## S10-2

# Identifying Functionally Active Microbial Microorganism from a Coal Tar Contaminated Site and Genetic Studies on its Naphthalene Degradation

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Microorganisms maintain the biosphere by catalyzing biogeochemical processes, including biodegradation of organic chemical pollutants. Therefore, identification of the microorganisms and genes responsible for catalyzing biodegradation in terrestrial and aquatic areas is one of the major goals of microbial ecology. Yet, seldom have the responsible agents and their respective genes been identified. Investigations aimed at linking identity with biodegradation activity of microorganisms in soils and waters have relied on multidisciplinary, often indirect, approaches. Among them, DNA and rRNA-based stable isotope probing (SIP) allows us to directly link distinct members of microbial populations to defined metabolic processes. In SIP, active microbial populations metabolize $^{13}$C-labeled substrates added to contaminated sediment and the $^{13}$C-carbon is incorporated into biomass, including biomarkers such as nucleic acids. After extraction, $^{13}$C-labelled nucleic acids are resolved from unlabelled nucleic acids. Subsequently, the structure of labelled and unlabelled communities is resolved by analyzing rRNA genes. Thus, we implemented field respiration and SIP procedures 7 which released $^{13}$C-labeled naphthalene to coal tar waste-contaminated sediment where flowing groundwater has deposited naphthalene.

DNA was extracted for CsCl density gradient ultracentrifugation to separate $^{12}$C- from $^{13}$C-DNA and constructed a clone library using the $^{13}$C-DNA fraction. After examining enzyme restriction digests of 95 clones derived from the $^{13}$C-labeled DNA 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. 1). 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*

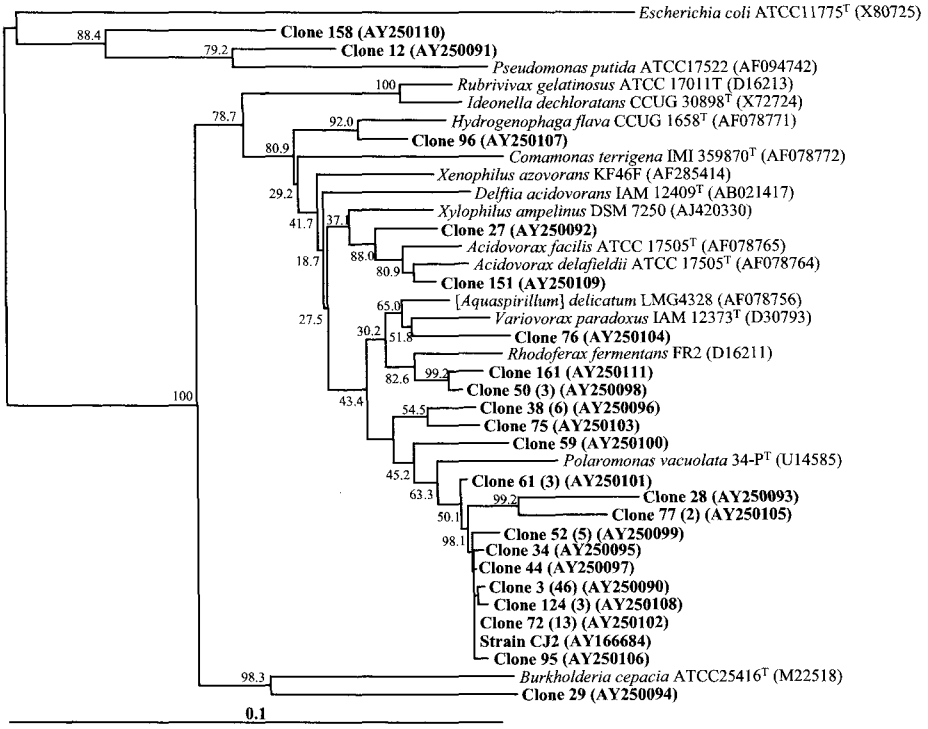


Fig. 1. Phylogenetic analysis of 95 cloned bacterial 16S rRNA genes from the sediment- derived $^{13}$C-DNA fraction.

*vacuolata* (93.2% identity). We isolated a microorganism related to *P. vacuolata* from seep sediment and named the naphthalene degrader, strain CJ2 as *Polaromonas naphthalenivorans*, sp. nov.

We investigated on its growth, physiology, and regulation of naphthalene degradation opreon. At first, the entire naphthalene degradation pathways and additional flanking regions of strain CJ2 were sequenced by the 'genome walking strategy' from a nagAc region. As the results, the naphthalene catabolic genes were split into two clusters, comprising nagRAaGHAbAcAdBFCQEDJI'orf1trpA and Rorf2I"KL. Sequence comparison revealed that the naphthalene metabolic genes were closely related to those of Rastoniasp. U2, which uses the gentisate pathway to central metabolites, but three open reading frames including nagY (chemotaxis-like gene), nagM, and nagN, which are present in Rastonia sp. U2, were deleted and two copies of gentisate dioxygenase (nagI) and two regulators (LysR-type and MarR-type transcriptional regulators) were present in two respective clusters (Fig. 2). To verify the putative role of nagR (LysR type) and R2 (MarR type) regulators in P. naphthalenivorans CJ2, these regulatory genes were disrupted using a Campbell-type single-crossover homologous recombination. As the results, the

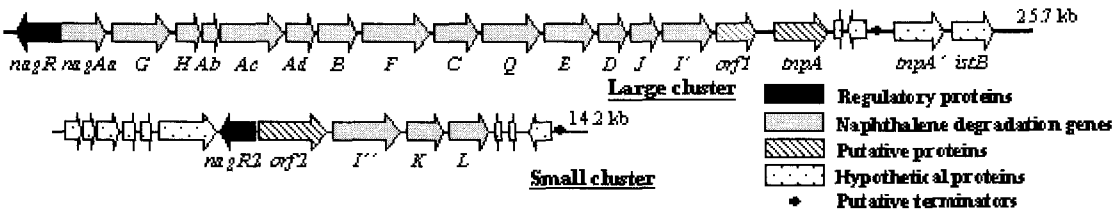


Fig. 2. Physical maps of naphthalene degradation genes from *P. naphthalenivorans* strain CJ2.

nagR mutant strain, CJN110, showed serious growth defect on MSB agar with naphthalene, but the MarR-type mutant strain, CJM110 grew faster than wild type, showing that the second regulator R2 regulates the expression of the second cluster negatively. Transcriptional fusion studies of R2-orf2::lacZ showed that the gene expression of second cluster was not induced in the presence of naphthalene, salicylate, or gentisate.

Although aerobic biodegradation of naphthalene has long been described, the majority of cultured hosts that have contributed to understanding both the biochemistry and genetics of naphthalene biodegradation fall into a readily cultivatable broad class of *Pseudomonas*-like Gram-negative bacteria. It is significant that the bacterium that we found to be active *in situ* is not related to *Pseudomonas*. Rather, the group responsible for naphthalene degradation is phylogenetically remote. Here we discovered a new group of bacteria responsible for *in situ* metabolism of an environmental pollutant, naphthalene using field-based DNA stable isotopic probing (SIP) at a coal tar contaminated site and isolated a microorganism, strain CJ2, responsible for the degradation of naphthalene. This investigative strategy (screening suing stable isotope and its genetic studies) 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.

## References

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2. Padmanabhan, P. Padmanabhan, S., DeRito, C., Gray, A., Gannon, D., Snape, J. R., Tsai, C. S., Park, W., Jeon, C. & Madsen, E. L. Respiration of $^{13}$C-labeled substrate added to soil in the field and subsequent 16S rRNA gene analysis of $^{13}$C-labeled soil DNA. *Appl. Environ. Microbiol.* 69, 1614-1622 (2003).