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Rhamnolipid Stimulates Uptake of Hydrophobic Compounds by *Pseudomonas aeruginosa*

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The biodegradation of hexadecane by five biosurfactant-producing bacterial strains (*Pseudomonas aeruginosa* UG2, *Acinetobacter calcoaceticus* RAG1, *Rhodococcus erythropolis* DSM 43066, *R. erythropolis* ATCC 19558, and strain BCG112) was determined in the presence and absence of exogenously added biosurfactants. The degradation of hexadecane by *P. aeruginosa* was stimulated only by the rhamnolipid biosurfactant produced by the same organism. This rhamnolipid did not stimulate the biodegradation of hexadecane by the four other strains to the same extent, nor was degradation of hexadecane by these strains stimulated by addition of their own biosurfactants. This suggests that *P. aeruginosa* has a mode of hexadecane uptake different from those of the other organisms. Rhamnolipid also enhanced the rate of epoxidation of the aliphatic hydrocarbon α,ω-tetradecadiene by a cell suspension of *P. aeruginosa*. Furthermore, the uptake of the hydrophobic probe 1-naphthylphenylamine by cells of *P. aeruginosa* was enhanced by rhamnolipid, as indicated by stopped-flow fluorescence experiments. Rhamnolipid did not stimulate the uptake rate of this probe in de-energized cells. These results indicate that an energy-dependent system is present in *P. aeruginosa* strain UG2 that mediates fast uptake of hydrophobic compounds in the presence of rhamnolipid.

The bioavailability of sparingly soluble hydrophobic compounds for microbial conversion usually is very low and often limits their rate of degradation in aqueous and soil systems (2, 36). The use of biosurfactants has been found to enhance degradation in various cases. The degradation of long-chain alkanes, for example, by *Pseudomonas aeruginosa* can be stimulated by rhamnolipid, a biosurfactant produced by the organism (15, 37). Insight into the mechanism of rhamnolipid-enhanced degradation of hydrophobic compounds is desirable for the application of this organism or its biosurfactant in bioremediation or biotransformation.

It has been reported that degradation of hexadecane by rhamnolipid-producing organisms is stimulated to a greater extent by rhamnolipid than by other biosurfactants (17) or synthetic surfactants (26). This is probably caused by the increase in cell surface hydrophobicity after extraction of lipopolysaccharides from the cellular envelope by rhamnolipid, which subsequently facilitates uptake via direct contact between cells and hydrocarbon droplets (1). In contrast, rhamnolipid failed to stimulate degradation of hydrophobic compounds by other strains or by mixed cultures which are not known to produce biosurfactants (15, 31). Thus, a high specificity seems to exist in the interaction between rhamnolipid and rhamnolipid-producing strains. It is not known whether such a specificity exists for other biosurfactant-producing bacteria and their biosurfactants in a similar way. Therefore, we have studied whether hexadecane biodegradation by *P. aeruginosa* and by four other biosurfactant-producing bacteria is stimulated by exogenous addition of their own and other biosurfactants. In order to obtain insight into how rhamnolipid stimulates hexadecane biodegradation by *P. aeruginosa*, we have determined whether rhamnolipid can directly enhance uptake of dissolved or solubilized substrate by using a hydrophobic fluorescent probe.

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MATERIALS AND METHODS

Bacterial strains and culture media. *Pseudomonas aeruginosa* UG2 was provided by J. T. Trevors (University of Guelph, Guelph, Canada) (5). *P. aeruginosa* PG201 was obtained from U. A. Ochsner (University of Colorado, Denver, Colo.) (30). The gram-positive bacterial strain BCG112 was provided by J. van der Waarde (Bioclear, Groningen, The Netherlands). This organism was isolated from contaminated soil with dioctylphthalate as the sole source of carbon and energy and produces a biosurfactant, as was shown by the emulsification of hexadecane and solubilization of pyrene in supernatants of cultures grown with hexadecane or dioctylphthalate. The strains *Acinetobacter calcoaceticus* RAG1 (ATCC 31012), *Rhodococcus erythropolis* ATCC 19558 (*Arthrobacter paraffineus*), and *P. aeruginosa* ATCC 15528 were obtained from the American Type Culture Collection (Rockville, Md.). *R. erythropolis* DSM 43066 was obtained from the Deutsche Sammlung für Microorganismen (Braunschweig, Germany). For production of biosurfactants, organisms were grown in media described in the original publications (see below). In all other experiments, a mineral salts medium was used that contained (per liter) 0.53 g of $Na_2HPO_4 \cdot 12H_2O$, 0.14 g of KH_2PO_4, 0.02 g of $MgSO_4 \cdot 7\ H_2O$, 0.1 g of $(NH_4)_2SO_4$, and 0.5 ml of a trace elements solution (18).

Biosurfactants. Rhamnolipid was produced by *P. aeruginosa* UG2 during growth with glucose (28, 34) and was isolated from the culture supernatant by a series of consecutive steps of acid precipitation and dissolution in 50 mM $NaHCO_3$. It was purified by column chromatography over Sephadex LH20 with methanol as the eluent (28). Emulsan, the lipo-heteropolysaccharide bioemulsifier produced by *A. calcoaceticus* RAG1, was isolated from a culture grown with ethanol by ammonium sulfate precipitation, dialysis, and washing with diethylether (33). The trehalose lipid produced by *R. erythropolis* DSM 43066 was isolated by extraction with pentane of the whole broth of a culture grown with hexadecane (23). The concentrated pentane extract was chromatographed over silica with petroleumether 40-60 and subsequently with increasing amounts of ethylacetate. The ethylacetate fractions that contained surface-active components were collected and concentrated. The lipid biosurfactants produced by *R. erythropolis* ATCC 19558 were isolated from a culture grown with hexadecane

(10). The culture broth was extracted with petroleumether 40-60, and the material that was concentrated at the aqueous-organic interface was collected, washed with water and petroleumether, and lyophilized. The biosurfactants were extracted from this crude product as lipids (19) and were lyophilized and redissolved in chloroform. The high-molecular-weight biosurfactant produced by strain BCG112 was isolated from a culture grown with hexadecane. The culture supernatant was dialyzed, concentrated by lyophilization, extracted with chloroform-methanol (1:2) to remove lipids (19), lyophilized, and resuspended in water.

Effect of biosurfactants on biodegradation of hexadecane. The effect of biosurfactants on the degradation of hexadecane by the biosurfactant-producing strains was determined by measuring at a specific time the residual amount of hexadecane present in cultures which had been incubated with or without added biosurfactants. Experiments were carried out in 8-ml tightly closed Pyrex culture tubes containing 1 ml of culture fluid and 7 ml of headspace. Substrate and biosurfactants were first added to empty flasks. An amount of hexadecane was added from a 1:10 (vol/vol) solution of hexadecane in pentane so that the initial amount of hexadecane in all flasks was 0.25 mg. Biosurfactants from *R. erythropolis* were added to a number of the flasks from a stock solution in ethylacetate or chloroform. All organic solvents were allowed to evaporate by incubation for 16 h in a sterile cabinet. Rhamnolipid, emulsan, or the biosurfactant from strain BCG112 was added to flasks from concentrated aqueous stock solutions. The concentration of the biosurfactants after addition of inoculated medium was 0.5 mg of biosurfactant ml^{-1} (rhamnolipid and emulsan) or twice the concentration that was present in the culture from which the biosurfactant was isolated (biosurfactants from *R. erythropolis* and strain BCG112). Precultures of the strains were grown for 3 days with hexadecane to a protein content of 30 to 70 μg of protein ml^{-1}. Mineral salts medium was inoculated with these precultures to a culture density of 3 μg of protein ml^{-1}. An amount of 1 ml of an inoculated medium was added to each flask so that all flasks contained 1 ml of aqueous phase and 7 ml of air. The amount of oxygen in the flasks was calculated to be sufficient to allow complete oxidation of hexadecane. The cultures were incubated at 30°C by end-over-end rotation at 1.4 rpm.

For each strain, the degradation of hexadecane was followed in time by sacrificing duplicate flasks of parallel cultures to which no surfactant had been added. At the time where approximately half of the substrate was degraded in these control cultures, the other cultures with this organism with and without added biosurfactant were analyzed for hexadecane. The amount of hexadecane was determined by extracting the whole contents of the culture flasks with 0.05 ml of 10% HCl, 2 ml of ethanol, and 1 ml of iso-octane. After vigorous extraction for 5 min and centrifugation at 2,600 × *g* (when necessary) to separate phases, the isooctane layer was analyzed by gas chromatography (GC). Three separate cultures were sacrificed and analyzed for hexadecane for each combination of biosurfactant and microorganism. The Student *t* test was used to determine whether significant differences existed between the amount of residual substrate in a culture flask containing a surfactant and that in the control cultures. It was verified that no hexadecane disappeared from the incubation flasks by abiotic processes by monitoring the amount of hexadecane in flasks that were not inoculated.

Microbial attachment to hydrocarbons. Cells were grown for 6 days on hexadecane or for 2 days on glucose or pyruvate, harvested by centrifugation, washed, and resuspended in mineral salts medium to an optical density at 400 nm of 0.7. Aliquots of 1.2 ml of cell suspension and 0.3 ml of hexadecane were placed in triplicate in 1.5-ml vials (inside diameter, 1.0 cm; height, 2.0 cm), closed, and vortexed for 60 s. The vials were incubated with septa facing down. After 30 min, samples of the aqueous phase were taken through the septa and the optical density was measured again. The microbial attachment to hydrocarbon was calculated as the percent decrease of optical density (38).

Conversion of α,ω-tetradecadiene by a cell suspension. *P. aeruginosa* UG2 was grown for 3 days on tetradecane to an optical density at 450 nm of 0.8 or a protein concentration of 0.17 mg ml^{-1}. Aliquots of 50 μl of a stock solution of α,ω-tetradecadiene in pentane (1:20, vol/vol) were added to 30-ml serum flasks. The pentane was allowed to evaporate. To each flask was added 15 ml of cell suspension that was supplemented with 4 mM succinate as a reducing cosubstrate and, where indicated, 0.5 mg of rhamnolipid ml^{-1}. The cell suspensions were incubated at 30°C with gyratory shaking at 200 rpm. Periodically, 1-ml samples were withdrawn and analyzed by GC or GC-mass spectrometry (MS) after extraction with 0.05 ml of 10% HCl, 2 ml of ethanol, and 1 ml of isooctane.

Uptake of 1-naphthylphenylamine. *P. aeruginosa* UG2 was grown for 3 days on hexadecane or for 1 day on glucose to a culture density of 0.15 mg of protein ml^{-1}. Cells were harvested and washed twice with mineral medium and resuspended in the same medium. Sonicated cell suspensions were prepared after dilution in 5 mM HEPES (pH 7.3) to a concentration of 0.05 mg of protein ml^{-1} by ultrasonication (Vibracell VC600; Sonic Materials Inc., Danbury, Conn.) in five sessions of 5 s using a microtip at maximal output. Cells were de-energized by treatment with 50 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and 30 mM NaN_3 (20). After 2 h of incubation at room temperature, CCCP and NaN_3 were removed by centrifugation and washing with fresh mineral medium (twice), since these agents interfered with the fluorescence of 1-naphthylphenylamine (NPN).

Uptake of NPN was determined by adding cells, sonicated cells, or de-energized cells to a cuvette containing HEPES (5 mM, pH 7.3) to yield a protein concentration of 0.05 mg ml^{-1} in a total volume of 2 ml. NPN was added to the cell suspension to a concentration of 3 μM from a stock solution in acetone (500 μM) (14), and the increase in fluorescence was monitored on a SLM Aminco SPF500-C spectrofluorometer at 30°C (excitation wavelength, 350 nm; emission wavelength, 420 nm; slit width, 5 nm). Subsequently, additions were made of glucose (2.8 mM), EDTA (0.5 mM) (14), and gentamicin (36 μM) (14). After the experiment, Triton X-100 was added to a final concentration of 1.5 mg ml^{-1} to completely solubilize NPN. The fluorescence of NPN was recorded, and the value was used to calculate the total amount of NPN added to the cuvette.

For the stopped-flow analysis of NPN uptake by whole cells, an Applied Photophysics model SX17MV stopped-flow spectrofluorometer was used at 30°C (excitation wavelength, 350 nm; cutoff filter, 375 nm). All solutions were made in HEPES buffer (5 mM, pH 7.3). In a typical experiment, one syringe contained a (de-energized) cell suspension (0.1 mg of protein ml^{-1}), and the other contained NPN (6 μM) and, when indicated, surfactant. Each reported trace is the average of results of three separate experiments with fresh cells loaded into the syringe prior to each experiment. It was verified that NPN exchange between micelles and medium was instantaneous on the accessible time scale (a few milliseconds). In a separate experiment, solutions of NPN (6 μM) and Triton X-100 (3 mg ml^{-1}) were mixed, and the fluorescence value of the resulting solution, which contains NPN dissolved in Triton X-100 micelles, was used to estimate the fraction of NPN taken up by the cells or solubilized by rhamnolipid in the other experiments. Reported rhamnolipid concentrations refer to concentrations in the reaction chamber. The observed rate constants, k_{obs1} and k_{obs2}, were determined by fitting the traces to a double exponential with a floating endpoint. The rate constants describing NPN uptake were determined by fitting the traces to a two-step consecutive reversible reaction scheme (see equation 1 [11]), using the computer program Scientist (version 2.0 for Windows; Micromath Scientific, Salt Lake City, Utah), assuming identical fluorescence factors for NPN in both kinetically distinguishable cellular domains (NPN_{C1} and NPN_{C2}) (equation 1) and for NPN solubilized in Triton X-100 micelles.

Analytical procedures. Culture densities were determined by measuring the optical density at 450 nm (Hitachi 100-60 spectrophotometer) and by determining the protein concentration with the Folin reagent after alkaline hydrolysis of the cell suspension. Hexadecane, α,ω-tetradecadiene, and 13,14-epoxy-1-tetradecene were analyzed by GC (Hewlett Packard model 6890) using an HP5 capillary column (Hewlett Packard) and a flame ionization detector or, for identification, a mass selective detector (Hewlett Packard type 5971).

Chemicals. Hexadecane (99%) and tetradecane were obtained from Acros (Geel, Belgium), α,ω-tetradecadiene and 1-naphthylphenylamine were obtained from Aldrich (Milwaukee, Wis.), gentamicin was obtained from Sigma (St. Louis, Mo.), and Triton X-100 was obtained from BDH Chemicals (Vancouver, Canada). A mixture of 13,14-epoxy-1-tetradecene and α,ω-diepoxytetradecane was synthesized by partial oxidation of α,ω-tetradecadiene with *m*-chloroperoxybenzoic acid (35).

RESULTS AND DISCUSSION

Effect of biosurfactants on the biodegradation of hexadecane. The effects of biosurfactants on the biodegradation of hexadecane by the biosurfactant-producing strains *P. aeruginosa, A. calcoaceticus,* and *R. erythropolis* and the bacterial strain BCG112 were determined (Table 1). The reported fractions of hexadecane remaining in the culture are the average values ± one standard deviation. Biodegradation of hexadecane by the rhamnolipid-producing strain *P. aeruginosa* UG2 was enhanced only by addition of its own biosurfactant. The other rhamnolipid-producing strains, PG201 and ATCC 15528, were also stimulated by this rhamnolipid (Table 1). Hexadecane degradation by *R. erythropolis* ATCC 19558 was modestly stimulated by the biosurfactants from *P. aeruginosa* and from

TABLE 1. Effect of biosurfactants on hexadecane degradation by biosurfactant-producing bacterial strains

Organism	Time (h)	Fraction of substrate left[c]					
		No added surfactant	*P. aeruginosa* biosurfactant	*A. calcoaceticus* biosurfactant	*R. erythropolis* DSM 43066 biosurfactant	*R. erythropolis* ATCC 19558 biosurfactant	Strain BCG112 biosurfactant
P. aeruginosa UG2	73	0.60 ± 0.10	0.00 ± 0.00[a]	0.65 ± 0.28	0.48 ± 0.02	0.69 ± 0.29	0.72 ± 0.06
P. aeruginosa ATCC15528	37	0.36 ± 0.01	0.01 ± 0.01[a]				
P. aeruginosa PG201	37	0.62 ± 0.15	0.00 ± 0.00[a]				
A. calcoaceticus RAG1	38	0.56 ± 0.12	0.60 ± 0.03	0.82 ± 0.04[b]	0.47 ± 0.01	0.69 ± 0.04	0.76 ± 0.12
R. erythropolis DSM 43066	62	0.26 ± 0.04	1.00 ± 0.04[b]	0.94 ± 0.01[b]	0.50 ± 0.06[b]	0.52 ± 0.00[b]	0.98 ± 0.06[b]
R. erythropolis ATCC 19558	29	0.71 ± 0.14	0.47 ± 0.02[a]	0.93 ± 0.21	0.44 ± 0.01[a]	0.58 ± 0.04	
Strain BCG112	17	0.49 ± 0.11	0.67 ± 0.21	0.83 ± 0.31	0.48 ± 0.02		0.85 ± 0.09[b]

[a] Biosurfactant stimulates hexadecane degradation ($P < 0.05$).
[b] Biosurfactant inhibits hexadecane degradation ($P < 0.05$).
[c] Values are averages ± 1 standard deviation.

R. erythropolis DSM 43066. Surprisingly, hexadecane degradation by *A. calcoaceticus, R. erythropolis* DSM 43066, and strain BCG112 was inhibited by exogenous addition of their own biosurfactants. The degradation of hexadecane by *R. erythropolis* was even inhibited by each of the biosurfactants added. In earlier studies with *Acinetobacter* species, including *A. calcoaceticus* RAG1, it was also found that nonionic surfactants did not stimulate mineralization of octadecane and oil (7, 9). Although degradation of long-chain alkanes by stationary phase *Rhodococcus* cells might be stimulated by surfactants, degradation by growing *Rhodococcus* cells was found to be inhibited or only slightly stimulated by synthetic surfactants or rhamnolipid (7, 8, 9), similar to what we observed for other biosurfactants.

The results show that rhamnolipid-producing organisms are stimulated mainly by exogenous addition of rhamnolipid and that the other biosurfactant-producing organisms investigated are not stimulated by their biosurfactants in a similar way. It is possible that some surfactants induced a lag phase or were toxic to some organisms. However, the difference in behavior towards biosurfactants of these strains was probably caused by a different way of interaction with the substrate. This was subsequently investigated by determining the attachment of cells to hexadecane.

Microbial attachment to hydrocarbons. Attachment to hexadecane of *P. aeruginosa* UG2 cells grown with hexadecane was at a lower level than that of cells grown with glucose (Table 2), in agreement with earlier observations (6). In contrast, the attachment of the non-rhamnolipid-producing strains to hexadecane increased or was unaffected during growth with hexadecane compared to glucose. A higher attachment of hexadecane-grown cells to hexadecane may result in enhanced degradation of hydrocarbons if uptake requires physical contact between the cells and hydrocarbon droplets (6). The fact that biosurfactants produced by *R. erythropolis* strains are cell bound also suggests that their function is to enhance attachment of the cells to the substrate (16).

The mode of hydrocarbon uptake may be different for various microorganisms (9, 12, 25). Differences may even exist between different strains of *P. aeruginosa* (38). Degradation of hydrophobic hydrocarbons by strains that take up the substrate after attachment to droplets is generally not stimulated by surfactants (9). This would suggest that *P. aeruginosa* does not take up hydrophobic substrates via direct contact, since the organism is stimulated by several surfactants (6, 9, 25, 26). The opposite effect of rhamnolipid on biodegradation of hexadecane by *P. aeruginosa* and on conversion by other organisms (Table 1) may be caused by differences in uptake mechanisms. Because rhamnolipid and other surfactants enhance hexadecane degradation by *P. aeruginosa*, we aimed to determine whether this stimulation is caused by enhanced uptake of the substrate and, more specifically, whether the biosurfactant directly enhances uptake of dissolved or solubilized substrate.

Conversion of α,ω-tetradecadiene by *P. aeruginosa*. To determine whether rhamnolipid stimulates the degradation of a hydrophobic substrate by *P. aeruginosa* by a general positive effect on the uptake of a hydrophobic substrate, we measured the oxidation of α,ω-tetradecadiene by a cell suspension. The substrate was present in an amount that exceeded its aqueous solubility, similar to the situation in the degradation experiments with hexadecane. Incubation with hexadecane-grown cells yielded a product with the same GC retention time as chemically synthesized 13,14-epoxy-1-tetradecene. Furthermore, the GC-MS spectra were similar and matched the spectra of aliphatic compounds with terminal epoxy and olefinic functionalities. Thus, the product was identified as 13,14-epoxy-1-tetradecene. The substrate α,ω-tetradecadiene is presumably converted by the same monooxygenase as hexadecane.

Addition of rhamnolipid enhanced the rate of epoxidation of α,ω-tetradecadiene by a cell suspension of *P. aeruginosa* UG2 (Fig. 1). Since it is unlikely that the surfactant influenced the monooxygenase activity, we propose that rhamnolipid enhanced the availability of the substrate to the cells, either by promoting the emulsification or solubilization of the substrate, by facilitating the uptake of surfactant-associated substrate (either solubilized or emulsified) by the cell, or by increasing

TABLE 2. Attachment of bacterial cells to hydrocarbons

Organism	Attachment to hexadecane (%) of[a]	
	Glucose-grown cells	Hexadecane-grown cells
P. aeruginosa UG2	58 ± 6	42 ± 1
A. calcoaceticus RAG1	75 ± 2[b]	81 ± 7
R. erythropolis DSM 43066	14 ± 3	30 ± 1
R. erythropolis ATCC 19558	14 ± 1	12 ± 12
Strain BCG112	30 ± 5	59 ± 8

[a] Values are averages ± 1 standard deviation.
[b] Cells were grown with pyruvate.

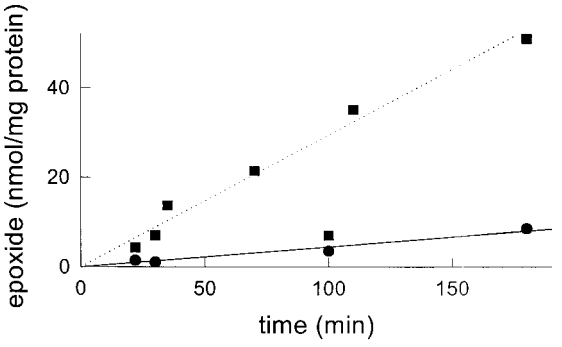

FIG. 1. Production of 13,14-epoxy-1-tetradecene from α,ω-tetradecadiene by cell suspensions of *P. aeruginosa* UG2. Combined data from three independent experiments are shown for cell suspensions with no added surfactant (●) and with 0.5 mg of rhamnolipid ml^{-1} (■). Lines were obtained by linear regression.

the attachment of the cells to the α,ω-tetradecadiene droplets.

Uptake of 1-naphthylphenylamine by cells of *P. aeruginosa* UG2. To determine whether rhamnolipid stimulated uptake of a micelle-solubilized hydrophobic compound by the cellular membrane, the hydrophobic fluorescent probe NPN was used. The fluorescence of this compound is much higher in a lipid environment than in an aqueous environment. Therefore, uptake can be measured by recording the increase in fluorescence (14). The probe was completely soluble at the concentration used, excluding the possibility that the measured uptake rate is influenced by the rate of solubilization or emulsification or by the attachment of the cells to NPN particles.

In the absence of rhamnolipid, uptake of NPN by whole cells of *P. aeruginosa* was slower than uptake by a sonicated cell suspension (Fig. 2A). This indicates the existence of a transport limitation that retards the accumulation of NPN in cell membranes of intact cells. Uptake of NPN was faster with glucose-grown cells than with hexadecane-grown cells (Fig. 2A). The hydrophilic lipopolysaccharide (LPS) layer probably is the component of the cellular envelope that restricts the uptake rate of hydrophobic compounds by cells to the greatest extent (13). Therefore, the low uptake rate with hexadecane-grown cells compared to that with glucose-grown cells suggests that the former have a more extended or hydrophilic LPS layer that is less permeable to hydrophobic compounds, which is in accordance with the observed decreased attachment to hexadecane of these cells compared to cells grown with glucose.

The amount of NPN that accumulated in cell membranes of *P. aeruginosa* was higher in the sonicated cell suspension than in intact cells (Fig. 2A) and increased more than twofold after the combined addition of the permeabilizing agents gentamicin and EDTA (Fig. 2B). De-energized cells took up more NPN than normal cells but, in contrast to normal cells, did not take up more NPN after the addition of EDTA and gentamicin (Fig. 2C). The amount of NPN in the cell membranes temporarily decreased upon addition of glucose, both for normal hexadecane-grown cells (Fig. 2B) and for de-energized hexadecane-grown cells (Fig. 2C). These findings are in agreement with the LPS layer functioning as a barrier for the uptake of hydrophobic compounds and with the presence in this strain of

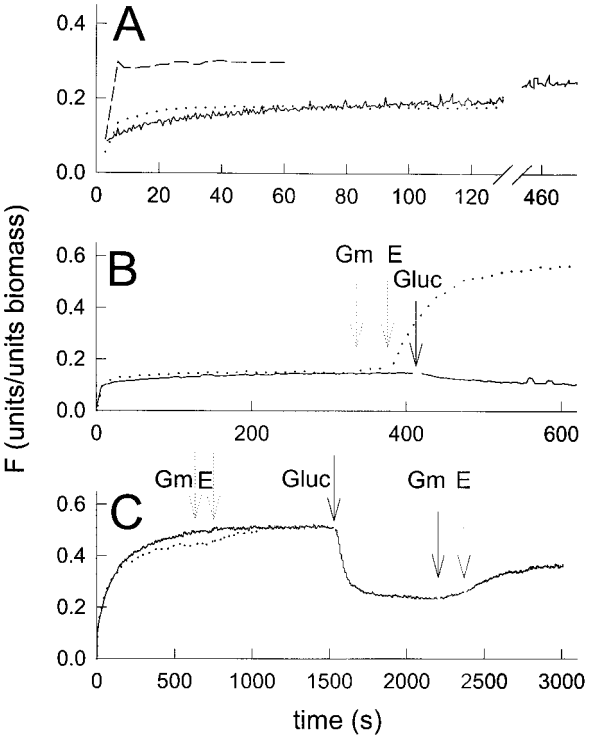

FIG. 2. Uptake of NPN by cells of *P. aeruginosa*. The increase in fluorescence after mixing of cells and probe was monitored. (A) Hexadecane-grown cells (line), glucose-grown cells (dotted line), and sonicated hexadecane-grown cells (dashed line). (B) Hexadecane-grown cells. In one experiment (line), glucose (Gluc) was added at the time indicated by the solid arrow. In another experiment (dotted line), gentamicin (Gm) and EDTA (E) were added at the time indicated by the dotted arrows. (C) Hexadecane-grown de-energized cells. In one experiment (line), glucose was added at the time indicated by the solid arrow followed by addition of gentamicin and EDTA. In another experiment (dotted line), gentamicin and EDTA were added at the time indicated by the dotted arrows.

an energy-dependent exporter that expels NPN, possibly the MexAB-OprM efflux system (22).

The kinetics of NPN uptake by hexadecane-grown cells was determined using stopped-flow fluorescence experiments (Fig. 3). The use of the stopped-flow apparatus enabled determination of uptake traces on the relevant time scale of 0.1 to 10 s. After the cells were rapidly mixed with NPN, an increase in fluorescence was observed. The trace could be fitted well ($r^2 > 0.999$) using a bi-exponential curve with two similar amplitudes and with a k_{obs1} value of 0.18 s^{-1} and a k_{obs2} value of 0.018 s^{-1}. The bi-exponential nature of the fluorescence trace shows that uptake of hydrophobic substrates involves at least two sequential steps.

The simplest equation describing NPN uptake in the absence of surfactant would be a process consisting of two consecutive reversible steps (equation 1 [11]):

$$NPN_{aq} \underset{k_{-1}}{\overset{k_1 \times C}{\rightleftharpoons}} NPN_{C1} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} NPN_{C2} \quad (1)$$

where NPN_{aq} represents NPN in the aqueous phase (millimolar), NPN_{C1} and NPN_{C2} represent NPN in two kinetically

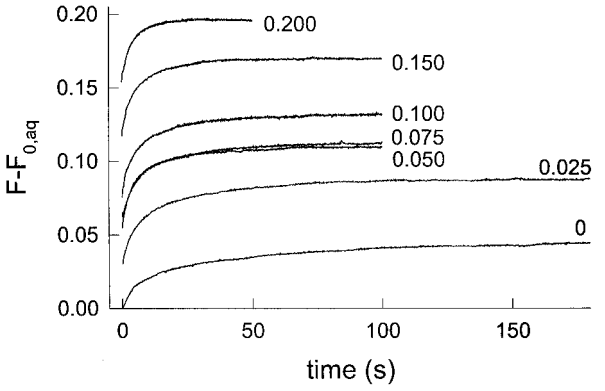

FIG. 3. Effect of rhamnolipid on the uptake kinetics of NPN by cells of *P. aeruginosa* as determined using stopped-flow analysis. Fluorescence traces were obtained after rapid mixing of cell suspensions with NPN containing various concentrations of rhamnolipid. The rhamnolipid concentration in the reaction chamber (in milligrams milliliter^{-1}) is given for each trace. $F_{0,aq}$ is the initial fluorescence for the trace with a rhamnolipid concentration of 0.

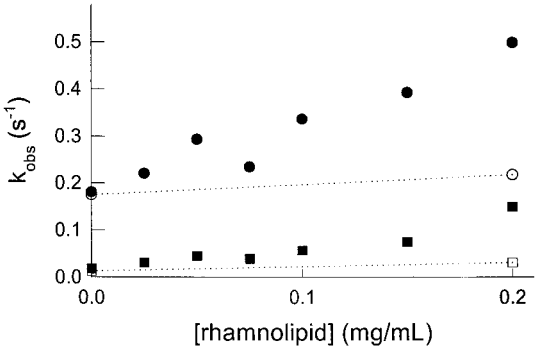

FIG. 4. Effect of rhamnolipid on the observed rate constants describing uptake of NPN by cells of *P. aeruginosa*. The observed rate constants k_{obs1} (circles) and k_{obs2} (squares) were derived from the traces presented in Fig. 3 and are plotted as a function of rhamnolipid concentration for normal cells (closed symbols) and for de-energized cells (open symbols).

distinguishable cellular domains (domains 1 and 2, respectively, millimoles milligram of protein^{-1}), and C is the concentration of cells (milligrams of protein milliliter^{-1}). The model is based on the assumption that the number of sites for NPN in both cellular domains is unlimited, as in a partitioning process, making the NPN concentration the only variable. We also assumed that NPN uptake followed first-order kinetics and that the NPN fluorescence factors for domains 1 and 2 and for NPN solubilized in Triton X-100 micelles were the same. By fitting the NPN uptake trace measured in the absence of rhamnolipid (Fig. 3), the microscopic rate constants (equation 1) were found to be the following: $k_1 = 0.15$ ml mg of protein^{-1} s^{-1}, $k_{-1} = 0.17$ s^{-1}, $k_2 = 0.0174$ s^{-1}, and $k_{-2} = 0.0191$ s^{-1}. These values could not be changed by more than 25% (k_1 or k_{-1}) or 50% (k_2 or k_{-2}) without losing good fit, indicating that they represent unique solutions.

The rate constants would allow growth in the absence of rhamnolipid with a specific growth rate of $V_{upt} \times Y$, where V_{upt} is the uptake flux through these steps in steady state (millimoles of substrate milligram^{-1} of protein minute^{-1}) and Y is the yield (milligrams of protein millimole^{-1} of substrate) (24). If conversion of NPN from domain 2 is fast and uptake is rate limiting, the uptake flux V_{upt} is equal to $[k_1k_2/(k_{-1}+k_2)] \times NPN_{aq}$. This is 2.4×10^{-6} mmol of NPN mg of protein^{-1} min^{-1} at an NPN_{aq} of 3 μM. Assuming a yield of 50 mg of protein mmol of substrate^{-1}, a specific growth rate of 0.01 h^{-1} is predicted. Based on the aqueous solubility of hexadecane and assuming that the rate constants for hexadecane uptake are the same as for NPN uptake, the maximum specific growth rate with hexadecane would be 10^{-5} h^{-1}. This predicted growth rate is much lower than the observed maximum specific growth rate of 10^{-2} h^{-1} for *P. aeruginosa* UG2 with hexadecane in the absence of rhamnolipid (29). This 3-orders-of-magnitude difference between the observed and estimated growth rates with hexadecane can hardly result from inaccurate assumptions of, e.g., the fluorescence factors. Therefore, it seems impossible that growth with hexadecane proceeds via uptake of dissolved substrate. Probably, hexadecane is taken up from submicron hydrocarbon droplets (3). Thus, the degradation of hydrophobic substrates is likely to be limited by substrate uptake, and rhamnolipid may accelerate biodegradation by enhancing the uptake rate. This is supported by analysis of the rate of hexadecane degradation by *P. aeruginosa* (29)

Effect of rhamnolipid on uptake of NPN. The effect of rhamnolipid on the uptake kinetics of NPN in hexadecane-grown cells was directly determined using stopped-flow fluorescence (Fig. 3). The initial value of NPN fluorescence increased with increasing rhamnolipid concentration, due to instantaneous partitioning of NPN into rhamnolipid micelles (Fig. 3). The difference in the initial fluorescence value of NPN and the value that resulted on complete solubilization of NPN with Triton X-100 (separate experiment) indicated that 100% (no rhamnolipid) to 73% (0.2 mg of rhamnolipid/ml) of NPN was present in the aqueous phase at time zero. The fluorescence subsequently increased in time at all concentrations of rhamnolipid, due to net transfer of NPN from the aqueous phase to the membranes. The fluorescent traces were also bi-exponential in the presence of rhamnolipid. Fitting of the traces again yielded two observed rate constants and two equal amplitudes ($r^2 \geq 0.999$). Both observed rate constants increased linearly with increasing rhamnolipid concentration up to the highest concentration measured of 0.2 mg/ml (Fig. 4). This directly shows that rhamnolipid can enhance uptake of a hydrophobic compound by cells of *P. aeruginosa*, as was recently suggested based on measurement of the amount of intracellular hexadecane in rhamnolipid-producing and rhamnolipid-deficient strains grown with hexadecane (4, 21).

The biosurfactant did not facilitate just one of the two steps in equation 1 and leave the other unaffected, since a good fit could not be obtained when using the values for k_1 and k_{-1} or for k_2 and k_{-2} from the experiment without rhamnolipid and accounting for solubilization of NPN by rhamnolipid by using rhamnolipid-water partitioning constants for NPN that were estimated from a linear free-energy relationship (27). Thus, it may be that the uptake process in the presence of rhamnolipid becomes more complex than suggested by equation 1, e.g., due to the direct uptake of NPN solubilized by rhamnolipid.

The observed rate constants for NPN uptake by de-ener-

gized cells in the absence of rhamnolipid were similar to those with normal cells, confirming that the cell walls were not permeabilized during de-energization (Fig. 4). The observed rate constants for NPN uptake by de-energized cells in the presence of rhamnolipid (0.2 mg ml^{-1}) were virtually the same as in the absence of rhamnolipid (Fig. 4), suggesting that rhamnolipid did not stimulate NPN uptake by de-energized cells. An increase in observed uptake rate constants in normal cells but not in de-energized cells would also be expected when rhamnolipid stimulated an energy-dependent exporter. However, stimulation of the exporter by rhamnolipid would reduce the concentration of NPN in the membrane at equilibrium. This is not observed, since the amplitude of the uptake trace did not decrease with increasing rhamnolipid concentration (Fig. 3). Therefore, these results indicate that rhamnolipid enhances uptake of NPN in an energy-dependent manner.

Since it was reported that rhamnolipid does not accumulate in the cell or partition into the cellular envelope of *P. aeruginosa* (38), its interaction with the cell must be short-lived and must most probably be restricted to the outermost cellular surface. Furthermore, it is likely that the LPS layer poses the most important barrier to uptake. Therefore, a rhamnolipid-facilitated uptake mechanism is probably mediated by components either in the LPS layer or in the outer membrane. Rhamnolipid extracts LPS from the cellular envelope (1). It has been suggested that this enables faster uptake of hydrophobic compounds due to the fact that cells become more hydrophobic and attach to hydrocarbon droplets more intensely (1). Since it is unlikely that extraction of LPS is enhanced by rhamnolipid in normal cells but not in de-energized cells, this hypothesis does not readily explain the observed energy dependence of the uptake system. Although it cannot be excluded that the extraction of LPS by rhamnolipid contributes indirectly to enhanced uptake of hydrophobic compounds, the present work strongly suggests that rhamnolipid also stimulates uptake of a solubilized hydrophobic compound by the cell membranes without promoting attachment of the cells to a separate phase of this compound.

Rhamnolipid-facilitated uptake in *P. aeruginosa*. Rhamnolipid stimulates uptake of hydrophobic compounds by *P. aeruginosa* in an energy-dependent manner. Kinetic analysis of NPN uptake by *P. aeruginosa* suggests that the uptake rate can limit the degradation rate of a hydrophobic compound. Exogenously added rhamnolipid or other biosurfactants did not stimulate the rate of hexadecane biodegradation by four organisms that produced these other biosurfactants. This shows that a mechanism that allows fast uptake of hydrophobic compounds in the presence of rhamnolipid is present in *P. aeruginosa* but not in the other strains. Ratledge previously suggested the existence of such a mechanism (32). It seems that rhamnolipid enhances hexadecane degradation by *P. aeruginosa* through a rhamnolipid-facilitated energy-dependent uptake mechanism. This, together with the observation that rhamnolipid-deficient mutants have a lower growth rate with hexadecane than wild-type *P. aeruginosa* (21), strongly supports a physiological role of rhamnolipid in hydrocarbon assimilation. The present work demonstrates that the influx of hydrophobic compounds into cells of *P. aeruginosa* UG2 is determined by an active efflux system, by regulation of attachment of cells to hydrocarbons, and by a rhamnolipid-facilitated uptake mechanism. The control of influx of hydrophobic compounds allows the organism to control the substrate availability and the toxicity caused by these compounds.

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