Polaromonas naphthalenivorans sp. nov., a naphthalene-degrading bacterium from naphthalene-contaminated sediment

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Strain CJ2^T, capable of growth on naphthalene as a sole carbon and energy source, was isolated from coal-tar-contaminated freshwater sediment. The Gram reaction of strain CJ2^T was negative. The cells were non-spore-forming, non-motile cocci (without flagella). The isolate was found to be an aerobic heterotroph capable of utilizing glucose and other simple sugars. Growth was observed between 4 and 25 °C (optimum, 20 °C) and between pH 6.0 and 9.0 (optimum, pH 7.0–7.5). The G + C content of the genomic DNA was 61.5 mol% and the major quinone was ubiquinone-8. The peptidoglycan of strain CJ2^T was determined as belonging to type A1-γ, meso-diaminopimelic acid. The major fatty acids of strain CJ2^T were 16:1ω7c (67.0%), 16:0 (19.6%), 18:1ω7c (~7.9%) and 10:0 3-OH (~2.5%). The polar lipids were phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol. Mycolic acid and glycolipids could not be detected. Comparative 16S rDNA analysis indicated that strain CJ2^T is related to the family Comamonadaceae and that the nearest phylogenetic relative was *Polaromonas vacuolata* 34-P^T (97.1% similarity). On the basis of the physiological and molecular properties, the naphthalene-degrading isolate was designated *Polaromonas naphthalenivorans* sp. nov. The type strain is CJ2^T (= ATCC BAA-779^T = DSM 15660^T).

Polycyclic aromatic hydrocarbon (PAH) contamination of soil, water or sediment is of concern because PAHs can be toxic and/or carcinogenic. Numerous studies have been conducted on PAH-degrading bacteria, and extensive information has accumulated on the physiology, biochemistry and genetic basis of PAH biodegradation by both Gram-positive and Gram-negative bacteria, especially for pseudomonads, because of their susceptibility to isolation and cultivation (Daane *et al.*, 2001; Fuenmayor *et al.*, 1998; Laurie & Lloyd-Jones, 1999; Serdar & Gibson, 1989; Simon *et al.*, 1993).

Coal-tar waste containing high concentrations of PAHs was buried at our study site approximately 40 years ago, contaminating sediments and groundwater in an area 300 m long by 60 m wide (Madsen *et al.*, 1991). Although we had

previously isolated a variety of Gram-negative and Gram-positive naphthalene-degrading bacteria from the contaminated site (Herrick *et al.*, 1997; Stuart-Keil *et al.*, 1998), a novel Gram-negative bacterium was discovered very recently. Here, we describe this bacterium, strain CJ2^T, as a novel species designated *Polaromonas naphthalenivorans* sp. nov.

The biogeochemistry of the study site has been reported previously (Bakermans *et al.*, 2002; Madsen *et al.*, 1991). Samples of surface freshwater sediment, bathed in groundwater flowing through naphthalene-rich coal-tar waste contamination, were diluted serially and spread on minimal salts base (MSB) agar medium (Stanier *et al.*, 1966) incubated in the presence of naphthalene vapour as the sole carbon source at 10 °C for 1 month. The isolate was routinely grown aerobically at 20 °C except where indicated otherwise. MSB containing 0.2% pyruvate (w/v), Luria–Bertani (LB) broth and oligotrophic medium PYGV (Staley, 1968) were used for growth evaluation of the isolate and related type strains. Broth cultures (5 ml, MSB containing 0.2% pyruvate) used to inoculate other media were inoculated with a loop of cells and then shaken (200 r.p.m.) overnight. These cells were diluted 1:20 for growth assays in other media. Growth was monitored by measuring the OD₆₀₀.

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Abbreviations: MSB agar, minimal salts base agar; PAH, polycyclic aromatic hydrocarbon; PHA, poly-β-hydroxyalkanoic acid.

The GenBank accession number for the 16S rDNA sequence of strain CJ2^T is AY166684.

Micrographs of strain CJ2^T, growth curves in various media and details of aromatic hydrocarbon metabolism are available as supplementary material in IJSEM Online.

Chromosomal DNA was isolated and purified by a method described previously (Cashion *et al.*, 1977). The G+C content was determined by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) using the method of Mesbah & Whitman (1989). DNA was hydrolysed and the resultant nucleotides were analysed by isocratic reverse-phase HPLC (using Shimadzu equipment).

The morphology of naphthalene-degrading isolate CJ2^T was observed using a Zeiss LSM 210 laser scanning microscope equipped for conventional phase-contrast and epifluorescence viewing or a Nikon E600 microscope equipped with differential interference contrast optics. Scanning electron microscopy was done at the Cornell Integrated Microscopy Center. Motility was observed at 12 and 36 h in wet mounts. Cells were measured and inclusion granules observed in a drop of culture mounted on glass slides coated with 1% (w/v) agarose or slides coated with a dried smear of 0.5 mg poly-L-lysine ml⁻¹ (to enhance cellular adhesion). The agarose-coated slides were used to enhance detection of intracellular granules and to make cellular measurements. For scanning electron microscopy, CJ2^T was fixed with 3% glutaraldehyde and 1% osmium tetroxide and dried with a critical-point dryer using liquid CO₂ as the transition fluid. The dried samples were sputter-coated with gold under a vacuum and then examined with a scanning electron microscope (S-4500; Hitachi). The Gram reaction was determined using the Difco Gram Stain kit according to the manufacturer's recommended protocol using light microscopy. Strain CJ2^T was physiologically characterized using API 20E tests (bioMérieux Vitek) as described by Logan & Berkeley (1984). Other phenotypic tests included those for catalase and oxidase activities and anaerobiosis. The temperature range for growth was determined on MSB solid medium, containing 0.2% pyruvate, that had been pre-incubated at the appropriate temperature for 2 h prior to inoculation. Cells were mounted on Formvar-coated copper grids and negatively stained with 3% ammonium molybdate or 1% potassium phosphotungstate broth (pH 7.0). Grids were examined in a Phillips 201 transmission electron microscope operated at 80 kV.

Cells grown in MSB-pyruvate broth for 12 h were fixed onto a glass microscope slide with poly-L-lysine and stained with the Nile pink component of Nile blue A for poly-β-hydroxyalkanoic acid (PHA) (Ostle & Holt, 1982; Takeda *et al.*, 2002) and 1% toluidine blue; this was followed by differentiation with 1% H₂SO₄ for polyphosphate (Murray *et al.*, 1994). In a separate preparation, cells were mounted in Gram's iodine/potassium iodide solution for the detection of polyglucose granules (Stanier *et al.*, 1986). Orange fluorescence observed in the highly refractile granules of the cells under short-wavelength epi-illumination established the presence of PHA in the granules (Takeda *et al.*, 2002). Metachromatic polyphosphate granules and dark blue polyglucose granules were observed under bright-field illumination.

Respiratory lipoquinones were extracted, purified and

analysed by the DSMZ using procedures described by Komagata & Suzuki (1987). The purified lipoquinones were dissolved in acetone and separated by isocratic reverse-phase HPLC by using methanol/isopropanol (50:50, v/v) as the eluting solvent. Whole-cell fatty acids were extracted and analysed by GC at the DSMZ and using the Microbial Identification System (MIDI; Microbial ID). The diamino acid of the peptidoglycan was determined by the DSMZ using methods described previously (Komagata & Suzuki, 1987), and the presence of free mycolic acid was tested according to the method of Tomiyasu & Yano (1984). The polar lipid profile was determined by the DSMZ using the integrated method for lipid analysis described by Minnikin *et al.* (1984).

The 16S rDNA was amplified by PCR using two universal primers as described previously (Lane, 1991). PCRs and transformation were conducted according to the modified method of Herrick *et al.* (1997). Sequencing of both strands of the 16S rRNA gene of strain CJ2^T was completed on an ABI Prism 377XL instrument (Applied Biosystems). The 16S rRNA gene sequences (~1500 bp) of the isolate were assembled using SeqMan (DNASTAR) and compared with available 16S rRNA sequences from GenBank using the BLAST program to determine its approximate phylogenetic affiliation. Sequence-similarity values were computed using DNADIST in PHYLIP version 3.5c (Cole *et al.*, 2003). The 16S rDNA sequence of strain CJ2^T was aligned with 16S rRNA gene sequences of representatives of related taxa by using CLUSTAL W software (Thompson *et al.*, 1994). Gaps at the 5' and 3' ends of the alignment were omitted from further analyses. Construction of phylogenetic trees was computed using two different methods of phylogenetic analysis [parsimony and distance (Kimura two-parameter correction)] available in the PHYLIP software package, version 3.6 (Felsenstein, 2002). The stability of relationships was assessed by using a bootstrap analysis of 1000 datasets through the PHYLIP package.

The procedures described by Daane *et al.* (2001) were used to assess utilization of a variety of aromatic compounds (naphthalene, phenanthrene, fluorene, pyrene and biphenyl) in MSB liquid medium. Each culture was tested in triplicate for each substrate. Cells were initially grown in 5 ml MSB containing 0.2% pyruvate (w/v). Each of the test aromatic substrates (1 μmole total; dissolved in the volatile solvent hexane) was added to sterile 50 ml serum bottles. After evaporation of the solvent, 10 ml MSB liquid medium was added prior to the addition of 100 μl cell inoculum. At time zero, killed controls were prepared by adding 200 μl poison (5% HCl, 0.25 M HgCl₂) to each of the serum-bottle treatments. Samples were incubated at 20 °C for 10 days. At each assay time (0 and 10 days), the serum-bottle samples were extracted with 10 ml ethyl acetate and this extract was analysed using a Hewlett Packard 6890 gas chromatograph with an HP-5MS column (30 m, 0.25 mm internal diameter, 0.25 μm film thickness) and an HP 5973 mass-selective detector. Results are reported as percentages remaining relative to killed controls.

On MSB-pyruvate and MSB-naphthalene media, strain CJ2^T produced circular, convex, beige colonies with smooth and glistening mucoid surfaces. No diffusible pigments were observed. The morphology of strain CJ2^T was examined by using scanning electron microscopy and phase-contrast light microscopy (see Supplementary Fig. A in IJSEM Online). The cells were non-motile cocci that varied from 1 to 4 µm in diameter. Cells occurred singly or in pairs and often formed small clusters of loosely adherent cells (see Supplementary Fig. A). The cells were Gram-negative and formed large, readily recognizable PHA granules, which fluoresced orange after being stained with Nile pink. Dark-blue granules indicative of polyglucose (Stanier *et al.*, 1986) were distributed outside large refractive PHA granules in iodine/potassium iodine-mounted cells. Small metachromatic polyphosphate granules were observed in peripheral regions of cells after toluidine blue staining. These results suggest that strain CJ2^T has the unusual ability to store, during exponential growth, all three of the common types of prokaryotic cellular reserve materials (Stanier *et al.*, 1986).

Negatively stained cells were difficult to observe under the transmission electron microscope as they did not readily adhere to the Formvar film. None of the cells observed possessed flagella. Since we did not observe motility in wet mounts of cells at different stages of growth, we assume that CJ2^T did not produce flagella under the growth conditions we employed.

Chemical assays of MSB broth supplied with several PAH compounds indicated that, like the two pseudomonad reference strains, strain CJ2^T metabolized 100 % of the naphthalene provided (Supplementary Table in IJSEM Online). Strain CJ2^T was originally recognized by its large (0.5 cm) mucoid colonies on MSB-naphthalene. In the absence of naphthalene vapour, this growth did not occur. Unlike the naphthalene-degrading reference strains *Pseudomonas putida* NCIB 9816-4 and *Pseudomonas putida* G7, colonies of strain CJ2^T failed to produce an obvious zone of clearing when sprayed with an ethereal solution of phenanthrene as described by Kiyohara *et al.* (1982). Strain CJ2^T was found to be an aerobic heterotroph capable of utilizing glucose and other simple sugars. Growth in MSB-naphthalene and MSB-pyruvate agar media was observed

between 4 and 25 °C, and the optimum temperature was about 20 °C. However, growth was not detectable at 30 °C. The optimum pH of strain CJ2^T on MSB containing 0.2 % pyruvate was about 7.0–7.5. The bacterium was oxidase- and catalase-positive. Its growth rate was markedly diminished in rich (100 % and 20 % LB) media, relative to MSB-pyruvate and oligotrophic PYGV media (Staley, 1968) (Supplementary Fig. B). Gelatinase and citrate-utilization tests were positive. Arginine was hydrolysed by strain CJ2^T but ONPG was not. Strain CJ2^T did not produce lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase, urease, hydrogen sulfide, indole or acetoin and also did not produce acid from D-glucose, mannitol, sucrose or arabinose. Growth was not significant on MSB-glucose plates incubated anaerobically; furthermore, anaerobic growth on this medium was not enhanced by added sodium nitrate.

Strain CJ2^T grows readily on MSB agar with naphthalene vapour as the sole source of carbon and energy at 10 °C. At temperatures above 20 °C, growth does not occur, possibly because the vapour pressure of naphthalene is toxic. At 20 °C, growth of strain CJ2^T on naphthalene vapour is not robust and may be influenced by the presence of capsular material, which may mitigate toxicity. Strain CJ2^T does not grow in MSB broth with naphthalene crystals in the medium.

The major quinone found in the strain CJ2^T was ubiquinone-8. The fatty acid profile of strain CJ2^T was characterized by the presence of 16:1ω7c (67.0 %), 16:0 (19.6 %), 18:1ω7c (~7.9 %) and 10:0 3-OH (~2.5 %). The fatty acid profile did not match any in the MIDI identification system. The G + C content of strain CJ2^T was 61.5 mol%. The major peptidoglycan of strain CJ2^T was determined as belonging to type A1-γ, *meso*-diaminopimelic acid. The polar lipids were phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol. Neither mycolic acid nor glycolipids could be detected in cells of strain CJ2^T. The fatty acid composition and the lipoquinone are typical of the β-Proteobacteria.

Phylogenetic analysis of the 16S rDNA sequence showed that strain CJ2^T was closely related to members of the family

Fig. 1. Phylogenetic position of strain CJ2^T among related bacteria. The tree was produced using the neighbour-joining algorithm and was calculated using ~1500 bases of the 16S rRNA gene. *Escherichia coli* was used as an outgroup. The scale bar represents 0.1 changes per nucleotide position.

Table 1. Characteristics of strain CJ2^T and other genera in the family Comamonadaceae

Data for other genera were taken from Wen *et al.* (1999). +, Present in all species; -, absent in all species; Q, ubiquinone; RQ, rhodoquinone; NA, no data available.

Characteristic	Strain CJ2 ^T	<i>Polaromonas</i>	[<i>Aquaspirillum</i>]	<i>Rhodoferrax</i>	<i>Variovorax</i>	<i>Xylophilus</i>	<i>Comamonas</i>
Morphology	Cocci	Rods	Spirilla or curved rods	Curved rods	Rods	Rods	Rods or spirilla
Flagella	-	One, polar	Bipolar tufts or 1-2 flagella at only one pole	One, polar	Peritrichous	One, polar	Polar or bipolar tufts
Pigments	-	-	-	-	+	+	-
Oxidase	+	+	+	+	+	-	+
Psychrophilic growth	-	+	-	-	-	-	-
Major quinone system	Q-8	Q-8	Q-8	Q-8+RQ-8	Q-8	Q-8+RQ-8	Q-8
Major cellular fatty acids	16:1 ω 7c, 16:0, 18:1 ω 7c	16:1 ω 7c, 16:0, 18:1 ω 7c, 18:1 ω 9t or 18:1 ω 12t	16:0, 16:1	16:0, 16:1	16:0, 16:1, 18:1	NA	16:1 ω 7c, 16:0, 18:1 ω 7c
Major 3-OH acid	10:0	NA	10:0	8:0	10:0	NA	10:0
DNA G+C content (mol%)	61.5	52-57	56-62	59-61	66-68	68-69	63-66

Comamonadaceae (Fig. 1). The nearest known relatives of strain CJ2^T were *Polaromonas vacuolata* 34-P^T (97.1% similarity), *Aquaspirillum delicatum* LMG 4328 (95.1%) and *Variovorax paradoxus* IAM 12373^T (94.2%). Table 1 features several phenotypic traits of strain CJ2^T that can differentiate it from phylogenetically related strains of the family *Comamonadaceae* of the β -*Proteobacteria* (Wen *et al.*, 1999). Two other phenotypic features that distinguish strain CJ2^T from all other strains of the *Comamonadaceae* are its coccoid and unflagellated (non-motile) morphology. The closest known relative of CJ2^T clearly is *Polaromonas vacuolata* 34-P^T, especially with regard to 16S rDNA sequence and major cellular fatty acids; however, the latter shows growth at lower temperatures, a lower G+C content, flagellation and rod-shaped cells (Irgens *et al.*, 1996). On the basis of its distinctive physiological, morphological and other key traits, it is proposed that strain CJ2^T represents a novel species, for which the name *Polaromonas naphthalenivorans* sp. nov. is proposed.

Description of *Polaromonas naphthalenivorans* sp. nov.

Polaromonas naphthalenivorans (naph.thal.en.i.vo'rans. N.L. neut. n. *naphthalenum* naphthalene; L. part. adj. *vorans* consuming; N.L. neut. adj. *naphthalenivorans* naphthalene-consuming).

Cells are non-motile, presumably non-flagellated, Gram-negative, aerobic cocci of variable size (1-4 μ m), usually found individually or in small clusters of loosely adherent cells. Oxidase- and catalase-positive. The optimum growth temperature and pH are respectively 20 °C and 7-7.5. Many simple sugars are utilized. PHA, polyglucose and polyphosphate granules accumulate as storage material. Chemo-organotrophic. The G+C content is approximately 61.5 mol% and ubiquinone-8 is the major quinone. Cells form circular, convex colonies with smooth and glistening surfaces. Pigment is not produced. Growth is not observed at 30 °C. The growth rate is much lower in LB medium than in MSB or oligotrophic media. Large (0.5 cm) mucoid colonies are produced with naphthalene vapour as the carbon-source medium at 10 °C. Naphthalene is efficiently removed from solution. Tests for gelatinase, citrate utilization and arginine hydrolase are positive. ONPG is not hydrolysed. Assays for lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase and urease are negative. Hydrogen sulfide, indole and acetoin are not produced. Acid, but no gas, is produced from D-glucose, mannitol, sucrose and arabinose. The major cellular fatty acids are 16:1 ω 7c (67.0%), 16:0 (19.6%), 18:1 ω 7c (~7.9%) and 10:0 3-OH (~2.5%). The major peptidoglycan is type A1- γ , *meso*-diaminopimelic acid. The polar lipids are phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol. Mycolic acid and glycolipids are not detected.

The type strain is CJ2^T (=ATCC BAA-779^T=DSM 15660^T), which was isolated from coal-tar-contaminated surface sediments from South Glens Falls, NY, USA.

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