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Interfacial effects in a two-phase partitioning bioreactor: degradation of polycyclic aromatic hydrocarbons (PAHs) by a hydrophobic *Mycobacterium*

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Abstract

The growth of *Mycobacterium* PYR-1 on polycyclic aromatic hydrocarbons (PAHs) was examined in a two-phase partitioning bioreactor (TPPB). TPPBs are characterized by a cell-containing aqueous phase, and an immiscible and biocompatible organic phase that partitions toxic/insoluble substrates to the cells based on their metabolic demand and the thermodynamic equilibrium of the system. Particular emphasis was placed on assessing the nature of the organism's interaction with the aqueous-organic interface. Results showed that agitation rate affected cell growth and PAH degradation rates, while substrate concentration did not, two characteristics of systems exhibiting an interfacial uptake mechanism. *Mycobacterium* PYR-1 associated exclusively with the aqueous-organic interface and contact angle measurements confirmed the organism's high hydrophobicity. This preferential association with the organic phase was also shown to reduce or eliminate the uptake of a water-soluble substrate (glucose) by the organism from the aqueous phase. Moreover, detailed examination using fluorescence microscopy revealed that, in addition to associating with the aqueous-organic interface, this bacterium exists exclusively on the organic side of the interface. This is the first report of an organism actively growing in the organic phase of a TPPB and may have implications on the future study of hydrophobic organisms in TPPBs and in the natural environment.

Keywords: Mycobacterium; PAH; Degradation; Partitioning Bioreactor; Hydrophobicity

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs), many of which possess carcinogenic, mutagenic and/or teratogenic properties, are ubiquitous chemicals, produced via natural and anthropogenic sources. Their abundance and toxicity have resulted in numerous potential treatment methods, recently focusing on the use of two-phase partitioning bioreactors (TPPBs) [1–6]. TPPBs are characterized by an immiscible organic phase loaded with a target substrate that partitions to microorganisms contained in the aqueous phase, based on equilibrium considerations and the real-time demand of the organisms. Guiysse et al. [5], and Daugulis and Janikowski [4] note that the hydrophobic nature of PAHs makes them ideal candidates for degradation in TPPB systems, since large masses and high surface areas can be generated by dissolving PAHs in a dispersed organic phase, and the highest PAH degradation rates recorded in the literature have been achieved in TPPBs [2,6].

Déziel et al. [7] have proposed three predominant methods of microbial substrate uptake in TPPBs: uptake of dissolved substrate in the aqueous phase, production of biosurfactants to enhance uptake of hydrophobic substrates, and uptake of substrate by direct contact with the organic phase. This final mode of uptake appears to be significant in the degradation of hydrophobic compounds, including PAHs, as studies have found that PAH degradation rates of microbes adhering to the aqueous-organic interface in a TPPB are often significantly higher than those of microbes suspended in the aqueous phase [2,7,8].

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The degradation of insoluble substrates via direct contact has been studied extensively, especially during the early 1970s when significant efforts were focused on the use of hydrocarbon substrates for the production of single cell protein (SCP). It had been demonstrated that agitation rate, impeller speed and dispersed phase volume affected degradation rates, presumably because they affect the interfacial area [9–15]. The uptake of substrate from the organic phase by the cells is the rate-limiting step, and growth curves in these systems are often linear, rather than exponential [11,15–17], reflecting fixed aqueous/organic surface areas at a particular set of conditions. It has also been shown that when organisms associate with the organic phase, their uptake of compounds in the aqueous layer can be affected [8,18].

In an earlier study [19] we showed that the selection and use of a bioavailable (i.e. one that can be metabolized) solvent, bis(ethyl hexyl) sebacate (BES), did not interfere with PAH degradation in a TPPB, but observed that cells of *Mycobacterium* were almost exclusively associated with the BES phase. The goal of this study was to examine the nature of the interaction between *Mycobacterium* PYR-1, a hydrophobic organism, and the aqueous-organic interface in a TPPB system.

2. Materials and methods

2.1. Organism and growth conditions

Mycobacterium PYR-1 was obtained from Dr. C.E. Cerniglia, Division of Microbiology, National Center for Toxicological Research, FDA, Jefferson, AR, USA, and its properties have been described previously [20,21]. Stock cultures were maintained on agar plates coated with phenanthrene crystals. The agar contained, per litre of dH_2O: 15 g Bacto-agar, 1 g glucose, 0.38 g tryptone, 0.38 g yeast extract, 0.05 g $MgSO_4{\cdot}7H_2O$, 0.12 g NaCl, 0.48 g $(NH_4)_2SO_4$, 0.40 g $NaNO_3$, 0.90 g KH_2PO_4, four drops of 0.01% $FeCl_3$ solution and 2 ml of trace element solution containing, per litre: 0.30 g KI, 0.43 g $SnCl_2{\cdot}2H_2O$, 0.20 g LiCl, 0.80 g $CuSO_4{\cdot}5H_2O$, 2.10 g $AlK(SO_4)_2{\cdot}H_2O$, 0.55 g $NiCl_2$, 0.85 g $CoCl{\cdot}6H_2O$, 0.60 g boric acid, 0.37 g $MnSO_4{\cdot}H_2O$ and 0.30 g $FeSO_4{\cdot}7H_2O$. These plates were streaked with *Mycobacterium* PYR-1 cells and coated with 1 ml of a saturated solution of phenanthrene in *iso*-octane. The *iso*-octane was allowed to evaporate, leaving a lawn of phenanthrene crystals on the plate.

Liquid medium for inoculum purposes was the same composition as the agar medium, with no Bacto-agar and no phenanthrene. For bioreactor experiments, 250 ml of medium was inoculated with a single colony, incubated for 3 days at 30 °C and 165 rpm. This was then used to inoculate 2–500 ml portions of medium which were then incubated under the same conditions for an additional 3 days, combined, centrifuged at 3000 × *g* for 5 min, resuspended in 200 ml dH_2O and added to the reactor. The large inoculum was necessary as *Mycobacterium* PYR-1 grows relatively slowly on glucose, resulting in low cell densities. Bis(ethyl hexyl) sebacate BES, a bioavailable solvent (i.e. one that can be metabolized by this organism), was used in the TPPB bioreactors, as previously described [19].

2.2. Bioreactor conditions

Fermentations were carried out in New Brunswick Scientific Bioflo III fermentors equipped with Broadley James D100 Series Oxyprobes and FermProbes for monitoring dissolved oxygen and pH, respectively. The pH was controlled to within ±0.1 of the set point with 2 M KOH. The reactors were operated at 30 °C, a pH of 7.4, an agitation rate of 300 rpm and an aeration rate of 4.0 l/min. Biomass was measured as the cell dry weight of a 15 ml sample of well-mixed reactor broth. All experiments used 3 l of aqueous medium with the masses of $MgSO_4{\cdot}7H_2O$, NH_4SO_4 and $NaNO_3$ tripled and 500 ml of BES (the bioavailable TPPB delivery solvent), both of which were autoclaved at 121 °C for 20 min. Nutrient boluses, containing all of the components of 3 l of aqueous medium in 100 ml of dH_2O, were added periodically to each fermentation.

2.3. Effect of agitation rate on pyrene degradation

Two bioreactors were run in parallel, one reactor operated at 300 rpm and one at 500 rpm. Each reactor contained 500 ml of BES loaded with 0.55 g pyrene. Two 1 l portions of inoculum were prepared as described, combined, and then used to inoculate the reactors. The reactors were operated for 83 h and nutrient boluses were added to both reactors at 27.5 h.

2.4. Impact of PAH concentrations on phenanthrene degradation

Previous work [19] had shown that *Mycobacterium* PYR-1 is capable of degrading phenanthrene, pyrene, anthracene and naphthalene simultaneously, and the goal of this experiment was to determine whether different substrate amounts, with resulting different cell concentrations, would affect the phenanthrene degradation rate (alternatively, if the phenanthrene degradation rates remained the same irrespective of substrate or cell concentration, then availability, i.e. interfacial surface area, could be the limiting factor). Thus, three TPPB bioreactor experiments undertaken at different initial substrate loadings of: equal amounts of phenanthrene and pyrene, double the amount of phenanthrene and pyrene, and the original amount of phenanthrene and equal amounts of anthracene and naphthalene. The actual levels were: 0.496 g phenanthrene and 0.498 g of pyrene, 0.994 g of phenanthrene and 1.001 g pyrene, and 0.515 g phenanthrene, 0.513 g anthracene and 0.494 g naphthalene. All experiments were run under the described conditions at 300 rpm.

2.5. Quantification of hydrophobicity

The hydrophobicity of *Mycobacterium* PYR-1 was quantified using the method of van Loosdrecht et al. [22]. A lawn of bacteria was prepared on a sterile 0.22 μm filter and placed in a dessicator for approximately 2 h. The contact angle of a drop of 0.1 M NaCl with this surface was then recorded using a microscope with a goniometric eyepiece. The contact angle was measured on both the left and right side of three different drops, placed on different bacterial lawns.

2.6. Effect of the presence of immiscible solvent on the uptake of glucose from the aqueous phase

This experiment was conducted to determine whether the cells' affinity for the immiscible organic phase affected the uptake of a water-soluble substrate. Accordingly, 70 ml of aqueous medium supplemented with 1 g/l glucose was added to each of three 250 ml flasks. To the three flasks was added no solvent, 20 ml of 2,2,4,4,6,8,8-heptamethylnonane (HMN), a non-bioavailable solvent (i.e. one that is not metabolized), and 20 ml of bis(ethylhexyl) sebacate (BES), a bioavailable solvent, respectively. All three flasks were inoculated with 10 ml of bacterial culture and were incubated at 30 °C and 165 rpm for 72 h. Two millilitre samples were taken from the aqueous layer every 24 h and analyzed for their glucose content via the DNS assay [23]. After 72 h, the flasks were removed from the incubator and the biomass in each flask was quantified gravimetrically.

2.7. Microscopic examination of bioreactor broth

A fermentation was conducted under the described conditions, using 500 ml of BES loaded with 0.265 g of phenanthrene. One litre of inoculum was prepared as described and then labelled with FITC (fluorescein-5-isothiocyanate). The 2–500 ml inoculum portions were centrifuged, and decanted. The cells were resuspended in 200 ml of sterile aqueous medium and placed in a sterile 500 ml flask to which 1 ml of a 40 g/l FITC solution (prepared by dissolving 200 mg FITC in 5 ml DMSO) was added. The flask was then covered with aluminum foil and incubated at 30 °C and 165 rpm for 30 min. The solution was then centrifuged at 3000 × *g* for 5 min and decanted. The FITC-labelled cells were then resuspended in 200 ml of sterile aqueous medium and used to inoculate the reactor.

The reactor was covered in foil and left to run for 12 h, at which point a well-mixed 25 ml sample was taken and placed in a foil-covered test tube. Twenty-five microlitre of a 2 g/l solution of nile red (prepared by dissolving 20 mg of nile red in 10 ml of DMSO) was added to the sample, to label the organic phase.

The solution was examined with a LEICA TCS SP2 fluorescent confocal microscope. Both a regular microscope slide and a hanging drop microscope slide were prepared and examined at a magnification of 100 times with immersion oil. An argon ion laser at 488 nm and a green HeNe laser were used to excite the FITC and nile red in the samples, respectively. Fluoresence was collected at 500–535 nm (FITC) and 583–688 nm (nile red). The pinhole was set to 1 ary disk and a 1.4 numerical aperture was used. All data were acquired and analyzed using LEICA software, version 2.0.

2.8. Analytical methods

PAHs in the organic layer were quantified using fluorescence spectroscopy. PAH concentrations in the aqueous phase were assumed negligible. Fluorescence spectra were collected using a QuantaMaster QM1 fluorescence spectrometer, Photon Technology International, London, Ontario, Canada, equipped with a 75 W Xenon arc lamp and Czerney-Turner excitation and emission monochromators. Excitation and emission slits were set to 2 nm bandpass for all measurements. A solution sample holder was used to hold the quartz cuvettes in the path of the excitation radiation. The quartz cuvettes used were type 3H, with a path length of 10 mm, obtained from NSG Precision Cells, Farmingdale, New York, USA.

Data were collected from this system using the Felix software package, version 1.1. In order to be in the linear range of detection for PAHs, all samples taken from the organic layer were diluted by a factor of 10,000 in anhydrous ethanol. Synchronous scans were performed to generate unique peaks for each PAH. The detection conditions for the synchronous scan of each PAH (Δλ, peak maxima and integration area) were, in nm: phenanthrene (53.5, 345.2, 342–350), pyrene (36.0, 369.7, 366–375).

2.9. Chemicals

PAHs were purchased from Sigma–Aldrich Canada Ltd., Oakville, ON. The fluorescent labeling compounds, fluorescein-5-isothiocyanate and nile red were obtained from Molecule Probes Inc., Eugene, OR and Sigma–Aldrich Canada Ltd., respectively. BES and all other chemicals used were obtained from Fisher Scientific, Ottawa, ON.

3. Results

3.1. Effect of agitation rate on PAH degradation

It has been consistently observed in this laboratory that *Mycobacterium* PYR-1 associates with the organic layer in TPPB systems, and thus it is possible that interfacial area is a key factor affecting PAH degradation. To examine this, interfacial area was varied between two reactors by operating at different agitations rates: 300 rpm and 500 rpm. The amount of pyrene degraded and base consumed were monitored over time (Fig. 1). A much faster rate of pyrene

degradation was observed at 500 rpm versus 300 rpm (150 mg/l-day at 500 rpm and 84 mg/l-day at 300 rpm) with both rates showing considerable linearity. Base consumption to maintain constant pH (an indirect indicator of microbial activity) illustrates a similar trend and linearity, with a total of 85 ml of base consumed at 500 rpm and only 65 ml consumed at 300 rpm in the same time period. In the reactor at 500 rpm, the final biomass concentration (reflecting uptake of pyrene and BES) was 3.9 g/l compared to only 2.6 g/l at 300 rpm. The dissolved oxygen levels in each reactor are also indicated in Fig. 1, as a percentage of the dissolved oxygen present in a fully aerated system (i.e. agitation rate of 500 rpm and aeration rate of 4 l/min). In both reactors, the dissolved oxygen never falls below 75%. Oxygen is not considered to be limiting until it is below 20%, and thus oxygen was not limiting in either reactor and did not cause the different (linear) rates. All of these results point to a faster rate of cell growth and degradation when the interfacial area is increased. The linear trend in the data presented is also intriguing, and may be another indication of an interfacial uptake mechanism [11,15,16].

3.2. Effect of PAH concentration on PAH degradation

Phenanthrene degradation curves were generated in three different fermentations run under the same operational conditions (i.e. same agitation speed to provide the same interfacial area), but with different phenanthrene concentrations, as well as the availability of other simultaneously-utilizable PAH substrates. The linear sections of the phenanthrene degradation curves (occurring at different times and cell concentrations) are shown at the time at which they occurred in each fermentation (Fig. 2). Similar phenanthrene degradation rates (slopes) are observed despite different phenanthrene and cell concentrations, and statistical analysis of the data at the 99% confidence level indicated that the phenanthrene degradation rates are in fact the same. These results are consistent with a system exhibiting an interfacial uptake mechanism that is limited by the interfacial area [1].

3.3. Quantification of hydrophobicity

In the previous experiments, a strong association between the bacteria and the organic phase, BES, was observed and hypothesized to be a result of the hydrophobic nature of *Mycobacterium* PYR-1. The hydrophobicity of the bacterium was quantified via contact angle measurements (Table 1), and a contact angle of 90° was recorded. Bacteria exhibiting contact angles below 35° are considered to be hydrophilic bacteria, and angles greater than 70° indicate hydrophobic bacteria [24], making *Mycobacterium* PYR-1 very hydrophobic. Variance in the recorded results is likely the result of inhomogeneities in the bacterial lawn [24], as *Mycobacterium* PYR-1 forms aggregates in aqueous solution that may have inhibited the formation of a smooth surface.

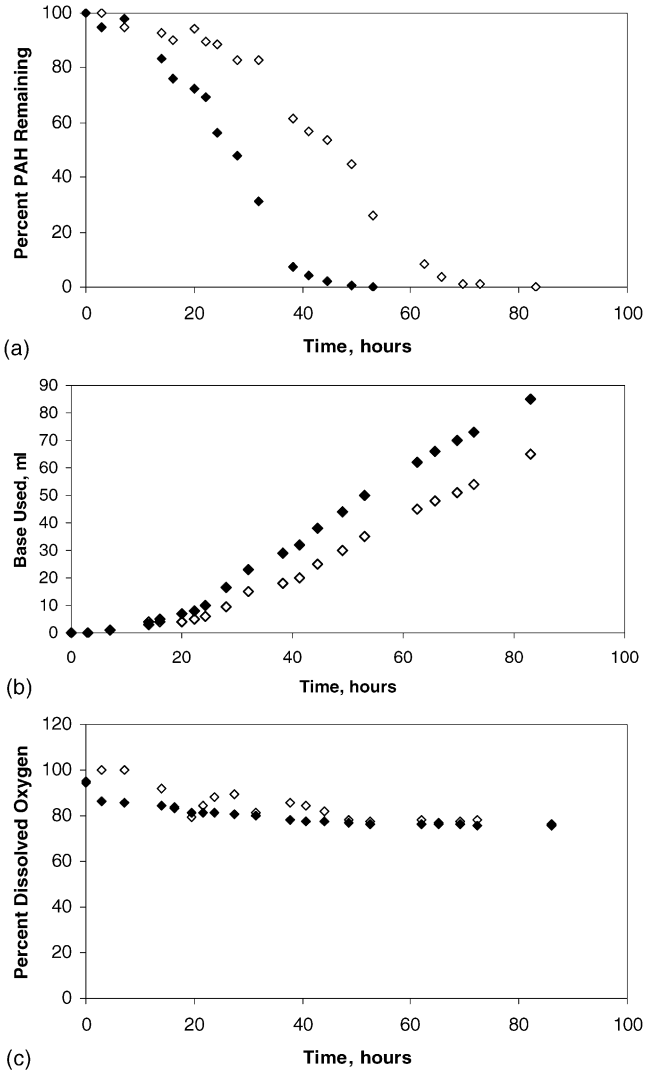

Fig. 1. Pyrene degradation (top), base consumption (middle) and percent dissolved oxygen (bottom) over time in the reactors at 300 rpm (◇) and 500 rpm (◆). The linear sections of the degradation curves illustrated correspond to degradation rates of 84 mg/l-day (300 rpm) and 150 mg/l-day (500 rpm), respectively.

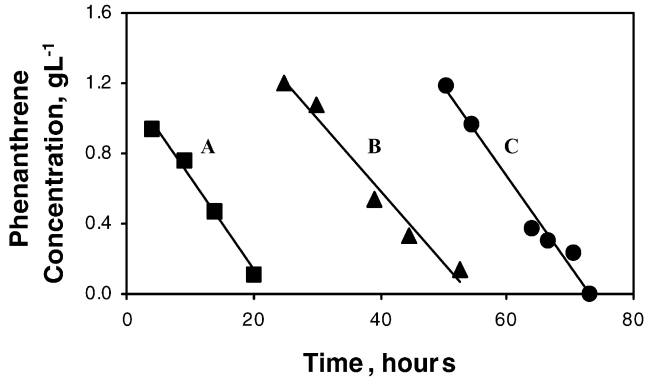

Fig. 2. Linear sections of phenanthrene degradation curves under varying biomass and phenanthrene concentrations. Curve (phenanthrene loaded, g; rate of degradation, mg/l-day): A: 0.496, 208; B: 0.994, 168; C: 0.515, 210.

Table 1
Table 4.5: Contact angle of a drop of 0.1 M NaCl with *Mycobacterium* PYR-1

Trial	Drop side (as seen through eyepiece)	Contact angle at time zero (degrees)
Drop 1	Left	88
Drop 1	Right	86
Drop 2	Left	94
Drop 2	Right	90
Drop 3	Left	88
Drop 3	Right	91
Average		90 ± 3

3.4. Uptake of glucose from the aqueous layer

The high hydrophobicity and affinity for the organic phase exhibited by *Mycobacterium* PYR-1 prompted an examination of the uptake of a water soluble substrate (glucose) by this organism in the presence of immiscible organic solvents, as other studies [8,18] have found that the rates of uptake of aqueous solutes can be affected. Glucose consumption was monitored in the presence of an immiscible solvent usable as a carbon source, bis(ethyl hexyl) sebacate (BES), a non-bioavailable immiscible solvent 2,2,4,4,6,8,8-heptamethylnonane (HMN) and no solvent (Fig. 3). In the presence of no solvent, glucose was completely consumed in 48 h. In the presence of a non-bioavailable solvent (HMN), *Mycobacterium* PYR-1 required 72 h to degrade the same amount of glucose. This impairment is not the result of any toxic effect of HMN on the bacteria, as it has been previously shown that HMN is not toxic to *Mycobacterium* PYR-1 [19]. The total biomass collected from these flasks were 29 and 30 mg, respectively, confirming that the bacteria degraded only the glucose in both flasks, and were not using any other carbon sources (i.e. the bacteria did not metabolize the HMN). In the presence of BES, glucose consumption ceased after 24 h, with only 30% of the glucose being degraded. Unlike the other flasks, a higher yield of 41 mg was collected in this flask, indicating that a substrate other than glucose (i.e. the BES) was consumed.

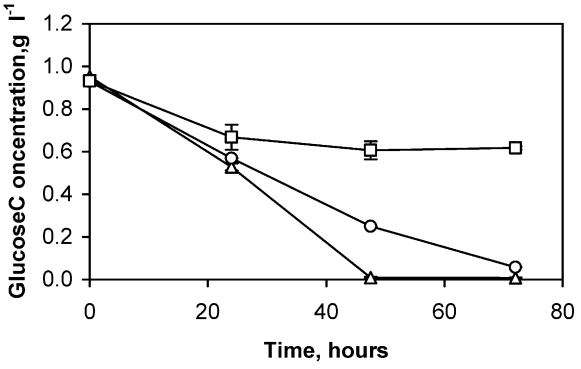

Fig. 3. Consumption of glucose in the presence of no solvent (△), HMN (○), and BES(□).

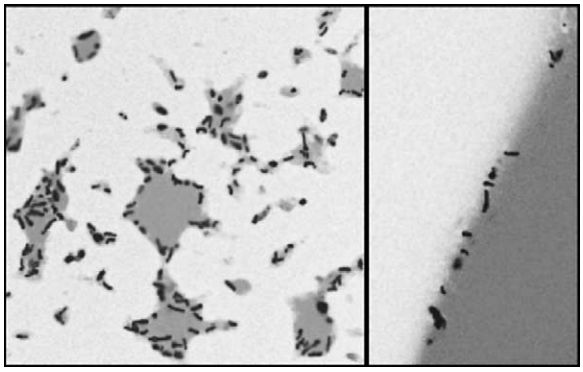

Fig. 4. Left: slide of *Mycobacterium* PYR-1 cells (black) grown in the presence of BES (gray) and aqueous medium (white). The black cells are located within the gray organic layer. Right: hanging drop slide of *Mycobacterium* PYR-1 cells contained within a BES droplet. Both images taken with a confocal microscope at 100× magnification with immersion oil.

3.5. Microscopic examination of bioreactor broth

In light of its hydrophobic nature and its apparent preference for hydrophobic solutes over aqueous solutes, the nature of the attraction between *Mycobacterium* PYR-1 and the organic phase was studied more closely. The system was fluorescently labelled (the bacteria were labelled green with FITC and the BES solvent was labelled red with nile red) and examined with a confocal microscope. In order to, obviate the high charges associated with printing colour images in scientific journals, the digital image obtained was then converted to black and white, making the aqueous layer white, the solvent layer gray and the bacteria black (Fig. 4). Both images clearly illustrate that the bacteria are associated exclusively with the organic layer. No bacteria, in these photographs or in others examined, were observed in the aqueous layer. The hanging drop slide shown in Fig. 4 reveals the true extent of the bacteria's relationship with the organic layer; the cells are located at the aqueous-organic interface in the TPPB, but on the organic side of the interface. Using the hanging drop slide technique, the location of the cells in the bioreactor broth is not altered and optical sections can be viewed with the confocal microscope. Thus, the image presented in Fig. 4 is a slice through an undisrupted droplet, and any bacteria visible in the gray organic layer are contained within it, not located at its upper or lower surface. This was confirmed to be moving vertically through the droplet using the confocal microscope, and seeing that the bacteria were in fact contained within the organic phase.

4. Discussion

Various factors affecting the degradation of PAHs by *Mycobacterium* PYR-1 in a TPPB, using BES as the organic

phase, were examined, and the results strongly suggest that PAH degradation by *Mycobacterium* PYR-1 involves an interfacial uptake mechanism. A larger interfacial area in the TPPB increased growth and degradation rates (Fig. 1), whereas substrate concentration (Fig. 2) did not affect PAH degradation rates, two attributes associated with interfacial uptake mechanisms [1]. Additionally, all PAH degradation rates had linear trends, which is consistent with cell growth involving metabolism via direct contact at the interface [13,15,16,17].

Interfacial uptake mechanisms have proven significant in many PAH degradation studies, as organisms adhering to organic solvents often have significantly higher PAH degradation rates than those suspended in the aqueous layer of a TPPB [7,8]. Many genera known for their PAH-degrading capabilities, including *Mycobacterium*, *Rhodococcus* and *Sphingomonas*, are also hydrophobic, a likely cause or consequence of their interfacial uptake mechanisms [25]. Both *Mycobacterium* and *Rhodococcus* species have glycolipids in their cell walls, and *Spingomonas* species have glycosphingolipids, which may promote their interaction with and uptake of hydrophobic PAHs, either directly or from an organic phase [24,26]. *Mycobacterium* species in particular are often quite hydrophobic [24], have very high growth rates on PAHs compared to other organisms [26], despite the fact that they are generally slow growers, and have been observed forming biofilms directly on PAH crystals [27,28].

The results presented here confirm that *Mycobacterium* PYR-1 in particular is a very hydrophobic organism, with contact angle measurements of 90°. This agrees with the results of Bastiaens et al. [24], who found other *Mycobacterium* species to be very hydrophobic, with contact angles ranging from 85° to 103°. However, the results indicate that their affinity for organic layers extends beyond a simple attraction. Uptake of glucose from the aqueous phase was inhibited in the presence of a solvent usable as a carbon source, but more interesting was the fact that the presence of even a non-toxic, non-bioavailable immiscible solvent (HMN) impaired glucose uptake (Fig. 3), implying that the attraction to the organic phase is at least partially passive. That is, the cells were not attracted to the organic (HMN) phase because of the presence of a possible substrate, but their affinity for and association with the organic phase was sufficient to reduce their uptake of an aqueous carbon source. Upon examination of the association between *Mycobacterium* PYR-1 and the BES organic layer (Fig. 4) it was clear that the hydrophobic organisms are not only attracted to the organic layer, they are located within it.

To our knowledge, this is the first report of an organism actively growing in the organic phase of a TPPB, and sheds new light on observations made in this study and in other studies. If an interfacial uptake mechanism is being used, in contrast to previous reports [1,11,15-17], the rate-limiting step would not be determined by bacteria on the aqueous side taking up PAHs from the organic layer, but rather by the bacteria being on the organic side and obtaining water and salts from the aqueous layer. This may explain why PAH degradation rates by *Mycobacterium* PYR-1 are so rapid while their glucose metabolism is relatively slow and hindered in the presence of immiscible solvents: they are surrounded by high concentration of PAHs in the organic layer of the TPPB, but the glucose is less accessible to them in the aqueous layer. The relatively slow growth of *Mycobacterium* PYR-1, and other *Mycobacterium* sp., in aqueous solutions may be explained by realizing that an aqueous milieu may not be their ideal environment.

There are many areas of potential work stemming from the understanding that *Mycobacterium* PYR-1 cells can locate in the organic layer of a TPPB system. A study of how these organisms are able to survive in an organic environment may yield valuable information (i.e. of transport systems, mechanisms for maintaining concentration gradients, PAH uptake systems, mechanisms for maintaining an aqueous intracellular environment, etc.) that could be useful in understanding the degradation of PAHs and other hydrophobic substrates by microorganisms. There may also be great potential for these bacteria as members of mixed populations in TPPB systems: the population in the organic layer could begin degradation of the target compounds, and easier-to-degrade intermediates could partition to the aqueous layer for degradation by other organisms.

In conclusion, *Mycobacterium* PYR-1 is capable of living within the organic layer of a TPPB, perhaps obtaining water and salts through an interfacial uptake mechanism. This has implications for the future use of TPPBs and the use of hydrophobic organisms in them. Living in the organic layer, there is the potential for improved degradation rates, cocultures that complement each other's degradative abilities and new methods of studying and describing PAH degradation mechanisms, all of which may be the topic of future studies.

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