APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1999, p. 251–259 Vol. 65, No. 1
0099-2240/99/$04.00+0

Polycyclic Aromatic Hydrocarbon Degradation by a New Marine Bacterium, *Neptunomonas naphthovorans* gen. nov., sp. nov.

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Received 16 June 1998/Accepted 19 October 1998

Two strains of bacteria were isolated from creosote-contaminated Puget Sound sediment based on their ability to utilize naphthalene as a sole carbon and energy source. When incubated with a polycyclic aromatic hydrocarbon (PAH) compound in artificial seawater, each strain also degraded 2-methylnaphthalene and 1-methylnaphthalene; in addition, one strain, NAG-2N-113, degraded 2,6-dimethylnaphthalene and phenanthrene. Acenaphthene was not degraded when it was used as a sole carbon source but was degraded by both strains when it was incubated with a mixture of seven other PAHs. Degenerate primers and the PCR were used to isolate a portion of a naphthalene dioxygenase iron-sulfur protein (ISP) gene from each of the strains. A phylogenetic analysis of PAH dioxygenase ISP deduced amino acid sequences showed that the genes isolated in this study were distantly related to the genes encoding naphthalene dioxygenases of *Pseudomonas* and *Burkholderia* strains. Despite the differences in PAH degradation phenotype between the new strains, the dioxygenase ISP deduced amino acid fragments of these organisms were 97.6% identical. 16S ribosomal DNA-based phylogenetic analysis placed these bacteria in the gamma-3 subgroup of the *Proteobacteria*, most closely related to members of the genus *Oceanospirillum*. However, morphologic, physiologic, and genotypic differences between the new strains and the oceanospirilla justify the creation of a novel genus and species, *Neptunomonas naphthovorans*. The type strain of *N. naphthovorans* is strain NAG-2N-126.

As a group, the bacteria are well-known for their metabolic diversity. One consequence of this diversity is the fact that many biohazardous or persistent anthropogenic chemical compounds are degraded by microbial activities. One group of compounds that are generally both biohazardous and stable are the polycyclic aromatic hydrocarbons (PAHs). PAHs are composed of fused aromatic rings in linear, angular, or cluster arrangements and are produced during the pyrolysis of organic material (1). Although some PAHs are toxic, carcinogenic, or teratogenic, a variety of bacteria can degrade certain PAHs completely to CO_2 and metabolic intermediates, en route gaining energy and carbon for cell growth.

Naphthalene, which is composed of two fused aromatic rings, has long been used in enrichment cultures to isolate PAH-catabolizing bacteria from soils and freshwater. Naphthalene-degrading bacteria commonly isolated from terrestrial environments include *Pseudomonas* and *Burkholderia* strains. In addition, naphthalene-degrading *Pseudomonas*, *Comamonas*, *Acinetobacter*, and *Sphingomonas* strains have been isolated from soil enrichment cultures by using other PAHs (29, 45). A well-studied example of naphthalene catabolism is the naphthalene degradation (*nah*) pathway of *Pseudomonas putida* NCIB 9816-4 and G7 (reviewed in reference 44). In these *Pseudomonas* strains, 16 *nah* genes are organized into two operons that encode enzymes specific to naphthalene catabolism. A positive transcriptional regulator of the *nah* genes, *nahR*, is encoded by a separate gene. NahR is activated by salicylate, an intermediate in naphthalene degradation. Four of the *nah* genes, *nahAa*, *nahAb*, *nahAc*, and *nahAd*, encode components of the naphthalene ring-hydroxylating dioxygenase, which catalyzes the incorporation of both atoms of molecular oxygen into adjacent positions of an aromatic ring, the first step in naphthalene catabolism. Since naphthalene-degrading *Pseudomonas* strains are abundant in PAH-contaminated terrestrial and freshwater sites (36), studies of these bacteria in the laboratory may yield information that is relevant to the dominant PAH degradation events at these sites.

In contrast, although naphthalene-degrading *Pseudomonas* (9, 42) and *Sphingomonas* (10, 45) strains have been enriched for and isolated from marine sediments, it is not clear that PAH-catabolizing *Pseudomonas*, *Burkholderia*, *Comamonas*, or *Sphingomonas* strains are abundant in the marine environment. In fact, the few studies that have focused on isolating numerically important PAH-degrading bacteria from marine sites, both polluted and nonpolluted, have identified members of completely different genera, including the genera *Cycloclasticus*, *Vibrio*, and *Pseudalteromonas* (11, 12, 20). All of these bacteria are obligately marine; thus, it seems possible that a significant portion of the PAH degradation that occurs in marine environments is degradation by obligately marine microorganisms. Importantly, little is known about how any of the obligately marine bacteria catabolize PAHs, such as naphthalene.

To understand more about PAH-degrading bacteria in marine ecosystems, Geiselbrecht et al. (11) assembled a collection of naphthalene- and phenanthrene-degrading bacteria from Eagle Harbor, a coal tar creosote-contaminated Environmental Protection Agency (EPA) superfund site in Puget Sound. These bacteria were identified as *Cycloclasticus*, *Vibrio*, and *Pseudalteromonas* species. The *Vibrio* isolate that was obtained represents a new species, "*Vibrio cyclotrophicus*" (21). Two other strains of PAH-degrading bacteria isolated from Eagle Harbor, strains NAG-2N-126 and NAG-2N-113, did not belong to the genera mentioned above. The purpose of this study was to describe the isolation of these two strains, characterize their PAH degradation properties, and place them into a meaningful taxonomic and phylogenetic framework.

(Part of this work was presented at the 96th General Meet-

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ing of the American Society for Microbiology, New Orleans, La., May 1996.)

MATERIALS AND METHODS

Media. Strains NAG-2N-126 and NAG-2N-113, as well as control *Oceanospirillum* strains, were grown in artificial seawater solution ONR7a (5) supplemented with an appropriate carbon source, in marine medium 2216 (Difco Laboratories, Detroit, Mich.), or in peptone-succinate-salt (PSS) medium (22).

Sediment sampling and strain isolation. Eagle Harbor sediments were obtained on 17 September 1993 at global positioning system coordinates 47°37.29′, 122°30.25′ and a depth of 15.5 m. The ambient sediment temperature was 16°C. Samples were obtained with a boxcore device from the University of Washington's R.V. *Clifford Barnes*. Sediment subcores were obtained by using sterile modified 60-ml syringes; the subcores were stored at 4°C until they were processed (within 24 h). Five milliliters of surficial sediment, representing approximately the top 1 cm, was diluted in 5 ml of ONR7a (a 1:2 dilution). After vigorous vortexing, this preparation was diluted 1:200; 100 μl of the resulting 10-ml 1:200 dilution was spread plated onto an ONR7a plate solidified with 0.8% agarose. After the plates were dried, they were inverted, and naphthalene crystals were placed in each lid. Naphthalene was the sole carbon and energy source in the plates. The plates were incubated, inverted and wrapped in Parafilm, at 15°C for approximately 4 weeks. The resulting colonies were picked and inoculated into ONR7a containing naphthalene crystals as the sole carbon source at a concentration of approximately 1 mg ml^{-1}. Purity was verified by restreaking colonies onto ONR7a-agarose-naphthalene plates.

PAH degradation experiments. PAH degradation experiments involved growing cultures with individual PAHs and monitoring the disappearance of the PAH with a gas chromatograph equipped with a flame ionization detector. Experiments were conducted in triplicate in 20-ml Balch tubes with Teflon-lined stoppers. PAHs (5 ppm of naphthalene, 5 ppm of 1-methylnaphthalene, 5 ppm of 2-methylnaphthalene, 5 ppm of biphenyl, 1 ppm of acenaphthene, 1 ppm of fluorene, 1 ppm of phenanthrene, 0.5 ppm of 2,6-dimethylnaphthalene) were delivered to the tubes in methylene chloride, and the methylene chloride was removed by evaporation. Five milliliters of ONR7a was added to each tube. The tubes were vortexed for 30 s and shaken for several days to allow the PAH to reach maximum solubility. The PAH concentrations in these experiments were near or below the saturation concentration of each PAH in seawater (25, 26).

The inoculum used for each tube was 50 μl of an exponential-phase culture (approximately 10^5 cells) grown in ONR7a supplemented with naphthalene as the sole carbon and energy source. Cultures were incubated in the dark on a rotary shaker at room temperature for 7 days, and then 1 ml of each culture was removed and extracted for 30 s with hexanes (1:1, vol/vol). The extract was analyzed with a Hewlett-Packard model 5890 Series II gas chromatograph equipped with a 30-m-long, 0.5-mm-diameter type DB-5 column. H_2 at a flow rate of 59.5 cm/s was the carrier gas; H_2, N_2, and air were supplied to the flame ionization detector. The initial oven temperature was 50°C, and the oven temperature was increased at a rate of 10°C/min until it was 250°C. The injector and detector temperatures were maintained at 110 and 150°C, respectively. PAH peaks were identified and integrated by using Hewlett-Packard Chemstation software. Control tubes contained no inoculum. All PAHs were the highest quality obtainable from Sigma Chemical Co., St. Louis, Mo.

PAH mixture experiments were carried out similarly. A mixture of the eight PAHs listed above was used, and each PAH was added to a final concentration of 1 ppm in ONR7a. The tubes were inoculated with cells and incubated as described above. One milliliter of the culture was removed after 2.5 and 24 h for analysis.

Dioxygenase ISP sequencing and phylogenetic analysis. DNA was isolated by using an Instagene kit (Bio-Rad, Hercules, Calif.) and late-exponential-phase cells grown in marine medium 2216. Naphthalene dioxygenase iron-sulfur protein (ISP) gene fragments were amplified by using PCR and the following two degenerate primers: pPAH-F (GGYAAYGCNAAAGAATTCGTNTGYWSH TAYCAYGGITGGG; [*Pseudomonas putida* G7 *nahAc* [38] amino acid positions 93 to 107) and pPAH-NR700 (CCAGAATTCNGTNGTRTTHGCATCRATS GGRTKCCA; *P. putida* G7 *nahAc* amino acid positions 316 to 327). The following PCR program was used: 35 cycles consisting of 1 min at 94°C (initial denaturation at 96°C), 1.5 min at 42°C, and 3 min at 72°C. The last step of the last cycle was continued for 7 min. Salts and free nucleotides were removed from the PCR product by using Ultrafree-MC filter units (Millipore, Bedford, Mass.), and the PCR product was then digested with *Eco*RI, isolated by agarose gel electrophoresis, and purified by using glass wool spin columns. The purified product was cloned into pBluescript II KS^+ (Stratagene, La Jolla, Calif.) by using standard techniques (35). Dioxygenase genes were sequenced with a *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Foster City, Calif.) and primers T3 and T7.

The following dioxygenase ISP sequences (GenBank accession numbers are given in parentheses) were retrieved from GenBank: *P. putida* G7 *nahAc* (M83947), *Burkholderia* sp. strain DNT *dntAc* (U62430), *Pseudomonas aeruginosa* PaK1 *pahAc* (D84146), *P. putida* JS42 *ntdAc* (U49504), *P. putida* F1 *todC1* (J04996), *Burkholderia cepacia* LB400 *bphA1* (M86348), *P. putida* KKS102 *bphA1* (D17319), *Comamonas testosteroni bphA1* (U47637), *Rhodococcus* sp. strain RHA1 *bphA1* (D32142), *Rhodococcus* sp. strain M5 *bpdB* (U27591), *Cycloclasticus pugetii* PS-1 *xylC1* (AF092998), and *Cycloclasticus* sp. strain 1P-32 *nahAc* (AF053737). The data set was aligned by using Pileup, which was obtained from the PHYLIP package, version 3.2 (8); PHYLIP was accessed through the Genetics Computer Group (13). The aligned data set was analyzed by performing a heuristic parsimony analysis with PAUP, version 3.0s (40). The data set was resampled 100 times by using a random sequence addition. The deduced amino acid alignments used for this analysis can be obtained in a variety of formats via the World Wide Web at the following URL: http://weber.u.washington.edu/~staley.

16S rDNA sequencing and phylogenetic analysis. 16S rRNA genes were amplified by PCR by using universal primers and the following program: 32 cycles consisting of 1.5 min at 94°C, 1 min at 42°C, and 4 min at 72°C. The last step of the last cycle was continued for 10 min (19). The 16S ribosomal DNA (rDNA) PCR product was cloned and sequenced by using the protocol described above for naphthalene dioxygenase genes, except that the PCR product was cloned into the *Not*I site in pBluescript II KS^+. 16S rDNA fragments (*Escherichia coli* nucleotides 28 to 1491 [2]) were sequenced by using 16S rDNA-specific sequencing primers (5).

The 16S rDNA sequence of strain NAG-2N-126 was examined with the Ribosomal Database Project (RDP) SIMILARITY_RANK program (24), which suggested that this strain is related to the genus *Oceanospirillum*. Representative sequences of members of the gamma-3 subgroup of the class *Proteobacteria*, which were used in the phylogenetic analyses, were obtained from the RDP or from GenBank. 16S rDNA sequences were initially aligned with similar sequences by using the RDP version 5.0 ALIGN_SEQUENCE program. The aligned sequences were imported into SeqApp (15), and manual adjustments were made to the aligned data set. The NAG-2N-126 16S rDNA sequence was projected onto the *Oceanospirillum linum* 16S rRNA secondary structure model (18) to check for correct alignment of homologous nucleotides.

The data set was initially analyzed with PAUP, version 3.0s (40). The single most-parsimonious tree produced by a heuristic analysis was used to determine the transition-to-transversion ratio by using the MacClade 3.05 State Changes and Stasis command (27). The resulting ratio, 1.05, was specified in maximum-likelihood and neighbor-joining analyses. The programs used in the neighbor-joining analysis, NEIGHBOR and SEQBOOT, were obtained from the PHYLIP package (8). The fastDNAml program was obtained from the RDP (7, 31). The tree shown in Fig. 3 was constructed by using the TREEVIEW program (32). The 16S rDNA alignment used for these analyses can be obtained in a variety of formats via the World Wide Web at the following URL: http://weber.u.washington.edu/~staley.

Microscopy. Late-exponential-phase cells of strain NAG-2N-126 grown on marine medium 2216 were concentrated by centrifugation and resuspension in 0.1 volume of half-strength ONR7a. Cells were pipetted onto Formvar-coated 200-mesh copper grids and allowed to settle for 10 min. Excess liquid was blotted from each preparation, and the cells were stained with 1.0% phosphotungstic acid. Cells were viewed with a JEOL transmission electron microscope at 60 kV.

Phenotypic tests. The pHs and salinities which allowed growth were examined in ONR7a broth supplemented with 0.1% peptone. For pH determinations, media were prepared with alternative buffers at concentrations of 25 mM near their pK_a values. The following buffers were used: 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 5.5; *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES), pH 6.5; 3-[*N*-tris(hydroxymethyl)methylamino]-2-hydroxypropanesulfonic acid (TAPSO), pH 7.6; tris(hydroxymethyl)methylaminopropanesulfonic acid (TAPS), pH 8.5; and 2-(*N*-cyclohexylamino)-ethanesulfonic acid (CHES), pH 9.5. Salinities were adjusted by varying the concentrations of the inorganic salts in ONR7a (the NH_4Cl, Na_2HPO_4, $FeCl_2$, and TAPSO concentrations were not varied). The salinities tested were 0.35, 1.05, 1.75, 3.5, 7.0, and 10.5%. Cultures were incubated at 24°C with shaking and were observed daily for 5 days. The temperature range for growth was determined on solid marine medium 2216 which was preincubated at the appropriate temperature for 2 h prior to inoculation. The temperatures tested included 4, 15, 24, 30, 37, and 42°C.

Gram stain reactions were determined by the method of Manafi and Kneifel (28). Routine phenotypic tests, including tests to determine catalase and oxidase activities, reduction of possible electron acceptors, and production of extracellular enzymes, were conducted as described previously (14). Nitrate reduction assays were performed with 0.1 and 0.01% $NaNO_3$. Tween 80 was used for the lipase test. Poly-β-hydroxybutyrate inclusions were visualized with Sudan black and were verified by purification and spectral analysis. Luminescence was tested by growing strains on solid marine medium 2216 supplemented with 3% glycerol.

For the carbon source utilization tests, late-exponential-phase cells were added to ONR7a containing the carbon source of interest at a concentration of 0.1% in microtiter wells. Growth was monitored by measuring the increase in turbidity at 600 nm with an automated microplate reader (model EL311sx; BIO-TEK, Winooski, Vt.) and Delta Soft II software (BioMetallics, Princeton, N.J.) after 2, 4, and 7 days of incubation at room temperature. Growth was defined as two or more cell doublings more than the negative control, which contained no carbon source. Each test was carried out in triplicate. Under these conditions, *O. linum* ATCC 11336 was not able to use any of the carbon sources tested as a sole carbon and energy source, a result that is consistent with other reports (22).

Carbohydrate acidification and fermentation tests were performed as described previously (14) in PSS medium. Tests were conducted in triplicate, and

TABLE 1. Degradation of single PAHs by *Neptunomonas* strains

PAH	% recovery[a]	
	Strain NAG-2N-113	Strain NAG-2N-126
Napththalene	0[b]	0[b]
1-Methylnaphthalene	34 ± 2	20 ± 4
2-Methylnaphthalene	12 ± 7[b]	1 ± 1[b]
2,6-Dimethylnaphthalene	0	107 ± 37
Biphenyl	91 ± 14	89 ± 7
Acenaphthene	102 ± 6	92 ± 5
Phenanthrene	0[b]	92 ± 17
Fluorene	80 ± 19	90 ± 8

[a] Percentage of the PAH remaining after 7 days (mean ± standard deviation; $n = 3$). The levels of PAH recovery from control tubes containing no bacteria were 85 to 100%.

[b] PAH used as a sole carbon and energy source.

O. linum was used as a negative control. Tubes were observed daily for 2 weeks and were considered positive if they became acidic at any time during the incubation period. The fermentation tubes contained 0.15% agar, were inoculated at approximately 42°C, and were covered with 2 to 3 mm of mineral oil. In most instances, growth occurred mostly near the surfaces of the fermentation tubes; nevertheless, such tubes were considered positive.

Antibiotic resistance determination. Resistance to antibiotics was determined in microtiter wells containing marine medium 2216 and serially diluted antibiotics. The sensitivity level of an antibiotic was defined as the concentration at which the antibiotic reduced the level of growth to less than one-half the level of growth without an antibiotic. Turbidities were determined after 2 days with the microplate reader. The antibiotics tested were chloramphenicol, kanamycin, ampicillin, and streptomycin.

Determination of G+C content of DNA. The guanine-plus-cytosine content of genomic DNA was determined by the thermal denaturation method (14). *E. coli* and *O. linum* were used as reference organisms.

Nucleotide sequence accession numbers. The *Neptunomonas naphthovorans* NAG-2N-126 16S rDNA sequence has been deposited in the GenBank database under accession no. AF053734. The naphthalene dioxygenase ISP sequences of NAG-2N-126 and NAG-2N-113 have been deposited in the GenBank database under accession no. AF053736 and AF053735, respectively.

RESULTS

Isolation of strains. Both NAG-2N-126 and NAG-2N-113 were obtained as part of a direct plating effort to compare the effectiveness of plate counts and the effectiveness of a most-probable-number (MPN) approach for enumeration of PAH-degrading bacteria. Since the sediments used were diluted prior to plating (final dilution, 1:20,000), isolation of these bacteria indicates that they were relatively numerous in Eagle Harbor, which is a highly contaminated EPA superfund site (3). It is important to note that creosote was visible as a free contaminant in these sediments. The two isolates were obtained from the same sediment boxcore sample but different plates.

PAH degradation experiments. Both strains were tested for the ability to degrade individual PAHs which are common in creosote and other petroleum products (30) (Table 1). Consistent with the results of sole-carbon-source tests, strains NAG-2N-126 and NAG-2N-113 degraded naphthalene and 2-methylnaphthalene; NAG-2N-113 also degraded 1 ppm of phenanthrene and 0.5 ppm of 2,6-dimethylnaphthalene. Interestingly, 1-methylnaphthalene was degraded by both strains, although the strains did not grow with 1-methylnaphthalene as a sole carbon source. The other PAHs that were tested were not significantly transformed in the single-PAH experiments.

The new strains were also tested for the ability to degrade the components of a mixture of eight PAHs (Fig. 1). Despite the differences in the ability to degrade single PAHs observed in the experiment described above, NAG-2N-126 and NAG-2N-113 produced almost identical results in the PAH mixture

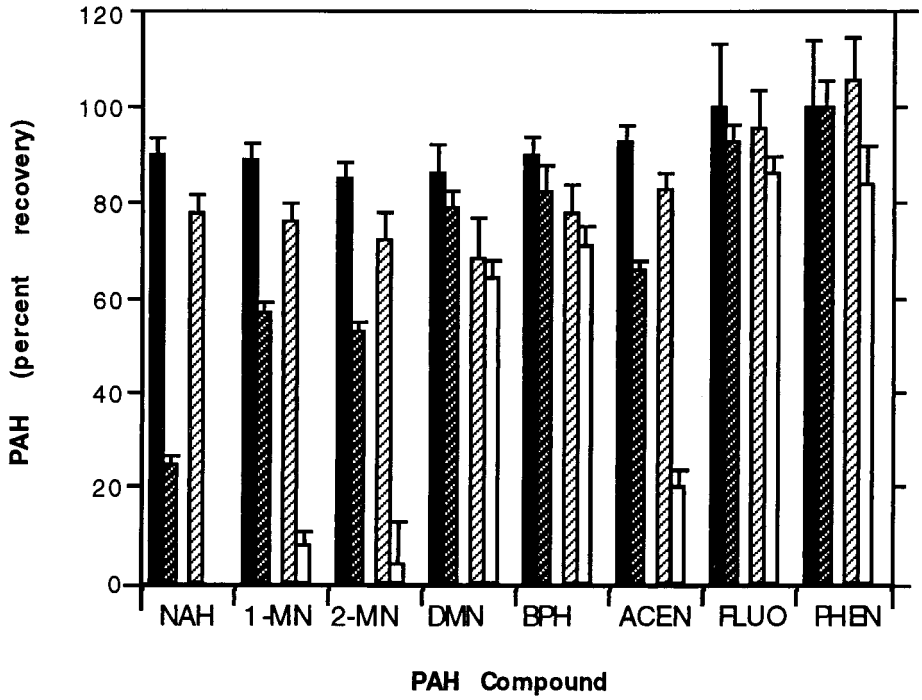

FIG. 1. Results of a mixed-PAH degradation experiment performed with NAG-2N-113. Exponential-phase cells were added to ONR7a containing a mixture of eight PAHs, each at a concentration of 1 ppm. One milliliter of the culture was removed after 2.5 and 24 h of incubation, extracted with hexanes, and analyzed with a gas chromatograph equipped with a flame ionization detector. ■, negative control, 2.5 h; ▨, NAG-2N-113, 2.5 h; ▨, negative control, 24 h; □, NAG-2N-113, 24 h. Abbreviations: NAH, naphthalene; 1-MN, 1-methylnaphthalene; 2-MN, 2-methylnaphthalene; DMN, 2,6-dimethylnaphthalene; BPH, biphenyl; ACEN, acenaphthene; FLUO, fluorene; PHEN, phenanthrene. Error bars represent one standard deviation.

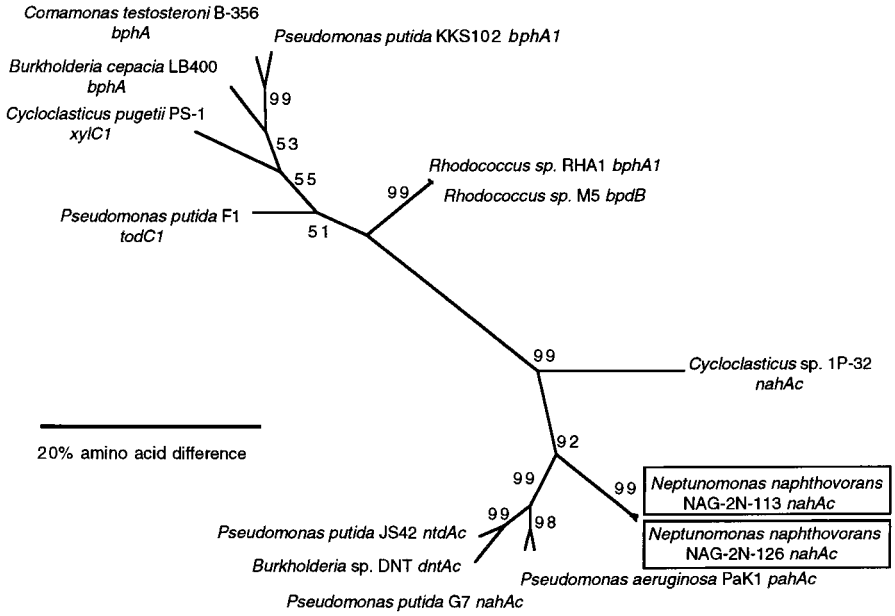

FIG. 2. Parsimony analysis of PAH dioxygenase ISP performed with deduced amino acid sequences. The numbers at the branch nodes are the percentages of bootstrap support for 100 resamplings. Scale bar = approximately 20% amino acid divergence.

experiments (data are not shown for NAG-2N-126). Both strains degraded naphthalene, singly methylated naphthalenes, and acenaphthene. Neither phenanthrene nor 2,6-dimethylnaphthalene was significantly degraded in 24 h by strain NAG-2N-113, even though these compounds were completely degraded in the single-PAH degradation experiments. This result may have been due to the short time course of the experiment.

Dioxygenase ISP sequence analysis. A low-stringency Southern hybridization experiment performed with the *P. putida* G7 *nahAb*, *nahAc*, and *nahAd* genes indicated that the new strains contained genes homologous to the genes that encode some of the prototypical naphthalene dioxygenase components (data not shown). Southern hybridization experiments performed with other dioxygenase probes representing the biphenyl and monoaromatic dioxygenase families, however, failed to reveal the presence of other dioxygenases in the new strains. Since Southern hybridization has limited power to recognize novel dioxygenase genes, we could not conclude from these experiments that the new strains only contain a single PAH dioxygenase.

Degenerate PCR primers were designed with the intent of amplifying approximately one-half of the *nahAc* gene, which encodes the naphthalene dioxygenase ISP. Degeneracies were introduced to allow binding to genes with high or low guanine-plus-cytosine contents. Using these primers and the PCR, we amplified and sequenced 630 bp (corresponding to 210 deduced amino acids) of a PAH dioxygenase large-subunit gene from NAG-2N-126 and NAG-2N-113. When deduced amino acid sequences were used, pairwise comparisons showed that the dioxygenases from NAG-2N-126 and NAG-2N-113 are 97.6% identical. The most closely related amino acid sequence, the *P. aeruginosa* PaK1 *pahAc* sequence (42), is 66% identical to the amino acid sequences of the dioxygenases from NAG-2N-126 and NAG-2N-113. The DNA guanine-plus-cytosine contents of the dioxygenase gene fragments from NAG-2N-126 and NAG-2N-113 were 42 and 40 mol%, respectively, indicating that these strains had not obtained these genes through a recent horizontal gene transfer event from bacteria with higher guanine-plus-cytosine contents.

A parsimony analysis produced the dendrogram shown in Fig. 2. This tree shows that the putative dioxygenases from the new strains, together with naphthalene dioxygenases from *Pseudomonas* strains, nitroaromatic dioxygenases from *Pseudomonas* and *Burkholderia* strains, and a putative naphthalene dioxygenase from a *Cycloclasticus* strain, form a monophyletic cluster that does not include the biphenyl and monoaromatic dioxygenases.

Genotypic analyses. Nearly complete (approximately 1,470 bp) 16S rDNA sequences of strains NAG-2N-126 and NAG-2N-113 were found to be identical. The NAG-2N-126 16S rDNA sequence was compared to sequences representing the RDP's *Oceanospirillum* assemblage and other members of the gamma-3 subgroup of the *Proteobacteria* (Table 2). Based on nucleotide identity alone, strain NAG-2N-126 is clearly affiliated with the oceanospirilla. The 16S rDNA sequence of NAG-2N-126 differs by 6.9% from the most closely related sequence, the sequence of *Oceanospirillum multiglobiferum*.

A maximum-likelihood phylogenetic analysis produced the tree shown in Fig. 3. To test the robustness of the dendrogram, the same data set was analyzed by neighbor-joining, maximum-likelihood, and parsimony methods, each of which was resampled 100 times (data not shown). The exact placement of strain NAG-2N-126 within the *Oceanospirillum* assemblage is not certain, since the bootstrap values for the node connecting it with the members of the genus *Oceanospirillum* and "*Oceanospirillum kriegii*" are low. However, the position of strain NAG-2N-126 shown in Fig. 3 appears to be correct since this position was favored over alternative positions in the majority of bootstrap resamplings with all three phylogenetic methods (data not shown).

The guanine-plus-cytosine content of the DNA of NAG-2N-126 was 46.3 ± 1 mol% (mean ± standard deviation; $n = 5$).

Colony and cell morphology. On solid PSS medium, NAG-2N-126 and NAG-2N-113 formed white, circular, convex colonies that had entire edges and were 3 mm in diameter. On solid marine medium 2216, both strains formed colonies similar to the colonies on PSS medium, except that the colonies were beige or light brown. When grown on solid marine me-

TABLE 2. 16S rDNA comparison of strain NAG-2N-126 and its closest relatives

Species	RDP abbreviation	GenBank accession no.	Source	No. of differences/% difference compared with strain NAG-2N-126
Oceanospirillum spp.				
O. linum	Osp.linum	M22365	ATCC 11336	117/8.1
O. multiglobiferum	Osp.multig	NA[c]	ATCC 33336	99/6.9
O. beijerinckii	Osp.beijer	NA	ATCC 12754	118/8.2
O. maris	Osp.maris	NA	ATCC 27649	136/9.4
O. japonicum[a]	Osp.japoni	NA	ATCC 19191	139/9.7
"*O. kriegii*"[b]	Osp.kriegi	NA	ATCC 27133	143/9.9
"*O. jannaschii*"[b]	Osp.jannas	NA	ATCC 27135	126/8.8
Halomonas elongata	Hlm.elong2	M93355	ATCC 33173	173/12.2
Marinomonas spp.				
M. vaga	Mrm.vaga	X67025	ATCC 27119	142/9.9
M. communis	Osp.commun	NA	ATCC 27118	131/9.1
Marinobacterium georgiense	NA	U58339	ATCC 700074	110/7.8
Other gamma-3 proteobacteria				
Vibrio splendidus	V.splendid	X74724	ATCC 33125	205/14.5
Vibrio fischeri	V.fischeri	X74702	ATCC 7747	202/14.2
Escherichia coli	E. coli	J01695	NA	231/15.9
Serratia marcescens	Ser.marces	M59160	ATCC 13880	228/15.8

[a] There is uncertainty concerning whether *O. japonicum* should be included in the genus *Oceanospirillum* (34).
[b] "*O. kriegii*" and "*O. jannaschii*" are not recognized members of the genus *Oceanospirillum* (23, 34).
[c] NA, not available.

dium 2216 containing naphthalene, both strains produced a diffusible brown pigment reminiscent of pigments produced by certain *Oceanospirillum* spp. when they are grown with aromatic amino acids (22, 23). Strain NAG-2N-113 produced a brilliant violet pigment when it was grown on solid marine medium 2216 supplemented with 3% glycerol.

NAG-2N-126 and NAG-2N-113 were examined by phase-contrast microscopy. Typical cells of each strain were straight rods, and very few cells were motile. The cells often clumped together, and India ink staining showed that a capsule was produced. Typical cells were 2 to 3 μm long and 0.7 to 0.9 μm in diameter. Electron microscopy of strain NAG-2N-126 showed that some cells had a single polar flagellum (Fig. 4).

Phenotypic characteristics. Strains NAG-2N-126 and NAG-2N-113 produced similar results in all tests. Both strains grew at pH values between 6.5 and 8.5 (optimum pH, 7.5) at salinities ranging from 1.75 to 7.0% (50 to 200% seawater). The temperature range for growth was 4 to 24°C. Temperatures below 4°C were not tested. Both strains were negative for DNase, amylase, gelatinase, and lipase activities; however, a phosphatase was produced. Nitrate was not reduced, nor was cysteine reduced to H_2S; however, selenite was reduced. The strains were gram negative, catalase and oxidase positive, and ampicillin resistant (sensitivity level, 78 μg/ml). Both strains produced poly-β-hydroxybutyrate granules. Neither strain was luminescent under the conditions tested.

The strains were tested for the ability to use a variety of aromatic and nonaromatic substrates as sole carbon and energy sources. NAG-2N-126 utilized naphthalene, 2-methylnaphthalene, D-fructose, glycerol, mannitol, D-arabitol, L-glutamate, L-proline, glycogen, DL-alanine, succinate, acetate, citrate, pyruvate, DL-lactate, DL-β-hydroxybutyrate, glutarate, *p*-hydroxybenzoate, L-serine, and D-glucuronate. Strain NAG-2N-113 used naphthalene, 2-methylnaphthalene, phenanthrene, D-glucose, D-fructose, glycerol, mannitol, D-arabitol, L-glutamate, L-proline, DL-alanine, succinate, acetate, citrate, pyruvate, L-serine, and DL-lactate. The following compounds were not used as sole carbon sources by either strain: 2,6-dimethylnaphthalene, 1-methylnaphthalene, biphenyl, acenaphthene, fluorene, L-arabinose, D-ribose, D-xylose, L-rhamnose, D-galactose, D-mannose, D-trehalose, D-melibiose, D-sucrose, D-lactose, D-maltose, D-raffinose, dextran sulfate, sorbitol, adonitol, methyl-D-glucopyranoside, ethanol, i-propanol, glycine, L-leucine, L-ornithine, L-arginine, tyrosine, gluconate, fumarate, D-galacturonate, DL-malate, malonate, propionate, DL-asparagine, L-hyroxyproline, *N*-acetylglucosamine, butyrate, valerate, adenine, D-glucosamine, caproate, tartrate, erythritol, quinate, and methanol. Strain NAG-2N-113 was not tested with D-gluconate or *p*-hydroxybenzoate.

Both strains fermented sucrose, D-glucose, D-mannose, L-arabinose, D-fructose, D-galactose, and mannitol. Only NAG-2N-126 fermented D-lactose and D-xylose. Neither strain fermented L-fucose, sorbitol, or dulcitol.

DISCUSSION

Strains NAG-2N-126 and NAG-2N-113 were isolated from coal tar creosote-contaminated marine sediment from Eagle Harbor, Washington. Since the sediment was diluted 20,000-fold prior to plating, the bacteria were probably present at concentrations greater than 2×10^4 cells/ml of sediment. It is interesting that even though these bacteria were relatively numerous, they were not isolated from high-dilution PAH-MPN tubes that were inoculated with sediment from the same box-core (11). Instead, *Cycloclasticus*, *Vibrio*, and *Pseudalteromonas* strains were isolated by the PAH-MPN protocol. This result reinforces a paradigm of environmental microbiology, that different isolation strategies can result in the isolation of different bacteria with the same physiological characteristic of interest. Thus, an integrated approach to bacterial isolation is recommended. Although the two strains were isolated from a dilute sediment sample, sequences related to the NAG-2N-126 or NAG-2N-113 sequence were not recovered from a 16S rDNA clonal library which was prepared from Eagle Harbor sediment (17). This result is not surprising since the direct microbial counts in sediment from the site exceeded the apparent concentration of strain NAG-2N-126 or NAG-2N-113 by several orders of magnitude (11).

PAH catabolism studies showed that strains NAG-2N-126 and NAG-2N-113 each degraded naphthalene and 2-methyl-

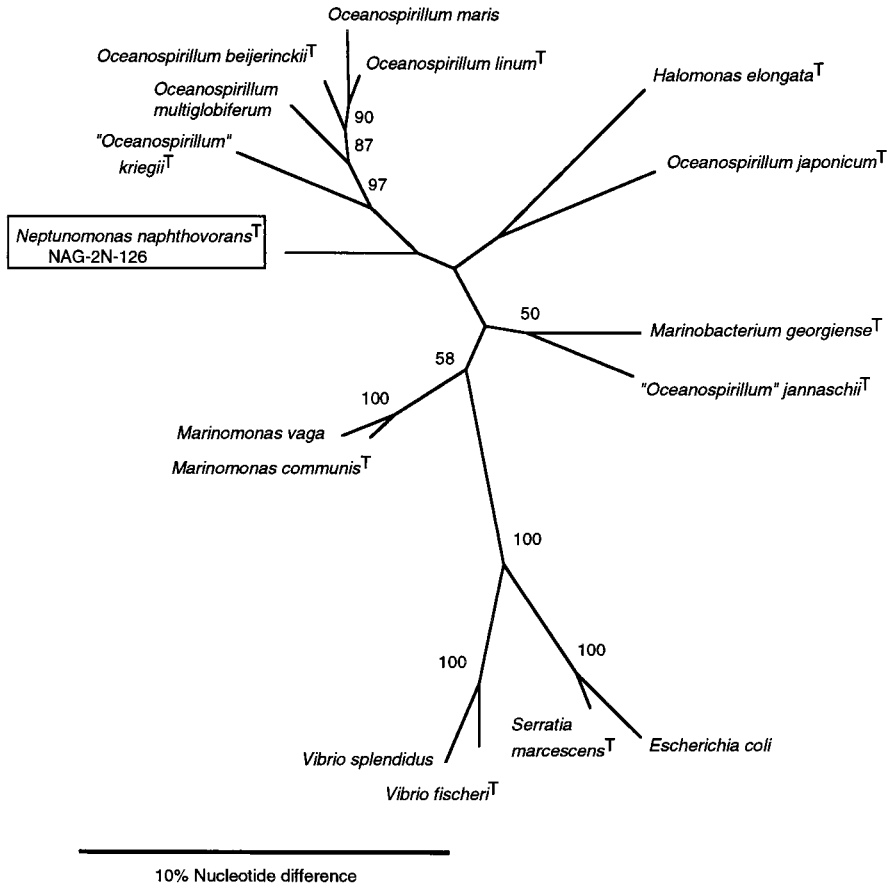

FIG. 3. 16S rDNA phylogenetic analysis of *N. naphthovorans* NAG-2N-126 and with other members of the gamma-3 subgroup of the *Proteobacteria*. The numbers at the branch nodes are bootstrap values based on 100 resamplings for maximum likelihood. Only bootstrap values greater than 50% are shown. Scale bar = approximately 10% nucleotide divergence. T = type strain.

naphthalene with concomitant growth. In addition, each strain significantly transformed 1-methylnaphthalene, although neither strain used this compound as a sole carbon and energy source. A possible explanation for this is that the initial PAH degradation enzymes of the strains transform 1-methylnaphthalene, causing removal of the PAH; however, one or more enzymes downstream in the catabolic pathway fails to recognize the transformed 1-methylnaphthalene, resulting in dead end products. This could cause unfavorable kinetics for complete 1-methylnaphthalene removal.

Interestingly, there were differences in the PAH degradation profiles of strains NAG-2N-126 and NAG-2N-113, despite the high level of similarity of the putative naphthalene dioxygenase large subunits (level of amino acid identity, 97.6%). Strain NAG-2N-126 could not degrade phenanthrene, but strain NAG-2N-113 not only transformed phenanthrene but grew with phenanthrene as a sole carbon and energy source. In addition, strain NAG-2N-113 transformed 2,6-dimethylnaphthalene. This observation may be similar to the scenario described by Erickson and Mondello (6) for *B. cepacia* LB400 and "*Pseudomonas pseudoalcaligenes*" KF707. Very few amino acid differences in the biphenyl dioxygenase large subunits of these strains (level of amino acid identity, 95.6%) resulted in dramatically different abilities to degrade polychlorinated biphenyl. By changing the amino acids by site-directed mutagenesis and documenting changes in polychlorinated biphenyl degradation patterns, Erikson and Mondello showed that the catabolic differences were due to a few key amino acids. Alternatively, strain NAG-2N-113 may contain more than one dioxygenase, which results in the degradation of a wider range of aromatic substrates. Elucidation of the basis for the difference in phenanthrene and 2,6-dimethylnaphthalene catabolism by strains NAG-2N-126 and NAG-2N-113 will require a functional analysis of the dioxygenases of these organisms.

Although there were differences in the ability to degrade single PAHs, NAG-2N-126 and NAG-2N-113 produced similar results when they were incubated with a mixture of eight PAHs. Interestingly, both strains degraded acenaphthene only when they were incubated with other PAHs, even though the cells used in the single-PAH experiments were induced with naphthalene. Perhaps naphthalene or other PAHs are required to keep the PAH degradation enzymes induced. The PAH mixture experiments were important since PAHs are typically found as complex mixtures in the environment, such as creosote-contaminated sites, which contain 150 to 200 distinct aromatic compounds (30).

A phylogenetic analysis based on deduced amino acids encoded by putative PAH dioxygenase ISP genes of strains NAG-2N-126 and NAG-2N-113 indicated that the PAH dioxygenases are related to naphthalene dioxygenases (Fig. 2). This result is not surprising since these bacteria were isolated by using naphthalene and since PAH catabolism experiments indicated that they degrade naphthalenes but not a broad range of PAHs. Other dioxygenases in the naphthalene dioxygenase phylogenetic cluster include prototypical *Pseudomonas* naphthalene dioxygenases (38, 42), *Burkholderia* nitroaromatic di-

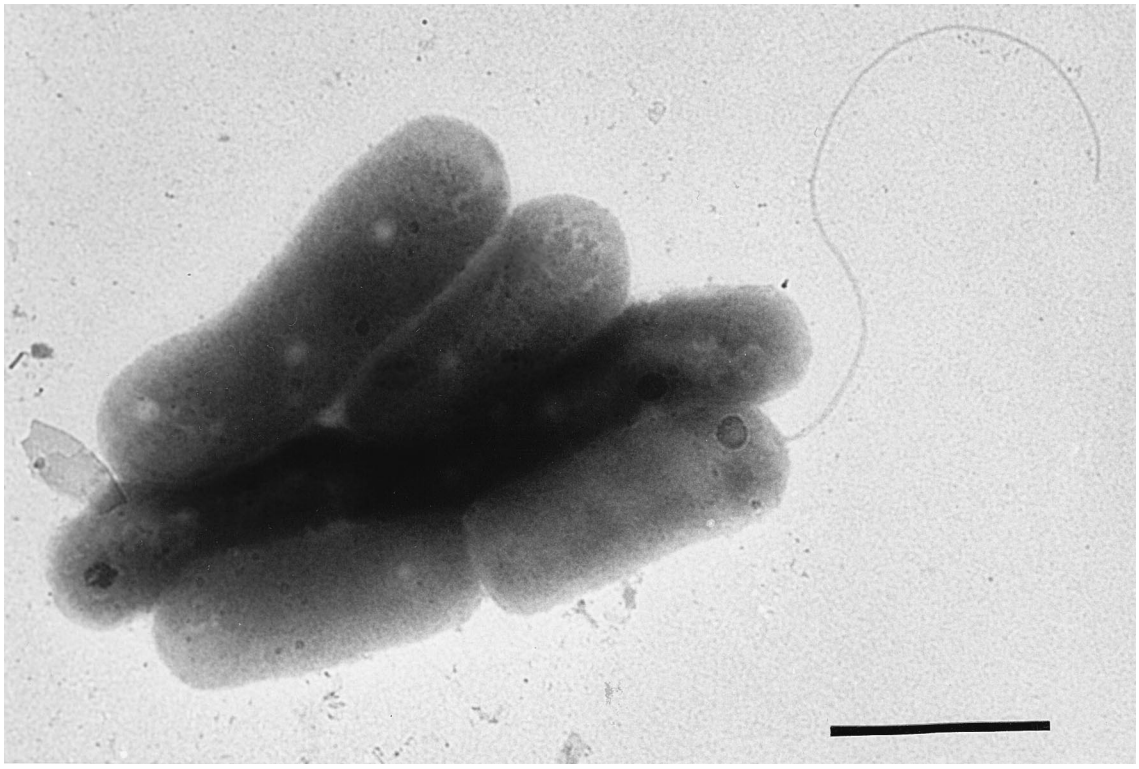

FIG. 4. Electron micrograph of negatively stained *N. napthovorans* NAG-2N-126. Bar = 2 μm.

oxygenases (33, 39), and a putative dioxygenase from *Cycloclasticus* spp. (12). All of the dioxygenases in this group that have been characterized transform naphthalene, and some also degrade phenanthrene (37). Strains NAG-2N-126 and NAG-2N-113 also produce indigo from indole, another characteristic of the naphthalene dioxygenase family. Since the cloned dioxygenases of strains NAG-2N-126 and NAG-2N-113 are most similar to naphthalene dioxygenases and the aromatic substrates of the new strains are similar to the aromatic substrates of naphthalene dioxygenases, this suggests that the cloned genes encode functional dioxygenases; however, further study will be required to show this.

The isolation of naphthalene degradation genes from the new marine strains suggests that their naphthalene degradation system is similar to the prototypical *Pseudomonas nah* pathway. Members of the genus *Cycloclasticus*, another marine PAH-degrading genus, have also been shown to contain genes homologous to known PAH degradation genes of terrestrial bacteria. The *Cycloclasticus xylC1* and *xylC2* genes, which were first isolated from an Alaskan strain, "*Cycloclasticus oligotrophus*" RB1 (43), have recently been shown to be present in *Cycloclasticus* strains from Puget Sound and the Gulf of Mexico (12). The *xylC1* and *xylC2* genes encode enzymes that are related to the biphenyl dioxygenase large and small subunits, respectively. A second dioxygenase ISP gene related to the naphthalene dioxygenases has also recently been isolated from *Cycloclasticus* strains (12). Thus, at least some marine bacteria have PAH degradation systems that involve components homologous to components of the prototypical PAH degradation pathways.

However, while there are recognizable similarities between putative PAH degradation genes of obligately marine bacteria and known PAH-degrading dioxygenase ISP genes, the levels of homology are relatively low. For instance, the *xylC1* and *xylC2* genes of "*C. oligotrophus*" RB1 are 65.7 and 63.3% identical to "*P. pseudoalcaligenes*" KF707 *bphA1* and *bphA2*. However, the corresponding ferredoxin and ferredoxin reductase genes, which are presumably required for dioxygenase activity, are not located near the *xylC* genes, as they are in most known PAH catabolic systems (43). Furthermore, naphthalene type dioxygenase ISP genes retrieved from Puget Sound and the Gulf of Mexico *Cycloclasticus* strains exhibit only 45.3% amino acid identity with their closest relative, *P. aeruginosa* PaK1 *pahAc* (12). Thus, the study of obligately marine bacteria has greatly expanded the known diversity of ISP sequences. Further study of the PAH catabolic systems of the obligately marine bacteria may reveal significant differences in gene organization and regulation, if not differences in the biochemical pathways for PAH catabolism, compared to the prototypical PAH degradation systems.

Phylogenetic analyses in which the nearly complete 16S rDNA sequence of strain NAG-2N-126 was used clearly indicated that this strain is related to members of the genus *Oceanospirillum* in the gamma-3 subgroup of the *Proteobacteria*. However, the 16S rDNA sequence of the new strain was not closely related to any known sequence (levels of identity, ≤93.1%). Accordingly, strain NAG-2N-126 was not monophyletic with the members of the genus *Oceanospirillum*, including *O. linum*, *O. maris*, *O. beijerinckii*, and *O. multiglobiferum*, and on a purely phylogenetic basis it is not clear that the new strains should be included in the genus *Oceanospirillum*. The taxonomy of the bacteria in the *Oceanospirillum* genetic cluster is complicated. Although both "*O. kriegii*" and "*O. jannaschii*" were originally placed in the genus *Oceanospirillum*, they are not recognized members of the genus (23, 34). In addition, it has been suggested that *O. japonicum* should be excluded from the genus *Oceanospirillum* based on low levels of similarity to the other *Oceanospirillum* spp. as determined by rRNA hybrid-

TABLE 3. Phenotypic comparison of *Neptunomonas* strains and related organisms

Characteristic	*Neptunomonas*	*Oceanospirillum*[a]	*O. japonicum*	"*O. kriegii*"	*Marinobacterium*
Cell shape	Straight rods	Spirilla	Spirilla	Straight and curved rods	Straight rods
Motility	+[b]	+	+	+	+
Flagellar arrangement	Single polar	Bipolar tufts	Bipolar tufts	Single polar	Single polar
Cell diam (μm)	0.7–0.9	0.3–1.2	0.8–1.4	0.8–1.2	0.5–0.7
PHB accumulation[c]	+	+	+	+	−
Na^+ requirement	+	+	+	+	+
NO_3 reduced to NO_2	−	D	−	−	−
Oxidase activity	+	+	+	+	+
Catalase activity	+	D	W or −	NR	+
Enzyme activities					
Amylase	−	−	−	NR	−
Gelatinase	−	D	D	NR	−
Lipase	−	NR	NR	+	+
Phosphatase	+	D	W	NR	NR
Acid produced from carbohydrates	+	−	−	−	−
Carbohydrates fermented	+	−	−	−	−
Temp (°C)	4–30	2–41	10–43	20–35	4–41
Water-soluble brown pigment	+	D	−	NR	NR
DNA G+C content (mol%)	46	45–50	45	NR	54.9

[a] The data include data for the members of the genus *Oceanospirillum* described by Krieg (23), except that data for *O. japonicum* are given separately because there is uncertainty concerning whether this organism should be included in the genus (34).
[b] +, positive; −, negative; D, results depend on the species or strain used; W, weak; NR, not reported.
[c] PHB, poly-β-hydroxybutyrate.

ization and multilocus enzyme electrophoresis experiments (34). Our phylogenetic placement of *O. japonicum* further suggests that it should be excluded from the genus *Oceanospirillum*.

Consistent with their phylogenetic placement, strains NAG-2N-126 and NAG-2N-113 share many phenotypic properties with *Oceanospirillum* spp. (Table 3). However, there are some major phenotypic differences between strains NAG-2N-126 and NAG-2N-113 and *Oceanospirillum* spp., suggesting that the new strains do not belong to the genus *Oceanospirillum*. Morphologically, strains NAG-2N-126 and NAG-2N-113 are rod shaped and have only a single polar flagellum; *Oceanospirillum* spp. are helical and have bipolar tufts of flagella (22, 23). Physiologically, the Puget Sound strains utilize carbohydrates, both in the presence and in the absence of oxygen, and produce acid in both cases. *Oceanospirillum* spp. fail to utilize carbohydrates and are obligately aerobic. In addition, strains NAG-2N-126 and NAG-2N-113 generally have a broader nutritional profile than the oceanospirilla have.

Other genera that are phylogenetically similar to the new strains are the genera *Balneatrix* (4) and *Marinobacterium* (16). Although we were not able to obtain the 16S rDNA sequence of *Balneatrix alpica*, this bacterium is obviously not highly related to strains NAG-2N-126 and NAG-2N-113 by virtue of its ability to reduce nitrate, its high DNA G+C content, and its inability to grow in media containing more than 1% NaCl. The genus *Marinobacterium* is obligately aerobic, has a high DNA G+C content, and does not produce poly-β-hydroxybutyrate. Other phenotypic differences between the new strains and their relatives are summarized in Table 3. The phylogenetic and phenotypic differences between strains NAG-2N-126 and NAG-2N-113 and their closest relatives justify the creation of a novel genus and species, *Neptunomonas naphthovorans*.

Description of *Neptunomonas* gen. nov. *Neptunomonas* (Nep-.tu.no.mo′nas. Rom. myth. Neptune, the Roman god of the sea; Gr.n. *monas*, unit; M.L. n. *Neptunomonas*, Neptune's bacterium.) Gram-negative rod-shaped bacteria that belong to the gamma-3 subgroup of the *Proteobacteria* based on phylogenetic analyses of 16S rRNA genes. Cells of the type species are approximately 0.7 to 0.9 by 2.0 to 3.0 μm and are motile by means of a single polar flagellum. Facultatively aerobic. Oxidase and catalase positive. May utilize amino acids, carbohydrates, organic acids, sugar alcohols, and some PAHs as sole carbon and energy sources. Cells require sodium ions for growth.

The DNA G+C content is 46 mol%.

The type and only species of the genus is *Neptunomonas naphthovorans*.

Description of *Neptunomonas naphthovorans* sp. nov. *Neptunomonas naphthovorans* (naph.tho.vo′rans. Chem. n. naphthalene, a white crystalline hydrocarbon [$C_{10}H_8$]; L. masc. *vorus*, devouring; L. part. *naphthovorans*, naphthalene devouring). Rod-shaped bacteria that are motile by means of a single polar flagellum. Growth occurs in defined media containing ammonium salts as a nitrogen source. Facultatively aerobic. Marine; requires at least 50% seawater salinity for growth. Catalase and oxidase positive. Uses some amino acids, carbohydrates, organic acids, and sugar alcohols, as well as certain PAHs, for growth. Nitrate is not reduced. Colonies are small, convex, and entire and produce a brown diffusible pigment when the organism is grown on rich media supplemented with naphthalene. The temperature range is ≤4 to 24°C. The DNA G+C content is 46 mol%.

The type strain, *N. naphthovorans* NAG-2N-126, was isolated from Eagle Harbor, a creosote-contaminated EPA superfund site in Puget Sound, Washington.

N. naphthovorans NAG-2N-126 and NAG-2N-113 have been deposited in the American Type Culture Collection as strains ATCC 700637 and ATCC 700638, respectively.

ACKNOWLEDGMENTS

This research was supported by grants N00014-91-J-1792 and N00014-91-J-1578 from the Office of Naval Research University Research Initiative in Bioremediation to the University of Washington under the auspices of the Marine Bioremediation Program. Additional support was provided by National Institutes of Health Biotechnology Fellowship GM-0837-05 to B.P.H. and by Sigma Xi Grants-in-Aid of Research to B.P.H. We also thank the National Oceanic and Atmospheric Administration's Sanctuaries and Reserves Division for continuing support of B.P.H.

We thank members of the University of Washington's Marine Bioremediation Program for useful discussions concerning this work. We

also thank Jeanne Poindexter for hands-on help with the electron microscopy. In addition, we thank Rob Sanford and Joanne Chee-Sanford for help with gas chromatography. We thank John Gosink for plentiful advice concerning phylogenetic analyses. Finally, we thank Matthew Stoecker for critically evaluating the manuscript.

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