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Application of a Reverse Transcription-PCR assay to monitor regulation of the catabolic *nahAc* gene during phenanthrene degradation

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Abstract

Biodegradation of polycyclic aromatic hydrocarbons (PAH), such as phenanthrene, in environmental samples is often limited by low bioavailability which results from a combination of low aqueous solubility and/or high sorption. The purpose of this study was to investigate the influence of agents that increase PAH bioavailability on expression of the PAH catabolic gene *nahAc*. Phenanthrene was used as a model PAH and *Pseudomonas putida* PpG7, which contains the NAH7 plasmid that encodes the genes responsible for naphthalene and phenanthrene degradation, was used as a model degrader. PAH bioavailability was altered by the addition of two biosurfactants, rhamnolipid and hydroxypropyl-β-cyclodextrin (HPCD). Gene expression was determined by extraction of bacterial mRNA followed by RT-PCR amplification of two transcripts; *nahAc*, a naphthalene dioxygenase gene, and *rpoD*, a housekeeping gene. Results indicate that the lag period preceding *nahAc* gene induction decreased from 312 to 48 h in the presence of biosurfactants. Expression of the *nahAc* gene, as measured by RT-PCR, in the presence of surfactants was bimodal on a temporal basis, indicating that induction stopped briefly during biodegradation. Cessation of induction could have resulted from the up-regulation of alternate pathways or the accumulation of toxic intermediates. In contrast, expression of the *rpoD* gene was maintained throughout the duration of each experiment. This research demonstrates that the use of a gene expression assay to monitor the impact of substrate bioavailability on substrate utilization provides unique information concerning the biodegradation process that cannot be obtained from more traditional biodegradation assays such as cell growth or substrate disappearance. Gene expression assays also have the potential for use in assessing the impact of other environmental factors on biodegradation.

Introduction

Successful bioremediation depends on achieving high rates of microbial activity. Yet there are numerous environmental factors that can activate or repress gene expression and thereby modulate microbial activity (Daubaras & Chakrabarty 1992). For example, biodegradation of polyaromatic hydrocarbons (PAH) is often constrained due to their low bioavailability (low aqueous solubility and high sorption by soil) (NRC 1993; Maier 2000). We used conditions of differing bioavailability as the basis for a study that was performed to determine how gene expression patterns relate to measured biodegradation behavior. We hypothesized that if gene expression patterns differ from measured biodegradation behavior, study of gene expression may offer insights into how specific catabolic genes are regulated.

Several recent studies have demonstrated that gene expression can be successfully measured at the transcriptional level. These studies involve either the use of a reporter gene (King et al. 1990; Heitzer et al. 1992, 1994; Burlage et al. 1994), the extraction of RNA followed by direct detection by gene probe

(Fleming et al. 1993; Ogunseitan & Olsen 1993; Sanseverino et al. 1993; Jeffrey et al. 1994; Jeffery & Barkay 1996) or RT-PCR (Ogram et al. 1994; Selvartnam et al. 1995; Wilson et al. 1999). The goal of this study was to use an RT-PCR assay to evaluate the regulation of the *nahAc* gene during phenanthrene degradation. Key to this evaluation, was the development of an RT-PCR baseline control assay to allow comparison of *nahAc* expression in response to variations in substrate bioavailability.

In this study, *Pseudomonas putida* G7 (PpG7) was used as a model degrader (Sanseverino et al. 1993). This organism carries the NAH7 plasmid that encodes for the genes responsible for naphthalene and phenanthrene degradation. Phenanthrene was used as a model PAH due to its relatively low vapor pressure (0.018 pa at 25 °C) and water solubility (1.3 mg/L). Biodegradation of phenanthrene was monitored in the absence and presence of two biosurfactants: rhamnolipid and HPCD, that were used to increase phenanthrene bioavailability (Maier 2000). Gene expression was detected by extraction of mRNA followed by RT-PCR amplification of the *nahAc* gene. The *nahAc* gene encodes for the large subunit of the iron-sulfur protein component of naphthalene dioxygenase, the enzyme responsible for the first step in the degradation of some PAH including naphthalene and phenanthrene. An internal housekeeping gene (*rpoD*) was chosen as the baseline control for evaluation of expression of the *nahAc* gene. The *rpoD* gene is a single copy housekeeping gene that encodes for σ_{70}, the housekeeping sigma factor for transcription initiation (Fujita et al. 1995).

Materials and methods

Chemicals

Phenanthrene (purity >98%) and sodium salicylate were purchased from Aldrich Chemical Co. (Milwaukee, WI). Analytical-grade hydroxypropyl-β-cyclodextrin (HPCD) (purity >99%) was supplied by Cerestar USA, Inc. (Hammond, IN). Rhamnolipid (a mixture of monorhamnolipid and dirhamnolipid) was produced by *Pseudomonas aeruginosa* IGB83 and purified as described previously (Zhang & Miller 1992, 1994).

Bacterial strain and media

PpG7 was kindly provided by Dr. Gary Sayler (University of Tennessee, Knoxville Tennessee). The organism was maintained at 24 °C on mineral salts medium (MSM) agar, specifically Bushnell-HAAS plates (Difco Laboratories, Detroit, MI) using naphthalene vapors as the sole carbon source. The organism was transferred monthly. PpG7 was screened for its ability to produce surfactants under the conditions of these experiments. Results from a general drop collapse assay (Bodour & Miller-Maier 1998) showed that PpG7 does not produce surfactants. More specifically, results from a PCR assay developed in our laboratory for the *rhlB* rhamnolipid gene which encodes the rhamnosyltransferase involved in rhamnolipid biosynthesis (Ochsner et al. 1994) showed that PpG7 does not produce rhamnolipid. Thus, PpG7 does not produce surfactants under the conditions used in this study.

Bioavailability of phenanthrene

The solubility of phenanthrene in surfactant systems was determined as previously described (Wang et al. 1998). Briefly, 2 ml of a mixture of phenanthrene and [9-^{14}C]phenanthrene (Sigma Chemical, St. Louis, MO) in chloroform was added to triplicate 250 ml flasks for each treatment. The chloroform was evaporated leaving a mass of 12.5 mg phenanthrene with specific activity 0.082 mCi/mmol. A 25 ml aliquot of water containing no surfactant, 10,000 mg/L HPCD, or 1,000 mg/L rhamnolipid was added to each flask. Flasks were shaken for 3 days at room temperature, allowed to settle, and a 50 μl aliquot was removed and assayed for radioactivity using liquid scintillation counting (Packard Tri-Carb Co., Model 1600 TR, Meriden, CT).

RNA extractions

Total RNA was extracted from bacterial cultures (1 ml) using Purescript®RNA Isolation Kits (Gentra Systems Inc., Minneapolis, MN) according to the manufacturer's instructions yielding a 30 ml aliquot of total RNA. Following all extractions, total RNA samples were stored at −80 °C until further analysis. Prior to all amplifications, total RNA was treated with DNase under the following conditions: 2.5 μl of 5X buffer (250 mM Tris-HCl, 250 mM KCl, 50 mM $MgCl_2$, 50 mM DTT and 2.5 mM spermidine); 0.5 μl of RNase inhibitor (20 U/μl) (Perkin Elemer, Branchburg, NJ), 10 μl total RNA, 2–3 μl of DNase (1 U/μl)

(Promega, Madison, WI) and 30 μl of DEPC-treated water. Samples were incubated at 37 °C for 35 min, followed by a 65 °C incubation for 20 min to inactivate the DNase.

RT-PCR assay

Reverse transcriptase amplification of the *nahAc* and *rpoD* transcripts was performed using a GeneAmp®RNA PCR kit (Roche, Branchburg, NJ). The internal housekeeping gene (*rpoD*) was assayed in concert with the *nahAc* degradative transcript. It served as a reference point to evaluate changes in *nahAc* expression, to detect relative differences in the integrity of individual RNA samples, and to indicate the presence of any reverse transcriptase inhibitors. For each sample, 4–6 μl of an RT mixture containing 2.5 mM $MgCl_2$; 1X PCR buffer II; 1 mM each of dGTP, dCTP, dATP and dTTP; 1U Rnase inhibitor; 2.5 U reverse transcriptase; and either the antisense *nahAc* (0.25 pmol) or *rpoD* primers (1.70 pmol) were added to 5 μl of DNased total RNA. Reverse transcription was carried out in a PTC-100 thermocycler (MJ Researchers Inc., Watertown, MA), with incubations at 25°C for 5 min, 42 °C for 60 min, 99 °C for 5 min, and 4 °C for 5 min. After completion of the RT step, the mixture was combined with the PCR mixture containing either the upstream *nahAc* (0.25 pmol) or *rpoD* primer (1.70 pmol), 2.5 mM $MgCl_2$, 1X PCR Buffer, and 2.5 U of Taq 2000 (Promega, Madison, WI). Amplification of the final 25 μl reaction was performed at 95 °C for 2 min, followed by 35 cycles of 95 °C for 15 sec, 55 °C for 30 sec, 72 ∘C for 45 sec, a final extension at 72 °C for 2 min, and a 4 °C soak. As a control, all Dnased RNA samples were subjected to PCR prior to RT analysis.

The primers used to detect the *nahAc* transcript were those described by Herrick et al. (1993). Briefly, the upstream NAHAcC1 primer 5′GTT TGC AGC TAT CAC GGC TGG GGC TTC GGC 3′ and the downstream NAHAcC3 primer TTC GAC AAT GGC GTA GGT CCA GAC CTC GGT 3′ PCR were used to detect a 701 bp region of the *nahAc* gene. The primers used to detect the *rpoD* housekeeping gene were designed using the GCG (Genetics Computer Group) sequence analysis software package (Madison, WI) and the Oligo 5.0 primer analysis software program (National Biosciences, Inc.). The primers used to amplify a 579 bp segment of the *rpoD* gene were the upstream UPRPOD primer 5′CCA TCG CCA AGA AGT ACA CCA ACC G 3′ and the downstream LOWRPOD primer 5′CGT CAA ACT GCT TGC CCA CCT CTT C 3′. Specificity of the primers was confirmed by direct sequencing of amplified products (University of Arizona, Laboratory of Molecular Systematic and Evolution Sequencing Facility). Sequencing was performed using an ABI 377 automated sequencer. BLAST was used to search the NCBI (National Center for Biotechnology Information) database for sequence homology. Amplified products had a 95% and 97% homology to *P. putida rpoD* and *nahAc* genes, respectively.

The sensitivity of the RT-PCR assay was determined by assaying for the presence of target transcripts from bacterial cells ranging in concentration from 1 CFU/ml to10^8 CFU/ml. Cells were grown on 500 mg/L salicylate for 17 h until the cell concentration was 10^8 CFU/ml. The cells were serially diluted, the RNA extracted and assayed using RT-PCR as described.

Analysis of amplified products

All amplified products were separated by gel electrophoresis using a 1or 2% agarose gel. Gels were stained with either ethidium bromide or SYBR Gold nucleic acid gel stain (Molecular Probes, Eugene, OR), and visualized with a UV transilluminator connected to an Alpha Imager 2000 system (Alpha Innotech Corp., Sunnydale, CA). RNA samples stored at −80 °C were run periodically on gels to show that no RNA degradation occurred during storage.

Biodegradation studies

The RT-PCR assay was evaluated in an initial biodegradation study using salicylate as the substrate. Salicylate was chosen because it is water soluble and it is an inducer of the *nah* operon including the *nahAc* gene (Burlage et al. 1990). Triplicate 250 ml flasks containing 50 ml mineral salts broth (MSB) and 500 mg/L salicylate were inoculated with approximately 10^7 CFU PpG7, and incubated at room temperature with gyratory shaking at 200 rpm. Periodically, samples were removed to determine CFU and salicylate concentration. CFU were determined by culturable plate counts on R2A agar. Enumeration occurred after 48 h incubation at room temperature. For each salicylate concentration, 1 ml samples were stored in microcentrifuge tubes to which 0.5 ml 1 N NaOH was added. Immediately prior to analysis, samples were centrifuged for 1 min at 14,000 rpm to pellet cells. Salicylate concentration in the supernatant was

subsequently determined by UV spectrophotometry at 296 nm.

The biodegradation of phenanthrene was quantified in two ways: (1) direct measurement of phenanthrene loss, used to determine substrate utilization; and (2) plate counts, for cell mass evaluation. For the substrate utilization experiments, which were conducted in triplicate, 8.75 mg of phenanthrene was dissolved in chloroform and added to 250-mL flasks. The solvent was allowed to evaporate overnight, leaving a thin coating of phenanthrene covering the sides and bottom of the flasks. Bushnell-HAAS broth (25 ml) containing no additive, 1000 mg/L rhamnolipid or 10,000 mg/L HPCD was added to each flask. Samples were inoculated with a 72 hour preculture to achieve a final cell density of approximately 10^7 CFU/ml, and incubated at 200 rpm on a orbital shaker at room temperature.

At the beginning and end of each experiment, a set of triplicate samples was sacrificed and the contents of each flask were serially extracted twice with 50 ml of chloroform. Three drops of concentrated HCl were added to samples with rhamnolipid to precipitate the rhamnolipid and reduce emulsification during solvent extraction. The extracts were combined and concentrated with a Rotavapor Evaporation System (Büchi Co., Switzerland) to approximately 1 ml, after which the volume was adjusted to 5 ml with HPLC-grade methanol. The amount of phenanthrene in the extracts was quantified by high performance liquid chromatography (Waters, Milford, MA). All samples were filtered through glass wool prior to HPLC analysis. HPLC analysis was performed isocratically using a mobile phase of 5% water (HPLC-grade) and 95% acetonitrile UV (HPLC- grade) and a Nova-Pak C18 - 4 μm reverse phase column, 3.9 $\times$ 150 mm (Water Corp., Milford, MA). The flow rate was 1 ml/min and the wavelength used for detection of phenanthrene was 250 nm.

Results

RT-PCR assay

To show that the transcript of interest, rather than any contaminating DNA was being amplified by the RT-PCR assay, total RNA extracts were subjected to several treatments prior to amplification. The extracts were either not treated, RNased, DNased, or both DNased and RNased, prior to PCR and RT-PCR amplification. Amplification products were detected after PCR only from those samples which were untreated or RNased (Table 1). Following RT-PCR amplification, products were detected from those samples which either had no treatment, the RNase treatment or the DNase treatment. No products were detected on samples that were subjected to both DNase and RNase treatment. These amplification results show that both DNA and RNA were amplified, but treatment of RNA extracts with DNase prior to RT-PCR ensures that only RNA is amplified and detected.

Table 1. Effects of Dnase and Rnase treatment of total RNA on PCR results

Enzymatic treatment	Transcript	Amplification results[1]	
		RT-PCR (mRNA)	PCR
No treatment	*nahAc*	+	+
Rnase	*nahAc*	+	+
Dnase	*nahAc*	+	−
Rnase and Dnase	*nahAc*	−	−
No treatment	*rpoD*	+	+
Rnase	*rpoD*	+	+
Dnase	*rpoD*	+	−
Rnase and Dnase	*rpoD*	−	−

[1] (+) indicates that the transcript was detected following amplification, (−) indicates that no transcript was detected following amplification.

The sensitivity of the RT-PCR assay was determined by monitoring target transcripts from cells ranging in concentration from 1 CFU/ml to 10^8 CFU/ml. Cells were grown on 500 mg/L salicylate for 17 h until the cell concentration reached 10^8 CFU/ml. At this time point, the cells were serially diluted, the RNA extracted, and the RT-PCR assay conducted. Results showed that detection of the *nahAc* and *rpoD* transcripts required a minimum of 3.7 $\times$ 10^5 CFU which is equivalent to approximately 1 ng of total mRNA.

Gene expression, salicylate disappearance, and CFU data from a salicylate degradation experiment are compared in Figure 1. Figure 1b shows that the *nahAc* transcript was detected at 12, 16, and 19 h, but not at 39 h. In contrast, the *rpoD* housekeeping gene was detected at all time points (Figure 1c). The presence of the *nahAc* transcript closely mirrored growth in the medium, both in terms of increased cell numbers, and decreased salicylate concentrations (compare Figure 1b to Figure 1a). These data show that cell numbers peaked and the majority of the salicylate was utilized by approximately 18 h, at which time the *nahAc* transcript was still detectable. After

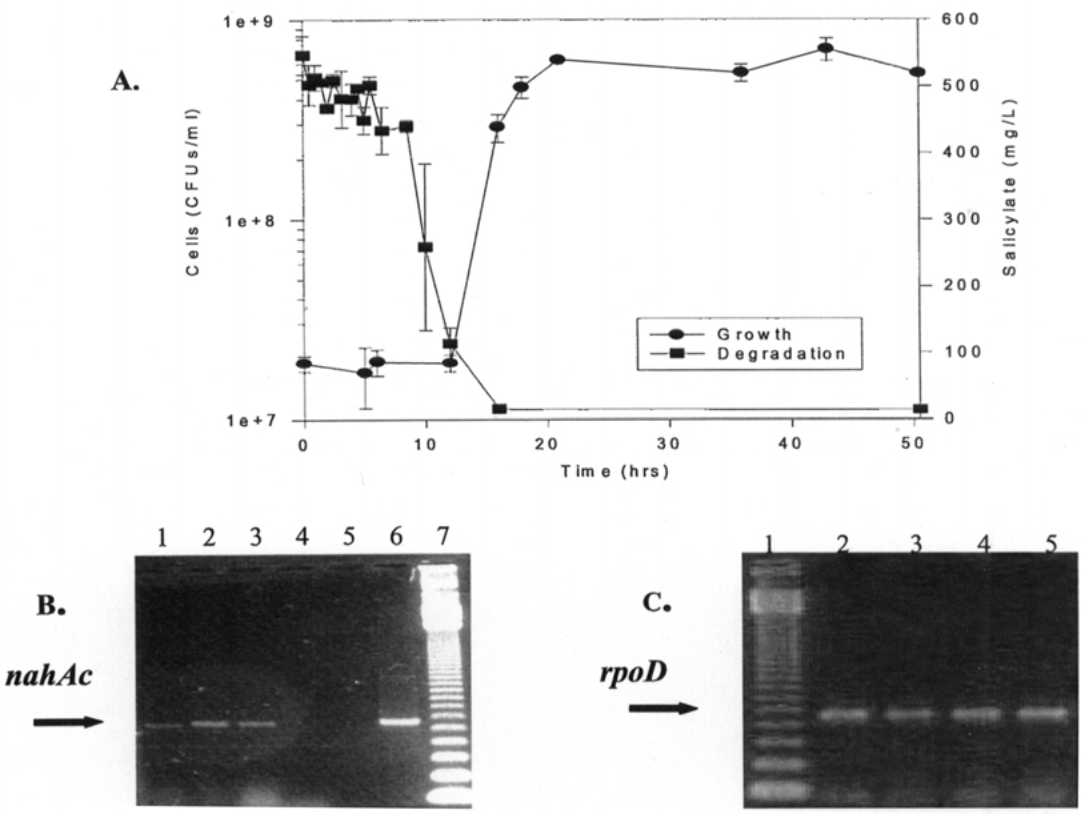

Figure 1. Detection of changes in gene expression. Cells were grown on 500 mg/L salicylate and monitored for *nahAc* and *rpoD* activity. (A) Salicylate degradation (■) and cell growth (●). (B) RT-PCR detection of the701 bp *nahAc* mRNA transcript; lane 1, 12 h; lane 2, 16 h; lane 3, 19 h; lane 4, 39 h; lane 5, negative control; lane 6, positive control; lane 7, 123 bp ladder. (C) RT-PCR detection of the 579 bp *rpoD* mRNA transcript; lane 1, 123 bp ladder; lane 2, 12 h; lane 3, 16 h; lane 4, 19 h; lane 5, 39 h; positive and negative controls not shown.

this time, intracellular levels of salicylate would be expected to decline to background levels, resulting in a return to basal levels of *nahAc* transcription which are not detected by this assay. Thus at 39 h, the *nahAc* transcript was not detected (Figure 1b, lane 4).

Preliminary studies were done to see whether basal, background levels of the nahAc transcript could be detected when cells were gown on LB or nutrient broth. No nahAc transcripts were detected in either medium at any stage of growth while the rpoD transcript was consistently detected in both media at all growth stages (data not shown).

Apparent solubility of phenanthrene

The aqueous solubility of phenanthrene was measured to be 1.3 mg/L. Addition of either surfactant increased the apparent solubility of phenanthrene. For rhamnolipid (1,000 mg/L), the apparent solubility of phenanthrene was increased 18-fold to 23.9 mg/L. For HPCD (10,000 mg/L), the increase was 15-fold to 20 mg/L. For rhamnolipid, the apparent solubility of phenanthrene is increased through the association of phenanthrene molecules with micellar rhamnolipid aggregates. For HPCD, phenanthrene associates with the hydrophobic interior of HPCD molecules. It has been demonstrated previously that the increase in apparent phenanthrene solubility in the presence of either rhamnolipid or HPCD results in increased phenanthrene bioavailability to degrading cells (Zhang et al. 1997; Wang et al. 1998).

Expression of nahAc during biodegradation of phenanthrene

Phenanthrene disappearance, cell growth, and *nahAc/rpoD* gene expression were measured simultaneously in a series of experiments to evaluate the impact of surfactants on phenanthrene degradation. Phenanthrene mass was measured at the beginning

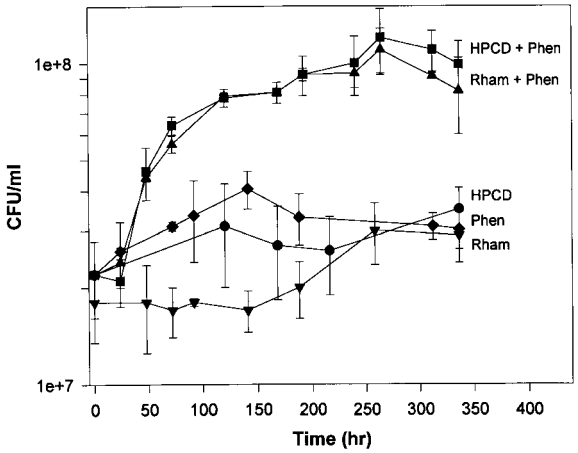

Figure 2. Effect of biosurfactants on phenanthrene biodegradation as evaluated by culturable counts. Five treatments were tested: phenanthrene (8.75 mg in 50 ml MSM) alone (⧫), phenanthrene with either: 10^3 mg/L rhamnolipid (△) or 10^4 mg/L HPCD (■), rhamnolipid alone (▽), and HPCD alone (●).

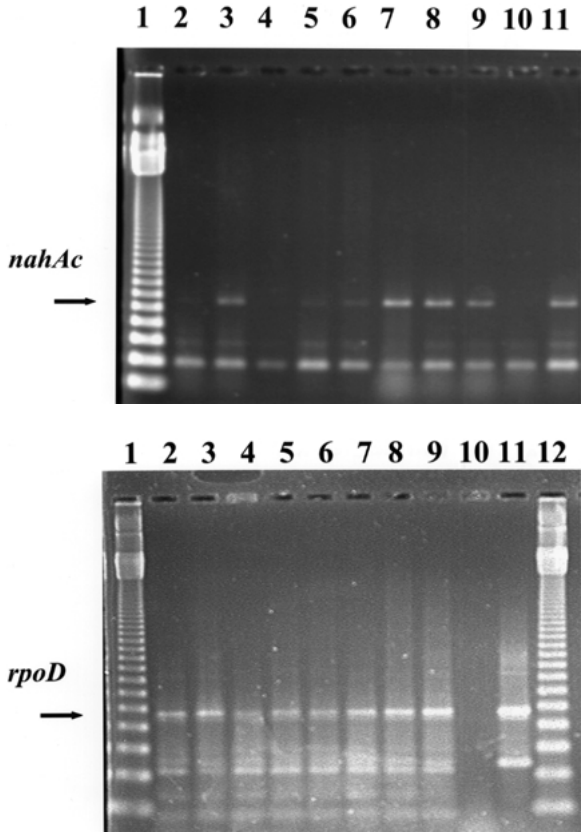

Figure 3. RT-PCR detection of *nahAc* (701 bp) and *rpoD* (579 bp) mRNA transcripts during phenanthrene biodegradation. (A) This gel shows the results of RT-PCR mRNA detection of *nahAc* transcripts in the presence of 10^4 mg/L of HPCD. Lane 1, 123 bp ladder; Lane 2, 0 h; Lane 3, 48 h; Lane 4, 96 h; Lane 5, 144 h; Lane 6, 196 h; Lane 7, 246 h; Lane 8, 312 h; Lane 9, 336 h; Lane 10, negative control; Lane 11, positive control. (B) This gel shows the results of RT-PCR mRNA detection of *rpoD* housekeeping gene transcripts in the presence of 10 mg/L of HPCD. Lane 1, 123 bp ladder; Lane 2, 0 h; Lane 3, 48 h; Lane 4, 96 h; Lane 5, 144 h; Lane 6, 196 h; Lane 7, 246 h; Lane 8, 312 h; Lane 9, 336 h; Lane 10, negative control; Lane 11, positive control; Lane 12, 123 bp ladder.

and the end of the 14 day experiment (336 h). Phenanthrene loss as measured by HPLC was greater in both the rhamnolipid and HPCD treatments, with only 17.4% and 26.9% phenanthrene remaining respectively after 336 h. In contrast, when there was no surfactant present, 91.4% of the phenanthrene still remained after 336 h.

Cell growth was measured by culturable plate counts (Figure 2). Little or no increase in cell number was detected for the three control treatments: phenanthrene alone, rhamnolipid alone, and HPCD alone. However, there was an increase in cell number to approximately 1×10^8 CFU/ml for phenanthrene/rhamnolipid and phenanthrene/HPCD treatments. These results suggest that both surfactants caused an increase in the rate of phenanthrene degradation. In addition, these data confirm that neither surfactant was used as a carbon or energy source.

Gene expression was measured by determining levels of both the *nahAc* and *rpoD* transcripts. Figure 3 shows an example of RT-PCR detection of both the *nahAc* (Figure 3a) and the *rpoD* housekeeping (Figure 3b) transcripts during biodegradation of phenanthrene in the presence of HPCD. Both gels show nonspecific bands in addition to the putative *nahAc* or *rpoD* products. Therefore, the two putative products were excised and sequenced to confirm their identity. Figure 4 shows a qualitative summary of all the gene expression assays. These results show that induction of the *nahAc* transcript was observed after 48 h of exposure to phenanthrene in the presence of either rhamnolipid or HPCD (Figures 4b and 4d). In contrast, induction of the *nahAc* transcript was not detected until 312 h when no surfactant was present (Figure 4a). This suggests that the increase in bioavailable phenanthrene due to the presence of the surfactants resulted in earlier induction of the *nahAc* gene and subsequent detection of the *nahAc* transcript. In the control treatments containing only surfactant but no phenanthrene, the *nahAc* transcript was not detected at any time point (Figures 4c and 4e).

Interestingly, in both the rhamnolipid and the HPCD treatments, induction of the *nahAc* gene ceased for a period of time (at 96 h for HPCD and at 196 h

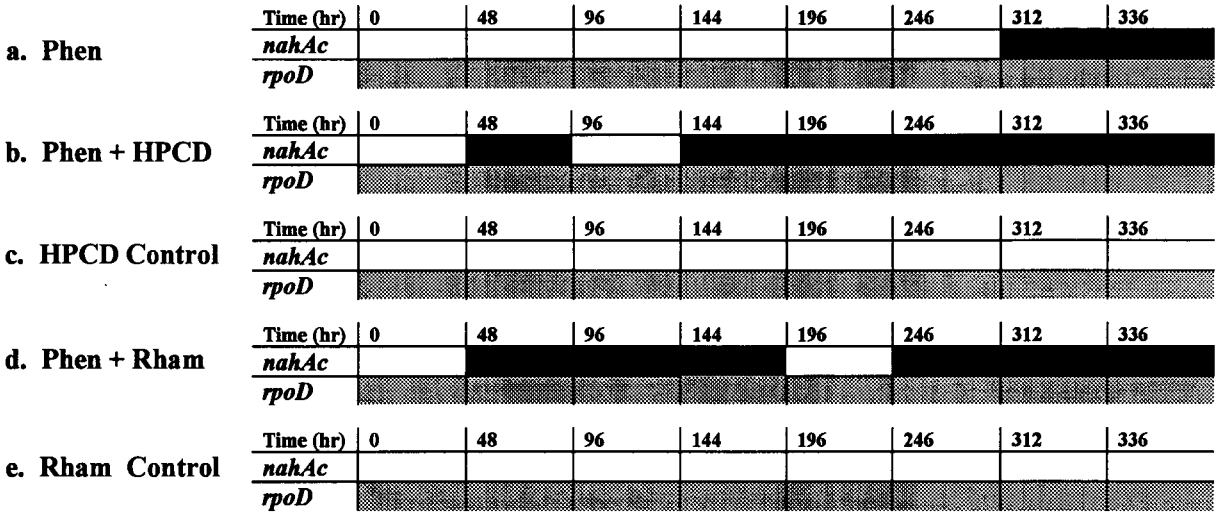

Figure 4. Qualitative detection of *nahAc* mRNA to *rpoD* mRNA as a function of time in the presence of (a) 8.75 mg phenanthrene alone, (b) 8.75 mg phenanthrene and 10,000 mg/L HPCD, (c) 10,000 mg/L HPCD alone, (d) 8.75 mg phenanthrene and 1,000 mg/L rhamnolipid, and (e) 1,000 mg/L rhamnolipid alone. White rectangles indicate that no transcript was detected. Black rectangles indicate that the *nahAc* transcript was detected, and gray rectangles indicate that the *rpoD* transcript was detected.

for rhamnolipid) (Figures 4b and 4d). After this brief cessation of induction, the *nahAc* transcript was again detected after 144 and 246 hours respectively. This bimodal expression pattern was not observed in the absence of surfactants. However, since the induction of gene expression was delayed in this sample, it may not have been assayed for long enough to observe a cessation period (Figure 4a). The *rpoD* housekeeping transcript was detected at all time points regardless of treatment (Figure 4). In the three control samples that contained only rhamnolipid, only HPCD, or only phenanthrene, the *rpoD* bands were not as intense as in those samples that contained both phenanthrene and surfactant (data not shown). This is not surprising since cell growth was minimal in all of these controls, with cell numbers remaining close to the threshold of detection for the mRNA assay (10^7 CFU/ml).

Discussion

This research demonstrates that the use of RT-PCR to monitor the impact of environmental variables such as bioavailability on substrate utilization at the transcriptional level, can provide information concerning the biodegradation process that is not apparent from more traditional biodegradation assays. Specifically, the gene expression assay used in this work was able to show temporal changes in *nahAc* expression as phenanthrene was degraded, i.e., induction vs. lack of induction, that were not observed in the classical growth curves based on increasing cell mass (compare Figures 2 and 4). Thus, monitoring gene expression may allow detection of subtle changes in the expression of degradative genes due to temporal changes in cell physiology, or changes in toxicity associated with accumulation of intermediates. As such, gene expression assays may be useful for evaluating the impact of other environmental factors (e.g., microbial competition, abiotic nutrient limitations, and terminal electron acceptor status) on biodegradation, as well as for understanding the general regulation of catabolic pathways.

This assay also allows simultaneous monitoring of the impact of changing environmental factors on the regulation of a specific response, e.g., biodegradation (*nahAc*) and a global response, e.g., *rpoD*. Results showed that for the most part, *rpoD* gene expression patterns mimicked cell growth but that the expression of the *nahAc* genes were influenced by factors other than substrate bioavailability. Thus, results of a gene expression assay need to be interpreted carefully to understand and differentiate potential influences on cell activity. This suggests that it is important to include baseline controls such as the *rpoD* gene used in this study to aid in the interpretation of gene expression.

The differences seen in the gene expression patterns of the *nahAc* transcript may be due to several factors. The NAH7 enzymes that initially act to meta-

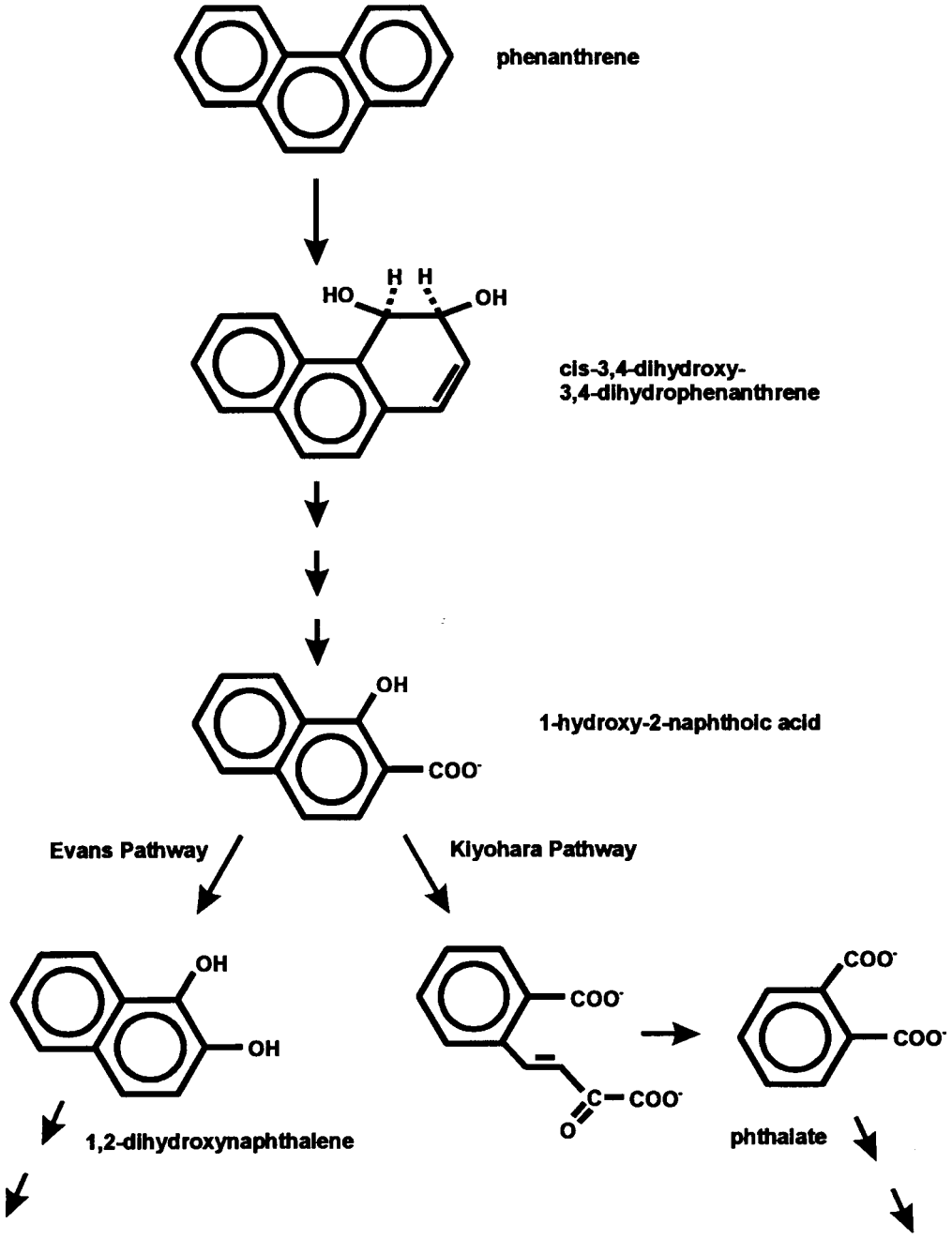

Figure 5. Catabolic pathways for the degradation of phenanthrene.

bolize naphthalene in PpG7 can also act to metabolize phenanthrene (Sanseverino et al. 1993; Sutherland et al. 1995). The NAH7 plasmid has two operons involved in naphthalene metabolism, the *nah* operon which encodes for the degradation of naphthalene to salicylate, and the *sal* operon which encodes for the degradation of salicylate to pyruvate and acetaldehyde. During phenanthrene biodegradation, NAH7 enzymes metabolize the first aromatic ring resulting in the formation of a 1-hydroxy-2-naphthoic acid intermediate (Figure 5). At least two possible pathways for degradation of this intermediate have been identified. In the first pathway the intermediate is decarboxylated to 1,2-dihydroxynaphthlene, which then

re-enters the catabolic pathway at the ring cleavage step (Davies & Evans 1964). In this case phenanthrene is metabolized via the upper NAH pathway twice to remove the first two aromatic rings, and via the lower SAL pathway once for the last aromatic ring. The second possibility is that the 1-hydroxy-2-naphthoic acid intermediate is cleaved between the hydroxyl and carboxyl substituents on the aromatic ring (Kiyohara & Nagao 1978). This is followed by the removal of the aliphatic side chain and the formation of phthalate, which is metabolized through protocatechuate to tricarboxylic acid intermediates (Goyal & Zylstra 1997). Thus, the NAH7 genes may or may not participate in metabolism of the intermediate. If the NAH7 genes do not participate, once enough of the intermediate 1-hydroxy-2-naphthoic acid has been formed, PpG7 may cease induction of the upper NAH pathway in order to induce the pathway required for metabolism of the intermediate. This metabolic shift would require a period of acclimation to allow buildup of the necessary enzymes for metabolism of the intermediate. Once metabolism of the 1-hydroxy-2-naphthoic acid has been initiated, up regulation of the upper NAH pathway would have to occur to maintain sufficient intermediate levels, and to allow for optimal degradation.

Another possibility to explain the disappearance and reappearance of the *nahAc* transcript is that there was a build up of toxic intermediates following the initial breakdown of phenanthrene, resulting in decreased cellular activity. Given time, the cells either adapted to the toxicity or slowly metabolized the toxic intermediates such that the concentration of the toxic intermediates decreased (Sikkema et al. 1995). After recovery from toxicity, the cellular metabolism would again increase. The changes in the metabolism of the cell due to toxic intermediates is supported by the varying banding intensities seen during the monitoring of the *rpoD* housekeeping gene. Toxicity effects were previously observed at high salicylate levels (1000 mg/L), which resulted in an increased lag period, and delayed induction of the *nahAc* transcript (Marlowe 1999). Thus, it seems likely that the influence of toxic intermediates would influence gene expression until the cells either recovered or adapted. The results presented here suggest that this assay is sensitive enough to evaluate changes in gene expression due to shifts in the metabolism and physiology of the cell.

The fact that the *nahAc* transcript was not detected for a short period at different times with each surfactant is intriguing. While there was no difference in CFUs produced during phenanthrene degradation in the presence of the two surfactants, the rhamnolipid treatment caused a slightly greater loss of phenanthrene than the HPCD treatment (17.4% vs 26.9% phenanthrene remaining). In terms of gene expression, the disappearance of the *nahAc* transcript occurred at a later time period in the rhamnolipid treatment (196 h) than in the HPCD treatment (96 h). While the interaction of surfactants with microorganisms is not yet well defined, surfactant function has been shown to be a factor of both the enhanced solubility of the phenanthrene, and the bioavailability of the phenanthrene within the surfactant micelles (Guha & Jaffe 1996; Zhang et al. 1997). In this study, it seems likely that the bioavailability of the phenanthrene within rhamnolipid micelles and the HPCD molecule was different. Thus, the build up of intermediates may have differed depending on the surfactant present.

In summary, the results presented here suggest that the use of gene expression assays can add to our understanding of the overall biodegradation process. Such assays could also be used to study the effects of environmental variables on gene regulation especially when the results are compared to a baseline control such as the *rpoD* housekeeping gene. Quantitation of gene expression will be an important step in further understanding gene regulation, and may be possible using recently developed techniques such as competitive RT-PCR (Meckenstock et al. 1998; McGrath et al. 2000).

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