Tropicibacter naphthalenivorans gen. nov., sp. nov., a polycyclic aromatic hydrocarbon-degrading bacterium isolated from Semarang Port in Indonesia

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An aerobic, Gram-negative, motile bacterium, strain C02^T, was isolated from seawater obtained from Semarang Port in Indonesia. Cells of strain C02^T were peritrichously flagellated and rod-shaped. Strain C02^T was able to degrade naphthalene, alkylnaphthalenes and phenanthrene. 16S rRNA gene sequence analysis revealed that this strain was affiliated with the family *Rhodobacteraceae* in the class *Alphaproteobacteria* and was related most closely to *Marinovum algicola* FF3^T (95.7 % similarity) and *Thalassobius aestuarii* JC2049^T (95.2 %). The DNA G+C content of strain C02^T was 64.6 mol%. The major cellular fatty acids were $C_{18:1}\omega7c$ (50.9 % of the total), $C_{16:0}$ (17.9 %), 11 methyl $C_{18:1}\omega7c$ (14.7 %), $C_{18:1}\omega9c$ (2.9 %) and $C_{19:0}$ cyclo $\omega8c$ (2.4 %), and the predominant respiratory lipoquinone was ubiquinone-10. Based on physiological, chemotaxonomic and phylogenetic data, strain C02^T is suggested to represent a novel species of a new genus, for which the name *Tropicibacter naphthalenivorans* gen. nov., sp. nov. is proposed. The type strain of *Tropicibacter naphthalenivorans* is C02^T (=JCM 14838^T=DSM 19561^T).

Contamination of the marine environment with petroleum hydrocarbons is of great public concern owing to their toxicity to humans and marine organisms (Malins *et al.*, 1985; Meador *et al.*, 1995). A number of hydrocarbon-degrading bacteria have been isolated from the marine environment and characterized (e.g. Kasai *et al.*, 2002a, b; Ozaki *et al.*, 2006), although information regarding hydrocarbon-degrading bacteria from tropical waters is relatively scarce (Chaillan *et al.*, 2004; Zhuang *et al.*, 2003; Zinjarde & Pant, 2002). We have recently isolated a substantial number of marine bacteria from seawater obtained from Semarang Port in Indonesia, and demonstrated that some of the isolates were capable of degrading hydrocarbons (Harwati *et al.*, 2007). The present study characterizes one of these Indonesian isolates, designated strain C02^T, affiliated with the class *Alphaproteobacteria*. Based on the results of polyphasic examinations, including phenotypic, chemotaxonomic and phylogenetic analyses, we propose that this strain represents a novel species of a new genus.

The ability of strain C02^T to degrade hydrocarbons in crude oil was examined in 10 ml ONR7a medium (Dyksterhouse *et al.*, 1995) supplemented with 1 mg heat-treated Arabian Light crude oil ml^{-1} (Dutta & Harayama, 2000). Cells of strain C02^T grown in 10 ml marine broth 2216 (MB; Difco) up to an optical density at 600 nm of approximately 1 were harvested by centrifugation (8000 ***g***, 10 min), washed twice with ONR7a medium and inoculated on to the medium in 50-ml tubes fitted with Teflon-lined caps. The tubes were incubated at 30 °C on a reciprocal shaker (at 90 r.p.m.) for 4 weeks. Cultures were prepared in triplicate. Non-inoculated samples were incubated similarly and served as controls. Following incubation, oil components were extracted by using chloroform, and hydrocarbon losses were analysed via GC-MS (GC-MS-QP5000; Shimadzu) as described by Kasai *et al.* (2002b). The percentage biodegradation was calculated as described by Dutta & Harayama (2000). Strain C02^T degraded 88.1 ± 1.1 % (mean ± SE) of total naphthalene, 56.0 ± 10.4 % of total C_1-alkylnaphthalenes, 22.5 ± 8.6 % of total C_2-alkylnaphthalenes and 14.5 ± 2.7 % phenanthrene, while alkanes, $C_{3–4}$-alkylnaphthalenes, $C_{0–4}$-alkyldibenzothiophenes, $C_{1–6}$-alkylphenanthrenes and $C_{0–3}$-alkylfluorenes were not significantly degraded (<10 %) (C_0–C_6 represent total carbon numbers of branched alkyl groups).

The cell morphology of strain C02^T was examined by transmission electron microscopy (Beveridge *et al.*, 1994) and motility was examined under a phase-contrast microscope. Gram staining and oxidase and catalase tests

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain C02^T is AB302370.

were performed according to the procedures of Smibert & Krieg (1994). Growth was tested at 30 °C in MB unless otherwise stated. Salinity requirements were tested by using modified MB (Sohn *et al.*, 2004) supplemented with 0–20 % (w/v) NaCl at 30 °C. The pH range for optimal growth was determined on solid media containing MB whose pH was adjusted to 5.5–9.5. Solid media contained 1.5 % (w/v) Bactoagar (Difco). The presence of poly-β-hydroxyalkanoate was detected by using Sudan Black according to the procedures of de Lima *et al.* (1998). Susceptibility to antibiotics was determined on agar plates containing MB (MA plates) in the presence of the following antibiotics (concentrations given in parentheses; μg ml^{-1}): ampicillin (50, 100, 150 and 200), chloramphenicol (20), gentamicin (50), kanamycin sulfate (20), nalidixic acid (20, 50, 100 and 200), spectinomycin (7.5, 15 and 20), streptomycin (20) and tetracycline (10). API ZYM, API 20NE (bioMérieux) and Microlog GN2 microplates (Biolog) were used for physiological and biochemical characterization according to the manufacturers' instructions.

Cells of strain C02^T were Gram-negative rods (1.1–3.4 μm in length and 0.1–0.7 μm in width), motile by means of peritrichous flagella (Fig. 1). Strain C02^T formed white colonies on MA plates. The strain was oxidase-positive but catalase-negative. It reduced nitrate to nitrite. Cells contained poly-β-hydroxyalkanoate. Growth of strain C02^T was observed between 10 and 43 °C, with optimum growth at 37 °C. It grew within a pH range of 6.5–8.5, with optimum growth at pH 7.6. Strain C02^T showed essential requirements for NaCl, as no growth was observed in media lacking NaCl. It grew at NaCl concentrations from 1 to 15 %, with optimum growth at 5 % NaCl. The physiological and biochemical characteristics of strain C02^T are presented in detail in the species description below. It was susceptible to ampicillin, chloramphenicol, gentamicin, nalidixic acid, kanamycin, spectinomycin, streptomycin and tetracycline.

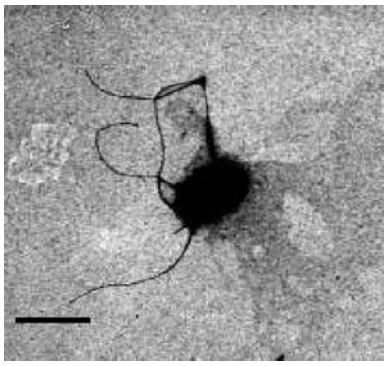

Fig. 1. Transmission electron micrograph of a cell of strain C02^T. Bar, 1 μm.

Table 1. Cellular fatty acid contents of strain C02^T and closely related taxa

Taxa: 1, strain C02^T; 2, *Antarctobacter heliothermus* EL-219^T (data from Labrenz *et al.*, 1998); 3, *Sagittula stellata* E-37^T (Gonzalez *et al.*, 1997); 4, *Ruegeria atlantica* 1480^T (Rüger & Höfle, 1992); 5, *Marinovum algicola* FF3^T (Lafay *et al.*, 1995; Martens *et al.*, 2006); 6, *Thalassobius aestuarii* JC2049^T (Yi & Chun, 2006); 7, *Roseovarius tolerans* EL-172^T (Labrenz *et al.*, 1999); 8, *Roseobacter denitrificans* DSM 7001^T (Labrenz *et al.*, 1999); 9, *Phaeobacter daeponensis* TF-218^T (Yoon *et al.*, 2007). Values are percentages of the total fatty acids; components that represented <0.5 % in all strains were omitted. ND, Not detected.

Fatty acid	1	2	3	4	5	6	7	8	9
Straight-chain									
$C_{10:0}$	ND	ND	ND	2.1	ND	ND	ND	ND	ND
$C_{12:0}$	ND	ND	ND	2.6	ND	ND	ND	ND	1.2
$C_{16:0}$	17.9	2.5	8.6	6.9	1.6	6.8	6.2	2.2	8.6
$C_{17:0}$	0.5	ND	ND	ND	ND	1.4	ND	ND	0.6
$C_{18:0}$	1.7	1.0	6.8	1.0	2.7	3.0	0.8	2.3	2.4
Unsaturated									
$C_{16:1}$	ND	0.8	ND	ND	ND	ND	0.8	ND	ND
$C_{18:1}\omega7c$	50.9	83.2	*	71.2	86.7	68.0	70.2	87.7	57.7
$C_{18:1}\omega9c$	2.9	ND	ND	ND	ND	ND	ND	ND	ND
Hydroxy									
$C_{10:0}$ 3-OH	1.4	ND	ND	0.4	ND	1.3	ND	1.8	1.7
$C_{12:0}$ 3-OH	0.7	ND	ND	4.7	1.6	ND	ND	ND	2.6
$C_{12:1}$ 3-OH	1.2	3.1	3.6	ND	ND	ND	3.6	ND	ND
$C_{14:0}$ 2-OH	0.5	ND	ND	ND	ND	ND	ND	2.9	ND
$C_{16:0}$ 2-OH	ND	ND	ND	3.4	ND	3.8	ND	ND	5.6
11 Methyl $C_{18:1}\omega7c$	14.7	ND	ND	7.1	3.2	12.0	ND	ND	16.6
$C_{19:0}$ cyclo $\omega8c$	2.4	2.4	ND	ND	ND	ND	ND	ND	ND

*Major fatty acid, but not quantified.

Cellular fatty acids and quinone were analysed at the TechnoSuruga Laboratory Co., Ltd from cells grown in MB for 24 h. The major cellular fatty acids of strain C02^T were $C_{18:1}\omega7c$ (50.9 % of the total), $C_{16:0}$ (17.9 %), 11 methyl $C_{18:1}\omega7c$ (14.7 %), $C_{18:1}\omega9c$ (2.9 %) and $C_{19:0}$ cyclo $\omega8c$ (2.4 %) (detailed results are given in Table 1). The major lipoquinone was ubiquinone-10 (approximately 88 % of the total).

The 16S rRNA gene sequence of strain C02^T (1363 bp) was determined previously (Harwati *et al.*, 2007). Searches for similar 16S rRNA gene sequences were conducted by using the GenBank and RDP (Maidak *et al.*, 1999) databases. Phylogenetic analysis was performed by using CLUSTAL_X (version 1.83) (Thompson *et al.*, 1997), and a phylogenetic tree was constructed by using the neighbour-joining plot program within the MEGA software package (version 3.0) (Kumar *et al.*, 2004). In the neighbour-joining phylogenetic tree, strain C02^T formed a separate branch within the family *Rhodobacteraceae* (Fig. 2). 16S rRNA gene sequence analysis revealed that strain C02^T was related most closely to *Marinovum algicola* FF3^T (95.7 % similarity) and *Thalassobius aestuarii* JC2049^T (95.2 %). The latter two taxa belong to the *Roseobacter* clade, which is known as one of the most abundant groups in the marine environment (Buchan *et al.*, 2005). The DNA G+C content of strain C02^T as determined by the method of Katayama-Fujimura *et al.* (1984) was 64.6 mol%.

Phenotypic characteristics of strain C02^T that can be used to differentiate it from closely related members of the *Roseobacter* clade are detailed in Table 2. Based on phylogeny, fatty acid composition and phenotypic characteristics, we conclude that strain C02^T represents a novel species of a new genus, for which the name *Tropicibacter naphthalenivorans* gen. nov., sp. nov. is proposed.

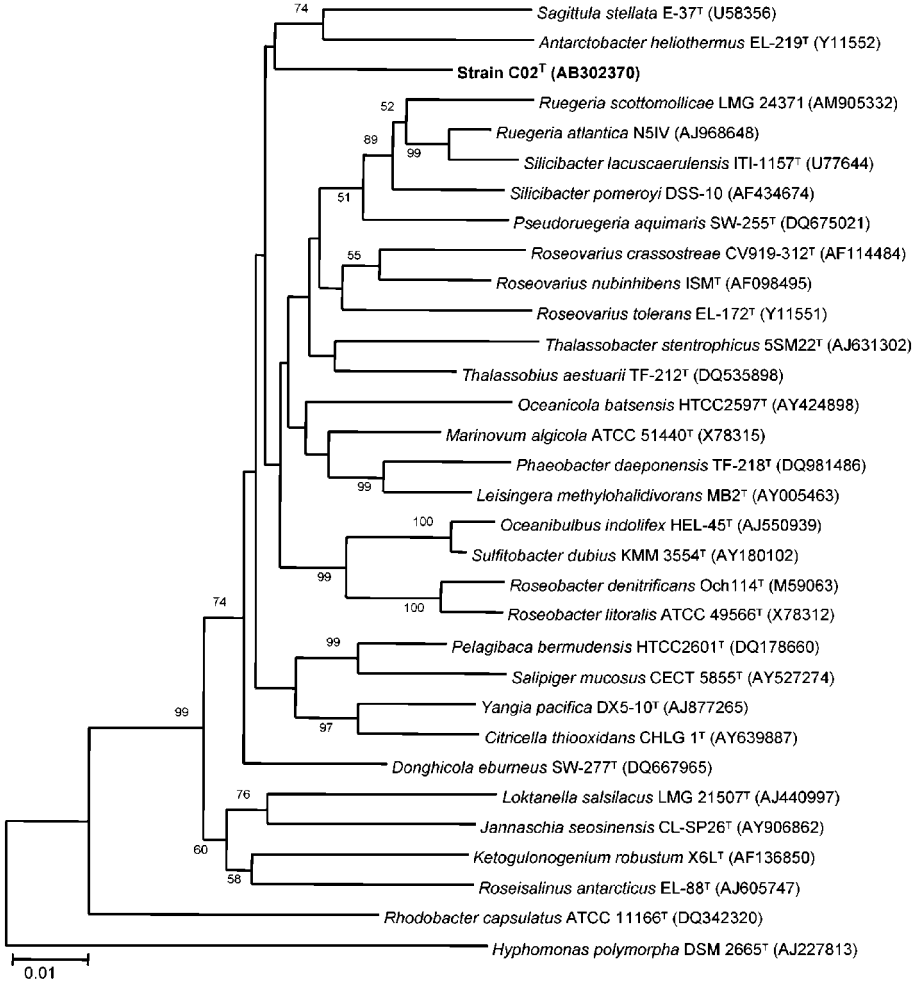

Fig. 2. Phylogenetic relationships between strain C02^T and other members of the family *Rhodobacteraceae*. The tree was constructed by using the neighbour-joining algorithm. Numbers at nodes are bootstrap percentages based on 1000 replications; only values >50 % are shown. Bar, 1 % estimated sequence divergence.

Table 2. Differential phenotypic characteristics between strain C02^T and closely related taxa of the *Roseobacter* clade

Taxa: 1, strain C02^T; 2, *Antarctobacter heliothermus* EL-219^T (data from Labrenz *et al.*, 1998); 3, *Sagittula stellata* E-37^T (Gonzalez *et al.*, 1997); 4, *Ruegeria atlantica* 1480^T (Rüger & Höfle, 1992); 5, *Marinovum algicola* FF3^T (Lafay *et al.*, 1995; Martens *et al.*, 2006); 6, *Thalassobius aestuarii* JC2049^T (Yi & Chun, 2006); 7, *Roseovarius tolerans* EL-172^T (Labrenz *et al.*, 1999); 8, *Sulfitobacter dubius* KMM 3554^T (Ivanova *et al.*, 2004); 9, *Roseobacter denitrificans* Och 114^T (Shiba, 1991); 10, *Phaeobacter daeponensis* TF-218^T (Yoon *et al.*, 2007). +, Positive; −, negative; +/−, variable; ND, no data available; NF, not found; p, polar; sp, subpolar.

Characteristic	1	2	3	4	5	6	7	8	9	10
Cell morphology	Rod	Rod	Rod	Rod	Ovoid	Ovoid	Rod	Rod	Rod or ovoid	Ovoid
Pigmentation	None	Brown–yellow	Cream	Beige	Brown–beige	Cream	Red	Yellow	Red	None
Motility	+	+/−	+*	−	+	−	+/−	+	+	+
Flagella	Peritrichous	1–3, sp	+/?	−	1–2, sp	−	NF	1, sp	1–3, sp	1, p
Salt growth range (%)	1–15	1–10	ND†	1–11	0.6–12	1–7	1–13	1–12	ND	>0−8
pH optimum	7.6	6.9–7.8	7.5	7	7.5	7	6.2–9.0	7.5–8.0	7.0–8.0	7–8
Temperature growth range (°C)	10–43	ND	10–41	5–30	10–37	15–35	3–43.5	10–30	ND	4–42
Temperature optimum (°C)	37	16–26	30	25	25–30	35	8.5–33.5	25	20–30	37
NO_3^- to NO_2^-	+	+	−	+	−	−	−	+	+	+
Indole production	+	−	−	ND	−	−	−	ND	+	ND
β-Galactosidase	+	ND	ND	−	+	−	ND	ND	ND	+
DNA G+C content (mol%)	64.6	62.3–62.8	65.0	55	60	61	63.3–63.4	63.7	59.6	64.9

*Motility was assumed as flagella were found in suspension, but motile cells were not seen.
†Not determined, but required NaCl for growth (Gonzalez *et al.*, 1997).

Description of *Tropicibacter* gen. nov.

Tropicibacter [Trop.ic.i.bac.ter. L. adj. *tropicus* tropical, pertaining to the tropical zone of the Earth; N.L. masc. n. *bacter* (from Gr. n. *bakterion*) rod; N.L. masc. n. *Tropicibacter* a rod belonging to the tropical zone].

Cells are Gram-negative, motile by means of peritrichous flagella and rod-shaped (1.1–3.4 μm long and 0.1–0.7 μm wide). Require sodium ions for growth. Positive for oxidase and nitrate reduction. Contain poly-β-hydroxyalkanoate. The major ubiquinone is Q-10. The predominant fatty acids are $C_{18:1}\omega7c$, $C_{16:0}$ and 11 methyl $C_{18:1}\omega7c$. The type species is *Tropicibacter naphthalenivorans*.

Description of *Tropicibacter naphthalenivorans* sp. nov.

Tropicibacter naphthalenivorans (naph.tha.le.ni.vo′rans. N.L. neut. N. *naphthalenum* naphthalene; L. part. adj. *vorans* devouring; N.L. part. adj. *naphthalenivorans* degrading naphthalene).

The description is identical to that for the genus, with the following additions. Colonies on MA are circular, slightly convex, smooth, yellowish–white and 2.0–3.0 mm in diameter after 3 days incubation at 37 °C. Growth occurs at temperatures of 10–43 °C (optimum 37 °C), at pH 6.5–8.5 and at NaCl concentrations of 1–15 % (optimum 5 %). Able to degrade $C_{0–2}$-alkylnaphthalene and phenanthrene. Susceptible to (μg ml^{-1}) ampicillin (50), chloramphenicol (20), gentamicin (50), kanamycin (20), nalidixic acid (20), spectinomycin (7.5), streptomycin (20) and tetracycline (10). Positive for nitrate reduction, protease, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydratase, α-galactosidase, β-galactosidase, α-glucosidase and β-glucosidase. Negative for glucose fermentation, arginine dihydrolase, alkaline phosphatase, cystine arylamidase, trypsin, chymotrypsin, β-glucuronidase, α-mannosidase and α-fucosidase. The following Biolog GN2 test substrates score as positive: dextrin, Tween 80, L-arabinose, D-fructose, D-glucose, D-lactose, maltose, D-mannose, D-sorbitol, sucrose, lactic acid, succinic acid, L-aspartic acid, L-alanine, L-proline, serine, inosine, uridine, turanose, xylitol, trehalose, inosine, uridine, cellobiose, melibiose, L-rhamnose and acetic acid. The predominant fatty acids are $C_{18:1}\omega7c$, $C_{16:0}$, 11 methyl $C_{18:1}\omega7c$, $C_{18:1}\omega9c$ and $C_{19:0}$ cyclo $\omega8c$.

The type strain, C02^T (=JCM 14838^T=DSM 19561^T), was isolated from seawater in Semarang Port, Java, Indonesia. The DNA G+C content of the type strain is 64.6 mol%.

Acknowledgements

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