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

Brian P. Hedlund and James T. Staley

Department of Microbiology, University of Washington, Box 357242, Seattle, WA 98195-7242, USA

Strain P-2P44T 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-2P44T 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-2P44T degraded several other PAHs, though it was unable to use them as sole sources of carbon and energy. Phylogenetic analyses confirmed that strain P-2P44T is a member of the genus Vibrio, most closely related to Vibrio splendidus. However, strain P-2P44T shared only 98.3% 16S rDNA identity and 35% DNA–DNA reassociation with the type strain of V. splendidus. Strain P-2P44T differed phenotypically from V. splendidus. Together, these differences indicated that strain P-2P44T represents a novel species in the genus Vibrio, for which the name Vibrio cyclotrophicus sp. nov. is proposed; the type strain is P-2P44T (= ATCC 700982T = PICC 106644T).

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

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-2P44T, 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-2P44T, and we propose a new species, Vibrio cyclotrophicus to include strain P-2P44T.

METHODS

Strains and media. Vibrio splendidus (biovar 1) ATCC 33125T and Oceanospirillum linum ATCC 11336T were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained on the complex marine medium 2216 (Difco). Neptunomonas naphthovorans ATCC 700637T was revived from our own frozen stocks. Pseudomonas putida G7 was a gift from G. Sayler and was maintained on Luria broth. Strain P-2P44T 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-2P44T 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
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 analyzed 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 subreplicates) 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\(^{az}\) and *O. limun* ATCC 11336\(^T\) served as references.

**DNA–DNA hybridization.** The levels of DNA–DNA hybridization in pure cultures of strain P-2P44\(^T\) and *V. splendidus* ATCC 33125\(^T\) were determined using the thermal reannealing method (Zakrzewska-Cerwinska et al., 1988). DNA (60 mg ml\(^{-1}\)) in 5x SSC and 20% DMSO was sheared by sonication in a model 4710 sonicator (Cole-Palmer) 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 centrifuged and resuspended in a 1/10 volume of high-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% 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 NH\(_4\)Cl, Na\(_2\)HPO\(_4\), FeCl\(_3\) and TAPSO (3[N-tris(hydroxymethyl)methylamino]-2-hydroxy-propanesulfonic acid; Sigma)]. The salinities tested were 0.5, 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\(^{az}\) or *P. putida* G7 controls (Endsley et al., 1983). Nitrate-reduction assays were attempted with 0.1% and 0.01% NaNO\(_3\). 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 (BioMetals) 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 µm × 1.5–2.5 µm, with some cells elongating to 5 µm 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...
pulvinate colonies with entire edges. On the solid, rich, medium marine agar 2216, P-2P44T 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-2P44T. The following PAH compounds failed to serve as sole carbon sources for P-2P44T: 1-methylnaphthalene, 2,6-dimethylnaphthalene, biphenyl, acenaphthene, fluorene, anthracene and fluoranthene. Although unable to grow with the aforementioned PAH compounds, P-2P44T 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-2P44T, 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-2P44T by using GC. As shown in Table 1, strain P-2P44T 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-2P44T 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-126T and P. putida G7 turned blue or violet, strain P-2P44T did not produce indigo or related coloured isomers.

Phenotypic tests

P-2P44T 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 ω-ornithine and ω-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 1. Results of PAH-degradation experiments

<table>
<thead>
<tr>
<th>PAH</th>
<th>Initial concn (p.p.m.)</th>
<th>Recovery (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthalene</td>
<td>5</td>
<td>0 ± 0†</td>
</tr>
<tr>
<td>1-Methylnaphthalene</td>
<td>5</td>
<td>55 ± 4</td>
</tr>
<tr>
<td>2-Methylnaphthalene</td>
<td>5</td>
<td>4 ± 2†</td>
</tr>
<tr>
<td>2,6-Dimethylnaphthalene</td>
<td>0–5</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Biphenyl</td>
<td>5</td>
<td>78 ± 2</td>
</tr>
<tr>
<td>Acenaphthene</td>
<td>1</td>
<td>104 ± 3</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>1</td>
<td>4 ± 3†</td>
</tr>
<tr>
<td>Fluorene</td>
<td>1</td>
<td>98 ± 4</td>
</tr>
</tbody>
</table>

* Each value is given as the mean percentage of the parent PAH remaining after 7d (±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.
Table 2. Comparison of phenotypic differences between Vibrio splendidus ATCC 33125<sup>T</sup> and strain P-2P44<sup>T</sup>

Both strains were positive for gelatinase, lipase, glucose, xylose*, D-galactose, cellobiose, D-glucuronate, acetate, D-lactate, pyruvate, D-alanine, 1-serine, 1-glutamate, 1-arginine, 1-citrulline, 1-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.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>ATCC 33125&lt;sup&gt;T&lt;/sup&gt;</th>
<th>Strain P-2P44&lt;sup&gt;T&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luminescence</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Alginase activity</td>
<td>V</td>
<td>–</td>
</tr>
<tr>
<td>Curved rods</td>
<td>100%</td>
<td>&lt;10%</td>
</tr>
<tr>
<td>Temperature range (°C)</td>
<td>4–35</td>
<td>4–37</td>
</tr>
<tr>
<td>Utilization of:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Maltose</td>
<td>+</td>
<td>V</td>
</tr>
<tr>
<td>Glycine</td>
<td>–*</td>
<td>+</td>
</tr>
<tr>
<td>Requires growth factor†</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>

* 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.

Strain P-2P44<sup>T</sup> fermented cellobiose, D-mannitol, sucrose, trehalose, and D-glucose; however, gas production 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<sup>T</sup> and its closest relatives

<table>
<thead>
<tr>
<th>Species</th>
<th>RDP short ID</th>
<th>Strain</th>
<th>GenBank no.</th>
<th>No. differences/% difference from strain P-2P44&lt;sup&gt;T&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vibrio splendidus</td>
<td>V.splendid</td>
<td>ATCC 33125&lt;sup&gt;T&lt;/sup&gt;</td>
<td>X74724</td>
<td>26/1-8</td>
</tr>
<tr>
<td>Vibrio splendidus</td>
<td>V.splendi2</td>
<td>SCB8</td>
<td>Z31657</td>
<td>32/2-2</td>
</tr>
<tr>
<td>Vibrio aestuarianus</td>
<td>V.aestuari</td>
<td>ATCC 35048</td>
<td>X74689</td>
<td>50/3-6</td>
</tr>
<tr>
<td>Vibrio tapetis</td>
<td>V. tapetis</td>
<td>CECT 4600&lt;sup&gt;T&lt;/sup&gt;</td>
<td>Y08430</td>
<td>55/3-8</td>
</tr>
<tr>
<td>Vibrio vulnificus</td>
<td>V.vulnuli3</td>
<td>ATCC 27562</td>
<td>X74727</td>
<td>59/4-1</td>
</tr>
<tr>
<td>Vibrio natriegens</td>
<td>V.natrieg4</td>
<td>ATCC 14098&lt;sup&gt;T&lt;/sup&gt;</td>
<td>X74714</td>
<td>59/4-4</td>
</tr>
<tr>
<td>Vibrio mediterranei</td>
<td>V.mediterr</td>
<td>CIP 103203&lt;sup&gt;T&lt;/sup&gt;</td>
<td>X74710</td>
<td>66/4-7</td>
</tr>
<tr>
<td>Listonella anguillarum</td>
<td>Lsn.angu22</td>
<td>ATCC 19264&lt;sup&gt;T&lt;/sup&gt;</td>
<td>X16895</td>
<td>67/4-7</td>
</tr>
<tr>
<td>Vibrio diabolicus</td>
<td>V.diabolic</td>
<td>CNCM 1-1629&lt;sup&gt;T&lt;/sup&gt;</td>
<td>X99762</td>
<td>66/4-6</td>
</tr>
<tr>
<td>Vibrio scophthalmi</td>
<td>V.scophtha</td>
<td>CECT 4638&lt;sup&gt;T&lt;/sup&gt;</td>
<td>U46579</td>
<td>69/4-8</td>
</tr>
<tr>
<td>Vibrio pectenicida</td>
<td>V.pectenic</td>
<td>CIP 105190&lt;sup&gt;T&lt;/sup&gt;</td>
<td>Y13830</td>
<td>68/4-8</td>
</tr>
<tr>
<td>Vibrio fluvialis</td>
<td>V.fluvial3</td>
<td>ATCC 33809&lt;sup&gt;T&lt;/sup&gt;</td>
<td>X76335</td>
<td>75/5-2</td>
</tr>
</tbody>
</table>

Fig. 2. Neighbour-joining tree showing strain P-2P44<sup>T</sup> 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.
Determinant of the G+C content of the DNA

The G+C content of the DNA for P-2P44T was 38.8±0 mol% (mean±SD; n = 5).

DNA–DNA hybridization

The DNA–DNA reassociation between DNA from P-2P44T and V. splendidus was 35±6% (mean±SD; n = 4).

Phylogeny

Table 3 and Fig. 2 show that strain P-2P44T is a member of the genus Vibrio. The closest relative of strain P-2P44T 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-2P44T as a member of a new species of Vibrio (Wayne et al., 1987). In addition, strain P-2P44T 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-2P44T 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-2P44T. 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’cloatro’phi’cus. Gr. n. kyklos circle or ring; Gr. adj. trophe’klos 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, lecinthinase, phophatase 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 ≥0 µg ampicillin ml⁻¹, but resistant to novobiocin (MIC = 80 µg ml⁻¹), polymyxin B (MIC = 80 µg 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-2P44T, was isolated from Eagle Harbor, a creosote-contaminated EPA Superfund site in Puget Sound, Washington, USA. The type strain, V. cyclotrophicus P-2P44T, has been deposited in the American Type Culture Collection, as number 700982T, and the Pasteur Institute Culture Collection, as number 106644T.

ACKNOWLEDGEMENTS

This research was supported by an Office of Naval Research University Research Initiative in bioremediation to the University of Washington, grants N00014-91-J-1792 and N00014-91-J-1578, under the auspices of the Marine Bioremediation Program. This work was also supported through a National Institutes of Health Biotechnology Fellowship to B.P.H. (no. GM-0837-05) and Sigma Xi Grants-in-Aid of Research to B.P.H. We also thank the National Oceanic and Atmospheric Association’s Sanc-
taries and Reserves Division for its continuing support of the primary author. We thank members of the University of Washington’s Marine Bioremediation Program for useful discussions, Jeanne Poindexter for assistance with the electron microscopy, Matt Stoecker for advice about DNA–DNA hybridization, and Rob Sanford and Joanne Chee-Sanford for help with GC methods. Finally, we thank Everett P. Greenberg for testing P-2P44’ for luminescence.

REFERENCES


