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ARTICLE

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Use of a Whole-Cell Biosensor to Assess the Bioavailability Enhancement of Aromatic Hydrocarbon Compounds by Nonionic Surfactants

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ABSTRACT: The whole-cell bioluminescent biosensor *Pseudomonas putida* F1G4 (*Pp*F1G4), which contains a chromosomally-based *sep-lux* transcriptional fusion, was used as a tool for direct measurement of the bioavailability of hydrophobic organic compounds (HOCs) partitioned into surfactant micelles. The increased bioluminescent response of *Pp*F1G4 in micellar solutions (up to 10 times the critical micellar concentration) of Triton X-100 and Brij 35 indicated higher intracellular concentrations of the test compounds, toluene, naphthalene, and phenanthrene, compared to control systems with no surfactants present. In contrast, Brij 30 caused a decrease in the bioluminescent response to the test compounds in single-solute systems, without adversely affecting cell growth. The decrease in bioluminescent response in the presence of Brij 30 did not occur in the presence of multiple HOCs extracted into the surfactant solutions from crude oil and creosote. The effect of the micellar solutions on the toluene biodegradation rate was consistent with the bioluminescent response in single-solute systems. None of the surfactants were toxic to *Pp*F1G4 at the doses employed in this study, and *Pp*F1G4 did not produce a bioluminescent response to the surfactants nor utilize them as growth substrates. TEM images suggest that the surfactants did not rupture the cell membranes. The results demonstrate that for *Pseudomonas putida* F1, nonionic surfactants such as Triton X-100 and Brij 35, at doses between 2 and 10 CMC, may increase the bioavailability and direct uptake of micellar phase HOCs that are common pollutants at contaminated sites.

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KEYWORDS: biosensor; aromatic hydrocarbons; surfactants; bioavailability; micelles; biodegradation

Introduction

Although bioremediation is a cost-effective and promising technology for the treatment of sites contaminated with hydrophobic organic compounds (HOCs), the success of bioremediation has often been limited by a lack of compound bioavailability (Alexander, 2000). Even when microorganisms that can readily degrade HOCs are present, the compounds may not be accessible for assimilation by these organisms, or bioavailable, due to such factors as low aqueous solubility, strong binding and/or sequestration into soil organic matter and porous media matrices, and retarded rates of dissolution from nonaqueous phase liquids (NAPLs) (Bosma et al., 1997). Hence, the conventional hypothesis has been that microbial uptake of HOCs occurs only in the aqueous phase, and that compounds from a nonaqueous phase (e.g., NAPL, sorbed state, or micellar pseudophase) are made available only once they have partitioned to the aqueous phase (Ghoshal et al., 1996; Volkering et al., 1995; Weissenfels et al., 1992). However, a growing number of studies have argued the possibility of a direct uptake mechanism for sorbed and separate phase compounds, whereby certain bacterial strains can directly utilize NAPL-phase, sorbed, or micellar-phase compounds (Guerin and Boyd, 1992; Guha et al., 1998; Mihelcic et al., 1993; Ortega-Calvo and Alexander, 1994). Most of these studies aimed at assessing bioavailability have relied upon quantification of microbial degradation rates in comparison to rates of partitioning and dissolution or desorption in corresponding abiotic systems. If the rate at which microorganisms acquire organic compounds exceeds the mass transfer rate from the nonaqueous phase determined in the absence of bacteria, then it is assumed that a direct uptake

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mechanism is involved. However, the very presence of bacterial cells in biotic systems may affect the rates of mass transfer, and thus abiotic systems may not constitute a good experimental control.

In order to overcome this shortcoming, we developed the whole-cell bioluminescent biosensor *Pseudomonas putida* F1G4 (*Pp*F1G4) as a tool for *direct* measurement of microbial bioavailability (Phoenix et al., 2003). In this study, *Pp*F1G4 is used to investigate the direct uptake of HOCs partitioned into surfactant micelles. *Pp*F1G4 produces a bioluminescent response that is proportional to the intracellular concentration of HOCs. By comparing the intensity of the bioluminescent response in systems containing only an aqueous phase, and in systems where HOCs are present in both the aqueous phase and in a surfactant micellar phase, it is possible to determine the total bioavailable concentration of HOCs from both phases. As long as the true aqueous phase concentration of HOCs is the same in both systems, a stronger bioluminescent response in the surfactant systems suggests that HOCs from the micellar phase are also entering the cell and are therefore directly bioavailable. This is the first report of an assessment of direct microbial uptake of HOCs in surfactant micelles using a whole-cell biosensor.

Influence of Surfactants on Biodegradation

Surfactants are amphipathic molecules that form aggregates called micelles at concentrations above a specific threshold value known as the critical micelle concentration (CMC). At concentrations below the CMC, surfactant molecules exist as monomers. With increasing surfactant doses, the concentration of monomers increases until the CMC is reached, and thereafter, the concentration of monomers remains constant, and any additional surfactant molecules introduced into solution go towards the formation of more micelles. The tendency of HOCs to partition into the hydrophobic core of micelles results in enhanced solubilization, and thus, bulk aqueous concentrations exceeding the compound's solubility limit can be attained when HOCs are present in excess of aqueous solubility (Rouse et al., 1994).

Although surfactants can increase the apparent solubility of sparingly soluble HOCs, and hence have the potential to enhance the bioavailability of these compounds, the effect of surfactants on biodegradation is unclear. A number of studies have reported that nonionic surfactants enhanced the biodegradation of HOCs. Some of these studies concluded that substrates in the micellar pseudophase were not bioavailable, and that enhanced biodegradation was due only to the ability of the surfactant to increase the dissolution rate of the compound to the aqueous phase (Mulder et al., 1998; Volkering et al., 1995), while others proposed that HOCs present within the micellar pseudophase were indeed bioavailable (Guha and Jaffe, 1996a,b; Guha et al., 1998; Liu et al., 1995; Tiehm, 1994). The reported increases in mineralization vary from less than 10% to more than 300%, and this wide range of responses can be explained by the fact that different nonionic surfactants, surfactant doses, and bacterial strains were used in these studies. Conversely, other studies have reported inhibition of biodegradation in the presence of nonionic surfactants (Laha and Luthy, 1991, 1992). This observed toxicity may be explained by the interaction of surfactants with the lipid components of the cell membrane, and with proteins essential to the functioning of the cell (Helenius and Simons, 1975).

To date, the approach used in studies investigating the direct uptake of micellar-phase HOCs has been to measure the rates of surfactant-aided solubilization of HOCs from their pure phases and the rates of biodegradation of the HOCs, with the assumption that dissolution rates do not change in the presence of microorganisms (Guha and Jaffe, 1996a,b; Guha et al., 1998; Willumsen and Arvin, 1999). However, this approach does not provide a direct assessment of micellar phase HOCs taken up by the cells.

The objective of this study was to test the hypothesis that the direct availability of micellar phase HOCs can be determined by measuring the intracellular concentration of HOCs in an equilibrated system where there is no net mass transfer from the bulk aqueous phase to the cells. In the test systems employed, the *Pp*F1G4 cells used glucose as a carbon source, rather than the target HOCs (toluene, naphthalene, and phenanthrene) whose bioavailability was being assessed. Because the cells did not degrade the HOCs, there was no sustained mass transfer of HOCs between the bulk solution and the *Pp*F1G4 cells. Thus equilibrium partitioning of the HOCs between the true aqueous phase and the surfactant micelles was maintained even in the presence of the cells, and the HOC concentrations in the aqueous and micellar phases in the test systems did not change with time. Nonionic surfactants (Triton X-100, Brij 30, and Brij 35) were used in this study because they are in general less toxic to bacteria (Volkering et al., 1998). This assessment of direct uptake of micellar-phase HOCs is unique in that it does not require characterization of HOC mass transport rates, and is based on a direct microbial response to intracellular concentrations of HOCs.

Description of Biosensor

Pseudomonas putida F1 (*Pp*F1) is an indigenous soil bacterium that is capable of growth on either toluene, ethylbenzene, or benzene as the sole source of carbon and energy, due to the degradative genes that are encoded in the *tod* operon (Zylstra and Gibson, 1989). *Pp*F1 is a strain that can tolerate a solvent shock (Huertas et al., 1998, 2000). In a previous study, we confirmed that solvent tolerance is conferred by the *sepABC* gene cluster, which codes for proteins that show a high homology to bacterial proteins known to be involved in solvent efflux or multidrug pumps (Phoenix et al., 2003).

The development of the whole-cell bioluminescent biosensor *Pp*F1G4, which contains a chromosomally-based

sep-lux transcriptional fusion, was reported in a previous paper (Phoenix et al., 2003). For the construction of this recombinant strain, the *luxCDABE* operon cloned from the terrestrial bacterium *Photorhabdus luminescens* (formerly *Xenorhabdus luminescens*) (Frackman et al., 1990) was employed. When *Pp*F1G4 is exposed to an inducing compound, the bioluminescence system is activated and the cell produces an intensity of visible light ($\lambda = 495$ nm) that is directly related to its level of exposure. This biosensor allows for direct measurement of microbial bioavailability, since transport of the inducing compound into the cell is a prerequisite for activation of the *sep* genes, and consequently, for light output. In our previous study, it was determined that the repressor protein SepR controls the regulation of the *sep* genes in *Pp*F1 (Phoenix et al., 2003), probably by binding to the promoter/operator region and blocking transcription. Thus, an inducer molecule must first enter the cell, where it can bind to the repressor, Sep R, releasing it from the operator and inducing transcription of the bioluminescence genes.

The wide diversity of whole-cell biosensors has been extensively reviewed (Daunert et al., 2000; Keane et al., 2002). While other engineered bioluminescent bacteria have been developed for the detection of specific organic compounds, they are all based on fusions with promotors from specific catabolic pathways. The biosensor *Pp*F1G4 is unique in that it is not based on a catabolic promoter but is nonetheless inducible by a broad range of aromatic hydrocarbon compounds (Phoenix et al., 2003). Therefore, this bioreporter strain is ideally suited for the assessment of bioavailability since the range of HOCs that can be tested is not limited to growth substrates. By adding glucose as a growth substrate in the bioluminescence assays, the mineralization of biodegradable inducers (e.g., toluene) is severely, if not totally, reduced, thus maintaining a relatively constant aqueous concentration of the test compound for the duration of the experiments. A recent study with *Pseudomonas putida* TOD102 showed that alternative carbon sources hindered the rate of toluene biodegradation due to a phenomenon known as metabolic flux dilution, a form of noncompetitive competition in which the utilization of a carbon source in a mixture is proportional to its relative availability (Lovanh and Alvarez, 2004). In the bioluminescence assays, the concentration of glucose was 110 times higher than that of toluene.

Materials and Methods

Organism and Culture Conditions

The construction of biosensor strain *Pp*F1G4 and the characterization of its sensing system are described in detail in a prior study (Phoenix et al., 2003). For the bioluminescence assays and cell growth experiments, cultures of *Pp*F1G4 were grown in Minimal M9 media (Miller, 1992) supplemented with glucose (2.2 g/L or 0.2 wt%) and an appropriate trace metal solution (Stanier et al., 1966). For the toluene biodegradation experiments, toluene was added to the M9 media, instead of glucose, as the sole carbon source. In all cases, the bacterial cells were grown at 30°C on an orbital shaker at 250 rpm.

Chemicals

The three surfactants used in this study, Triton X-100, Brij 30, and Brij 35, were obtained from Sigma–Aldrich Canada Ltd. (Oakville, ON), and used without any further purification. Some properties of these surfactants are listed in Table I. The compounds toluene, naphthalene, and phenanthrene were also procured from Sigma–Aldrich Ltd., and had purities of 99.8%, 99+%, and 96+%, respectively. HPLC grade methanol (99.8%) was obtained from Caledon Laboratories Ltd. (Georgetown, Ontario, Canada). The coal tar creosote was obtained from Kopper Industries, Carbon Materials and Chemicals Division (Pittsburg, PA), and the Brent Blend crude oil was obtained from the Petro Canada Refinery in Montreal.

Analytical Procedures

The concentrations of the test compounds in aqueous or micellar solutions were quantified with an Agilent 1100 series high-pressure liquid chromatograph (HPLC), fitted with a Vydac 201 TP52, 5 μm, 250 mm × 2.1 mm specialty reverse-phase column. Toluene was detected with a diode array UV detector at 262 nm for concentrations above 50 mg/L, and at 203 nm for concentrations below 50 mg/L. Naphthalene was detected with the diode array UV detector at 220 nm, and phenanthrene was detected using the fluorescence detector with excitation at 280 nm and emission at

Table I. Nonionic surfactants used in this study.

Surfactant	Structure	Average MW	HLB[a]	CMC[b] (mg/L)
Triton X-100 ($C_8PE_{9.5}$)	Alkyl phenol ethoxylate ether: $C_8H_{17}C_6H_4O(CH_2CH_2O)_nH$, $n = 9.5$	625	13.5	43.0
Brij 30 ($C_{12}E_4$)	Alkyl ethoxylate ether: $C_{12}H_{25}(CH_2CH_2O)_nOH$, $n = 4$	363	9.7	10.6
Brij 35 ($C_{12}E_{23}$)	Alkyl ethoxylate ether: $C_{12}H_{25}(CH_2CH_2O)_nOH$, $n = 23$	1,200	17.0	39.6

[a]Hydrophile–lipophile balance (HLB) values from Hill and Ghoshal (2002).
[b]CMC values from Guha and Jaffe (1996b).

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389 nm. The mobile phase consisted of acetonitrile and water. When surfactants were present, the run time for each sample was extended to 33 min from 10 min. All aqueous samples were diluted 3:1 in HPLC grade methanol and centrifuged at 4,000 rpm (1,240*g*) for 10 min prior to analysis. The dilution in methanol served several purposes: to precipitate the salts in the M9 media, to release HOCs from surfactant micelles, and to lyse any suspended bacterial cells.

Absorbance measurements to estimate cell numbers were performed on a Pharmacia Biotech Ultrospec 2000 UV/Visible spectrophotometer at 600 nm. If necessary, cell suspension samples were diluted with M9 media prior to analysis to obtain absorbance measurements in the linear range between 0.1 and 0.5.

Determination of Micelle-Water Equilibrium Partition Coefficients (K_{mc})

The procedure used to determine the micelle-water equilibrium partition coefficients (K_{mc}) for Triton X-100, Brij 30, and Brij 35 and each of the three test compounds was based on the methods described in Guha and Jaffe (1996a). A known volume of a concentrated methanol stock solution of either naphthalene or phenanthrene was placed in a series of acid-washed, 10-mL centrifuge tubes, so that when the methanol evaporated, the mass of crystals remaining was at least four times in excess of solubility (the presence of excess crystals was confirmed visually at the end of each experiment). The tubes were then filled with M9 media and the appropriate volume of stock surfactant solution (100 CMC) to attain various surfactant concentrations up to 10 CMC. The capped tubes were placed on an orbital shaker at 30°C and 75 rpm for 7 days to achieve equilibrium. After this time, they were centrifuged at 5,000 rpm (1,370*g*) for 30 min in order to settle the crystals, and aliquots of the supernatants were removed with a gas-tight syringe and then diluted in methanol for analysis. The syringe was preconditioned by rinsing it with each solution prior to sampling.

The solubilization of a HOC by a surfactant can be expressed as (Guha and Jaffe, 1996a):

$$C_{mic} = S_{mc} K_{mc} C \tag{1}$$

where

$$S_{mc} = \begin{cases} S - \text{CMC} & \text{for } S > \text{CMC} \\ 0 & \text{for } S \leq \text{CMC} \end{cases} \tag{2}$$

and C_{mic} is the concentration of HOC partitioned into micelles (mg/L water); S_{mc} the surfactant concentration in micellar form (mg/L water); K_{mc} the partition coefficient of HOC between the micellar pseudophase and the aqueous phase (L water/mg); C the concentration HOC truly dissolved in the aqueous phase (mg/L water); and S is the total surfactant concentration (mg/L water). When the HOC in question is present in excess of solubility, the concentration of HOC in the bulk aqueous phase (C_{bulk}) is equal to the sum of the concentration for aqueous solubility and the concentration in the micellar pseudophase, excluding any pure phase HOC that may be present in the bulk phase:

$$C_{bulk} = S_{mc} K_{mc} C_{sol} + C_{sol} \tag{3}$$

Therefore, K_{mc} can be determined from the slope of the straight-line plot of C_{bulk} versus S_{mc}.

In an attempt to determine the K_{mc} for the three surfactants and toluene, a similar procedure was followed, except that instead of adding crystals, a layer of toluene (100 μL) was deposited on top of the surfactant solutions. However, inconsistent results were obtained each time the experiment was repeated. Large variability in the micellar partitioning coefficients in NAPL-Brij 30 surfactant solution systems have also been reported elsewhere (Bernardez and Ghoshal, 2004).

Bioluminescence Assays

For all experiments described below, an overnight culture from a frozen glycerol stock of *Pp*F1G4 was used to prepare 350 mL of sub-culture, which was grown to an A_{600} of 0.3 ($\sim 5 \times 10^9$ cfu/mL). The cells were then harvested and resuspended in 2.5 mL M9 media.

Experiments With HOCs in Single-Solute Systems

Each reactor received a total volume of 75 mL of M9 media supplemented with glucose and the appropriate volume of stock surfactant solution (100 CMC) to attain various surfactant concentrations (0, 0.2, 0.4, 0.8, 2, 4, 6, 8, or 10 CMC). Each reactor was then inoculated with 250 μL of cell suspension, resulting in a cell density with an A_{600} of 0.15 ($\sim 2.5 \times 10^9$ cfu/mL). Unlike toluene, naphthalene and phenanthrene are solid at room temperature, thus concentrated stock solutions of these compounds were prepared in methanol, and the appropriate volume was measured with a syringe and added to the reactors. In all cases, the volume of methanol added to the reactors was negligible (less than 0.1% of the total volume). Unless specified otherwise in the results, the true aqueous concentrations of naphthalene and phenanthrene in the reactors were 3.3 and 1.2 mg/L, respectively, while the bulk concentration of toluene in all reactors was the same, at 20 mg/L, since it was not possible to determine the K_{mc} values for toluene and the three surfactants. As a result, the bulk concentration of toluene remained constant across all reactors, and the true aqueous concentration decreased with increasing surfactant doses. It should be noted that the bulk concentration of toluene was well below its aqueous solubility limit (526 mg/L), and thus there was no toluene as NAPL present in any of the reactors. The aqueous concentrations of the HOCs in the experiments were chosen based on considerations for

ensuring a measurable range of bioluminescence readings in the surfactant-free as well as in the micellar surfactant solutions. The reactors were incubated at 30°C and 250 rpm for a period of 2 h before bioluminescence was measured. All bioluminescence experiments were conducted in replicate (minimum triplicate). Each experiment was repeated with a fresh culture of *Pp*F1G4.

Experiments With HOCs Partitioned From Multicomponent NAPLs

The surfactant solutions were first equilibrated with the multicomponent NAPLs (creosote or Brent Blend crude oil) in order to allow for partitioning of the HOCs from the NAPL phase. A series of 120-mL capacity glass vials received a total volume of 90 mL, consisting of M9 media and the appropriate volume of stock surfactant solution (100 CMC) to attain various surfactant concentrations (0, 0.2, 0.4, 0.8, 2, 4, 6, 8, or 10 CMC). After either 0.85 mL crude oil or creosote was added to each vial, they were sealed with aluminum foil, securely capped, and covered in Parafilm to prevent losses. The vials were then incubated at 30°C and agitated at 75 rpm for a period of 5 days (120 h). In previous partitioning studies, it has been shown that 96 h are sufficient to attain equilibrium (Hill and Ghoshal, 2002). Once equilibrium had been attained, 75 mL of each equilibrated surfactant solution was transferred to a reactor and supplemented with glucose. Care was taken to avoid transferring any NAPL droplets in the solution phase from the equilibration vials into the reactors. Reactors were inoculated and incubated in a manner identical to the single-solute systems.

Bioluminescence Measurements

To measure the intensity of the light emitted by the biosensor cells, the reactors were placed inside a light-proof box with one of the optical windows flush up against the tip of a liquid light cable (Fig. 1). This cable was connected to an optical power meter (OPM) (Oriel Instruments, Stratford, CT), consisting of a photomultiplier tube, power supply, and readout. A data acquisition program was used to collect 20 readings at 5-s intervals for each sample, and the mean bioluminescence was calculated from these values. In order to supply adequate oxygen to the cells to ensure a steady bioluminescent output, the reactors were continuously stirred with a magnetic stir bar when placed in the light-proof box, which was equipped with a magnetic stir plate. Stirring of the cell suspensions provided sufficient aeration to avoid decay of the light signal during the measurement process. After the bioluminescence readings were taken, the A_{600} of the cell suspensions were measured to verify that the cell numbers in each reactor were comparable. The concentrations of the test compounds were measured after the bioluminescence readings to ensure that no losses had occurred.

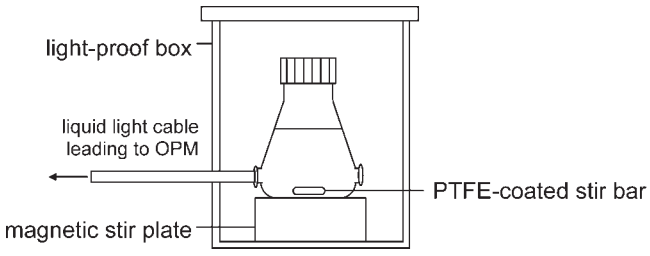

Figure 1. Light-proof box and glass reactor with optical windows used in bioluminescence assays.

Biodegradation Experiments

For this series of experiments, overnight cultures from a frozen glycerol stock of *Pp*F1G4 were grown with 100 μL toluene as the sole source of carbon in 300 mL of M9 media. After 16 h growth, the cells were harvested and resuspended in 1.25 mL M9 media. A series of 50-mL culture tubes was filled with 30 mL of solution, consisting of M9 media and the appropriate volume of stock surfactant solution (100 CMC) to attain various surfactant concentrations (0, 0.2, 0.8, 4, or 8 CMC). Each tube was inoculated with 100 μL of cell suspension (except for the abiotic controls), resulting in a cell density with an A_{600} of 0.3 ($\sim 5 \times 10^9$ cfu/mL), and spiked with toluene, yielding an initial aqueous concentration of 45 mg/L (approximately double the concentration of toluene used for the bioluminescence assays). The tubes were tightly sealed with open-top caps and teflon-lined septa, and then well mixed on a vortex. Each septum was punctured with a syringe in order to extract an aliquot to determine the exact initial concentration of toluene. The holes were immediately sealed with a drop of fast-drying glue (Seal-All, Eclectic Products, Inc., Pineville, LA), to prevent losses due to volatilization. The tubes were incubated at 30°C and 250 rpm, and samples were extracted with a syringe at 30 and 60 min. After each sampling event, the same precautions were taken to prevent losses.

Cell Growth of *Pp*F1G4 in the Presence of Surfactants

An overnight culture from a frozen glycerol stock of *Pp*F1G4 was grown. A series of 50-mL culture tubes was filled with 30 mL of solution, consisting of M9 media and the appropriate volume of stock surfactant solution (100 CMC) to attain various surfactant concentrations (0, 2, or 10 CMC). The tubes were then supplemented with glucose (2.2 g/L or 0.2%) as sole source of carbon. The overnight culture was harvested and then distributed equally among all the tubes, yielding an initial A_{600} of 0.14 ($\sim 2.3 \times 10^9$ cfu/mL). The tubes were tightly capped, and then incubated at 30°C and 250 rpm for a period of 24 h. After this time, the A_{600} of the cultures were determined.

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Electron Microscopy

*Pp*F1G4 cells were grown in the same way as for the bioluminescence assays. After the 2-h incubation period, instead of measuring bioluminescence, the bacterial samples were harvested and prepared for electron microscopy. The cells were prefixed overnight by addition of an equal volume of 2.5% (v/v) gluteraldehyde in 0.1 M sodium cacodylate buffer directly to the concentrated liquid cultures, followed by three washings in 0.1 M sodium cacodylate buffer. The washed cells were fixed for 2 h at 4°C in a solution of 1% osmiumtetraoxide in 1.5% KFeCN, and then chemically dehydrated by repeated washings with increasing concentrations of acetone in water (30%, 50%, 70%, 80%, 90%, and 100%). Dehydrated samples were infiltrated by successive treatment with a series of acetone:Epon mixtures (1:1, 1:2, and 1:3), and then embedded in pure Epon. Samples were left to polymerize at 58°C for 48 h, and then thin sections were cut and stained. The cells were examined using a JEOL model JEM-2000 transmission electron microscope at an operating voltage of 80 kV.

Results and Discussion

Micelle-Water Equilibrium Partition Coefficients (K_{mc})

For the bioluminescence assays conducted in the presence of surfactants, the micelle-water equilibrium partition coefficients (K_{mc}) were used to calculate the mass of naphthalene or phenanthrene to be added to each reactor so that the *true* aqueous phase concentration would be the same in each reactor (3.3 and 1.2 mg/L, respectively) regardless of the concentration of surfactant. At surfactant doses above the CMC, the *bulk* aqueous concentrations of naphthalene and phenanthrene were increased due to the increasing mass of polycyclic aromatic hydrocarbons (PAHs) partitioned into the surfactant micelles. The values of K_{mc} for the three surfactants and naphthalene and phenanthrene obtained from solubilization experiments (Fig. 2), are given in Table II. These results are similar to values reported in the literature (Guha and Jaffe, 1996a; Guha et al., 1998). The small differences may be attributed to the higher amounts of mineral salts in the aqueous phase in the reactors and the higher temperature maintained in this study.

The presence of micelles in cell suspensions at doses higher than the CMC was verified by dye solubilization tests with 4-dimethylaminoazobenzene (methyl yellow) according to methods described elsewhere (Lopes and Loh, 1998). Methyl yellow is a hydrophobic dye that is completely insoluble in water, but will partition into surfactant micelles, thus imparting color to the aqueous phase.

Bioluminescent Response to Three Target Compounds

Figure 3 illustrates how the bioluminescent response of *Pp*F1G4 is affected by the concentration of the inducing compound (toluene, naphthalene, or phenanthrene), in the absence of surfactants. The values on the *x*-axes are concentrations measured after the 2-h incubation period, and represent the actual measured aqueous concentrations. For both toluene and naphthalene, the biosensor exhibits a concentration-dependent response, and the magnitude of the maximum bioluminescent signal is slightly higher for naphthalene than for toluene. For increasing concentrations of toluene (Fig. 3A), there is an initial increase in bioluminescence up to around 20 mg/L, followed by a gradual decrease of light production at higher concentrations. Thus, high concentrations of toluene have an inhibitory effect on

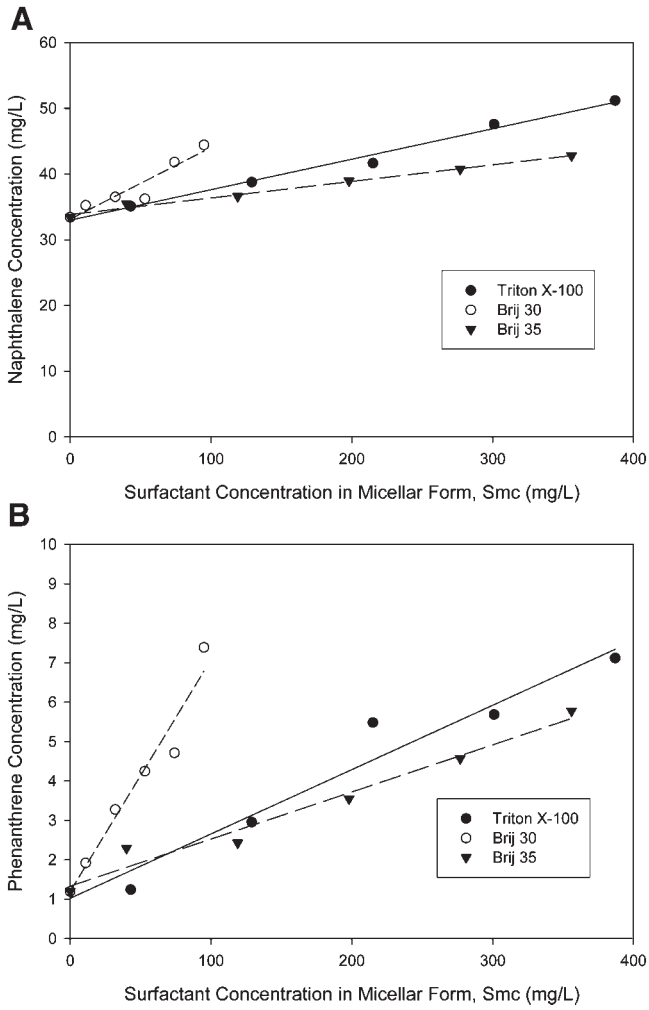

Figure 2. Equilibrium bulk concentration of naphthalene and phenanthrene in M9 media at 30°C as a function of the concentration of surfactant in micellar form. Symbols are experimental data and lines represent linear regression.

Table II. Micellar partition coefficients, K_{mc} (L/mg).

	Triton X-100	Brij 30	Brij 35
Naphthalene	0.0014	0.0033	0.0008
Phenanthrene	0.0136	0.0490	0.0099

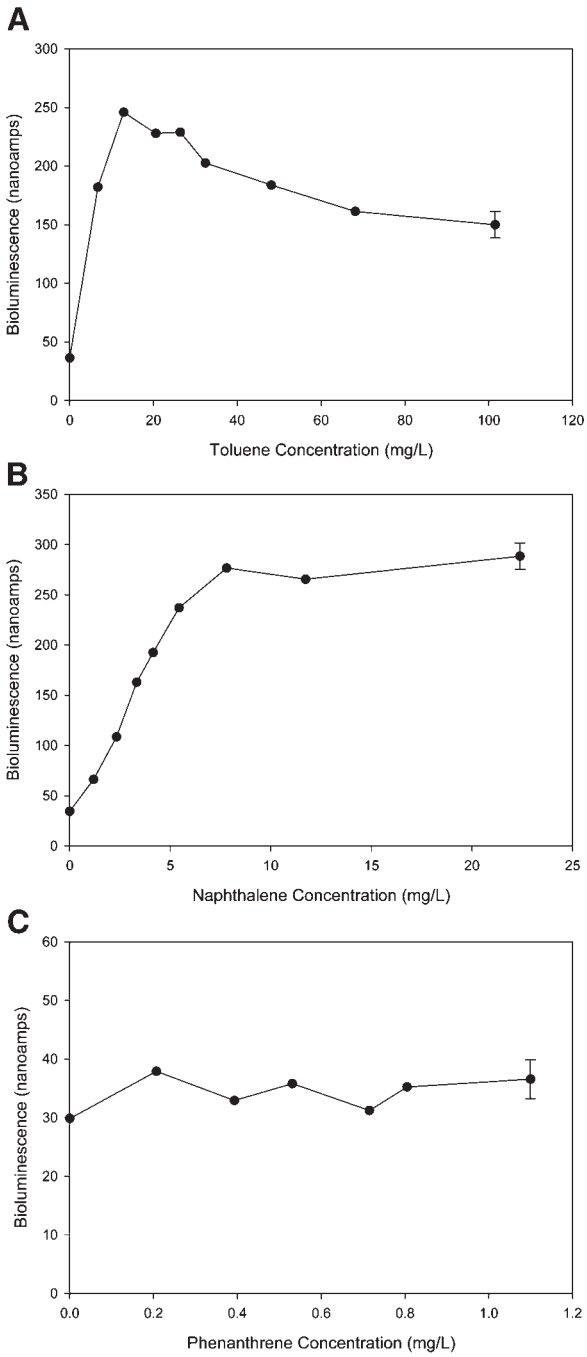

Figure 3. Bioluminescent response of *Pp*F1G4 to increasing concentrations of toluene, naphthalene or phenanthrene, as measured in nanoamps by the optical power meter (OPM). The error bar in each panel represents the average standard error of the mean bioluminescence values for that data set.

the biosensor's ability to produce a bioluminescent signal. The same initial increase in bioluminescence is seen in response to increasing concentrations of naphthalene (Fig. 3B); however, a plateau is attained rather than a decrease in response. No inhibition of light production is observed, probably because the range of naphthalene concentrations that can be tested is limited by the relatively low aqueous solubility of naphthalene (~33 mg/L) compared to that of toluene (~526 mg/L). The fact that there is no significant response to phenanthrene above the background bioluminescence level (Fig. 3C) can be attributed to compound's extremely low aqueous solubility (~1.2 mg/L). The aqueous concentrations of phenanthrene available to the biosensor in the absence of surfactants are simply too low to have any effect.

Bioluminescent Response to Single Target Compounds in Surfactant Solutions

Figure 4A and B shows that Triton X-100 and Brij 35 significantly enhanced the bioluminescent response of *Pp*F1G4 to naphthalene and phenanthrene. The horizontal line in the figures represents the bioluminescent response to the target compound in a surfactant-free system and to the true aqueous phase concentration of 3.3 mg/L for naphthalene and 1.2 mg/L for phenanthrene. Data points above this line indicate a bioluminescent response greater than for the true aqueous phase concentration of the PAHs. For the Triton X-100 and the Brij 35 systems containing naphthalene or phenanthrene, the bioluminescent response increased gradually and reached a plateau. The increasing bioluminescent response with surfactant dose suggests that the PAHs partitioned into the micellar phase were available to the *Pp*F1G4 cells and were inducing the *sep* genes. If micellar phase PAHs were not bioavailable, the bioluminescent response would have been unchanged with surfactant dose, because the pure aqueous phase PAH concentration was identical in all surfactant systems and in the surfactant-free systems. The bioluminescent response to toluene was somewhat different in that it increased rapidly to the maximum levels at sub-CMC surfactant doses. This is expected because the bulk phase toluene concentration of 20 mg/L was unchanged in all systems. If micellar phase toluene was not bioavailable, the bioluminescent response would have decreased with increasing surfactant concentrations; however, no such decrease was observed. The plateau in the bioluminescent response to toluene and to the two PAHs that occurred at doses above the CMC could be caused by a limitation in the mass transport of oxygen, required for the bioluminescence reaction, from the bulk solution to the cell, or because the bioluminescence genes were maximally expressed under those conditions. Furthermore, the plateau in bioluminescent response may also be related to surfactant sorption on the cell walls, which has been reported to plateau at or just above the CMC (Brown and Al Nuaimi, 2005).

While Brij 30 actually inhibited the bioluminescent response, as shown in Figure 4C, cell numbers were not adversely affected. The A_{600} values of the cell suspensions measured at the end of the experiments indicated that cell growth was the same over the 2-h period, regardless of the surfactant used. The bioluminescent response of *Pp*F1G4 to

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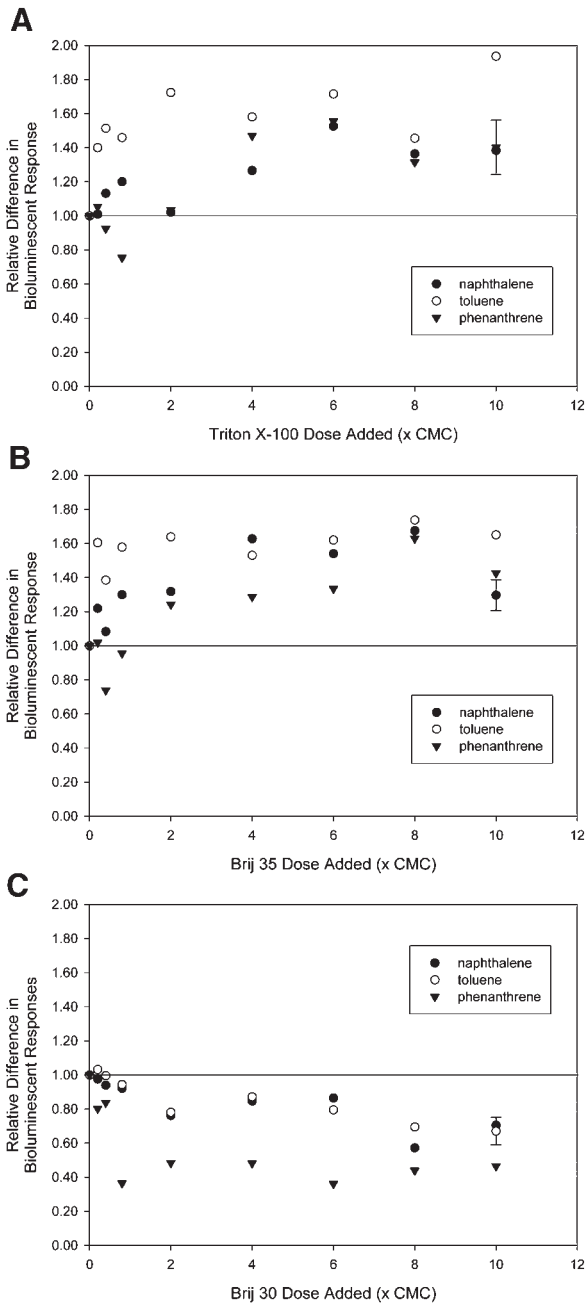

Figure 4. Change in bioluminescent response of *Pp*F1G4 to target compounds as a function of surfactant concentration. The horizontal line indicates the response of *Pp*F1G4 to the target compounds when no surfactants are present. The error bars represent the average standard error over multiple experiments (performed at least in triplicate).

the individual test compounds toluene, naphthalene, and phenanthrene in Brij 30 solutions was suppressed compared to the controls with no surfactant present. In those experiments, the true aqueous concentrations of naphthalene and phenanthrene in the control reactors and in the test reactors containing Brij 30 were the same. Thus, even if the test compounds solubilized within the Brij 30 micelles were not bioavailable, the biosensor cells should have responded with an intensity of bioluminescent response corresponding to the response obtained for the control, which had the same true aqueous concentration of the test compound. However, it is clear from Figure 4C that light production in all systems, including those containing sub-CMC levels of Brij 30, was inhibited.

In other studies involving biosensors, a reduction in the bioluminescent response is usually attributed to toxic effects (Hay et al., 2000; Heitzer et al., 1994; Kelly et al., 2000; Kurittu et al., 2000). Indeed, the Microtox^{TM} toxicity assay is based on the premise that any inhibition of cellular metabolism due to toxicity results in a decrease in the light emission of the affected cells. However, three lines of evidence suggest that toxicity was probably not a factor in the inhibition of light production observed in Brij 30 solutions containing single HOCs: (1) A_{600} measurements taken after the bioluminescence assays showed that cell numbers had actually increased, to a similar extent as when Triton X-100 or Brij 35 were present, and thus were not adversely affected over the 2-h incubation period; (2) in cell-growth experiments, the biosensor's growth on glucose in the presence of Brij 30 was higher than the growth attained when no surfactants were present, as discussed in the following paragraph; and (3) there was no inhibition of light production in Brij 30 solutions containing multiple HOCs partitioned from NAPLs such as crude oil and creosote, which are complex mixtures of aliphatic, cyclic hydrocarbons, and PAHs, as discussed below.

When *Pp*F1G4 was incubated in systems with glucose as the carbon source, with or without surfactants at various doses, the cell growth over 24 h as measured by an increase in absorbance was greater or unchanged in systems with surfactants compared to those without (Fig. 5). At doses of 2 and 10 CMC none of the surfactants tested adversely affected growth of *Pp*F1G4 cells, and growth was higher at 2 CMC

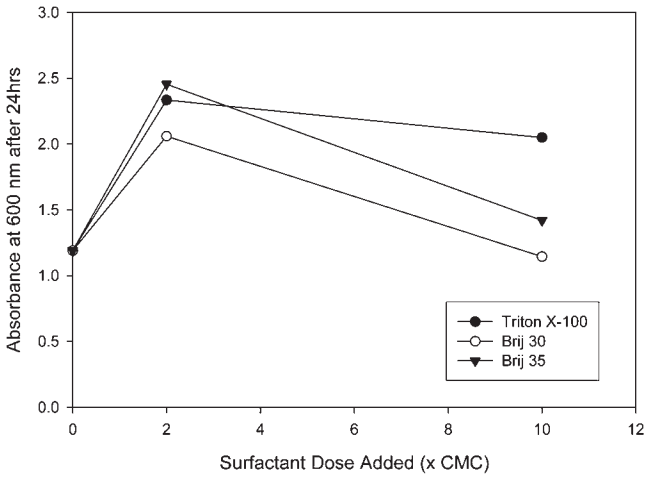

Figure 5. Effect of surfactants on growth of *Pp*F1G4 after a 24-h incubation period with an initial inoculum size (A_{600}) of 0.14 ($\sim 2.3 \times 10^9$ cfu/mL).

than at 10 CMC. The increase in growth was however lower for Brij 30 than for the two other surfactants, likely because the flux of glucose into cells in the presence of micellar solutions of Brij 30 was lower than in the presence of Triton X-100 or Brij 35. It should be noted that the incubation time of 24 h is significantly higher than the 2 h that was employed for the bioluminescence tests. As mentioned earlier, the increases in absorbance measured over a 2-h period were very similar for all three surfactants.

To assess the bioluminescence in response to solutes partitioned from the crude oil and creosote, the surfactant solutions were first equilibrated with the multicomponent NAPLs. Then, the equilibrated surfactant solutions were introduced into the reactors where the cells were added and the bioluminescence assays were carried out. Figure 6 shows the bioluminescent response of biosensor *Pp*F1G4 to surfactant solutions that were preequilibrated with either creosote or Brent Blend crude oil. The horizontal line in the figures represents the bioluminescent response in surfactant-free aqueous solutions that had been equilibrated with the multicomponent NAPLs. The most striking observation is that all three surfactants, Triton X-100, Brij 35, and Brij 30, produced very similar results, in contrast to the single-solute systems described previously. The suppression of bioluminescence does not occur in systems where multiple HOCs have partitioned into Brij 30 solutions.

While it is conceivable that the Brij 30 monomers are directly involved in the suppression of light production, either by inhibiting the bioluminescence reaction (through interaction with essential reaction components such as luciferase), or by binding to the SepR repressor in such a manner as to block transcription of the *lux* genes, these are unlikely scenarios, since there is no evidence of this occurring in Brij 30 solutions with multiple HOCs partitioned from creosote and crude oil.

It is more likely that the suppression of light production in Brij 30 solutions containing single HOCs was due to the interaction of Brij 30 molecules with cell membranes. Brij 30 has several distinguishing characteristics that differentiate it from Triton X-100 and Brij 35. Brij 30 micelles yield a larger core volume and a smaller shell volume than Triton X-100 or Brij 35 micelles (Bernardez and Ghoshal, 2004). Of the three surfactants tested, Brij 30 has the lowest hydrophile–lipophile balance (HLB) number (Table I) and thus is the most hydrophobic. Brij 30 has been reported to have a significantly higher sorption capacity than Brij 35 on cells of a *Sphingomonas* sp., and Brij 30 surfactant monomers formed surface aggregates such as hemimicelles, admicelles or bilayers on the cells, whereas Brij 35 was adsorbed in monolayers (Brown and Al Nuaimi, 2005). It is possible that specific interactions of Brij 30 molecules with the *Pp*F1G4 cell membranes, because of the particular Brij 30 properties described above, altered their structure in such a manner as to hinder the mass flux of HOCs into the cells in the single solute systems. Brown and Jaffé (2001) observed changes in cell-wall characteristics with respect to the distribution of membrane proteins for the same *Sphingomonas* sp.

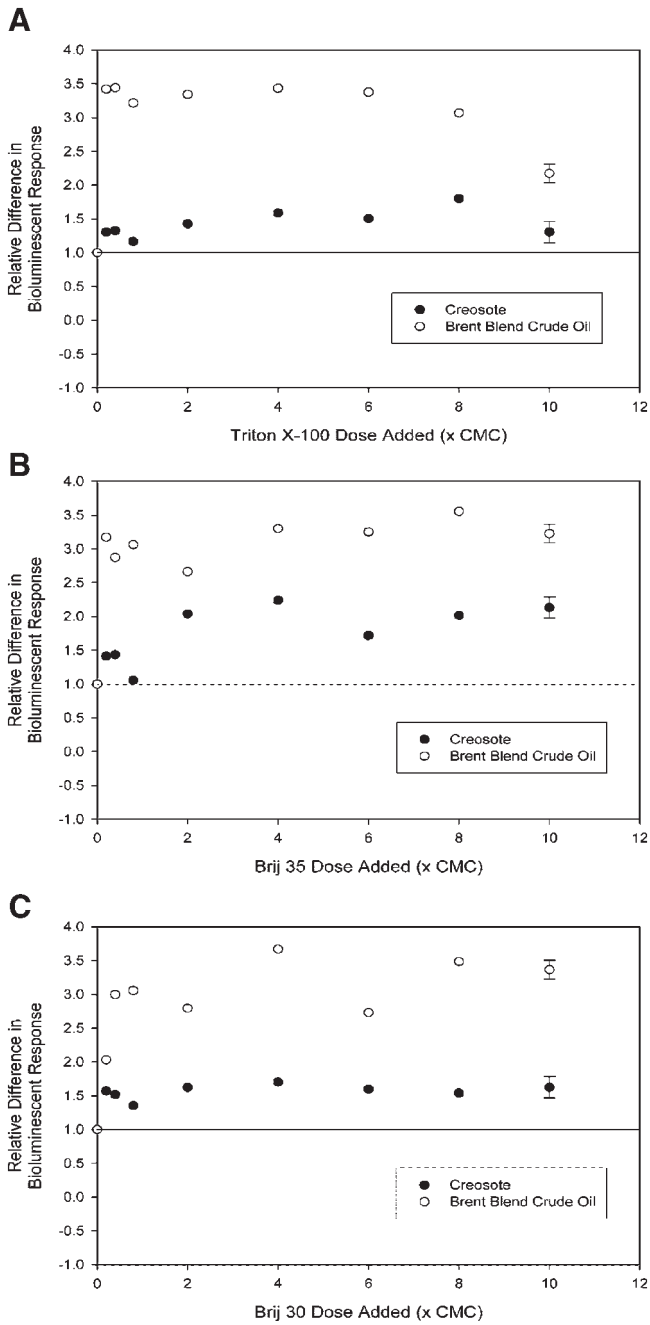

Figure 6. Change in bioluminescent response of *Pp*F1G4 to equilibrated solutions containing HOCs partitioned from multicomponent NAPLs as a function of surfactant concentration. The horizontal line indicates the response of *Pp*F1G4 to HOCs partitioned from multicomponent NAPLs when no surfactants were present. The error bars represent the average standard error over multiple experiments (performed at least in triplicate).

employed by Brown and Al Nuaimi (2005). At a surfactant concentration of 4 CMC, the cell-wall characteristics were more significantly altered for Brij 30 than for Brij 35, for the cell incubation times of 1–2 h employed by Brown and Al Nuaimi (2005) and by this study. However, in this study, the negative effects of Brij 30 were eliminated in the presence of

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multiple HOCs partitioned from the crude oil and creosote. The partitioning of multiple solutes may have altered the surfactant aggregate structures in such a way that the mass flux was no longer hindered.

It should be noted that *Pp*F1G4 did not produce a bioluminescent signal above background levels in response to methanol or to any of the surfactants alone (Keane, 2003). Hence, the surfactants Triton X-100 and Brij 35 do not provoke a response on their own, but rather assist in making the inducing compounds toluene, naphthalene, and phenanthrene more bioavailable to the cells. Furthermore, false positive bioluminescence readings, such as those reported with the naphthalene biosensor strain *P. fluorescens* HK44 due to a physiological phenomenontermed the "solvent effect" (Heitzer et al., 1998), can be ruled out with *Pp*F1G4 cells. In a previous study, it was determined that a "solvent effect" is not possible with biosensor *Pp*F1G4, since the cells are not aldehyde-limited (Phoenix et al., 2003). Thus, all light production can be correlated to induction of the *sep* genes, and to increased bioavailability. Since bioluminescence is indeed sensitive to a host of physiological and environmental factors (Blouin et al., 1996; Dorn et al., 2003; Neilson et al., 1999), precautions were taken to keep experimental conditions, such as cell numbers, oxygen tension, and temperature, as constant as possible in all systems in an experiment and also in the replicate experiments conducted on different days.

The results from this study indicate that micellar-phase compounds are bioavailable, in agreement with findings from other studies (Guha and Jaffe, 1996a,b; Guha et al., 1998; Liu et al., 1995; Tiehm, 1994). While the aqueous phase concentration of the sparingly soluble compound phenanthrene was too low to produce a significant response in the absence of surfactants (Fig. 3C), there was a clear increase in the light signal of *Pp*F1G4 at doses of Triton X-100 and Brij 35 above the CMC (Fig. 4A and B). The bioluminescent response to phenanthrene increased gradually with increasing surfactant doses, suggesting greater intracellular concentrations as a result of greater micellar concentrations of phenanthrene. As mentioned earlier, the true aqueous concentration of phenanthrene was the same in all systems.

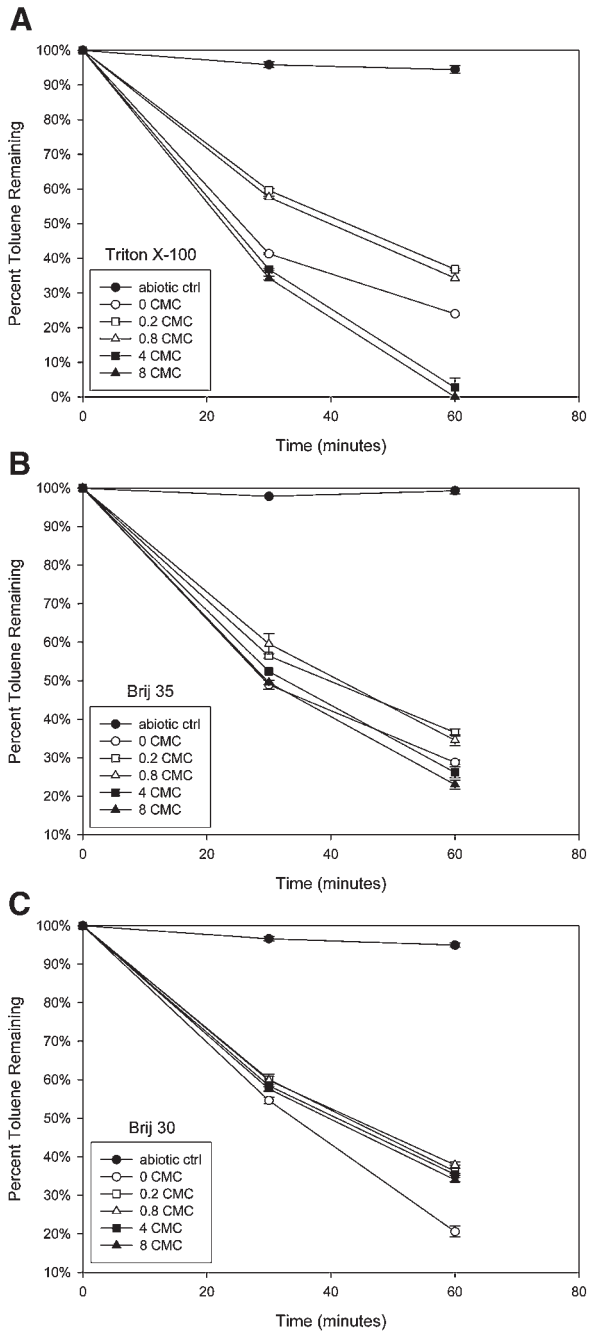

Figure 7. Effect of surfactant concentration on the biodegradation of toluene by *Pp*F1G4. The error bars represent the standard error for replicate experiments (performed at least in triplicate).

Biodegradation of Toluene in Surfactant Solutions

Further evidence that micellar-phase compounds are directly bioavailable to the cells is provided by the results from the toluene biodegradation tests (Fig. 7). Of the three test compounds in this study, *Pp*F1G4 is capable of utilizing only toluene as a growth substrate, and thus biodegradation tests could only be performed for toluene. The biodegradation rate of toluene by *Pp*F1G4 was affected by the addition of different concentrations (0, 0.2, 0.8, 4, or 8 CMC) of the three surfactants (Fig. 7). The total toluene concentration in each test system was 45 mg/L. Concentrations of Triton X-100 greater than the CMC significantly enhanced the biodegradation rate of toluene (Fig. 7A). A similar, but less pronounced effect was also observed with Brij 35 (Fig. 7B). However, biodegradation of toluene was inhibited at sub-CMC concentrations of both these surfactants, suggesting that the enhancement in bioavailabilty is associated with the presence of micellar-phase toluene, after a 60-min incubation period. The biodegradation of toluene was inhibited

by all concentrations of Brij 30, compared to when no surfactant was present (Fig. 7C). The results in Figure 7 support the findings from the bioluminescence assays: Triton X-100 and Brij 35 micellar solutions appear to increase the bioavailability of single-solute HOCs, while Brij 30 has the opposite effect. The fact that the biodegradation of toluene was inhibited by all concentrations of Brij 30, compared to when no surfactant was present, may be attributed to limitations in the mass flux of toluene across the cell membrane.

Surfactant-Bacterial Cell Interactions

It is well known that nonionic surfactants bind to cell membranes and that this interaction alters membrane properties (Brown and Al Nuaimi, 2005; Florence et al., 1984; Glover et al., 1999; Helenius and Simons, 1975; Lichtenberg et al., 1983). According to Helenius and Simons (1975), when surfactants penetrate into the membrane, they alter its molecular organization, leading to an increase in membrane permeability, and, at higher surfactant concentrations, to lysis and eventually, membrane solubilization, resulting in the formation of mixed micellar structures containing both lipids and proteins. Some studies have reported on the permeabilization of cell membranes by Triton X-100 in particular, as evidenced by the rapid release of colored biodegradation intermediates from the cytoplasm into the growth medium (Willumsen and Arvin, 1999; Willumsen and Karlson, 1998), and by transmission electron micrographs clearly showing an inflated periplasmic space and an uneven distribution of the cytoplasm adjacent to the cell envelope (Willumsen and Karlson, 1998).

A number of studies investigating the effect of surfactants on microbial biodegradation specifically cite the interaction of surfactants with cell membranes as being an important factor, be it beneficial (Mihelcic et al., 1993; Van Hoof and Rogers, 1992) or inhibitory (Laha and Luthy, 1991, 1992) to pollutant degradation. The different responses observed reflect the complexity of the interactions between surfactants and cell membranes, and underline the fact that these effects are strain-specific and/or surfactant-specific. In a study of the effects of various surfactants on cell membranes, Glover et al. (1999) found that enhanced membrane fluidity due to surfactant action was not directly correlated with the biocidal activity of the surfactants. Thus, while high concentrations of surfactants are invariably toxic, appropriate doses of compatible surfactants have the potential to increase the rate and extent of flux of substrate molecules into the cell through membrane fluidization, without damaging the integrity of the cells.

The general consensus in the literature is that it is primarily the monomers, and not the micelles, that bind to the cell membranes and are responsible for altering the bacterial membrane structure (Florence et al., 1984; Helenius and Simons, 1975; Van Hoof and Rogers, 1992). Once the membrane structure has been altered by the binding of surfactant monomers, the transport of micellar-phase compounds into the cell is facilitated by fusion of the micelles with the cell membrane, as hypothesized by Guha and Jaffe (1996b). Evidence for this mechanism has been reported by Miller and Bartha (1989). In that study, it was found that a *Pseudomonas* isolate was able to uptake liposomes, vesicles consisting of phospholipids bilayers ranging in diameter from 20 to 50 nm, which were used to encapsulate water-insoluble alkanes, much in the same manner as surfactant micelles solubilize hydrophobic compounds within their core. The authors suggested that the liposomes delivered the substrates by passing through the cell-wall and fusing with the cell membrane, hence overcoming the transport limitation inherent to insoluble compounds.

In an attempt to capture physical evidence of changes in the cell membrane due to the presence of surfactants, transmission electron micrographs (TEMs) were taken to compare cells that had been exposed to surfactants and those that had not. TEMs for the control (*Pp*F1G4 not exposed to surfactants or HOCs) and for *Pp*F1G4 incubated in the presence of Triton X-100 at 10 CMC and toluene, and in the presence of Brij 30 at 10 CMC and a mixture of the three target compounds, are shown in Figure 8. The TEMs show that the outer membranes do not appear to be significantly

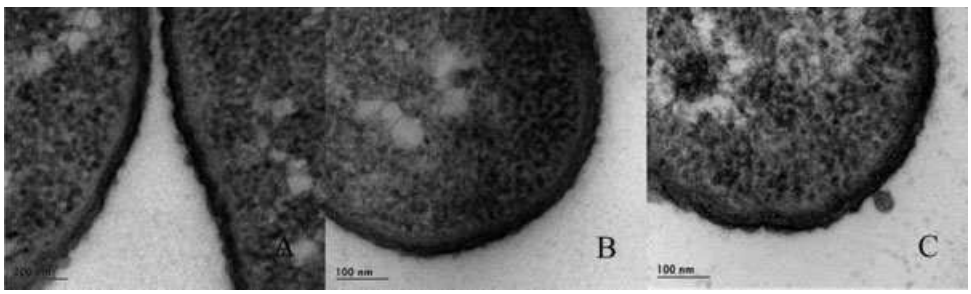

Figure 8. Transmission electron micrographs depicting the cell membrane region of *Pp*F1G4 grown in M9 media containing (**A**) no surfactants or HOCs; (**B**) Triton X-100 at 10 CMC and toluene; (**C**) Brij 30 at 10 CMC and a mixture of all three target compounds. The bar in the bottom left is 100 μm in length.

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different after exposure to surfactants compared to the control, and there is no evidence of membrane rupture. Some membrane vesicles can be observed blebbing off from the cell membranes. During normal growth, certain gram-negative bacteria are known to produce membrane vesicles that bleb off into the culture medium, although some studies have reported that vesicle formation is enhanced as a result of disturbances in growth or in outer membrane integrity, such as starvation or exposure to antibiotics (Beveridge et al., 1997; Zhou et al., 1998). However, in our study, the membrane vesicles were observed in the cells representing the control as well as in the surfactant- and HOC-exposed cells, and thus this phenomenon is not likely to be significant. Based on these images, it can be surmised that exposure to the surfactants at the doses used in this study did not result in visible changes in the membrane structure such as those reported by Willumsen and Karlson (1998). Subtle changes in membrane structure, undetectable in the TEMs, cannot be ruled out.

This research elucidates how certain nonionic surfactants such as Triton X-100 and Brij 35 may increase the bioavailability of HOCs through direct uptake, and thus have the potential to enhance biodegradation rates. The specific results from this study may not be generalized for other microorganisms, nonionic surfactants and HOCs because direct uptake is influenced by the characteristics of a particular microorganism, surfactant or HOC. For example, contrary to this study, Guha and Jaffe (1996a,b) observed that phenanthrene biodegradation and direct uptake was enhanced in the presence of Brij 30 and not Brij 35 and this may be attributable to the different microorganisms used in that study. In this study, unknown interactions between microorganisms, Brij 30, and HOC solutes reduced the bioluminescence and toluene biodegradation rates of *Pp*F1G4 to levels below those observed for the surfactant-free controls. The reduction in bioluminescence was not attributable to the toxicity of Brij 30 or of toluene to the cells. Further work is needed to understand the surfactant, HOC, and cell–wall interactions that influence bioluminescence and bioavailability.

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