Paracoccus stylophorae sp. nov., isolated from the reef-building coral *Stylophora pistillata*

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A bacterial strain, designated KTW-16^T, was isolated from the reef-building coral *Stylophora pistillata*, collected from southern Taiwan. Strain KTW-16^T was a Gram-negative, facultatively anaerobic, pale-yellow, non-motile short rod. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain KTW-16^T belonged to the genus *Paracoccus* in the *Alphaproteobacteria* and exhibited 93.7–96.9% 16S rRNA gene sequence similarity with type strains of species of the genus *Paracoccus* (96.9% with *Paracoccus alcaliphilus* JCM 7364^T). Strain KTW-16^T grew at 15–40 °C (optimum 35 °C), at pH 6.0–10.0 (optimum pH 8.0) and with 0–9% NaCl (optimum 5%). The predominant cellular fatty acids were C_{18:1}ω7c, C_{19:0} cyclo ω8c and C_{18:0}. The major respiratory quinone was Q-10 and the DNA G+C content was 69.1 mol%. The polar lipid profile consisted of phosphatidylglycerol, diphosphatidylglycerol, phosphatidylcholine and several unknown polar lipids. The physiological and biochemical tests allowed clear phenotypic differentiation of the isolate from the type strains of already described *Paracoccus* species. It is evident from the genotypic, phenotypic and chemotaxonomic analysis that strain KTW-16^T should be classified in a novel species of the genus *Paracoccus*, for which the name *Paracoccus stylophorae* sp. nov. is proposed. The type strain is KTW-16^T (=LMG 25392^T =BCRC 80106^T).

The genus *Paracoccus*, first proposed by Davis *et al.* (1969), belongs to the family *Rhodobacteraceae* of the order *Rhodobacterales* in the *Alphaproteobacteria*. At the time of writing, the genus *Paracoccus* comprises 30 recognized species. Members of the genus *Paracoccus* are Gram-negative and oxidase- and catalase-positive and have a high metabolic versatility, a large amount of C_{18:1}ω7c and Q-10 as the predominant respiratory quinone. Members of this genus have been isolated from soil (Siller *et al.*, 1996; Tsubokura *et al.*, 1999), bioreactors (La *et al.*, 2005), activated sludge (Liu *et al.*, 2006), root nodules (Deng *et al.*, 2011), cornetfish (Kim *et al.*, 2010) and the marine environment (Berry *et al.*, 2003; Kim *et al.*, 2006; Liu *et al.*, 2008; Chen *et al.*, 2011), indicating that members of the genus *Paracoccus* may exist in various environments. At least one species has been identified as an occasional

pathogen of humans (Daneshvar *et al.*, 2003; Wallet *et al.*, 2010). The aim of the present study was to determine the taxonomic position of a *Paracoccus*-like strain, designated KTW-16^T, isolated from the reef-building coral *Stylophora pistillata*, collected off the coast of southern Taiwan.

A coral sample (50 g) was collected in a sterile bag at a depth of 3–8 m from the Kenting coast, Pingtung County, southern Taiwan (21° 52' 35" N 120° 43' 29" E), stored at 4 °C and transported to the laboratory within 3–4 h. The coral sample was ground to powder and plated on marine agar 2216 (MA; BD Difco) using the standard dilution-plating method. After incubation at 25 °C for 5 days, strain KTW-16^T was purified from a single colony. The strain was preserved at –80 °C as a 20% (v/v) glycerol suspension in marine broth 2216 (MB; BD Difco) or by lyophilization with 20% (w/v) skimmed milk. The most closely related type strain, *Paracoccus alcaliphilus* JCM 7364^T, was obtained from the Japan Collection of Microorganisms and used as a reference strain for phenotypic and genotypic tests.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain KTW-16^T is GQ281379.

A supplementary figure and a supplementary table are available with the online version of this paper.

Morphology of cells from lag, exponential and stationary phase was observed by phase-contrast microscopy (DM 2000; Leica). The motility of cells was tested by the hanging drop and semi-solid agar methods. The Spot Test flagella stain (BD Difco) was used for flagellum staining. The Gram stain set S kit (BD Difco) and the Ryu non-staining KOH method (Powers, 1995) were adopted for testing the Gram reaction. Poly- β -hydroxybutyrate granule accumulation was observed under light microscopy after staining with Sudan black. Colony morphology was examined using a stereoscopic microscope (SMZ 800; Nikon). The pH range for growth (at intervals of 1 pH unit) was examined in MB using biological buffers as follows: glycine/HCl, pH 3.0–4.0; citrate/ Na_2HPO_4 , pH 4.0–6.0; phosphate buffer, pH 6.0–8.0; and glycine/NaOH, pH 9.0–12.0. The pH was adjusted prior to sterilization and post-sterilization controls revealed only minor changes in pH. Growth with 0, 0.5 and 1.0–15.0 % (w/v) NaCl (in increments of 1.0 % NaCl) was tested in NaCl-free MB prepared according to the formula of the medium except that the NaCl concentration was altered as required. Growth at 4–45 °C was measured in MB. Cellular growth was determined by measuring the OD₆₀₀ of cultures. Anaerobic cultivation was performed on MA using the Oxoid AnaeroGen system.

Genomic DNA was isolated using a kit and the 16S rRNA gene sequence was analysed as described previously (Chen *et al.*, 2001). In brief, primers FD1 (5'-AGAGTTTGA-TCCTGGCTCAG-3') and RD1 (5'-CAGGCCTAACACATGCAA GTC-3') were used for PCR amplification. These primers correspond to nucleotide positions 8–27 and 1524–1540, respectively, of the *Escherichia coli* 16S rRNA gene and can be used to amplify nearly full-length sequences of the 16S rRNA gene. After purification of the PCR products, direct sequencing was performed using sequencing primers FD1, RD1, 520F and 800R (Anzai *et al.*, 1997) and an automated DNA sequencer (ABI Prism 3730; Applied Biosystems). An almost-complete 16S rRNA gene sequence (1401 nt) of strain KTW-16^T was compared against sequences available from the EzTaxon server (Chun *et al.*, 2007), the Ribosomal Database Project (Maidak *et al.*, 2001) and the GenBank database (<http://www.ncbi.nlm.nih.gov/BLAST/>). Sequence analysis was performed using BioEdit (Hall, 1999) and MEGA version 3.1 (Kumar *et al.*, 2004) after multiple alignment of the data by CLUSTAL X (Thompson *et al.*, 1997). Distances (corrected according to Kimura's two-parameter model; Kimura, 1983) were calculated and clustering was performed using the neighbour-joining method (Saitou & Nei, 1987). Maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Kluge & Farris, 1969) trees were generated using the treeing algorithms contained in the PHYLIP software package (Felsenstein, 1993). In each case, bootstrap values were calculated on the basis of 1000 replications. A comparison of the 16S rRNA gene sequence of strain KTW-16^T with those of members of the order *Rhodobacterales* in the *Alphaproteobacteria* showed that the strain fell within the evolutionary radiation occupied by the

genus *Paracoccus*. In the neighbour-joining phylogenetic tree, strain KTW-16^T formed a separate lineage within the genus *Paracoccus* and similar topologies were obtained in phylogenetic trees generated using the maximum-parsimony and maximum-likelihood algorithms (Fig. 1). According to the pairwise sequence comparisons, strain KTW-16^T exhibited 93.7–96.9 % 16S rRNA gene sequence similarity with type strains of species of the genus *Paracoccus*. Strain KTW-16^T was closely related to *P. alcaliphilus* JCM 7364^T (96.9 % 16S rRNA gene sequence similarity), *Paracoccus homiensis* DD-R11^T (96.5 %), *Paracoccus zeaxanthinifaciens* ATCC 21588^T (96.2 %) and *Paracoccus denitrificans* DSM 413^T (95.9 %). 16S rRNA gene sequence similarity between strain KTW-16^T and other members of the order *Rhodobacterales* was below 96.6 %.

The DNA G + C content of strain KTW-16^T was estimated as described by Mesbah *et al.* (1989). The nucleoside mixture was separated by means of HPLC. The DNA G + C content of strain KTW-16^T was 69.1 ± 1.0 mol%. Isoprenoid quinones were purified by the methods outlined in Minnikin *et al.* (1984) and analysed by HPLC as described by Collins (1985). The major isoprenoid quinone of strain KTW-16^T was ubiquinone 10 (Q-10).

Biomass of strain KTW-16^T and *P. alcaliphilus* JCM 7364^T was obtained after aerobic growth on MA at 30 °C for 3 days. Fatty acid methyl esters were prepared, separated and identified according to the instructions for the Microbial Identification System (Microbial ID; MIDI) (Sasser, 1990). The predominant fatty acids of strain KTW-16^T were C_{18:1} ω 7c (49.4 %), C_{19:0} cyclo ω 8c (22.7 %) and C_{18:0} (12.1 %). The fatty acid profile of strain KTW-16^T was similar to those of the type strains of other *Paracoccus* species, including *P. alcaliphilus* JCM 7364^T, *P. homiensis* DD-R11^T, *P. zeaxanthinifaciens* and *P. denitrificans* DSM 413^T, although there were differences in the proportions of some components: for example, in strain KTW-16^T, the quantities of C_{19:0} cyclo ω 8c and C_{18:0} were higher and the quantity of C_{18:1} ω 7c was lower (Table 1).

Polar lipids were extracted and analysed by two-dimensional TLC according to Tindall (1990) and Altenburger *et al.* (1996). Strain KTW-16^T exhibited a complex polar lipid profile consisting of phosphatidylglycerol (PG), diphosphatidylglycerol (DPG), phosphatidylcholine (PC), an unknown aminolipid (AL1), an unknown glycolipid (GL1) and four unknown phospholipids (PL2–5) (Supplementary Fig. S1, available in IJSEM Online). This profile was very similar to that of *P. alcaliphilus* JCM 7364^T: both the isolate and the reference strain contained PG, DPG, PC, AL1, GL1 and PL2–4. However, unknown phospholipid PL1 was absent in strain KTW-16^T but present in *P. alcaliphilus* JCM 7364^T and unknown phospholipid PL5 was present in strain KTW-16^T but absent in *P. alcaliphilus* JCM 7364^T. Thus, there were differences as well as similarities in the polar lipid profiles between the isolate and the reference strain.

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of strain KTW-16^T in the genus *Paracoccus*. Bootstrap values (>50%) based on 1000 replications are shown at branch nodes (above, neighbour joining; below, maximum parsimony). Filled circles indicate that the corresponding nodes were also recovered in trees generated using the maximum-likelihood and maximum-parsimony algorithms. Open circles indicate that the corresponding nodes were also recovered in the tree generated with the maximum-parsimony algorithm. *Roseobacter denitrificans* OCh 114^T was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

Strain KTW-16^T was examined for a broad range of phenotypic properties. Catalase, oxidase, DNase, lipase and hydrolysis of starch, casein and Tweens 20, 40, 60 and 80 were determined according to standard methods (Gerhardt *et al.*, 1994; MacFaddin, 2000). Hydrolysis of alginate (1% w/v sodium alginate) was tested on MA (Hosoya *et al.*, 2009). The API 20 NE, API ZYM (bioMérieux) and GN2 MicroPlate (Biolog) systems were used to determine the biochemical properties, enzyme activities and carbohydrate utilization pattern of strain KTW-16^T. API ZYM tests were performed according to the manufacturer's recommendation and were read after 4 h at 37 °C. For GN2 MicroPlate and API 20 NE tests, because strain KTW-16^T required 5% NaCl for optimum growth, cells were suspended in artificial seawater (containing l⁻¹ distilled water: 24 g NaCl, 5.1 g MgCl₂, 4 g Na₂SO₄, 1.1 g CaCl₂, 0.7 g KCl, 0.2 g NaHCO₃, 0.1 g KBr, 0.027 g H₃BO₃, 0.024 g SrCl₂, 0.003 g NaF; Lyman & Fleming, 1940) and results were read after 72 h at 30 °C.

Sensitivity to antibiotics was by spreading cells (0.5 McFarland standard) on MA and placing antibiotic discs on the surface of the agar (µg per disc): ampicillin (10), chloramphenicol (30), gentamicin (10), kanamycin (30), nalidixic acid (30), novobiocin (30), penicillin G (10), rifampicin (5), streptomycin (10) and tetracycline (30). The effects of antibiotics on cell growth were assessed after 3 days at 30 °C. The diameter of each disc was 8 mm and the strain was considered susceptible if the diameter of the inhibition zone was >13 mm, partly susceptible at 10–12 mm and resistant at <10 mm, as described by Nokhal & Schlegel (1983).

The physiological, biochemical and morphological characteristics of strain KTW-16^T are given in the species description, Tables 1 and 2, Supplementary Table S1 and Supplementary Fig. S1. Phenotypic examinations revealed many common traits between the isolate and its closest relatives, *P. alcaliphilus* JCM 7364^T, *P. homiensis* DD-R11^T, *P. zeaxanthinifaciens* R-1512^T and *P. denitrificans* DSM 413^T. All strains were Gram-negative, heterotrophic and oxidase- and catalase-positive. They contained large amounts of C_{18:1}ω7c, the main respiratory quinone was Q-10 and the DNA G+C content was 63–69 mol%. However, strain KTW-16^T could be differentiated clearly from all of the reference strains by its inability to grow at 10 °C, its ability to assimilate *N*-acetylglucosamine and maltose (Table 2), some differences in phospholipid components (Supplementary Fig. S1), its ability to utilize 2-aminoethanol, 2,3-butanediol, α-D-glucose 1-phosphate and D-glucose 6-phosphate and its inability to utilize succinic acid monomethyl ester, β-hydroxybutyric acid, DL-lactic acid and L-proline as sole carbon sources (Supplementary Table S1). Strain KTW-16^T could also be differentiated from each of the reference strains by a number of features: for example, from *P. alcaliphilus* JCM 7364^T by hydrolysis of gelatin, aesculin and DNA; from *P. homiensis* DD-R11^T by colony colour, motility, maximum NaCl concentration for growth, the presence of poly-β-hydroxybutyrate granules, reduction of nitrate to nitrite, β-galactosidase and hydrolysis of starch; from *P. zeaxanthinifaciens* R-1512^T by colony colour, NaCl concentration range for growth, reduction of nitrate to

Table 1. Cellular fatty acid compositions of strain KTW-16^T and closely related members of the genus *Paracoccus*

Strains: 1, *Paracoccus stylophorae* sp. nov. KTW-16^T; 2, *P. alcaliphilus* JCM 7364^T; 3, *P. homiensis* DD-R11^T; 4, *P. zeaxanthinifaciens* R-1512^T; 5, *P. denitrificans* DSM 413^T. Data in columns 1 and 2 were obtained in this study and those in columns 3–5 were taken from Chen *et al.* (2011). All strains were grown on MA at 30 °C for 3 days. Values are percentages of total fatty acids; fatty acids that made up <1% of the total in all strains are not shown.

Fatty acid	1	2	3	4	5
C _{10:0} 3-OH	2.0	4.4	4.1	7.8	4.3
C _{11:0} 2-OH	—	—	—	—	1.1
anteiso-C _{12:0}	—	—	—	—	3.2
anteiso-C _{13:0}	—	—	—	—	1.5
C _{16:0}	1.8	1.7	—	2.1	16.2
iso-C _{17:0} 3-OH	—	1.1	—	—	—
C _{18:0}	12.1	8.3	4.4	6.0	4.9
C _{18:0} 3-OH	1.0	—	—	1.9	—
C _{18:1} ω7c	49.4	70.9	80.4	72.8	57.1
C _{18:1} ω9c	—	—	—	—	1.4
11-Methyl C _{18:1} ω7c	5.2	1.0	5.1	—	—
C _{19:0} cyclo ω8c	22.7	5.4	—	—	—
Summed features*					
2	2.0	4.6	1.7	5.4	3.6
3	—	—	—	2.8	1.2

*Summed features represent groups of two or three fatty acids that cannot be separated by GLC using the Microbial Identification System. Summed feature 2 consisted of C_{14:0} 3-OH and/or iso-C_{16:1}. I. Summed feature 3 consisted of C_{16:1}ω7c and/or C_{16:1}ω6c.

nitrite, β-galactosidase and hydrolysis of gelatin, urea and Tweens 20 and 80; and from *P. denitrificans* DSM 413^T by colony colour, maximum NaCl concentration for growth, reduction of nitrate to nitrite and nitrite to nitrogen and hydrolysis of gelatin, aesculin and urea.

Strain KTW-16^T was halophilic and could reduce nitrate. Most members of the genus *Paracoccus* are not halophilic and cannot reduce nitrate. Only ten species are halophilic, *Paracoccus haeundaensis*, *P. seriniphilus*, *P. fistulariae*, *P. halophilus*, *P. zeaxanthinifaciens*, *P. homiensis*, *P. saliphilus*, *P. aestuarii*, *P. marinus* and *P. isopora*, and, of these, only the first three can reduce nitrate. Colony colour distinguished strain KTW-16^T from *P. haeundaensis* and *P. seriniphilus*; colonies of strain KTW-16^T were pale yellow whereas *P. haeundaensis* and *P. seriniphilus* form orange-red and creamy white colonies, respectively. Strain KTW-16^T could be distinguished from *P. fistulariae* by several phenotypic features, such as the ability to hydrolyse Tweens 20, 40, 60 and 80, the presence of esterase lipase (C8) and the absence of esterase (C4), naphthol-AS-BI-phosphohydrolase and α-glucosidase. Therefore, strain KTW-16^T is a member of the genus *Paracoccus* but can be separated from other members of the genus by some phenotypic differences.

Table 2. Differential characteristics of strain KTW-16^T and closely related members of the genus *Paracoccus*

Strains: 1, *Paracoccus stylophorae* sp. nov. KTW-16^T; 2, *P. alcaliphilus* JCM 7364^T; 3, *P. homiensis* DD-R11^T; 4, *P. zeaxanthinifaciens* R-1512^T; 5, *P. denitrificans* DSM 413^T. Data in columns 1 and 2 were obtained in this study, unless stated otherwise; those in columns 3–5 were taken from Chen *et al.* (2011). All strains are Gram-negative and oxidase- and catalase-positive. PHB, Poly-β-hydroxybutyrate.

Characteristic	1	2	3	4	5
Colony colour*	PY	PY	DY	DO	CW
Motility	—	—	+	—	—
Growth at/with:					
NaCl (% w/v)	0–9	0–8	0–14	3–8	0–7
pH	6–10	6–10	5–9	6–9	6–8
Temperature (°C)	15–40	10–40	10–40	10–40	10–40
PHB accumulation	+	+	—	+	+
Reduction of nitrate to:					
Nitrite	+	+	—	—	—
N ₂	—	—	—	—	+
β-Galactosidase	—	—	+	+	—
Hydrolysis of:					
Gelatin	+	—	+	—	—
Aesculin	+	—	+	+	—
Urea	—	—	—	+	+
Tweens 20 and 80	—	—	—	+	—
Starch	—	—	+	—	—
DNA	+	—	+	+	+
Assimilation of:					
Glucose	+	+	—	—	—
Arabinose	+	+	—	—	—
Mannose	+	+	—	—	—
Mannitol	+	+	—	—	—
N-Acetylglucosamine	+	—	—	—	—
Maltose	+	—	—	—	—
Malate	+	+	—	—	—
DNA G + C content (mol%)	69.1	64.4†	63.0	67.6	66.5

*CW, Creamy white; DO, deep orange; DY, deep yellow; PY, pale yellow.

†Data from Urakami *et al.* (1989).

On the basis of 16S rRNA gene sequence comparisons, strain KTW-16^T occupies a distinct position within the genus *Paracoccus*. This phylogenetic insight is supported by the unique combination of chemotaxonomic characteristics and biochemical traits of the isolate (Tables 1 and 2). Therefore, it is clear from the phylogenetic and phenotypic data that strain KTW-16^T constitutes a novel member of the genus *Paracoccus*. The name *Paracoccus stylophorae* sp. nov. is proposed for this taxon.

Description of *Paracoccus stylophorae* sp. nov.

Paracoccus stylophorae (sty.lo.pho'ra.e. N.L. gen. n. *stylophorae* of *Stylophora*, referring to the isolation of the type strain from a coral belonging to the genus *Stylophora*).

Cells are Gram-negative, facultatively anaerobic, short rods (0.9–1.0 µm wide and 1.0–1.2 µm long) and are non-motile. Colonies on MA are pale yellow, circular and convex with entire edges, 0.4–0.5 mm in diameter on MA after 24 h incubation at 30 °C. Growth occurs at 15–40 °C, with 0–9 % NaCl and at pH 6.0–10.0. Optimum growth occurs at 35 °C, with 5 % NaCl and at pH 8.0. Poly-β-hydroxybutyrate granule accumulation is observed. Positive for oxidase, catalase and DNase activities. Negative for lipase (corn oil) activity and hydrolysis of casein, starch, alginate and Tweens 20, 40, 60 and 80. With API 20 NE, positive for reduction of nitrate, hydrolysis of aesculin and gelatin and assimilation of glucose, arabinose, mannose, mannitol, *N*-acetylglucosamine, maltose, gluconate, malate, citrate and phenylacetate, but negative for production of indole, fermentation of *D*-glucose, arginine dihydrolase, urease and β-galactosidase and assimilation of caprate and adipate. With API ZYM, positive for alkaline phosphatase, esterase lipase (C8), leucine arylamidase and acid phosphatase, but negative for esterase (C4), lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, naphthol-AS-BI-phosphohydrolase, α- and β-galactosidase, β-glucuronidase, α- and β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. With GN2 MicroPlates, utilizes Tweens 40 and 80, *D*-galactonic acid lactone, *L*-histidine, urocanic acid, 2-aminoethanol, 2,3-butanediol, glycerol, *DL*-α-glycerol phosphate, α-*D*-glucose 1-phosphate and *D*-glucose 6-phosphate; all other substrates included in the Biolog GN2 MicroPlate are not utilized. Sensitive to rifampicin, nalidixic acid, kanamycin, chloramphenicol, gentamicin, novobiocin, tetracycline, ampicillin and penicillin G, but resistant to streptomycin. The predominant cellular fatty acids are *C*_{18:1ω7c}, *C*_{19:0} cyclo *ω*8c and *C*_{18:0}. The major respiratory quinone is Q-10. The polar lipids are phosphatidylglycerol, diphosphatidylglycerol, phosphatidylcholine, an unknown aminolipid, an unknown glycolipid and four unknown phospholipids. The DNA G + C content of the type strain is 69.1 mol%.

The type strain, KTW-16^T (=LMG 25392^T =BCRC 80106^T), was isolated from the reef-building coral *Stylophora pistillata*, collected off the coast of southern Taiwan.

Acknowledgements

This study was funded by grants from the Academia Sinica (thematic grant 20082010), Taipei, Taiwan, Republic of China.

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