

Vibrio cyclotrophicus sp. nov., a polycyclic aromatic hydrocarbon (PAH)-degrading marine bacterium

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Strain P-2P44^T was isolated from creosote-contaminated marine sediments by using a most-probable number procedure in which phenanthrene was the sole carbon and energy source. Growth experiments showed that P-2P44^T utilized several two- and three-ring polycyclic aromatic hydrocarbons (PAHs) as substrates, including naphthalene, 2-methylnaphthalene and phenanthrene. Additionally, gas-chromatography experiments showed that P-2P44^T degraded several other PAHs, though it was unable to use them as sole sources of carbon and energy. Phylogenetic analyses confirmed that strain P-2P44^T is a member of the genus *Vibrio*, most closely related to *Vibrio splendidus*. However, strain P-2P44^T shared only 98.3% 16S rDNA identity and 35% DNA–DNA reassociation with the type strain of *V. splendidus*. Strain P-2P44^T differed phenotypically from *V. splendidus*. Together, these differences indicated that strain P-2P44^T represents a novel species in the genus *Vibrio*, for which the name *Vibrio cyclotrophicus* sp. nov. is proposed; the type strain is P-2P44^T (= ATCC 700982^T = PICC 106644^T).

Keywords: *Vibrio*, polycyclic aromatic hydrocarbons, marine

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are compounds that consist of two or more fused aromatic rings. PAH contamination of the environment is of concern because some PAHs are toxic, carcinogenic or teratogenic. Nevertheless, PAHs are a rich source of carbon and energy and are exploited by some bacteria as growth substrates.

To extend our knowledge of marine PAH-degrading bacteria, several phenanthrene-, naphthalene- and biphenyl-degrading strains were isolated from polluted sediments in Eagle Harbor, a creosote-contaminated Environmental Protection Agency (EPA) Superfund site in Puget Sound, Washington, USA (Geiselbrecht *et al.*, 1996). One strain, P-2P44^T, was shown to belong to the genus *Vibrio* on the basis of whole-cell fatty acid analyses and 16S rDNA phylogenetic analyses (Geiselbrecht *et al.*, 1996). In the present study, we provide a phenotypic, as well as a further phylogenetic,

description of P-2P44^T, and we propose a new species, *Vibrio cyclotrophicus* to include strain P-2P44^T.

METHODS

Strains and media. *Vibrio splendidus* (biovar 1) ATCC 33125^T and *Oceanospirillum linum* ATCC 11336^T were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained on the complex marine medium 2216 (Difco). *Neptunomonas naphthovorans* ATCC 700637^T was revived from our own frozen stocks. *Pseudomonas putida* G7 was a gift from G. Saylor and was maintained on Luria broth. Strain P-2P44^T was maintained on the defined medium ONR7a (Dyksterhouse *et al.*, 1995), with added phenanthrene as the sole source of carbon and energy, or on marine medium 2216 (Difco).

PAH growth-substrate experiments. We tested the ability of P-2P44^T to grow using several PAHs as sole sources of carbon and energy. PAHs were added to 4 ml ONR7a in 20 ml test tubes at levels above saturation. The inoculum consisted of 50 µl exponential-phase cells grown on naphthalene. All growth assays were performed in triplicate. Growth was scored after 1 week by measuring an increase in turbidity at 600 nm.

PAH-degradation experiments. PAH-degradation experiments entailed incubating dilute phenanthrene-grown

Abbreviations: PAHs, polycyclic aromatic hydrocarbons; RDP, Ribosomal Database Project

cultures in Balch tubes with ONR7a and individual PAHs. No other carbon or energy sources were present in these experiments. The disappearance of a PAH compound was determined by GC/flame-ionization detection after 7 d, as described previously (Hedlund *et al.*, 1999).

Phylogenetic analysis. The sequence of the 16S rRNA gene of strain P-2P44^T (GenBank accession no. U57919) was determined previously (Geiselbrecht *et al.*, 1996). The SEQUENCE MATCH program from the 'On-line analyses' section of the Ribosomal Database Project (RDP; Maidak *et al.*, 1999) web site was used to determine which sequences in the RDP database were most closely related to that from P-2P44^T. The 12 most closely related sequences were downloaded from the 'Sequence Alignments' archive in the 'Download' section of the RDP web site. All sequences used were as complete (or nearly as complete) as that from P-2P44^T (*Escherichia coli* nucleotides 28–1490). Small adjustments in the alignment were made using the SEQAPP program (Gilbert, 1992). A distance matrix was created using PAUP software (Swofford, 1991). A heuristic bootstrap analysis in PAUP (Swofford, 1991) (random input order; 10 sub-replicates) was used to find the most parsimonious tree. The most parsimonious tree was analysed using the MACCLADE State Changes and Stasis command to determine the transition-to-transversion ratio, which was 1.08 (Maddison & Maddison, 1992). That ratio was specified in distance analyses. Bootstrap replications were produced using SEQBOOT; and that data set was analysed using DNADIST (jumbled input; 10 sub-replicates) and NEIGHBOR (Kimura two-parameter correction), both of which were obtained through the PHYLIP package (Felsenstein, 1989).

Determination of the G + C content of the DNA. The G + C content of the genomic DNA was determined using the thermal denaturation method (Gerhardt *et al.*, 1994). *E. coli* DH5 α and *O. linum* ATCC 11336^T served as references.

DNA–DNA hybridization. The levels of DNA–DNA hybridization between strain P-2P44^T and *V. splendidus* ATCC 33125^T were determined using the thermal reannealing method (Zakrzewska-Cerwinska *et al.*, 1988). DNA (60 ng ml⁻¹) in 5 \times SSC and 20% DMSO was sheared by sonication in a model 4710 sonicator (Cole–Parmer) for 3 min at a setting of 5. Hybridization was determined using a Response II spectrophotometer (Gilford) by heating the samples to 97 °C for 10 min and reducing the temperature to 65 °C; this temperature was held for 6 h. The procedure was performed in quadruplicate.

Microscopy. Strain P-2P44^T was grown in Marine Broth 2216 until it reached late-exponential phase. Cells were concentrated by centrifugation and resuspension in a 1/10 volume of half-strength ONR7a, pipetted onto Formvar-coated 200-mesh copper grids, and allowed to settle for 10 min. Liquid was blotted from the preparation. The cells were stained with 1.0% phosphotungstic acid (w/v) and viewed with a JEOL transmission electron microscope at 60 kV.

Phenotypic testing. The pHs and salinities that allowed growth were examined in ONR7a broth with 0.1% Bacto peptone (Difco). For pH determinations, media were prepared with alternative buffers at 25 mM concentrations near their pK_a values, as described (Dyksterhouse *et al.*, 1995). Salinities were adjusted by varying the inorganic salt concentrations [except NH₄Cl, Na₂HPO₄, FeCl₂ and TAPSO (3[*N*-tris(hydroxymethyl)methylamino]-2-hydroxypropanesulfonic acid; Sigma)]. The salinities tested were 0.35,

1.05, 1.75, 3.5, 7.0 and 10.5% (w/v). Cultures were incubated at 24 °C with shaking and observed daily for 5 d. For pH and salinity testing, growth was defined as being from 50% of that which occurred in media with 3% NaCl (w/v) at pH 7.0. The temperature range for growth was determined on solid 2216 medium that had been preincubated at the appropriate temperature for 2 h prior to inoculation. The temperatures used included the following: 4, 15, 24, 30, 37 and 42 °C.

Routine phenotypic tests, including catalase, oxidase, reduction of possible electron acceptors, and production of extracellular enzymes, were conducted as described and included *V. splendidus* ATCC 33125^T, *E. coli* DH5 α or *P. putida* G7 controls (Endsley *et al.*, 1983). Nitrate-reduction assays were attempted with 0.1% and 0.01% NaNO₃. Tween 20 and Tween 80 were used for the lipase test. Cystine–lactose–electrolyte-deficient agar was used to determine whether NaCl was required for growth.

For carbon-source tests, late-exponential-phase cells grown on 0.1% glucose and 0.01% peptone were diluted into ONR7a containing 0.01% peptone and then dispensed into microtitre wells. Peptone was used because *V. splendidus* ATCC 33125^T requires an unknown growth factor. Carbon sources dissolved in water were added to a final concentration of 0.1%. Because of solubility problems, cellobiose was added at 0.025% and *myo*-inositol at 0.05%. Growth was monitored by measuring an increase in turbidity at 600 nm, using Automated Microplate Reader EL311sx (BIO-TEK) and DELTA SOFT II software (BioMetallics) after 2, 4 and 7 d incubations at room temperature. Growth was scored as positive if the turbidity reached 150% of that of the mean of the negative control wells at any time during the assay. Each test was carried out in triplicate.

Antibiotic-resistance determination. Resistance to antibiotics was determined in microtitre wells containing 2216 medium and serially diluted antibiotics. Antibiotic MICs were defined as the concentrations at which the antibiotics reduced growth to less than half the level of growth without any antibiotics. Turbidities were determined after 2 d with the microplate reader. The antibiotics tested were polymyxin B, novobiocin, ampicillin and streptomycin.

RESULTS

Microscopy

V. cyclotrophicus P-2P44^T cells were examined by phase-contrast (Fig. 1a) and transmission electron microscopy (Fig. 1b). Typical cells were rod-shaped, with less than 10% of cells being curved. A high percentage of cells were motile during exponential growth, a few cells being motile during stationary phase. Cells possessed either one or two polar or subpolar flagella. Exponential-phase cells measured 0.6 μ m \times 1.5–2.5 μ m, with some cells elongating to 5 μ m during stationary phase. Some cells formed involution bodies during stationary phase.

Colony and cell morphology

When grown on solid ONR7a medium containing 0.8% agarose and phenanthrene, supplied in the vapour phase, P-2P44^T formed small, brown, circular

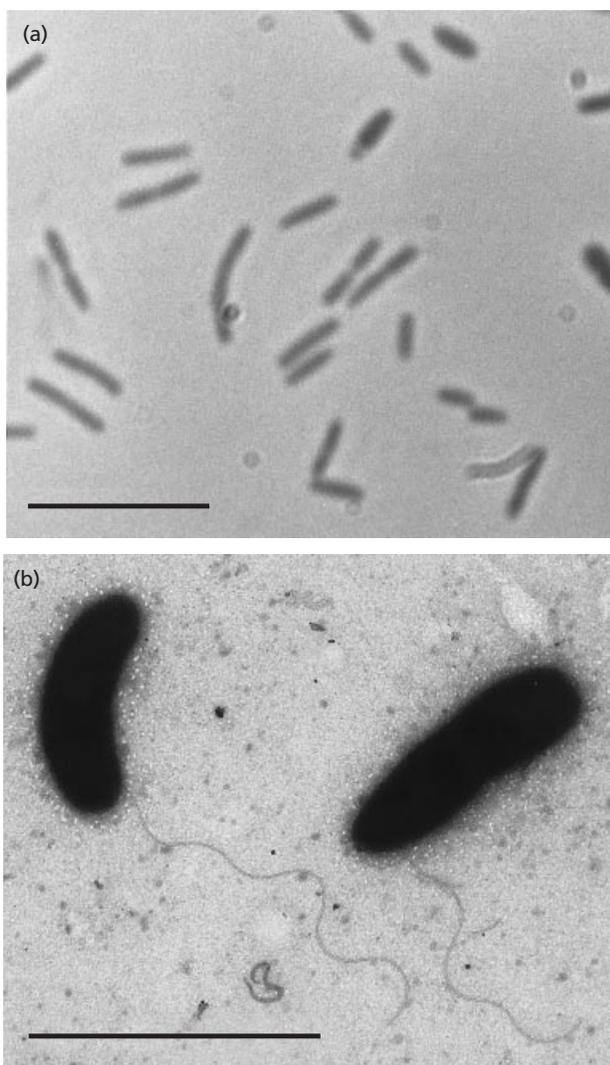


Fig. 1. (a) Phase-contrast micrograph showing strain P-2P44^T; bar, 10 μm. (b) Transmission electron micrograph of strain P-2P44^T; bar, 2 μm.

pulvinate colonies with entire edges. On the solid, rich, medium marine agar 2216, P-2P44^T formed cream-coloured, circular, flat colonies with entire edges that reached 4 mm in diameter.

PAH-degradation experiments

Naphthalene, 2-methylnaphthalene and phenanthrene supported growth of strain P-2P44^T. The following PAH compounds failed to serve as sole carbon sources for P-2P44^T: 1-methylnaphthalene, 2,6-dimethylnaphthalene, biphenyl, acenaphthene, fluorene, anthracene and fluoranthene. Although unable to grow with the aforementioned PAH compounds, P-2P44^T did produce bright yellow intermediates characteristic of partial degradation of biphenyl and fluorene and a dark orange intermediate from acenaphthene. Since *V. splendidus* is the closest known relative of strain P-

Table 1. Results of PAH-degradation experiments

PAH	Initial concn (p.p.m.)	Recovery (%)*
Naphthalene	5	0 ± 0†
1-Methylnaphthalene	5	55 ± 4
2-Methylnaphthalene	5	4 ± 2†
2,6-Dimethylnaphthalene	0.5	0 ± 0
Biphenyl	5	78 ± 2
Acenaphthene	1	104 ± 3
Phenanthrene	1	4 ± 3†
Fluorene	1	98 ± 4

* Each value is given as the mean percentage of the parent PAH remaining after 7 d ± SD (n = 3). Control tubes containing no bacteria gave 80–100% recovery for all PAHs.

† These PAHs were used as sole carbon and energy sources.

2P44^T, we tested whether *V. splendidus* also could use PAHs as sole carbon sources under the conditions described above. However, no growth or coloured intermediate production was observed.

To examine PAH degradation by *V. cyclotrophicus* more rigorously, we quantified the PAH-degradation phenotype of strain P-2P44^T by using GC. As shown in Table 1, strain P-2P44^T significantly degraded naphthalene, 1-methylnaphthalene, 2-methylnaphthalene, 2,6-dimethylnaphthalene and phenanthrene. Although the strain produced coloured intermediates from biphenyl, fluorene and acenaphthene in the growth experiment, no significant degradation was shown in the GC analyses.

Many bacteria that catabolize naphthalene produce indigo when naphthalene-induced cells are given indole in the vapour phase (Endsley *et al.*, 1983). P-2P44^T was grown on solid 2216 medium with naphthalene; indole crystals were added to the lid of the Petri dish when the cells were in mid-exponential-phase growth. Although colonies of the control strains, *N. naphthovorans* NAG-2N-126^T and *P. putida* G7 turned blue or violet, strain P-2P44^T did not produce indigo or related coloured isomers.

Phenotypic tests

P-2P44^T was catalase- and oxidase-positive. Nitrate was not reduced to nitrite. Tests were negative for indole production, Voges–Proskauer, H₂S production, β-galactosidase, urease, alginate, chitinase, sulfatase and L-ornithine and L-lysine decarboxylase. Activities of the following enzymes were detected: protease (casein hydrolysis), gelatinase, lecithinase, phosphatase and lipase (Tween 20, Tween 80). Weak arginine dihydrolase activity was present. Growth on solid 2216 medium occurred between 4 °C and 37 °C; no growth occurred at 42 °C. Growth in ONR7a medium with 0.1% peptone occurred at NaCl concentrations be-

Table 2. Comparison of phenotypic differences between *Vibrio splendidus* ATCC 33125^T and strain P-2P44^T

Both strains were positive for gelatinase, lipase, glucose, xylose*, D-galactose, cellobiose, D-glucuronate, acetate, DL-lactate, pyruvate, D-alanine, L-serine, L-glutamate, L-arginine, L-citrulline, L-proline and the presence of a single polar flagellum. Both strains were negative for L-arabinose, propionate, butyrate, sorbitol, *myo*-inositol, ethanol, *p*-hydroxybenzoate, aminobutyrate and gas production from glucose. +, Positive; -, negative; v, variable.

Characteristic	ATCC 33125 ^T	Strain P-2P44 ^T
Luminescence	+	-
Alginate activity	v	-
Curved rods	100 %	< 10 %
Temperature range (°C)	4-35	4-37
Utilization of:		
Sucrose	+	-
Maltose	+	v
Glycine	-*	+
Requires growth factor†	+	-

* These results are different from those reported by Reichelt *et al.* (1976).

† A cofactor or vitamin other than biotin, thiamin, nicotinic acid or pantothenic acid is required.

tween 1.75 and 10 %, with maximum growth occurring with 7 % NaCl. NaCl concentrations above 10 % were not tested. The pH range for growth was 6.5-9.5. No luminescence was detected. Strain P-2P44^T was sensitive to 10 µg ampicillin ml⁻¹, but resistant to novobiocin (MIC = 80 µg ml⁻¹), polymyxin B (MIC = 80 U ml⁻¹) and streptomycin (MIC = 40 µg ml⁻¹).

Strain P-2P44^T fermented cellobiose, D-mannitol, sucrose, trehalose, and D-glucose; however, gas pro-

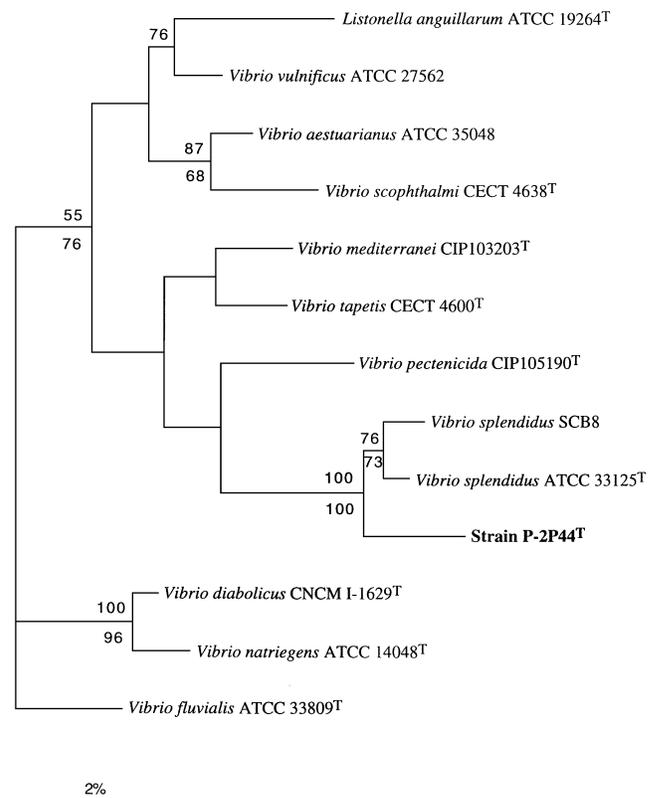


Fig. 2. Neighbour-joining tree showing strain P-2P44^T and related *Vibrio* strains. Numbers above and below tree nodes represent the percentage bootstrap support for 100 resamplings using neighbour-joining and parsimony, respectively. Values below 50 % are not shown. Bar, approximately 2 % nucleotide divergence.

duction was not observed. L-Arabinose, *myo*-inositol, D-mannose, salicin and sorbitol were not fermented. Data for the carbon-source tests are summarized in Table 2.

Table 3. 16S rDNA sequence comparison between strain P-2P44^T and its closest relatives

Species	RDP short ID	Strain	GenBank no.	No. differences/% difference from strain P-2P44 ^T
<i>Vibrio splendidus</i>	V.splendid	ATCC 33125 ^T	X74724	26/1.8
<i>Vibrio splendidus</i>	V.splendi2	SCB8	Z31657	32/2.2
<i>Vibrio aestuarianus</i>	V.aestuari	ATCC 35048	X74689	50/3.6
<i>Vibrio tapetis</i>	V. tapetis	CECT 4600 ^T	Y08430	55/3.8
<i>Vibrio vulnificus</i>	V.vulnifi3	ATCC 27562	X74727	59/4.1
<i>Vibrio natriegens</i>	V.natrieg4	ATCC 14048 ^T	X74714	59/4.4
<i>Vibrio mediterranei</i>	V.mediterr	CIP 103203 ^T	X74710	66/4.7
<i>Listonella anguillarum</i>	Lsn.angu22	ATCC 19264 ^T	X16895	67/4.7
<i>Vibrio diabolicus</i>	V.diabolic	CNCM I-1629 ^T	X99762	66/4.6
<i>Vibrio scophthalmi</i>	V.scophtha	CECT 4638 ^T	U46579	69/4.8
<i>Vibrio pectenica</i>	V.pecten	CIP 105190 ^T	Y13830	68/4.8
<i>Vibrio fluvialis</i>	V.fluvial3	ATCC 33809 ^T	X76335	75/5.2

Determination of the G + C content of the DNA

The G + C content of the DNA for P-2P44^T was 38.8 ± 0 mol % (mean ± SD; n = 5).

DNA–DNA hybridization

The DNA–DNA reassociation between DNA from P-2P44^T and *V. splendidus* was 35 ± 6% (mean ± SD; n = 4).

Phylogeny

Table 3 and Fig. 2 show that strain P-2P44^T is a member of the genus *Vibrio*. The closest relative of strain P-2P44^T is *V. splendidus* (biovar 1).

DISCUSSION

In recent years, several genera of marine bacteria have been reported to catabolize PAHs for carbon and energy: these include *Marinobacter* (Gauthier *et al.*, 1992), *Cycloclasticus* (Dyksterhouse *et al.*, 1995), *Pseudoalteromonas* (Hedlund *et al.*, 1996) and *Neptunomonas* (Hedlund *et al.*, 1999). In addition to these genera (which require sodium ions), several other genera capable of PAH degradation, including *Sphingomonas*, *Pseudomonas*, *Burkholderia* and *Mycobacterium*, have been isolated from marine or estuarine sites (Berardesco *et al.*, 1998). Thus, a diverse group of bacteria may participate in the degradation of PAH molecules in marine and estuarine sediment environments.

The genus *Vibrio* was first described as a phenanthrene-degrading organism by West and colleagues (Okpokwasili *et al.*, 1984; West *et al.*, 1984), who isolated large numbers of strains by spreading Chesapeake Bay samples onto complex media each with a phenanthrene surface layer. Some of the strains were identified by numerical taxonomy as *Vibrio parahaemolyticus* and *Vibrio fluvialis*, but others could not be assigned to any particular species. Using a similar isolation strategy, Berardesco *et al.* (1998) isolated phenanthrene-degrading *Vibrio* strains from Boston Harbour, but the strains were not identified to the species level.

In this paper, we present the first detailed characterization of a phenanthrene-degrading *Vibrio* strain. The strain is closely related to *V. splendidus*, but low levels of DNA–DNA reassociation are sufficient to consider strain P-2P44^T as a member of a new species of *Vibrio* (Wayne *et al.*, 1987). In addition, strain P-2P44^T differs from the type strain of *V. splendidus* by virtue of its ability to degrade naphthalenes and phenanthrene, its lack of luminescence, its cell shape and its differences in carbon-source utilization (outlined in Table 2).

Additionally, this is the first detailed description of the PAH-degradation phenotype of a *Vibrio* strain. P-2P44^T can use naphthalene or 2-methylnaphthalene in

addition to phenanthrene as a sole carbon and energy source. It is noteworthy that the PAHs susceptible to degradation by *Neptunomonas* and *Cycloclasticus* strains were determined under conditions identical to those used for strain P-2P44^T. In those studies, *Neptunomonas* degraded a narrower group of PAHs, mainly naphthalene and methylated naphthalenes (Hedlund *et al.*, 1999). In contrast, *Cycloclasticus* strains degraded all of the PAHs shown in Table 1 (Geiselbrecht *et al.*, 1998). These PAH-degradation differences among bacteria from a single environment probably reflect diversity in the PAH-catabolic systems and general physiology, and possibly impart ecological differences that are currently not understood. More complete descriptions of other PAH-degrading *Vibrio* strains are to be encouraged to determine the diversity of PAH-degrading *Vibrio* strains and their PAH-degradation capabilities.

Description of *Vibrio cyclotrophicus* sp. nov.

Vibrio cyclotrophicus (cy'clo.tro'phi.cus. Gr. n. *kyklos* circle or ring; Gr. adj. *trophikós* pertaining to food).

Rod-shaped bacteria motile via one or two subpolar or polar flagella. Cells measure 0.6 µm × 1.5–2.5 µm. Growth occurs in defined media using ammonium salts as the sole nitrogen source. Facultatively anaerobic. Requires at least 1.75% NaCl for growth. Catalase- and oxidase-positive. Uses some amino acids, carbohydrates, organic acids and sugar alcohols for growth, as well as certain PAHs, as summarized in Table 2. Protease, gelatinase, lecithinase, phosphatase and lipase activities are present. Nitrate is not reduced. Colonies are small, convex and entire. The temperature range is 4–37 °C. The pH range is 6.5–9.5. The G + C content of the DNA is 39 mol %. The type strain is sensitive to 10 µg ampicillin ml⁻¹, but resistant to novobiocin (MIC = 80 µg ml⁻¹), polymyxin B (MIC = 80 U ml⁻¹) and streptomycin (MIC = 40 µg ml⁻¹). A phylogenetic analysis using 16S rRNA gene sequences indicates that the type strain is closely related to *Vibrio splendidus*. The type strain, *V. cyclotrophicus* P-2P44^T, was isolated from Eagle Harbor, a creosote-contaminated EPA Superfund site in Puget Sound, Washington, USA. The type strain, *V. cyclotrophicus* P-2P44^T, has been deposited in the American Type Culture Collection, as number 700982^T, and the Pasteur Institute Culture Collection, as number 106644^T.

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