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Quantification of catechol 2,3-dioxygenase gene homology and benzoate utilization in intertidal sediments

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ABSTRACT: The catechol 2,3-dioxygenase (C23O) gene, *xylE*, from the TOL plasmid pathway was used to probe naturally occurring bacterial communities in an intertidal microbial mat. Bound probe was quantified by densitometric analysis of slot blots using colorimetric detection of hybridization between the probe and total DNA extracts from the sediments. The C23O gene encodes the key ring-breaking enzyme of the toluene degradation pathway, of which benzoate is an intermediate. Radiotracer experiments using ^{14}C-benzoate detected benzoate mineralization in these sediments, corroborating the presence of both the genetic potential and *in situ* activity for this transformation.

KEY WORDS: Catechol 2,3-dioxygenase · Quantitative hybridization · Microbial mats · Benzoate utilization

INTRODUCTION

Bacteria play a vital role in degrading aromatic hydrocarbons into less toxic forms. Catechol (1,2-dihydroxybenzene) is a central intermediate in the aerobic bacterial degradation of a wide variety of aromatic compounds, such as benzene, toluene, ethylbenzene, xylene (BTEX), naphthalene, and phenol (Dagley 1986). The primary way by which bacteria convert catecholic substrates into readily usable Krebs cycle intermediates is via aromatic ring cleavage dioxygenases, of which there are 2 types: intradiol dioxygenases cleave *ortho* to the hydroxyl groups while extradiol dioxygenases cleave *meta* to the hydroxyl groups. The toluene degradation pathway of *Pseudomonas putida* mt-2, encoded by the TOL plasmid pWWO, has been the most comprehensively studied *meta*-cleavage pathway (Burlage et al. 1989, Assinder & Williams 1990). Catechol 2,3-dioxygenase (C23O), encoded by the *xylE* gene, plays a key role in this pathway, catalyzing the essential ring-opening step. The *xylE* gene shares significant sequence identity with C23O genes from a number of other *meta*-cleavage pathways (Eltis & Bolin 1996), including those for naphthalene (*nahH*) and phenol (*dmpB*) degradation (Ghosal et al. 1987, Bartilson & Shingler 1989), both of which are also plasmid-encoded.

The prevalence of catabolic genes in known cultivated species and partially characterized isolates is often extrapolated to infer the occurrence of such genotypes in the environment. However, culturable species are not necessarily representative of the natural microbial assemblages. In recent years, the use of molecular techniques to detect specific genes directly, especially in contaminated soil and sediment environments, has become more widespread. In particular, hybridization of catabolic gene probes and PCR amplification using specific primers have been used successfully to detect target genes in DNA extracted directly from the environment (Holben et al. 1988, Sayler & Layton 1990, Walia et al. 1990, Tsai & Olson 1991, Holben et al. 1992, Herrick et al. 1993, Joshi & Walia 1996), supplementing and sometimes circumventing the need for isolation and culturing of microorganisms. Of course, depen-

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dence upon cultures for probe development means that our environmental analyses are still somewhat constrained by the culture bias, but rapid progress has nevertheless been made with this approach.

In this study, we used a digoxygenin-labeled *xylE* gene probe to quantify gene sequences homologous to the C23O gene in total DNA extracted from a microbial mat community. The study site was Elkhorn Slough, an estuary opening onto Monterey Bay, California, USA, closely neighboring several potential sources of aromatic hydrocarbons: fuel hydrocarbons from a nearby boat harbor and agricultural runoff from adjacent fields. A wide variety of organic contaminants have been previously detected in Elkhorn Slough sediments (Rice et al. 1993), including the pesticide dichlorodiphenyltrichloroethane (DDT), polychlorinated biphenyls (PCBs), phthalic acid esters (PAEs), and polycyclic aromatic hydrocarbons (PAHs). Aromatic compounds containing a benzene ring occur not only in many organic contaminants and xenobiotics (e.g. herbicides and pesticides) but also naturally in the amino acids phenylalanine and tyrosine and in many plant-derived compounds, such as humic and fulvic acids. In environments where aromatic hydrocarbons are present, bacteria harboring genes for degradation pathways may have a selective advantage (reviewed by Leahy & Colwell 1990). The objectives of this study were to quantify the genetic potential and the *in situ* capacity for aromatic hydrocarbon degradation in these intertidal sediments, without the need for culturing of microorganisms.

MATERIALS AND METHODS

Study site. Elkhorn Slough Reserve, located 40 km south of Santa Cruz, California, contains several wetland microhabitats and a variety of microbial mat communities. The sampling site for this study was in the lower slough, less than a mile from Monterey Bay, and subject to frequent tidal incursions. Samples for the hybridization study were collected in June 1994 from a crumbly, green mat in which oxidized sediments were present to a depth of several cm. Three core samples were collected with cut-off 5 ml glass syringes and returned to the laboratory for DNA extraction. Core samples for the benzoate utilization experiments were collected using small Teflon cores from the same site in November 1993. At this time, sediments were oxidized in the upper few cm and nitrate was present throughout the cores (Golet 1997).

Nucleic acid extraction and hybridization. DNA was extracted from the top 4 cm of each core. Core samples were extruded in 0.5 cm increments, weighed (wet wt), transferred to 1.7 ml microcentrifuge tubes containing 0.8 ml of 0.5 M EDTA, and stored at –6°C. The DNA was subsequently extracted using an alkaline lysis protocol (Ausubel et al. 1987), modified as follows: vigorous vortexing to resuspend mat particles, as well as treatment with pronase, DNase-free RNase, and numerous phenol-chloroform extractions to purify the DNA from organic contaminants. Prior to phenol-chloroform extractions, samples from each depth interval were pooled to obtain an 'average' sample.

The concentration of DNA in the sediment extracts was determined by comparing the ethidium bromide fluorescence of each sample to that of a dilution series (1 to 100 ng) of a DNA standard (1 kb ladder from Bethesda Research Laboratories, Inc., Gaithersburg, MD, USA) prepared the same way (Maniatis et al. 1982). Each sample was prepared by adding 4 µl of ethidium bromide (0.5 µg ml^{-1}) to 1 µl of each extract on a UV transilluminator. The quantification of DNA in each spot was performed using a gel documentation system, the IS-1000 Digital Imaging System, with multi-ID software version 1.97 (Alpha Innotech Corporation). The standard curve of control DNA extracts versus fluorescence (r^2 = 0.993) was used to calculate the final DNA concentration in the mat DNA extracts, as described previously (Zhou et al. 1996). The DNA extracts were visualized by electrophoresis in 1.0% agarose gels in 1X TAE [40 mM Tris, 5 mM sodium acetate, 1 mM EDTA (pH 7.8)].

The digoxygenin-labeled *xylE* probe was generated as follows. *Escherichia coli* (J831/pxylE; a gift from Daryl Dwyer, Department of Civil and Mineral Engineering, University of Minnesota), was grown at 37°C on Luria Bertani medium supplemented with ampicillin (100 µg ml^{-1}). The pxylE plasmid was isolated by standard protocols (Ausubel et al. 1987) and restriction digested according to manufacturer's instructions [Boehringer Mannheim (BM), Indianapolis, IN, USA]. The pxylE plasmid contains a 550 bp *Sal* I fragment of the *xylE* gene, from pWWO-EB62, in pUC19 (Ramos-Gonzalez et al. 1991). The 550 bp DNA fragment was purified from an agarose gel using the GENECLEAN® kit (BIO 101, La Jolla, CA, USA) and labeled with digoxygenin-dUTP by random priming according to the manufacturer's directions (BM). *Pseudomonas putida* mt-2 (ATCC 33015), which carries the TOL plasmid (pWWO), was grown overnight at 30°C on ATCC medium #1271 supplemented with sodium succinate to 15 mM. Plasmid DNA, extracted from *P. putida* using a standard alkaline lysis protocol (Ausubel et al. 1987), was used as a positive control for some hybridization experiments. DNA concentration and purity were determined spectrophotometrically, and purified DNA was visualized by agarose gel electrophoresis.

The quantitative hybridization protocol was adapted from Kerkhof (1992). Purified DNA from environmen-

tal samples was digested with the restriction enzymes *Eco*RI and *Bgl*I (BM) at 37°C for 2 h, diluted to 50 µl with 6X SSC, denatured by boiling for 15 min, and filtered onto Nytran membrane (Schleicher and Schuel, Inc., Keene, NH, USA) using a slot blot manifold (Hoefer Scientific Instruments, San Francisco, CA, USA). To allow for quantification, a 5-fold dilution series (2.1 to 1340 pg DNA) of purified pxylE DNA was also filtered onto some blots. DNA was cross-linked to membranes by baking at 80°C for 2 h in a vacuum oven. In addition to slot blots, a colony blot was performed (Maniatis et al. 1982) to determine the specificity of the *xylE* probe against several bacterial strains encoding related extradiol dioxygenase genes.

Blots were prehybridized for 3 to 4 h in hybridization solution [0.3% SDS, 5X SSC, 3% (w/v) powdered milk, 0.5 mg sheared salmon sperm DNA ml^{-1}]. Hybridizations were performed overnight at 65°C with probe concentrations of ~100 ng ml^{-1}; blots were then washed under stringent conditions: twice for 20 min in 2X SSC-0.1% SDS at 65°C and twice for 20 min in 0.2X SSC-0.1% SDS at 65°C. Bound digoxygenin-labeled probe was detected colorimetrically using the Genius protocol (BM), yielding a blue or purple precipitate. A Digital Imaging System was used to obtain a digitized image of the blots and to determine the color intensity of each slot. A standard curve of *xylE* DNA detection was produced by quantification of the dilution series of pxylE ($r^2 = 1.00$), and the slope and intercept were calculated by the least-squares method.

Benzoate uptake and oxidation. Sediment cores (2 cm deep × 2.4 cm inner diameter; 7 replicates: 5 live, 2 killed) were collected from the upper surface of the mat using beveled Teflon coring tubes and transported to the laboratory on ice. Seawater collected from the site was sterile filtered through a 0.2 µm polycarbonate filter and amended with sodium benzoate (Sigma) for a final concentration of 10 µM benzoate. Selected cores were autoclaved for 20 min at 121°C for dead controls. *Pseudomonas putida* mt-2 (ATCC 33015, a benzoate-degrading strain) was used as a positive control. *P. putida* cells were grown overnight in benzoate-amended, heterotrophic medium (5 mM sodium benzoate, 0.1 g peptone, 0.1 g yeast extract, 1 ml trace metal solution, 1000 ml sterile seawater) and harvested by centrifugation (8 min at 8000 × *g*). Cell pellets were resuspended in 150 ml benzoate-amended (10 µM) sterile seawater, and 10 ml aliquots were transferred to Teflon tubes for positive controls. Seawater-only and medium-only controls were run with initial experiments to assess loss of radiolabeled substrate due to adsorption. The benzoate-amended seawater was then poured (10 ml) over the live and dead mat cores in the Teflon tubes. ^{14}C-benzoate (specific activity, 13.3 mCi $mmol^{-1}$, Sigma) was added with a micropipettor to each tube for a final concentration of 4.6 nCi ^{14}C ml^{-1}. The tubes were stoppered with a headspace of ambient air, sealed with electrical tape, and incubated at ambient temperature (about 22°C) for 24 h. Preliminary time course experiments showed this incubation period to be optimal for CO_2 production (data not shown) and for diffusion of ^{14}C-substrate to a depth of at least 5 cm in the core (Hogan & Ward 1998).

After 24 h, incubations were stopped by injecting 3 drops 1 N NaOH into the overlying water in each tube. $^{14}CO_2$ in particulate (cell), liquid (overlying water and supernatant) and gas (CO_2) phases was measured. To collect the liquid fraction, the tubes were unstoppered, the liquid was poured into 160 ml serum vials and the vials were set aside. Cores were extruded from the tubes using a micromanipulator, and ten 2 mm slices were transferred to separate 15 ml Corning tubes. The mat slices were washed 3 times with 3 ml sterile seawater and centrifuged between washes (5 min at 4000 × *g*). The washed sediment was counted for the particulate component, and washes were combined with the liquid fraction in serum vials.

To collect the CO_2 fraction, a 25 mm diameter GF/F glass fiber filter was moistened with 100 µl phenylethylamine (Kodak) and suspended over the liquid in the 160 ml serum vials containing the incubation medium; 300 µl concentrated H_2SO_4 was then injected into the liquid through the stopper. Vials were gently swirled and then allowed to degas undisturbed for at least 20 h. The filters and aliquots (1 ml) of the liquid in the serum vials were transferred to 7 ml scintillation vials. Scintillation fluid (5 ml, Scinti-Safe 30, Fisher Scientific Corp.) was added to each vial containing microbial mat slurries, supernatant, or CO_2 filters. Vials were counted for 10 min each using a Packard Model APL.06 Prias scintillation counter. Distribution of ^{14}C in the 3 phases was quantified using a standard quench curve after subtracting values for killed controls. Incorporation and mineralization rates were calculated as nmol ^{14}C m^{-2} d^{-1} and presented as averages of 5 replicate cores.

RESULTS AND DISCUSSION

DNA in sufficient quantity and adequate quality to probe was obtained from all core samples (Fig. 1). The quantitative dot assay method resulted in a linear correlation between fluorescence and DNA concentration ($r^2 = 0.993$), and this standard curve was used to determine the amount of DNA in mat extracts that were filtered onto each slot. The quantity of DNA in each slot ranged from 151 to 361 ng and derived from an average of approximately 0.15 g wet wt of sediment. Based on average cell numbers and DNA content, we con-

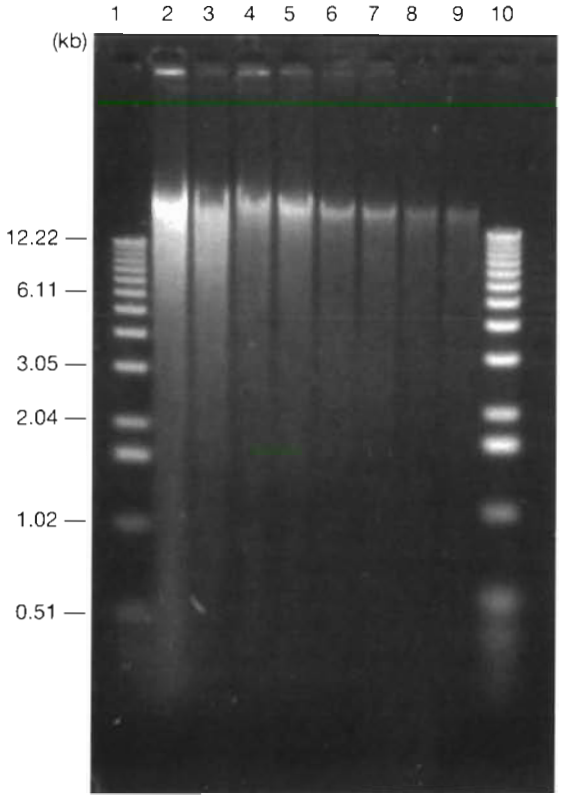

Fig. 1. Agarose gel of DNA extracts (10 µl) from environmental samples of increasing depths. Lanes: 2, 0–0.5 cm; 3, 0.5–1.0 cm; 4, 1.0–1.5 cm; 5, 1.5–2.0 cm; 6, 2.0–2.5 cm; 7, 2.5–3.0 cm; 8, 3.0–3.5 cm; 9, 3.5–4.0 cm. Lanes 1 and 10, 1 kb ladders (BRL)

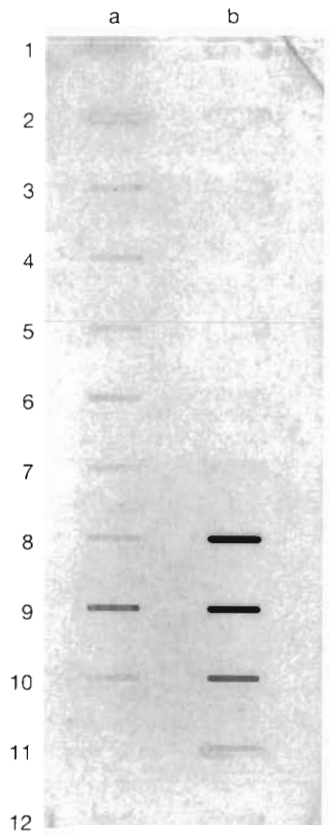

Fig. 2. Detection of homologous C23O genes in a slot blot. (a) Source of DNA in slots: DNA extracts from different depths in the microbial mat. Slots: 1 and 2, mat DNA from trial extractions; 3 to 10, DNA extracts from sequential 5 mm depths in the mat (slot 3, 0–5 mm; slot 4, 6–10 mm, etc.); 11 and 12, 6X SSC. (b) Slots 1 to 7 represent negative controls. Slots: 1, 2, and 7, 1 µg of salmon sperm DNA; 3 to 6, 6X SSC. Slots 8 to 12 represent a dilution series of pxylE, corresponding to the following amounts of *xylE* DNA. Slots: 8, 1340 pg; 9, 268 pg; 10, 53.6 pg; 11, 10.7 pg; 12, 2.1 pg

sider the DNA extraction yield (1.01 to 2.41 µg DNA g^{-1} wet wt sediment) to be less than 50%. Quantification of DNA extraction yield, however, is impossible to determine for these samples, given the complex nature of the microbial community (comprised of eukaryotes as well as bacteria). To make comparisons among depths, the extraction efficiency was assumed to be constant, but because the efficiency is unknown, we did not account for extraction loss in the calculations.

Hybridization between the *xylE* probe and a dilution series of purified pxylE DNA (Fig. 2) was used to determine the detection limit for the assay and to produce a standard curve ($r^2 = 1.00$) for the quantification of DNA homologous to the C23O gene in environmental samples. The detection limit of the probe was determined from the standard curve of the difference in color intensities between the pxylE dilution series and the salmon sperm DNA negative control. This limit, calculated from the linear regression as the amount of DNA corresponding to 1/2 the color intensity of the adjusted lowest detectable dilution, was 0.076 pg, which is in close agreement with the colorimetric detection limit of approximately 0.1 pg suggested for the Genius system. Using the calculated molecular weight of the *xylE* gene fragment (5.92×10^{-7} pg), the detection limit of the probe corresponds to 1.28×10^4 gene fragments. The regression analysis was performed only on the linear portion of the data (2.1 to 53.6 pg), while saturation of color intensity detection occurred somewhere between 53.6 and 268.0 pg. The quantity of homologous C23O gene abundance detected in all of the environmental DNA samples fell in the linear range of detection.

The specificity of the *xylE* probe was tested through a colony blot hybridization experiment, under the stringent conditions used for field samples, with 3 different strains known to carry extradiol dioxygenase genes with different identities to *xylE*: *Pseudomonas putida* mt-2 [pWWO (*xylE*: 100%)]; *P. putida* AC10 [NAH7 (*nahH*: 81%)]; and *Arthrobacter globiformis*

CM-2 (*mndD:* 46%) (Boldt et al. 1995). Only the 2 *P. putida* strains gave positive hybridization signals (data not shown), indicating that the *xylE* probe was specific for more highly conserved C23O genes, many of which are known to be plasmid-encoded (Ghosal et al. 1987, Bartilson & Shingler 1989, Assinder & Williams 1990). In addition to the highly conserved C23O genes found in many *Pseudomonas* species (Williams & Sayers 1994), closely related genes have also been found in a wide variety of other Gram-negative and Gram-positive organisms, including species of *Alcaligenes, Acinetobacter, Achromobacter, Sphingomonas, Burkholderia,* and *Rhodococcus* (Candidus et al. 1994, Kim & Zylstra 1995, Moon et al. 1995, Kim et al. 1996, Fujii et al. 1997, Moon et al. 1997). Although lowering the stringency of hybridization and wash conditions might have allowed the detection of less homologous C23O sequences, the more stringent conditions used in these experiments generally yield the most reproducible and conservative results from quantitative hybridization experiments. The hybridization conditions were designed not to make the probe specific for any one particular bacterial species but rather to detect the C23O functional gene in any bacterial host.

Preliminary slot blot hybridizations demonstrated that the *xylE* probe could detect target sequences in mat DNA extracts and positive controls (purified TOL plasmid and pxylE DNA), while negative controls (salmon sperm DNA and buffer alone) were undetectable. Subsequent blots (Fig. 2) contained a depth profile of purified mat DNA extracts, which were thoroughly quantified, and a dilution series of pxylE to allow for quantification of homologous C23O gene abundance. The detection of C23O gene homology in these intertidal sediments is consistent with the occurrence of aromatic hydrocarbon degradation (see below).

The total number of copies of homologous C23O gene fragments detected at each depth was estimated from the calculated molecular weight of the 550 bp *xylE* fragment and the total amount of homologous DNA quantified in each slot. The estimated number of copies of C23O genes detected on the blot ranged from 1.2×10^6 to 7.5×10^7 per depth interval (from 0.15 g wet wt of sediment). The vertical distribution of C23O gene abundance in the mat showed a peak in the 3–3.5 cm interval (Fig. 3), unlike the distribution of total DNA (Fig. 4), which was maximal in the 0.5–1.0 cm interval. The average number of copies of homologous C23O genes detected throughout the 4 cm mat core was approximately 1.3×10^8 copies g^{-1} wet wt of sediment. The abundance of bacteria in sediment samples from this site was in the range of 5×10^8 to 1.4×10^9 g^{-1} wet wt in the uppermost 5 mm of the sediment (Hogan & Ward 1998). From these data, it appears that a considerable fraction of this microbial community had the genetic potential for the degradation of aromatic hydrocarbons.

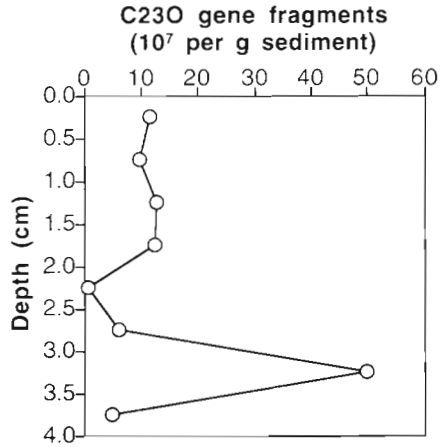

Fig. 3. Depth distribution of homologous catechol 2,3-dioxygenase (C23O) gene fragments in the mat per gram wet weight of sediment

Unlike the abundance of homologous C23O gene sequences, it is more difficult to accurately express the amount of total DNA extracted from the mats in terms of the relative number of fragments or genomes. For this reason, it is useful to consider the amount of C23O gene DNA at each depth as the percentage of total DNA. A single 550 bp fragment in a typical bacterial genome of 6×10^6 bp represents 0.009%, while this same 550 bp fragment in the 117 kb TOL plasmid would represent 0.47%. This calculated theoretical range of 0.009 to 0.47% overlaps with the observed range of 0.0004 to 0.026% (average value, 0.0054%). A value of 0.009% or greater would suggest that every bacterial cell had at least 1 or more copies of the C23O gene per genome, whereas values significantly less

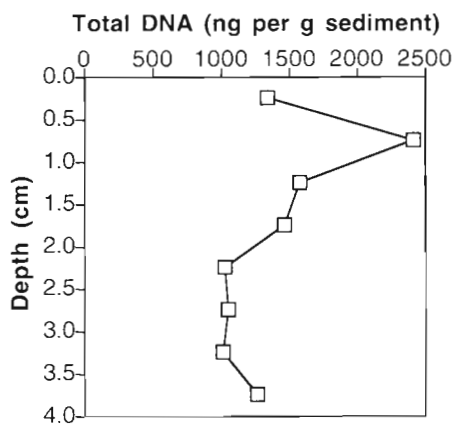

Fig. 4. Depth distribution of total DNA in the mat per gram wet weight of sediment

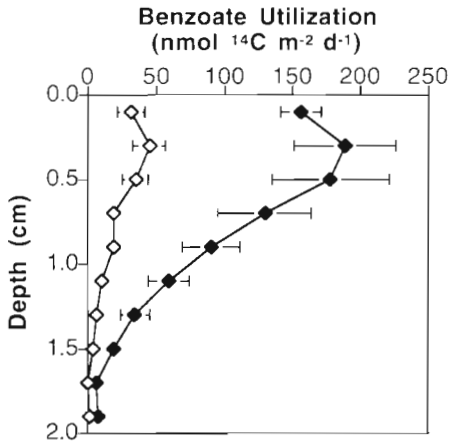

Fig. 5. ^{14}C-benzoate uptake (◇) and oxidation (◆) in Elkhorn Slough mat cores; horizontal bars indicate the standard deviation of the mean of 5 replicate cores

than this (e.g. 0.0004%) would suggest that only a small fraction of the cells carried this gene. The presence of more than 1 copy of the gene per cell implies that it is plasmid-encoded. In particular, the peak in homologous C23O gene sequences (0.026%) at the 3–3.5 cm interval coincides with relatively low total DNA concentration, suggesting greater prevalence of plasmids in this depth interval than elsewhere. Although it is not possible from our data to distinguish between possibilities ranging from a single dominant aromatic-degrading species carrying the gene on a high copy number plasmid to plasmids uniformly dispersed throughout the community, our data are consistent with the supposition that these genes are predominantly located on plasmids. While we cannot entirely discount the possibility that our probe detected genes that are similar but not identical to C23O, these targets also represent hydrocarbon-degrading capability in the natural population.

In a recent study, Joshi & Walia (1996) used PCR primers to amplify C23O gene sequences from hydrocarbon-degrading bacteria isolated from gasoline contaminated groundwater. By using primers designed from conserved regions of the C23O genes from *xylE*, *nahH*, and pAW313, amplification was observed only in those bacterial isolates showing C23O enzyme activity. Thus, molecular probes, whether used in PCR amplification or quantitative hybridization, can be powerful tools for assessing the catabolic potential of bacterial communities.

The *meta*-cleavage pathway genes of the TOL plasmid encode the enzymes for the conversion of benzoate, via catechol, to central metabolites. Thus, the detection of *in situ* microbial benzoate utilization, under aerobic conditions, strongly suggests the presence of microorganisms harboring catabolic genes, like C23Os. In these sediments, benzoate utilization (CO_2 production plus particulate incorporation) was detected throughout the 20 mm core depth (Fig. 5). The amount of substrate remineralized always exceeded the amount incorporated into cells (associated with the particulate fraction), and both terms showed a discrete subsurface maximum in the 2–4 mm interval (190 and 45 nmol m^{-2} d^{-1}, respectively). The particulate fraction represented 32% of the benzoate utilization at the depth of the subsurface maximum. The differences in benzoate utilization between the surface (above 4 mm) and depth (below 4 mm) were highly significant ($p < 0.001$). Absorption of ^{14}C-benzoate to sample tubes was negligible (<1%). Absorption to killed mat controls was much greater (30 to 80% of the live core value depending on depth). Incorporation rates reported in Fig. 5 were corrected at all depths by subtracting the average of duplicate killed controls from the corresponding live sample.

The benzoate utilization data were collected on a different date but from the same site as the hybridization data and clearly demonstrate the presence of benzoate utilization capability in the sediments in which C23O gene homology was detected. Since we have observed great variability within replicate samplings from the same date at this site and even greater variability between dates, it is not expected that the hybridization data and the utilization data should be directly comparable. The depth of oxygen penetration can vary on diel and seasonal time scales; so, although the vertical arrangement of activities and chemical gradients in the mat are similar at different times, the vertical extent of the oxic/anoxic gradients can vary. Thus, bacterial processes may be correlated by their relative position in the gradient rather than by their exact depth. In the 2 sets of experiments reported here, the hybridization data have a 5 mm resolution and extend throughout the upper 40 mm of the core, while the benzoate utilization data have a 2 mm resolution and were sampled only down to 20 mm. The maximum rate of benzoate utilization was detected in the upper 2 to 4 mm of the core, while the maximum C23O gene homology abundance was between 30 and 35 mm. Due to the different sampling times, it is not possible to directly correlate the presence of genetic potential with *in situ* activity at the different depths; nevertheless, both methods corroborate the presence of the target gene/enzyme at this site.

The data in this paper demonstrate the potential for quantitative determination of the distribution and abundance of particular genes of metabolic importance in the environment. They represent the *in situ* genetic capability of the community rather than that of the relatively small portion of the community that might be detected in enrichments of cultivable strains.

The quantification method is no more complicated than standard slot blots and does not require the use of radioactivity. Less expensive densitometric quantification systems (Shea 1994) have also been used in our laboratory with comparable precision. The combination of data on genetic potential and *in situ* activity distributions should be a powerful tool for investigation of the regulation of bacterially mediated transformations in the environment.

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