane concentrations were 4 mM.
[d] The dR-Me and dR-A concentrations were 0.05 mM.
[e] Biodegradation was inhibited by dirhamnolipid addition.

that showed that a reduction in surface tension of the fluid phase in a stirred soil bioreactor was correlated with 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 a low concentration are reduced.

The results of this study demonstrate that surfactant effects on hydrocarbon biodegradation depend to some degree on surfactant structure, the physical state of the alkane, the amount of alkane dispersion and emulsification, and the degrading isolate. 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. This difference can be attributed to the carboxylic acid group (pK_a, 5.6), which confers a negative charge on the dR-A at a neutral pH. This charge caused an enhanced interaction of the rhamnolipid with water and a weaker interaction between the rhamnolipid and hexadecane by comparison with those of the dR-Me. Thus, the dR-A was less effective at reducing surface tension between hexadecane and water. A second result of the 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 that the true cmc was not reached.

Physical state. The physical state of the *n*-alkane serving as the substrate 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 that 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 the dispersion of the liquid alkane than that of 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 dyne/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 sizes 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 a 10^5-fold increase in hexadecane dispersion to 0.4 mM, or ~90 mg/liter (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 effect with the dR-Me, the dR-A caused an increase of only 10^3-fold in hexadecane dispersion, to 0.01 mM (~2 mg/liter). The resulting effect on alkane degradation was an enhancement, but this enhancement was two- to threefold 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 distribution in the particle sizes of the octadecane 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 than those formed in the presence of the dR-A. The resulting effects on biodegradation of octadecane were several. (i) By comparison with those for hexadecane, the increases in octadecane biodegradation were much smaller. (ii) In contrast to that with hexadecane, only the biodegradation of slow degraders was enhanced. (iii) For the slow degraders, there was a 1.5- to 2-fold greater enhancement of octadecane biodegradation with the dR-Me than with the dR-A.

Degrading isolate. This study investigated biodegradation by two types of bacteria: slow degraders that exhibit relatively low cell surface hydrophobicity and a relatively low inherent rate of

alkane biodegradation and fast degraders that have a higher cell surface hydrophobicity and a higher inherent rate of alkane biodegradation. After a consideration of all alkane-surfactant combinations examined, it was apparent that 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 for which the presence of the surfactant inhibited degradation of the alkane relative to that with 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 ongoing work in our laboratory.

In summary, dirhamnolipids have exciting potential for the 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 the use of 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 that 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 esters. The current method used by most researchers for the isolation of rhamnolipids (acid precipitation) selects for the dR-A 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.

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