Enhanced Octadecane Dispersion and Biodegradation by a 
Pseudomonas Rhamnolipid Surfactant (Biosurfactant)

YIMIN ZHANG AND RAINA M. MILLER*

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

Received 20 March 1992/Accepted 20 July 1992

A microbial surfactant (biosurfactant) was investigated for its potential to enhance bioavailability and, hence, the biodegradation of octadecane. The rhamnolipid biosurfactant used in this study was extracted from culture supernatants after growth of Pseudomonas aeruginosa ATCC 9027 in phosphate-limited proteose peptone-glucose-ammonium salts medium. Dispersion of octadecane in aqueous solutions was dramatically enhanced by 300 mg of the rhamnolipid biosurfactant per liter, increasing by a factor of more than 4 orders of magnitude, from 0.009 to >250 mg/liter. The relative enhancement of octadecane dispersion was much greater at low rhamnolipid concentrations than at high concentrations. Rhamnolipid-enhanced octadecane dispersion was found to be dependent on pH and shaking speed. Biodegradation experiments done with an initial octadecane concentration of 1,500 mg/liter showed that 20% of the octadecane was mineralized in 84 h in the presence of 300 mg of rhamnolipid per liter, compared with only 5% octadecane mineralization when no surfactant was present. These results indicate that rhamnolipids may have potential for facilitating the bioremediation of sites contaminated with hydrocarbons having limited water solubility.

The bioavailability of an organic compound is a function of its solubility, mobility, and sorption or desorption in the environment. Bioavailability may be the critical limiting factor controlling biodegradation rates for many organic compounds having low water solubility. These compounds resist biodegradation at least in part because of limited transport into cells, as demonstrated by Miller and Bartha (16) in a study of alkane biodegradation. This work showed that transport limitations could be overcome by encapsulation of alkanes (C_{14} and C_{36} compounds) into small unilamellar vesicles (liposomes). Encapsulation increased the aqueous dispersion of the alkanes by intercalation of the alkanes into the vesicle phospholipid bilayers, which were approximately 20 to 50 nm in diameter. Furthermore, encapsulation facilitated the delivery of alkanes to bacterial membrane-bound enzymes, most likely via fusion of the vesicles with cell membranes. While the liposome-encapsulated alkanes were efficiently degraded by a hydrocarbon-utilizing Pseudomonas sp., the high cost of encapsulation precludes its use in bioremediation. However, this work suggested that further investigation into methods to increase the aqueous dispersion of recalcitrant compounds is necessary.

Relatively low concentrations of surfactants can also enhance the aqueous dispersion of slightly water-soluble compounds (11). Surfactants can be synthetic (detergents) but are also naturally produced by plants, animals, and many different microorganisms (24). A property that all surfactants have in common is that they reduce the surface tension of a liquid medium. For example, distilled water has a surface tension of 73 dyn/cm. An effective microbially produced surfactant (biosurfactant) can lower this value to <30 dyn/cm (12). The amount of surfactant needed to achieve the lowest possible surface tension is defined as the critical micelle concentration (CMC). Alternatively, the CMC can be defined as the surfactant concentration at which the concentration of a free monomer ceases to increase and any further monomer added will form micellar structures (1). In the process of micelle formation, the surfactant molecules that aggregate to form micelles have the ability to surround slightly soluble molecules, a process that effectively disperses or emulsifies them into the aqueous phase (21). The CMCs of biosurfactants typically range from 1 to 200 mg/liter (12).

We postulated that biosurfactants would enhance the aqueous dispersion of organic compounds having limited water solubility in two ways: (i) biosurfactants would decrease the surface and interfacial tensions in the culture medium, thereby increasing the aqueous dispersion of organic compounds at the molecular level, and (ii) physical biosurfactant interactions with slightly water-soluble organic compounds would increase their aqueous dispersion, the increase below the CMC being due to hydrophobic interactions between biosurfactant monomers and slightly soluble organic compounds (22) and the increase above the CMC being due to biosurfactant encapsulation of slightly soluble organic compounds into micellar or bilayer aggregates. In this report, we quantitate the ability of a rhamnolipid to increase the aqueous dispersion of a slightly soluble model organic compound and to enhance the rate of biodegradation of that model organic compound. The use of biosurfactants is attractive because they are natural products, they are biodegradable, and they have potential for use in in situ remediation.

The biosurfactant used in this study was a rhamnolipid produced by Pseudomonas aeruginosa ATCC 9027. We chose this biosurfactant because (i) it is a glycolipid, which is the most commonly isolated type of biosurfactant (4), and (ii) members of the genus Pseudomonas are common soil microorganisms. A previous characterization of rhamnolipids produced by various Pseudomonas spp. (5–7, 9, 10, 19) indicated that a mixture of rhamnolipids containing either one or two rhamnose residues and two lipid chains is produced (Fig. 1) (13, 18). A slightly water-soluble alkane, n-octadecane (C_{18}), was chosen as a model compound for several reasons. The water solubility of octadecane is very low, 0.006 mg/liter (21), and a study of octadecane would allow a comparison with results obtained from previous
octadecane liposome encapsulation studies (16). In this study, the effects of rhamnolipid concentration, pH, and shaking speed on the aqueous dispersion of octadecane were determined. Octadecane mineralization rates with *P. aeruginosa* ATCC 9027 in the presence of various concentrations of rhamnolipid were determined by measuring 14CO2 evolution from the radioactive substrate. Experiments were performed at 23°C, below the melting point of octadecane, to maintain the compound in a solid physical state.

**MATERIALS AND METHODS**

**Biosurfactant production.** *P. aeruginosa* ATCC 9027 was obtained from the American Type Culture Collection (Rockville, Md.). The culture was maintained on *Pseudomonas* agar P (Difco, Detroit, Mich.) slants and transferred monthly. The organism was grown as described by Mulligan et al. (17) to induce biosurfactant production. In brief, *P. aeruginosa* was inoculated into 25 ml of Kay’s minimal medium, composed of NH4H2PO4 (0.3%), K2HPO4 (0.2%), glucose (0.2%), FeSO4 (0.5 mg of Fe per liter), and MgSO4 · SO4 (0.1%), in a 125-ml flask (23). The preculture was incubated with gyratory shaking at 250 rpm for 24 h at 37°C, and then 2 ml was used to inoculate 200 ml of phosphate-limited proteose peptone-glucose-ammonium salts (PPGAS) medium in a 1,000-ml flask. PPGAS medium is composed of NH4Cl (0.02 M), KCl (0.02 M), Tris–HCl (0.12 M), glucose (0.5%), proteose peptone (1%), and MgSO4 · SO4 (0.0016 M), adjusted to pH 7.2 (3). This flask was incubated at 37°C with gyratory shaking at 250 rpm, with periodic aseptic removal of samples to monitor surface tension. The initial surface tension of PPGAS medium was 66 dyn/cm. The surface tension of the medium started to decrease at 6 h, coinciding with the production of a blue pigment. By 48 h, the surface tension of the medium reached 29 dyn/cm and did not decline further. Rhamnolipid was harvested at 60 h.

**Rhamnolipid extraction and measurement.** Rhamnolipid was recovered from the culture supernatant after the removal of cells by centrifugation at 6,800 × g for 20 min. Rhamnolipid was then precipitated by acidification of the supernatant to pH 2.0 and centrifugation at 12,100 × g for 20 min. The precipitate was dissolved in 0.05 M bicarbonate (8.6), reacidified, and recentrifuged at 12,100 × g for 20 min. Following centrifugation, the precipitate was extracted with chloroform–ethanol (2:1) three times. The organic solvent was evaporated on a rotary evaporator, and the yellowish oily residue was dissolved in 0.05 M bicarbonate (pH 8.6). The biosurfactant concentration was then estimated independently by surface tension measurement and by rhamnose measurement.

Surface tension was measured with a SensaDyne (Milwaukee, Wis.) 6000 surface tensiometer, which uses the maximum bubble pressure technique. This technique avoids errors that can be caused by foam or debris on the solution surface by measuring surface tension within the body of the test fluid (20). The sensitivity of the surface tension measurements was <1 mg/liter for rhamnolipid concentrations between 0 and 50 mg/liter, and the measurements were found to be reproducible to ±0.1 dyn/cm. Rhamnolipid was also quantified by measurement of L-rhamnose by the 6-deoxyhexose method (2). In brief, biosurfactant solutions were treated with 70% sulfuric acid and boiled for 10 min. After the samples had cooled, thioglycolic acid was added to a final concentration of 0.059%. Samples were incubated in the dark for 3 h and then measured spectrophotometrically at 420 nm. Standard curves were prepared with L-rhamnose obtained from Sigma (St. Louis, Mo.).

**Effect of pH on surface tension.** Rhamnolipid was diluted to 50 mg/liter in 0.1 M pH 7.0 phosphate buffer. The solution pH was adjusted by the addition of HCl or NaOH, and the surface tension was measured at 25°C.

**Aqueous dispersion tests.** Octadecane (99% pure) was purchased from Aldrich Chemical Co. (Milwaukee, Wis.). [14C]Octadecane, labeled at the C-1 position (specific activity, 3.6 mCi/mmol; 98% pure), was obtained from Sigma. Appropriate amounts of a mixture of octadecane and [14C]octadecane (0.78 g/liter, with a specific activity of 15 μCi/mmol for rhamnolipid dispersion tests; 1 mg/liter, with a specific activity of 1.1 mCi/mmol for determination of the solubility of octadecane in water) in chloroform were added to test tubes (16 by 100 mm). The chloroform was evaporated to allow the octadecane to coat the bottom of the test tubes. After evaporation of the solvent, 1 ml of rhamnolipid solution was added. The test tubes were incubated at 37°C in a water bath for 30 s, 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 at an appropriate shaking speed. After 24 h, 0.1 ml was carefully removed from the bottom of the test tubes to avoid floating of any particulate octadecane on the top of the liquid surface and 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).

To confirm that this method did not exaggerate dispersion results and also to characterize the size of the dispersed octadecane particles, we filtered some octadecane-rhamnolipid solutions through 30,000- or 300,000-molecular-weight (MW)-cutoff membranes (Millipore Corp., Bedford, Mass.) and determined the radioactivity in the filtrates.

Rhamnolipid solutions were prepared in 0.1 M pH 7.0 phosphate buffer. Tests were done in triplicate, with the exception of the test for octadecane solubility in water, for which five replicates were done. Each experiment was repeated twice.

**Biodegradation tests.** The biodegradation of octadecane and rhamnolipid was determined by measurement of the increase in the protein concentration as an indication of cell growth and by rhamnolipid determination (based on rhamnose). Octadecane mineralization was measured by the quantification of 14CO2 and 14C-volatile compounds. For protein and rhamnose determinations, octadecane was dissolved in chloroform, and the mixture was used to coat the bottom of 250-ml flasks. The chloroform was evaporated, and 50 ml of mineral salts medium (16) containing rhamnolipid was added to each flask. The flasks were then inoculated with a 5% inoculum of *P. aeruginosa* ATCC 9027.
grown in Kay's minimal medium at 37°C for 24 h. The flasks were incubated with gyratory shaking (200 rpm) at 23°C. Periodically, samples were taken from each flask for rhamnolipid and protein determinations. For rhamnolipid analyses, 1-ml samples were filtered through a 0.22-μm-pore-size filter, and L-rhamnose was quantified. For protein analyses, 1-ml samples were heated for 10 min with 0.1 ml of 1 N NaOH (to lyse cells), and the protein content was determined by the method of Lowry et al. (14).

For mineralization experiments, a mixture of octadecane and [14C]octadecane (1.55 g/liter; specific activity, 0.37 μCi/mmole) was used to coat the bottom of modified 125-ml micro-Fernbach flasks (Wheaton, Milville, N.J.), designed for the collection of 14CO2 and 14C-volatile compounds (15). The solvent was evaporated, and 20 ml of mineral salts medium (16) containing rhamnolipid was added to each flask. 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 14CO2 and 14C-volatile organic compounds (15).

RESULTS

Effect of rhamnolipid on surface tension. Figure 2 shows the dependence of surface tension on the biosurfactant concentration. Surface tension decreased rapidly from 72 to 30 dyn/cm with small increases in the rhamnolipid concentration up to 50 mg of rhamnolipid per liter. Further increases in the rhamnolipid concentration only slowly reduced the surface tension from 30 to 29 dyn/cm. Once the surface tension reached 29 dyn/cm, the further addition of rhamnolipid had no effect. The CMC was determined from a semilog plot of surface tension versus rhamnolipid concentration to be 40 mg/liter (data not shown). The data in Fig. 2 were fitted ($y = ax^c$, where $a = 29.5$ dyn/cm, $b = 0.117$ dyn/cm, and $c = 42.5$ mg liter$^{-1}$). However, two separate linear functions described a better fit ($r^2 = 0.999$) for the data at rhamnolipid concentrations of <5 mg/liter and >50 mg/liter (see solid lines and inset). These functions are described by the equation $y = a + bx$, where $a = 72.1$ dyn/cm and $b = -4.26$ dyn/cm/mg/liter for 0 to 5 mg of rhamnolipid per liter and $a = 29.6$ dyn/cm and $b = -0.00385$ dyn/cm/mg/liter for 50 to 200 mg of rhamnolipid per liter. The close fit obtained for the data with these two different linear functions implies that there are different behaviors for the rhamnolipid in solution. Low rhamnolipid concentrations (<5 mg/liter) have a strong effect on surface tension, while high concentrations (>50 mg/liter) have a negligible effect.

Dispersion of octadecane in the biosurfactant solution. As shown in Figure 3, rhamnolipid dramatically increased the aqueous dispersion of octadecane. For instance, in the presence of 50 mg of rhamnolipid per liter, the aqueous dispersion of octadecane was 125 mg/liter, an increase of more than 4 orders of magnitude over the solubility of octadecane in pure water (0.009 ± 0.005 mg/liter). The data in Fig. 3 suggest that there are two mechanisms of dispersion, one at low rhamnolipid concentrations (region 1) and another at higher concentrations (region 2). At low rhamnolipid concentrations (1 to 50 mg/liter), there was a sharp increase in octadecane dispersion. After this sharp increase, there was a linear dependence of octadecane dispersion on biosurfactant concentration from 50 to 500 mg/liter. Rhamnolipid also caused gross visible changes in the physical state of the excess insoluble octadecane. Instead of several large flakes of octadecane floating on the liquid surface, in the presence of rhamnolipid, the insoluble octadecane was dispersed as fine particles on the surface of the solution.

Estimation of sizes of octadecane-rhamnolipid aggregates. For estimation of the sizes of the octadecane-rhamnolipid aggregates, a solution containing 200 mg of rhamnolipid per...
was gates between of dispersed passed through rhamnolipid of octadecane. The concentrations of octadecane that were measured as described in Materials and Methods. Symbols: •, experimental data; ---, best-fit linear functions at ≤5 and ≥50 mg of rhamnolipid per liter.

![Graph: Rhamnolipid Concentration (mg/l)](image)

**FIG. 3.** Effect of rhamnolipid concentration on the aqueous dispersion of octadecane. Solutions containing rhamnolipid and [14C]octadecane (0.78 g/liter) were incubated with gyroratory shaking at 100 rpm and 23°C for 24 h. The aqueous dispersion of octadecane was measured as described in Materials and Methods. Symbols: •, experimental data; ---, best-fit linear functions at ≤5 and ≥50 mg of rhamnolipid per liter.

![Graph: Effect of pH on Surface Tension](image)

**FIG. 4.** Effect of pH on the surface tension of rhamnolipid solutions. The pH of rhamnolipid solutions (50 mg/liter) in 0.1 M phosphate buffer was varied, and the surface tension was measured at 25°C.

>40 dyn/cm. Visually, the rhamnolipid solutions were clear above pH 7.0 but became turbid below this pH. Precipitation of rhamnolipid was not apparent until the pH was decreased below 5.0.

As might be expected given the effect of pH on the surface activity of rhamnolipids, octadecane dispersion in the presence of rhamnolipids was also influenced by pH (Fig. 5). Maximum enhancement of aqueous octadecane dispersion occurred at approximately pH 7.0, with dispersion decreasing with an increase in pH. Octadecane dispersion also decreased from pH 7.0 to 6.0 but unexpectedly increased again from pH 6.0 to 5.5. Below pH 5.5, experimental error in the measurement of octadecane dispersion became very high because of the precipitation of rhamnolipid.

**Rhamnolipid-enhanced mineralization of octadecane.** As shown in Fig. 6, *P. aeruginosa* ATCC 9027 did not degrade rhamnolipid either alone or in the presence of octadecane, as measured on the basis of protein (Fig. 6A) or rhamnolipid (Fig. 6B) concentrations. The culture medium used in these experiments was adjusted to the appropriate pH and incubated with gyroratory shaking at 100 rpm and 23°C for 24 h. The aqueous dispersion of octadecane was measured as described in Materials and Methods.

![Graph: Effect of pH on Octadecane Dispersion](image)

**FIG. 5.** Effect of pH on octadecane dispersion in rhamnolipid solutions. Solutions containing rhamnolipid (200 mg/liter) and [14C]octadecane (0.78 g/liter) were adjusted to the appropriate pH and incubated with gyroratory shaking at 100 rpm and 23°C for 24 h. The aqueous dispersion of octadecane was measured as described in Materials and Methods.
experiments was a mineral salts medium amended with 1.55 g of octadecane per liter. Figure 7 shows the mineralization of octadecane by *P. aeruginosa* ATCC 9027 in the presence of various concentrations of rhamnolipid. *P. aeruginosa* mineralized 5.1% of the substrate in 84 h with no rhamnolipid present. Rhamnolipid increased octadecane mineralization to 10.2% (100 mg/liter), 12.8% (125 mg/liter), and 19.9% (300 mg/liter). Surface tension measurements of the culture supernatants showed that *P. aeruginosa* ATCC 9027 did not produce rhamnolipid during growth on octadecane in the mineral salts medium.

**DISCUSSION**

Rhamnolipid-enhanced dispersion of octadecane. The results showed that a rhamnolipid biosurfactant enhanced the aqueous dispersion of octadecane by more than 4 orders of magnitude, from 0.009 to 320 mg/liter. As shown in Fig. 3, the dramatic increase in octadecane dispersion seemed to be due to two different mechanisms, one at low (region 1) and one at high (region 2) rhamnolipid concentrations. In region 1, the octadecane/rhamnose ratio was 3.7:1. In this region, octadecane solubility may be due to the rapid decrease in surface tension and/or may be due to hydrophobic interactions between octadecane and the hydrophobic tails of rhamnolipid monomers (22). Taken together, the results in Fig. 2 and 3 suggest that a lowering of surface tension may be the dominant mechanism by which octadecane dispersion is enhanced at low rhamnolipid concentrations. Examination of these figures shows that the effect of low rhamnolipid concentrations on octadecane dispersion (Fig. 3) mirrors the effect of low rhamnolipid concentrations on surface tension (Fig. 2). In both cases, low rhamnolipid concentrations have a strong effect on surface tension and octadecane dispersion and increasing rhamnolipid concentrations have a diminishing effect on both surface tension (Fig. 2) and the relative enhancement of apparent octadecane dispersion (Fig. 3).

At rhamnolipid concentrations higher than the CMC (region 2, Fig. 3), the octadecane/rhamnose ratio decreased to 0.3:1, resulting in a slower rate of octadecane dispersion with increasing rhamnolipid concentrations. At these higher rhamnolipid concentrations, increasing octadecane dispersion is most likely due to physical association with rhamnolipid aggregates. The physical interaction between octadecane and rhamnolipids seems to be analogous to the previously described intercalation of octadecane into phospholipid bilayers during liposome formation (16). For rhamnolipids, the case is more complex, because a number of
different rhamnolipid structures that are dependent on pH can form (8). The type of rhamnolipid structure formed is especially sensitive to pH in the range of 6.0 to 7.0, most likely because of the rhamnosyl moiety, which has a pKₐ of 5.6 (8). At a low pH (<6.0), the rhamnosyl moiety is at least 50% uncharged and rhamnolipids form liposome-like vesicles. Between pH 6.0 and 6.6, rhamnolipids form either lamella-like structures or lipid aggregates, and above pH 6.8, when the rhamnosyl moiety is negatively charged, micelles form.

The effect of pH on octadecane dispersion seems to be correlated with the type of rhamnolipid aggregate that is present in solution. The dispersion of octadecane was highest at pH 7.0 (Fig. 5), corresponding to rhamnolipids being in a micellar formation. We speculate that in this case, the octadecane was intercalated into the rhamnolipid micelles, which may then have fused with the outer membrane of the cell in a manner similar to that described for the liposome delivery of octadecane (16). Octadecane dispersion decreased dramatically from pH 7.0 to 6.0 (Fig. 5), corresponding to particulate and lamellar rhamnolipid structures. From pH 6.0 to 5.5, octadecane dispersion increased again, corresponding to rhamnolipids forming liposome-like vesicle structures.

In comparison with liposome encapsulation of octadecane by phospholipids, which increased the aqueous dispersion of octadecane by more than 6 orders of magnitude, to 7.5 g/liter, rhamnolipid enhancement of octadecane dispersion to 0.32 g/liter is a modest 4 orders of magnitude (16). There are several possible reasons for the large difference in the solubilization of octadecane by rhamnolipids and phospholipids. First, the octadecane-rhamnolipid association was driven by a much smaller energy input (shaking) than the octadecane-phospholipid association (sonication). In fact, sonication of rhamnolipid-octadecane solutions does increase octadecane dispersion to levels comparable to those achieved with liposome encapsulation (data not shown). Second, the octadecane/rhamnolipid ratio in biosurfactant experiments (3:1) was higher than the octadecane/phospholipid ratio in liposome experiments (1:1).

Effect of rhamnolipid-enhanced octadecane dispersion on mineralization. The results in Fig. 7 indicate that mineralization rates can be increased significantly by rhamnolipid-enhanced octadecane dispersion. The enhancement of mineralization rates was linearly dependent on the rhamnolipid concentration at a statistically significant level (P = 0.05) for the tested range of 0 to 300 mg of rhamnolipid per liter. However, the fourfold increase in mineralization was not nearly as high as the >10⁴-fold increase in the aqueous dispersion of octadecane. Therefore, it is clear that although rhamnolipids increase the dispersion of octadecane, the octadecane is still not freely bioavailable.

A large proportion (45%) of the dispersed octadecane was shown to be associated with aggregates that passed through a 300,000-MW filter but not through a 30,000-MW filter. The remainder of the dispersed octadecane was able to pass through a 30,000-MW filter. Further work will be necessary to determine the optimal aggregate size for biodegradation.

It should be emphasized that these experiments represent a realistic environmental scenario in that the octadecane was not mixed with the rhamnolipid prior to inoculation with P. aeruginosa ATCC 9027. Therefore, initial octadecane dispersion was very low and maximum dispersion was reached only after approximately 10 h. Figure 7 shows that very little biodegradation occurred at any rhamnolipid concentration until approximately 10 h.

The results of this study have exciting implications for the bioremediation of sites containing recalcitrant compounds having limited water solubility. The rhamnolipid used in this study served as an effective dispersion agent, increasing the mineralization of octadecane fourfold in a pure-culture batch experiment. Although shaking was used in batch experiments, rhamnolipids can significantly increase the aqueous dispersion of octadecane to 75 mg/liter even without energy input (shaking). Thus, rhamnolipids may have the potential to facilitate the biodegradation of hydrocarbons in contaminated soil as well as aqueous environments. Future studies are needed to examine (i) rhamnolipid-hydrocarbon interactions with bacterial cells, (ii) the effects of rhamnolipids on the rates of biodegradation of slightly water-soluble organic compounds in soil and aqueous environments, and (iii) the fate of rhamnolipids in the environment.

ACKNOWLEDGMENTS

This research was supported by the Subsurface Science Program, Office of Health and Environmental Research, U.S. Department of Energy. Support was also provided by the University of Arizona Agricultural Experiment Station ( Hatch Project ARZT 136452 H21-032).

REFERENCES