Environmental Microbiology (2006) **8**(1), 178–182 doi:10.1111/j.1462-2920.2005.00871.x

Brief report

Isolation and characterization of *Pseudoalteromonas* strains with divergent polycyclic aromatic hydrocarbon catabolic properties

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Summary

Fifteen strains of polycyclic aromatic hydrocarbon (PAH)-catabolizing bacteria, identified as *Pseudoalteromonas* spp. were isolated from Eagle Harbor, Puget Sound, USA, using a most probable number procedure in which naphthalene or phenanthrene was the sole carbon and energy source. Despite having identical 16S rDNA sequences, some catabolized many PAHs, whereas others oxidized only naphthalenes. A putative naphthalene 1,2-dioxygenase gene fragment was polymerase chain reaction-amplified from the naphthalene-degrading strains and shown to be almost identical to a gene present in *Neptunomonas naphthovorans*, suggesting horizontal transfer.

Introduction

Approximately 2.3×10^5 metric tons of polycyclic aromatic hydrocarbons (PAHs) are released into aquatic systems every year in the USA, the ultimate reservoirs for which are estuarine and coastal marine sediments (Kennish, 1992). Polycyclic aromatic hydrocarbon-degrading microbes can be enriched at contaminated sites to at least 1.5×10^7 cells (g dry wt)$^{-1}$, several orders of magnitude more than in comparable non-contaminated sites (Geiselbrecht *et al*., 1996; 1998). The phylogenetic and biodegradative diversity of marine and estuarine PAH-degrading microbes has been the subject of several investigations, leading to the isolation of PAH-degrading members of the marine bacterial genera *Cycloclasticus* (Chung and King, 2001; Dyksterhouse *et al*., 1995; Geiselbrecht *et al*., 1996; 1998), *Neptunomonas* (Hedlund *et al*., 1999), *Vibrio* (Hedlund and Staley, 2001), *Marinobacter* (Hedlund *et al*., 2001) and *Lutibacterium* (Chung and King, 2001), in addition to those commonly found in terrestrial habitats such as *Pseudomonas*, *Paenibacillus*, *Rhodococcus*, *Tsukamurella*, *Arthrobacter* and *Sphingomonas* (Daane *et al*., 2001).

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Enumeration of naphthalene- and phenanthrene-degrading bacteria by a PAH-most probable number (MPN) method

In this study, Eagle Harbor, a creosote-contaminated EPA Superfund Site (Brenner *et al*., 2002), was sampled on 1 March 1999 with a boxcore device that was lowered from the University of Washington's R. V. *Clifford A. Barnes*. Samples were stored on ice for less than 24 h, and surface sediment (top 1 cm) was serially diluted into triplicate test tubes containing medium ONR7a at 4°C (Dyksterhouse *et al*., 1995) and naphthalene or phenanthrene crystals as a sole carbon source. After 6 weeks' incubation at 15°C, MPN results were tabulated (de Man, 1975). Naphthalene degrading bacteria numbered 2.86×10^6 cells (g dry wt)$^{-1}$ (95% confidence interval 0.4–4.8×10^6 cells (g dry wt)$^{-1}$). Phenanthrene degrading bacteria numbered 8.58×10^7 cells (g dry wt)$^{-1}$ (95% confidence interval 1.0–10.1×10^7 cells (g dry wt)$^{-1}$). These concentrations are similar to a previous report of PAH-degraders in Eagle Harbor in September 1993 using the same method. At that time naphthalene degrading bacteria in four different boxcores ranged from 8.0×10^4 cells (g dry wt)$^{-1}$ to 1.5×10^7 cells/g (g dry wt)$^{-1}$ and phenanthrene degrading bacteria ranged from 9.0×10^6 cells (g dry wt)$^{-1}$ to 5.0×10^7 cells (g dry wt)$^{-1}$ (Geiselbrecht *et al*., 1996). By comparison, concentrations of naphthalene and phenanthrene degrading bacteria, as determined by the same method, were lower than 1×10^5 cells (g dry wt)$^{-1}$ in several non-contaminated sediments in Puget Sound (Geiselbrecht *et al*., 1996) and the Gulf of Mexico (Geiselbrecht *et al*., 1998).

Isolation and description of naphthalene-degrading *Pseudoalteromonas* strains

Naphthalene MPN tubes were used as a source for PAH-degrading bacteria. After 2 weeks' incubation, diluted samples from positive tubes were spread onto solidified Marine Broth 2216 (Difco Laboratories, Detroit, MI, USA) amended with naphthalene in the vapour phase. Once colonies were visible, indole crystals were added to the inverted Petri dish lids, causing most colonies to turn blue resulting from production of indigo (Ensley *et al*., 1983). All colonies that turned blue, indicative of naphthalene dioxygenase activity, were superficially similar. Thirteen colonies from three different dilutions were isolated by picking and re-streaking. To group the isolates, 16S rRNA genes were polymerase chain reaction (PCR)-amplified as described (Geiselbrecht *et al*., 1996), digested with *Msp1* and *Tsp5091*, separately, and separated on a 1.5% agarose gel. All restriction patterns were identical, indicating they were closely related (data not shown); thus, it appears that *Pseudoalteromonas* may be the only organism that was enriched in the naphthalene MPN tubes that was able to grow on marine agar 2216 and produce indigo from indole. It should be noted that this method of isolation is very selective because it specifically excludes known PAH-degrading organisms previously isolated from Eagle Harbor including *Cycloclasticus*, *Vibrio* and some *Pseudoalteromonas* strains (Geiselbrecht *et al*., 1996; B. P. Hedlund, unpubl. obs.). The 16S rDNA from one strain, EH-2-1, was sequenced (*Escherichia coli* nucleotides 28-1491), aligned with similar sequences using the Ribosomal Database Project (Maidak *et al*., 1999) and Genedoc (Nicholas and Nicholas, 1991), and analysed phylogenetically (Fig. 1; van de Peer and de Wachter, 1994). The sequence was identical to that from two PAH degrading bacteria previously isolated from Eagle Harbor using a phenanthrene-MPN approach: P-1P41 and P-2P45 (Geiselbrecht *et al*., 1996). These strains were isolated from the highest positive dilution MPN tubes (2×10^5), which was the same dilution from which a PAH-degrading *Vibrio* strain was isolated, but order of magnitude higher than MPN tubes from which *Cycloclasticus* strains were isolated. The *Pseudoalteromonas* strains were originally thought to be *Vibrio* strains based on membrane fatty acid analyses (Geiselbrecht *et al*., 1996); however, the 16S rRNA genes from the PAH-degrading strains were identical to those from two named species, *Pseudoalteromonas distincta* and *Pseudoalteromonas elyakovii*, and within four nucleotides of three other species: *Pseudoalteromonas nigrifaciens, Pseudoalteromonas atlantica* and *Pseudoalteromonas espejiana*. Even though members of the genus *Pseudoalteromonas* are metabolically diverse, they are not well known for their ability to biodegrade petroleum hydrocarbons or other organic pollutants. However, one notable exception is a study by Melcher and colleagues (2002) in which isolates that grew with phenanthrene as a sole carbon and energy source were identified by phenotypic tests as *Pseudoalteromonas*.

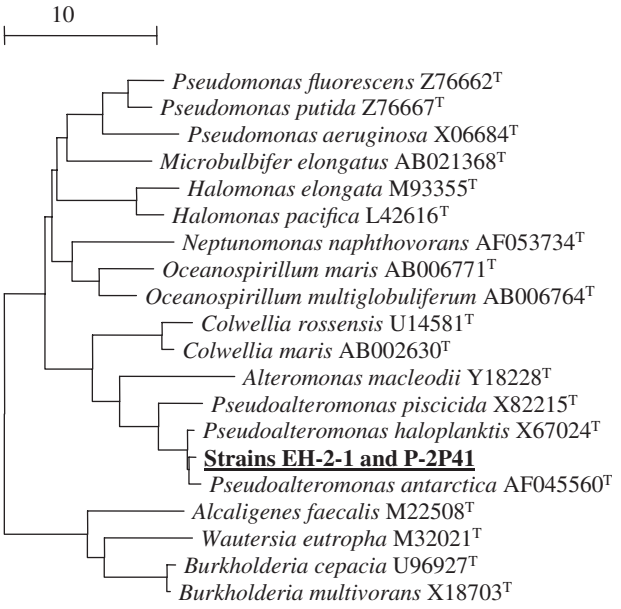

Fig. 1. 16S rDNA phylogenetic analysis of the PAH-degrading *Pseudoalteromonas* strain EH-21 within the Gamma proteobacteria, using members of the Beta proteobacteria as an out-group. The neighbour-joining tree was produced using a Kimura 2-parameter correction using TREECON (van de Peer and de Wachter, 1994). Similar phylogenies were obtained using maximum-likelihood and parsimony (data not shown). Bar represents approximately 10% nucleotide sequence difference.

The new *Pseudoaltermonas* strains, EH-2-1 and EH-3-5, were phenotypically compared with P-1P41 and P-2P45. All isolates were highly motile Gram-negative rods that measured approximately 2–3 μm by 1 μm and failed to accumulate granules of storage compounds such as poly β-hyroxybutyrate. They grew at 30°C but only poorly at 37°C. Some isolates produced a diffusible brown pigment when grown on 2216 with naphthalene vapours. The strains were non-fermentative. For carbon source tests, late exponential-phase cells were added to ONR7a containing 0.1% of a carbon source in triplicate microtitre wells. Growth was monitored by measuring turbidity at 600 nm using Automated Microplate Reader EL311sx (BIO-TEK, Winooski, VT) and Delta Soft II software (BioMetallics, Princeton, NJ) after 2-, 4- and 7-day incubations at room temperature. Growth was defined as 2 or more turbidity doublings more than the negative control, which contained no carbon source. Control organisms were *Oceanospirillum linum* (ATCC 11336) and *Vibrio splendidus* (ATCC 33125). The following compounds were used by all strains: D-glucose, mannitol, L-glutamate, L-serine, L-proline, DL-alanine, citrate, acetate and β-hydroxybu-

tyrate. Glycerol, succinate, L-arginine and D-arabitol were not used.

All isolates grew on naphthalene as a sole carbon source and representative strains were tested for their ability to grow on other PAH crystals, which were added to ONR7a broth in triplicate test tubes. The tubes were monitored for obvious increases in turbidity or for the production of coloured compounds. P-1P41 and P-2P45 also used phenanthrene, 1-methylnaphthalene, 2-methylnaphthalene, 2,6-dimethylnaphthalene and biphenyl. Strains P-1P41 and P-1P45 also produced coloured intermediates indicative of partial degradation of acenaphthene and fluorene, yet these compounds did not support growth of the organisms. Anthracene and fluoranthene were not used. In contrast, EH-2-1 and EH-3-5 only utilized naphthalene and 2-methylnaphthalene for growth.

Activity on PAH compounds

Polycyclic aromatic hydrocarbon degradation experiments were carried out as described (Hedlund *et al*., 1999) to better compare the PAH catabolic activities of the isolates with other bacteria. Briefly, triplicate 20-ml Balch tubes with Teflon™-lined stoppers containing 5 ml of ONR7a and a single PAH compound were inoculated with approximately 1×10^5 late exponential phase cells and the disappearance of the PAH was monitored using GC/FID. This analysis confirmed that, despite their phylogenetic similarity, the strains had dramatically different PAH catabolic activities (Table 1).

Genetic basis of aromatic degradation activity

To investigate the genetic basis for differences in PAH oxidation activities, degenerate primers, pPAH-F and pPAH-NR700, were used to PCR amplify aromatic dioxygenase large subunit gene fragments from the four strains, as described (Hedlund *et al*., 1999). Polymerase chain reaction products were only obtained from strains EH-2-1 and EH-3-5. Products were cloned using a T/A cloning kit (Invitrogen, San Diego, CA), sequenced using the *Taq* DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA) and T3 and T7 primers, aligned with other sequences using Genedoc (Nicholas and Nicholas), and phylogenetically analysed (Fig. 2; van de Peer and de Wachter, 1994). The products were identical to each other and similar to known naphthalene 1,2-dioxygense genes, particularly *nahAc* from *Neptunomonas naphthovorans* ATCC 700637^T (Hedlund *et al*., 1999). Only two nucleotide differences were detected between the *Pseudoalteromonas* and *Neptunomonas* dioxygenase genes, each of which led to amino acid changes. The similarity between the dioxygenase genes in these two genera is consistent with similarities in their PAH degradation activities (Hedlund *et al*., 1999). Neither produced yellow compounds characteristic of *meta*-cleavage of catechol. Both had narrow aromatic degradation spectra: naphthalene, singly methylated naphthalenes and indole. As the dioxygenase genes are much more similar than their 16S rRNA genes, 0.3% versus 14.0% divergence (Figs 1 and 2), it is likely they were passed horizontally between the two lineages.

A separate attempt was made to identify aromatic dioxygenase genes from the *Pseudoalteromonas* isolates. Southern hybridization experiments with *Eco*RI- and *Bam*HI-restricted DNA and the following dioxygenase probes were conducted under low stringency conditions: *Pseudomonas putida* G7 *nahAc* (Simon *et al*., 1993), *N. naphthovorans* ATCC 700637^T *nahAc* (Hedlund *et al*., 1999), *Rhodococcus globerulus* P6 *bphA* (Asturias *et al*., 1995) and *Cycloclasticus pugetii* PS-1^T *xylC1* (Geiselbrecht *et al*., 1996) (data not shown). The two *nahAc* probes hybridized with DNA from EH-2-1 and EH-3-5; however, none hybridized with DNA from P-1P41 or P-2P45, even though control hybridizations were able to detect dioxygenase genes with as little as 60% DNA sequence identity

Table 1. PAH degradation by representative *Pseudoalteromonas* strains.

PAH	Initial concentration (p.p.m.)	% recovery[a]	
		strain P-1P41	strain EH-2-1
Naphthalene	5	**0 ± 0**[b,c]	**0 ± 0**[c]
1-Methylnaphthalene	5	**5 ± 8**[c]	**19 ± 7**
2-Methylnaphthalene	5	**30 ± 26**[c]	**0 ± 0**[c]
2,6-Dimethylnaphthalene	0.5	**0 ± 0**[c]	77 ± 6
Biphenyl	5	**28 ± 16**[c]	81 ± 12
Acenaphthene	1	87 ± 34	83 ± 14
Phenanthrene	1	**0 ± 0**[c]	103 ± 10
Fluorene	1	**27 ± 13**	105 ± 14

a. Values are given as the per cent of the parent PAH remaining after 7 days ± SD ($n = 3$). Control tubes containing no bacteria gave 80–100% recovery for all PAHs.
b. Numbers in bold represent clear cases of PAH degradation.
c. These PAHs are used as sole carbon and energy sources in ONR7a broth with a crystalline PAH added.

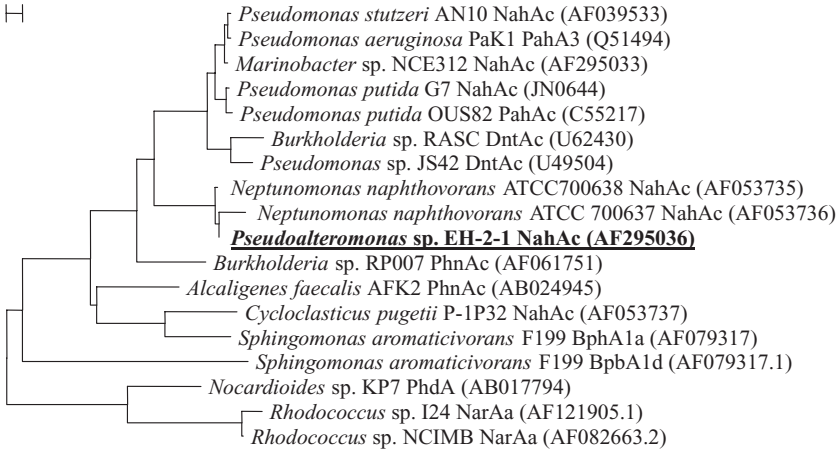

Fig. 2. Neighbour-joining tree of aromatic dioxygenase large subunit amino acid sequences generated using TREECON (van de Peer and de Wachter, 1994). Similar phylogenies were obtained using parsimony (data not shown). Bar represents approximately 10% amino acid sequence difference.

with the probes. These negative results suggest P-1P41 and P-2P45 may have aromatic dioxygenases that are divergent from those that are well characterized.

Acknowledgements

The primary author was supported by the Helen Riaboff Whiteley Endowment and the Public Health Service, National Research Service Award, T32 G07270, from the National Institute of General Medical Sciences. We are also grateful to the Sea Grant Program at the University of Washington for their support. We thank Allison D. Geiselbrecht for comments and discussions.

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