Zhongshania antarctica gen. nov., sp. nov. and *Zhongshania guokunii* sp. nov., gammaproteobacteria respectively isolated from coastal attached (fast) ice and surface seawater of the Antarctic

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Two Gram-negative, motile, aerobic, catalase- and oxidase-positive, rod-shaped strains, designated ZS5-23^T and ZS6-22^T, were respectively isolated from Antarctic coastal attached (fast) ice and surface seawater samples. Both strains could grow at 4–35 °C (optimum 30 °C) and in the absence of NaCl. Analyses of 16S rRNA gene sequences revealed that strains ZS5-23^T and ZS6-22^T were closely related to each other (99.0 % sequence similarity) and belonged to the class *Gammaproteobacteria*, with their closest relatives being *Spongiibacter* and *Melitea* species (93.1–94.3 % sequence similarity). The predominant cellular fatty acids in both strains were C_{17:1}ω8c, C_{17:0} and summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH). Genomic DNA G + C contents of strains ZS5-23^T and ZS6-22^T were 51.5 and 51.8 mol%, respectively. The DNA–DNA relatedness between strains ZS5-23^T and ZS6-22^T was 50.9 %. Strains ZS5-23^T and ZS6-22^T could be differentiated from each other and from *Spongiibacter* and *Melitea* species by differences in a number of phenotypic properties. Based on the data presented, strains ZS5-23^T and ZS6-22^T represent two novel species in a new genus in the class *Gammaproteobacteria*, for which the names *Zhongshania antarctica* gen. nov., sp. nov. (the type species) and *Zhongshania guokunii* sp. nov. are proposed. The type strain of *Zhongshania antarctica* is ZS5-23^T (=KACC 14066^T =CCTCC AB 209246^T) and that of *Zhongshania guokunii* is ZS6-22^T (=KACC 14532^T =CCTCC AB 209247^T).

The genus *Spongiibacter* in the class *Gammaproteobacteria* was proposed by Graeber *et al.* (2008) and currently comprises two species: *Spongiibacter marinus* (type species) and *S. tropicus*. The type strain of *S. marinus* was isolated from a marine sponge collected from the Norwegian coast (Graeber *et al.*, 2008), while that of *S. tropicus* was obtained from a *Synechococcus* culture derived from tropical surface water of the Pacific Ocean (Hwang & Cho, 2009). The genus

Melitea in the class *Gammaproteobacteria* was created by Urios *et al.* (2008b) and currently contains a single species, *Melitea salexigens*, whose type strain was isolated from coastal seawater of the Mediterranean Sea (Urios *et al.*, 2008b). In 2007, in the course of a study on the diversity of culturable bacteria in various Antarctic samples, two strains, designated ZS5-23^T and ZS6-22^T, were isolated from coastal attached (fast) ice and surface seawater samples, respectively. Preliminary analysis of 16S rRNA gene sequences revealed that the two strains shared a high sequence similarity of 99.0 % and both were phylogenetically most closely related to *Spongiibacter* and *Melitea* species, with moderately high sequence similarities of 93.1–94.3 %. In this study, the two strains are characterized further taxonomically by using a

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains ZS5-23^T and ZS6-22^T are respectively FJ889619 and FJ889678.

A supplementary figure is available with the online version of this paper.

polyphasic approach and, on the basis of the results obtained, they are proposed to be allocated to a new genus, *Zhongshania* gen. nov., which is phylogenetically adjacent to the genera *Spongiibacter* and *Melitea* within the class *Gammaproteobacteria*, as representatives of two novel species.

Antarctic coastal attached (fast) ice and surface seawater samples were collected from the coastal area off the Chinese Antarctic Zhongshan Station, East Antarctica (69° 22' 12" S 76° 22' 20" E), in March 2007. Fast ice samples were collected using a MARKII ice auger and surface seawater samples were collected using sterile plastic bottles. Ice samples were cut into 10 cm sections using a sterile saw and then melted at 4 °C in sterile plastic bottles. The melted ice samples and surface seawater samples were spread directly onto seawater agar and incubated at 4 °C for 6–8 weeks. Individual colonies that appeared on the plates were picked and purified by successive streaking three times on fresh agar medium. Two isolates, ZS5-23^T and ZS6-22^T, were routinely cultivated on a solid medium containing 0.5 % tryptone (Oxoid), 0.1 % yeast extract (Oxoid), 1.5 % agar and artificial seawater (hereafter marine agar) or in a liquid medium containing 0.5 % tryptone, 0.1 % yeast extract and artificial seawater (hereafter marine broth) at 30 °C and preserved at –80 °C in marine broth supplemented with 20 % glycerol. The artificial seawater was prepared with synthetic sea salt (Marine Life Reef Salt, QingDao, China) and contained approximately (g l⁻¹): Na⁺, 9.1; K⁺, 0.35; Mg²⁺, 1.25; Ca²⁺, 0.43; Sr²⁺, 0.0095; Cl⁻, 17.1; SO₄²⁻, 2.4; Br⁻, 0.06; F⁻, 0.001; and HCO₃⁻, 0.145.

Growth at 10, 15, 20, 25, 30, 35, 37 and 40 °C and at pH 5.0–10.0 (at 0.5 pH unit intervals, buffered with MES, MOPS, Tris or CHES and incubated at 30 °C) was measured in marine broth. The NaCl concentration range for growth was determined (0–10 %, in 0.5 % increments, w/v) at 30 °C in a modified marine broth where the artificial seawater was replaced with distilled water containing appropriate amounts of NaCl. Growth under anaerobic conditions was examined in marine broth 2216 (Difco) in an anaerobic chamber (Forma 1029; Thermo Electron) at 28 °C for 20 days. Gram-staining was performed following a standard procedure (Hucker's method) (Murray *et al.*, 1994). Cell morphology was observed by transmission electron microscopy (JEM-100CX II). Cells for observation were grown in marine broth at 30 °C for 5 days and negatively stained with a 1.0 % phosphotungstic acid solution.

Oxidase activity was examined using commercial oxidase test strips (Merck). Catalase activity was evaluated through observing bubble production in a 3 % H₂O₂ solution. Hydrolysis of starch and Tweens 40 and 80 was determined on marine agar containing 1 % (w/v) substrate at 30 °C based on the methods described in Smibert & Krieg (1994). DNA hydrolysis was tested on DNase test agar (Oxoid) prepared with artificial seawater. Utilization of various carbohydrates (1 %, w/v) as sole carbon and energy sources

was determined in a basal medium containing 0.054 % NH₄Cl, 3 % NaCl, 0.3 % MgCl₂·6H₂O, 0.2 % K₂SO₄, 0.02 % K₂HPO₄, 0.001 % CaCl₂, 0.0006 % FeCl₃·6H₂O, 0.0005 % Na₂MoO₄·7H₂O, 0.0004 % CuCl₂·2H₂O, 0.6 % Tris and distilled water as described by Shieh *et al.* (2004). Carbohydrate compounds tested included arabinose, glucose, fructose, xylose, mannose, trehalose, cellobiose, lactose, maltose, sucrose, raffinose, glycerol, mannitol, acetate, citrate, pyruvate, *N*-acetylglucosamine and Tweens 40 and 80. Other biochemical traits were determined using API 20NE strips (bioMérieux) according to the manufacturer's instructions except that cells were suspended in artificial seawater. API 20NE strips were read after 72 h of incubation at 30 °C. Three reference strains, *S. tropicus* DSM 19543^T, *S. marinus* DSM 17750^T and *M. salexigens* DSM 19753^T, were also used in the above tests; they were purchased from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) and routinely cultivated on marine agar or in marine broth at 30 °C.

Genomic DNA for use as the template for PCR amplification of the 16S rRNA gene was extracted using a bacterial genomic DNA isolation kit (BioTeke). The 16S rRNA gene was amplified using primers 27F and 1492R (Lane, 1991). The PCR products were ligated into pMD 18-T vector (TaKaRa) and then sequenced using an automated DNA sequencer (Applied Biosystems model 3730) at Biosune Inc. (Shanghai, China). The 16S rRNA gene sequence obtained was compared with those of all type strains of species with validly published names in the EzTaxon database (<http://www.eztaxon.org>; Chun *et al.*, 2007) and those in the GenBank sequence database using BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to determine the approximate phylogenetic affiliation and to select sequences of related species for further phylogenetic analysis. Pairwise 16S rRNA gene sequence similarity values were calculated through the EzTaxon server 2.1 using the global alignment algorithm (<http://www.eztaxon.org>; Chun *et al.*, 2007). Sequences of strains ZS5-23^T and ZS6-22^T were aligned with those of closely related type strains using the CLUSTAL_X 1.81 program (Thompson *et al.*, 1997). Phylogenetic trees were constructed using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods with MEGA 4.0 software (Tamura *et al.*, 2007) and using the maximum-likelihood (Felsenstein, 1981) method with the TreePuzzle 5.2 program (Schmidt *et al.*, 2002). Bootstrap analyses based on 1000 replications were performed to evaluate tree topologies. Evolutionary distances for the neighbour-joining method were computed using the model of Jukes & Cantor (1969).

Cellular fatty acids of strains ZS5-23^T and ZS6-22^T and reference strains *S. marinus* DSM 17750^T, *S. tropicus* DSM 19543^T and *M. salexigens* DSM 19753^T were analysed simultaneously at the Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences (Beijing, PR China), following the instructions of the Sherlock Microbial Identification System. For analyses of

cellular fatty acids, strains were all grown in marine broth for 5 days at 30 °C. Genomic DNA G+C content was determined by the thermal denaturation method (Marmur & Doty, 1962) using a Beckman DU800 spectrophotometer with a high-performance temperature controller. Genomic DNA of *Escherichia coli* K-12 was used as the reference. DNA–DNA hybridization between strains ZS5-23^T and ZS6-22^T was carried out based on the renaturation rate method described by De Ley *et al.* (1970) with the modifications described by Huß *et al.* (1983) and using the above-mentioned spectrophotometer.

Nearly full-length 16S rRNA gene sequences of strain ZS5-23^T and ZS6-22^T (both 1501 bp) were determined and the pairwise sequence similarity between them was 99.0 %. Searching the GenBank and EzTaxon databases revealed that both strains were most closely related to species of the genera *Spongiibacter* and *Melitea*, members of the class *Gammaproteobacteria*. The two strains shared the highest 16S rRNA gene sequence similarities with the type strain of *S. tropicus* (93.7 and 94.3 % for strains ZS5-23^T and ZS6-22^T, respectively), followed by those of *S. marinus* (93.1 and 93.7 %) and *M. salexigens* (93.1 and 93.4 %). They also exhibited 90–93 % 16S rRNA gene sequence similarity with the type strains of *Dasania marina* (Lee *et al.*, 2007), *Cellvibrio japonicus* (Humphry *et al.*, 2003), *Saccharophagus degradans* (Ekborg *et al.*, 2005), *Haliea salexigens* and *H. rubra* (Urios *et al.*, 2008a, 2009), but they showed <90 % sequence similarity with other species in the class *Gammaproteobacteria*. In phylogenetic trees inferred with the neighbour-joining, maximum-parsimony and maximum-likelihood methods (Fig. 1 and Supplementary Fig. S1, available in IJSEM Online), strains ZS5-23^T and ZS6-22^T, together with *Spongiibacter* and *Melitea* species, formed a clade with >98 % bootstrap support in the class *Gammaproteobacteria* but, within the clade, the two novel strains formed a robust branch supported by a high bootstrap value of 100 % that was obviously separate from the branch formed by *Spongiibacter* and *Melitea* species, indicating the phylogenetic novelty of the two strains at the genus level. DNA–DNA relatedness between strains ZS5-23^T and ZS6-22^T was 50.9 %, less than the threshold value of 70 % recommended to discriminate species, suggesting that they represent two genetically distinct species, despite

the high 16S rRNA gene sequence similarity (99.0 %) (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994; Stackebrandt *et al.*, 2002).

The DNA G+C contents of strains ZS5-23^T and ZS6-22^T were 51.5 and 51.8 mol%, respectively, significantly below the DNA G+C content reported for *Spongiibacter* and *Melitea* species (57.2–69.1 mol%; Table 1), the closest relatives. The fatty acid profiles of strains ZS5-23^T and ZS6-22^T were essentially similar, both containing C_{17:1}ω8c, summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH), C_{17:0}, C_{15:0}, C_{18:1}ω7c and C_{16:0} as major components (>5 %) (Table 2). However, the proportions of the above components differed considerably between the two strains, especially the three most abundant (>10 %), C_{17:1}ω8c (37.6 % in strain ZS5-23^T versus 22.5 % in strain ZS6-22^T), summed feature 3 (12.5 versus 21.8 %) and C_{17:0} (15.9 versus 10.9 %) (Table 2). The fatty acid profiles of the two strains clearly differed from that of *S. tropicus* DSM 19543^T, the closest relative, in the proportions of C_{16:0} (4.3–5.1 versus 14.9 %), C_{17:0} (10.9–15.9 versus 1.7 %), C_{17:1}ω8c (22.5–37.6 versus 5.8 %) and C_{18:1}ω7c (5.5–7.7 versus 37.6 %); they also differed from those of *S. marinus* DSM 17750^T and *M. salexigens* DSM 19753^T in the proportions of summed feature 3 (12.5–21.8 versus 3.2–5.7 %) and the presence of unknown ECL 11.799 (Table 2).

Morphological, physiological and biochemical characteristics of strains ZS5-23^T and ZS6-22^T are given in the genus and species descriptions and some of them are also shown in Table 2. Cells of both strains were Gram-negative rods that were motile with a single polar flagellum (Fig. 2). Strain ZS5-23^T could be differentiated phenotypically from strain ZS6-22^T by showing growth at >8 % NaCl and possessing a relatively strong ability to hydrolyse Tween 40 and being unable to utilize glycerol (Table 1). There were also a range of characteristics, as shown in Table 1, that allowed differentiation of both strains from *Spongiibacter* and *Melitea* species, such as flagellum formation, salt, temperature and pH ranges for growth, hydrolysis of Tweens 40 and 80 and DNA, reduction of nitrate, patterns of carbohydrate utilization and DNA G+C content.

In summary, the data presented here from our polyphasic taxonomic study, including phylogenetic analysis of 16S

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the phylogenetic positions of strains ZS5-23^T and ZS6-22^T, *Spongiibacter* and *Melitea* species and representatives of related genera. Percentage bootstrap values (1000 replicates) are indicated at nodes. Bar, 0.02 substitutions per nucleotide position.

Table 1. Differential characteristics of strains ZS5-23^T and ZS6-22^T and type strains of *Melitea* and *Spongiibacter* species

Strains: 1, strain ZS5-23^T; 2, strain ZS6-22^T; 3, *S. tropicus* DSM 19543^T (unless indicated, data from Hwang & Cho, 2009); 4, *S. marinus* DSM 17750^T (Graeber *et al.*, 2008); 5, *M. salexigens* DSM 19753^T (Urios *et al.*, 2008b). +, Positive; –, negative; w, weakly positive.

Characteristic	1	2	3	4	5
Flagellum	Single, polar	Single, polar	None	Single, polar	Single, polar
Temperature for growth (°C)					
Range	4–35	4–35	10–40	10–40	15–37
Optimum	30	30	30–35	20–30	30
NaCl concentration for growth (% w/v)					
Range	0–9.0	0–7.5	1.0–9.0	1.0–7.0	0.7–7.0
Optimum	0.5–3.0	2.5–3.5	3.0–4.0	3.0	2.8
pH for growth					
Range	5.5–9.0	5.5–9.0	6.0–9.5	6.5–9.5	6.0–10
Optimum	7.0–7.5	6.5–7.5	7.0–8.0	7.0–7.5	8.0
Nitrate reduction to nitrite	+	+	–*	w*	w*
Hydrolysis of:					
Tween 40	+	w	–*	+	+
Tween 80	–	–	+	+	w*
DNA	–	–	–*	–*	+
Utilization of:					
Fructose	+	+	–*	–*	–*
Mannitol	+	+	–*	–*	–*
Glycerol	–	+	–*	–*	–*
DNA G + C content (mol%)	51.5	51.8	57.7	69.1	57.0
Isolation source	Antarctic fast ice	Antarctic surface seawater	<i>Synechococcus</i> culture	Boreal sponge	Seawater

*Data from this study.

rRNA gene sequences, phenotypic characterization, DNA–DNA hybridization experiments and analyses of fatty acid profiles and genomic G + C contents, indicate that strains ZS5-23^T and ZS6-22^T should be assigned to a new genus in the class *Gammaproteobacteria* as the type strains of two novel species, for which the names *Zhongshania antarctica* gen. nov., sp. nov. and *Zhongshania guokunii* sp. nov. are proposed.

Description of *Zhongshania* gen. nov.

Zhongshania (Zhong.sha'ni.a. N.L. fem. n. *Zhongshania* named after the Chinese Antarctic Zhongshan Station).

Cells are Gram-negative, aerobic rods. Motile with a single polar flagellum. Catalase- and oxidase-positive. Nitrates are reduced to nitrites. NaCl is not needed for growth. Predominant fatty acids include C_{17:1}ω8c, summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH) and C_{17:0}. The genus belongs phylogenetically to the class *Gammaproteobacteria*. The type species is *Zhongshania antarctica*.

Description of *Zhongshania antarctica* sp. nov.

Zhongshania antarctica (an.tarc'ti.ca. L. fem. adj. *antarctica* southern, of the Antarctic).

Description is as for the genus plus the following characteristics. Cells are straight rods, 0.4–0.5 µm in diameter and 0.8–1.5 µm long. Colonies grown on marine agar at 30 °C for 7 days are white, circular (0.5–1.2 mm in diameter) and convex with smooth surfaces. Grows at 4–35 °C (optimum at 30 °C), but not at or above 37 °C. Grows in 0–9.0 % NaCl (w/v, optimum at 0.5–3.0 %) and at pH 5.5–9.0 (optimum at pH 7.0–7.5). Hydrolyses Tween 40 but not starch, Tween 80 or DNA. Able to utilize fructose, mannitol, pyruvate and Tweens 40 and 80 as sole carbon and energy sources but not glucose, sucrose, maltose, mannose, lactose, xylose, raffinose, trehalose, arabinose, cellobiose, *N*-acetylglucosamine, acetate, glycerol or citrate. In API 20NE tests, positive for nitrate reduction and assimilation of mannitol, but negative for indole production, hydrolysis of aesculin, arginine dihydrolase, gelatinase, urease, β-galactosidase, acid production from glucose and assimilation of arabinose, D-glucose, mannose, maltose, gluconate, capric acid, adipic acid, malic acid, citrate, phenylacetic acid and *N*-acetylglucosamine. The predominant cellular fatty acids of the type strain are C_{17:1}ω8c, C_{17:0}, summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH), C_{18:1}ω7c and C_{15:0}. The DNA G + C content of type strain is 51.5 mol%.

The type strain is ZS5-23^T (=KACC 14066^T =CCTCC AB 209246^T), isolated from Antarctic coastal fast ice.

Table 2. Cellular fatty acid compositions of strains ZS5-23^T and ZS6-22^T and type strains of *Melitea* and *Spongiibacter* species

Strains: 1, strain ZS5-23^T; 2, strain ZS6-22^T; 3, *S. tropicus* DSM 19543^T; 4, *S. marinus* DSM 17750^T; 5, *M. salexigens* DSM 19753^T. All data were obtained in this study under identical conditions. Values are percentages of total fatty acids. —, Not detected; ECL, equivalent chain-length.

Fatty acid	1	2	3	4	5
C _{9:0}	0.2	—	0.6	2.0	1.5
C _{10:0}	0.2	0.2	2.5	0.3	0.5
C _{11:0}	1.3	1.1	—	1.1	0.6
C _{12:0}	0.4	0.9	1.0	0.2	0.4
C _{13:0}	0.4	0.9	—	0.5	0.3
C _{14:0}	0.5	1.4	2.0	0.2	0.6
C _{15:0}	6.0	9.8	1.9	4.8	6.9
C _{16:0}	4.3	5.1	14.9	3.6	4.6
C _{17:0}	15.9	10.9	1.7	11.4	15.4
C _{18:0}	0.8	1.5	1.9	0.9	1.0
iso-C _{17:0}	0.4	—	—	0.9	1.7
C _{9:0} 3-OH	0.3	0.5	—	0.4	0.3
C _{10:0} 3-OH	1.1	2.1	4.7	0.6	1.2
iso-C _{11:0} 3-OH	0.6	1.1	0.2	2.9	3.5
C _{11:0} 3-OH	3.7	3.7	1.0	4.6	3.1
C _{12:0} 3-OH	0.3	0.6	0.7	—	—
C _{15:1} ω6c	0.8	2.5	—	0.4	0.5
C _{15:1} ω8c	0.3	0.4	—	0.2	—
C _{17:1} ω6c	0.8	0.6	—	0.5	0.6
C _{17:1} ω8c	37.6	22.5	5.8	53.6	36.0
C _{18:1} ω7c	7.7	5.5	37.6	3.9	9.5
C _{18:1} ω9c	0.6	1.5	2.5	1.0	0.9
Summed feature 1*	0.3	—	—	—	—
Summed feature 3*	12.5	21.8	15.3	3.2	5.7
Unknown ECL 11.799	2.0	2.8	—	—	—

*Summed features are groups of two or three fatty acids that could not be separated by GLC. Summed feature 1 comprised C_{13:0} 3-OH and/or iso-C_{15:1} H; summed feature 3 comprised C_{16:1}ω7c and/or iso-C_{15:0} 2-OH.

Description of *Zhongshania guokunii* sp. nov.

Zhongshania guokunii (guo.ku'ni.i. N.L. gen. masc. n. *guokunii* of Guo Kun, named after Guo Kun, who made a significant contribution to the Chinese Antarctic expedition).

Description is as for the genus plus the following characteristics. Cell are straight rods, 0.4–0.5 µm in diameter and 0.8–1.5 µm long. Colonies grown on marine agar at 30 °C for 7 days are white, circular (0.5–1.2 mm in diameter) and convex with smooth surfaces. Grows at 4–35 °C (optimum at 30 °C), but not at or above 37 °C. Grows in 0–7.5 % NaCl (w/v, optimum at 2.5–3.5 %) and at pH 5.5–9.0 (optimum at pH 6.5–7.5). Hydrolyses Tween 40 (weakly) but not starch, Tween 80 or DNA. Able to utilize fructose, glycerol, mannitol, pyruvate and Tweens 40 and 80 as sole carbon and energy sources but

Fig. 2. Transmission electron micrograph of negatively stained cells of strains ZS5-23^T (a) and ZS6-22^T (b) cultured in marine broth at 30 °C for 5 days. F, Flagellum. Bars, 1 µm.

not glucose, sucrose, maltose, mannose, lactose, xylose, raffinose, trehalose, arabinose, cellobiose, *N*-acetylglucosamine, acetate or citrate. In API 20NE tests, positive for nitrate reduction and assimilation of mannitol, but negative for indole production, hydrolysis of aesculin, arginine dihydrolase, gelatinase, urease, β-galactosidase, acid production from glucose and assimilation of arabinose, glucose, mannose, maltose, gluconate, capric acid, adipic acid, malic acid, citrate, phenylacetic acid and *N*-acetylglucosamine. The predominant cellular fatty acids of the type strain are C_{17:1}ω8c, summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH), C_{17:0}, C_{15:0} and C_{18:1}ω7c. The DNA G+C content of the type strain is 51.8 mol%.

The type strain is ZS6-22^T (=KACC 14532^T =CCTCC AB 209247^T), isolated from Antarctic coastal surface seawater.

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