Wangia profunda gen. nov., sp. nov., a novel marine bacterium of the family *Flavobacteriaceae* isolated from southern Okinawa Trough deep-sea sediment

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Abstract

An orange-pigmented, Gram-negative, nonmotile, strictly aerobic and oxidase- and catalase-positive bacterium (SM-A87^T) was isolated from the deep-sea sediment of the southern Okinawa Trough area. The main fatty acids were i15 : 0, i17 : 0 3OH, i15 : 1 G, i17 : 1ω9c, 15 : 0, i15 : 0 3OH and summed feature 3 (comprising i-15 : 0 2OH and/or 16 : 1ω7c). MK-6 was the predominant respiratory quinone. DNA G+C content was 35.8 mol%. Flexirubin-type pigments were absent. Phylogenetic analyses based on 16S rRNA gene sequences revealed that strain SM-A87^T formed a distinct lineage within the family *Flavobacteriaceae*, with $< 93\%$ sequence similarity to the nearest strain of genus *Salegentibacter*. Moreover, strain SM-A87^T could be distinguished from the nearest phylogenetic neighbors by a number of chemotaxonomic and phenotypic properties. On the basis of polyphasic analyses, it is proposed that strain SM-A87^T be classified in a novel genus and a new species in the family *Flavobacteriaceae*, designated *Wangia profunda* gen. nov., sp. nov. The type strain is SM-A87^T (CCTCC AB 206139^T = DSM 18752).

Introduction

The family *Flavobacteriaceae*, belonging to the phylum *Bacteroidetes* [formerly *Cytophaga–Flavobacterium–Bacteroides*, (CFB)], includes a number of marine bacteria (Bowman *et al.*, 1998; Nedashkovskaya *et al.*, 2003, 2005a, b). Some members of the family have been reported to be able to decompose complex polysaccharides and other biomacromolecules (Bernardet *et al.*, 2002). In recent years, several new genera of the *Flavobacteriaceae* have been described, e.g. *Mesonia*, *Gramella*, *Leeuwenhoekiella*, *Nonlabens*, *Stenothermobacter*, *Dokdonia* and *Sandarakinotalea* (Nedashkovskaya *et al.*, 2003, 2005a, b; Lau *et al.*, 2005a, b, 2006; Yoon *et al.*, 2005; Khan *et al.*, 2006). In this study, the bacterial strain SM-A87^T originating from marine sediment is proposed to represent a novel genus of the family *Flavobacteriaceae*.

Materials and methods

Isolation of the strain and culture condition

Bacteria were isolated on marine agar 2216 medium (Difco) from deep-sea sediment samples taken from near the southern Okinawa Trough at a water depth of 1245 m using core sampler. Strain SM-A87^T was isolated from the subseafloor sediments at 2 mbsf (meters below seafloor). The *in situ* temperature, pH and chlorinity of the sediment or porewater were 4.7 °C, 7.35, and 533 mmol kg^{-1}, respectively. Besides strain SM-A87^T, more than 300 other bacterial strains were also isolated from the surface and subseafloor (down to 8.6 mbsf) sediments. After primary isolation, the purified isolate was cultivated on an agar medium composed of 10 g L^{-1} peptone, 5 g L^{-1} yeast extract (both Oxoid), 15 g L^{-1} agar and artificial sea water (Bian *et al.*, 2006) (referred to as marine agar hereafter) at 25 °C. The isolate was stored at -70 °C in marine broth (composing of 10 g L^{-1} peptone, 5 g L^{-1} yeast extract and artificial sea water) supplemented with 20% (v/v) glycerol.

DNA isolation, PCR amplification, sequencing of the 16S rRNA gene and phylogenetic analysis

Genomic DNA extraction, PCR and 16S rRNA gene sequencing followed the procedures of Kim *et al.* (1998). The nearly complete 16S rRNA gene sequence of strain SM-A87^T (1493 nucleotides) has been deposited in the

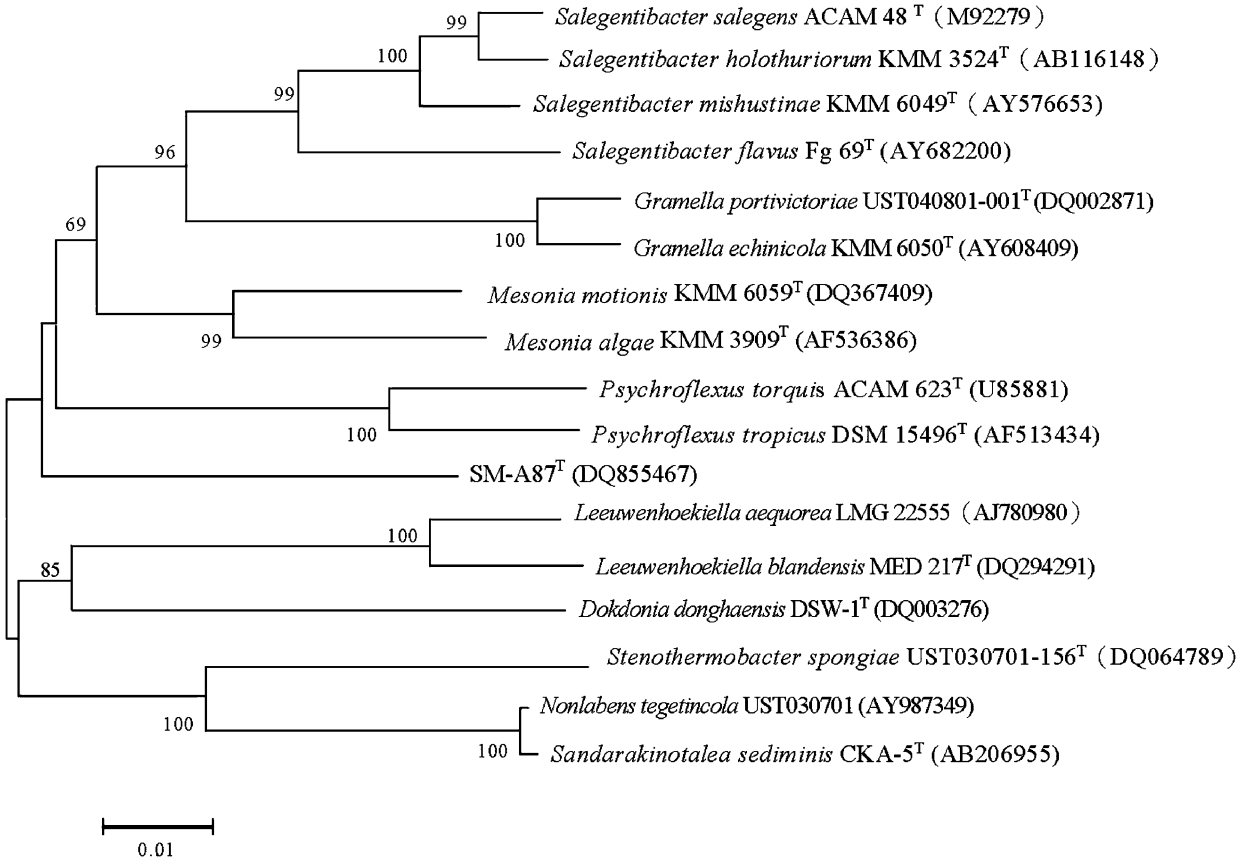

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences of strain SM-A87^T and members of related genera in the family *Flavobacteriaceae*. The tree was generated by the neighbor-joining method (Saitou & Nei, 1987). Numbers at nodes indicate bootstrap values (%) (only values > 50% are shown). Bar, 0.01 substitutions per nucleotide position.

GenBank database under accession number DQ855467. It was aligned with its nearest neighboring sequences retrieved from GenBank, and only the valid published strains were considered. Phylogenetic trees were constructed using the MEGA software package (version 3.1) with three different methods (neighbor-joining, maximum-parsimony and UPGMA). Phylogenetic distances were calculated from the model of Jukes & Cantor (1969) and bootstrap analysis was performed with 1000 replicates by MEGA package.

Phenotypic study

Cell morphology was examined using scanning electron microscopy (SEM) according to the method of Neu *et al.* (2001) at different growth phases. Gliding motility was determined using the methods of Bowman (2000) and Bernardet *et al.* (2002). The strain growth temperature (4–45 °C) and pH (3–12) were tested in marine broth by measuring $OD_{660\,nm}$ after 24 h incubation (Ivanova *et al.*, 2004). The requirement for NaCl (0–15%) was determined on marine agar except that the artificial sea water was replaced by NaCl solutions at different concentrations. The presence of flexirubin-type pigments was examined using 20% KOH (w/v) as described by Bernardet *et al.* (2002). Oxidative or fermentative utilization of glucose was determined according to the method of Lemos *et al.* (1985). Sensitivity to antibiotics was tested using the disc-diffusion method as described by Ivanova *et al.* (2004). Other physiological and biochemical properties were tested using standard procedures as described by Gerhardt *et al.* (1994). The commercial systems API 20E, API ZYM (both from bioMerieux) and MicroPlate GN2 (Biolog) were used to test the substrate oxidation profile, nitrate reduction and production of H_2S, indole and acetoin. The manufacturer's instructions were followed except that cells for inoculation of API 20E and MicroPlate GN2 systems were suspended in artificial sea water (Khan *et al.*, 2006). The GC content of DNA was determined by HPLC (Mesbah *et al.*, 1989). Chemotaxonomic analyses were carried out by Dr Brian Tindall (Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH,

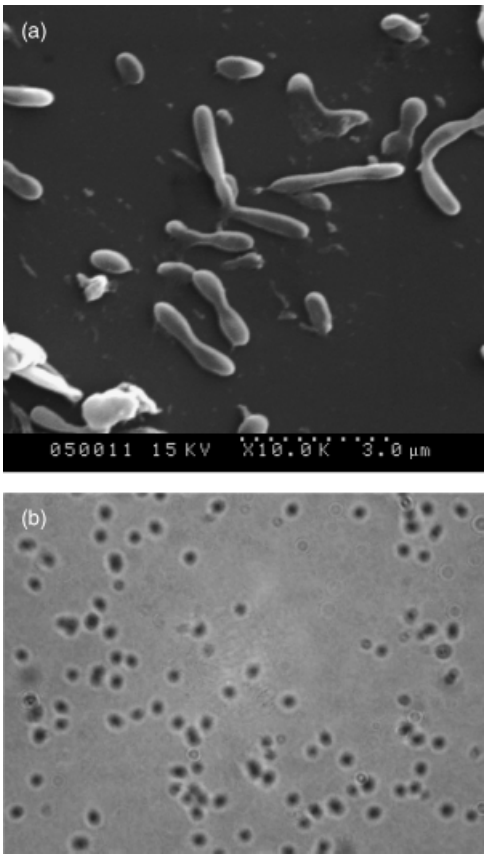

Fig. 2. Micrographs of strain SM-A87^T cultured on marine agar at 28 °C for 24 h taken by SEM (a) and coccoid bodies in aging culture taken by phase contrast microscopy (b).

Braunschweig, Germany). The analysis of cellular fatty acids was carried out according to the standard protocol of the Sherlock microbial identification system. Analysis of quinones was performed by HPLC. Polar lipids were determined by thin layer chromatography.

Results and discussion

16S rRNA gene phylogeny

The neighbor-joining phylogenetic tree revealed that strain SM-A87^T formed a distinct lineage within the family *Flavobacteriaceae* (Fig. 1). Trees based on maximum-parsimony and UPGMA methods showed essentially the same topology. Strain SM-A87^T had 92.9% 16S rRNA gene sequence similarity (99 nucleotides differences) to its nearest neighbor *Salegentibacter holothuriorum*, and 91.8% and 91.5% to *Mesonia algae* and *Gramella portivictoriae*, respectively. Therefore, according to phylogenetic analyses, strain SM-A87^T should be classified as a novel genus and species in the family *Flavobacteriaceae*.

Phenotypic characteristics

After 48 h cultivation at 28 °C on marine agar, the colonies were yellow to orange and circular, about 1–3 mm in diameter, and were adherent to the agar. Cells were rod-shaped and ranged from 0.3 to 0.6 µm in width and from 1.5 to 3.3 µm in length and were nonmotile. Cells in old cultures might form coccoid bodies (Fig. 2).

The DNA G+C content of strain SM-A87^T was 35.8 mol%, an intermediate value among members of the family *Flavobacteriaceae* (Bernardet *et al*., 2002). The main respiratory quinone was MK-6, in accordance with all members of the family *Flavobacteriaceae*. The strain synthesized mainly terminally branched iso- and anteiso-fatty acids together with diagnostic amounts of iso-branched 2-hydroxy and 3-hydroxy fatty acids. The dominant fatty acids were i-15 : 0 (22.73%), summed feature 3 (comprising i-15 : 0 2OH and/or 16 : 1ω7c) (14.59 %), i-17 : 0 3OH (14.52%), i-15 : 1 G (9.68 %), i-17 : 1ω9c (9.01%), 15 : 0 (7.45%) and i-15 : 0 3OH (3.92%) (total more than 77%). Strain SM-A87^T contained the characteristic fatty acids of the family; however, the higher content of i-17 : 0 3OH, the absence of a-17 : 1ω9c and the presence of 15 : 0 3OH distinguished it from related genera (Table 1). The only identified phospholipid was phosphatidylethanolamine.

Strain SM-A87^T could be differentiated from members of the nearest genus, *Salegentibacter*, by (1) the formation of coccoid bodies in aging cultures, (2) growth without Na^+ and with 12% NaCl, (3) being able to hydrolyze casein and unable to hydrolyze starch, (4) producing acid from arabinose, (5) production of H_2S and (6) being resistant to ampicillin. Other characteristics that differentiate strain SM-A87^T from members of related genera are shown in Table 2. Results of polyphasic analysis supported the description of Strain SM-A87^T as a new genus and species in the family *Flavobacteriaceae* for which the name *Wangia profunda* is proposed.

Description of *Wangia* gen. nov.

Wangia [Wan'gi.a. N.L. fem. n. *Wangia* of Wang, named in honor of Zu-Nong Wang, who has made great contributions to the development of microbiology in China]

Cells are Gram-negative, rod-shaped, nonmotile, non-spore-forming, strictly aerobic, oxidase- and catalase-positive. Flexirubin-type pigments are absent. MK-6 is the predominant respiratory quinone. The main fatty acids are i15 : 0, Summed Feature 3 (comprising i-15 : 0 2OH and/or 16 : 1ω7c), i-17 : 0 3OH, i-15 : 1 G, i-17 : 1ω9c, 15 : 0 and i-15 : 0 3OH. On the basis of 16S rRNA gene sequence analysis, the genus *Wangia* is a member of the family *Flavobacteriaceae* in the phylum *Bacteroidetes*. The type species is *Wangia profunda*.

Table 1. Comparison of major cellular fatty acids of SM-A87^T and related genera

Fatty acids	SM-A87^T	*S. salegens*	*M. algae*	*G. echinicola*	*P. torquis* ($n=4$)	*L. aequorea* ($n=6$)	*S. spongiae*	*N. tegetincola*
15 : 0	7.45	4.5	4.8	7.1	4.2 ± 0.6	–	–	–
i-15 : 0	22.73	23.7	21.2	14.4	1.1 ± 0.3	18.2 ± 1.1	38.1 ± 0.5	33.1 ± 6. 4
a-15 : 0	1.67	11.6	4.2	7.6	35.2 ± 4.4	4.5 ± 0.4	6.2 ± 0.7	3.8 ± 2.3
i-15 : 1 G	9.68	23.7	7.9	1.2	–	7.6 ± 1.6	–	–
15 : 1ω6c	2.44	0.6	1.8	1.9	–	–	–	–
15 : 0 3OH	1.95	–	–	–	2.5 ± 0.7	–	–	–
i-15 : 0 3OH	3.92	–	3.2	1.3	0.3 ± 0.2	2.1 ± 0.2	6.2 ± 0.3	5.4 ± 3.0
i-16 : 0	1.64	7.6	6.3	13.1	6.0 ± 1.2	3.8 ± 1.2	1.9 ± 0.4	6.8 ± 0.4
i-16 : 0 3OH	1.61	0.3	6.0	5.9	15.4 ± 1.0	3.4 ± 0.9	3.4 ± 0.3	5.7 ± 3.1
i-17 : 0 3OH	14.52	3.0	14.6	6.7	0.2 ± 0.1	12.7 ± 1.0	11.2 ± 0.3	13.7 ± 5.5
17 : 1ω6c	1.52	0.8	2.4	3.6	–	2.1 ± 0.4	1.7 ± 0.3	2.2 ± 0.4
i-17 : 1ω9c	9.01	–	5.1	3.5	–	18.8 ± 2.9	5.7 ± 1.4	3.5 ± 0.8
a-17 : 1ω9c	–	–	1.9	2.0	–	1.6 ± 0.4	–	–
i-17 : 1ω7c	–	13.2	–	–	–	–	–	–
Summed feature 3	14.59	9.4	5.1	11.4	–	9.4 ± 1.6	8.8 ± 1.2	7.5 ± 3.6
Unknown	2.67	–	3.3	4.6	–	tr	4.0 ± 1.1	6.1 ± 1.8

Values are percentages of total fatty acids. Data for *P. torquis*, *N. tegetincola*, *L. aequorea* and *S. spongiae* are means ± SD. *n*, number of strains studied. Summed feature 3, comprising i-15 : 0 2OH and/or 16 : 1ω7c.

−, not detected; tr, trace amount (< 1%).

Data from Dobson *et al*. (1993), Nedashkovskaya *et al*. (2003, 2005a, b), Bowman *et al*. (1998), Lau *et al*. (2005a, b, 2006).

Table 2. Characteristics differentiating strain SM-A87^T from related members of the family *Flavobacteriaceae*

Characteristic	1	2	3	4	5	6	7	8	9
Coccoid bodies in aging cultures	+	−	−	−	+	−	−	−	−
Gliding motility	−	−	v	+	+	−	−	+	+
Growth at									
4 °C	+	v	+	+	+	−	+	v	−
37 °C	+	v	v	v	v	+	−	+	−
Growth with									
0% NaCl	+	−	−	−	−	−	−	+	−
12% NaCl	+	v	+	v	v	−	−	+	−
Hydrolysis of									
Casein	+	v	v	v	ND	−	+	+	−
Gelatin	+	+	+	+	−	+	−	+	+
Starch	−	+	−	+	+	+	−	+	+
Acid from									
Glucose	+	v	v	v	v	−	−	v	−
Arabinose	+	−	−	−	−	−	−	−	−
H_2S production	−	+	v	−	ND	−	−	−	−
Susceptible to									
Ampicillin	−	+	+	+	ND	+	−	ND	+
Tetracycline	+	+	−	+	ND	+	+	+	+
Streptomycin	−	−	−	−	ND	+	−	ND	+
DNA G+C content (mol%)	35.8	36.8–40.4	32.7–36.1	39.6–39.9	32.6–35	33.6	38.3	35–42.5	41.0

1, SM-A87^T; 2, *Salegentibacter* (4) (in parentheses is the number of species compared); 3, *Mesonia* (2); 4, *Gramella* (2); 5, *Psychroflexus* (2); 6, *Nonlabens* (1); 7, *Dokdonia* (1); 8, *Leeuwenhoekiella* (2) 9, *Stenothermobacter* (1).

−, negative; +, positive; ND, not determined; v, variable.

Data from Dobson *et al*. (1993), Nedashkovskaya *et al*. (2003, 2004, 2005a, b, c, 2006), Ivanova *et al*. (2006), Lau *et al*. (2005a, b, 2006), Bowman *et al*. (1998), Donachie *et al*. (2004), Yoon *et al*. (2005), Pinhassi *et al*. (2006).

Description of *Wangia profunda* sp. nov.

Wangia profunda (pro.fun'da L. adj. description of the environment where the stain was isolated).

Description is as for the genus plus the following. On marine agar, colonies are circular, 1–3 mm in diameter, convex with smooth surfaces and yellow to orange in color. Rods are from 0.3 to 0.6 μm in width and from 1.5 to 3.3 μm

in length, coccoid bodies appear in aging cultures. Non-diffusible yellow pigments are produced. Growth occurs at 4–38 °C (25–30 °C optimum), at pH 5.0–8.5 and in the presence of 0–12% NaCl (3%, optimum). Growth is not observed on MacConkey agar. Hydrolyzes gelatin, casein and Tweens 20, 40, 80, but not agar, starch, cellulose (CM-cellulose or filter paper) and chitin. Positive for the following enzyme activities: ONPG (2-nitrophenyl-β-D-galactopyranoside) and gelatinase (API 20E), alkaline and acid phosphatase, trypsin, leucine arylamidase, valine arylamidase, naphthol-AS-BI-phosphohydrolase, β-galactosidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase (API ZYM). Negative for the following enzyme activities: arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and tryptophan deaminase (API 20E), esterase (C4), esterase lipase (C8), lipase (C14), cystine arylamidase, α-chymotrypsin, α-galactosidase, β-glucuronidase, α-mannosidase and α-fucosidase (API ZYM). Acids are produced from glucose, sucrose and arabinose, but not from mannose, inositol, sorbitol, rhamnose, melibiose and amygdalin (API 20E). Oxidizes D-melibiose, acetic acid, α-cyclodextrin, D-fructose, dextrin, citric acid, D-galactose, D-raffinose, gentiobiose, α-D-glucose, D-sorbitol, D-galacturonic acid, sucrose, 2-aminoethanol, α-D-lactose, D-trehalose, 2,3-butanediol, lactulose, turanose, glycerol, L-arabinose, maltose, L-threonine, D,L-α-glycerol phosphate, pyruvic acid methyl ester, glycyl-L-aspartic acid, D,L-carnitine, α-D-glucose-1-phosphate, D-cellobiose, D-mannose and D-glucose-6-phosphate (MicroPlates). Does not oxidize i-erythritol, *p*-hydroxyphenyl-acetic acid, bromosuccinic acid, L-histidine, urocanic acid, β-methyl-D-glucoside, *cis*-aconitic acid, itaconic acid, succinamic acid, hydroxyl-L-proline, inosine, L-fucose, D-psicose, α-ketobutyric acid, glucuronamide, L-leucine, uridine, formic acid, α-ketoglutaric acid, L-alaninamide, L-ornithine, Tween 40, L-rhamnose, D-galactonic acid lactone, α-ketovaleric acid, D-alanine, L-phenylalanine, phenylethylamine, Tween 80, DL-lactic acid, L-alanine, L-proline, putrescine, *N*-acetyl-D-galactosamine, *m*-inositol, D-gluconic acid, malonic acid, L-alanyl-glycine, L-pyroglutamic acid, *N*-acetyl-D-glucosamine, D-glucosaminic acid, propionic acid, L-asparagine, D-serine, adonitol, D-glucuronic acid, quinic acid, L-aspartic acid, L-serine, xylitol, α-hydroxybutyric acid, D-saccharic acid, L-glutamic acid, D-arabitol, D-mannitol, β-hydroxybutyric acid, sebacic acid, succinic acid mono-methyl ester, γ-hydroxybutyric acid, succinic acid, glycyl-L-glutamic acid and γ-aminobutyric acid (MicroPlates). Sensitive to tetracycline, cephalosporin, erythromycin and resistant to streptomycin, ampicillin, gentamicin, neomycin and kanamycin. Nitrate is reduced to N_2. H_2S is not produced. Indole and acetoin (Voges–Proskauer reaction) production are negative. Phosphatidylethanolamine is the only phospholipid identified. DNA G+C content is 35.8 mol%.

The type strain is SM-A87^T (CCTCC AB 206139^T = DSM 18752), isolated from the deep-sea sediment of the southern Okinawa Trough area.

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Author Contribution

Co first author, Zhao Dian-Li and Qin Qi-Long contributed equally to the work in this manuscript.

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