Rubritalea sabuli sp. nov., a carotenoid- and squalene-producing member of the family *Verrucomicrobiaceae*, isolated from marine sediment

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The taxonomic status of a verrucomicrobial strain isolated from marine sediment was established based on a polyphasic examination. The novel isolate, strain YM29-052^T, was obligately aerobic, Gram-negative, non-motile, coccoid or rod-shaped and chemoheterotrophic. Phylogenetic analyses based on 16S rRNA gene sequences demonstrated that the new isolate shared approximately 94–99 % sequence similarity with members of genus *Rubritalea* of the family *Verrucomicrobiaceae* within the phylum '*Verrucomicrobia*'. Genomic DNA–DNA hybridization between strain YM29-052^T and *Rubritalea squalenifaciens* HOact23^T showed relatedness of <70 %, the value commonly accepted as the threshold for the phylogenetic definition of a species. Strain YM29-052^T produces carotenoid compounds that render the cell biomass a pink colour; the strain also contains squalene. The cell-wall peptidoglycan of the novel strain contains muramic acid and meso-diaminopimelic acid. The DNA G + C content of strain YM29-052^T was 47.7 mol%; MK-8 and MK-9 were the major menaquinones. The presence of iso-C_{14:0}, iso-C_{16:0} and C_{16:1ω7c} as major cellular fatty acids supported the identification of the novel isolate as a member of the genus *Rubritalea*. On the basis of polyphasic taxonomic evidence, it was concluded that strain YM29-052^T should be classified within a novel species of the genus *Rubritalea*, for which the name *Rubritalea sabuli* sp. nov. is proposed. The type strain is YM29-052^T (=MBIC08323^T =KCTC 22127^T).

The family *Verrucomicrobiaceae* is part of the phylum '*Verrucomicrobia*' (Hedlund *et al.*, 1997; Hugenholtz *et al.*, 1998) and consists of seven genera with validly published names, *Prostheco bacter* (Staley *et al.*, 1976; Hedlund *et al.*, 1996), *Verrucomicrobium* (Schlesner, 1987), *Akkermansia* (Derrien *et al.*, 2004) and *Rubritalea* (Scheuermayer *et al.*, 2006) together with the new genera *Luteolibacter*, *Persicirhabdus* and *Roseibacillus*, described in the accompanying paper (Yoon *et al.*, 2008). The genus *Rubritalea* was the first marine member of the family to be described and includes the species *Rubritalea marina* (Scheuermayer *et al.*, 2006), *R. squalenifaciens* (Kasai *et al.*, 2007), *R. spongiae* (Yoon *et al.*, 2007b) and *R. tangerina* (Yoon *et al.*, 2007b). The three latter species were found to be squalene- and carotenoid-producing bacteria. Recently, a novel acyl glyco-carotenoid, diapolycopenedioic acid xylosyl ester, possessing antioxidative activities was discovered in *R.*

squalenifaciens HOact23^T (Shindo *et al.*, 2007). A novel marine sediment isolate belonging to the genus *Rubritalea* is the subject of a polyphasic taxonomic characterization in this study.

Strain YM29-052^T was isolated from sediment samples collected from the shore of Cape Oa, on the island of Pohnpei, part of the Federated States of Micronesia (GPS location 06° 52' 07.4" N 158° 20' 24.7" E), in October 2006. Samples (0.5–1 cm³) were homogenized with a glass rod in 5 ml sterile seawater. A 50 µl sample of the homogenate was applied to the surface of an agar isolation medium (medium P; Yoon *et al.*, 2007a). Strain YM29-052^T appeared after incubation for 30 days at 25 °C. The pink-pigmented colony was purified on marine broth 2216 (Difco) containing 1.5 % agar after being cultivated for 7–10 days.

In the present study, we attempted to elucidate the phylogenetic position of strain YM29-052^T using a polyphasic taxonomic approach including 16S rRNA gene

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Rubritalea sabuli* YM29-052^T is AB353310.

sequence analysis. In parallel, we performed physiological, biochemical and chemotaxonomic analyses to characterize the novel isolate.

The temperature range and pH range for growth were determined by incubating the isolate on marine agar 2216 (MA; Difco). The NaCl concentration for growth was determined on 1/2-strength R2A agar (Difco) containing 0–10 % (w/v) NaCl. Gram-staining was performed as described by Murray *et al.* (1994). Cell morphology was observed using light microscopy (BX60; Olympus). Cells of strain YM29-052^T on MA were coccoid shaped, 0.6–0.8 µm in diameter, and rod shaped, 0.5–1.0 µm wide and 1.2–1.5 µm long. Motility of cells due to flagella or gliding was not observed, while cell division occurred by binary fission only. Growth under anaerobic conditions was determined after 2 weeks of incubation in an AnaeroPack (Mitsubishi Gas Chemical Co.) on MA. Catalase activity was determined by bubble formation in a 3 % H₂O₂ solution. Oxidase activity was determined by cytochrome oxidase paper (Nissui Pharmaceutical Co.). API 20NE and API ZYM strips (bioMérieux) were used to determine physiological and biochemical characteristics, and were read after

24 h incubation at 30 °C and 4 h incubation at 37 °C, respectively. The substrate oxidation range of strain YM29-052^T was determined using Biolog MicroPlates. Determination of the respiratory quinone system and cellular fatty acid composition were carried out as described previously (Katsuta *et al.*, 2005).

DNA was extracted and purified according to the method of Marmur (1961) from cells grown on MA and the DNA base composition was determined by using the HPLC method of Mesbah *et al.* (1989). DNA–DNA hybridizations were carried out with photobiotin-labelled probes in microplate wells as described by Ezaki *et al.* (1989). The hybridization temperature was set at 48 °C. Hybridization was performed using five replications for each; of the values obtained, the highest and lowest for each sample were excluded and the means of the remaining three values are quoted as DNA–DNA relatedness values. Cell walls were prepared by the methods described by Schleifer & Kandler (1972), and amino acids in an acid hydrolysate of the cell wall were identified by TLC (Harper & Davis, 1979) and by HPLC, as their phenylthiocarbamoyl derivatives, with a model LC-10AD HPLC apparatus (Shimadzu)

Fig. 1. Neighbour-joining tree showing the phylogenetic relationship of strain YM29-052^T and related species of the family Verrucomicrobiaceae within the phylum 'Verrucomicrobia' based on 16S rRNA gene sequences. Numbers at nodes are percentage bootstrap values derived from 1000 replications. The sequence of *Escherichia coli* ATCC 11775^T was used as an outgroup. Bar, 2 % estimated difference in nucleotide sequence.

equipped with a Wakopak WS-PTC column (Wako Pure Chemical Industries) (Yokota *et al.*, 1993).

An approximately 1500 bp fragment of the 16S rRNA gene was amplified from the extracted DNA by using bacterial universal primers 27F and 1492R specific to the 16S rRNA gene (*Escherichia coli* numbering system; Weisburg *et al.*, 1991). To ascertain the phylogenetic position of the new isolate, the 16S rRNA gene sequence of strain YM29-052^T was compared with sequences obtained from GenBank (National Center for Biotechnology Information; <http://www.ncbi.nlm.nih.gov>). Multiple alignments of the sequences were performed using CLUSTAL_X (version 1.83) (Thompson *et al.*, 1997). Alignment gaps and ambiguous bases were not taken into consideration when 1123 bases of the 16S rRNA gene sequence were compared. Aligned sequences were analysed by the MEGA3.1 software (Kumar *et al.*, 2004). Evolutionary distances [distance options according to the Kimura two-parameter model (Kimura, 1983)] and clustering with the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods were determined by using bootstrap values based on 1000 replications (Felsenstein, 1985). Similarity values were calculated using the same software.

Comparative analysis of 16S rRNA gene sequences revealed that strain YM29-052^T belonged to the genus *Rubritalea*, with the *Rubritalea* sequence cluster supported by a 100% bootstrap value in both neighbour-joining (Fig. 1) and maximum-parsimony (not shown) trees. Analysis of 16S rRNA gene sequences showed that the sequence of strain YM29-052^T was most similar to that of the marine bacterium *R. squalenifaciens* HOact23^T (99.6% similarity), followed by *R. marina* Pol012^T (95.6%), *R. tangerina* YM27-005^T (95.1%) and *R. spongiae* YM21-132^T (94.9%). All other cultivated species of the family *Verrucomicrobiaceae* with validly published names were related more distantly, showing 16S rRNA gene sequence similarities of less than 90%.

DNA–DNA hybridization values between strain YM29-052^T and *Rubritalea squalenifaciens* HOact23^T were approximately 26%. This result suggests strongly that strain YM29-052^T should be classified within a distinct species (Wayne *et al.*, 1987).

Analysis of pigments produced by strain YM29-052^T was performed by HPLC/PAD/APCI (photodiode array detection/atmospheric pressure chemical ionization) MS (ThermoFinnigan) of the crude acetone extract from frozen cells. The carotenoid in the extract of strain YM29-052^T showed the same UV-visible absorption spectrum as that of the novel carotenoid produced by *R. squalenifaciens* HOact23^T (Shindo *et al.*, 2007); however, the retention time in HPLC was different. The molecule-related ion peak of the carotenoid could not be identified in the MS spectrum because of the presence of many peaks from other components in the extract. We conclude that the carotenoid produced by strain YM29-052^T was different from that produced by *R. squalenifaciens* HOact23^T. Squalene was also detected in the new isolate.

The predominant cellular fatty acids of the novel strain were iso-C_{14:0} (49.4%), iso-C_{16:0} (29.1%) and C_{16:1ω7c} (7.0%), which are similar to those of other members of the genus *Rubritalea* (Table 1). However, strain YM29-052^T is distinguished from the other species in the genus *Rubritalea* by the different proportions of C_{16:0}, C_{17:0}, C_{18:0} and anteiso-C_{15:0}. Furthermore, strain YM29-052^T could be distinguished from the other species of the genus *Rubritalea* by the phenotypic characteristics given in Table 2. Cell walls of the isolate were prepared by disrupting cells followed by heating with 3% SDS, washing and centrifugation. Amino acid analysis of the cell-wall hydrolysate indicated the presence of muramic acid and meso-diaminopimelic acid in the cell-wall peptidoglycan of strain YM29-052^T.

Based on the results of the phylogenetic analysis and its biochemical and physiological properties, strain YM29-052^T isolated from marine sediment in Pohnpei, Federated States of Micronesia, should be classified as the type strain of a novel species of the genus *Rubritalea*, for which we propose the name *Rubritalea sabuli* sp. nov.

Description of *Rubritalea sabuli* sp. nov.

Rubritalea sabuli (L. gen. n. *sabuli* of sand).

Cells are Gram-negative, non-motile, obligately aerobic and coccoid (0.6–0.8 µm in diameter) or rod shaped (0.5–1.0 × 1.2–1.5 µm). Neither cellular gliding movement nor

Table 1. Comparison of the cellular fatty acid contents of strain YM29-052^T (*Rubritalea sabuli* sp. nov.) and related *Rubritalea* type strains

Strains: 1, strain YM29-052^T; 2, *R. marina* Pol012^T (data from Scheuermayer *et al.*, 2006); 3, *R. squalenifaciens* HOact23^T (Kasai *et al.*, 2007); 4, *R. spongiae* YM21-132^T (Yoon *et al.*, 2007b); 5, *R. tangerina* YM27-005^T (Yoon *et al.*, 2007b). Values are percentages of total fatty acids; values <1% are not shown. ND, Not described; –, not detected; tr, trace.

Fatty acid	1	2	3	4	5
Saturated					
C _{14:0}	tr	ND	tr	tr	2.9
C _{15:0}	1	4.5	1.9	2.6	1.4
C _{16:0}	2.5	23	4.4	7.3	23.1
C _{17:0}	–	ND	tr	2.8	1.1
C _{18:0}	–	ND	tr	tr	2.4
Unsaturated					
C _{15:1ω6c}	2.1	ND	1	2	tr
C _{16:1ω7c}	7	21.5	7	11.8	12.7
C _{18:1ω7c}	–	ND	–	–	1.5
Branched					
iso-C _{14:0}	49.4	22	43.1	40.6	35.5
iso-C _{16:0}	29.1	ND	20.6	16.1	12.2
anteiso-C _{15:0}	5.1	6	18.1	12.9	4.7
anteiso-C _{17:0}	1.3	ND	1.6	2	–

Table 2. Physiological and biochemical characteristics of strain YM29-052^T (*R. sabuli* sp. nov.) in comparison with related *Rubritalea* type strains

Strains: 1, strain YM29-052^T; 2, *R. marina* Pol012^T (data from Scheuermayer *et al.*, 2006); 3, *R. squalenifaciens* HOact23^T (Kasai *et al.*, 2007); 4, *R. spongiae* YM21-132^T (Yoon *et al.*, 2007b); 5, *R. tangerina* YM27-005^T (Yoon *et al.*, 2007b).

Characteristic	1	2	3	4	5
Isolation source	Marine sediment	Marine sponge	Marine sponge	Marine sponge	Sea hare
Cell shape	Coccoid or rod	Coccoid or rod	Rod	Coccoid or rod	Coccoid or rod
Colony colour	Pink	Red	Reddish pink	Reddish pink	Reddish orange
O ₂ requirement	Obligately aerobic	Aerobic	Obligately aerobic	Obligately aerobic	Facultatively anaerobic
pH range	6.5–8.5	6.8–8.2	7.5–8.5	6.5–8	6.5–8.5
Temperature range (°C)	10–37	8–30	15–37	4–37	15–37
NaCl range (%)	0–8	1.4–3.8	1–4	1–7	0–9
Production of:					
Catalase	+	–	+	+	–
Oxidase	–	+	+	+	+
Nitrate reduction	–	+	+	+	–
Oxidation of:					
Acetic acid	+	+	–	+	–
Dextrin	+	+	–	–	–
Cellobiose	+	+	–	–	–
D-Glucuronic acid	+	–	+	–	–
D-Mannose	+	+	–	–	+
D-Mannitol	–	–	–	+	–
Melibiose	+	+	+	+	–
Formic acid	–	+	–	–	–
N-Acetyl-D-galactosamine	+	–	+	–	–
N-Acetyl-D-glucosamine	+	–	+	–	–
D-Sorbitol	–	–	–	+	–
Trehalose	+	–	+	–	–
L-Fructose	+	–	–	+	–
L-Fucose	–	–	–	+	+
D-Glucose	+	+	+	+	–
cis-Aconitic acid	–	–	–	+	+
Citric acid	–	–	–	–	+
α-Ketobutyric acid	–	–	–	+	–
α-Ketoglutaric acid	–	–	–	+	–
Succinic acid	+	–	–	–	+
Inosine	–	–	–	+	–
Glucose 1-phosphate	–	–	–	+	+
Glucose 6-phosphate	–	–	–	–	+
Enzyme activity of:					
Esterase (C4)	–	+	+	–	–
Esterase lipase (C8)	–	+	+	–	–
Leucine arylamidase	+	+	+	–	+
Trypsin	–	+	–	–	–
Chymotrypsin	–	–	+	–	–
N-Acetyl-β-glucosaminidase	+	–	+	–	+
α-Fucosidase	–	–	–	+	–
DNA G + C content (mol%)	47.7	50.9	52.6	48	50.3
Major quinones	MK-8, MK-9	MK-8, MK-9	MK-8, MK-9, MK-10	MK-8, MK-9	MK-8, MK-9

swarming growth is observed. Colonies grown on MA are circular, convex and pink in colour. The temperature range for growth is 10–37 °C, with optimal growth at 30–37 °C and no growth at 4 or 45 °C. The pH range for growth is 6.5–8.5. NaCl is not required for growth, but is tolerated up to 8 % (w/v). Catalase reaction is positive, whereas

oxidase is negative. Nitrate is not reduced. Alkaline phosphatase, leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and *N*-acetyl-β-glucosaminidase tests are positive, but esterase (C4), esterase lipase (C8), lipase (C4), valine arylamidase, cystine arylamidase, trypsin, chymotrypsin, α-galactosidase,

β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, α -mannosidase and α -fucosidase tests are negative. Dextrin, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, L-arabinose, cellobiose, D-fructose, D-galactose, gentiobiose, D-glucose, D-lactose, lactulose, maltose, D-mannose, melibiose, methyl β -D-glucoside, trehalose, furanose, methyl pyruvate, acetic acid, D-glucuronic acid, succinic acid, alaninamide, L-alanine, L-alanyl glycine, L-glutamic acid, L-serine and L-threonine are oxidized. The major respiratory menaquinones are MK-8 and MK-9. The cell-wall peptidoglycan contains muramic acid and meso-diaminopimelic acid. Major fatty acid components (>1.0 %) include iso-C_{14:0} (49.4 %), anteiso-C_{15:0} (5.1 %), C_{15:1 ω 6c} (2.1 %), C_{15:0} (1.0 %), iso-C_{16:0} (29.1 %), C_{16:1 ω 7c} (7.0 %), C_{16:0} (2.5 %) and anteiso-C_{17:0} (1.3 %). The G+C content of the genomic DNA of the type strain is 47.7 mol%.

The type strain is YM29-052^T (=MBIC08323^T =KCTC 22127^T), which was isolated from marine sediment in Pohndpei.

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