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Biodegradation by an *Arthrobacter* Species of Hydrocarbons Partitioned into an Organic Solvent

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An *Arthrobacter* strain mineralized naphthalene and *n*-hexadecane dissolved in 2,2,4,4,6,8,8-heptamethylnonane. The extent of mineralization increased with greater volumes of solvent. Measurements under aseptic conditions of the partitioning of naphthalene into the aqueous phase from the solid phase or from heptamethylnonane showed that the rates were rapid and did not limit mineralization. The rate of mineralization of hexadecane was rapid, although partitioning of the compound into aqueous solution was not detected. The *Arthrobacter* sp. grown in media with or without heptamethylnonane did not excrete products that increased the aqueous solubility of naphthalene and hexadecane. Measurements of the number of cells in the aqueous phase showed that the *Arthrobacter* sp. attached to the heptamethylnonane-water interface, but attachment was evident even without a substrate in the heptamethylnonane. Tests with small inocula of the *Arthrobacter* sp. demonstrated that at least a portion of naphthalene or hexadecane dissolved in heptamethylnonane was degraded by cells attached to the solvent-water interface. The cells did not adhere in the presence of 0.1% Triton X-100. The surfactant prevented mineralization of the hexadecane initially dissolved in heptamethylnonane, but it increased the rate and extent of mineralization of naphthalene initially dissolved in heptamethylnonane. The data show that organic solvents into which hydrophobic compounds partition affect the biodegradation of those compounds and that attachment of microorganisms to the organic solvent–water interface may be important in the transformation.

Toxic waste sites, industrial effluents, and natural waters into which petroleum has spilled contain nonaqueous, hydrophobic phases. These phases are composed of organic chemicals present at concentrations above their solubilities in water as well as chemicals of moderate aqueous solubility that partition into organic liquids. Organic solvents have been demonstrated to affect the leaching and sorption of hydrophobic chemicals (5, 12), but the effects of such solvents on the means by which bacteria obtain the chemicals for degradation are poorly understood.

In the metabolism of sparingly soluble compounds, bacteria may utilize the chemical present in the aqueous phase, or they may assimilate the substrate only when in association with the insoluble phase of the chemical. Use of the compound by assimilation from the solution phase is dependent on chemical equilibria or the bacterial excretion of substances that increase dispersion of the compound. Naphthalene and bibenzyl (20) and phenanthrene (21) were found to be used by some bacteria only from the dissolved state. Dissolution rates have been hypothesized to limit the rates of bacterial mineralization of palmitic acid (19) and phenanthrene (17). Alternatively, bacteria may excrete compounds that increase either the aqueous solubility of the substrate (3, 6) or the rate at which it partitions to the aqueous phase (17). Furthermore, hydrophobic microorganisms or those with specialized cellular projections (9, 15) may utilize an insoluble compound by direct contact with it.

It has been suggested that bacterial adherence is required for growth on liquid hydrocarbons when emulsification of the substrate and cell densities are low (16). Under similar conditions, bacteria capable of degrading substrates partitioned into a nonbiodegradable hydrocarbon solvent might need to be attached to the organic solvent–water interface to utilize their substrates. The presence of an organic solvent would influence whether bacteria attach to the organic-aqueous interface. Wodzinski and Larocca (22) have found that liquid but not solid diphenylmethane is used by cells of a *Pseudomonas* sp. at the aqueous-organic interface and that another strain of *Pseudomonas* utilizes naphthalene at the water-heptamethylnonane interface but not at the surface of naphthalene crystals.

Because of the ecological or toxicological importance of environmental pollution with sparingly soluble organic pollutants and the paucity of knowledge of how microorganisms assimilate many of these compounds, a study was conducted to assess the role of an organic solvent in determining the means by which bacteria degrade two hydrophobic substrates.

MATERIALS AND METHODS

Medium. The buffered inorganic salts solution used contained (per liter of deionized water) 900 mg of KH_2PO_4, 100 mg of K_2HPO_4, 100 mg of NH_4NO_3, 100 mg of $MgSO_4 \cdot 7H_2O$, 100 mg of $CaCl_2 \cdot 2H_2O$, and approximately 10 mg of $FeCl_3$. The pH was 5.7.

Chemicals. Naphthalene was obtained from Fisher Scientific, Rochester, N.Y. *n*-Hexadecane, 2,2,4,4,6,8,8-heptamethylnonane, di(2-ethylhexyl) phthalate, dichloromethane, and Triton X-100 were purchased from Aldrich Chemical Co., Milwaukee, Wis. *n*-[1-^{14}C]hexadecane (61 mCi/mmol), [1(4,5,8)-^{14}C]naphthalene (4.5 mCi/mmol), and sodium [^{14}C]bicarbonate (58.7 mCi/mmol) were obtained from Amersham Corp., Arlington Heights, Ill. The radiochemical purities of naphthalene and hexadecane were 97.1 and 98%, respectively.

Organisms. A bacterium capable of using naphthalene or

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n-hexadecane as its sole source of carbon and energy was isolated from Kendaia clay loam by using an enrichment medium (at pH 7.0) containing 0.1% naphthalene, 0.08% K_2HPO_4, 0.02% KH_2PO_4, and the other inorganic salts listed above. The initial enrichments were incubated for 2 to 3 weeks at 30°C on a rotary shaker operating at 100 rpm; 0.5 ml of this enrichment was transferred aseptically to a second flask containing inorganic salts and naphthalene. After this solution became turbid (7 to 10 days), the enrichment was transferred serially twice, and then portions were streaked on a medium containing 0.3% (wt/vol) Trypticase soy broth, 1.5% agar, and the inorganic salts. The culture grew in a liquid medium containing 0.01% naphthalene and inorganic salts at pH 5.7. The culture was identified by standard procedures (10).

To prepare inocula, the cultures were grown at 30°C on a rotary shaker operating at approximately 120 rpm in the 0.005% naphthalene–salts medium and harvested in early stationary phase by centrifugation at 4°C. The pellets were washed, the cells were resuspended in buffer, and the entire centrifugation and washing procedure was repeated. The final pellet was resuspended in inorganic salts solution. The cultures used in the experiments were incubated at 21 to 24°C on a reciprocal shaker operating at 60 strokes per min.

For most experiments, the inoculum size was chosen so that little or no growth would have to occur for 10 μg of substrate per ml of water to be rapidly metabolized. The drop plate method (8) was used to measure growth in tests involving simultaneous determinations of mineralization. In experiments in which population density was being measured in media supplemented with Triton X-100, the first dilution tube at all sampling times contained the surfactant, whether the bacteria were from cultures in which the surfactant was present or absent.

Measurement of mineralization. The substrates dissolved in2,2,4,4,6,8,8-heptamethylnonaneordi(2-ethylhexyl)phthalate were added to 250-ml biometer flasks (Bellco Glass, Inc., Vineland, N.J.) to give 80,000 to 120,000 dpm of the radiolabeled compound and sufficient unlabeled chemical to reach 10 μg of substrate per ml of water. The substrate-solvent mixture (250 or 25 μl) was added to 25 ml of inoculated inorganic salts solution, and the substrate-solvent mixture was present at the surface of the aqueous phase. In some experiments, the test hydrocarbons were dissolved in dichloromethane, which was allowed to evaporate. Although the organic chemicals were not sterilized, growth was not evident when agar plates were streaked with samples of sterilized salts solution that had been shaken with the chemicals.

The side arm of the flask contained 2.2 ml of 0.5 M NaOH to trap evolved CO_2. The flask was sealed with two silicone-rubber stoppers covered with Teflon tape. Periodically, the NaOH was removed through a stainless-steel cannula fitted with a 2-cm length of Teflon tubing and replaced with fresh NaOH. The NaOH solution and approximately 3.8 ml of Liquiscint scintillation cocktail (National Diagnostics, Inc., Sommerville, N.J.) were added to a 7-ml plastic scintillation vial and mixed. Radioactivity was determined with a liquid scintillation counter (model LS 7500; Beckman Instruments, Inc., Irvine, Calif.). Mineralization tests were conducted with duplicate flasks and with single uninoculated controls.

Mass balances were performed at the end of the experiments by pouring the remaining contents of the flasks into scintillation vials. The flasks were rinsed with 0.5% Triton X-100, and this mixture was also placed in a scintillation vial. In studies of hexadecane mineralization, the flasks were rinsed first with heptamethylnonane and then with Triton X-100. The ^{14}C recovered was thus the radioactivity in these vials plus that in the $^{14}CO_2$, and these values were compared with the radioactivity added to the flasks.

Rates of partitioning to the aqueous phase. The volume of sterile salts solution and the amount and method of addition of hydrocarbons were identical to those used in studies of mineralization. Biometer flasks or 250-ml Erlenmeyer flasks of the same size and shape as the main portion of the biometer flasks were used. Subsamples (0.5 ml) of the aqueous phase were taken at intervals with a 1-ml syringe attached to a 22-gauge needle. When the test compound was initially dissolved in heptamethylnonane, contact between the needle and the droplet of this hydrocarbon was avoided. If the droplet had spread to cover the surface of the liquid, the needle was inserted while air was slowly expelled from the syringe. Needles were discarded before placing the sample into a scintillation vial. Distilled water (1.0 ml) and scintillation fluid (3.0 ml) were added to the 0.5-ml sample in each vial. Duplicate flasks were used for the measurements.

Solubilization assay. To determine whether the bacterium produced surfactants that solubilized naphthalene or hexadecane, the organism was grown in 100 ml of medium in 250-ml Erlenmeyer flasks that were incubated at 23°C on a reciprocating shaker operating at 60 strokes per min. To measure the equilibrium concentration of the substrate, a 5.0-ml subsample of the culture fluid was passed through a 0.2-μm-pore-size Teflon syringe filter to remove the cells. The filtrate was added to a glass vial in tests of naphthalene solubilization or to a 50-ml Teflon centrifuge tube in tests of hexadecane solubilization. The glass vials contained 2.50 mg of naphthalene, including 100,000 dpm of the labeled compound. This amount of chemical is more than 15 times the amount potentially in solution at saturation. Samples of uninoculated inorganic salts solution also were shaken with excess naphthalene. The vials were shaken at 29°C on a rotary shaker operating at 120 rpm for 60 h. The samples were then allowed to stand at 22°C for 3 h. The solid naphthalene was removed by filtration through Teflon syringe filters, and the radioactivity in the filtrate was counted to determine the amount of naphthalene in the solution.

The filtrate from hexadecane-grown cultures was added to centrifuge tubes with 235 ng of hexadecane (containing 111,000 dpm of the labeled compound), which is more than 47 times the amount potentially dissolved in distilled water. Tubes containing the culture filtrates and inorganic salts solution with 0, 0.0001, 0.001, 0.01, or 0.1% Triton X-100 were shaken for 16 to 24 h at 29°C on a rotary shaker operating at 140 rpm. The samples were then centrifuged at about 7,700 × *g* at 20°C for 20 min. Because nonsolubilized hexadecane was expected to rise to the surface, 1.0-ml portions of the surface liquid in the centrifuge tube were removed to avoid contamination of the underlying liquid. The radioactivity then was counted by using two 1.0-ml samples from each centrifuge tube.

Enumeration of bacteria in the aqueous phase. Two methods were used to count bacteria not attached to the heptamethylnonane-water interface. In both methods, 0.50-ml portions of a 25-ml culture grown in a 250-ml Erlenmeyer flask were removed. The first method was the drop plate counting technique. If the shape of a curve depicting cell number versus time appeared to be linear, the line of best fit was determined by linear regression analysis. In the second method, the cells were labeled with ^{14}C and their numbers were determined by scintillation counting. To prepare labeled cells, the cultures were grown in a 2-liter Erlenmeyer flask containing 1 liter of inorganic salts solution, 50 mg of

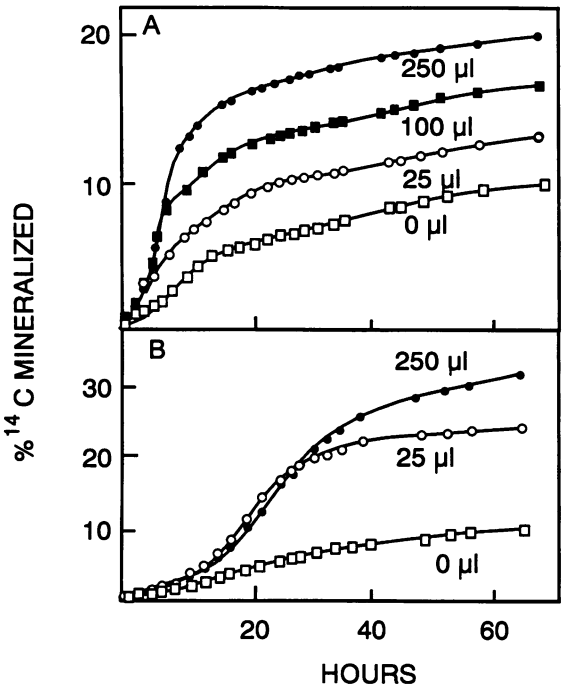

FIG. 1. Mineralization of naphthalene (A) or hexadecane (B) dissolved in 0, 25, 100, or 250 µl of heptamethylnonane. The inoculum contained 3.1×10^6 (A) or 1.2×10^7 (B) *Arthrobacter* sp. cells per ml.

unlabeled substrate, and 2 µCi of ^{14}C-labeled naphthalene. The cells incorporated ^{14}C from the labeled naphthalene as they grew. They were subsequently harvested and washed. A factor for converting radioactivity to cell numbers was determined by plate counting of the inoculum and simultaneously measuring the radioactivity of the inoculum.

RESULTS

Characterization of the isolate. The naphthalene-utilizing isolate forms long and short rods during the early stages of growth and cocci during the stationary phase. The bacterium is gram positive, catalase positive, and not acid fast. It was identified as a member of the genus *Arthrobacter* and designated strain R1. This organism is capable of utilizing naphthalene or hexadecane but not 2,2,4,4,6,8,8-heptamethylnonane or di(2-ethylhexyl) phthalate as a sole carbon and energy source for growth. Some rods have been observed microscopically to move into the heptamethylnonane phase, but it is unclear whether these organisms were metabolically active.

Mineralization of hydrocarbons initially dissolved in heptamethylnonane. Naphthalene or hexadecane (250 µg) was dissolved in 25, 100, or 250 µl of heptamethylnonane. The solvent then was added as a drop to the surface of 25 ml of inorganic salts solution. The substrate was also added in dichloromethane (i.e., with no heptamethylnonane), which left a film of substrate at the bottom of the flask after the solvent was allowed to evaporate.

The extent of mineralization of naphthalene was affected by the volume of heptamethylnonane added to the cultures (Fig. 1A). The more solvent present, the greater was the percentage of compound mineralized. The mineralization curves appeared to consist of two phases. Although not depicted, mineralization was measured until 231 h. At this time, 31, 26, 21, and 17% of the ^{14}C were recovered when 250, 100, 25, and 0 µl, respectively, of heptamethylnonane were used. The extents of mineralization of hexadecane also increased with the volume of heptamethylnonane in which the substrate was dissolved (Fig. 1B). Although the initial rates of degradation were similar for the cultures containing 0, 25, or 250 µl of heptamethylnonane, the maximum rates of degradation were greater with the solvent present. The recovery at 101 h of the initial ^{14}C was 86% when the hexadecane was dissolved in 250 µl of solvent, but the recovery decreased to 48 and 31% with 25 and 0 µl of heptamethylnonane, respectively. The small extent of naphthalene or hexadecane mineralization in cultures with little or no organic solvent present probably resulted from the slow volatilization or sorption of the substrates to sides of the flasks and the consequent decreased substrate availability to the bacteria. The final pH of all cultures after 100 to 110 h was between 5.6 and 5.8.

To determine the rate of diffusion of CO_2 to the NaOH trap, $NaH^{14}CO_3$ was added to the inorganic salts solution in duplicate biometer flasks to a concentration of 1.0 ng/ml. The diffusion of 90% of the $^{14}CO_2$ from the labeled bicarbonate to the NaOH took 1.5 to 2 h. Thus, mineralization may have been somewhat more rapid than the previous data indicate.

Partitioning of substrate to aqueous phase. The rates of partitioning of naphthalene and hexadecane to the aqueous phase were measured in sterile solution to ascertain whether the rate of utilization was determined by transfer of the chemical to the aqueous phase. The tests were conducted in flasks containing 0, 25, or 250 µl of heptamethylnonane. Naphthalene (250 µg) was added to 25 ml of inorganic salts solution in each flask. With no heptamethylnonane present, the dissolution of naphthalene into the aqueous phase was so rapid that essentially all was in the aqueous phase in the first sample, which was taken after 15 min (Fig. 2). The decrease in concentration in the aqueous phase with time in uninoculated flasks probably reflects volatility of the compound. The rate of movement of the compound to the aqueous phase was slower when the chemical was initially dissolved in heptamethylnonane than when only solid naphthalene was present. Nevertheless, the slowest partitioning rate, which was evident in flasks containing 250 µl of solvent, was more rapid than the mineralization rate. Thus, the metabolism of naphthalene was not limited by the rate of movement of the compound from the solid or organic-solvent phase to the aqueous phase.

The partitioning of *n*-hexadecane to the aqueous phase could not be detected, presumably because its solubility in water, less than 1 ng/ml (4, 18), was below the sensitivity of the method of measurement. Therefore, the mineralization rates shown in Fig. 1B exceeded the rate of partitioning of the compound into solution in uninoculated media.

Solubilization assay. Tests of the ability of the *Arthrobacter* sp. to produce extracellular solubilizers when grown in medium containing 10 or 100 µg of naphthalene per ml, with an inoculum of 1.4×10^5 or 1.4×10^7 cells per ml, and in the presence or absence of heptamethylnonane were conducted. Samples from the cultures were taken at 7.0, 17.5, 32, and 48 h; the cells were removed by filtration; and the solubility of the substrate in the filtrate was determined. Because the concentration of naphthalene in the aqueous phase did not increase with filtrates prepared from cultures of increasing age, the values for the four culture ages were averaged. For cultures with no heptamethylnonane, the equilibrium con-

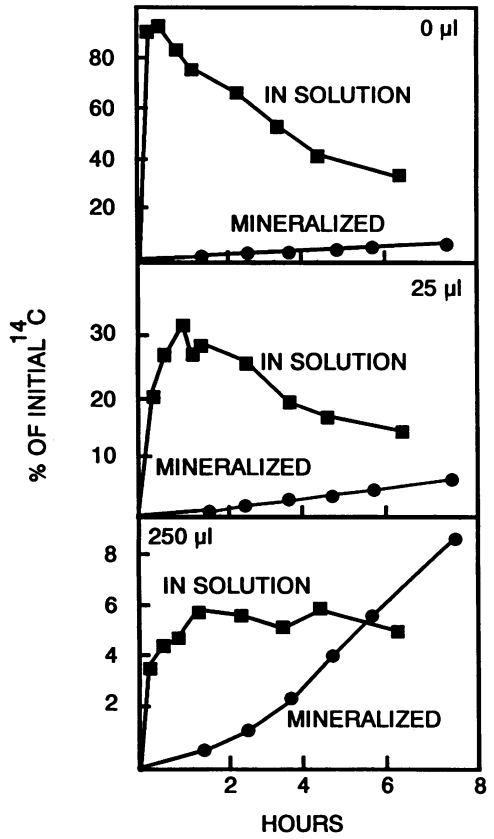

FIG. 2. Partitioning of naphthalene to the aqueous phase and rates of naphthalene mineralization. The substrate was initially dissolved in 0, 25, or 250 μl of heptamethylnonane.

centrations of naphthalene in the aqueous phase were 26, 27, 28, and 26 μg/ml if the initial substrate concentrations and cell densities were 10 μg and 1.4×10^5 cells per ml, 10 μg and 1.4×10^7 cells per ml, 100 μg and 1.4×10^5 cells per ml, and 100 μg and 1.4×10^7 cells per ml, respectively. If heptamethylnonane was present in the original culture medium, the analogous equilibrium concentrations of naphthalene in the aqueous phase were 30, 29, 29, and 31 μg/ml, respectively. The solubility of naphthalene in uninoculated salts solution was 29 μg/ml. These values are close to the reported solubility of naphthalene, namely, 31.9 μg/ml of water (14).

The ability of the *Arthrobacter* sp. to excrete products solubilizing hexadecane when the bacterium was grown on hexadecane was also tested. Media containing 10 μg of hexadecane per ml with or without heptamethylnonane were inoculated with 4.8×10^5 or 4.8×10^7 cells per ml. Samples of culture fluid were taken at 4.5, 16.5, 30, and 44 h; the cells were removed by filtration; and the solubility of hexadecane in the filtrate was determined. The values for the solubilities of hexadecane in filtrates taken at all culture ages were less than 0.2 ng/ml, except for one anomalous value of 0.63 ng/ml. The values of less than 0.2 ng/ml are much lower than the solubility of hexadecane even in uninoculated inorganic salts solutions, namely, 1.6 ± 1.3 ng/ml. Hexadecane solubility in distilled water at 25°C is reported to be 0.9 ng/ml (18) and 0.02 ng/ml (4). In the presence of a known solubilizing agent, the values for hexadecane solubility in uninoculated salts solution were 1.3 ± 0.4, 7.3 ± 6.2, 45.8 ± 2.0, and 48.7 ± 6.6 ng/ml in the presence of 0.0001, 0.001, 0.01, and 0.1% Triton X-100, respectively, under conditions in which 47 ng/ml was the total concentration of hexadecane available for solubilization.

TABLE 1. Population of the *Arthrobacter* sp. in the aqueous phase in the presence of heptamethylnonane

Time (h)	Mean cell no. (10^6/ml) ± SD in:		
	Naphthalene + solvent	Hexadecane + solvent	Solvent only
0.0	17 ± 8	17 ± 8	17 ± 8
2.0	13.6 ± 0.3	13.4 ± 0.6	13.3 ± 0.9
4.5	10.6 ± 0.2	8.8 ± 1.3	9.7 ± 1.3
10.5	6.6 ± 0.3	6.5 ± 0.2	6.5 ± 0.4
20.2	5.5 ± 0.1	4.4 ± 1.8	5.1 ± 0.7
35.5	4.4 ± 0.7	3.0 ± 1.3	4.0 ± 0.6

Bacteria in the aqueous phase. To determine whether the *Arthrobacter* sp. became attached to the solvent-water interface, 4.3×10^8 *Arthrobacter* sp. cells were introduced into flasks to which were added 25 ml of water and 250 μl of heptamethylnonane containing 250 μg of naphthalene or hexadecane or no substrate. The cell density was sufficiently high that little growth presumably occurred on the small amount of substrate provided. Liquid scintillation counting of ^{14}C-labeled organisms was initially used to estimate cell density, and the radioactivity was determined with duplicate flasks. The factor for converting radioactivity to cell numbers was 7.68×10^3 cells per dpm. The initial cell density was determined by plate counting. The numbers of cells in the aqueous phase at various periods of time are shown in Table 1. The number of *Arthrobacter* sp. cells in the aqueous phase decreased with time whether the cells were incubated with naphthalene in heptamethylnonane, hexadecane in heptamethylnonane, or only heptamethylnonane. The rate of decrease did not appear to be affected by whether substrate was present.

The number of bacteria in the aqueous phase also was determined by plate counts when small inocula were used. In one instance, 7.2×10^5 cells per ml were added to flasks containing 25 ml of water and 250 μg of hexadecane initially dissolved in 250 μl of heptamethylnonane. After an initial increase, the number of *Arthrobacter* sp. cells in the aqueous phase decreased as mineralization proceeded (Fig. 3A). The numbers of cells eventually fell to 1.4×10^5/ml. When each flask received 7.2×10^4 *Arthrobacter* sp. cells per ml, the population size in the aqueous phase remained relatively constant (Fig. 3B). When the substrate was naphthalene dissolved in heptamethylnonane and 1.9×10^6 cells were added per ml, the cell density also decreased with time (Fig. 3C). In cultures without heptamethylnonane, approximately 3.7×10^6 cells of the *Arthrobacter* sp. per ml of solution appeared in the aqueous phase during the mineralization of 10 μg of naphthalene per ml of solution. With heptamethylnonane present, the density of cells in the aqueous phase after 50 to 60 h was about 1 order of magnitude lower. During the mineralization of hexadecane, the cell density was up to 2 orders of magnitude lower than that noted during the mineralization of soluble naphthalene.

Prevention of adherence. Triton X-100 was not toxic to the *Arthrobacter* sp. at the experimental concentration. To determine whether Triton X-100 could remove cells adhering to the heptamethylnonane-water interface, 25 μl of Triton X-100 was added to separatory funnels containing 25 ml of

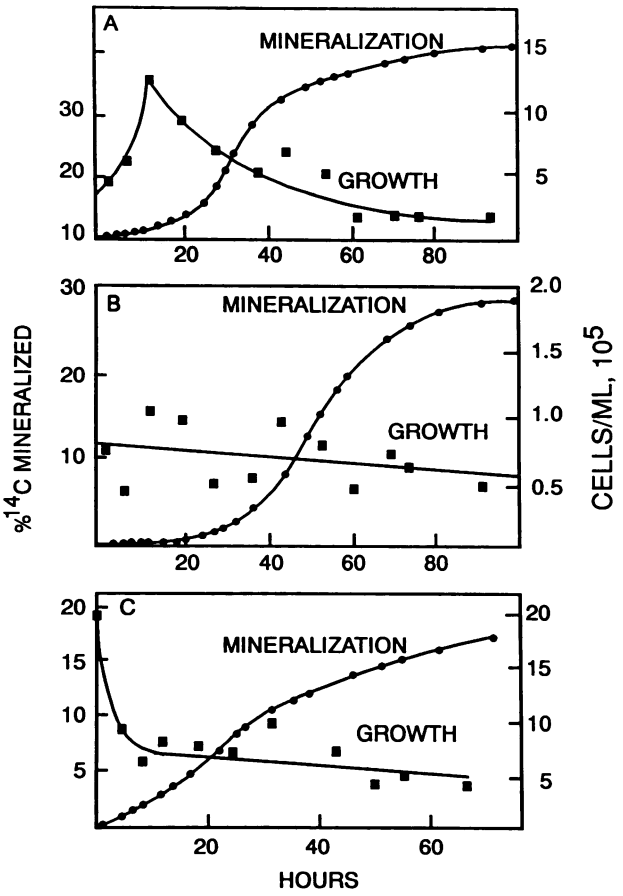

FIG. 3. Number of *Arthrobacter* sp. cells in the aqueous phase during the mineralization of hexadecane in heptamethylnonane by an inoculum of 7.2×10^5 cells per ml (A), hexadecane in heptamethylnonane by an inoculum of 7.2×10^4 cells per ml (B), and naphthalene by an inoculum of 1.9×10^6 cells per ml (C).

Arthrobacter sp. cultures that had been growing for about 5 h on 1 μg of naphthalene per ml of water. The substrate was initially dissolved in 250 μl of heptamethylnonane. Counts of cells in the aqueous phase prior to and after the addition of Triton X-100 showed that the number of *Arthrobacter* sp. cells in the aqueous phase increased following addition of Triton X-100. Thus, the counts before and after addition of the surfactant were $(1.2 \pm 0.1) \times 10^7$ and $(1.7 \pm 0.1) \times 10^7$ cells per ml in one funnel and $(1.0 \pm 0.1) \times 10^5$ and $(1.4 \pm 0.0) \times 10^5$ cells per ml in the second.

Because Triton X-100 appeared to remove cells of the *Arthrobacter* sp. attached to the heptamethylnonane-water interface, it also might prevent the bacterium from adhering to the interface. An experiment was thus conducted in which Triton X-100 (1 μl/ml of water) was added to flasks containing 10 μg of hexadecane or naphthalene per ml of water. The flasks were inoculated with 4.2×10^7 cells per ml. The substrates were initially dissolved in 250 μl of heptamethylnonane. The mineralization of hexadecane was rapid in the absence of the surfactant (Fig. 4A). Almost 30% of the substrate was mineralized. However, hexadecane added in heptamethylnonane was not mineralized if 0.1% Triton X-100 was present. Small droplets appeared within 30 min in inoculated or uninoculated flasks shaken with the substrate dissolved in heptamethylnonane, Triton X-100, and the

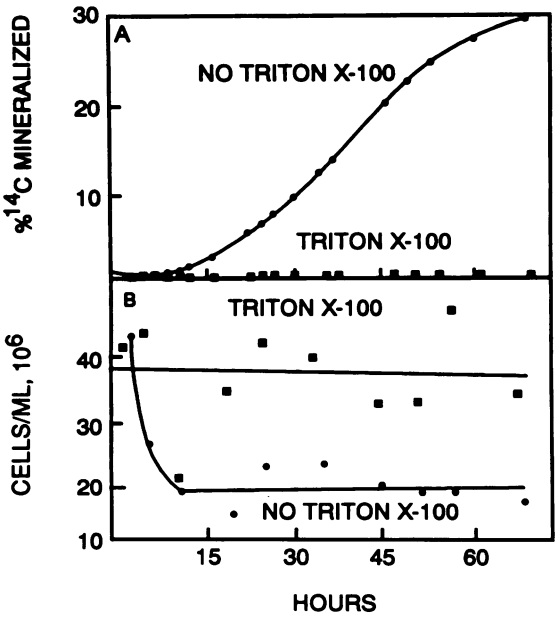

FIG. 4. Mineralization of hexadecane dissolved in heptamethylnonane (A) and accompanying populations of *Arthrobacter* sp. in the aqueous phase (B) in the presence and absence of Triton X-100.

Arthrobacter sp. At 26 h, the water phase in uninoculated flasks contained up to 700 ng of dispersed hexadecane per ml, which was measured by inserting a needle below the heptamethylnonane slick. Many droplets were probably of solubilized size. The amount of heptamethylnonane dispersed with the hexadecane was not determined. During the mineralization of hexadecane dissolved in heptamethylnonane in the absence of 0.1% Triton X-100, the number of *Arthrobacter* sp. cells in the aqueous phase decreased from 4.2×10^7 to approximately 2.0×10^7 cells per ml (Fig. 4B). In the presence of Triton X-100, however, the population density after 15 h, and possibly even earlier, was essentially the same as at 0 h. Statistical analysis indicated that the averages of the counts after 15 h were significantly different in the presence and absence of Triton X-100 ($P < 0.05$).

A study was also conducted to assess the effect of Triton X-100 on the mineralization of naphthalene initially dissolved in heptamethylnonane. The substrate was present in concentrations equivalent to 10 μg/ml of water. Triton X-100 enhanced the rate and extent of mineralization of naphthalene by the *Arthrobacter* sp. (Fig. 5A). This contrasts with the influence of the surfactant on the mineralization of hexadecane. The rapid mineralization of naphthalene in the presence of the surfactant shows that Triton X-100 was not toxic to the *Arthrobacter* sp. The recovery of ^{14}C at 71 h was 69 and 71% for naphthalene-degrading cultures without and with Triton X-100, respectively.

The numbers of *Arthrobacter* cells in the aqueous phase decreased from 4.2×10^7 to approximately 1.8×10^7/ml during the degradation of naphthalene in media without Triton X-100 (Fig. 5B). A decrease of similar magnitude was evident when hexadecane was the substrate. No such decline was observed in the presence of the surfactant. The averages of the counts after 15 h in the presence and absence of Triton X-100 were significantly different ($P < 0.05$).

Di(2-ethylhexyl) phthalate as solvent. To determine whether the identity of the solvent in which the substrate

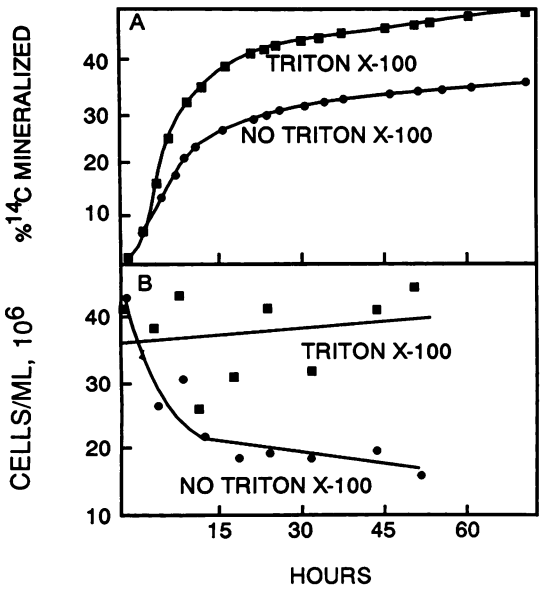

FIG. 5. Mineralization of naphthalene dissolved in heptamethylnonane (A) and accompanying populations of *Arthrobacter* sp. in the aqueous phase (B) in the presence and absence of Triton X-100.

was dissolved would influence bacterial utilization of the test compound, di(2-ethylhexyl) phthalate also was used as a solvent for *n*-hexadecane. Mineralization of 250 μg of hexadecane dissolved in 250 μl of either di(2-ethylhexyl) phthalate or heptamethylnonane was measured in media inoculated with 1.2×10^7 *Arthrobacter* sp. cells per ml. The results are shown in Fig. 6. A one-tailed *t* test of the means of CO_2 formation showed that the extent of mineralization of hexadecane at all times from 12.1 to 38.1 h was greater when the compound was initially dissolved in the phthalate ($P < 0.05$). The amount of hexadecane that partitioned in 32 h from di(2-ethylhexyl) phthalate to water in sterile solution was too small to be detected.

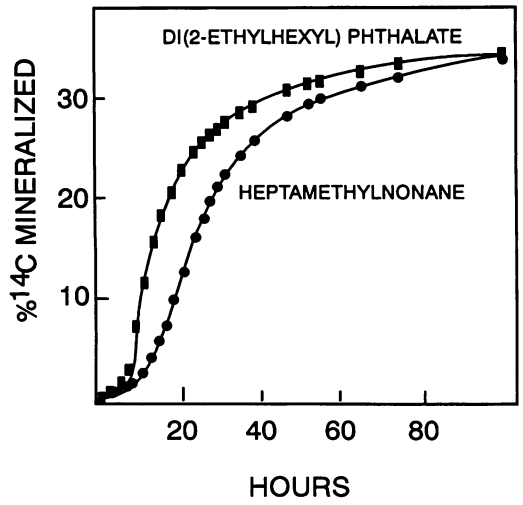

FIG. 6. Mineralization of hexadecane initially dissolved in di(2-ethylhexyl) phthalate or heptamethylnonane. The substrate was present to give the equivalent of 10 μg/ml of aqueous phase.

DISCUSSION

n-Hexadecane and naphthalene initially dissolved in 2,2,4,4,6,8,8-heptamethylnonane appeared to be mineralized by bacteria attached to the solvent. When radiolabeled cells mineralized naphthalene and hexadecane, the marked decrease in radioactivity in the aqueous phase indicated attachment of *Arthrobacter* sp. cells to the heptamethylnonane-water interface. Furthermore, when a small inoculum was used and naphthalene mineralization was essentially complete, the final cell density in the aqueous phase was almost an order of magnitude lower when heptamethylnonane was present than when solvent was absent, suggesting that many of the cells causing the degradation were attached to the solvent-water interface. In addition, with certain inoculum sizes, the number of cells in the aqueous phase decreased during the degradation of naphthalene or hexadecane in heptamethylnonane. Because the decreases in the number of labeled cells in the aqueous phase were similar for cultures containing solvent with naphthalene, hexadecane, or no substrate, adherence of the *Arthrobacter* sp. to the solvent was not related to the presence of a C source for the bacteria. The adherence of bacterial cells to a hydrocarbon-water interface has been shown to be a prerequisite to the production of emulsifying or solubilizing agents (13). However, an increase in cell numbers in the aqueous phase would have been expected to coincide with the appearance of surfactants that solubilize the hydrocarbons. Bacterial attachment to the solvent-water interface in the present study appears to result in direct utilization of the substrate from the insoluble phase and not to be a prelude to the production of solubilizing agents.

The bacteria did not excrete substances that increased the solubility of naphthalene or hexadecane. Indeed, the solubility of naphthalene in water is sufficiently high that such substances might have little effect on the chemical equilibrium between the heptamethylnonane and water phases. Microbially produced surfactants also might have no effect on the means by which the *Arthrobacter* sp. initially assimilates hexadecane if most of the cells metabolizing this alkane are attached to the organic solvent-water interface.

Microbial utilization of *n*-hexadecane by direct contact of cells with the substrate has been demonstrated previously (11, 16), but few studies have dealt with the altered availability of substrates more soluble in the aqueous phase when the compound preferentially partitions into an organic solvent. The organic solvent provides an interface to which the population of bacteria may attach, and at least part of the mineralization of naphthalene noted in this study was brought about by cells located at the solvent-water interface. It is uncertain whether the bacteria used naphthalene from the organic solvent phase, the water phase, or both. Wodzinski and Larocca (22) demonstrated that adherent cells of a *Pseudomonas* sp. used only the portion of naphthalene dissolved in heptamethylnonane and not that in the water phase.

Surfactants can prevent the adherence of cells to an organic-aqueous interface. Goswami et al. (7) used an alkyl aryl polyglycol ether to prevent the attachment of an *Arthrobacter* species to β-sitosterols. Bacterial growth on the substrate was markedly inhibited. Aiba et al. (1) observed a decrease in specific growth rate of *Candida guillermondii* when Tween 20 was used to prevent a large percentage of the cells from adhering to dodecane and tetradecane. In the present study, Triton X-100 completely prevented the mineralization of hexadecane that had been dissolved in hep-

tamethylnonane. The surfactant prevented adherence of most of the bacteria to the heptamethylnonane-water interface and hence prevented direct contact of the cells with hexadecane. In support of this view are the findings that the number of bacteria in the aqueous phase decreased just prior to the rapid phase of mineralization in the absence of Triton X-100, whereas with the surfactant present, the number of bacteria in the aqueous phase remained essentially constant. In addition to altering the solvent-water interface, Triton X-100 may act by some effect on cell membranes (2).

The *Arthrobacter* sp. apparently could not use hexadecane in heptamethylnonane that was solubilized by Triton X-100. Because of its relatively high partitioning into water, naphthalene was available in the aqueous phase as well as in the solvent phase. This may be the basis for the different effect of the surfactant on mineralization of naphthalene. Triton X-100 increased both the rate and extent of mineralization of naphthalene that had initially been dissolved in heptamethylnonane. Although the cells presumably were prevented from adhering to the solvent-water interface in the presence of the surfactant, sufficient naphthalene was present in aqueous solution not to limit degradation. The *Arthrobacter* sp., when not attached to the interface, probably was free of the spatial and nutrient limitations that may be associated with the solvent-water interface so that mineralization was enhanced.

Surfactants are often used to increase dispersion of oil following spills in marine waters. If the components of the oil that are of greatest ecological concern require that bacteria adhere prior to biodegradation, the use of surfactants at the site may be detrimental. Surfactants may prevent the adherence of microorganisms and consequently may prevent biodegradation of certain constituents of oil.

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REFERENCES

1. **Aiba, S., V. Moritz, J. I. Someya, and K. L. Haung.** 1969. Cultivation of yeast cells by using *n*-alkanes as the sole carbon source. (I) Batch culture. J. Ferm. Technol. **47**:203–210.
2. **Breuil, C., and D. J. Kushner.** 1980. Effects of lipids, fatty acids, and other detergents on bacterial utilization of hexadecane. Can. J. Microbiol. **26**:223–231.
3. **Cameotra, S. S., H. D. Singh, and J. N. Baruah.** 1984. Demonstration of extracellular alkane-solubilizing factor produced by (*Endomycopsis lipolytica*) YM. Biotechnol. Bioeng. **26**:554–556.
4. **Coates, M., D. W. Connell, and D. M. Barron.** 1985. Aqueous solubility and octan-1-ol to water partition coefficients of aliphatic hydrocarbons. Environ. Sci. Technol. **19**:628–632.
5. **Fu, J.-K., and R. G. Luthy.** 1986. Effect of organic solvent on sorption of aromatic solutes onto soils. J. Environ. Eng. **112**: 346–366.
6. **Gibbons, J. A., and M. Alexander.** 1989. Microbial degradation of sparingly soluble organic chemicals: phthalate esters. Environ. Toxicol. Chem. **8**:283–291.
7. **Goswami, P. C., H. D. Singh, S. D. Bhagat, and J. N. Baruah.** 1983. Mode of uptake of insoluble solid substrates by microorganisms. I. Sterol uptake by an *Arthrobacter* species. Biotechnol. Bioeng. **25**:2929–2943.
8. **Hoben, H. J., and P. Somasegaran.** 1982. Comparison of the pour, spread, and drop plate methods for enumeration of *Rhizobium* spp. in inoculants made from presterilized peat. Appl. Environ. Microbiol. **44**:1246–1247.
9. **Käppeli, O., P. Walther, M. Mueller, and A. Fiechter.** 1984. Structure of the cell surface of the yeast *Candida tropicalis* and its relation to hydrocarbon transport. Arch. Microbiol. **138**:279–292.
10. **Keddie, R. M., M. D. Collins, and D. Jones.** 1986. Genus *Arthrobacter* Conn and Dimmick 1974, 300^{AL}, p. 1288–1301. *In* P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 2. The Williams & Wilkins Co., Baltimore.
11. **Nakahara, T., L. E. Erickson, and J. R. Gutierrez.** 1977. Characteristics of hydrocarbon uptake in cultures with two liquid phases. Biotechnol. Bioeng. **19**:9–25.
12. **Nkedl-Kizza, P., P. S. C. Rao, and A. G. Hornsby.** 1987. Influence of organic cosolvents on leaching of hydrophobic organic chemicals through soils. Environ. Sci. Technol. **21**: 1107–1111.
13. **Panchal, C. J., and J. E. Zajic.** 1978. Isolation of emulsifying agents from a species of *Corynebacterium*. Dev. Ind. Microbiol. **19**:569–576.
14. **Pearlman, R. S., S. H. Yalkowsky, and S. Banerjee.** 1984. Water solubilities of polynuclear aromatic and heteroaromatic compounds. J. Phys. Chem. Ref. Data **13**:555–562.
15. **Rosenberg, M., E. A. Bayer, J. Delarea, and E. Rosenberg.** 1982. Role of thin fimbriae in adherence and growth of *Acinetobacter calcoaceticus* RAG-1 on hexadecane. Appl. Environ. Microbiol. **44**:929–937.
16. **Rosenberg, M., and E. Rosenberg.** 1981. Role of adherence in growth of *Acinetobacter calcoaceticus* RAG-1 on hexadecane. J. Bacteriol. **148**:51–57.
17. **Stucki, G., and M. Alexander.** 1987. Role of dissolution rate and solubility in biodegradation of aromatic compounds. Appl. Environ. Microbiol. **53**:292–297.
18. **Sutton, C., and J. Calder.** 1974. Solubility of higher-molecular weight *n*-paraffins in distilled water and seawater. Environ. Sci. Technol. **8**:654–657.
19. **Thomas, J. M., J. R. Yordy, J. A. Amador, and M. Alexander.** 1986. Rates of dissolution and biodegradation of water-insoluble organic compounds. Appl. Environ. Microbiol. **52**:290–296.
20. **Wodzinski, R. S., and D. Bertolini.** 1972. Physical state in which naphthalene and bibenzyl are utilized by bacteria. Appl. Microbiol. **23**:1077–1081.
21. **Wodzinski, R. S., and J. E. Coyle.** 1974. Physical state of phenanthrene for utilization by bacteria. Appl. Microbiol. **27**: 1081–1084.
22. **Wodzinski, R. S., and D. Larocca.** 1977. Bacterial growth kinetics on diphenylmethane and naphthalene-heptamethylnonane mixtures. Appl. Environ. Microbiol. **33**:660–665.