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Application of Reverse Transcriptase PCR for Monitoring Expression of the Catabolic *dmpN* Gene in a Phenol-Degrading Sequencing Batch Reactor

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A modified freeze-thaw method in combination with reverse transcriptase PCR was developed for monitoring gene expression in activated sludge. The sensitivity of the methodology was determined by inoculating nonsterile activated sludge samples with 3-chlorobenzoate-degrading *Pseudomonas putida* PPO301(pRO103), which contains the catabolic *tfdB* gene. *tfdB* mRNA was detected in 10 mg of activated sludge inoculated with 10^4 CFU of the target organism. This technique was subsequently utilized to analyze the in situ expression of the catabolic *dmpN* gene in a sequencing batch reactor (SBR) bioaugmented with phenol-degrading *P. putida* ATCC 11172. Greatest *dmpN* expression was observed 15 min after maximum phenol concentration was reached in the reactor and 15 min after the start of aeration. Decreased phenol concentrations in the reactor corresponded to reduced levels of *dmpN* expression, although low levels of *dmpN* mRNA were observed throughout the SBR cycle. These results indicate that concentration of phenol in the reactor and the onset of aeration stimulated transcriptional activity of the *dmpN* gene. The information obtained from this study can be used to alter SBR operational strategies so as to lead to more effective bioaugmentation practices.

In recent years, there has been a rapid increase in the environmental release of harmful chemicals. Currently, several commercialized chemical, biological, physical, and thermal processes are employed for the degradation of chemical compounds such as phenol. Biological processes are attractive because mixed populations of microorganisms break down or transform organic pollutants to innocuous substances. Bioaugmented processes have been utilized for the degradation of environmental pollutants and detoxification of wastes either in situ or in contained bioreactors (14, 20). In bioaugmented systems, which are being utilized to improve biological treatment, the organic contaminant is contained within a reactor together with a large population of viable microorganisms to which an organism that has a specific degradative capacity is added.

Analysis of microbial communities within bioaugmented reactors is an essential step in understanding the fate of introduced microorganisms, the expressions of specific catabolic genes, and the mechanisms controlling the behavior of the indigenous population or the added microorganisms in mixed-culture systems. Such investigations enable the determination of the potential roles played by microorganisms in environmental processes. A current limitation to evaluating microbial activity in the environment is the lack of a sensitive and rapid technique for monitoring in situ gene expression. In the past few years, several microbial ecological studies have investigated gene expression at the transcriptional level either with the help of reporter genes or by direct mRNA extraction and detection by gene probing (6, 7, 12). Methods to detect in situ gene expression have only recently been reported (4, 16, 19).

The goals of this study were (i) to determine the applicability and detection limit of a modified freeze-thaw method in combination with reverse transcriptase PCR (RT-PCR) for monitoring gene expression in activated sludge and (ii) to apply the above methodology in examining the effects of phenol and aeration on the in situ expression of the catabolic *dmpN* gene in mixed microbial populations. The mixed-culture system utilized in this study was a bioaugmented sequencing batch reactor (SBR). SBRs, which are suspended-growth, activated sludge systems, have been effectively utilized for the degradation of organic pollutants (5). In this study, a laboratory scale SBR was bioaugmented with the phenol-degrading bacterium *Pseudomonas putida* ATCC 11172 containing the *dmpN* gene, which codes for phenol hydroxylase, an enzyme involved in the conversion of phenol to catechol (11). It has been hypothesized that this enzyme is induced by the primary substrates of the pathway, namely, phenol and cresols (1).

A number of studies have been instrumental in characterizing the genes encoding phenol dissimilation and in determining how these genes function in pure culture (8, 9, 11). However, our study is the first report of the expression of the *dmpN* gene under conditions representative of a mixed-culture environment.

MATERIALS AND METHODS

Organism. The organism used for determining the sensitivity of the freeze-thaw and RT-PCR methodology, *P. putida* PPO301(pRO103), was a gift from R. H. Olsen (University of Michigan, Ann Arbor). Plasmid pRO103, a derivative of the 80-kb, broad-host-range plasmid pJP4 (10), encodes genes for the degradation of 2,4-D-2-*methyl*-4-chlorophenoxyacetic acid and 3-chlorobenzoate (3-CB) (3). The *tfdB* gene of plasmid pRO103 codes for 2,4-D-dichlorophenol hydroxylase, which is involved in the degradation of 3-CB (3). 3-CB induces *tfdB* gene expression and thus the synthesis of *tfdB* mRNA. Cells were grown to exponential phase in Trypticase soy medium (BBL, Cockeysville, Md.) containing 3 mM 3-CB (10). The concentration of bacteria for inoculation was determined by plating dilutions, in triplicate, on Trypticase soy agar (BBL) supplemented with 3 mM 3-CB and incubating the plates at room temperature (23 to 25°C) for 24 to 48 h.

The organism used for pure culture studies and for bioaugmenting SBR 1 was *P. putida* ATCC 11172, which degrades phenol by the aerobic *meta* cleavage pathway (9). The strain, obtained from the American Type Culture Collection (Rockville, Md.), was chosen for the study because of the presence of the *dmpN*

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gene, which is not found in the municipal sludge used to start up the laboratory scale reactors (see below). The cells were grown to mid-exponential phase in nutrient broth containing 0.05% phenol before either initiation of pure-culture studies or addition to SBR 1. In order to confirm the initial number of cells in the cultures, dilutions were plated onto nutrient agar plates containing 0.05% phenol and incubated at room temperature for 48 to 72 h.

Evaluation of *dmpN* expression in pure culture. In order to determine the impact of aeration and phenol on transcription of the *dmpN* gene, 10^7 CFU of *P. putida* ATCC 11172 was washed twice in phosphate buffer (13) and resuspended in reactor buffer (78.6 mg of ammonium chloride, 20 mg of sodium phosphate [dibasic], and 20 mg of potassium phosphate [monobasic]). To mimic SBR conditions (see below), the organisms were starved of phenol for 5 h (a 3-h shaking period and a 2-h static period) prior to addition of phenol.

At the end of the starvation period, 2.5×10^8 CFU of *P. putida* ATCC 11172 was placed into a serum bottle with reactor buffer containing 50 ppm of phenol and (i) aerated at room temperature by shaking on a rotary shaker at 150 rpm (two bottles), (ii) incubated at room temperature without shaking (static controls, two bottles), or (iii) incubated anoxically with nitrogen gas (two bottles). Two-milliliter samples were removed from the bottles at various times and frozen immediately at −80°C until analyzed. Phenol concentrations were analyzed by high-pressure liquid chromatography (HPLC) (see below). For analysis of *dmpN* expression by RT-PCR and the determination of *dmpN* DNA by PCR, 1.5-ml samples were centrifuged in a microcentrifuge for 10 min. The pellet was resuspended in 100 μl of diethylpyrocarbonate-treated sterile water (13) and subjected to freeze-thaw lysis as described below.

Reactor operation. The inoculum used to start the two 1-liter reactors (SBR 1 and SBR 2) consisted of mixed liquor from a secondary aeration tank from the South Bend Municipal Wastewater Treatment Plant (South Bend, Ind.). This inoculum was chosen for our study because of the absence of indigenous phenol- and CB-degrading organisms. The reactors were run on two cycles per day; each cycle lasted 12 h (5). Each cycle consisted of the following phases: a 1-h fill (addition of feed), a 9-h "react" (during which the reactors were aerated), a 1-h "settle" (separation of solids and liquids), a 30-min draw (removal of treated wastewater), and a 30-min idle period. Following establishment of steady state on day 19, 7×10^{11} CFU of *P. putida* ATCC 11172 (approximately 20% of the existing indigenous population) resuspended in sterile feed (see below) was added to SBR 1 during the idle phase. SBR 2 served as the unaugmented but otherwise identical control reactor. Mixed-liquor-suspended solids in the reactors were maintained at 2,000 mg/liter by wasting of excess sludge. The sterile feed was composed of methanol (6.7 mg/liter), sodium acetate (100 mg/liter), sodium succinate (66.6 mg/liter), glucose (400 mg/liter), sodium propionate (26.7 mg/liter), sodium phosphate (dibasic; 20 mg/liter), potassium phosphate (monobasic; 20 mg/liter), and ammonium chloride (78.6 mg/liter). During the fill phase of a cycle, 500 ml of sterile feed was added to the reactors. The in situ *dmpN* expression experiments were carried out on day 34, 15 days after bioaugmentation of SBR 1.

Inoculation of test samples with target bacteria. For determining the sensitivity of freeze-thaw and RT-PCR, exponential-phase cells of *P. putida* PPO301(pRO103) were collected by centrifugation (10 min at 5,000 × *g*) and washed once in sterile dechlorinated water. The nonsterile activated sludge samples (10 mg) were amended with 3 mM 3-CB to induce expression of the *tfdB* gene when *P. putida* PPO301(pRO103) was added. An uninoculated, nonsterile activated sludge sample also amended with 3 mM 3-CB served as the control. Samples were mixed with 2 ml of 100 mM sodium phosphate buffer (pH 8) (13) by shaking at 150 rpm for 15 min at room temperature. Following centrifugation at 5,000 × *g* for 10 min, the slurry biomass pellet was resuspended in 100 μl of DEPC-treated sterile water and subjected to freeze-thaw as described below.

Processing of samples from reactors. For the bioaugmentation studies, 3-ml samples (6 mg of biomass) of mixed liquor (biomass in suspending liquid) were removed from the reactors on day 34 at various times during a cycle and frozen immediately at −80°C until analyzed. Samples were withdrawn at −135 (135 min prior to onset of the aeration phase, which is 75 min prior to the fill phase), −35 (during the fill phase and 35 min prior to onset of aeration), 0, 15, 45, 90, 150, and 270 min following the start of aeration phase. The biomass was separated by centrifugation, and the pellets were resuspended in 1 ml of 100 mM sodium phosphate buffer (pH 8) (13) by shaking at 150 rpm for 15 min at room temperature. The remaining clear supernatant was assumed to consist of non-floc-associated cells. Following a second centrifugation at 5,000 × *g* for 10 min, the pellets were resuspended in 100 μl of DEPC-treated sterile water. Nucleic acids were extracted by freeze-thaw lysis as described below.

Isolation of total nucleic acids from activated sludge. Bacterial cells were lysed by freeze-thawing (2) with certain modifications. Samples were frozen in an ethanol–dry-ice bath for 30 s and then thawed at 50°C for 1 min. Following six cycles of freeze-thawing, samples were heated at 85°C for 5 min to inactivate inhibitors of PCR. Nucleic acids were separated from cellular debris and sludge particles by DEPC-equilibrated Sephadex G-200 spin columns (17, 18) before treatment with DNase. DNA was eliminated from the extracts by the addition of 3 U of DNase (Promega Corp., Madison, Wis.) and incubating the samples at 37°C for 15 min. Effectiveness of freeze-thaw lysis was determined as described by Bej et al. (2). Efficiency of RNA recovery was determined by inoculating 10 mg of sterile sludge (activated sludge from SBR 1 autoclaved for 30 min at 121°C) with various concentrations of either *P. putida* PPO301(pRO103) or *P. putida* ATCC 11172 as described by Tsai et al. (19).

Amplification of *P. putida* PPO301(pRO103) and *P. putida* ATCC 11172 RNA by RT-PCR. Reverse transcription and subsequent amplification of target mRNA were performed with a Gene Amp RNA PCR kit (Perkin Elmer-Roche, Norwalk, Conn.) with the following modifications. Briefly, 10 μl of an RT mixture (made up according to the manufacturer's instructions) containing 5 mM $MgCl_2$; 1× PCR buffer II; 1 mM each dGTP, dATP, dTTP, and dCTP; 2.5 μM random hexamers; 1 U of an RNase inhibitor; and 2.5 U of RT was added to 10 μl of DNase-treated samples. Reverse transcription was carried out in a Thermolyne DNA thermal cycler (Fisher Scientific, Itasca, Ill.) at 25°C for 10 min, 42°C for 30 min, 99°C for 5 min, and 4°C for 5 min.

For detection of *P. putida* PPO301(pRO103) by PCR, a 231-bp region located within the *tfdB* gene was amplified with two primers (synthesized by the BioCore Facility, University of Notre Dame). *tfdB1* (5′-GCCGAATGACCTAATGGGC GAGAACACC-3′) and *tfdB2* (5′-CTGTTCGTGTCCAAGATACTCTGTGTG G-3′) were both 28-mers. *P. putida* ATCC 11172 was detected with primers for a 199-bp region located within the *dmpN* gene. *dmpN1* (5′-ATCACCGACTGG GACAAGTGGGAAGACC-3′) was a 28-mer, while *dmpN2* (5′-TGGTATTCC AGCGGTGAAACGGCGG-3′) was a 25-mer. Sequences for both genes were obtained from GenBank. The finished RT mixture was combined with the PCR master mix containing either *tfdB1* and *tfdB2* primers or *dmpN1* and *dmpN2* primers (0.3 μM) and 2.5 U of AmpliTaq DNA polymerase and amplified as described below. A second 10-μl aliquot of each DNase-treated sample (which served as the non-RT control) was combined with 90 μl of the PCR master mix made up as follows: 2 mM $MgCl_2$; 1× PCR buffer II; 1 mM each dGTP, dATP, dTTP, and dCTP; 0.15 μM each appropriate primer, and 2.5 U of AmpliTaq DNA polymerase.

Target DNA (or cDNA) was amplified with initial denaturation of the DNA at 95°C for 2 min and then 40 cycles of three-step PCR amplifications consisting of denaturation at 95°C for 1 min, primer annealing at either 60°C [for *P. putida* PPO301(pRO103)] or 50°C (for *P. putida* ATCC 11172), and primer extension at 72°C for 1 min. Samples were incubated at 72°C for 5 min at the end of amplification to complete the extension reaction.

Analysis of RT-PCR products. RT-PCR products were visualized by gel electrophoresis using a 2% horizontal agarose gel (Sigma Chemical Co., St. Louis, Mo.) with TAE buffer (13). Gels were stained in a solution of ethidium bromide, visualized with a UVP UV Transilluminator (UVP, Inc., San Gabriel, Calif.), and photographed. Amplified *tfdB* products were verified by slot blot DNA hybridization with a [γ-^{32}P]ATP (ICN Biomedicals Inc., Costa Mesa, Calif.)-labeled internal probe (TFDB-INT [5′-GATTACGACGAATGTTCGCCGACCT-3′]) as described previously (15). Binding of the radioactive probe was detected by autoradiography with Fuji RX film (Fisher) at −80°C for 24 to 48 h.

Evaluation of *dmpN* gene expression by densitometry. Gels containing RT-PCR-amplified samples from the reactors were analyzed with an Ultra Scan XL densitometer (Pharmacia-LKB, Piscataway, N.J.). Data from scanned gels were collected and evaluated by the Image Master software program (Pharmacia) as suggested in the manufacturer's instructions.

Sampling of reactors and determination of phenol concentrations. Phenol concentrations were determined by HPLC. Samples were filtered through 0.45-μm-pore-size Whatman WCN type cellulose nitrate filters (Fisher) prior to analysis. A Beckman Ultrasphere octyldecyl silane 5-mm column (4.6 mm by 15 cm) was used with a Waters M45 pump and a Waters 484 detector set at 254 nm. A Hewlett-Packard HP3394 integrator determined the peak areas. The mobile phase consisted of 1:1 methanol and 1% acetic acid. For the pure-culture experiment, samples were withdrawn, in triplicate, from duplicate serum bottles at 0 (just prior to addition of phenol), 15, 45, 90, 150, and 270 min after the addition of phenol and start of aeration (by shaking). Time course studies with the reactors were performed to determine phenol dissimilation during the aeration phase of a cycle. For these studies, samples were withdrawn on day 34, in triplicate, from the reactors at 0 (just prior to start of aeration), 15, 30, 45, 60, 75, 90, 120, 150, 180, 210, 240, 360, and 480 min after the onset of aeration. Samples were centrifuged at 8,000 rpm for 10 min at 4°C. The supernatants were filtered and analyzed by HPLC, as described above.

RESULTS AND DISCUSSION

Sensitivity of RT-PCR. Extraction of nucleic acids by freeze-thawing and amplification by RT-PCR resulted in the detection of *tfdB* mRNA from 10^5 CFU of *P. putida* PPO301 (pRO103) in 10 mg of nonsterile activated sludge biomass (Fig. 1). Amplified *tfdB* product was observed in 10 mg of activated sludge supplemented with as few as 10^5 CFU of the target organism (lane J). Because only 10% (10 of 100 μl) of each DNase-treated extract was used for RT-PCR, the detection sensitivity was calculated to be 10^4 CFU/10 mg of activated sludge. No amplified products were observed in reaction mixtures with nucleic acid from a 10-mg nonsterile sludge sample inoculated with 10^4 CFU (lane I). One reason for this obser-

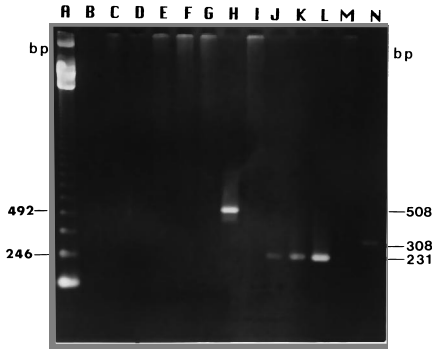

FIG. 1. Nucleic acids from 10-mg nonsterile activated sludge samples inoculated with various concentrations of *P. putida* PPO301(pRO103) were amplified by RT-PCR with primers for the *tfdB* gene. Lanes: A, 123-bp DNA ladder size marker; B, RT-PCR reagent blank; C to F, PCR-amplified DNase-treated controls at 10^4, 10^5, 10^6, and 10^7 CFU/10 mg, respectively; G, DNase-treated uninoculated activated sludge control; H, positive control lambda DNA for PCR; I to L, RT-PCR-amplified samples at 10^4, 10^5, 10^6, and 10^7 CFU/10 mg, respectively; M, uninoculated activated sludge control; N, positive mRNA control for RT-PCR.

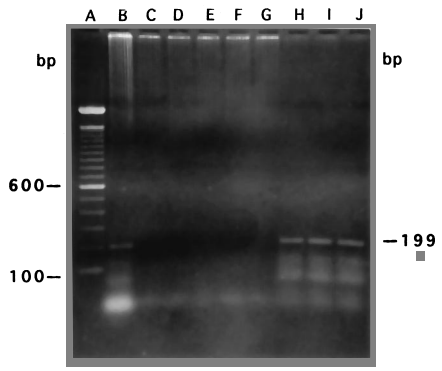

FIG. 3. Nucleic acids from pure-culture samples of *P. putida* ATCC 11172 were amplified either by RT-PCR or by PCR with primers for the *dmpN* gene. Samples were withdrawn 45 min after the addition of phenol and are representative of the results obtained at other sampling times. Lanes: A, 100-bp DNA ladder size marker; B, RT-PCR-amplified nucleic acid from an aerated sample; D, RT-PCR-amplified nucleic acid from a static sample; F, RT-PCR-amplified nucleic acid from an anoxic sample; C, E, and G, PCR-amplified DNase-treated controls from the aerated, static, and anoxic samples, respectively; H to J, PCR-amplified nucleic acids from the aerated, static, and anoxic samples, respectively.

vation could be that the number of cells in the sample (10^3 CFU) is probably below the detection limit for mRNA by this technique because of reasons such as inefficient RNA recovery and the efficiency of cDNA generation by RT. No amplified products were observed in the PCR reagent control (lane B), DNase-treated controls (lanes C to G), or an uninoculated, nonsterile 10-mg sludge sample (lane M). Amplified products were also absent in purified nucleic acid extracts treated with RNase (data not shown). Our detection sensitivity was 2 orders of magnitude greater than that of Tsai et al. (19), whose detection limit was 8×10^8 CFU/g of soil and was comparable to that of Fleming et al. (4), whose detection limit was 10^6 CFU/g of soil.

RT-PCR products were verified by slot blot hybridization with the radiolabeled TFDB-INT oligonucleotide probe (Fig. 2). Positive signals were observed for nonsterile activated sludge samples inoculated with 10^5 to 10^7 CFU of *P. putida* PPO301 (pRO103), representing 10^4 to 10^6 CFU/10 mg. No signals were observed in lanes corresponding to activated sludge supplemented with 10^4 CFU of *P. putida* PPO301(pRO103) representing 10^3 CFU in the sample. A reason for this could be the absence of sufficient target mRNA in this sample, which resulted in very few RT-PCR-amplified products that could not

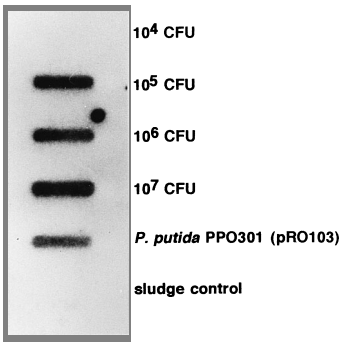

FIG. 2. RT-PCR mixtures containing nucleic acids from inoculated, nonsterile activated sludge samples were analyzed by slot blot hybridization with the radiolabeled TFDB-INT probe. The concentrations of *P. putida* PPO301 (pRO103) cells in 10 mg of activated sludge before amplification by RT-PCR are indicated. Purified DNA (10 μg) from *P. putida* PPO301(pRO103) was used as the positive control.

be visualized either by gel electrophoresis or hybridization techniques. A signal was also absent from the uninoculated activated sludge control, as expected.

DNA in the samples was successfully eliminated by treatment with DNase as indicated by the absence of amplified products in the DNase-treated controls. No RT-PCR-amplified product was observed in RNase-treated samples, verifying that the target nucleic acid was RNA. Amplified *tfdB* product was not observed in the uninoculated activated sludge control, indicating that indigenous populations contained no sequences similar to *tfdB*.

The modified freeze-thaw lysis method resulted in efficient lysis of *P. putida* PPO301(pRO103) and *P. putida* ATCC 11172. Cells were lysed within six cycles of freeze-thawing. These results were similar to those obtained by Bej et al. (2). The recovery efficiencies of total RNA from 10 mg of sterile activated sludge seeded with 5×10^7 CFU of either *P. putida* PPO301(pRO103) or *P. putida* ATCC 11172 were 75% ± 6% and 73% ± 5%, respectively, compared with RNA extraction from the same cell density of pure cultures. This indicates that approximately 25 to 27% of RNA was degraded or not extractable during the extraction procedures. The recovery of total RNA by freeze-thawing was more efficient than the phenol-chloroform method utilized by Tsai et al. (19), whose efficiency of recovery of total RNA from seeded, sterile soil was 60% ± 5%.

Evaluation of *dmpN* expression in pure culture. Total nucleic acids were isolated from pure-culture samples by freeze-thaw lysis, and *dmpN* expression was analyzed by RT-PCR. *dmpN* expression in the samples at 45 min after the addition of phenol can be seen in Fig. 3. The results obtained at this time point were representative of those obtained during other sampling times. This study demonstrated the presence of *dmpN* expression only in samples that had been aerated (lane B). No *dmpN* expression was observed in the static control (lane D), in the anoxic control (lane F), or in DNase-treated controls (lanes C, E, and G), as expected. However, amplification of *dmpN* DNA by PCR revealed the presence of *P. putida* ATCC 11172 in all three samples (lanes H to J). Analysis of phenol concentrations by HPLC in the pure-culture samples showed that, by 270 min, 95% of the phenol was removed in the

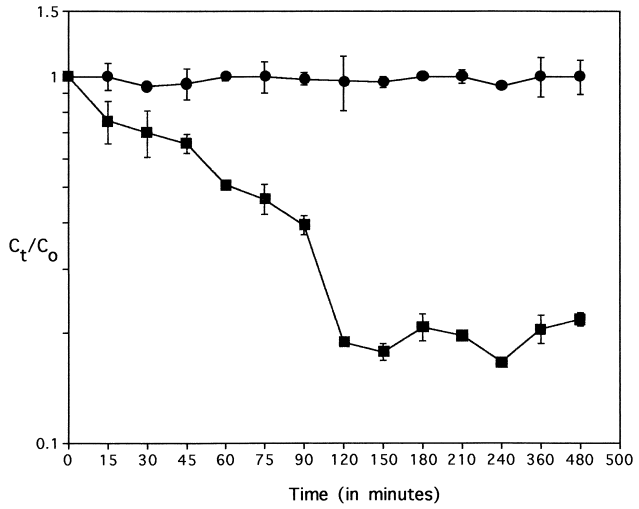

FIG. 4. Time course of phenol removal on day 34 in bioaugmented SBR 1 (■) and unaugmented SBR 2 (●) during the aeration phase. C_t, concentration of phenol at various sampling times; C_o, concentration of phenol at time zero, just prior to onset of aeration. Each datum point is an average of three samples.

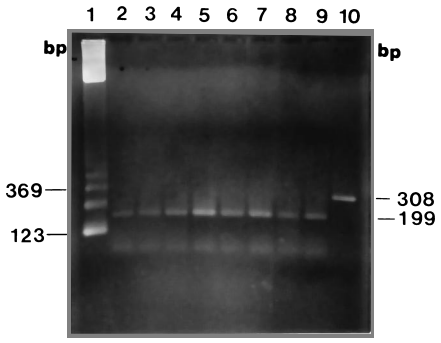

FIG. 5. Total RNAs from 6-mg activated sludge samples from bioaugmented SBR 1 were amplified by RT-PCR. Lane 1, 123-bp DNA ladder size marker. Samples were removed from the SBR 135 min prior to onset of aeration phase, 75 min prior to the fill phase (lane 2), 35 min prior to onset of aeration, during the fill phase (lane 3), at the start of aeration (lane 4), 15 min after start of aeration (lane 5), 45 min after the start of aeration (lane 6), 90 min after start of aeration (lane 7), 150 min after the start of aeration (lane 8), and 270 min after start of aeration (lane 9). Lane 10, positive-control RNA.

aerated samples. In contrast, in the static and anoxic controls, only 5% of the phenol was removed by 270 min (data not shown). These results indicate that a combination of aeration and phenol was required for *dmpN* expression by the pure culture.

Comparison of phenol removal in the bioaugmented and unaugmented reactors during the aeration phase. Phenol removal in SBRs 1 and 2 during the aeration phase can be seen in Fig. 4. These experiments were carried out on day 34, 15 days following bioaugmentation of SBR 1. Results obtained on day 34 were representative of those observed on other days. In the unaugmented SBR 2, phenol concentrations remained unchanged throughout the aeration phase. This was representative of the base case or performance in the absence of bioaugmentation. In the bioaugmented SBR 1, 85% of the phenol was degraded within 2.5 h after the start of aeration. Though phenol concentrations in SBR 1 never reached 0, our results indicate that bioaugmentation with phenol-degrading *P. putida* ATCC 11172 resulted in a significant decrease in phenol concentration compared with the unaugmented SBR 2.

Expression pattern of *dmpN* in SBR 1 during a cycle. Total nucleic acids from the activated sludge samples were isolated by rapid freeze-thaw lysis, and *dmpN* mRNA was amplified by RT-PCR. DNA in the samples was eliminated by treatment with DNase. After the addition of *P. putida* ATCC 11172 to SBR 1 on day 19, *dmpN* gene sequences and mRNA were observed weekly in SBR 1 sludge by PCR and RT-PCR. No *dmpN* sequences or mRNA was observed in the unaugmented SBR 2 or in SBR 1 prior to addition of *P. putida* ATCC 11172 (data not shown).

On day 34, experiments were initiated to determine if differential *dmpN* expression was observed in activated sludge from SBR 1 during a cycle and if this correlated with phenol concentrations in the reactor (Fig. 5). These experiments demonstrated a rapid induction of *dmpN* expression with maximal transcript accumulation 15 min after the start of the aeration phase (lane 5). Basal levels of *dmpN* expression were observed in the reactor during the settle (lane 2) and fill phases (lane 3). By 270 min (lane 9), *dmpN* expression had decreased to levels observed prior to the start of aeration (lanes 2 and 3). No amplified products were observed in DNase-treated non-RT samples or in samples from unaugmented SBR 2 (data not shown). These results combined with those in Fig. 1 indicate that, during aeration, the level of *dmpN* expression is proportional to the concentration of phenol in SBR 1 following an initial lag in expression. The results of the mixed-culture experiments correspond to those obtained for pure-culture studies. Thus, a decrease in phenol concentration due to metabolism in SBR 1 resulted in reduced expression of *dmpN*.

Evaluation of *dmpN* expression by densitometry. Densitometry scanning of the agarose gel containing RT-PCR-amplified samples from SBR 1 indicates that maximum *dmpN* expression was observed 15 min after the start of aeration (Fig. 6). As measured by relative optical density, an almost sevenfold increase in gene expression was observed at this time compared with gene expression before the start of aeration. By 270 min of aeration, *dmpN* expression had decreased to levels observed

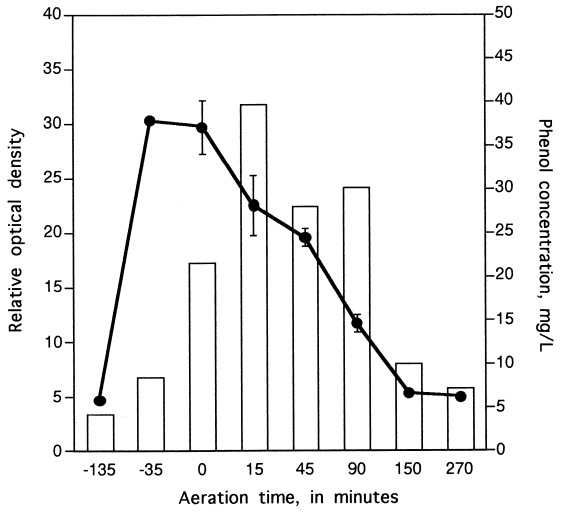

FIG. 6. The effect of phenol (●) on the expression of the *dmpN* gene in the bioaugmented SBR 1 was evaluated by densitometer scanning of RT-PCR-amplified products from Fig. 4. Samples for analysis were withdrawn 135 min prior to onset of the aeration phase (−135) (75 min prior to the fill phase), during the fill phase 35 min prior to onset of aeration (−35), at the onset of aeration (0 min), and 15, 45, 90, 150, and 270 min following the start of the aeration phase. For phenol concentrations, each datum point is an average of three samples.

prior to aeration. It is interesting that *dmpN* RNA levels never reach 0 even after a cycle is complete. This indicates the possibility of a long-lived *dmpN* RNA species or continued low level of RNA synthesis. One explanation for this observation would be the occurrence of spontaneous constitutive mutants for the *dmpN* gene and the presence of these cells in a subset of the population. A more likely explanation would be that residual phenol in the reactor stimulated a basal level of *dmpN* expression.

Phenol concentrations in SBR 1 at similar times are also shown in Fig. 6. Maximum phenol concentration was observed in the reactor at the end of the feeding cycle, approximately 35 min before the start of aeration. Since there is no mixing or aeration during the feeding cycle, it is expected that the phenol concentration would increase during this phase. The beginning of the aeration phase coupled with a large resident phenol concentration resulted in rapid synthesis of *dmpN* mRNA. When phenol concentrations in the reactor decreased, so did expression of the *dmpN* gene; however, *dmpN* mRNA synthesis or the lifetime of the mRNA coincided with the phenol concentration. Basal levels of expression were observed prior to aeration and at the end of aeration; this correlates well with the trace concentrations of phenol in the reactor. These results, which agree with those obtained for the pure-culture studies, indicate that phenol concentrations in the reactor and the onset of aeration stimulated a major burst in transcriptional activity of the *dmpN* gene.

The presence of significant quantities of *dmpN* mRNA during phenol removal implies that mRNA synthesis of this gene is prolonged until trace levels of phenol are reached. Clearly, the onset of aeration coupled with a high phenol concentration is the stimulus for *dmpN* RNA synthesis. Since the reactor cycle is of a "feast or famine" nature, the production of large amounts of mRNA is probably the result of the sudden availability of phenol after a period of starvation. The use of RT-PCR allows the determination of when the *dmpN* gene is transcribed and when transcription has ceased or diminished. This information can be used to alter the SBR cycle to enhance metabolic activity or to time the addition of more substrate or additional organisms, thereby leading to increased effectiveness of bioaugmentation practices.

Detection of specific RNA from environmental samples depends on a sequence of events including cell lysis, successful isolation of RNA, and the steps involved in reverse transcription and amplification. An impairment of any of these steps will result in diminished or no amplified product. Little is known, also, about the efficiency of the latter steps as they apply to the method utilized in our experiments. RNA typically is susceptible to degradation by RNases during extraction procedures and is more difficult to recover than DNA. We have overcome this problem by carrying out cell lysis in the presence of DEPC, which prevents the degradation of nucleic acids by nucleases.

The combination of freeze-thaw lysis and RT-PCR for the detection of specific RNA, presumably mRNA, in environmental samples will help answer important questions regarding the maintenance and physiological status of a given gene in mixed cultures. Since this technology does not require prior culturing of organisms, it should also be useful for the analysis of viable but nonculturable cells in environmental samples.

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