Croceicoccus marinus gen. nov., sp. nov., a yellow-pigmented bacterium from deep-sea sediment, and emended description of the family *Erythrobacteraceae*

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A Gram-negative, aerobic, neutrophilic, coccoid bacterium, strain E4A9^T, was isolated from a deep-sea sediment sample collected from the East Pacific polymetallic nodule region. 16S rRNA gene sequence analysis showed that the isolate was related to the type strain of *Altererythrobacter epoxidivorans* (96.0 % sequence similarity). Lower 16S rRNA gene sequence similarities were observed with other members of the genera *Altererythrobacter* (94.7 %), *Erythrobacter* (94.0–95.4 %), *Erythromicrobium* (94.8 %) and *Porphyrobacter* (94.6–95.1 %) of the family *Erythrobacteraceae*. Phylogenetic analysis including all described species of the family *Erythrobacteraceae* and several members of the family *Sphingomonadaceae* revealed that the isolate formed a distinct phylogenetic lineage with the family *Erythrobacteraceae*. Chemotaxonomic analysis revealed ubiquinone-10 as the predominant respiratory quinone, anteiso-$C_{15:0}$, iso-$C_{14:0}$ and iso-$C_{15:0}$ as major fatty acids, and phosphatidylglycerol as the major polar lipid. The DNA G+C content was 71.5 mol%. The isolate contained carotenoids, but no bacteriochlorophyll *a*. On the basis of phenotypic and genotypic data presented in this study, strain E4A9^T represents a novel species in a new genus in the family *Erythrobacteraceae* for which the name *Croceicoccus marinus* gen. nov., sp. nov. is proposed; the type strain is E4A9^T (=CGMCC 1.6776^T=JCM 14846^T).

The family *Erythrobacteraceae* (order *Sphingomonadales*, class *Alphaproteobacteria*) was proposed by Lee *et al.* (2005) based on a comprehensive phylogenetic analysis and, at present, it comprises four recognized genera: *Altererythrobacter* (Kwon *et al.*, 2007), *Erythrobacter* (Shiba & Simidu, 1982), *Erythromicrobium* (Yurkov *et al.*, 1994) and *Porphyrobacter* (Fuerst *et al.*, 1993).

Abbreviation: BChl *a*, bacteriochlorophyll *a*.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain E4A9^T is EF623998.

Additional figures showing the phylogenetic tree based on 16S rRNA gene sequences constructed using the maximum-parsimony method, the two-dimensional TLC of the total polar lipids of strain E4A9^T and absorption spectra of cells of strain E4A9^T are available with the online version of this paper.

Members of the family *Erythrobacteraceae* are aerobic and produce pink, orange or yellow pigments. Ubiquinone-10 is the major respiratory quinone. Most species in the family have been isolated from various aquatic environments such as freshwater, seawater, marine mats or sediment, a hot spring, seaweed, a starfish and coral (Shiba & Simidu, 1982; Fuerst *et al.*, 1993; Yurkov *et al.*, 1994; Hanada *et al.*, 1997; Denner *et al.*, 2002; Hiraishi *et al.*, 2002; Rainey *et al.*, 2003; Yoon *et al.*, 2003, 2004a, b, 2005a, b, 2006; Ivanova *et al.*, 2005; Kwon *et al.*, 2007). This study focuses on the description of strain E4A9^T, isolated from a deep-sea sediment sample. Based on the taxonomic data, it is proposed that this strain be included in a new genus within the family *Erythrobacteraceae*.

The deep-sea sediment samples were collected by a multicorer from the East Pacific polymetallic nodule region (8° 22′ 38″ N 145° 23′ 56″ W) at a depth of

5280 m (temperature 2 °C, salinity 34 ‰) from the vessel *Da Yang Yi Hao*. On board the ship, all sediment samples were subsampled aseptically and stored at −20 °C until use.

Approximately 100 mg sediment subsample was incubated for 3 days in ZoBell marine-Casamino acids (ZMCA) medium, which contained (per litre distilled water): NaCl, 19.45 g; $MgCl_2$, 8.8 g; Na_2SO_4, 3.24 g; $CaCl_2$, 1.8 g; KCl, 0.55 g; $NaHCO_3$, 0.16 g; $C_6H_5FeO_7 . 5H_2O$, 0.1 g; KBr, 0.08 g; $CsCl_2$, 34 mg; H_3BO_3, 22 mg; Na_2SiO_3, 4.0 mg; NaF, 2.4 mg; NH_4NO_3, 1.6 mg; Na_3PO_4, 8.0 mg; peptone (Difco), 0.5 g; yeast extract (Difco), 0.1 g; and Casamino acids (Difco), 0.1 g; pH 7.2 (ZoBell, 1941). The liquid was then plated on ZMCA agar plates using a tenfold dilution series method. After several days of aerobic incubation at 25 °C, a yellow colony, designated strain E4A9^T, was picked. The strain was purified by repeated restreaking; purity was confirmed by the uniformity of colony morphology. Unless otherwise stated, strain E4A9^T was maintained on rich (R) medium at 28 °C. R medium contained (per litre distilled water): NaCl, 30 g; $MgSO_4 . 7H_2O$, 2.46 g; KCl, 1.5 g; $CaCl_2 . 2H_2O$, 0.15 g; NaBr, 0.1 g; $FeCl_2 . 2H_2O$, 0.016 g; yeast extract (Difco), 2.0 g; peptone (Difco), 5.0 g; and Casamino acids (Difco), 1.0 g; pH 7.2.

The 16S rRNA gene was amplified and analysed as described previously (Xu *et al.*, 2007b). The sequence was compared with closely related sequences of reference organisms from the FASTA and EZTAXON services (Chun *et al.*, 2007). Sequence data were aligned with CLUSTAL W 1.8 (Thompson *et al.*, 1994). Phylogenetic trees were constructed by the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony methods (Fitch, 1971) with the MEGA3 program package (Kumar *et al.*, 2004) and by the maximum-likelihood method (Felsenstein, 1981) with the TREEPUZZLE 5.2 program. Evolutionary distances were calculated according to the algorithm of the Kimura two-parameter model (Kimura, 1980) for the neighbour-joining method.

The almost complete 16S rRNA gene sequence (1450 nt) of strain E4A9^T was obtained. Comparisons with sequences from representative bacteria with validly published names indicated that strain E4A9^T belonged to the order *Sphingomonadales*. The strain was most closely related to members of the genera *Altererythrobacter* (94.7–96.0 % similarity), *Erythrobacter* (94.0–95.4 %), *Erythromicrobium* (94.8 %) and *Porphyrobacter* (94.6–95.1 %) in the family *Erythrobacteraceae* and moderately related to members of the genera *Blastomonas* (94.9 %), *Novosphingobium* (93.5–95.5 %), *Sandaracinobacter* (91.1 %), *Sandarakinorhabdus* (91.4 %), *Sphingobium* (91.3–92.2 %), *Sphingomonas* (90.4–95.2 %), *Sphingopyxis* (92.7–95.4 %), *Sphingosinicella* (93.1–93.2 %) and *Zymononas* (91.8 %) in the family *Sphingomonadaceae*. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain E4A9^T formed a distinct phylogenetic lineage with respect to the family *Erythrobacteraceae* and could not be associated with any of the recognized genera in the family (Fig. 1). The topologies of the phylogenetic trees constructed using the maximum-likelihood and maximum-parsimony algorithms also supported the notion that the isolate could represent a genus that was phylogenetically distinct from closely related genera in the family *Erythrobacteraceae* (Fig. 1 and Supplementary Fig. S1, available in IJSEM Online).

The optimal conditions for growth were determined in R medium containing various NaCl concentrations (0, 0.5, 1.0, 3.0, 5.0, 7.5, 10.0, 15.0 and 20.0, w/v). The pH range for growth was determined by adding MES (pH 5.0–6.0), PIPES (pH 6.5–7.0), Tricine (pH 7.5–8.5) or CAPSO (pH 9.0–10.0) to R medium at concentrations of 25 mM. The temperature range for growth was determined by incubating cells at various temperatures (4–48 °C). Cell morphology and motility were examined by optical microscopy (BX40; Olympus) and electron microscopy (S260, Cambridge; JEM-1230, JEOL). Pigment absorption spectrum analysis was performed by the method described by Rainey *et al.* (2003) using a Beckman Coulter DU 800 spectrophotometer.

Single carbon source assimilation tests were performed using a basal medium (Kämpfer *et al.*, 1991). The basal medium contained (per litre distilled water): NaCl, 9.0 g; $MgSO_4 . 7H_2O$, 0.5 g; $CaCl_2 . 2H_2O$, 0.1 g; K_2HPO_4, 1.74 g; KH_2PO_4, 1.36 g; $(NH_4)_2SO_4$, 5 g; yeast extract (Difco), 0.02 g; peptone (Difco), 0.02 g; vitamin mixture solution, 1 ml; mineral salts mixture solution, 5 ml; and 25 mM PIPES; pH 7.2. All carbon sources (w/v: 0.2 % sugars; 0.1 % alcohols; 0.1 % organic acids; or 0.1 % amino acids) were filter-sterilized before addition to the basal liquid medium. The vitamin mixture contained (per litre distilled water): biotin, 2 mg; folic acid, 2 mg; pyridoxine hydrochloride, 10 mg; riboflavin, 5 mg; thiamine, 5 mg; nicotinic acid, 5 mg; pantothenic acid, 5 mg; vitamin B_{12}, 0.1 mg; *p*-aminobenzoic acid, 5 mg; and thioctic acid, 5 mg (Wolin *et al.*, 1963). The mineral salts mixture contained (per litre distilled water): nitrilotriacetic acid, 1.5 g; $MgSO_4 . 7H_2O$, 3.0 g; $MnSO_4 . 2H_2O$, 0.5 g; NaCl, 1.0 g; $FeSO_4 . 7H_2O$, 0.1 g; $CoCl_2 . 6H_2O$, 0.1 g; $CaCl_2 . 2H_2O$, 0.1 g; $ZnCl_2$, 0.13 g; $CuSO_4 . 5H_2O$, 0.01 g; $AlK(SO_4)_2$, 0.01 g; H_3BO_3, 0.01 g; Na_2MoO_4, 0.025 g; $NiCl_2 . 6H_2O$, 0.024 g; and $Na_2WO_4 . 2H_2O$, 0.025 g (Balch *et al.*, 1979). Modified R medium was also used to confirm the assimilation of substrates. Modified R medium contained (per litre distilled water): NaCl, 30 g; $MgSO_4 . 7H_2O$, 2.46 g; KCl, 1.5 g; $CaCl_2 . 2H_2O$, 0.15 g; NaBr, 0.1 g; $FeCl_2 . 2H_2O$, 0.016 g; and Casamino acids (Difco), 0.01 g; pH 7.2. Acid production was tested using modified MOF medium supplemented with 0.5 % sugars or alcohols (Leifson, 1963; Xu *et al.*, 2008). Biochemical tests were performed using the methods described by Mata *et al.* (2002).

Fatty acid methyl esters were obtained from cells grown in R medium for 24 h at 28 °C and analysed by using GC-MS (Kuykendall *et al.*, 1988). Isoprenoid quinones were

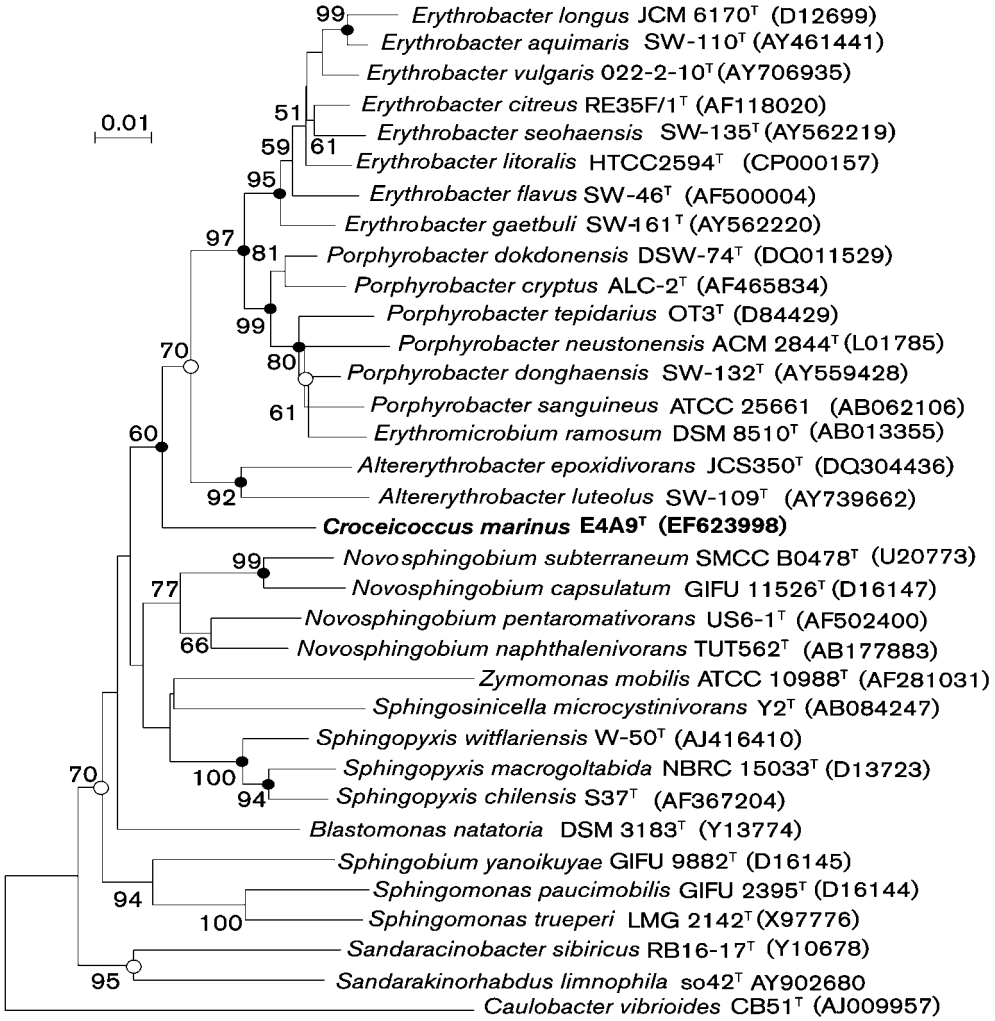

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships between the novel isolate and related taxa. Bootstrap values are based on 1000 replicates; only values >50 % are shown. Filled circles indicate that nodes were recovered with bootstrap values >50 % in both maximum-likelihood and maximum-parsimony trees. Open circles indicate nodes were recovered with bootstrap values >50 % in the maximum-parsimony tree. Bar, 0.01 substitutions per nucleotide position.

extracted from freeze-dried cells (200 mg) with chloroform/methanol (2 : 1) and analysed by reversed-phase HPLC. Total lipids were extracted by the modified method of Kamekura & Kates (1988). Phospholipids and glycolipids were separated on silica gel plates (10 × 10 cm) by TLC and analysed according to Xu *et al.* (2007a). The purified DNA was hydrolysed with P1 nuclease and the nucleotides were dephosphorylated with calf intestine alkaline phosphatase (Mesbah & Whitman, 1989). The G+C content of the resulting deoxyribonucleosides was determined by reversed-phase HPLC and calculated from the deoxyguanosine/thymidine ratio (Mesbah & Whitman, 1989).

Strain E4A9^T was Gram-negative, chemoheterotrophic, facultatively aerobic, catalase-positive and oxidase-negative. Cells were motile, irregular cocci that possessed multifibrillar stalk-like fascicle structures on the cell surface (Fig. 2). An electron micrograph of negatively stained cells did not reveal flagella. Strain E4A9^T occurred in clusters of two to four cells (Fig. 2a). Cell division occurred via fission (Fig. 2b). The polar lipid profiles included phosphatidylglycerol and two unidentified glycolipids as the major compounds and minor to trace amounts of phosphatidylcholine and an unidentified phospholipid (see Supplementary Fig. S2, available in IJSEM Online). The major fatty acids (>1 %) of strain E4A9^T were anteiso-$C_{15:0}$ (73.3 %), iso-$C_{15:0}$ (8.3 %), iso-$C_{14:0}$ (7.0 %), iso-$C_{16:0}$ (4.1 %), $C_{16:0}$ (2.5 %), anteiso-$C_{13:0}$ (1.8 %) and anteiso-$C_{15:0}$ (1.3 %). Detailed results of the phenotypic and biochemical tests are given in the species description.

Strain E4A9^T could be differentiated from members of other genera of the family *Erythrobacteraceae* by several

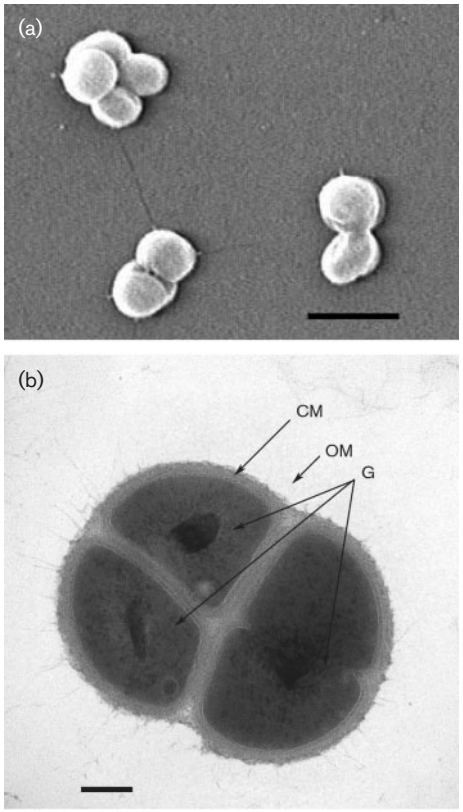

Fig. 2. Scanning electron micrograph (a; bar, 2 μm) and transmission electron micrograph of an ultrathin section (b; bar, 0.2 μm) of exponentially grown cells of strain E4A9^T showing the unique cell division pattern. OM, Outer membrane; CM, cytoplasmic membrane; G, intracellular granules.

phenotypic characteristics and DNA G+C content, as well as by its fatty acid constituents (Table 1). Members of the genera *Erythromicrobium* and *Porphyrobacter*, as well as *Erythrobacter longus* and *Erythrobacter litoralis*, synthesized bacteriochlorophyll *a* (BChl *a*) under aerobic conditions and displayed absorption peaks at about 800 and 870 nm. The absence of characteristic peaks between 770 and 900 nm in membrane preparations and their extracts indicated that strain E4A9^T lacked BChl *a* and differed from most species within the family *Erythrobacteraceae* (see Supplementary Fig. S3, available in IJSEM Online). The DNA G+C content of strain E4A9^T was 71.5 mol%, which was 11–17 mol% higher than those of the members of the genus *Altererythrobacter*. The major fatty acid constituents of strain E4A9^T were short- and branched-chain fatty acids, unlike those found in other members of the family *Erythrobacteraceae* (Table 1). The biochemical characteristics and carbon source utilization patterns also clearly differentiated strain E4A9^T from members of other genera of the family *Erythrobacteraceae* (Table 1).

On the basis of the phenotypic and phylogenetic data presented in this study, strain E4A9^T represents a novel species in a new genus within the family *Erythrobacteraceae* for which the name *Croceicoccus marinus* gen. nov., sp. nov. is proposed.

Emended description of the family *Erythrobacteraceae* Lee *et al.* 2005

The description is identical to that given by Lee *et al.* (2005) with the following amendments. Gram-negative, rod-shaped or pleomorphic coccoid bacteria. Cells contain carotenoids and most species contain BChl *a*.

Description of *Croceicoccus* gen. nov.

Croceicoccus [Cro.ce.i.coc′cus. L. adj. *croceus* yellow, golden; N.L. masc. n. *coccus* (from Gr. masc. n. *kokkos*) grain or berry; N.L. masc. n. *Croceicoccus* referring to a yellow coccoid-shaped bacterium].

Gram-negative and non-spore-forming cocci. Divide by binary division. Capable of producing multifibrillar stalk-like fascicle structures on the cell surface. Contains carotenoids, but no BChl *a*. Aerobic and chemoheterotrophic. No growth occurs anaerobically in the light. Ubiquinone-10 is the major respiratory quinone. The polar lipid profiles comprise phosphatidylglycerol, two unidentified glycolipids, phosphatidylcholine and an unidentified phospholipid. The phylogenetic position is in the α-4 subgroup of the class *Alphaproteobacteria*. The type species is *Croceicoccus marinus*.

Description of *Croceicoccus marinus* sp. nov.

Croceicoccus marinus (ma.ri′nus. L. masc. adj. *marinus* of or belonging to the sea, marine).

Shows the following characteristics in addition to those given in the genus description. Cells are motile and occur in pairs. Young cultures show pleomorphic coccoid cells (0.8–1.0 μm). Colonies on complex agar containing peptone, Casamino acids and yeast extract are 1–2 mm in diameter, circular, smooth, elevated, opaque and yellow-pigmented after 48 h at 25 °C. The methanol-soluble pigment is characterized by absorption maxima at 399, 423 and 452 nm. Growth occurs at NaCl concentrations of 0–10.0 % (w/v), with optimum growth at 0–1.0 % (w/v) NaCl. Grows at pH 6.0–9.0 and 4–42 °C; optimum growth is at pH 7.0 and 25 °C. Oxidase-negative and catalase-positive. Does not grow on MacConkey agar or cetrimide agar. Aesculin, casein, gelatin, starch, Tween 20, Tween 80 and tyrosine are hydrolysed. Lysine decarboxylase and the Voges–Proskauer reaction are positive. Negative for DNase, gluconate oxidation, indole production, methyl red test, *o*-nitrophenyl-β-D-galactopyranosidase, ornithine decarboxylase and urease. H_2S is not produced from thiosulfate. The following substrates are utilized for growth: acetate, L-alanine, ethanol, fumarate, glucose, L-glutamate, glycerol, L-histidine, lactate, malate, maltose, D-mannose, propionate, pyruvate, L-serine, succinate, sucrose and trehalose.

Table 1. Phenotypic characteristics that differentiate strain E4A9^T from other related members of the family *Erythrobacteraceae*

Strains/taxa: 1, strain E4A9^T; 2, *Altererythrobacter* (Yoon *et al.*, 2005b; Kwon *et al.*, 2007); 3, *Erythrobacter* (Shiba & Simidu, 1982; Yurkov *et al.*, 1994; Denner *et al.*, 2002; Yoon *et al.*, 2003, 2004a, 2005a; Ivanova *et al.*, 2005); 4, *Erythromicrobium* (Yurkov *et al.*, 1994; Rainey *et al.*, 2003); 5, *Porphyrobacter* (Fuerst *et al.*, 1993; Hanada *et al.*, 1997; Hiraishi *et al.*, 2002; Rainey *et al.*, 2003; Yoon *et al.*, 2004b, 2006). Data refer to all species within the genera. +, Positive; −, negative; (+) and (−), positive or negative results, respectively, reported for half or more species, but no data available for the others; NA, no data available; NA (−), negative results reported for some species, but no data available for most species; V, variable reaction; V (+) and V (−), variable, but more than half of the species display positive or negative results, respectively.

Characteristic	1	2	3	4	5
Cell shape*	C	O/R	R	R	C/O/R
Colour of colony†	Y	Y	Y/O/R	O	O/R
Motility	+	−	V (−)	+	V
Presence of BChl *a*	−	−	V (−)	+	+
Growth in NaCl (%):					
Range	0–10	0–9	0.1–9.0	NA	0–7
Optimum	0–1	2	0.2–5.0	NA	1–2
Growth pH:					
Range	6.0–9.0	5.5–8.5	6.0–10.0	NA	6.0–9.0
Optimum	7.0	6.5–8.0	6.5–8.5	7.0–8.5	6.5–8.5
Growth temperature (°C):					
Range	4–42	4–40	10–43	NA	10–55
Optimum	25	30–35	25–37	25–30	30–50
Catalase	+	+	+	+	(+)
Oxidase	−	+	+	+	V (+)
H_2S production	−	−	(−)	NA	NA (−)
Hydrolysis of:					
Aesculin	+	V	V (+)	NA	V (+)
Casein	+	(+)	(−)	NA	V (−)
Gelatin	+	−	V (−)	−	V (−)
Starch	+	(+)	V (−)	−	V (+)
Tween 80	+	+	(+)	−	V (+)
Acid production from:					
Glucose	+	(−)	V (−)	NA	NA (−)
Maltose	+	(−)	V (+)	NA	V
Sucrose	+	(−)	V (−)	NA	NA (−)
Trehalose	+	V	V (−)	NA	NA (−)
Utilization of:					
Acetate	+	−	(+)	+	V (−)
Cellobiose	−	(+)	NA	NA	V (+)
Citrate	−	−	V (−)	+	(−)
Ethanol	+	NA	V (−)	+	NA (−)
Glutamate	+	−	V (−)	+	V
Glycerol	+	NA	NA	−	NA (−)
Lactate	+	NA	V (−)	+	(−)
Malate	+	−	V (−)	+	V (−)
Maltose	+	(+)	NA (−)	+	V (+)
Mannose	+	(−)	NA	NA	V (−)
Pyruvate	+	V	V (+)	+	V (+)
Succinate	+	−	V (+)	+	V
Sucrose	+	(+)	NA (−)	+	V (+)
Xylose	−	(+)	NA (−)	NA	V (+)
Major cellular fatty acids	anteiso-$C_{15:0}$	$C_{18:1}\omega7c$, $C_{16:1}\omega7c$	$C_{18:1}\omega7c$, $C_{17:1}\omega6c$	$C_{17:1}\omega6c$, $C_{18:1}\omega7c$	$C_{18:1}\omega7c$, $C_{17:1}\omega6c$
DNA G+C content (mol%)	71.5	54.5–60.3	60–67	62.5–68.5	63.8–66.8

*C, Cocci; O, oval; R, rod.

†Y, Yellow; O, orange; R, red.

The following compounds are not utilized as sole carbon sources: L-arabinose, L-arginine, L-aspartate, cellobiose, citrate, L-cysteine, formate, D-fructose, D-galactose, glycine, gluconate, inositol, isoleucine, lactose, lysine, malonate, mannitol, L-methionine, raffinose, rhamnose, ribose, salicin, L-sorbitol, sorbose, L-valine and xylose. Acid is produced from: glucose, maltose, sucrose and trehalose. Susceptible to ampicillin (10 μg), bacitracin (0.04 U), cefalexin (30 μg), ceftriaxone (30 μg), chloramphenicol (30 μg), erythromycin (15 μg), gentamicin (10 μg), minocycline (30 μg), neomycin (30 μg), novobiocin (30 μg), penicillin (10 μg), streptomycin (10 μg) and tetracycline (30 μg), but not to kanamycin (30 μg), nitrofurantoin (300 μg), nystatin (100 μg) or tobramycin (10 μg). Major fatty acids are anteiso-$C_{15:0}$, iso-$C_{14:0}$ and iso-$C_{15:0}$.

The type strain, E4A9^T (=CGMCC 1.6776^T=JCM 14846^T), was isolated from a deep-sea sediment sample collected from a polymetallic nodule region in the East Pacific Ocean. The DNA G+C content of the type strain is 71.5±0.2 mol% (as determined by HPLC).

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