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

Jaewoo Yoon,¹ Yoshihide Matsuo,² Satoru Matsuda,² Kyoko Adachi,² Hiroaki Kasai² and Akira Yokota¹

Correspondence

Jaewoo Yoon
aa57058@mail.ecc.u-tokyo.ac.jp

¹Institute of Molecular and Cellular Biosciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-Ku, Tokyo 113-0032, Japan

²Marine Biotechnology Institute Co. Ltd, 3-75-1, Heita, Kamaishi, Iwate 026-0001, Japan

A polyphasic taxonomic study was performed on strain YM26-026^T, 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 YM26-026^T 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 YM26-026^T was 54.0 mol%. Menaquinone-7 was the major quinone and C_{14:0} and C_{18:1 ω 9c} were the major fatty acids. On the basis of polyphasic taxonomic studies, it was concluded that strain YM26-026^T 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 YM26-026^T (=MBIC08280^T=KCTC 12870^T).

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; Vanderkerckhove *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 *Verrucomicrobiaceae* (subdivision 1), *Opitutaceae* (subdivision 4) and 'Xiphinematobacteriaceae' (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 *Opitiales* containing the family *Opitutaceae*, was formally proposed recently for the classification of subdivision 4 (Choo *et al.*, 2007). At present, the genera *Alterococcus*, *Coralimargarita*, *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 YM26-026^T 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° 52' 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 μ l sample of the homogenate was applied to the surface of an agar isolation medium (medium 'P'; Yoon *et al.*, 2007b). Strain YM26-026^T appeared after incubation for

Abbreviation: TEM, transmission electron microscopy.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain YM26-026^T is AB292183.

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 YM26-026^T 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 YM26-026^T 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 phenylthio-carbamoyl 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 YM26-026^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 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 YM26-026^T 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 YM26-026^T showed the

Fig. 1. Transmission electron micrograph of a negatively stained cell of strain YM26-026^T. Bar, 500 nm.

Fig. 2. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequence analysis showing the position of strain YM26-026^T in relation to representative 16S rRNA gene sequences that include the currently known cultivated phylogenetic diversity within the class Opitutae of the phylum 'Verrucomicrobia'. Numbers at nodes are percentage bootstrap values derived from 1000 replications. The sequence of *Escherichia coli* ATCC 11775^T was used as an outgroup. Subdivisions of the phylum 'Verrucomicrobia' are shown as SD 1–SD 6. Bar, 2% sequence divergence.

highest similarity (87.4%) to '*Fucophilus fucoidanolyticus*' strain SI-1234, followed by the marine bacteria *Coraliomargarita akajimensis* 04OKA010-24^T (87.1%), *Puniceicoccus vermicola* IMCC1545^T (87.1%), *Pelagicoccus albus* YM14-201^T (85.1%), *Pelagicoccus litoralis* H-MN57^T (84.7%) and *Pelagicoccus mobilis* 02PA-Ca-133^T (84.2%) belonging to the family Puniceococcaceae in the order Puniceococcales. Strain YM26-026^T also shared similarities of 84.1% to *Opitutus terrae* PB90-1^T and 83.7% to *Alterococcus agarolyticus* ADT3^T, 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 YM26-026^T was found to be related to the genera *Coraliomargarita*, *Pelagicoccus*, *Puniceicoccus* and '*Fucophilus*', which are members of the family Puniceococcaceae. Thus, on the basis of the phylogenetic data presented, strain YM26-026^T should be classified as representing a

novel genus and species of the phylum 'Verrucomicrobia', family Puniceococcaceae (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 YM26-026^T were C_{14:0} (38.7%) and C_{18:1ω9c} (43.3%). In addition, on the basis of the fatty acid composition, strain YM26-026^T could be differentiated from *Coraliomargarita akajimensis* 04OKA010-24^T, *Puniceicoccus vermicola* IMCC1545^T and species of the genus *Pelagicoccus*, the phylogenetically related taxa, indicating that strain YM26-026^T probably represents an independent genus of the family Puniceococcaceae 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).

Table 1. Cellular fatty acid contents of strain YM26-026^T (*Cerasicoccus arenae* gen. nov., sp. nov.) and related taxa

Strains: 1, strain YM26-026^T; 2, *Coralimargarita akajimensis* 04OKA010-24^T; 3, *Puniceicoccus vermicola* IMCC1545^T; 4, *Pelagicoccus mobilis* 02PA-Ca-133^T; 5, *Pelagicoccus albus* YM14-201^T; 6, *Pelagicoccus litoralis* H-MN57^T. Data are from this and previous studies (Choo *et al.*, 2007; Yoon *et al.*, 2007a, b). Data are expressed as percentages of total fatty acids. Fatty acids representing less than 1 % are not shown. —, Not detected; tr, trace.

Fatty acid	1	2	3	4	5	6
Saturated						
C _{14:0}	38.7	24.2	4.9	5.8	4	4.4
C _{16:0}	2.3	3.3	7.9	23.3	23.8	14.3
C _{17:0}	—	1.3	7	2.1	tr	1.3
C _{18:0}	1.5	15.6	24.7	tr	1.2	1.4
Unsaturated						
C _{18:1} ω9c	43.3	23.5	—	—	—	—
Branched						
iso-C _{14:0}	3.5	8.2	5.3	2.1	tr	1.1
anteiso-C _{15:0}	tr	3	30.9	29.8	37.5	38.1
anteiso-C _{17:0}	—	tr	3.6	tr	3.9	1.4
2-Hydroxy						
C _{14:0} 2-OH	4.4	—	—	—	—	—
3-Hydroxy						
C _{12:0} 3-OH	—	1.3	2.1	tr	tr	tr
C _{16:0} 3-OH	1.3	tr	—	tr	1.4	tr
C _{18:0} 3-OH	—	—	2.5	—	—	—

Strain YM26-026^T also showed distinct biochemical and physiological features that distinguished it from other cultivated members of the class *Opitutae* (Table 2). The major quinone was menaquinone-7 (MK-7). The G+C content of the DNA of strain YM26-026^T was 54.0 mol%. The major fatty acids of strain YM26-026^T were C_{14:0} (38.7 %) and C_{18:1}ω9c (43.3 %) (Table 1). The cell wall was prepared by disrupting cells, followed by heating with 3 % SDS, washing and centrifugation. Amino acid analysis of the cell-wall hydrolysate indicated the absence of muramic acid and diaminopimelic acid in the cell wall, suggesting that the strain does not contain peptidoglycan in the cell wall.

Strain YM26-026^T, 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* IMCC1545^T). Moreover, strain YM26-026^T 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 YM26-026^T 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 arenae* gen. nov., sp. nov. for strain YM26-026^T.

Description of *Cerasicoccus* gen. nov.

Cerasicoccus (Ce.ra.si.coc'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 cocci, 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 C_{14:0} and C_{18:1}ω9c. The type species is *Cerasicoccus arenae*.

Description of *Cerasicoccus arenae* sp. nov.

Cerasicoccus arenae (a.ren'ae. L. gen. n. *arenae* 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, L-fucose, D-arabitol, L-arabitol, erythritol, mannitol, sorbitol, glycerol, D-arabinose, ribose, D-xylose, L-xylose, adonitol, methyl β-D-xylopyranoside, sorbose, rhamnose, dulcitol, inositol, methyl α-D-mannopyranoside, N-acetylglucosamine, amygdalin, arbutin, salicin, 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

Table 2. Characteristics that differentiate strain YM26-026^T (*Cerasicoccus arenae* gen. nov., sp. nov.) from members of other genera in the class *Opitutae*, within the phylum 'Verrucomicrobia'

Strains: 1, strain YM26-026^T; 2, *Coralimargarita akajimensis* 04OKA010-24^T; 3, *Puniceicoccus vermicola* IMCC1545^T; 4, *Pelagicoccus mobilis* 02PA-Ca-133^T; 5, *Pelagicoccus albus* YM14-201^T; 6, *Pelagicoccus litoralis* H-MN57^T; 7, *A. agarolyticus* BCRC 19135^T; 8, *O. terrae* DSM 11246^T. Data are from this and previous studies (Shieh & Jean, 1998; Chin *et al.*, 2001; Choo *et al.*, 2007; Yoon *et al.*, 2007a, b). +, Positive; w, weakly positive; –, negative; ND, no data.

Characteristic	1	2	3	4	5	6	7	8
Isolation source	Marine sand	Seawater	Sea polychaete	Seawater	Seawater	Seawater	Hot spring	Rice paddy soil
Cell diameter (µm)	0.8–1.0	0.5–1.2	0.6–1.0	0.5–0.7	0.8–1.2	1.0–1.2	0.8–0.9	0.4–0.6
Colony colour	Pale-pink	White	Pale-red	White	White	White	White	Non-pigmented
Flagella	–	–	–	+	–	–	+	+
Motility	–	–	–	+	–	–	+	+
Anaerobic growth	–	–	+	–	+	–	+	–
NaCl requirement	–	+	+	+	+	+	+	ND
Growth in 8 % (w/v) NaCl	+	–	+	–	–	–	–	–
Catalase	+	–	–	–	+	+	+	–
Oxidase	+	+	–	+	+	+	+	–
Nitrate reduction	–	–	–	–	–	–	ND	+
Temperature range for growth (°C)	10–30	20–30	8–37	20–37	15–37	4–30	38–58	10–37
pH range for growth	6.0–9.0	7.0–9.0	5.0–12.0	7.0–9.0	6.5–9.0	6.5–9.0	7.0–8.5	5.5–9.0
Hydrolysis of:								
Agar	–	–	–	+	–	–	+	ND
DNA	–	w	–	+	+	–	–	ND
Starch	+	–	ND	+	–	–	w	+
Urea	–	+	–	+	–	–	ND	–
Acid production from:								
D-Arabinose	–	–	–	–	–	–	–	+
Cellobiose	–	–	+	+	–	–	+	+
Glucose	+	–	+	–	–	–	+	+
Lactose	+	–	+	+	–	–	+	+
Mannitol	–	+	–	–	–	–	–	+
Mannose	+	+	–	–	–	–	w	+
Melibiose	–	–	–	–	–	+	–	+
Resistant (µg ml ^{–1}) to:								
Ampicillin (1–1000)	+	+	+	+	+	+	–	ND
Penicillin G (1–1000)	+	+	+	+	+	+	–	ND
Cell wall components								
Muramic acid	–	–	–	–	–	–	ND	ND
meso-Diaminopimelic acid	–	–	–	–	–	–	ND	ND
DNA G+C content (mol%)	54	53.9	52.1 ± 0.5	57.4	57.2	56.4	65.5–67.0	74
Major quinone	MK-7	MK-7	MK-7	MK-7	MK-7	MK-7	ND	ND

and α -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}ω9c (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.

Acknowledgements

We thank Atsuko Katsuta, Ayako Matsuzaki, Tomomi Haga, and Yukiko Itazawa for their technical assistance. This work was supported by the New Energy and Industrial Technology Development Organization (NEDO).

References

Chin, K.-J., Hahn, D., Hengtsmann, U., Liesack, W. & Janssen, P. H. (1999). Characterization and identification of numerically abundant

culturable bacteria from the anoxic bulk soil of rice paddy microcosms. *Appl Environ Microbiol* **65**, 5042–5049.

Chin, K.-J., Liesack, W. & Janssen, P. H. (2001). *Opitutus terrae* gen. nov., sp. nov., to accommodate novel strains of the division 'Verrucomicrobia' isolated from rice paddy soil. *Int J Syst Evol Microbiol* **51**, 1965–1968.

Choo, Y.-J., Lee, K., Song, J. & Cho, J.-C. (2007). *Puniceicoccus vermicola* gen. nov., sp. nov., a novel marine bacterium, and description of *Puniceicoccaceae* fam. nov., *Puniceoccales* ord. nov., *Opitutaceae* fam. nov., *Opitiales* ord. nov. and *Opitutae* classis nov. in the phylum 'Verrucomicrobia'. *Int J Syst Evol Microbiol* **57**, 532–537.

Dedysh, S. N., Pankratov, T. A., Belova, S. E., Kulichevskaya, I. S. & Liesack, W. (2006). Phylogenetic analysis and in situ identification of bacteria community composition in an acidic *Sphagnum* peat bog. *Appl Environ Microbiol* **72**, 2110–2117.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.

Fitch, W. M. (1971). Toward defining the course of evolution: minimum change for a specific tree topology. *Syst Zool* **20**, 406–416.

Garrrity, G. M. & Holt, J. G. (2001). The road map to the *Manual*. In *Bergey's Manual of Systematic Bacteriology*, 2nd edn, vol. 1, pp. 119–166. Edited by D. R. Boone, R. W. Castenholz & G. M. Garrrity. New York: Springer.

Harper, J. J. & Davis, G. H. G. (1979). Two-dimensional thin-layer chromatography for amino acid analysis of bacterial cell walls. *Int J Syst Bacteriol* **29**, 56–58.

Haukka, K., Heikkinen, E., Kairesalo, T., Karjalainen, H. & Sivonen, K. (2005). Effect of humic material on the bacterioplankton community composition in boreal lakes and mesocosms. *Environ Microbiol* **7**, 620–630.

Haukka, K., Kolmonen, E., Hyder, R., Hietala, J., Vakkilainen, K., Kairesalo, T., Haario, H. & Sivonen, K. (2006). Effect of nutrient loading on bacterioplankton community composition in lake mesocosms. *Microb Ecol* **51**, 137–146.

Hedlund, B. P., Gosink, J. J. & Staley, J. T. (1997). *Verrucomicrobia* div. nov., a new division of the bacteria containing three new species of *Prostheobacter*. *Antonie Van Leeuwenhoek* **72**, 29–38.

Hugenholtz, P., Goebel, B. M. & Pace, N. R. (1998). Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *J Bacteriol* **180**, 4765–4774.

Janssen, P. H., Schuhmann, A., Mörschel, E. & Rainey, F. A. (1997). Novel anaerobic ultramicrobacteria belonging to the Verrucomicrobiales lineage of bacterial descent isolated by dilution culture from anoxic rice paddy soil. *Appl Environ Microbiol* **63**, 1382–1388.

Joseph, S. J., Hugenholtz, P., Sangwan, P., Osborne, C. A. & Janssen, P. H. (2003). Laboratory cultivation of widespread and previously uncultured soil bacteria. *Appl Environ Microbiol* **69**, 7210–7215.

Kanokratana, P., Chanapan, S., Pootanakit, K. & Eurwilaichitr, L. (2004). Diversity and abundance of Bacteria and Archaea in the Bor Khlung hot spring in Thailand. *J Basic Microbiol* **44**, 430–444.

Katsuta, A., Adachi, K., Matsuda, S., Shizuri, Y. & Kasai, H. (2005). *Ferrimonas marina* sp. nov. *Int J Syst Evol Microbiol* **55**, 1851–1855.

Kimura, M. (1983). *The Neutral Theory of Molecular Evolution*. Cambridge: Cambridge University Press.

Kumar, S., Tamura, K. & Nei, M. (2004). MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* **5**, 150–163.

Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from micro-organisms. *J Mol Biol* **3**, 208–218.

Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.

Murray, R. G. E., Doetsch, R. N. & Robinow, C. F. (1994). Determinative and cytological light microscopy. In *Methods for General and Molecular Bacteriology*, pp. 21–41. Edited by P. Gerhardt, R. G. E. Murray, W. A. Wood & N. R. Krieg. Washington, DC: American Society for Microbiology.

Rappé, M. S. & Giovannoni, S. J. (2003). The uncultured microbial majority. *Annu Rev Microbiol* **57**, 369–394.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Sakai, T., Ishizuka, K. & Kato, I. (2003). Isolation and characterization of a fucoidan-degrading marine bacterium. *Mar Biotechnol* **5**, 409–416.

Schleifer, K. H. & Kandler, O. (1972). Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol Rev* **36**, 407–477.

Shieh, W. Y. & Jean, W. D. (1998). *Alterococcus agarolyticus*, gen. nov., sp. nov., a halophilic thermophilic bacterium capable of agar degradation. *Can J Microbiol* **44**, 637–645.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.

Vandekerckhove, T. T. M., Willems, A., Gillis, M. & Coomans, A. (2000). Occurrence of novel verrucomicrobial species, endosymbiotic and associated with parthenogenesis in *Xiphinema americanum*-group species (Nematoda, Longidoridae). *Int J Syst Evol Microbiol* **50**, 2197–2205.

Weisburg, W. G., Barns, S. M., Pelletier, D. A. & Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* **173**, 697–703.

Yokota, A., Tamura, T., Nishii, T. & Hasegawa, T. (1993). *Kineococcus aurantiacus* gen. nov., sp. nov., a new aerobic, Gram-positive, motile coccus with meso-diaminopimelic acid and arabinogalactan in the cell wall. *Int J Syst Bacteriol* **43**, 52–57.

Yoon, J., Yasumoto-Hirose, M., Katsuta, A., Sekiguchi, H., Matsuda, S., Kasai, H. & Yokota, A. (2007a). *Coralimargarita akajimensis* gen. nov., sp. nov., a novel member of the phylum 'Verrucomicrobia' isolated from seawater in Japan. *Int J Syst Evol Microbiol* **57**, 959–963.

Yoon, J., Yasumoto-Hirose, M., Matsuo, Y., Nozawa, M., Matsuda, S., Kasai, H. & Yokota, A. (2007b). *Pelagicoccus mobilis* gen. nov., sp. nov., *Pelagicoccus albus* sp. nov. and *Pelagicoccus litoralis* sp. nov., three novel members of subdivision 4 within the phylum 'Verrucomicrobia', isolated from seawater by *in situ* cultivation. *Int J Syst Evol Microbiol* **57**, 1377–1385.