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Analysis of Microbial Communities Responsible for Naphthalene Biodegradation in a Coal Tar Waste-contaminated Site

Che Ok Jeon and Eugene L. Madsen[1]

KRIBB, 52 Oeundong, Yusong, Daejeon 305-333, Republic of Korea; [1]Department of Microbiology, Cornell University, Ithaca, NY, 14853-8101, U.S.A

Microorganisms maintain the biosphere by catalyzing biogeochemical processes, including biodegradation of organic chemical pollutants. Yet, seldom have the responsible agents and their respective genes been identified. Here we used field-based stable isotopic probing (SIP) to discover a new group of bacteria responsible for in situ metabolism of an environmental pollutant, naphthalene. We released ^{13}C-labeled naphthalene in a contaminated study site, documented $^{13}CO_2$ evolution, created a library of 16S rRNA gene clones from ^{13}C-labeled sediment DNA, identified a unique taxonomic cluster (92 of 95 clones) from the microbial community involved in metabolism of the added naphthalene, and isolated a novel bacterium from site sediment whose 16S rRNA gene matched that of the dominant member (48%) of the clone library. Moreover, we discovered that this bacterium hosts the sequence of a naphthalene dioxygenase gene, prevalent in site sediment, detected before only in environmental DNA. This investigative strategy may have general application for elucidating the bases of many biogeochemical processes; hence for advancing knowledge and management of ecological and industrial systems that rely on microorganisms.

Introduction

Although microorganisms isolated from natural environments often exhibit the physiological potential for catalyzing biogeochemical process in laboratory assays, many longstanding methodological obstacles have prevented documenting the identity and activity of microorganisms in real-world habitats such as soil [1]. Prominent among these obstacles is the propensity for large diverse populations in the microbial community to change physiologically and in abundance after removal from their *in situ* context [1, 2]. Thus, investigations aimed at linking identity with biogeochemical activity of microorganisms in soils and waters have relied on multidisciplinary, often indirect, approaches. If active microorganisms are recognizable microscopically (either due to morphological distinctiveness or to phylogenetic probing for their 16S rRNA genes [3, 4], they can be simultaneously probed for evidence that the gene(s) that catalyze the biogeochemical process of interest are expressed (e.g., mRNA or key enzymes or metabolic intermediates; [5, 6]. Alternatively, some natural systems contain a substrate fortuitously labeled with a unique stable isotopic signature (e.g., methane in anaerobic marine sediments); this has allowed the signature to be directly traced to taxonomically recognizable membrane lipids or other microbial biomarkers in field-derived

samples [7, 8]. Another, perhaps compromised, path to simultaneously measure the identity and activity of microorganisms involves the use of model systems (typically samples of the habitat of interest incubated in the laboratory after addition of radioactive or stable isotopically-labeled substrates) that allow metabolic activity to be measured and the detection of label incorporated into active cells [9, 10].

Results and Discussion

We hypothesized that microorganisms were actively engaged in naphthalene biodegradation in a contaminated study site [11, 12]. Thus, we implemented field respiration and SIP procedures [2] which released ^{13}C-labeled naphthalene to coal tar waste-contaminated sediment where flowing groundwater has deposited naphthalene [13]. The small volumes of naphthalene-dosed sediment (approx. 50 cc) were covered by open-bottom glass chambers. Gas chromatography/mass spectrometry (GC/MS) analysis of headspace samples monitored the of evolution of $^{13}CO_2$ above background produced from sediment organic matter by the native microbial community. Net $^{13}CO_2$ rose steadily--amounting to 2.3% of the added naphthalene in 8 hours (Data not shown).

We then revisited the site, adding ^{13}C-naphthalene to sediment in 3 doses over a 54-hour period. Parallel control treatments added ^{12}C (unlabeled) naphthalene to adjacent sediments. The chambers were removed and DNA was extracted for CsCl density gradient ultracentrifugation to separate ^{12}C- from ^{13}C-DNA. When PCR primers designed to amplify the 16S rRNA gene were applied to the location of the CsCl gradient that corresponded with a ^{13}C-DNA standard, an amplicon was obtained from the ^{13}C-naphthalene-treated sediment, but not from the ^{12}C-naphthlene-treated sediment. To confirm that the ^{13}C-DNA fraction that was amplified by PCR was not simply a relic from the ^{12}C-band seemed prudent; we also conducted 16S rDNA-based T-RFLP analysis to allow comparison of population profiles between bulk soil DNA and both of the CsCl-separated (^{12}C- and ^{13}C-) DNA fractions from the soil that had been dosed with ^{13}C naphthalene. The t-RFLP population fingerprints (Fig. 1) showed that the ^{13}C-DNA fraction was readily distinguishable from and far simpler than (Fig. 1C) the other 2 population profiles, which matched one another as expected (Figs. 1A, B).

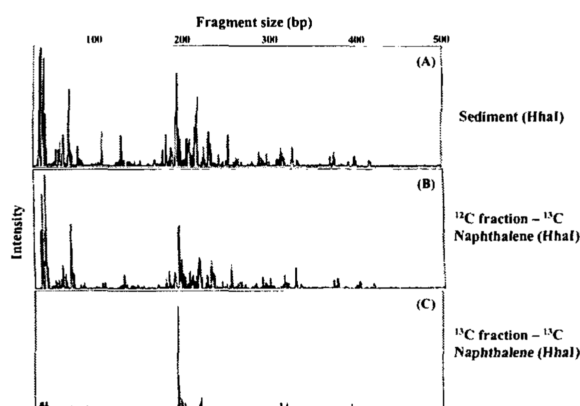

Fig 1. Comparison of the composition of the sediment microbial community before and after separation of ^{13}C- and ^{12}C-DNA pools. Populations reflected by t-RFLP analysis of 16S rRNA genes, in bulk coal tar-contaminated sediment (A) and CsCl gradient-ultracentrifuged preparations from the sediment ^{12}C-DNA (B) and ^{13}C-DNA (C) fractions.

After examining enzyme restriction digests of 95 clones derived from the ^{13}C-labeled DNA (Fig. 1C) and determining 22 full 16S rRNA gene sequences, we prepared a phylogenetic tree to examine the relationships between the cloned sequences and those in GenBank from related bacteria (Fig. 2). The vast majority of the clones (92 of 95) clustered in an unusual group associated with *Acidovorax, Variovorax, Rhodoferax,* and *Polaromonas* --members of the beta proteobacteria. Three outlying sequences were split between other Gram-negative species (*Pseudomonas* and *Burkolderia*). Interestingly, there was surprisingly little variation among the cloned 16S rDNA sequences: 84 of the 92 fell into the same tight clade whose closest cultured relative was *Polaromonoas vacuolata* (93.2% identity; [14]).

To grow and isolate the host of the 16S rRNA gene sequence that we found was dominant in the clone library, we plated dilutions of sediment samples on a naphthalene-based mineral salts medium (MSBN) media and incubated them at the *in situ* groundwater temperature of 10°C. Large (~4 mm diameter) mucoid colonies appeared after 2-4 weeks. When restreaked and purified, this culture (strain CJ2) produced large colonies in the presence of naphthalene and not in its absence. Strain CJ2 was unable to be isolated on MSBN media at temperatures above 20°C because at such temperatures the vapor pressure of naphthalene was toxic; thus, the 10°C incubation was crucial for isolation of strain CJ2. Remarkably, the 16S rRNA gene sequence of strain CJ2 was identical to that of the predominant clone (Clone 3) found in the SIP^{13}C-DNA library (Fig. 2) of microorganisms involved in naphthalene metabolism *in situ.*

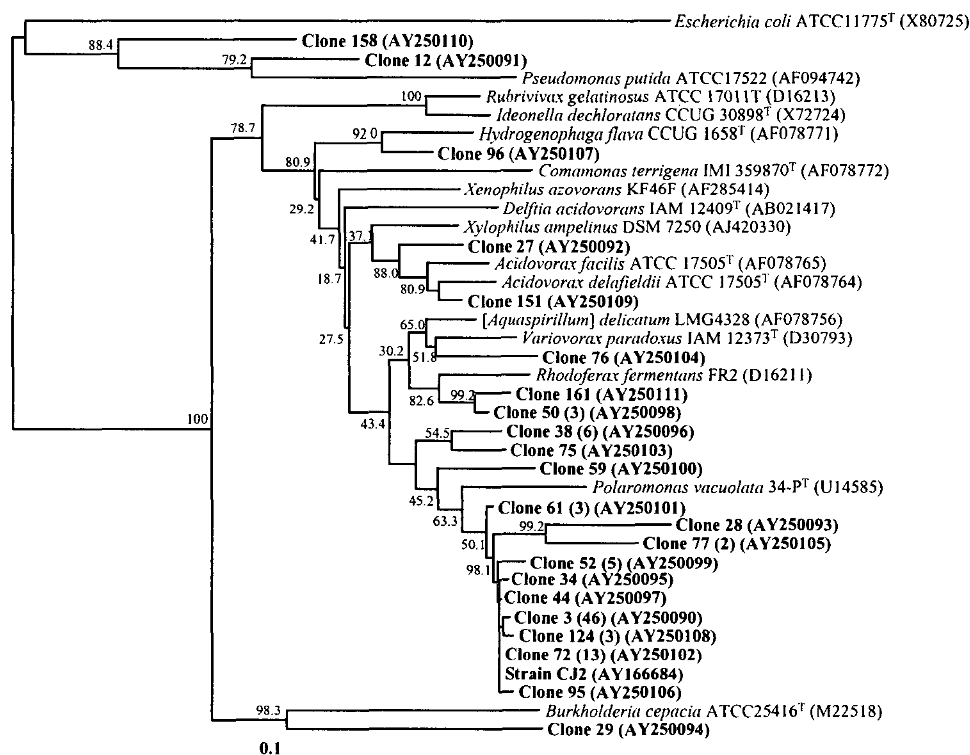

Fig 2. Phylogenetic analysis of 95 cloned bacterial 16S rRNA genes from the sediment-derived ^{13}C-DNA fraction and from isolated strain CJ2. The clones were screened by their enzyme restriction patterns and 22 full 16S rRNA genes representing these patterns were fully sequenced. In parenthesis, numbers indicate frequencies of clones exhibiting the same restriction pattern.

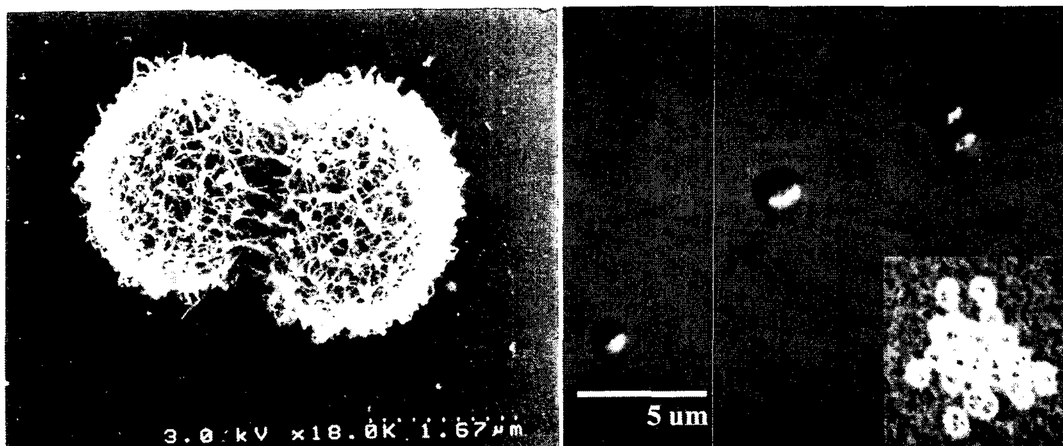

Fig 3. Photomicrographs of isolated naphthalene-degrading bacterium, strain CJ2. Note coccoid shape of 2 dividing cells, rough surface texture from extra-cellular polysaccharide, absence of flagella, and size (1-2 um) visible in scanning electron microscopy image (A). Alcian-blue staining at low pH reveals acidic nature of extracellular polysaccharide (B). Insert shows India ink-staining of capsular material.

Naphthalene utilization by strain CJ2 was confirmed in mineral salts media analyzed after 10 days by GC/MS. Furthermore, assays that used GC/MS to monitor biodegradation of the naphthalene component of the contaminated sediment in laboratory-incubated sediment slurries (10°C) showed that inoculation of strain CJ2 accelerated naphthalene loss. Thus, strain CJ2 has the potential to be metabolically active in a laboratory approximation of the field habitat. Strain CJ2 is a Gram-negative, coccoid bacterium (Fig. 3) with several phenotypic and genotypic characteristics (including absence of flagella, extra-cellular polysaccharide production, inability to grow on rich media, and intracellular accumulation of polyhydroxyalkanoic acids, polyphosphates, and glycogen [15]) unlike those of taxonomically related bacteria listed in Fig. 2.

We next focused on strain CJ2's naphthalene catabolic genes by using PCR primers specific for the terminal iron-sulfur component of naphthalene dioxygenase (*nah*Ac; [11]) to amplify, clone and sequence a 482 bp fragment of *nah*Ac. The dendrogram portraying the relationship of strain CJ2's *nah*Ac to related sequences (Fig. 4) reveals that strain CJ2 is host of a distinctive sequence (clade I in Fig 4). This central clade, with approximately 7% amino acid dissimilarity with clade II, was previously discovered among dioxygenase genes expressed in situ as mRNA sequences in groundwaters from the study site [11]. Until now, there has been no known host for this *nah*Ac allele. The *nahAc* sequence of strain CJ2 had 92.8% sequence identity to the closest previously-cultured host, *Comamonas testosteroni* H [16]. To verify that the *nah*Ac-like amplicon from stain CJ2 was present in the study site, we extracted DNA from strain CJ2's sediment of origin and used the *nah*Ac-specific primers to amplify, clone, and sequence related genes in the microbial community. Six of the twenty sediment-derived *nah*Ac clones that we obtained fell within clade I, matching the sequence of strain CJ2 (Fig.4).

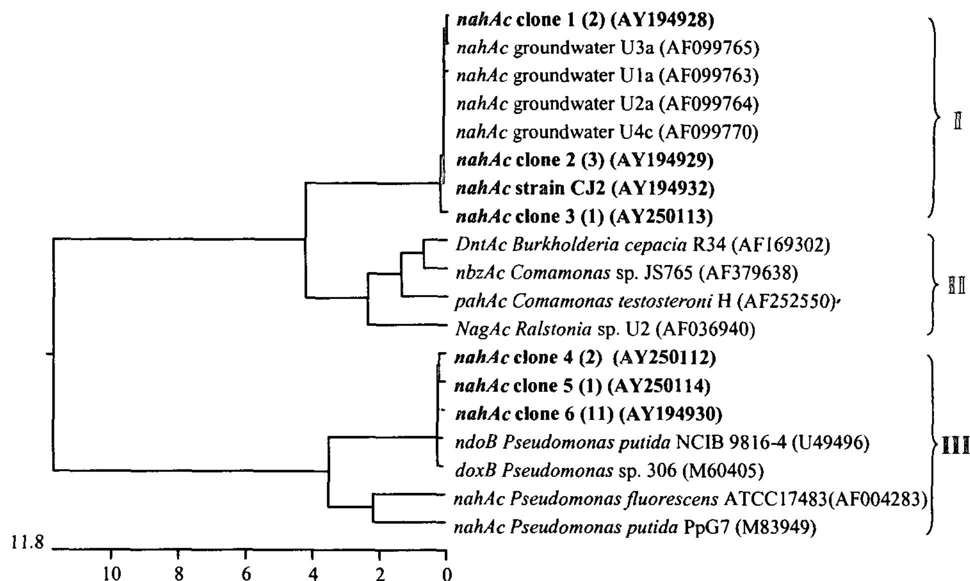

Fig 4. Comparison of naphthalene dioxygenase genes from strain CJ2, sediment DNA, and reference sequences. Cluster analysis examining the percent dissimilarity of 446 bp *nahAc*-like sequences from cultured microorganisms and amplicons from site sediment. Shown are partial *nahAc* sequences for 8 reference strains, new isolate CJ2, 20 clones from site sediment-derived DNA extracts, and 4 GenBank sequences previously cloned from well water mRNA taken from this study site. In parenthesis numbers indicate frequencies of clones found representing the same sequence.

This investigation relied on a field-based SIP technique to trace labeled C atoms from an added microbial substrate (naphthalene) into the DNA of naturally occurring populations involved in substrate metabolism. Data interpretation presumed that the 16S rRNA gene sequences found in ^{13}C-labeled DNA were indicative of microbial populations directly responsible for naphthalene biodegradation. But potential indirect incorporation of the ^{13}C (e.g., via excreted metabolites or other C transfers from biodegrading populations to adjacent community members) must be considered. In this implementation of SIP, indirect C incorporation was unlikely because the incubation period in the field was brief (about 2 days) and because our isolated bacterium, host of the predominant SIP-based 16S RNA gene sequence, possessed genotypic and phenotypic traits consistent with its role as an active member of the site's microbial community. Thus, this investigation allows us to link strain CJ2 to in situ metabolism of an environmental pollutant. The presence of the distinctive *nah*Ac allele in both strain CJ2 and its sediment of origin strongly suggests that this gene catalyzes naphthalene biodegradation *in situ*.

Although aerobic biodegradation of naphthalene has long been described, the majority of cultured hosts that have contributed to understanding both the biochemistry and genetics [17] of naphthalene biodegradation fall into a readily-cultivatable broad class of *Pseudomonas*-like Gram-negative bacteria [18]. It is significant that the bacterium that we found to be active in situ, and to contain the distinctive sediment-borne naphthalene dioxygenase gene, is not related to *Pseudomonas*. Rather, strain CJ2 is phylogenetically remote and features several unusual traits that argue for its novelty and perhaps explain why this strain has not been isolated before. Strain CJ2 joins the growing minority of cultured non-Psuedomonads [19] able to biodegrade naphthalene.

An emergent theme in current microbial ecology is the repartee and feedback that develops between culture- and non-culture-based inquiries [20]. This dialogue has routinely proven to be heuristic—leading to the discovery of both new taxonomic diversity in the microbial world and clues that allow initially mysterious hosts of environmentally-derived traits (e.g., 16S rRNA gene sequences or physiological activity) to be pursued and eventually brought into cultivation [21]; thus enabling further physiological, genetic, genomic, and ecological inquiries [22, 23].

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