Croceicoccus naphthovorans sp. nov., a polycyclic aromatic hydrocarbons-degrading and acylhomoserine-lactone-producing bacterium isolated from marine biofilm, and emended description of the genus Croceicoccus

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A polycyclic aromatic hydrocarbons-degrading and acylhomoserine-lactone-producing marine bacterium, designated strain PQ-2T, was isolated from marine biofilm collected from a boat shell at a harbour of Zhoushan island, Zhejiang Province, PR China. Strain PQ-2T is Gram-stain-negative, yellow-pigmented, non-motile and short rod-shaped. Optimal growth of strain PQ-2T was observed at 32°C, at pH 7.0 and in 2% (w/v) NaCl. The 16S rRNA gene sequence of strain PQ-2T showed highest similarity to Croceicoccus marinus E4A9T (96.3%) followed by Novosphingobium malaysiense MUSC 273T (95.6%) and Altererythrobacter marinus H32T (95.6%). Phylogenetic analysis with all species of the family Erythrobacteraceae with validly published names revealed that strain PQ-2T formed a phyletic line with Croceicoccus marinus E4A9T that was distinct from other members of the family Erythrobacteraceae. The sole respiratory quinone was ubiquinone 10 (Q-10). The predominant fatty acids were C18 : 1ω7c, C17 : 1ω6c and summed feature 3 (C16 : 1ω7c and/or iso-C15 : 02-OH). The genomic DNA G+C content was 61.7 mol%. In the polar lipid profile, phosphatidylethanolamine, phosphatidylcholine, phosphatidylglycerol, one unidentified phospholipid and one sphingoglycolipid were the major compounds; and another sphingoglycolipid was present in a minor amount. Based on the genotypic and phenotypic data, strain PQ-2T represents a novel species of the genus Croceicoccus, for which the name Croceicoccus naphthovorans sp. nov. is proposed. The type strain is PQ-2T (=CGMCC 1.12805T=NBRC 110381T). In addition, emended descriptions for the genus Croceicoccus and the species C. marinus are given.

The genus Croceicoccus, belonging to the family Erythrobacteraceae within the class Alphaproteobacteria, was established by Xu et al. (2009). At the time of writing, the genus Croceicoccus contained only the type species Croceicoccus marinus, which was isolated from deep-sea sediment (Xu et al., 2009). The genus Croceicoccus is characterized by Gram-stain-negative and non-spore-forming cocci, presence of carotenoids, absence of bacteriochlorophyll and ubiquinone-10 as the predominant respiratory quinone. This study focuses on the description of a novel species (represented by strain PQ-2T) that was isolated from marine biofilm.

A marine biofilm sample was collected from a boat shell at a harbour of Zhoushan island, Zhejiang, China (GPS position, 30°43′ 45.54 N 122°29′ 21.20 E). The sample was enriched with 50 mg phenanthrene l–1 as the sole carbon source in 100 ml marine minimal medium containing (g l–1): sea salt (30), KH2PO4 (1), K2HPO4 (1), NH4NO3 (1) at pH 7.2. After 9 days of incubation at 28°C, 10 ml culture broth was transferred to 100 ml fresh medium and enriched for another 9 days. Subsequently, a culture broth dilution series was spread on marine minimal agar [marine minimal medium supplemented with 1.5% (w/v) agar]. The polycyclic aromatic hydrocarbon (PAH) degraders were first screened using the clear zone method.
(Kiyohara et al., 1982) and then were tested for acylhomoserine-lactone (AHL)-producing ability by cross-feeding assay using AHL reporter strain Agrobacterium tumefaciens A136 (Huang et al., 2013). A yellow isolate which showed a clear zone in the phenanthrene-sprayed agar plate and induced coloration of A136, designated strain PQ-2T, was obtained. The isolate was further confirmed to be able to degrade PAHs with two or three rings, such as fluorene, fluoranthene and phenanthrene. Strain PQ-2T was able to degrade PAHs with two or three rings, such as fluorene, fluoranthene and phenanthrene. Strain PQ-2T was routinely cultivated on P5Y3 medium (g l\(^{-1}\)) containing: peptone (5), yeast extract (3), sea salt (30) at pH 7.0 or R medium (g l\(^{-1}\)) containing: NaCl (30), MgSO\(_4\cdot7\)H\(_2\)O (2.46), KCl (1.5), CaCl\(_2\cdot2\)H\(_2\)O (0.15), NaBr (0.1), FeCl\(_3\cdot2\)H\(_2\)O (0.016), yeast extract (2), peptone (5), Casamino acids (1) at pH 7.0. To make solid medium, 1.5 % agar was added.

The 16S rRNA gene sequence of strain PQ-2T was amplified by PCR using the universal primers 27F and 1492R as described by Huang et al. (2008). The PCR product was cloned into vector pMD19-T and sequenced. The almost full-length 16S rRNA gene sequence (1448 nt) was compared with closely related sequences of reference species from the EzTaxon-e server (Kim et al., 2012). Multiple sequence alignment was determined by the CLUSTAL W 1.8 (Thompson et al., 1994). EzEditor (a versatile sequence alignment editor for both rRNA- and protein-coding genes) was also used to align the 16S rRNA gene sequences based on their secondary structure information (Jeon et al. 2014). Phylogenetic analysis was performed with the software MEGAS.1 (Tamura et al., 2011) and evolutionary trees were reconstructed using both neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981) methods. The topology of the tree was evaluated by bootstrap analyses (Felsenstein, 1985) based on 1000 replications. The evolutionary distances were calculated using the Jukes–Cantor model for the neighbour-joining method (Jukes & Cantor, 1969). Strain PQ-2T exhibited the highest 16S rRNA gene sequence similarity to Croccicoccus marinus E4A9\(^T\) (96.3 %). Lower 16S rRNA gene sequence similarities were shown to members of the genera Altererythrobacter (92.9–95.6 %), Erythrobacter (93.3–95.4 %) and Porphyrobacter (94.2–94.5 %) in the family Erythrobacteraceae and to members of the genera Novosphingobium (92.5–95.6 %), Sphingopyxis (93.6–95.1 %), Sphingobium (91.4–94.9 %), Blastomonas (93.3–93.5 %) and Sphingomonas (90.2–93.9 %) in the family Sphingomonadaceae. The 16S rRNA gene sequence alignment based on secondary structure information showed a similar identity matrix pattern with slight differences. Specifically, based on the secondary structure, strain PQ-2T exhibited 96.6 % 16S rRNA gene sequence similarity with Croccicoccus marinus E4A9\(^T\). The neighbour-joining tree (Fig. 1) based on 16S rRNA gene sequence clearly showed strain PQ-2T clustered with Croccicoccus marinus E4A9\(^T\) and these two strains formed a distinct lineage within the family Erythrobacteraceae. The maximum-likelihood tree (Fig. S1, available with the online Supplementary Material) also confirmed its affiliation to the genus Croccicoccus. Erythromicrobium ramosum DSM 8510\(^T\) and some species of the genus Erythrobacter (Erythrobacter jejunesis CNU001\(^T\), Erythrobacter litoralis DSM 8509\(^T\) and Erythrobacter gaethuli SW-161\(^T\)) were not clustered with their genus, a problem that existed even after we adopted many statistical methods to reconstruct the phylogenetic tree. The taxonomic status of these separated species of the genus Erythrobacter may need to be re-evaluated. However, these problems in the genus Erythrobacter do not affect our conclusion that strain PQ-2T belongs to the genus Croccicoccus.

Cell morphology was examined using an optical microscope (SZM-168; Motic) and a transmission electron microscope (JEM-1230; JEOL) with cells grown for 3 days at 28 °C on P5Y3 agar (Huo et al., 2010). Cell motility was examined in semi-solid medium using the stab cultivation method (Dong & Cai, 2001). Salt requirement for growth was tested in R broth with various NaCl concentrations [0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8 and 9 % (w/v)]. Growth at 4, 10, 15, 25, 28, 32, 37, 45, 50 and 55 °C was tested in P5Y3 broth. The pH range for growth was determined in R broth that was adjusted to pH 4.0–10.0 (at 0.5 pH unit intervals) using appropriate biological buffers (MES for pH 4.0–6.5, PIPES for pH 7.0–7.5, Tricine for pH 8.0–9.0 and 3-(Cyclohexylamino)-2-hydroxy-1-propanesulfonic acid sodium salt CAPSO for pH 9.5–10.0) at a concentration of 50 mM. Evaluation of the pH values after autoclaving revealed only minor changes. Growth was also tested on MacConkey agar (Difco) and cetrimide agar (Difco) at 28 °C. After 3 days of incubation at 28 °C on P5Y3 agar, colonies are 0.5–1 mm in diameter, convex, opaque, circular, smooth and yellow-pigmented. Cells were non-motile and short rod-shaped (0.5–0.6 μm × 0.8–1.2 μm) (Fig. S2). Growth occurred at 15–50 °C (optimum, 32 °C) and at pH 6.5–9.5 (optimum, pH 7.0). Growth was detected in the presence of 0.5–8 % (w/v) NaCl; optimal growth occurred with 2 % (w/v) NaCl.

For physiological and biochemical tests, Croccicoccus marinus E4A9\(^T\) was used as reference strain and cultivated under the same conditions as strain PQ-2T. Pigment absorption spectrum analysis was performed by the method described by Xu et al. (2009). Cultures in the exponential phase of growth were scraped from the P5Y3 agar plates and resuspended in sterile water. Three millilitres of methanol/acetone (7:2, v/v) solution was added per millilitre of bacterial suspension and shaken vigorously for 1 h. The disrupted suspension was centrifuged to sediment cells and debris and the absorption spectrum of the supernatant was examined on a UV-5100B UV/Vis scanning spectrophotometer (Metash; absorption spectrum from 300 to 1000 nm). Catalase activity was determined by assessing bubble production by cells in 3 % (v/v) H\(_2\)O\(_2\) and oxidase activity was determined using 1 % (w/v) \(N, N\)-dimethyl-p-phenylenediamine dihydrochloride solution (Dong & Cai, 2001). Other physiological and biochemical characteristics, including enzyme activities, were determined at 28 °C using API 20 NE and API ZYM strips (bioMérieux). The utilization of single carbon sources was tested on a basal medium as described by Xu.
et al. (2009). All carbon sources (w/v: 0.2% sugars, 0.1% alcohols, 0.1% organic acids and 0.1% amino acids) were filter-sterilized before adding to the basal liquid medium. Resistance to antibiotics was tested using the disc-diffusion method (Dong & Cai, 2001) including following antibiotic discs (μg unless otherwise stated): ampicillin (10), bacitracin (0.04 IU), penicillin (10 IU), kanamycin (30), neomycin (30), erythromycin (15), nystatin (100), streptomycin (10),
tobramycin (10), carbenicillin (100), amoxicillin (10), cefoxitin (30), cefotaxime (30), novobiocin (30), macro-
dantin (300), chloromycetin (30), tetracycline (30), rifampi-
cin (5) and polymyxin (300 IU). The detailed physiological
and biochemical characteristics of strain PQ-2<sup>T</sup> are given in the
species description. A comparison of the phenotypic
characteristics between PQ-2<sup>T</sup> and *Croceicoccus marinus*
E4A9<sup>T</sup> is listed in Table 1.

Respiratory quinones were extracted from freeze-dried cells
(200 mg) with chloroform/methanol (2:1, v/v) and analysed by
HPLC-MS as described by Komagata & Suzuki (1987). The
polar lipids of strain PQ-2<sup>T</sup> and the reference strain
*Croceicoccus marinus* E4A9<sup>T</sup> were extracted and analysed by
Leibniz-Institute DSMZ (Braunschweig, Germany). For
cellular fatty acid analysis, strain PQ-2<sup>T</sup> and the reference
strain *Croceicoccus marinus* E4A9<sup>T</sup> were grown on R agar
plates at 28 °C for 72 h and fresh cells were harvested from
late-exponential growth phase according to the protocol
given by MIDI. Fatty acids were saponified, methylated and
identified by using the TSBA6 database of the MIDI system
(Sasser, 1990). The genomic DNA G+C content was determined
by reversed-phase HPLC method (Mesbah & Whitman, 1989).
Genomic DNA of strain PQ-2<sup>T</sup> was extracted and purified
with a genomic DNA isolation kit (MoBio). The purified
DNA was enzymically degraded into nucleosides by P1
nuclease and then treated using calf intestine alkaline phos-
phatase. The genomic DNA G+C content was determined
by reversed-phase HPLC and calculated from the deox-
yyguanosine : thymidine ratio.

Ubiquinone 10 (Q-10) was the sole respiratory quinone, in
line with *Croceicoccus marinus* E4A9<sup>T</sup>. The major polar
lipids profile of PQ-2<sup>T</sup> was composed of phosphatidy-
lethanolamine, phosphatidylcholine, phosphatidyglycerol,
one unidentified phospholipid and one sphingoglycolipid.
Furthermore, another sphingoglycolipid of minor amount
was identified. All these polar lipids were found in
*Croceicoccus marinus* E4A9<sup>T</sup>. However, another uniden-
tified phospholipid with moderate amount was only
detected in *Croceicoccus marinus* E4A9<sup>T</sup> (Fig. 2).

The major fatty acids (>10%) of strain PQ-2<sup>T</sup> were
C<sub>18:1ω7c</sub> (43.2%), C<sub>17:1ω6c</sub> (20.2%) and summed feature
3 (C<sub>16:1ω7c</sub> and/or iso-C<sub>15:0</sub> 2-OH) (12.3%). Compared
with *Croceicoccus marinus* E4A9<sup>T</sup>, strain PQ-2<sup>T</sup> had lower
amounts of C<sub>17:1ω6c</sub> and C<sub>15:0</sub> 2-OH but higher amounts
of C<sub>18:1ω7c</sub> and summed feature 3 (Table 2). The genomic
DNA G+C content of strains PQ-2<sup>T</sup> and *Croceicoccus marinus*
E4A9<sup>T</sup> were 61.7 and 64.5 mol%, respectively. These
values are different to that reported for previously
(Xu *et al.*, 2009). The description for *Croceicoccus marinus*
was emended and the fatty acid profile and the genomic
DNA G+C content was corrected.

In sum, PQ-2<sup>T</sup> could be assigned to the genus *Croceicoccus*
by the following characteristics. First and most importantly,
family Erythrobacteraceae. Second, the main chemotaxonomic characteristics between PQ-2T and the type species Croceicoccus marinus E4A9T were similar, including the major fatty acids and polar lipids profile, the sole respiratory quinone. Other common characteristics are listed in Table 1 in detail. Strain PQ-2T could also be differentiated from Croceicoccus marinus E4A9T on the basis of some phenotypic characteristics, including cell shape, presence of flagella, nitrate reduction, enzyme activities in the API tests, utilization of substrates, antibiotic susceptibility and the genomic DNA G+C content (Table 1). Based on the discussion above, strain PQ-2T should be classified as a novel species of the genus Croceicoccus, for which the name Croceicoccus naphthovorans sp. nov. is proposed.

Emended description of the genus Croceicoccus Xu et al. 2009

In addition to the characteristics reported by Xu et al. (2009), the following properties are observed. The polar lipid profiles comprise phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, sphingoglycolipid and unidentified phospholipid. The genomic DNA G+C content is 61.7–64.5 mol%.

Emended description of Croceicoccus marinus Xu et al. 2009

In addition to the characteristics reported by Xu et al. (2009), the following properties are observed. Cells are motile by means of polar flagella. Positive for oxidase production and negative for gelatin degradation. The following substrates are utilized for growth: L-arabinose, cellobiose and xylose. Major fatty acids are C_{17:1}ω6c, C_{18:1}ω7c and C_{15:0} 2-OH. The genomic DNA G+C content is 64.5 mol% (as determined by HPLC).

**Description of Croceicoccus naphthovorans sp. nov.**

*Croceicoccus naphthovorans* (naph.tho.vo’rans. Gr. n. naphtha oil; L. part. vorans devouring; N.L. part. adj. naphthovorans oil-degrading).

Cells are aerobic, Gram-stain-negative, non-motile and short rod-shaped (0.5–0.6 μm × 0.8–1.2 μm). After 3 days of incubation at 28 °C on P5Y3 agar, colonies are 0.5–1 mm in diameter, convex, opaque, circular, smooth and yellow-pigmented. Does not grow on MacConkey agar or cetrimide agar. Growth occurs at 15–50 °C (optimum, 32 °C), at pH 6.5–9.5 (optimum, pH 7.0) and with 0.5–8 % (w/v) NaCl (optimum, 2 %, w/v). Utilizes a variety of PAHs with two or three rings, including fluorene, fluoranthene and phenanthrene, as the sole carbon and energy sources. Contains carotenoids, but no bacteriochlorophyll a. The methanol-soluble pigment is characterized by absorption maxima at 450 and 478 nm. Positive for catalase and oxidase, hydrolysis of aesculin, nitrate reduction, alkaline phosphatase, leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase. Weakly positive for esterase, esterase lipase, valine arylamidase, trypsin and α-glucosidase reaction. Negative for indole production, fermentation of glucose, urease, gelatinase and arginine dihydrolase, lipase, cystine arylamidase, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. Utilizes cellobiose, D-fructose, galactose, glucose, glycerol, inositol, D-mannose, raffinose, rhamnose, sucrose, trehalose, L-alanine, L-glutamate, isoleucine, L-valine, citrate, propionate and pyruvate as sole carbon sources, but not L-arabinose, ethanol, maltose, mannitol, ribose, salicin, L-sorbitol, xylose, L-arginine, L-cysteine, L-histidine, lysine, L-methionine, L-serine, fumarate, gluconate,

Fig. 2. Two-dimensional TLCs showing the total polar lipids of strain: (a) PQ-2T and (b) Croceicoccus marinus E4A9T. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PL, phospholipid; SGL, sphingoglycolipid.
Table 2. Fatty acid composition of strain PQ-2T and reference strain Croceicoccus marinus E4A9T

<table>
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<th>Fatty acid (%)</th>
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<th>3</th>
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<tr>
<td>Saturated</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>C14:0</td>
<td>TR</td>
<td>ND</td>
<td>TR</td>
</tr>
<tr>
<td>C15:0</td>
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<td>1.8</td>
<td>3.5</td>
</tr>
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<td>C16:0</td>
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<td>1.3</td>
<td>1.5</td>
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<td>TR</td>
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<tr>
<td>iso-C17:0</td>
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<td>ND</td>
<td>TR</td>
</tr>
<tr>
<td>Hydroxy</td>
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<tr>
<td>C13:0-2-OH</td>
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<td>TR</td>
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<tr>
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<td>ND</td>
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<td>iso-C16:0-3-OH</td>
<td>1.4</td>
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<td>Unsaturated</td>
<td></td>
<td></td>
<td></td>
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<tr>
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<td>ND</td>
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<tr>
<td>C18:10:7c</td>
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<td>*Summed feature 3</td>
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*Summed features comprise two or three fatty acids that cannot be separated by the MIDI identification system. Summed feature 3 contained C16:10:7c and/or iso-C15:0-2-OH.

malate or malonate. Susceptible to ampicillin, penicillin, kanamycin, neomycin, carbenicillin, amoxicillin, cefoxitin, cefotaxime, novobiocin, macrodantin, chloromycetin and rifampicin. The sole respiratory quinone is ubiquinone 10 (Q-10). The predominant fatty acids are C18:1(ω7c), C17:1(ω6c) and summed feature 3 (C16:1(ω7c) and/or iso-C15:0-2-OH). In the polar lipid profile, phosphatidylethanolamine, phosphatidylycholine, phosphatidylglycerol, one unidentified phospholipid and one sphingoglycolipid are the major compounds; another sphingoglycolipid is present in minor amount.

The type strain PQ-2T (=CGMCC 1.12805T=NBRC 110381T) was isolated from marine biofilm collected from a boat shell at a harbour of Zhoushan island in Zhejiang Province, PR China. The genomic DNA G+C content of the type strain is 61.7 mol% (as determined by HPLC).

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References


