Cerasicoccus arenae gen. nov., sp. nov., a carotenoid-producing marine representative of the family Puniceicoccaceae within the phylum 'Verrucomicrobia', isolated from marine sand

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A polyphasic taxonomic study was performed on strain YM26-026T, which was isolated from acid-treated sediment in Kamaishi, Japan. The bacterial cells were pale-pink-pigmented, Gram-negative, obligately aerobic, non-spore-forming, spherical and non-motile. Phylogenetic analysis based on 16S rRNA gene sequences showed that the novel isolate was a member of the phylum 'Verrucomicrobia' and shared approximately 84–87% sequence similarity with strains of the class Opitutae that have been cultivated to date. Strain YM26-026T produced pale-pink pigments of carotenoid. β-Lactam antibiotic susceptibility tests and amino acid analysis of cell-wall hydrolysates indicated that the novel isolate did not contain muramic acid or diaminopimelic acid in the cell wall, suggesting that the strain lacks peptidoglycan. The G+C content of the DNA of strain YM26-026T was 54.0 mol%. Menaquinone-7 was the major quinone and C14:0 and C18:1ω9c were the major fatty acids. On the basis of polyphasic taxonomic studies, it was concluded that strain YM26-026T represents a new genus of the family Puniceicoccaceae within the phylum 'Verrucomicrobia', for which the name Cerasicoccus arenae gen. nov., sp. nov. is proposed. The type strain is YM26-026T (=MBIC08280=KCTC 12870T).

A naturally distributed and abundant phylogenetic group, the phylum 'Verrucomicrobia' (Hedlund et al., 1997; Hugenholtz et al., 1998), to date has not been studied taxonomically. The phylum comprises six informal monophyletic subdivisions (Hugenholtz et al., 1998; Vandekerckhove et al., 2000) of which three are recognized in the second edition of Bergey's Manual of Systematic Bacteriology (Garrity & Holt, 2001) as the families Puniceicoccaceae (subdivision 1), Opitutaceae (subdivision 4) and Xiphinemaotobacteriaceae (subdivision 2). Since the six informal monophyletic subdivisions of this phylogenetic group were first proposed, the names of only a few species belonging to subdivisions 1 and 4 have been validly published. The class Opitutae, comprising two orders: Puniceicoccales containing the family Puniceicoccaceae and Opitutales containing the family Opitutaceae, was formally proposed recently for the classification of subdivision 4 (Choo et al., 2007). At present, the genera Alterococcus, Coraliomargarita, Opitutus, Pelagicoccus, Puniceicoccus and 'Fucophilus' (Janssen et al., 1997; Shieh & Jean, 1998; Chin et al., 1999, 2001; Sakai et al., 2003; Choo et al., 2007; Yoon et al., 2007a, b) have been described as members of the class Opitutae. The name of the latter micro-organism, 'Fucophilus fucoidanolyticus' strain SI-1234, which is able to degrade fucoidan and was isolated from sea cucumbers (Sticopus japonicus), has not yet been validly published. However, in spite of the abundance of members of the phylum 'Verrucomicrobia' in nature (Joseph et al., 2003; Rappé & Giovannoni, 2003; Kanokratana et al., 2004; Haukka et al., 2005; Dedysh et al., 2006; Haukka et al., 2006), to date few representatives have been cultivated and most of them have yet to be cultured and described.

Strain YM26-026T was isolated from a sediment sample collected from the shore of the Gulf of Touni, Touni-cho, Kamaishi, Iwate, Japan (depth 60 cm; GPS location: 39°11′ 20.36″ N, 141°32′ 12.96″ E) in August 2006. The samples (0.5–1 cm³) were washed gently with 0.1 M HCl for 5 min, neutralized using sterile seawater and then 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., 2007b). Strain YM26-026T appeared after incubation for
30 days at 25 °C. A pale-pink-pigmented colony was purified on marine broth 2216 (Difco) containing 1.5 % agar by cultivation for 7–10 days.

In the present study, we attempted to elucidate the phylogenetic position of strain YM26-026T by using a polyphasic taxonomic approach, including 16S rRNA gene sequence analysis. In parallel, we performed physiological, biochemical and chemotaxonomic analyses to characterize the novel isolate. Based on these data, it is proposed that the isolate represents a novel genus of the family Puniceicoccaceae within the phylum 'Verrucomicrobia'.

The temperature range and pH range for growth were determined by incubating the isolate on 1/5 strength marine agar 2216 (Difco). The NaCl concentration for growth was determined using a salt-tolerance test medium containing: 1 % tryptone, 0.3 % yeast extract, 0.9 % MgCl₂.6H₂O, 0.9 % MgSO₄.7H₂O, 0.2 % CaCl₂.2H₂O, 0.06 % KCl and 1.5 % agar, with 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) and transmission electron microscopy (TEM). For TEM, cells were mounted on Formvar-coated copper grids and negatively stained with 1 % (w/v) aqueous uranyl acetate. Grids were observed using a JEOL 1011 TEM (JEOL) operated at 100 kV. In the course of TEM, various cell sizes were observed. Cells of strain YM26-026T grown on 1/5 strength marine agar 2216 were spherical and mostly 0.8–1.0 μm in diameter. The cells did not bear flagella or appendages. No motility was seen by light microscopy. Cells divided by means of binary fission (Fig. 1). Growth under anaerobic conditions was determined after 2 weeks incubation in an AnaeroPack (Mitsubishi Gas Chemical Co., Inc.) on 1/5 strength marine agar 2216. Catalase activity was determined by the observation of bubble formation in a 3 % H₂O₂ solution. Oxidase activity was determined using cytochrome oxidase paper (Eiken Chemical Co., Ltd). API 20E, API 50 CH and API ZYM strips (bioMérieux) were used to determine physiological and biochemical characteristics. The API 20E and API 50 CH tests were read after 72 h incubation at 30 °C and the API ZYM tests were read after 4 h incubation at 37 °C. Determination of the respiratory quinone system was carried out as described previously (Katsuta et al., 2005). GC analysis of the cellular fatty acid methyl esters was performed using a culture grown on 1/5 strength marine agar at 27 °C for 4 days, according to the instructions of the Microbial Identification System (MIDI; Microbial ID). DNA was prepared according to the method of Marmur (1961) from cells grown on 1/5 strength marine agar 2216 and the DNA base composition was determined by using the HPLC method of Mesbah et al. (1989). The sensitivity of the novel isolate to the β-lactam antibiotics ampicillin and penicillin G was tested on 1/5 strength marine agar 2216, using 8 mm paper discs (Advantec) at concentrations of 1, 10, 100, 500 and 1000 μg ml⁻¹. Cell walls were prepared using the methods described by Schleifer & Kandler (1972), and the amino acids in an acid hydrolysate of the cell walls were identified by TLC (Harper & Davis, 1979) and HPLC, as their phenylthiocarbamoyl derivatives, with a model LC-10AD HPLC apparatus (Shimazu) 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 extracted DNA by using bacterial universal primers specific for the 16S rRNA gene: 27F and 1492R (Escherichia coli numbering system; Weisburg et al., 1991). To ascertain the phylogenetic position of the novel isolate, the 16S rRNA gene sequence of strain YM26-026T 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 for the comparison of the 1166 bases of the 16S rRNA gene nucleotides. Aligned sequences were analysed using MEGA3.1 software (Kumar et al., 2004). The 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 a bootstrap analysis based on 1000 replications (Felsenstein, 1985). The similarity values were calculated using the same software.

Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain YM26-026T belongs to the class Opitutae within the phylum 'Verrucomicrobia', with bootstrap confidence values of 78 % using the neighbour-joining method (Fig. 2) and 44 % using maximum-parsimony (data not shown). Analysis of the 16S rRNA gene sequences showed that the sequence of strain YM26-026T showed the

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**Fig. 1.** Transmission electron micrograph of a negatively stained cell of strain YM26-026T. Bar, 500 nm.
highest similarity (87.4 %) to ‘Fucophilus fucoidanolyticus’ strain SI-1234, followed by the marine bacteria Coraliomargarita akajimensis 04OKA010-24T (87.1 %), Puniceicoccus vermicola IMCC1545T (87.1 %), Pelagiococcus albus YM14-201T (85.1 %), Pelagiococcus litoralis H-MN57T (84.7 %) and Pelagiococcus mobilis 02PA-Ca-133T (84.2 %) belonging to the family Puniceicoccaceae in the order Puniceicoccales. Strain YM26-026T also shared similarities of 84.1 % to Opitutus terrae PB90-1T and 83.7 % to Alterococcus agarolyticus ADT3T, belonging to the family Opitutaceae in the order Opitutales. All other cultivated species of the phylum ‘Verrucomicrobia’ with validly published names were more distantly related, with 16S rRNA gene sequence similarities of less than 80 %. Based on the divergence of the 16S rRNA gene sequences, strain YM26-026T was found to be related to the genera Coraliomargarita, Pelagiococcus, Puniceicoccus and ‘Fucophilus’, which are members of the family Puniceicoccaceae. Thus, on the basis of the phylogenetic data presented, strain YM26-026T should be classified as representing a novel genus and species of the phylum ‘Verrucomicrobia’, family Puniceicoccaceae (Fig. 2).

The pale-pink pigment that accumulated in the cells was extracted from freeze-dried cells using acetone and analysed by HPLC/PAD (photodiode array detection). The pigment had a UV-VIS spectrum with absorption maxima at 480 and 510 nm, characteristic for carotenoid. As shown in Table 1, the predominant cellular fatty acids of strain YM26-026T were C14:0 (38.7 %) and C18:1v9c (43.3 %). In addition, on the basis of the fatty acid composition, strain YM26-026T could be differentiated from Coraliomargarita akajimensis 04OKA010-24T, Puniceicoccus vermicola IMCC1545T and species of the genus Pelagiococcus, the phylogenetically related taxa, indicating that strain YM26-026T probably represents an independent genus of the family Puniceicoccaceae within the phylum ‘Verrucomicrobia’. When the novel isolate was grown in the presence of increasing concentrations (1–1000 µg ml⁻¹) of the β-lactam antibiotics ampicillin and penicillin G, they showed a remarkable resistance (Table 2).
Strain YM26-026T, an obligately aerobic bacterium, was isolated from marine sand and was able to tolerate 8 % NaCl (w/v), whereas other members of the class Opitutae, originating from rice paddy soil, thermal springs and seawater, could only tolerate up to 1–5 % (w/v) NaCl (with the exception of Puniceicoccus vermicola IMCC1545T). Moreover, strain YM26-026T could also be distinguished by the following characteristics: colony colour, production of catalase and oxidase, oxygen requirement, NaCl requirement and temperature range for growth. Some differential characteristics were obtained from the API 50 CH results, including acid production from D-arabinose, cellobiose, glucose, lactose, mannitol, mannose and melibiose (Table 2).

Based on the results of the phylogenetic analysis and its biochemical and physiological properties, strain YM26-026T should be classified as representing a novel species in a new genus belonging to the family Puniceicoccaceae within the phylum ‘Verrucomicrobia’. We therefore propose the name Cerasicoccus areniae gen. nov., sp. nov. for strain YM26-026T.

### Description of Cerasicoccus gen. nov.

*Cerasicoccus* (Ce.ra.si.co.cus) L. neut. n. *cerasum* a cherry; Gr. masc. n. *kokkos* berry; N.L. masc. n. *Cerasicoccus* referring to the pale-pink colour of the bacterium).

Cells are coccis, Gram-negative and obligately aerobic. Cells lack flagella and are non-motile. Spores are not formed. Catalase- and oxidase-positive. Nitrate is not reduced. The major respiratory quinone is MK-7. Predominant cellular fatty acids are C14:0 and C18:1ω9c. The type species is *Cerasicoccus areniae*.

### Description of Cerasicoccus areniae sp. nov.

*Cerasicoccus areniae* (a.ren’ae) L. gen. n. *areniae* of sand).

Main characteristics are the same as those given for the genus. In addition, cells are 0.8–1.0 μm in diameter. Neither cellular gliding movement nor swarming growth is observed. Colonies on 1/5 strength marine agar medium are circular, convex and pale-pink-pigmented. Temperature range for growth is 10–30 °C, with optimal growth at 25–30 °C; no growth occurs at 4 or 45 °C. pH range for growth is 6–9. Seawater and NaCl are not required for growth, but up to 8 % (w/v) NaCl can be tolerated. Growth occurs in the presence of ampicillin (1–1000 μg ml⁻¹) and penicillin G (1–1000 μg ml⁻¹). Aesculin and starch are hydrolysed, but agar, DNA, gelatin and urea are not. *O-Nitrophenyl β-D-galactosidase* (ONPG) and tryptophan deaminase are positive, but acetoin, citrate utilization, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, hydrogen sulfide and indole production are negative. Acid is produced from L-arabinose, D-lyxose, galactose, glucose, fructose, mannose, methyl α-D-glucopyranoside, aesculin, ferric citrate, lactose, gentiobiose, D-turanose and 5-ketogluconate, but not from trehalose, D-tagatose, D-fucose, D-arabitol, L-arabitol, erythritol, mannitol, sorbitol, glycerol, D-arabinose, ribose, D-xylose, L-xylene, adonitol, methyl β-D-xylopyranoside, sorbose, rhamnose, dulcitol, inositol, methyl α-D-mannopyranoside, N-acetylglucosamine, amygdalin, arbutin, salinan, cellobiose, maltose, melibiose, sucrose, inulin, melezitose, raffinose, starch, glycogen, xylitol, gluconate or 2-ketogluconate. Alkaline phosphatase, naphthol-AS-BI-phosphohydrolase and β-galactosidase are positive, but acid phosphatase, α-galactosidase, α-glucosidase, leucine arylamidase, valine arylamidase, trypsin, esterase (C4), esterase lipase (C8), lipase (C4), cystine arylamidase, chymotrypsin, β-glucuronidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase.
and \(\alpha\)-fucosidase are negative. Usual components of bacterial cell walls such as muramic acid and diaminopimelic acid are not detected. Major fatty acids (>1.0%) include iso-C\(_{14:0}\) (3.5%), C\(_{14:0}\) (38.7%), C\(_{14:0}\) 2-OH (4.4%), C\(_{16:0}\) (2.3%), C\(_{16:0}\) 3-OH (1.3%), C\(_{18:1\alpha9c}\) (43.3%), C\(_{18:0}\) (1.5%) and C\(_{20:0}\) (1.5%). The G+C content of the DNA of the type strain is 54.0 mol%.

The type strain is YM26-026\(^T\) (=MBIC08280\(^T\)=KCTC 12870\(^T\)), which was isolated from acid-treated sediment in Kamaishi, Japan.

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### References


