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Rhamnolipid (Biosurfactant) Effects on Cell Aggregation and Biodegradation of Residual Hexadecane under Saturated Flow Conditions

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The objective of this research was to evaluate the effect of low concentrations of a rhamnolipid biosurfactant on the in situ biodegradation of hydrocarbon entrapped in a porous matrix. Experiments were performed with sand-packed columns under saturated flow conditions with hexadecane as a model hydrocarbon. Application of biosurfactant concentrations greater than the CMC (the concentration at which the surfactant molecules spontaneously form micelles or vesicles [0.03 mM]) resulted primarily in the mobilization of hexadecane entrapped within the sand matrix. In contrast, application of biosurfactant concentrations less than the CMC enhanced the in situ mineralization of entrapped hexadecane; however, this effect was dependent on the choice of bacterial isolate. The two *Pseudomonas* isolates tested, R4 and ATCC 15524, were used because they exhibit different patterns of biodegradation of hexadecane, and they also differed in their physical response to rhamnolipid addition. ATCC 15524 cells formed extensive multicell aggregates in the presence of rhamnolipid while R4 cells were unaffected. This behavior did not affect the ability of the biosurfactant to enhance the biodegradation of hexadecane in well-mixed soil slurry systems but had a large affect on the extent of entrapped hexadecane biodegradation in the sand-packed-column system that was used in this study.

Water flushing of soils or subsurface materials is inefficient in the removal of nonaqueous-phase liquids (NAPLs) (e.g., nonpolar hydrocarbons and chlorinated solvents), which can be retained as relatively immobile and discontinuous globules (5, 9, 17). The NAPL retained within a porous matrix, referred to as residual NAPL, represents a long-term source for the contamination of subsurface water. Removal of entrapped NAPL can be enhanced by the use of surfactants, including synthetic surfactants (5, 13) and surfactants of biological origin (1, 2, 4, 16). Surfactant-enhanced removal of residual hydrocarbon requires surfactant concentrations greater than the critical micelle concentration (CMC), which is the concentration where the surfactant molecules spontaneously aggregate into micelles or vesicles. Surfactant-enhanced flushing of contaminated soil also requires that the surfactant-mobilized NAPL be collected and treated.

An alternative to flushing strategies is to promote the in situ biodegradation of entrapped contaminants. There have been several reports showing that biodegradation of hydrocarbons that have low solubility or that may be sorbed by soil particles can be enhanced by the addition of biosurfactants (3, 8, 12, 18). The effect of biosurfactant addition is not yet well understood. Similar to synthetic surfactants, biosurfactants can increase the bioavailability of hydrocarbons by increasing hydrocarbon solubility and desorption. But there is an additional level of complexity when a biosurfactant is added to the system, because biosurfactants can influence surface properties of degrading cells, resulting in enhanced hydrocarbon utilization (19). For this reason, biosurfactants can be effective at very low concentrations (20). However, there is little information available concerning the use of low concentrations of biosurfactant in systems that contain porous media.

There were two objectives of this study. The first was to examine the influence of biosurfactant concentrations less than the CMC on in situ biodegradation of residual NAPL under saturated flow conditions. The second objective was to determine whether biosurfactant effects are isolate specific. To address these objectives, saturated flow experiments were carried out with a sand-packed column containing residual NAPL and one of two hydrocarbon-degrading *Pseudomonas aeruginosa* isolates. Both of these isolates exhibit an enhanced rate of hydrocarbon (hexadecane) biodegradation in the presence of less than the CMC of biosurfactant; however, they differ in their pattern of growth on hexadecane. The biosurfactant chosen was a mixture of mono- and dirhamnolipids produced by an environmental isolate, *P. aeruginosa* R4.

MATERIALS AND METHODS

Cultures. A variety of *P. aeruginosa* isolates were used in this study. These isolates can be divided into two groups based on their patterns of growth on hydrocarbons (19). Two of the isolates, one representative of each group, were studied more intensively. These were *P. aeruginosa* R4, a cantaloupe root epiphyte, and *P. aeruginosa* ATCC 15524, originally isolated from soil. R4 was maintained on PTYG agar (peptone, 5 g liter^{-1}; tryptone, 5 g liter^{-1}; yeast extract, 10 g liter^{-1}; glucose, 10 g liter^{-1}; $MgSO_4 \cdot 7H_2O$, 0.6 g liter^{-1}; and $CaCl_2 \cdot 2H_2O$, 0.07 g liter^{-1}), and ATCC 15524 was maintained on nutrient agar (Difco, Detroit, Mich.). Inocula were prepared by growing R4 at room temperature in mineral salts medium (MSM) containing 0.2% glucose and by growing ATCC 15524 at room temperature in nutrient broth. After 48 h, the cells were washed twice in MSM and then suspended in 40 ml of MSM for at least 48 h to ensure that all alternative carbon sources had been utilized before the cells were exposed to hexadecane. The MSM consisted of 1.0 g of KH_2PO_4, 1.0 g of Na_2HPO_4, 0.5 g of NH_4NO_3, 0.5 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.02 g of $CaCl_2 \cdot 2H_2O$, 0.002 g of $FeCl_3$, and 0.002 g of $MnSO_4 \cdot 2H_2O$ per liter of water.

All of the isolates studied can produce rhamnolipid under appropriate conditions. However, it should be noted that neither R4 nor ATCC 15524 produced rhamnolipid during the experiments outlined below.

Biosurfactant production, extraction, and purification. For biosurfactant production, R4 was grown in MSM containing 20 g of glucose liter^{-1} (11). Biosur-

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factant was extracted, purified, and characterized as described previously (18–20) and then quantified by surface tension measurement with a Model 21 Fisher Scientific tensiomat (11). R4 rhamnolipid is a mixture of mono- and dirhamnolipids, the composition of which was analyzed by high-performance liquid chromatography (HPLC). Based on HPLC results, the average molecular weight of R4 rhamnolipid is estimated to be 564. The CMC of R4 rhamnolipid in MSM was determined to be 0.03 mM.

Biosurfactant solutions used in this study were prepared in two separate ways. Biosurfactant solutions of a low concentration (between 0.01 and 0.1 mM) were prepared by filter sterilizing the supernatant fluid from glucose-grown R4 cultures and then diluting it into MSM. When a higher biosurfactant concentration was required, the rhamnolipid that had been extracted and purified from the supernatant was dissolved in MSM. The concentrations of rhamnolipid in each solution were standardized by surface tension measurement. A preliminary study showed that there was little difference between the two rhamnolipid preparations in their ability to promote hexadecane mineralization (data not shown).

Chemicals. Hexadecane, radiolabeled hexadecane (*n*-[1-^{14}C]hexadecane, 2.2 mCi/mmol), and D-[^{14}C]glucose (specific activity, 251 mCi/mmol) were obtained from Sigma (St. Louis, Mo.).

Column studies. Column studies were performed to examine the biodegradation of hexadecane entrapped within a porous medium. A stainless steel column (length, 7 cm; diameter, 2.1 cm; Alltech, Deerfield, Ill.) was packed with autoclaved, oven-dried 40/50-mesh Accusand (North Kato Supply, Mankato, Minn.) by the following procedure. Sand was placed into the column to a height of 2 cm, and then 10 μl of [^{14}C]hexadecane (0.095 μCi μl^{-1}) was added with a Hamilton syringe. The remaining sand was packed in 1-cm layers, and each layer was thoroughly mixed to distribute the hexadecane throughout the column. The packed column was saturated from the bottom up with 0.01 M NaCl by gradually increasing the flow rate from 0.03 ml min^{-1} to 0.3 ml min^{-1} over 3 days by using a metering pump (Fluid Metering Inc., Oyster Bay, N.Y.). During the saturation period, the column effluent was collected and the amount of radioactivity released was determined with a Beckman Model 1600 TR liquid scintillation counter (Packard Instruments, Meriden, Conn.). No more than 3% of the radioactivity initially loaded into the column was removed during column saturation; the remainder was entrapped within the column.

An initial study was used to determine the concentration of biosurfactant required to mobilize the entrapped hexadecane under sterile conditions. In this study, biosurfactant solutions in MSM were flushed sequentially (at a flow rate of 0.3 ml min^{-1}) through a column containing entrapped [^{14}C]hexadecane. Sterility within the column was maintained by adding $HgCl_2$ (50 mg liter^{-1}) to the biosurfactant solution. The biosurfactant treatments were 0.015 mM for 138 pore volumes, 0.13 mM for 62 pore volumes, and 1.5 mM for a final 62 pore volumes. The removal of [^{14}C]hexadecane from the column was monitored by collecting 12-ml effluent fractions and transferring 1 ml into liquid scintillation cocktail (Scintiverse BD; Fisher Scientific) for determination of radioactivity.

The next series of column experiments examined the mineralization of entrapped [^{14}C]hexadecane. Columns containing entrapped [^{14}C]hexadecane were inoculated by loading 20 pore volumes of R4 or ATCC 15524 cell suspension (10^7 CFU ml^{-1} in MSM) into the column at a flow rate of 0.3 ml min^{-1}. The columns were then treated with MSM containing between 0 and 1.0 mM biosurfactant, depending on the experiment, for up to 500 pore volumes. Column effluent fractions (12 ml) were collected in test tubes containing 1 ml of 1 M NaOH to inhibit microbial activity and to trap CO_2, and every fifth fraction was subsampled (1 ml) for liquid scintillation analysis.

Selected column effluent fractions were examined to determine the form in which radioactivity was being removed from the column. Mineralization of [^{14}C]hexadecane produces $^{14}CO_2$, and the amount of $^{14}CO_2$ in the column effluent was determined by quantifying the amount of radioactivity that was purged from the effluent following acidification with 1 ml of a H_2SO_4 solution (20% [vol/vol]). The presence of [^{14}C]hexadecane-free product in the column effluent was determined by thin-layer chromatography (TLC) following the method of Harvey et al. (6). Briefly, column effluent was extracted with methylene chloride, and the solvent extract was reduced in volume to 100 μl. An aliquot of the solvent extract (10 μl) was spotted onto a TLC plate (Whatman Silica Gel AL-SIL-G), and the hexadecane was separated with pentane as the mobile phase. A control lane was spotted with pure [^{14}C]hexadecane to determine the migration of hexadecane. Strips were cut from each lane and transferred into liquid scintillation cocktail in order to determine the presence of [^{14}C]hexadecane.

Cell aggregate formation. The isolates used in this study showed differing characteristics with regard to growth on hexadecane, particularly in the presence of biosurfactants. One striking difference between the two groups of isolates studied pertained to the ability to form multicell aggregates. The ability to form cell aggregates was quantified with ^{14}C-labeled microorganisms. ^{14}C-labeled cells were prepared by adding D-[^{14}C]glucose (final concentration, 2 g liter^{-1}; specific activity, 4.5 μCi mmol^{-1}) into Kay's minimal medium. The medium was inoculated and incubated at 37°C for 24 h and then filtered through a Whatman no. 1 paper filter (pore size, 11 μm) to remove any clumped cells. The cells in the filtrate were inoculated into 10 ml of MSM (1.5×10^8 cells ml^{-1}) containing varying concentrations of rhamnolipid (0, 0.01, and 0.1 mM) and hexadecane (0 and 1.0 μl ml^{-1}). Samples were incubated with gyratory shaking (200 rpm) at room temperature for 4 h and then were filtered through Whatman no. 1 paper filters (diameter, 25 mm) and washed with MSM three times. The filters containing the cell aggregates were added to 10 ml of Scintiverse BD, and radioactivity was determined. In some cases, the composition of the multicell aggregates was examined by phase-contrast microscopy.

Hexadecane mineralization in a well-mixed batch system. Isolate-specific mineralization of hexadecane was determined in a slurry containing MSM and 20 g of Accusand 40/50 mesh. The sand was placed in 125-ml micro-Fernbach flasks, autoclaved, and then oven dried. [^{14}C]hexadecane (10 μl; specific activity, 3,500 dpm μl^{-1}) was added to the dry sand and thoroughly mixed in order to coat the sand particles. MSM (9 ml) containing 0.01 or 0.1 mM biosurfactant was then added along with an inoculum (0.1 ml) of R4 or ATCC 15524 cell suspension to establish an initial cell density of 10^7 CFU ml^{-1}. The flasks were incubated at room temperature on a gyratory shaker at 100 rpm. The production of $^{14}CO_2$ was determined after 1, 2, 3, 5, 7, 9, 12, and 15 days by purging the headspace through a series of traps containing 10 ml of Oxosol (National Diagnostics, Atlanta, Ga.) and then quantifying the radioactivity.

RESULTS

Column studies. A series of individually packed columns were used to determine the effect of rhamnolipid on biodegradation of residual hexadecane under saturated flow conditions. The physical properties of these columns were as follows: the bulk density was between 1.73 and 1.75 g cm^{-3}, the porosity was between 0.33 and 0.35, and the pore volume was between 8.1 and 8.4 ml.

The ability of the biosurfactant to remove entrapped hexadecane by mobilization was tested under sterile conditions by treating a column with 0.015 mM (for 138 pore volumes), 0.13 mM (for 62 pore volumes), and 1.5 mM (for 62 pore volumes) biosurfactant, representing a total flushing volume of 2.3 liters. Hexadecane mobilization was not promoted by the lowest biosurfactant concentration, 0.015 mM, which was 0.5 times the CMC. Addition of 0.13 mM biosurfactant (4.3 times the CMC) resulted in the mobilization of less than 3% of the residual hexadecane in 62 pore volumes. However, 1.5 mM rhamnolipid, which was 50 times the CMC, resulted in the rapid mobilization of almost 75% of the residual hexadecane within 62 pore volumes (data not shown). These results confirm that a biosurfactant concentration greater than the CMC is required for the mobilization of residual hexadecane.

Since it was apparent that entrapped hydrocarbon could be physically removed by biosurfactant concentrations greater than the CMC, the focus of the biodegradation studies was to evaluate whether biosurfactant concentrations less than the CMC could promote hydrocarbon removal by biodegradation. The biodegradation of residual hexadecane within sand-packed columns was first examined with *P. aeruginosa* R4. The results of two column studies are shown in Fig. 1. The first 20 pore volumes represent the period in which the cell suspension was loaded into each column. After loading of the cell suspension was completed, one column was flushed with MSM alone (column experiment R4-MSM) and the second column was flushed with MSM containing 0.01 mM biosurfactant (column experiment R4-B01). In the absence of rhamnolipid, there was a lag phase until 80 pore volumes, and then radioactivity was detected in effluent fractions, indicating that R4 was mineralizing entrapped hexadecane. Biodegradation continued until 53% of the radioactivity was removed from the column at 454 pore volumes. The addition of 0.01 mM biosurfactant decreased the lag phase for hexadecane biodegradation to 25 pore volumes and increased the rate of biodegradation compared to the control (see the slope of each curve shown in Fig. 1). Total removal of radioactivity by biodegradation in the presence of biosurfactant was 58% after only 310 pore volumes. Table 1 shows the proportion of radioactivity found as $^{14}CO_2$ and [^{14}C]hexadecane in selected fractions from each column. Up to 47.9% of the released radioactivity was $^{14}CO_2$, confirming the mineralization of hexadecane. There was no

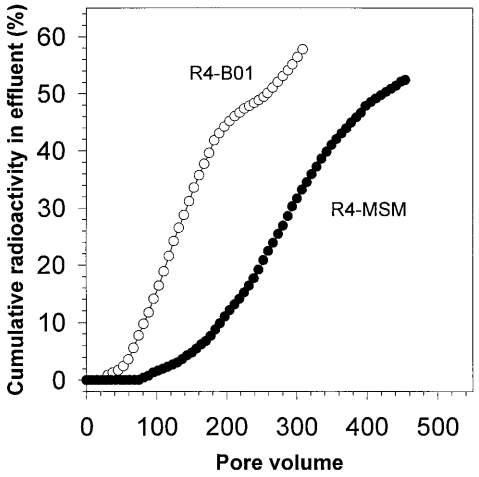

FIG. 1. Cumulative release of radioactivity from sand-packed columns during the mineralization of residual [^{14}C]hexadecane by *P. aeruginosa* R4. The first 20 pore volumes represent the loading of the R4 cell suspensions, after which one column was flushed with MSM alone (R4-MSM) and the other was flushed with MSM containing 0.01 mM biosurfactant (R4-B01).

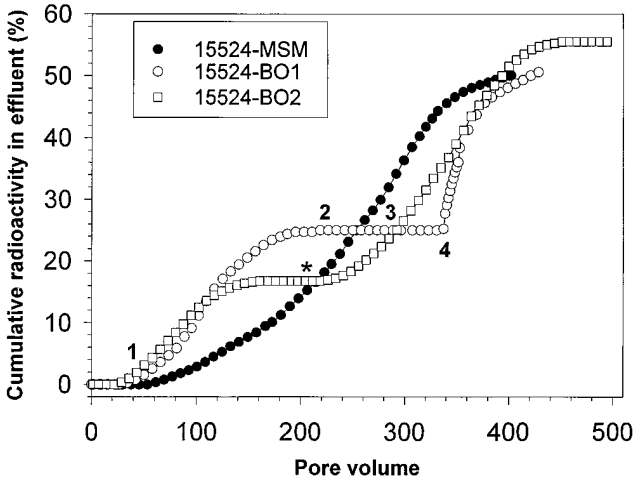

FIG. 2. Cumulative release of radioactivity from sand-packed columns during the mineralization of residual [^{14}C]hexadecane by *P. aeruginosa* ATCC 15524. The first 20 pore volumes represent loading of the ATCC 15524 cell suspensions. Various treatments followed depending on the experiment. For column experiment 15224-MSM, only MSM was used. For 15524-B01, 0.01 mM biosurfactant was loaded beginning at the point indicated by the numeral 1, 0.03 mM biosurfactant was loaded beginning at the point indicated by the numeral 2, 0.1 mM biosurfactant was loaded beginning at the point indicated by the numeral 3, and 1.0 mM biosurfactant was loaded beginning at the point indicated by the numeral 4. For 15524-B02, 0.1 mM biosurfactant was loaded beginning at pore volume 1 and then MSM was loaded beginning at the point indicated by the asterisk.

[^{14}C]hexadecane detected, indicating, as expected, no removal by mobilization. The remaining radioactivity removed from the column was probably in the form of undefined cellular metabolites, as discussed in the paper by Herman et al. (7).

A similar series of column experiments were performed with ATCC 15524 (Fig. 2). When the column was treated with MSM alone (column experiment 15524-MSM), hexadecane biodegradation began after 53 pore volumes, and 50% of the radioactivity was removed from the column after 402 pore volumes. In the presence of 0.01 mM biosurfactant (column experiment 15524-B01), the lag phase for hexadecane biodegradation was reduced to 35 pore volumes and the initial rate of biodegradation increased. However, a plateau in release of radioactivity occurred after 180 pore volumes, when only 25% of the radioactivity had been removed from the column. Therefore, at pore volume 218 the concentration of biosurfactant loaded into the column was increased to 0.03 mM (CMC) for 58 pore volumes. This increase did not stimulate the resumption of hexadecane biodegradation. The biosurfactant concentration was increased further at pore volume 276 to 0.1 mM (three times the CMC) for 61 pore volumes, but again no increase in hexadecane biodegradation occurred. Finally, at pore volume 337, 1.0 mM (30 times the CMC) biosurfactant was applied to the column for 90 pore volumes. With this treatment, there was a rapid increase in removal of radioactivity, primarily as [^{14}C]hexadecane (Table 1).

This first ATCC 15524 column experiment showed that

TABLE 1. Analysis of selected column effluent fractions to determine the source of radioactivity

Column expt	Treatment	Pore volumes combined[a]	^{14}C as $^{14}CO_2$ (%)[b]	Presence of hexadecane
R4-MSM	MSM	255–258	42.8, 42.2	ND[c]
		283–286	37.7, 34.1	ND
R4-B01	0.01 mM Rham.[d]	154–158	30.0, 32.4	–
		162–181	21.5, 23.5	ND
		307–310	47.9, ND	–
15524-MSM	MSM	278–329	33.4, 35.1	ND
15524-B01	0.01 mM Rham.	96–113	36.2, 44.9	–
	1.0 mM Rham.	339–355	6.1, 9.6	+
15524-B02	0.01 mM Rham.	82–102	31.5, 35.2	–
	MSM	349–370	43.8, 49.5	–

[a] Range of pore volumes combined into one sample.
[b] Duplicate determinations were performed, and both values are shown.
[c] ND, not determined.
[d] Rham., rhamnolipid.

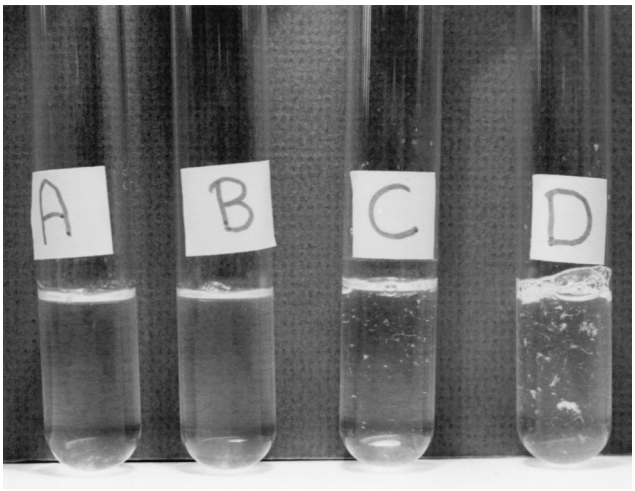

FIG. 3. Appearance of multicell aggregates when *P. aeruginosa* isolates were grown on hexadecane for 24 h. The photograph shows R4 in MSM containing 0 (tube A) and 0.1 (tube B) mM biosurfactant and shows ATCC 15524 grown in 0 (tube C) and 0.1 (tube D) mM biosurfactant.

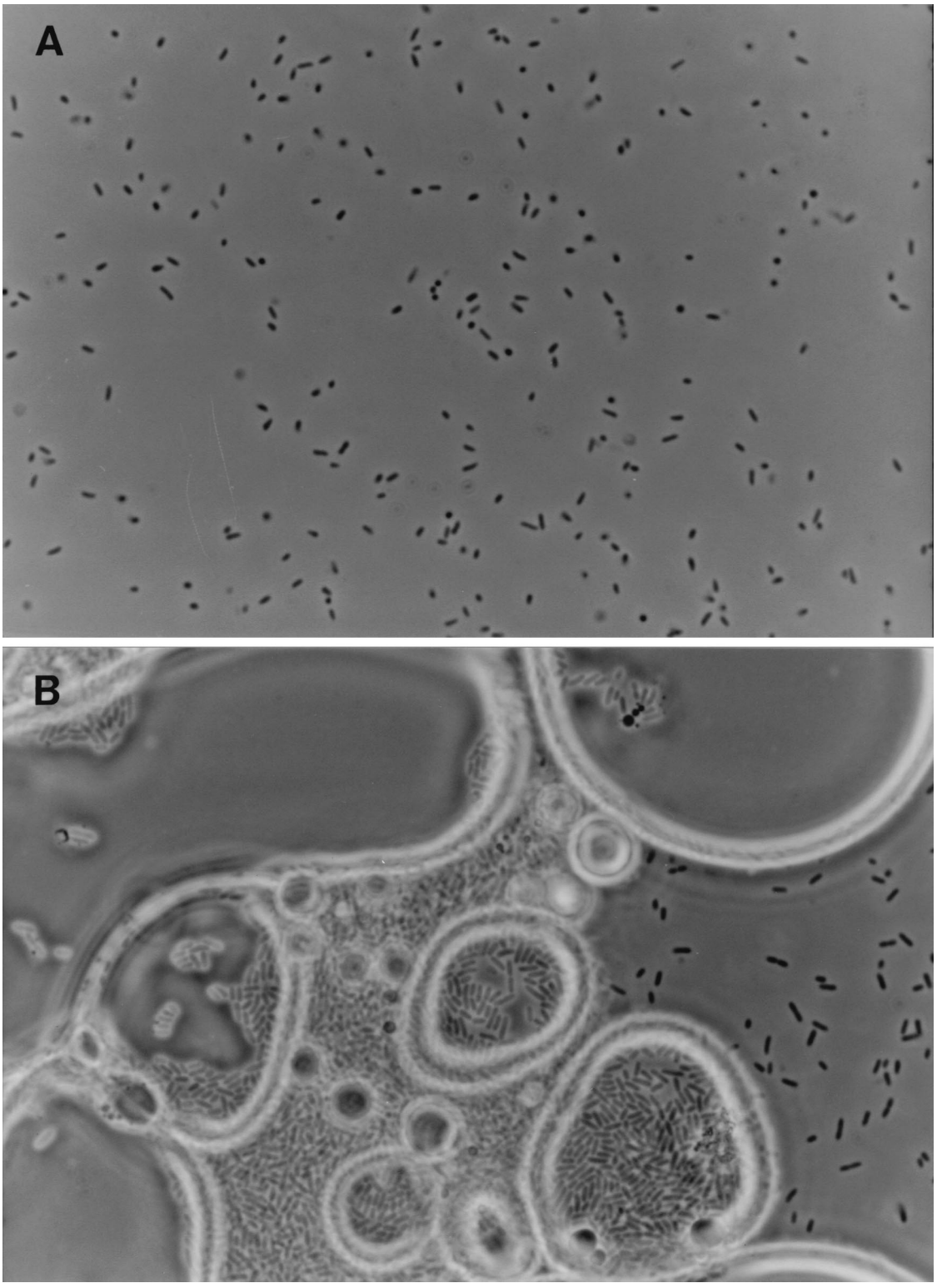

FIG. 4. Aggregation of ATCC 15524 in the absence (A) and presence (B) of biosurfactant. Pictures were taken with a phase-contrast microscope. Magnification, ×1,000.

while the biosurfactant could initially enhance the mineralization of residual hexadecane, the removal of radioactivity from the column by mineralization was limited to 25% and any further removal of radioactivity required the mobilization of residual hexadecane by a concentration of biosurfactant greater than the CMC. In the next column experiment, 15524-B02, the initial biosurfactant treatment was increased 10-fold to 0.1 mM. Mineralization of residual hexadecane was detected after 29 pore volumes; however, a plateau in hexadecane mineralization was again observed after 150 pore volumes, when only

17% of the radioactivity had been removed from the column. At this time the inlet reservoir was replaced with a solution of MSM to wash biosurfactant out of the column (pore volume 211). After a 20-pore-volume lag phase, mineralization of residual hexadecane resumed and continued until 56% of the hexadecane had been removed from the column after a total of 500 pore volumes. In this case, no [^{14}C]hexadecane was recovered and 33 to 47% of the radioactivity was in the form of $^{14}CO_2$ (Table 1).

Cell aggregation. A series of batch experiments were performed to try to explain the marked and repeatable difference in behavior between R4 and ATCC 15524 in the column studies. Recall that *P. aeruginosa* isolates were categorized into one of two groups in a previous investigation based upon differences in growth on hydrocarbons (19). We had casually observed in these experiments that group 2 isolates had a tendency to clump during growth on hydrocarbon in the presence of rhamnolipid while group 1 isolates did not. Therefore, aggregate formation by six isolates, three from each group, was quantified (Table 1). The results indicate that group 1 isolates, including R4, NRRL 3198, and ATCC 9027, had low aggregation in the presence of hexadecane alone (9.2% of the R4 cells were aggregated) and the amount of aggregation was unaffected by addition of rhamnolipid. In contrast, group 2 isolates, including ATCC 15524, ATCC 27853, and ATCC 15442, had a higher tendency to aggregate (25.6% for ATCC 15524) in the presence of hexadecane alone, and the addition of rhamnolipid greatly increased cell aggregation.

Direct observation of this phenomenon is shown in Fig. 3 and 4. Figure 3 is a direct comparison of R4 and ATCC 15524 showing the large clumps that form when ATCC 15524 is grown on hexadecane or on hexadecane and rhamnolipid. Microscopic examination of the ATCC 15524 culture revealed that the aggregates were composed of an abundance of cells surrounding hexadecane droplets (Fig. 4).

Comparison of batch and column conditions. A further experiment was performed to determine whether cell aggregation would affect hexadecane mineralization in a well-mixed batch system in a manner similar to the way it affected hexadecane mineralization in the column system (Fig. 1 and 2). Results from these experiments showed that for both R4 and ATCC 15524, addition of either 0.01 or 0.1 mM rhamnolipid stimulated the rate of mineralization of hexadecane (data not shown). The increase in mineralization rate directly corresponded to the biosurfactant concentration. These results indicate that in a well-mixed batch system cell aggregation does not impact mineralization.

TABLE 2. The effect of rhamnolipid on cell aggregation under nongrowth conditions

Strain	% Cell aggregation (mean ± SD) with:				
	C_{16} alone[a]	0.02 mM:		0.1 mM:	
		Rham.[b]	Rham. + C_{16}[a]	Rham.	Rham. + C_{16}[a]
Group 1					
NRRL 3198	5.9 ± 0.5	ND[c]	ND	3.3 ± 0.3	4.6 ± 0.0
ATCC 9027	7.4 ± 0.7	ND	ND	8.8 ± 2.2	10.0 ± 1.8
R4	9.2 ± 0.4	9.9 ± 1.2	9.2 ± 0.3	10.4 ± 1.0	8.5 ± 0.4
Group 2					
ATCC 27853	17.6 ± 2.4	ND	ND	34.6 ± 1.1	51.0 ± 3.8
ATCC 15524	25.6 ± 3.2	37.8 ± 2.4	49.6 ± 1.1	39.5 ± 5.5	71.4 ± 2.0
ATCC 15442	50.4 ± 5.2	ND	ND	96.3 ± 3.7	96.2 ± 2.1

[a] Hexadecane concentration was 3.4 mM.
[b] Rham., rhamnolipid.
[c] ND, not determined.

DISCUSSION

The results of this study show that biosurfactants can be used to enhance the removal of residual hexadecane: either entrapped hydrocarbon can be mobilized with high concentrations of biosurfactant or biodegradation can be promoted with low concentrations of biosurfactant. In the latter option, the response of the degrading isolate is important to the success of hydrocarbon removal. The isolates used in this study, R4 and ATCC 15524, were representative of two groups of hydrocarbon-degrading strains of *P. aeruginosa*. Although both groups of organisms show a positive response to biosurfactant addition in a well-mixed batch reactor, there was a varied response in a column system that more closely mimics a natural soil system. For one group, represented by R4, biosurfactant addition enhanced the rate of residual hydrocarbon biodegradation during the entire period of hydrocarbon degradation (Fig. 1). In contrast, ATCC 15524, which represents a second group of *P. aeruginosa* strains, initially showed an enhanced rate of mineralization in the presence of biosurfactant but then exhibited an abrupt plateau in biodegradation after 17 to 25% removal (Fig. 2). A major difference in the behavior of these two groups of bacteria is that only group 2 isolates form large multicell aggregates in the presence of a combination of hydrocarbon and biosurfactant (Table 2 and Fig. 3).

Previous studies have shown that surfactants can either promote or inhibit hydrocarbon biodegradation (3, 4, 14, 15, 19). A surfactant can increase hydrocarbon bioavailability by increasing the apparent aqueous solubility and/or desorption of the hydrocarbon. The increase in bioavailable hydrocarbon allows increased rates of biodegradation. However, biodegradation can also be influenced by an interaction between a surfactant and the hydrocarbon degrader (19). The mechanisms by which hydrocarbon biodegradation is inhibited are not completely understood, although a surfactant may be toxic to degrading cells or may serve as an alternate carbon source (10). The results of this study suggest that in situ hydrocarbon biodegradation may also be inhibited by a surfactant that promotes the physical aggregation of cells. The incomplete removal of residual hexadecane by ATCC 15524 may be due to the combined effect of cell aggregation and the discontinuous manner in which residual hexadecane is distributed within the porous matrix. For group 1 isolates that do not aggregate, there can be movement of bacteria with water flow from a colonized globule that is actively being degraded to an uncolonized globule. However, for bacteria like ATCC 15524 that have a strong aggregation tendency in the presence of biosurfactant, the ability to redistribute within the porous matrix may be restricted. Cell aggregation may limit the physical contact between residual hexadecane and the degrading cells. This hypothesis is supported by the fact that when MSM was added to the 15524-B02 column, mineralization resumed again, presumably because the removal of the rhamnolipid caused the 15524 cell aggregates to dissociate and redistribute within the column.

In summary, the results of this study reveal that low biosurfactant concentrations can, at least partially, promote the in situ bioremediation of residual hexadecane. The success of biosurfactant treatment will depend on the response of the microorganisms present, as was indicated by the differences evident between the two closely related *P. aeruginosa* isolates in this study. The results also reveal that the response of a hydrocarbon-degrading isolate to the presence of a biosurfac-

tant can be very different when tested in a soil slurry environment compared to a saturated-flow column system. While the exact nature of the interaction between a bacterium, a hydrocarbon, and a biosurfactant is not fully understood, the results of this study increase understanding of this interaction. Our laboratory is continuing to investigate the mechanism of interaction between biosurfactants, degrading cells, and the physical environment in order to evaluate the efficacy of biosurfactant application to the remediation of hydrocarbon-contaminated soil environments.

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