

Marinobacter strain NCE312 has a *Pseudomonas*-like naphthalene dioxygenase

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Abstract

One strain of bacteria, designated NCE312, was isolated from a naphthalene-digesting chemostat culture that was inoculated with creosote-contaminated marine sediment. The strain was isolated based on its ability to grow using naphthalene as a sole carbon source. In addition, the strain degraded 2-methylnaphthalene and 1-methylnaphthalene. Analysis of a 16S rRNA gene sequence from NCE312 placed the isolate in the genus *Marinobacter*. Degenerate PCR primers were used to amplify a fragment of a naphthalene 1,2-dioxygenase large subunit gene. A phylogenetic analysis indicated the *Marinobacter* naphthalene dioxygenase is similar to those from *Pseudomonas* and *Burkholderia* strains suggesting that the dioxygenase gene may have been transferred horizontally between these lineages of bacteria. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: *Marinobacter*; Polycyclic aromatic hydrocarbon; Naphthalene; Marine bacterium; Dioxygenase; Horizontal gene transfer

1. Introduction

Marinobacter is a widely distributed bacterium that has been isolated from coastal marine environments in Europe [1–3], Asia [4,5], North America [5], Africa [6] and Antarctica [7,8]. Culturable *Marinobacter* strains have been shown to comprise 0.01–10% of the microbial community in several pelagic and hydrothermal vent plume samples [9]. In addition, at least some bacteria classified as *Pseudomonas nautica* have been transferred to the genus *Marinobacter* based on phenotypic similarity and high DNA/DNA reassociation values [10]. *Marinobacter* strains are alike in their metabolism, using O₂ or NO₃⁻ to reduce selected organic acids and amino acids for carbon and energy. Some *Marinobacter* strains, including members of the only named species, *M. hydrocarbonoclasticus* and

M. aquaeolei, were isolated from petroleum hydrocarbon-contaminated environments. The type strain, *M. hydrocarbonoclasticus* ATCC 49280^T, was isolated from hydrocarbon-contaminated sediments in the Mediterranean Sea based on its ability to grow with crude oil as a carbon source. It was shown to degrade several aliphatic components of crude oil including hexadecane, eicosane, and heneicosane as well as the aromatic compound phenanthrene [1]. However, a later study indicated that the strain degrades naphthalene but not phenanthrene [10]. The finding that *M. hydrocarbonoclasticus* grows on either aliphatic or aromatic hydrocarbons as sole carbon sources is significant because bacteria with this ability have not otherwise been described. *M. aquaeolei*, isolated from an offshore oil well in Vietnam, is also known to degrade aliphatic and aromatic hydrocarbons; however, the particular compounds it degraded were not described [4].

The distribution of aromatic hydrocarbon degradation ability within the genus *Marinobacter* is unknown. Furthermore, *Marinobacter* strains have not been tested for the ability to degrade polycyclic aromatic hydrocarbon (PAH) molecules other than naphthalene and phenanthrene. Likewise, the mechanisms of aromatic degradation by *Marinobacter* strains have never been discussed. In the present study, we describe the isolation and study of a

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Marinobacter strain, NCE312, from creosote-contaminated marine sediment in Puget Sound, Washington, USA.

2. Materials and methods

2.1. Media

For routine bacterial growth, including the isolation of strain NCE312 and naphthalene growth experiments, cells were grown in the artificial seawater solution ONR7a [11] with naphthalene as a sole carbon source. The complex medium was marine broth 2216 (Difco).

2.2. 16S rRNA gene polymerase chain reaction (PCR), sequencing, and phylogenetic analysis

DNA was isolated from pure cultures using the InstaGene kit (Bio-Rad). 16S rRNA genes (16S rDNA) were amplified by PCR using primers specific for bacteria. Reactions were done in 25- μ l volumes as described previously [12]. The PCR product was cleaned using Ultrafree-MC columns (Millipore) and sequenced using 16S rDNA-specific sequencing primers [11] and the *Taq* DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems).

Sequence contigs were created using Genedoc [13]. Similar sequences were downloaded from the Ribosomal Database Project (RDP) [14] and aligned manually. Parsi-

mony and distance analyses were done using PAUP 3.0S and Treecon, respectively [15,16].

2.3. PAH degradation experiments

Approximately 1×10^5 naphthalene-grown cells were incubated with individual PAHs. After 1 week incubation PAH degradation was assayed using GC/FID as described [12]. Experiments were conducted in triplicate with control tubes that contained no inoculum.

2.4. Naphthalene dioxygenase PCR and sequencing

Naphthalene dioxygenase large subunit gene fragments were amplified using the PCR and the degenerate primers pPAH-F and pPAH-NR700 as described [12]. The product was ligated into pCR2.1TOPO, transformed into *Escherichia coli* (Invitrogen, Carlsbad, CA, USA), and sequenced via the *Taq* DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems). Dioxygenase phylogenies were created using programs described for 16S rDNA phylogenetics.

3. Results and discussion

3.1. Isolation of strain NCE312

Sediment was collected from Eagle Harbor, Puget

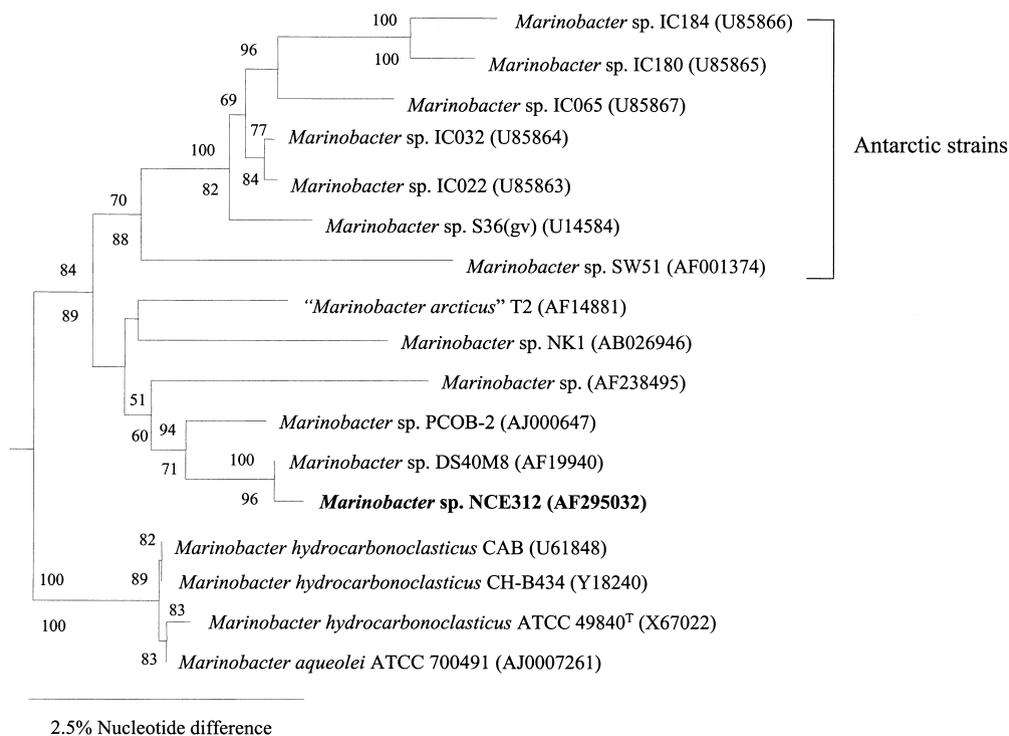


Fig. 1. Distance matrix tree prepared using a Kimura two-parameter correction and the neighbor-joining method of tree construction depicting the phylogeny of NCE312 and related *Marinobacter* strains. Numbers above and below nodes represent bootstrap percentages for 100 replications using distance and parsimony, respectively. Values below 50% are not shown. The NCE312 16S rDNA sequence has been deposited in GenBank (AF295032).

Sound, adjacent to the ruins of a wood treatment facility from which creosote seeped into the harbor. The sediment was used to inoculate an enrichment containing 300 ml of sterile artificial seawater with 10 mg ml⁻¹ naphthalene fed weekly as a sole carbon and energy source [17]. After several weeks, a portion of the enrichment was used to inoculate a 2-l suspended growth reactor, which was fed 200 mg l⁻¹ of naphthalene weekly, with a solid retention time of 15 days. A sample from the reactor was spread onto ONR7a solidified with 0.8% agarose. After the inoculum had dried, a thin layer of 0.8% agarose containing evenly precipitated naphthalene crystals was applied. After several days' incubation, a colony that cleared a halo in the opaque naphthalene overlay was picked and streaked onto ONR7a plates fed with vapor-phase naphthalene. Colonies were streaked iteratively until a pure culture was obtained.

3.2. Phylogenetic analysis of strain NCE312 and other *Marinobacter* strains

The nearly complete (1464 bp) 16S rDNA sequence from strain NCE312 was determined. The NCE312 16S rDNA was analyzed using the RDP Sequence Match program, which indicated the strain is most closely related to *Marinobacter* strains [14]. Therefore, we compared the 16S rDNA from NCE312 to those from *Marinobacter* strains that were available from the RDP or from the National Center for Biotechnology Information (NCBI). NCE312 shared 99.1% 16S rDNA sequence identity with *Marinobacter* sp. strain DS40M8, a strain isolated from the continental slope off the coast of West Africa, between the Canary and Cape Verde Islands [6]. The most closely related strains to NCE312 that have been characterized and named were *M. hydrocarbonoclasticus* ATCC 49480^T and *M. aquaeolei* ATCC 700491^T, each of which shared 96.2% 16S rDNA identity with NCE312. The most distantly related sequences to NCE312 that are still considered *Marinobacter* by the NCBI were from Antarctic sea ice and water.

Phylogenetic trees were created using parsimony and distance methods to better determine the relationship between strain NCE312 and other bacteria in the *Marinobacter* clade (Fig. 1). The phylogenetic trees show that NCE312 is a member of a clade of *Marinobacter* strains that is separate from *M. hydrocarbonoclasticus* and *M. aquaeolei* and from a group of Antarctic isolates. The distinctness of the clades is supported by bootstrap replications.

3.3. PAH degradation experiments

When grown on 2216 medium with naphthalene vapors, strain NCE312 produced bright yellow diffusible products suggesting *meta*-cleavage of catechol through the action of a NahH-type protein. When naphthalene-induced cells

Table 1
Results of *Marinobacter* NCE312 PAH degradation experiments

PAH	Initial concentration (ppm)	Recovery (%) ^a
Naphthalene	5	0 ± 0 ^b
1-Methylnaphthalene	5	32 ± 7
2-Methylnaphthalene	5	0 ± 0 ^b
2,6-Dimethylnaphthalene	0.5	87 ± 8
Biphenyl	5	80 ± 9
Acenaphthene	1	89 ± 10
Phenanthrene	1	98 ± 15
Fluorene	1	95 ± 16

^aValues are given as the percent of the parent PAH remaining after 7 days ± S.D. (*n* = 3). Control tubes containing no bacteria gave 80–100% recovery for all PAHs.

^bThese PAHs are used as sole carbon and energy sources.

were exposed to indole vapors, non-diffusible blue products were produced. These products were most likely indigo, which is produced by *Pseudomonas* naphthalene 1,2-dioxygenases and closely related enzyme systems [18].

Strain NCE312 was tested for the ability to grow using PAH compounds for sole carbon and energy sources. Significant growth occurred only on naphthalene and 2-methylnaphthalene. Little or no growth occurred on 1-methylnaphthalene, 2,6-dimethylnaphthalene, acenaphthene, biphenyl, phenanthrene, and fluorene. In addition, naphthalene-grown cells were tested for their ability to metabolize other PAH compounds. Table 1 shows that naphthalene, 1-methylnaphthalene and 2-methylnaphthalene were degraded by naphthalene-grown cells of NCE312. Our results are similar to those of Spröer et al. [10] who reported that *M. hydrocarbonoclasticus* ATCC 49480^T and other *Marinobacter* strains degrade naphthalene and not phenanthrene.

Many strains in the phylogenetic clade with NCE312 degrade naphthalene. To test whether naphthalene degradation was a general characteristic of the group, six additional strains that were isolated by Kaye and Baross [9] were tested for naphthalene degradation ability: Splume2.1814c, ASW1741a, Splume1.1802c, Splume3.1825c, ASW1747a, and Aplume1727a. These strains are in the *Marinobacter* clade with NCE312 (data not shown); however, they are not shown in Fig. 1 since only a third of their 16S rDNA was sequenced. The strains were isolated on media with a dilute complex source of carbon containing no aromatic substrates. None of the strains grew with naphthalene as a sole carbon source on ONR7a, nor did they produce *meta*-cleavage products on 2216 with naphthalene, or produce indigo when induced and exposed to indole vapors. Thus, it appears that *Marinobacter* strains in this clade that were not isolated on petroleum hydrocarbons do not generally have naphthalene catabolic abilities. This is similar to the pattern of naphthalene degradation in the genus *Pseudomonas* and suggests the genes encoding this ability are on unstable genetic elements.

No strains in the antarctic *Marinobacter* clade have

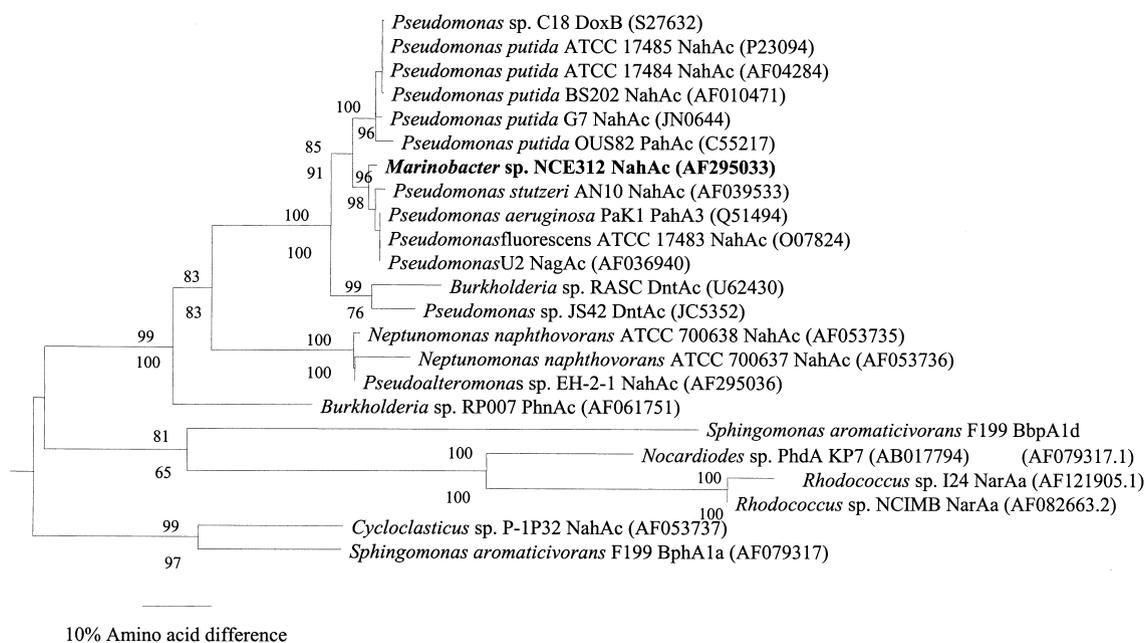


Fig. 2. Neighbor-joining tree showing the NCE312 naphthalene 1,2-dioxygenase and related proteins. Numbers at large nodes represent bootstrap percentages for 100 replications for distance (above) and parsimony (below). Bootstrap values for minor nodes are not shown. The NCE312 dioxygenase gene sequence has been deposited in GenBank (AF295033).

been tested previously for aromatic hydrocarbon catabolism. Six strains in this cluster were tested for naphthalene degradation: strain S-36W(gv) and five relatives from the arctic (N) and antarctic (S) that were identified as *Marinobacter* strains based on membrane fatty acid analyses: N22-B5, N21-B9, N21-F4, SPS1A, and SPS1B [19]. All of these strains were negative for naphthalene degradation.

3.4. Dioxygenase large subunit sequence analysis

Degenerate primers pPAHF and pPAH-NR700 [16] were used to PCR-amplify a fragment of the *nahAc* gene, which is predicted to encode a naphthalene 1,2-dioxygenase large subunit in strain NCE312. The most similar sequences in GenBank were from a well-studied naphthalene dioxygenase family in members of the genera *Pseudomonas*, *Burkholderia*, and *Neptunomonas*. More distantly related to the NCE312 dioxygenase were those from members of the genera *Alcaligenes*, *Cycloclasticus*, *Sphingomonas*, *Nocardiodes* and *Rhodococcus*. The close relationship between the NCE312 and *Pseudomonas* dioxygenases is consistent with the similarity in PAH substrate range for the respective organisms. *Pseudomonas stutzeri* AN10, for example, degrades naphthalene and 2-methylnaphthalene for carbon and energy [20]. Furthermore, both strain NCE312 and *P. stutzeri* AN10 convert indole to indigo. Fig. 2 shows the results of phylogenetic analyses of naphthalene 1,2-dioxygenase large subunit proteins and related sequences. The placement of the NCE312 sequence with *Pseudomonas* naphthalene dioxygenases is strongly supported by bootstrap analyses.

Since the naphthalene dioxygenase from NCE312 is similar to *Pseudomonas* naphthalene dioxygenases, the case for horizontal gene transfer was examined. Although 16S rDNAs are generally highly conserved, the 16S rDNAs from NCE312 and *P. stutzeri* AN10 were more highly divergent (10.9%) than their *nahAc* gene fragments (7.9%). This suggests the dioxygenase genes were transferred horizontally between the two lineages. Herrick et al. [21] used similar data to invoke that horizontal transfer of *nahAc* alleles had occurred between different *Pseudomonas* strains. Horizontal transfer of aromatic catabolic genes has been suggested previously [21–23]. In one example, a gene cluster containing *nahAaAbAcAdBFCEd* in *P. stutzeri* AN10 contained a much lower DNA% G+C content than the *P. stutzeri* genome [20]. Furthermore, a *tnpA1* gene homolog, predicted to encode a bacteriophage λ -type transposase, was discovered adjacent to the gene cluster. This information taken together is consistent with the modular theory for aromatic catabolism pathways [22,23] which explains that aromatic degradation genes can be transferred between bacteria by both transposition and conjugation events.

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