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Solvent-Augmented Mineralization of Pyrene by a *Mycobacterium* sp.†

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The biodegradation of polycyclic aromatic hydrocarbon pollutants is constrained, in part, by their solid physical state and very low water solubility. Searching for ways to overcome these limitations, we isolated from soil a bacterium capable of growing on pyrene as a sole source of carbon and energy. Acid-fast stain, morphology, and fatty acid profile identified it as a *Mycobacterium* sp. In a mineral salts solution, the isolate mineralized 50% of a 250-μg/ml concentration of [^{14}C]pyrene in 2 to 3 days. Detergent below the critical micelle concentration increased the pyrene mineralization rate to 154%, but above the critical micelle concentration, the detergent severely inhibited pyrene mineralization. The water-miscible solvent polyethylene glycol was inhibitory. The hydrophobic solvents heptamethylnonane, decalin, phenyldecane, and diphenylmethane were also inhibitory at several concentrations tested, but the addition of paraffin oil, squalene, squalane, tridecylcyclohexane, and *cis*-9-tricosene at 0.8% (vol/vol) doubled pyrene mineralization rates by the *Mycobacterium* sp. without being utilized themselves. The *Mycobacterium* sp. was found to have high cell surface hydrophobicity and adhered to the emulsified solvent droplets that also contained the dissolved pyrene, facilitating its mass transfer to the degrading bacteria. Cells physically adhering to solvent droplets metabolized pyrene 8.5 times as fast as cells suspended in the aqueous medium. An enhanced mass transfer of polycyclic aromatic hydrocarbon compounds to microorganisms by suitable hydrophobic solvents might allow the development of solvent-augmented biodegradation techniques for use in aqueous or slurry-type bioreactors.

Polycyclic aromatic hydrocarbons (PAHs) are hazardous byproducts of combustion processes and petroleum refining. Since many PAH compounds have high environmental persistence and some of them are carcinogenic (6), the disposal of PAH-containing refinery sludges is a highly regulated and expensive process (3). Efforts to render refinery sludges nonhazardous by the biodegradative removal or reduction of their PAH components are currently hampered by the very low rates of PAH biodegradation. The causes of this slow biodegradation are complex. All PAH compounds with two and three rings and some PAHs with four rings serve as growth substrates to some microorganisms (6), but the rest of the PAHs are only cometabolized in the presence of other primary substrates. Largely because of their low water solubility, even substrate-type PAH compounds are degraded only slowly. Liposome packaging has failed to increase PAH utilization rates, even though this technique was highly successful in the case of long-chain alkanes (15). Below their critical micelle concentrations (CMCs), some detergents were reported to increase the pseudosolubilization and biodegradation of some PAHs (12), but above their CMCs, detergents were inhibitory to PAH utilization. Some positive effects on biodegradation rates of naphthalene and hexadecane were noted by the use of an organic solvent (7).

From soil, we isolated a *Mycobacterium* strain capable of growth on pyrene as its only source of carbon and energy. Using this isolate, we attempted to augment pyrene mineralization rates by increasing the mass transfer of this hydrophobic solid to the degrading microorganism. The ultimate aim of our work was to increase PAH biodegradation rates in bioremediation processes.

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MATERIALS AND METHODS

Radiochemicals and chemicals. [4,5,9,10-^{14}C]pyrene (50 mCi/mmol) was purchased from Chemsyn Science Labs, Lenexa, Kans., and D-[U-^{14}C]glucose (292 mCi/mmol) was from Amersham (Elk Grove, Ill.). Their radiochemical purities, as determined by the manufacturers by radiochromatographic techniques, were 98% or higher. Unlabeled pyrene, 2,2,4,4,6,8,8-heptamethylnonane, and polyethylene glycol (average molecular weight, 600) were purchased from Sigma Chemical Co. (St. Louis, Mo.); phenanthrene, squalene, squalane, *cis*-9-tricosene, tridecylcyclohexane, diphenylmethane, decalin, and phenyldecane were from Aldrich (Milwaukee, Wis.); Oxosol C-14 scintillation fluid for CO_2 trapping was from National Diagnostics (Atlanta, Ga.); and Triton X-100 was from Research Products International (Elk Grove Village, Ill.). Paraffin oil (mineral oil) was manufactured by Squibb (Princeton, N.J.) and obtained from a local pharmacy.

Isolation and characterization of a *Mycobacterium* sp. Nixon sandy loam (5) was suspended in Stanier's basal medium (SBM), a mineral salts solution supplemented by the trace metal solutions Hutner's mineral base and Metals 44 but no vitamins (2). To this SBM solution, 0.25% (wt/vol) pyrene was added as a sole carbon and energy source. The inoculated medium was incubated for 2 weeks at 30°C with shaking (300 rpm). Serial transfers to fresh medium were made in a 1:100 ratio six times in 2-week intervals. The pyrene-degrading microbial enrichment culture was subsequently maintained in a 3.5-liter semicontinuous batch culture with an air sparger and magnetic stirring. From the growth medium (SBM with 0.1% pyrene), 50% of the volume was removed weekly and replaced with fresh medium. The *Mycobacterium* sp. was isolated from the described consortium on nutrient agar (Becton Dickinson, Cockeysville, Md.) plates with pyrene overlay by a technique modified from that of Heitkamp and Cerniglia (9). Dilutions of the consortium were first spread over the nutrient agar plates, and immediately afterwards, the plates were sprayed with a saturated solution of pyrene in diethyl ether. The plates were incubated for 30 days at 30°C in an incubator (Labrepco, Southampton, Pa.) that maintained high humidity. Colonies that produced clearing of the opaque pyrene layer were purified by restreaking, and one of the isolates was selected for the subsequent studies. In addition to morphology and staining characteristics, the fatty acid profile of the bacterium was used in its identification. The MIDI/Hewlett-Packard microbial identification system was used by Analytical Services Inc., Essex Junction, Vt.

Cell surface hydrophobicity of the isolate was determined by the BATH assay of Rosenberg (17). The bacterium was pregrown for 5 days in nutrient broth (Becton Dickinson), harvested and washed twice in SBM by centrifugation (10,400 × *g*, 10 min), and finally suspended in phosphate buffer (pH 7.0) containing urea (1.8 g/liter) and $MgSO_4$ (0.2 g/liter). The partition of the cells between this aqueous phase and *n*-hexadecane was measured as specified by the assay (17). For comparison, *Escherichia coli* pregrown in nutrient broth for 2 days was subjected to the same BATH assay.

Preparation of cell suspensions for PAH mineralization experiments. The *Mycobacterium* sp. was pregrown in nutrient broth containing 0.001% (wt/vol) pyrene with gyratory shaking (300 rpm) at 30°C for 5 days. The cells were

harvested by centrifugation (10,400 × *g*, 10 min), washed once in SBM, and resuspended in the same medium. The suspension was passed through a packed glass wool filter to remove any cell clumps and remaining pyrene crystals. The cell protein content of the suspension was determined and, if necessary, adjusted to the range of 20 to 30 μg of cell protein per ml. Modified 250-ml screw-cap flasks (14) were each charged with 6.2 mg of pyrene and 50,000 dpm (0.1 μg) of [^{14}C]pyrene. These additions were made from the same diethyl ether stock solution, 500 μl per flask. The solvent was evaporated and the flasks were steam-sterilized prior to the addition of the aqueous solutions. Glucose (6.2 mg unlabeled, 0.02 μg labeled, 100,000 dpm) was added to flasks as a filter-sterilized aqueous solution. To each of the prepared flasks, 25 ml of cell suspension was added, resulting in a concentration of 250 μg of substrate per ml. In the case of the pyrene, this was far above its aqueous solubility limit. All subsequent treatments and untreated controls that contained pyrene and cells only were made in triplicate flasks. Incubation was at 30°C with gyratory shaking (300 rpm). At appropriate time intervals, the headspace of the flasks was flushed through double Oxosol C-14 CO_2 traps (14). Because of the low volatility of pyrene, traps for volatilized substrate were not used.

Growth experiments on hydrophobic solvents. To investigate the mode of action of hydrophobic solvents on the rate of pyrene mineralization by the *Mycobacterium* sp., it was essential to determine whether any of these hydrocarbon solvents was used as a growth substrate by this bacterium. However, because of the emulsification of these solvents and the adherence of the *Mycobacterium* sp. cells to the solvent droplets, protein determination in the cell suspensions was not possible in the usual manner. The following procedure was developed to overcome this problem.

To 25 ml of SBM containing 200 μl of sterile hydrophobic solvent, an inoculum was added to result in an initial cell protein concentration of 52 μg/ml. Incubation conditions were the same as before (30°C, 300 rpm). At days 3 and 7, 300-μl aliquots were removed and combined with 900 μl of a solvent mixture consisting of ethanol-acetone-chloroform in a 100:100:8 ratio. The mixture was vortexed to break the emulsion, and the cells were sedimented in a microcentrifuge at 7,100 × *g* for 5 min. The supernatant was discarded, and the cell pellet was suspended and washed in 300 μl of water plus 900 μl of the solvent mixture. The cells were sedimented again, the supernatant was discarded, and any solvent residue was removed under a nitrogen stream. Finally, the cell pellet was suspended in 150 μl of water–50 μl of 1 N NaOH and used for protein determination in the usual manner (13). Growth on all solvents was evaluated in this manner with triplicate flasks.

Comparison of pyrene mineralization rates by cells attached to solvent droplets or suspended in the aqueous medium. Microscopic observation of the cultures that contained hydrophobic solvents indicated that many of the *Mycobacterium* sp. cells were attached to the emulsified solvent droplets while others were in aqueous suspension. In an attempt to quantify the ratio of adhering and suspended cells during growth, Erlenmeyer flasks were each charged with 25 ml of *Mycobacterium* sp. suspension in SBM (28 μg of protein per ml) and 200 μl of paraffin oil containing 5 mg of pyrene. The triplicate flasks were incubated under the usual conditions (30°C, 300 rpm). In daily intervals, samples were removed and centrifuged at low speed (2,160 × *g*) for 10 min. This resulted in an upper layer of oil emulsion with attached cells, a clear aqueous supernatant, and a cell pellet of previously suspended cells. The oil emulsion layer with the attached cells was carefully removed with a Pasteur pipette, the oil emulsion was broken with the previously described solvent mixture, and the protein of the attached cells was determined (13). The clear aqueous supernatant was discarded, and the cell pellet was washed, resuspended, and also used in protein determination. Although care was taken to use minimal force, it is recognized that centrifugation may have detached some previously solvent-attached cells. Therefore, the proportion of solvent-attached cells may have been underestimated, with a corresponding overestimation of the suspended cells.

The separated attached and unattached cells were also used in subsequent pyrene mineralization experiments. In these cases, attached and unattached cells were separated after 3 days of incubation with mineral oil that did not contain pyrene. The oil phase with the attached cells was added to 25 ml of fresh SBM. The cell pellet was also suspended in 25 ml of fresh SBM that either did or did not receive 200 μl of mineral oil. Pyrene (2 mg, 50,000 dpm) was added to each flask sterile 0.5-mm-diameter glass beads by means of coating with pyrene $^{14}CO_2$ evolution and measuring as described earlier.

Analytical procedure. Protein was determined by the Lowry method (13) with bovine serum albumin as a standard. Radiolabeled CO_2 trapped in Oxosol C-14 was counted in a Beta-Trac model 6895 instrument (TM Analytic, Elk Grove Village, Ill.). Counts of labeled PAH and glucose were made in Scintiverse BD scintillation fluid (Fisher Scientific, Springfield, N.J.). All counts were corrected for background and for counting efficiency by the channel ratio method.

The solubility of pyrene in water and in mineral oil was determined by adding excess pyrene of known radioactivity. After equilibration at room temperature with shaking for 24 h, any suspended material was removed by centrifugation and an aliquot of the supernatant was counted.

Partition of pyrene between water-paraffin oil and water-heptamethylnonane was determined by dissolving 1.0 mg of high-specific-activity pyrene (10^6 dpm/mg) per ml in the hydrophobic solvent. Equal volumes of H_2O were added, and the two phases were allowed to equilibrate at 30°C and 300 rpm for 6, 12, and 24 h. Subsequently, the aqueous and solvent phases were allowed to separate and counted for dissolved radioactivity individually.

Quality and statistical controls. $^{14}CO_2$ trapping efficiency was evaluated periodically by releasing known amounts of $^{14}CO_2$ from $Na_2{}^{14}CO_3$ by acid and counting the Oxosol C-14 traps. Trapping efficiency averaged 97% ± 3%. A typical radiocarbon balance at the end of 4-day pyrene mineralization experiments was 55.6% CO_2, 14.5% cell biomass, 20.5% dissolved in the aqueous medium, and 90.6% total recovered. For this reason, we terminated mineralization experiments when $^{14}CO_2$ evolution approached 50%. All experiments were performed in triplicate flasks. The error bars in the figures indicate 1 standard deviation. Initial rates of mineralization were calculated usually from time zero through two or more time points, as long as $^{14}CO_2$ evolution remained linear. The protein basis of specific activity was the time zero protein determined in each experiment. Because of the slow growth of the isolate on pyrene, the protein increase during the time period of rate measurements was negligible.

RESULTS AND DISCUSSION

Isolation, characterization, and growth of the *Mycobacterium* sp. From the soil enrichment, a bacterium that produced a clearing zone on the opaque pyrene-covered nutrient agar plate was purified and selected for the subsequent studies. The morphology and the Ziehl-Neelsen acid-fast staining (8) of the bacterium suggested that it belonged to the *Mycobacterium* group. The fatty acid profile of the bacterium confirmed this tentative identification, although the index of similarity of the isolate to the type strains in the database was only 0.113. Low similarity index values are not unusual for environmental isolates that degrade xenobiotics.

The cell surface hydrophobicity of the isolate was high, 65% of the *Mycobacterium* cells adhering to the *n*-hexadecane phase in the BATH assay (17) in comparison with only 2.7% for *E. coli*. The increase of cell biomass on pyrene was only 50 to 70% per week, making it difficult to obtain sufficient biomass for experiments. Somewhat surprisingly, cells with comparable or even higher pyrene mineralization activity (73 to 89 μg of pyrene per mg of protein per h) grew in nutrient broth containing only 0.001% (wt/vol) pyrene. On pyrene-spiked nutrient broth, the middle to late exponential growth phase was reached 4 to 5 days after inoculation. In addition to pyrene, phenanthrene served as a growth substrate, but radiolabeled chrysene and benzo(*a*)pyrene were not mineralized. None of the hydrophobic solvents that increased PAH utilization by the *Mycobacterium* sp. served as a growth substrate for this microorganism.

All reports on the use of pyrene as a growth substrate are relatively recent. In 1988, Heitkamp et al. isolated a *Mycobacterium* sp. that mineralized pyrene and some additional PAH compounds but that also required additional organic nutrients for growth (10). Nevertheless, this *Mycobacterium* sp. was used in determining the pathway of pyrene mineralization (11). In 1990, Weissenfels et al. (20) for the first time described the growth of a pure culture (*Alcaligenes denitrificans*) on the four-ring PAH fluoranthene. This report was followed in rapid succession by reports of growth on pyrene as the sole carbon and energy source by a *Rhodococcus* sp. in 1991 (19) and by a *Mycobacterium* sp. in 1993 (4). Our *Mycobacterium* isolate seems free of any vitamin requirements, and its ability for pyrene degradation does not decrease when it is grown on nutrient broth and low concentrations (0.001%) of pyrene. Its high surface hydrophobicity is consistent with the high mycolic acid content of mycobacterial cell walls (16).

The pyrene degradation rates reported for the *Mycobacterium* isolate of Boldrin et al. (4) can be compared with our results only roughly because of the differing measurement approaches and the unspecified cell densities. The pyrene degradation rates reported by Boldrin et al. ranged from 1 to 5 μg/ml/h, depending on the crystal size of pyrene. By calculating with the 55% conversion of pyrene to CO_2 observed in our

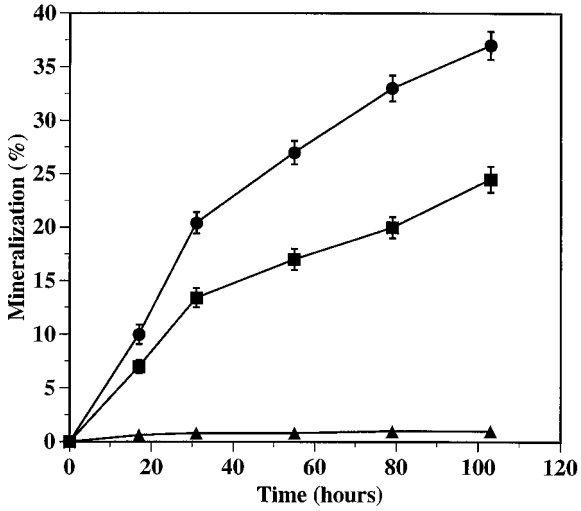

FIG. 1. Pyrene mineralization by the *Mycobacterium* sp. in the presence of Triton X-100 at 4 μl/ml, below the CMC (●), and at 46 μl/ml, above the CMC (▲), and in the absence of Triton X-100 (■). The initial mineralization rates with and without a low Triton X-100 concentration were 120 and 78 μg of pyrene per mg of protein per h, respectively.

TABLE 1. Effect of solvent additives on the initial rates of mineralization of pyrene by the *Mycobacterium* sp.

Solvent additive	Concn (μl/ml)	Mineralization rate	
		μg/mg of protein/h	%[a]
None (control)		81	100
PEG[b] (mol wt, 600)	4	29	35
Heptamethylnonane	2	25	31
	6	22	29
Phenyldecane	2	46	56
	4	26	32
	10	20	24
Diphenylmethane	4	8	10
Paraffin oil	4	116	143
	10	150	184
	16	112	137
Tridecylcyclohexane	8	182	225
cis-9-Tricosene	8	172	212
Squalane	8	172	211
Squalene	8	161	197

[a] Mineralization of pyrene by the *Mycobacterium* sp. without any additives (81 μg/mg of protein per h) was chosen as the basis (100%) for comparison. In reality, the rates listed above were derived from several separate experiments with their own somewhat higher or lower control values. For better comparison, the data were normalized to the single average control value shown above.

[b] PEG, polyethylene glycol.

work with the *Mycobacterium* sp., our degradation rate without paraffin oil was 4 μg/ml/h. The addition of paraffin oil increased this to 8 μg/ml/h.

Effects of detergents and water-miscible solvents on the rates of pyrene mineralization by the *Mycobacterium* sp. Detergents and water-miscible solvents were added to increase the solubility or pseudosolubilization of pyrene, thus potentially augmenting its mass transfer and mineralization rates. The nonionic detergent Triton X-100, when applied at 4 μl/ml, increased the mineralization rate of pyrene to 147% of the control (Fig. 1). When the detergent concentration was increased to 46 μl/ml, exceeding its CMC of 23 μl/ml (18), pyrene mineralization was completely inhibited. Tested at their respective CMCs, 1-decanesulfonic acid, glycodeoxycholic acid, and *n*-octyl-β-D-glucopyranoside were as inhibitory to pyrene mineralization as Triton X-100 was (data not shown). The effect of Triton X-100 and other detergents on pyrene mineralization was in good agreement with the results of Laha and Luthy (12), who investigated the effect of nonionic detergents on the mineralization of phenanthrene. They also observed stimulation below the CMC and severe inhibition above the CMC level. The inhibition did not appear to be due to toxicity but rather to the occlusion of PAH into micelles that, according to their theory, prevents PAH uptake by cells. The water-miscible solvent polyethylene glycol, added at 4 μl/ml, also inhibited the mineralization of pyrene. This was most likely due to toxicity, although we did not perform specific experiments to prove this. To save space, some of the negative or repetitive results like the polyethylene glycol data are summarized in Table 1.

Effects of hydrophobic solvents on the rates of PAH mineralization by the *Mycobacterium* sp. The addition of heptamethylnonane at concentrations similar to the amounts used by Efroymson and Alexander (7) inhibited the mineralization of pyrene and phenanthrene by the *Mycobacterium* sp. (Table 1). Similarly, the solvents decalin, diphenylmethane, and phenyldecane were also inhibitory. However, when paraffin oil was added at 8 μl/ml to SBM, the initial rate of pyrene mineralization by the *Mycobacterium* sp. was doubled (Fig. 2). When at the concentration of 250 μg of pyrene per ml the amount of added paraffin oil was varied (Table 1), the 8-μl/ml addition shown in Fig. 2 was found to be optimal. In subsequent experiments, tridecylcyclohexane, *cis*-9-tricosene, squalane, and squalene were used at the same 8-μg/ml concentration. During the first 24 h of incubation, these hydrophobic solvents doubled the rates of pyrene mineralization (Table 1). While the solvents enhanced the mineralization of the hydrophobic pyrene, the addition of paraffin oil had a marginally negative influence on the mineralization of water-soluble glucose (data not shown).

Our work with hydrophobic solvents was inspired by previous reports on the use of heptamethylnonane as an inert hydrophobic solvent for naphthalene and *n*-hexadecane (7, 21).

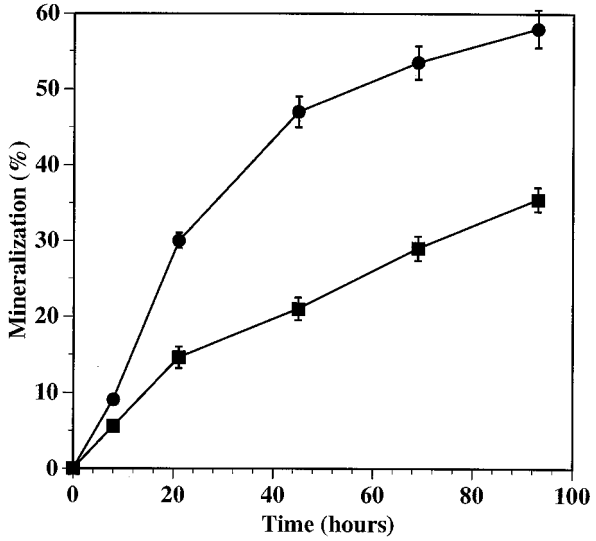

FIG. 2. Pyrene mineralization by the *Mycobacterium* sp. in the presence (●) and absence (■) of 8 μl of paraffin oil per ml. Initial mineralization rates were 163 and 81 μg of pyrene per mg of protein per h, respectively.

These reports demonstrated that the inclusion of this solvent at concentrations from 1 to 10 μl/ml increased the utilization of the hydrophobic substrates. The maximal stimulatory solvent concentration was not explored, but at 10 μl/ml, the hydrophobic solvent approximately doubled the rates of substrate mineralization. Indirect evidence suggesting that a portion of the microbial cells attached to the solvent-water interface was presented. The addition of Triton X-100 at 1 μl/ml prevented the mineralization of hexadecane but not of naphthalene.

We tested heptamethylnonane and several other hydrophobic solvents for augmenting pyrene mineralization without success. We did not specifically investigate the reasons for these negative results, but toxicity of the solvents to the *Mycobacterium* sp. is a likely explanation. When we tested these solvents at several concentration levels, the inhibition of pyrene mineralization increased with the solvent concentrations (Table 1). The opposite effect was observed by Efroymson and Alexander (7). In the majority of cases, non-aqueous-phase liquids act to sequester hydrophobic substrates and reduce their availability for biodegradation (1). Whether a particular substrate is sequestered by a non-aqueous-phase liquid depends at least in part on the partition coefficient of the substrate between the non-aqueous-phase liquid and the aqueous-phase liquid. Differing partition coefficients of hexadecane and naphthalene in the heptamethylnonane-aqueous phase system provided a satisfactory explanation for the different behaviors of the two substrates in the presence of both heptamethylnonane and Triton X-100 (7). In this case, the utilization of the hydrophobic hexadecane was prevented, but the less hydrophobic naphthalene continued to be utilized at an increased rate, presumably from the aqueous phase (7). With these findings in mind, we compared the partition of pyrene between water and mineralization-promoting paraffin oil with pyrene partition between water and inhibitory heptamethylnonane. In both of these cases, however, dissolution of pyrene in the aqueous phase was undetectable, and the partition behavior of pyrene into the two non-aqueous-phase liquids did not explain their opposite effects on pyrene utilization.

Paraffin oil is a complex mixture of liquid alkanes, and individual components of a mixture may contribute a variety of effects. For this reason, we sought to reproduce the effect of paraffin oil with defined hydrocarbon compounds. We formed the theory that a solvent suitable for our purpose should be a hydrocarbon in the C_{20} to C_{30} range that is a liquid at room temperature. Since *n*-alkanes starting with C_{18} are solid at room temperature, we selected hydrocarbons that branched and/or had some unsaturated bonds. We tested with success four such hydrocarbons (Table 1), and they should facilitate future mode-of-action studies on solvent-augmented pyrene utilization.

Rates of pyrene mineralization by suspended and solvent-attached *Mycobacterium* sp. cells. Microscopic observation showed that numerous *Mycobacterium* sp. cells adhered to the emulsified solvent droplets, forming an incomplete cell monolayer around the droplets, while other cells remained freely suspended in the aqueous portion of the SBM. The quantitative shift with time towards attached cells, after addition of pyrene (200 μg/ml) and paraffin oil (8 μl/ml) to the *Mycobacterium* sp. suspended in SBM, is shown in Fig. 3. While some growth occurred during the 4-day incubation period, raising the initial cell protein level from 28 to 33 μg/ml (a 20% increase), most of the attachment was by preexisting suspended *Mycobacterium* sp. cells. In spite of the vigorous shaking and the rapid formation of the solvent emulsion, cell attachment was a slow, gradual process that may have reflected changes in cell surface hydrophobicity with culture age. Since the separation of the oil phase involved centrifugation, the proportion of the attached cells represents a minimal value. The possibility exists that some less strongly attached cells were detached by the centrifugation process.

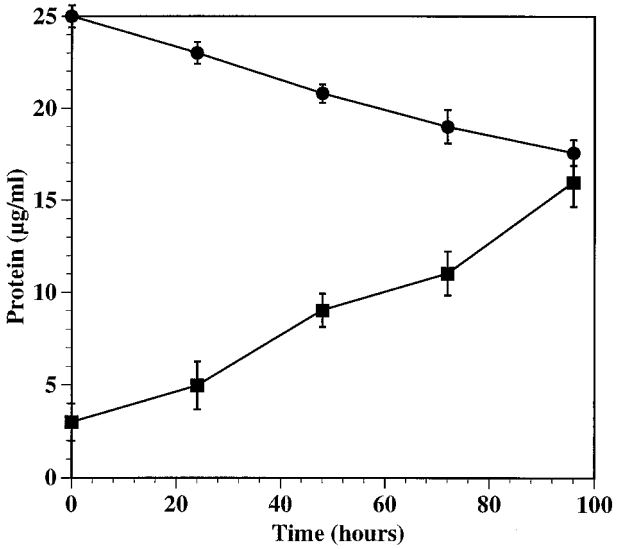

FIG. 3. Distribution of the *Mycobacterium* sp. biomass attached to paraffin oil droplets (■) or suspended in the aqueous medium (●).

When solvent-attached and suspended cells were separated and their pyrene mineralization rates were compared, the suspended cells mineralized pyrene on a milligrams-of-cell-protein basis at 40 μg/h, while the solvent-attached cells mineralized pyrene at 344 μg/h, i.e., at an 8.5 times higher rate (Fig. 4). For the solvent-attached cells, the rate calculations were based on the 9- to 18-h period, since the time required for the pyrene to be partitioned into the paraffin oil droplets caused an initial lag. The rate comparison was based on the performance of suspended cells in the presence of 8 μl of mineral oil per ml. Thus, some of the initially suspended cells may have attached

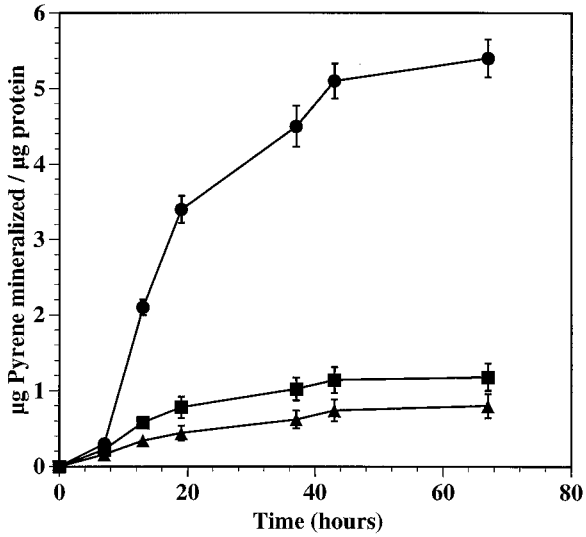

FIG. 4. Rates of pyrene mineralization by *Mycobacterium* sp. cells attached to paraffin oil droplets (●) or suspended in the aqueous medium in the presence (■) or absence (▲) of paraffin oil. The respective initial mineralization rates were 344, 40, and 20 μg of pyrene per mg of protein per h.

themselves to the paraffin oil droplets during the measurement period. When the paraffin oil addition was omitted, the performance of suspended cells was even lower (20 μg/mg of protein per h). Calculated on this basis, pyrene mineralization by solvent-attached cells was more than 1 order of magnitude higher.

Solubility and partitioning characteristics of pyrene at 30°C. The water solubility of pyrene was reported at room temperature to be 140 ng/ml (6). Our own measurement of pyrene water solubility at 30°C (184 ng/ml) was close to the reported value. Pyrene solubility in paraffin oil was measured by us at 25 mg/ml, i.e., at 5 orders of magnitude higher than in water. Not surprisingly, when pyrene was partitioned between equal volumes of paraffin oil and water, pyrene was undetectable in the water phase. In a similar partitioning experiment between water and heptamethylnonane, pyrene was also undetectable in the water phase. Since the partitioning experiments involved 10^6 dpm/ml and the reliable detection limit was 10^2 dpm, we concluded that in each case less than 0.001% of the pyrene was partitioned into the water phase. The opposite effects of paraffin oil and heptamethylnonane additions on pyrene mineralization were not explained by any differences in pyrene partitioning measured in the paraffin oil-water and heptamethylnonane-water systems.

Mode of action of hydrophobic solvents in promoting pyrene mineralization by the *Mycobacterium* sp. Although mechanistic studies on the utilization of hydrophobic substrates were not prime objectives of this work, here we also interpret our observations also from this point of view. None of the solvents that promoted pyrene mineralization served as a growth substrate for our *Mycobacterium* isolate, and the same solvents were ineffective in promoting utilization of a hydrophilic substrate such as glucose. The interaction between solvents and the *Mycobacterium* sp. was moderately specific. Paraffin oil and four selected liquid hydrocarbons in the C_{20} to C_{30} range were effective, while several other solvents, including heptamethylnonane (7), were inhibitory to PAH utilization by the *Mycobacterium* sp.

For solvents that promoted pyrene mineralization, measurements demonstrated that *Mycobacterium* cells gradually attached themselves to emulsified solvent droplets, and these attached cells mineralized pyrene about an order of magnitude faster than their suspended counterparts. From Fig. 4, it is evident that the mere presence of paraffin oil has little if any effect on PAH utilization, unless the *Mycobacterium* cells are actually attached to the solvent droplets. This rules out the possibility that the presence of solvents somehow modifies the cell wall or cell membrane of the *Mycobacterium* sp., rendering them more suitable for pyrene uptake.

By the preponderance of the evidence, we propose a mass transfer model for explaining the mode of action of hydrophobic solvents on pyrene mineralization. This model is similar to the one proposed by Efroymson and Alexander (7). It is supported by solubility and partition data for pyrene. Of 200 μg of pyrene per ml added to an aqueous medium, 99.93% remains a crystalline solid and only 0.07% is in aqueous solution. Under these circumstances, *Mycobacterium* cells have direct access only to the minute dissolved portion of the pyrene. In addition, they may interact with the crystalline surface of the undissolved solid. In both cases, mass transfer is likely to be slow.

Upon addition of 8 μl of solvent per ml, all crystalline pyrene was dissolved in it, and pyrene in the water phase became undetectable. With time, increasing numbers of the *Mycobacterium* sp. cells became attached to the paraffin oil, and the cells adhering to these droplets mineralized pyrene at least 8.5 times as fast as those suspended in the aqueous medium.

Prospects for application. The demonstrated principle of solvent-augmented pyrene biodegradation has a potential for lowering the costs and time requirements of biotreatment for refinery sludges that are classified as hazardous wastes because of their PAH levels (3). Paraffin oil is an inexpensive additive with such low toxicity that it is taken orally as a laxative. No prohibitive cost or environmental hazard appears to prevent its addition to aqueous or slurry-type bioreactors. While not degraded by the *Mycobacterium* sp., paraffin oil in a mixed culture is expected to degrade along with the PAH pollutants. Obviously, several hurdles need to be overcome to realize the potential of solvent-augmented PAH bioremediation. The adherence of the *Mycobacterium* sp. to paraffin oil may be specific. It needs to be demonstrated that the approach also works with the less controlled microbial community of refinery sludges in the presence of organic and inorganic particulates and on a mixture of PAH compounds. The potential benefits of solvent-augmented bioremediation appear to justify further exploration of this bioremediation principle.

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