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Bacterial Adhesion to Soil Contaminants in the Presence of Surfactants

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It has been proposed that addition of surfactants to contaminated soil enhances the solubility of target compounds; however, surfactants may simultaneously reduce the adhesion of bacteria to hydrophobic surfaces. If the latter mechanism is important for the biodegradation of virtually insoluble contaminants, then the use of surfactants may not be beneficial. The adhesion of a *Mycobacterium* strain and a *Pseudomonas* strain, isolated from a creosote-contaminated soil, to the surfaces of highly viscous non-aqueous-phase liquids (NAPLs) was measured. The NAPLs were organic material extracted from soils from two creosote-contaminated sites and two petroleum-contaminated sites. Cells suspended in media with and without surfactant were placed in test tubes coated with an NAPL, and the percentages of cells that adhered to the surface of the NAPL in the presence and absence of surfactant were compared by measuring optical density. Test tubes without NAPLs were used as controls. The presence of either Triton X-100 or Dowfax 8390 at a concentration that was one-half the critical micelle concentration (CMC) inhibited adhesion of both species of bacteria to the NAPLs. Both surfactants, when added at concentrations that were one-half the CMCs to test tubes containing previously adhered bacteria, also promoted the removal of the cells from the surfaces of the NAPL-coated test tubes. Neither surfactant was toxic to the bacteria. Further investigation showed that a low concentration of surfactant also inhibited the growth of both species on anthracene, indicating that the presence of a surfactant resulted in a reduction in the uptake of the solid carbon source.

Industrial chemicals, such as hydrocarbons, have been released into the soil environment as a result of mechanical failure, incineration practices, corrosion, leakage, accidental spillage, and improper disposal practices (5, 24, 37, 44). Biodegradation is an attractive method for remediating contaminated sites because of its economic viability and environmental soundness. One limitation of biodegradation, however, is that many hydrocarbons are poorly accessible to bacteria. Heavily contaminated soils contain a separate non-aqueous-phase liquid (NAPL), which may be present as droplets or films on soil surfaces. Biodegradation takes place more readily when the target contaminants are dissolved in an aqueous solution (14, 27, 31, 40, 48, 49), but many hydrocarbons are virtually insoluble in water and remain partitioned in the NAPL. Thus, there have been efforts to improve the bioavailability of hydrocarbons through the use of surfactants.

Both nonionic and anionic surfactants increase the solubility of hydrocarbons by forming micelles (10, 15–17, 35). The surfactants begin to assemble into micelles at the critical micelle concentration (CMC), and the interiors of the micelles provide a hydrophobic environment to solubilize nonpolar compounds, such as hydrocarbons. No enhancement of solubility is observed at concentrations below the CMC. While some research groups have found that the presence of surfactants enhances biodegradation (2–4, 7, 11, 20, 41, 45, 46), others have found that the presence of surfactants inhibits biodegradation (1, 7, 9, 11, 14, 18, 19, 41, 47). This discrepancy within the literature indicates that there is a need to understand the mechanism of biodegradation in the presence of surfactants.

Diffusion of hydrocarbons to bacteria for use as growth substrates can occur by several pathways. Hydrocarbons can dissolve from the NAPL into the aqueous phase. As the bacterial population increases, however, the rate of uptake increases while the rate of dissolution remains constant (40). If surfactant is present at a concentration above the CMC, hydrocarbons can dissolve from the micelles into aqueous solution. Direct interactions between cells and micelles can also occur. Both of these dissolution pathways rely on mixing and diffusion in the aqueous phase to bring the hydrophobic compounds to the bacteria. A more direct pathway is adhesion of the bacteria to the interface between the NAPL and the aqueous phase. A number of species of bacteria are able to degrade liquid hydrocarbons after adhering to the surfaces of droplets (8, 11, 23, 26, 31, 32). This direct contact between a bacterial cell and a target hydrocarbon can significantly increase the rate of diffusion into the cell, thereby enhancing growth and increasing the apparent rate of dissolution of the hydrocarbon. While few bacteria that grow on viscous tars or solid hydrocarbons have been identified, growth of a *Mycobacterium* species (previously described as a *Rhodococcus* species) as a biofilm on solid anthracene has been observed (43), suggesting that immediate proximity of a solid carbon source to a cell has the same benefit as it does in the case of liquid hydrocarbons.

The overall impact of addition of a surfactant on biodegradation depends on how the basic diffusion pathways are altered and whether the surfactant itself affects the cells. If the surfactant is neither toxic nor a growth substrate, it can either increase the rate of biodegradation by carrying hydrocarbons in relatively accessible micelles, or it can decrease the rate by inhibiting the adhesion of cells to the NAPL-water interface. The overall impact depends on the importance of each path-

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way. The presence of surfactants at concentrations above the CMC does inhibit adhesion of bacteria to the surfaces of droplets of liquid hydrocarbons and thus inhibits biodegradation (11, 26, 30). The sorption of surfactants to bacteria and to interfaces can either enhance or inhibit adhesion, depending on the nature of the surfaces and the surfactant itself (25). The alterations of surfaces depend only on the concentration of free surfactant; therefore, they are significant at concentrations below the CMC.

The purpose of this study was to study the adhesion of hydrocarbon-degrading bacteria to the surfaces of NAPL materials that occur in contaminated soils, with and without added surfactants. These NAPL materials are much more viscous than hydrocarbon liquids, such as hexadecane, which have been studied in the past. Our hypothesis was that sorption of surfactants to cells or to NAPL surfaces would inhibit bacterial adhesion to the NAPL surfaces. The adhesion of two polycyclic aromatic hydrocarbon (PAH)-degrading organisms to highly viscous NAPLs was measured in the presence of two surfactants, one nonionic (Triton X-100) and one anionic (Dowfax 8390). Because the sorption of a surfactant to surfaces depends on the free concentration of the surfactant in solution, modifications of surface properties are significant at concentrations below the CMC. So that we could concentrate on surface adhesion and avoid solubilizing the NAPL components in micelles, each surfactant was used at one-half its CMC. Growth of the bacteria on anthracene was used to determine whether surfactants had any influence on bacterial growth on a solid carbon source.

MATERIALS AND METHODS

NAPL extracts from contaminated soils. NAPL extracts from four different contaminated soils were used in the adhesion experiments. The soils originated from industrial sites in Edmonton, Alberta, Canada; Prince Albert, Saskatchewan, Canada; Devon, Alberta, Canada; and Montreal, Quebec, Canada. The Edmonton (EDM) and Prince Albert (PAA) soils were primarily contaminated with creosote, while the Devon (DEV) and Montreal (MTL) soils were primarily contaminated with petroleum hydrocarbons. The MTL soil had been partially bioremediated before the investigation was begun. The organic material was extracted from approximately 15 g of each soil with 200 ml of methylene chloride by using a Soxhlet extraction apparatus over a period of 8 to 10 h. The viscosity of each NAPL was determined at 23 and 60°C by using a Carri-Med CLS controlled stress rheometer. At 23°C, the stress was applied linearly from 0 to 4,000 dynes/cm^2 for the DEV and EDM NAPLs and from 0 to 1,000 dynes/cm^2 for the PAA NAPL. The viscosity of the MTL NAPL could not be determined at this temperature. At 60°C, the stress was applied linearly from 0 to 2,000 dynes/cm^2 for the MTL NAPL and from 0 to 100 dynes/cm^2 for the other NAPLs.

Bacteria. In this study we used two hydrocarbon-degrading bacterial strains isolated from a creosote-contaminated soil, a gram-positive *Mycobacterium* strain and a gram-negative *Pseudomonas* strain (13). The *Mycobacterium* strain was originally described as *Rhodococcus* sp. strain S1 based on chemotaxonomic data (43) but was recently reclassified as a *Mycobacterium* strain on the basis of 16S rRNA analysis (6). This analysis showed that this strain is closely related to, but not identical to, *Mycobacterium fortuitum*. The 16S rRNA sequence data has been deposited in the EMBO database under accession no. Y15709 (*Mycobacterium* sp. strain S1). The *Pseudomonas* strain was not characterized in as much detail as the *Mycobacterium* strain was.

The bacteria were grown in a medium containing (per liter) 1.33 g of KH_2PO_4, 2.67 g of K_2HPO_4, 1 g of NH_4Cl, 2 g of Na_2SO_4, 2 g of KNO_3, 0.01 g of $FeSO_4 \cdot 7H_2O$, and 1 ml of a trace metal solution (12). The pH was 7.2. Anthracene at a concentration of 500 mg/liter was added to the medium before it was autoclaved for 20 min at 121°C. Sterile $MgSO_4 \cdot 7H_2O$ was then added to the medium to a concentration of 2 g/liter. Flasks containing the medium, each of which also contained a 1.2-cm-diameter steel coil to prevent aggregation, were incubated at 27°C on a New Brunswick gyratory shaker at 200 rpm for 14 days. To test for purity, the *Mycobacterium* strain was streaked onto plate count agar obtained from Difco Laboratories, Detroit, Mich., while the *Pseudomonas* strain was streaked onto Trypticase soy agar obtained from Becton Dickinson and Company, Cockeysville, Md. Aseptic technique was used for all transfers. Each set of plates was incubated at 27°C for 7 days.

Chemicals. Both a nonionic surfactant and an anionic surfactant were used in this investigation. The nonionic surfactant was Triton X-100, obtained from Rohm and Haas Company of Canada Limited, West Hill, Ontario, Canada. The anionic surfactant was Dowfax 8390, obtained from Dow Chemical Company, Midland, Mich. The CMC of each surfactant was determined by using a tensiometer (model 70545; Central Scientific Company, Chicago, Ill.). Anthracene was obtained from Sigma Chemical Company, St. Louis, Mo., and was reported to be 99% pure. Anhydrous D-glucose was obtained from BDH Inc., Toronto, Ontario, Canada. Methylene chloride was obtained from EM Science, Gibbstown, N.J., and was determined to be more than 99% pure (42).

Adhesion experiments. To determine whether surfactants affect the adhesion of bacteria to NAPLs, each organic extract was dissolved in methylene chloride to a concentration of 1 g/liter. Each test tube was prepared by adding 5 ml of this solution. The test tubes were rotated in a roller test tube rack at 12 rpm, which allowed the methylene chloride to evaporate while the inside surface of each test tube became coated with the NAPL. The bacteria were harvested from the growth medium after 14 days by centrifugation at 16,200 × *g* for 10 min. Each pellet was resuspended three times in 50 mM potassium phosphate buffer (pH 7.2). The resulting suspension, which had an optical density at 600 nm (OD_{600}) of approximately 0.6, was then divided into three parts. Triton X-100 was added to one part of the bacterial suspension to a concentration of 0.12 mM, and Dowfax 8390 was added to a second part of the bacterial suspension to a concentration of 0.4 mM. Surfactant was not added to the third part of the bacterial suspension so that it could be used as a control. A 50 mM potassium phosphate buffer solution was also divided into three parts, which were used as blanks; one part contained 0.12 mM Triton X-100, one part contained 0.4 mM Dowfax 8390, and one part did not contain any surfactant. These blanks were used to account for any NAPL that came off the surfaces of the test tubes, which altered the measured OD_{600}. The OD_{600} of each of the six solutions was recorded. Each of the six solutions was transferred to test tubes containing one of the four NAPLs and to test tubes that did not contain any NAPL (5 ml per tube). Test tubes for each set of conditions were prepared in triplicate, and the total number of test tubes was 90.

All of the test tubes were placed in a roller test tube rack and then were rotated at 12 rpm for 3 h. This time period was long enough to allow the cells to adhere to the test tube surfaces but brief enough to avoid growth or metabolite production that might have altered adhesion. The test tubes were then vortexed at the lowest speed setting for 30 s so that any cells that settled at the bottom of the test tubes could be resuspended but adherent cells were left on the inner surfaces of the test tubes. Then there was a 10-min settling period, which allowed any NAPLs that came off the surfaces of the test tubes to resettle, while the unadhered bacteria remained in suspension. Samples were withdrawn by placing the tips of Pasteur pipettes halfway between the meniscus and the bottom of each test tube. Approximately 1 ml of each sample was withdrawn, and the OD_{600} was measured. This protocol was repeated for both bacterial strains.

The percentage of cells that adhered to the surface of each NAPL (or glass in the case of the controls) in the presence of surfactant *x*, where *x* was Triton X-100, Dowfax 8390, or no surfactant, was calculated as follows:

$$(\%\ \text{of cells adhered})_x = \frac{(\text{initial OD}_{600}\ \text{of cells})_x - ([\text{final OD}_{600}\ \text{of cells}]_x - [\text{final OD}_{600}\ \text{of buffer}]_x)}{(\text{initial OD}_{600}\ \text{of cells})_x}$$

We assumed that any cells that were not in suspension had adhered to the NAPL or glass surface. At the end of the experiment, samples of each bacterial strain suspended in test tubes without NAPL were examined with a microscope to ensure that the cells did not aggregate or flocculate.

The next step in this study was to determine whether addition of either surfactant resulted in removal of bacteria that adhered to the surfaces in the absence of surfactant. The test tubes were prepared with the NAPLs, and the bacteria were suspended in 50 mM potassium phosphate buffer to an OD_{600} of approximately 0.6, as described above. The initial OD_{600} of each bacterial suspension was recorded before the suspension was transferred to test tubes with and without NAPL (5 ml per test tube). The 50 mM potassium phosphate buffer was also transferred to test tubes with and without NAPL, and these test tubes were used as blanks. The test tubes were placed in a roller test tube rack and rotated at 12 rpm for 3 h to allow the bacteria to adhere to the surfaces. Triton X-100 and Dowfax 8390 were then added to several test tubes to concentrations of 0.12 and 0.4 mM, respectively. For each set of test tubes (one set containing each NAPL and a control set without any NAPL), there were three test tubes containing the following: cells, cells and Triton X-100, cells and Dowfax 8390, buffer, buffer and Triton X-100, and buffer and Dowfax 8390. As described above, the test tubes were vortexed at the lowest speed setting for 30 s and then left so that the cells could settle for 10 min before the final OD_{600} of each sample was determined. The percentages of cells that remained adhered to the NAPLs were then calculated. This procedure was repeated for both bacterial cultures.

Bacterial growth on solid anthracene. To determine whether the presence of surfactant had any effect on the ability of the bacteria to grow on a solid carbon source, sterile flasks containing 50 mg of anthracene, 90 ml of sterile growth medium, and 10 ml of a bacterial suspension were prepared aseptically. Flasks containing either Triton X-100, Dowfax 8390, or no surfactant (control) were prepared in quintuplicate. A 1-ml sample was taken aseptically from each flask at zero time and on days 1, 2, 3, 5, 7, 10, and 14, and the OD_{600} of each sample was recorded as a measure of bacterial growth. At the end of the experiment, the *Mycobacterium* strain was streaked onto plate count agar and the *Pseudomonas* strain was streaked onto Trypticase soy agar to ensure that the cultures remained

TABLE 1. Viscosities of NAPLs and hexadecane at 23 and 60°C

Substance	Viscosity (cP) at:	
	23°C	60°C
EDM creosote-derived NAPL	95	10
PAA creosote-derived NAPL	55	8.3
DEV petroleum-derived NAPL	120	21
MTL petroleum-derived NAPL	ND[a]	11,000
n-Hexadecane	3.0	1.6

[a] ND, not determined.

pure and uncontaminated. This experiment was repeated with the *Pseudomonas* strain and 50 mg of glucose instead of anthracene in order to determine the effect of surfactants on bacterial growth in the presence of a soluble carbon source.

RESULTS

Viscosity of the NAPLs. The viscosity of each NAPL was determined at 23 and 60°C, as shown in Table 1. Each NAPL was much more viscous than hexadecane (28), which is a typical liquid hydrocarbon that has been used in previous bacterial adhesion studies (30–33). The MTL NAPL was essentially solid at room temperature.

Determination of the surfactant CMCs. The CMC of Triton X-100 was determined to be 0.24 mM, a value which has been reported elsewhere (18). The CMC of Dowfax 8390 was determined to be 0.8 mM, which is 1 order of magnitude lower than the value which has been reported elsewhere (34). Throughout this study, the surfactants were used at one-half their CMCs (0.12 mM for Triton X-100 and 0.4 mM for Dowfax 8390).

Initial adhesion of bacteria to the NAPL interface. The percentages of cells that adhered to both the coated test tubes and the clean test tubes in the presence and absence of surfactant are shown in Table 2. Approximately 40% of the *Mycobacterium* cells adhered to the four NAPLs in the absence of surfactant, while approximately 50% of the *Mycobacterium* cells adhered to the surfaces of the clean glass test tubes in the absence of surfactant. Fewer cells adhered to the surfaces if either surfactant was present. In the presence of Triton X-100, only 5 to 17% of the cells adhered to the NAPLs, while in the presence of Dowfax 8390, only 11 to 36% of the cells adhered to the NAPLs. Similarly, in the absence of any NAPL, only 5 and 19% of the cells adhered to the glass in the presence of Triton X-100 and Dowfax 8390, respectively. Clearly, the *Mycobacterium* strain was able to adhere to both the glass test tube surfaces and the NAPLs, but the presence of either surfactant inhibited adhesion. Triton X-100 at a concentration that was one-half its CMC, however, inhibited adhesion more effectively than Dowfax 8390 at a concentration that was one-half its CMC. No correlation between adhesion and viscosity of the NAPLs was apparent. The OD_{600} was linearly proportional to the concentration of bacteria, with an OD_{600} of 0.1 corresponding to approximately 10^8 CFU/ml (43). Examination of the suspension with the microscope revealed that no aggregation or flocculation of the *Mycobacterium* cells occurred during this experiment.

Between 37 and 53% of the *Pseudomonas* cells adhered to the NAPLs in the absence of surfactant, while fewer than 30 and 20% of the *Pseudomonas* cells adhered to any of the NAPLs in the presence of Triton X-100 and Dowfax 8390, respectively. Similarly, approximately 39% of the cells adhered to the clean glass in the absence of surfactant, while only 16% of the cells adhered to the glass in the presence of Triton X-100 and only 11% of the cells adhered to the glass in the presence of Dowfax 8390. Thus, even though the *Pseudomonas* strain was able to adhere to the various surfaces used in this study, both surfactants inhibited adhesion. Dowfax 8390, however, inhibited adhesion more effectively than Triton X-100. No consistent relationship between adhesion behavior and viscosity of the NAPLs was observed, with or without surfactants. The *Pseudomonas* cells did not form aggregates or flocs during this experiment.

Mobilization of the NAPLs was enhanced by adding surfactant. If no surfactant was present, the NAPLs tended to remain on the surfaces of the test tubes. The MTL NAPL, which was the most viscous NAPL, remained adhered to the surfaces of the test tubes in the presence of both surfactants. Portions of the other three NAPLs were removed from the surfaces of the test tubes in the presence of both surfactants, although Triton X-100 was much more effective at mobilizing these NAPLs than Dowfax 8390 was. Withdrawing samples from locations halfway down the test tubes minimized the collection of NAPL droplets with the pipette. The use of control samples that contained NAPL and surfactant but no cells corrected for any effect that NAPL droplets in the samples had on the OD_{600} measurements.

Removal of bacteria from the NAPL interface. If low levels of a surfactant inhibit the adhesion of cells to surfaces, they may also result in removal of previously adhered cells by sorbing to cell or NAPL surfaces (25). Table 3 shows the effects of surfactants on adhesion of both strains. Between 31 and 52% of the *Mycobacterium* cells remained adhered to the surfaces if no surfactant was added to the test tubes, but fewer cells remained if either surfactant was added. After Triton X-100 was added, the percentages of cells that remained adhered to the surfaces ranged from 4 to 30%, while after of Dowfax 8390 was added, the percentages of cells that remained adhered to the surfaces ranged from 3 to 26%. Clearly, addition of either surfactant resulted in removal of *Mycobacterium* cells from the NAPLs.

If no surfactant was added, between 34 and 52% of the *Pseudomonas* cells remained adhered to the surfaces. Fewer cells remained adhered to the clean test tubes, the EDM NAPL test tubes, and the PAA NAPL test tubes after either surfactant was added (15 to 35 and 14 to 31% of the cells remained adhered after addition of Triton X-100 and Dowfax 8390, respectively), and only 15% of the cells remained adhered to the DEV NAPL test tubes after Triton X-100 was

TABLE 2. Adhesion of cells to NAPLs

NAPL	Solution	% of *Mycobacterium* cells adhered to surface	% of *Pseudomonas* cells adhered to surface
EDM	Buffer	41 ± 2[a]	53 ± 2
	Triton X-100	17 ± 6	13 ± 3
	Dowfax 8390	36 ± 3	6 ± 2
PAA	Buffer	44 ± 3	50 ± 1
	Triton X-100	6 ± 5	28 ± 3
	Dowfax 8390	11 ± 7	14 ± 2
DEV	Buffer	41 ± 2	43 ± 1
	Triton X-100	6 ± 5	25 ± 3
	Dowfax 8390	34 ± 2	15 ± 2
MTL	Buffer	39 ± 2	37 ± 1
	Triton X-100	5 ± 1	18 ± 1
	Dowfax 8390	24 ± 1	2 ± 1
None	Buffer	49 ± 3	39 ± 2
	Triton X-100	5 ± 1	16 ± 1
	Dowfax 8390	19 ± 2	11 ± 1

[a] Mean ± standard error (n = 3).

TABLE 3. Removal of adhered cells from NAPL surfaces

NAPL	Solution added	% of *Mycobacterium* cells adhered to surface	% of *Pseudomonas* cells adhered to surface
EDM	None	31 ± 2	52 ± 1
	Triton X-100	16 ± 3	26 ± 3
	Dowfax 8390	26 ± 2	14 ± 2
PAA	None	51 ± 1	50 ± 1
	Triton X-100	10 ± 5	35 ± 2
	Dowfax 8390	3 ± 3	31 ± 2
DEV	None	50 ± 3	43 ± 2
	Triton X-100	30 ± 3	15 ± 2
	Dowfax 8390	19 ± 2	46 ± 2
MTL	None	34 ± 1	37 ± 2
	Triton X-100	4 ± 1	36 ± 1
	Dowfax 8390	8 ± 1	36 ± 2
None	None	52 ± 2	34 ± 1
	Triton X-100	6 ± 3	16 ± 2
	Dowfax 8390	3 ± 1	20 ± 2

[a] Mean ± standard error (n = 3).

added. Conversely, virtually no cells were removed from the MTL NAPL test tubes after either surfactant was added, nor were any cells removed from the DEV NAPL test tubes after Dowfax 8390 was added. Thus, addition of either surfactant resulted in removal of *Pseudomonas* cells from surfaces in most, but not all, cases.

Growth of bacteria on solid anthracene. Data for the growth of the *Mycobacterium* strain on anthracene in the presence and absence of surfactants are shown in Fig. 1. In the absence of surfactant, growth occurred until approximately day 7, at which time the OD_{600} was 1.16. In the presence of Triton X-100, the cell concentration increased more slowly, reaching an OD_{600} of 0.82 on day 14. In the presence of Dowfax 8390, the OD_{600} reached a maximum value of 0.32 after day 10. In the absence of either surfactant, the liquid medium turned yellow as growth proceeded, but the medium remained clear when either surfactant was present. The yellow pigmentation did not interfere with OD_{600} measurements.

Figure 2 shows the data for the growth of the *Pseudomonas* strain on anthracene in the presence and absence of surfactant.

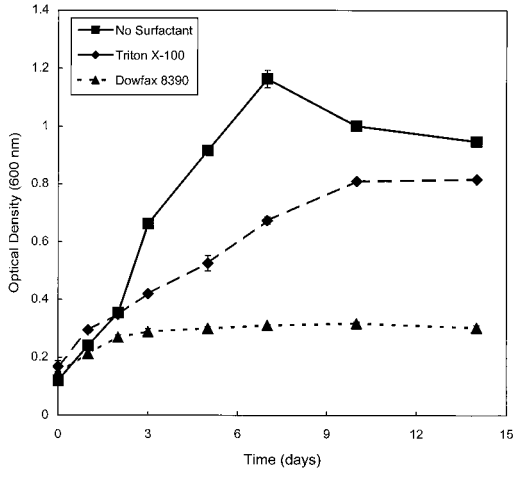

FIG. 1. Growth of the *Mycobacterium* strain on anthracene in the presence and absence of surfactants at concentrations that were one-half the CMCs. The error bars indicate standard deviations based on four or five replicates; where there are no error bars, the standard deviations were smaller than the symbols.

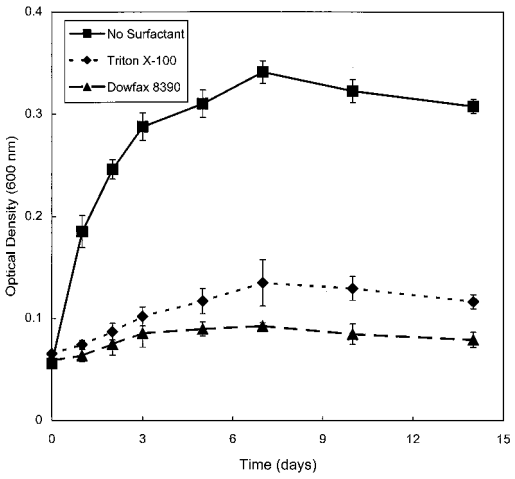

FIG. 2. Growth of the *Pseudomonas* strain on anthracene in the presence and absence of surfactants at concentrations that were one-half the CMCs. The error bars indicate standard deviations based on four or five replicates; where there are no error bars, the standard deviations were smaller than the symbols.

In the absence of surfactant, growth occurred until approximately day 7, at which point the OD_{600} was 0.34. Conversely, in the presence of Triton X-100, the OD_{600} increased to 0.14 after day 7 and then decreased, while in the presence of Dowfax 8390, the OD_{600} increased to 0.09 after day 7 before it decreased. As in the case of the *Mycobacterium* strain, the liquid medium turned yellow as growth progressed in the absence of either surfactant and remained clear if either surfactant was present. The OD_{600} measurements were not affected by the yellow pigmentation. Thus, each surfactant inhibited both the rate and the extent of growth of both strains even at a concentration that was one-half the CMC.

The suppression of growth in the presence of surfactants clearly showed that the surfactants were not growth substrates for the bacteria. In order to eliminate the possibility that the surfactants were toxic to the bacteria, the experiment described above was repeated by growing the *Pseudomonas* strain on glucose as a soluble carbon source. The *Pseudomonas* strain grew quite effectively within hours of inoculation regardless of the presence of surfactant, as shown in Fig. 3. Consequently, the inhibition of growth that is apparent in Fig. 2 cannot be attributed to any form of toxicity. The *Mycobacterium* strain did not grow on glucose; therefore, we cannot rule out the possibility that growth of this organism was inhibited by the surfactants.

DISCUSSION

In order for biodegradation to occur, the bacteria must have access to the target compounds, either by dissolution of the target compounds in the aqueous phase or by adhesion of the bacteria directly to the NAPL-water interface. Dissolution has been emphasized in the degradation of solid PAHs (3, 22, 46), while adhesion has been stressed in the case of liquid hydrocarbons, such as hexadecane (31, 32). The results of the present study show that bacteria were able to adhere to more viscous NAPL and solid surfaces; however, adhesion was inhibited in the presence of surfactants at concentrations below the CMCs. Addition of surfactants at low concentrations also resulted in removal of adhered bacteria from the NAPL-water interface. Furthermore, the presence of a surfactant had a deleterious effect on the growth of both the *Mycobacterium*

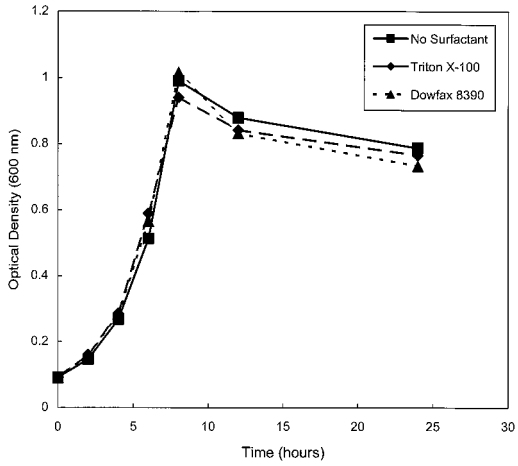

FIG. 3. Growth of the *Pseudomonas* strain on glucose in the presence and absence of surfactants at concentrations that were one-half the CMCs. The three sets of data are statistically equivalent (error bars are not shown).

strain and the *Pseudomonas* strain when solid anthracene was used as a carbon source.

Similar phenomena have been reported for adhesion of cells to droplets of low-viscosity liquid hydrocarbons. Addition of Triton X-100 at a concentration greater than its CMC inhibited adhesion of an *Arthrobacter* species to a heptamethylnonane-water interface, which in turn prevented degradation of both hexadecane and naphthalene in the heptamethylnonane phase (11, 26). Addition of surfactant also resulted in removal of previously adhered bacteria from the liquid substrates. In a similar study, addition of a nonionic surfactant caused yeast cells to detach from an *n*-alkane–water interface, leading to a decline in the growth rate of the culture (1). In another study, the presence of a surfactant had little effect on the growth rate of yeast cells on a soluble carbon source, although Triton X-100 at a concentration greater than its CMC did delay the onset of the exponential growth phase (21). The present study extended these findings to more viscous substrates and solid PAHs by showing that surfactants inhibit bacterial adhesion to the surfaces of these compounds and that surfactants inhibit bacterial growth on such carbon sources.

Although the presence of surfactant had a negative impact on the adhesion of both strains to the NAPLs, adhesion of the *Mycobacterium* strain was inhibited more in the presence of Triton X-100 than in the presence of Dowfax 8390. The reverse was true for the *Pseudomonas* strain. This result suggests that the relationship between surfactant charge and cell surface characteristics is more important in determining bacterial adhesion than the properties of the NAPL are. The cell walls of acid-fast bacteria, such as corynebacteria, mycobacteria, and *Nocardia* strains, contain mycolic acids, nocardols, and nocardones. These components do not carry a charge, but they increase the hydrophobicity and polarity of the cell surfaces, making the cells more likely to interact with surfactants that do not carry a charge (39). Conversely, the surfaces of gram-negative cells, such as *Pseudomonas* cells, carry a charge, indicating that these cells are more likely than gram-positive cells to interact with surfactants that carry a charge. Some evidence suggests that the positive charges on the surface of a bacterial cell play an important role in adhesion (38).

In the test tube experiments, the two strains had similar affinities for both the NAPLs and the glass surfaces. This observation was unexpected, because the NAPLs are hydrophobic and glass is hydrophilic. Hydrophobic interactions regulate the adhesion of bacteria to hydrocarbons (33). As summarized by Neu (25), surfactants can alter adhesion by adsorbing to the cell surface, to the hydrocarbon surface, or to both. If the hydrophobic ends of the surfactant molecules adsorb to hydrophobic surfaces, such as the NAPLs used in this study, then the hydrophilic ends remain in the aqueous phase. This adsorbed layer should make the surface more hydrophilic. Adsorption of surfactant to hydrophobic domains on the cell surface should give the same result. Either surface modification should decrease the hydrophobic interactions between the cells and the NAPL and reduce adhesion. Unfortunately, this mechanism cannot explain why the nonionic surfactant inhibited bacterial adhesion to glass.

An alternative explanation is that adsorption of the surfactants to the cells may have had a dispersive effect by increasing the steric hindrance to cell-cell and cell-surface interactions (36). This mechanism would account for the inhibition of adhesion of the bacteria to both hydrophobic and hydrophilic surfaces. This dispersion mechanism is important in some industrial applications (for example in the pulp and paper industry, where dispersants are used to reduce biofilm deposits) (29).

Regardless of which mechanism is responsible for the role of surfactants in reducing adhesion, the observations made in this study suggest why the previously published data on the benefits of surfactants in biodegradation of hydrophobic compounds are so contradictory. In the absence of toxicity, the net effect of addition of a surfactant to a contaminated soil depends on the benefits that result from enhanced solubility of target compounds versus the reduction in direct adhesion of bacteria to the NAPL. Consequently, the impact of surfactant addition on biodegradation depends on which mechanism is responsible for uptake of compounds in the highly viscous NAPL or solid phase.

The results of the bacterial growth experiment could be explained by two possible mechanisms. First, the surfactants may inhibit growth. Second, the interactions between the cells and the substrate may be altered in the presence of a surfactant. Since further investigation showed that neither surfactant affected the growth of the *Pseudomonas* strain on glucose, we concluded that the surfactants did not inhibit growth of this organism and did not serve as carbon sources. The adhesion studies clearly showed that surfactants reduced the adhesion of cells to NAPLs, independent of the viscosity of the hydrocarbon. Figures 1 and 2 clearly show that both bacterial strains were able to grow when solid anthracene was used as the sole carbon source and that growth was suppressed in the presence of a surfactant. If transient adhesion of cells to anthracene were a significant factor in the rate of uptake of anthracene by cells, then surfactant would inhibit both adhesion of the cells and cell growth on the solid PAH. Consequently, biodegradation would be inhibited. Transient adhesion would also be consistent with previous observations that cells do not easily adhere to PAHs to form biofilms (43, 48). Anthracene did not adhere well to glass; therefore, adhesion in the presence of surfactant could not be studied directly by using the techniques used for the NAPL materials. Further study is needed to determine whether transient adhesion is necessary for rapid uptake and growth on contaminants that are present in highly viscous or solid phases.

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