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Brevibacterium spongiae sp. nov., isolated from marine sponge *Hymeniacidon* sp.

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Abstract

A novel bacterial strain, designated as WHS-Z9^T, was isolated from marine sponge *Hymeniacidon* sp. collected from Weihai (37° 25′ N, 121° 58′ E), Shandong Province, PR China. Cells of strain WHS-Z9^T were Gram-stain-positive, non-spore-forming, non-motile, short-rod-shaped and light yellow-pigmented. The strain could grow at 10–40 °C (optimum, 20 °C), pH 4.5–9.5 (optimum, pH 8.5) and 2–14% (w/v) NaCl (optimum, 4%). The 16S rRNA gene sequence of strain WHS-Z9^T showed 98.7% similarity to that of *Brevibacterium epidermidis* NBRC 14811^T, 98.5% to *Brevibacterium sediminis* FXJ8.269^T and 98.4% to *Brevibacterium oceani* BBH7^T. The phylogenetic tree based on 16S rRNA gene sequences revealed that strain WHS-Z9^T was clustered with *Brevibacterium limosum* o2^T. The whole genome of WHS-Z9^T was approximately 4217721 bp in size with a G+C content of 65.2%. The average nucleotide identity (ANI) and digital DNA–DNA hybridization (dDDH) values among WHS-Z9^T and other *Brevibacterium* type strains were 83.3–85.5% (ANI based on BLAST), 86.4–87.9% (ANI based on MUMmer) and 41.9–57.5% (dDDH). Percentage of conserved protein values between the genomes of strain WHS-Z9^T and members of genera *Brevibacterium* were 76.8–82.9%, while the average amino acid identity (AAI) values were 83.7–87.0%. The dDDH, ANI, AAI and POCP values were below the standard cut-off criteria for the delineation of bacterial species. The sole respiratory quinone in strain WHS-Z9^T was MK-8(H_2), and the predominant fatty acids were anteiso-$C_{15:0}$ and anteiso-$C_{17:0}$. The major polar lipids of WHS-Z9^T consisted of diphosphatidylglycerol and glycolipid. The diagnostic cell-wall diamino acid of strain WHS-Z9^T was *meso*-diaminopimelic acid. Based on the data obtained in this study, strain WHS-Z9^T (=MCCC 1K07845^T=KCTC 49848^T) should be classified as the type strain of a novel species of the genus *Brevibacterium*, for which the name *Brevibacterium spongiae* sp. nov. is proposed.

The genus *Brevibacterium* was proposed by Breed in 1953 [1], for which the type species is *Brevibacterium linens*. At the time of writing, the genus *Brevibacterium* contains 36 species with validly published names (www.bacterio.net/brevibacterium.html) [2]. Members of the genus have been isolated from habitats such as sediments [3–5], cheese [6, 7], water [8] and baijiu [9]. *Brevibacterium* species are Gram-stain-positive, strictly aerobic, short rods. The major cellular fatty acids are anteiso-$C_{15:0}$ and anteiso-$C_{17:0}$, and the major menaquinone is MK-8(H_2) for most members of the genus *Brevibacterium* [10].

In this study, strain WHS-Z9^T, which was isolated from sponge *Hymeniacidon* sp. from Weihai (37° 25′ N, 121° 58′ E), Shandong province, PR China, is identified as belonging to a novel species of the genus *Brevibacterium* by using a polyphasic approach. The sponge sample was identified according to the cytochrome oxidase subunit I (COI) gene with 99.64% similarity to *Hymeniacidon perlevis*. The sponge sample's COI gene sequence was deposited into GenBank under accession number OP757661. The sponge was rinsed with ice-cold Ca^{2+}- and Mg^{2+}-free sterile artificial seawater (400 mM NaCl, 27.6 mM Na_2SO_4, 2.3 mM $NaHCO_3$, 8.9 mM KCl, 0.8 mM KBr, 0.4 mM H_3BO_3, 0.15 mM $SrCl_2$, 0.07 mM NaF) several times to remove organisms loosely attached on the sponge surface. Sponge surface tissue was removed by a sterile surgical blade. Clean sponge internal tissues were ground with a mortar and pestle aseptically. For micro culture, the ground sponge sample was diluted in cold sterile artificial seawater free of $Ca^{2+}$and Mg^{2+} for 1:200 as the bacterial inoculum. About 50 µl bacterial inoculum was added to 10 ml Ca^{2+}- and Mg^{2+}-free

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Abbreviations: AAI, average amino acid identity; ANI, average nucleotide identity; COI, cytochrome oxidase subunit I; dDDH, digital DNA–DNA hybridization; GBDP, genome BLAST distance phylogeny method; GGDC, Genome-to-Genome Distance Calculator; MA, marine agar; MB, marine broth; MK, menaquinone; PC, polycarbonate; POCP, percentage of conserved protein; TCI, tissue culture insert.
The GenBank accession numbers for the 16S rRNA gene sequence and the whole-genome sequence of strain WHS-Z9^T are OM970864 and CP093443, respectively.
Five supplementary figures and one supplementary table are available with the online version of this article.

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sterile artificial seawater; then, the mixture was filtered by a 0.2 µm polycarbonate (PC) membrane using a syringe filter holder. The tissue culture insert (TCI) was filled with 3 g ground sponge tissue. A TCI was then placed upside down in a sterile six-well multi-dish. Inoculated PC membranes were then placed on top of a sterile 25 mm TCI. The culture vessels were incubated at 20 °C in the dark for 7–10 days. After incubation for 7–10 days, the PC membrane was carefully placed into a centrifuge tube containing 5 ml ultrapure Milli-Q water with sterile tweezers. Then the sample was homogenized and diluted 10- to 1000- fold and spread on agar plates with 2216E medium (0.5% tryptone, 0.1% yeast extract and 0.01% $FePO_4$, pH 7.0–7.5; artificial seawater). After incubation for 2 weeks at 20 °C, each colony was selected and spread on marine agar (MA; Difco). The purified strain WHS-Z9^T was stored at −80 °C in marine broth (MB; Difco) with 30% (v/v) glycerol.

Genomic DNA was extracted using a TIANamp Bacteria DNA Kit (Tiangen Biotech) as described by the manufacturer. PCR amplification was performed using primers, 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). The PCR condition was as follows: denaturation at 95 °C for 10 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 120 s. At the end of the cycles, the reaction mixture was kept at 72 °C for 10 min and then cooled to 4 °C. The amplified gene fragments were recovered by agarose gel electrophoresis and then connected with plasmid pEASY -T5 Zero vector (TransGen Biotech). The plasmid pEASY -T5 Zero vector was introduced into DH5α cells for blue-white screening. The positive white clones on the plate were selected for culture, and the plasmids were extracted and sequenced at Sangon Biotech (Shanghai, PR China) according to Sanger *et al.* [11] to obtain the complete 16S rRNA gene sequence of WHS-Z9^T. The almost full-length 16S rRNA gene sequence of WHS-Z9^T (1449 bp) was determined. Then, 16S rRNA gene similarity was compared by EzBioCloud (www.ezbiocloud.net) [12]. Phylogenetic analysis was carried out using three tree-making algorithms (neighbour-joining, maximum-likelihood and minimum-evolution) using MEGA version X [13] after multiple alignments of sequences using the CLUSTAL_X program [14]. Regions including any gaps were removed. Evolutionary distance matrices of phylogenetic trees were calculated according to Kimura's two-parameter model [15]. Bootstrap analysis was performed with 1000 replications [16].

The neighbour-joining tree (Fig. 1) shows that strain WHS-Z9^T is clustered with *Brevibacterium limosum* o2^T. The cluster was further found to be stable when trees were reconstructed using the maximum-likelihood and maximum-parsimony algorithms (Figs S1 and S2, available in the online version of this article). Thus WHS-Z9^T was suggested to be affiliated with the genus *Brevibacterium*. In the phylogenetic analysis of 16S rRNA gene sequences, '*Brevibacterium renqingii*' REN4^T, *Brevibacterium limosum* o2^T, *Brevibacterium siliguriense* MB18^T, *Brevibacterium linens* CNRZ 918^T, *Brevibacterium permense* VKM Ac-2280^T, *Brevibacterium epidermidis* NBRC 14811^T and *Brevibacterium sediminis* FXJ8.269^T were selected as experimental controls. *B. limosum* o2^T, *B. epidermidis* NBRC 14811^T, *B. sediminis* FXJ8.269^T and '*B. renqingii*' REN4^T were obtained from the Marine Culture Collection of China (MCCC). *B. siliguriense* MB18^T was purchased from the Belgian Coordinated Collections of Microorganisms (BCCM). *B. linens* CNRZ 918^T was purchased from the NITE Biological Resource Center (NBRC). *B. permense* VKM Ac-2280^T was obtained from the Japan Collection of Microorganisms (JCM).

Whole-genome sequencing of WHS-Z9^T was performed on the DNBSEQ (BGI) and Nanopore (ONT) platforms. The high-quality paired-end reads were assembled using Canu version 1.5 and GATK version 1.6-13. The rRNA and tRNA genes of strain WHS-Z9^T were predicted using RNAmmer [17] and tRNAscan-SE, respectively [18]. The gene prediction for strain WHS-Z9 ^{T} was carried out using Glimmer (version 3.02) [19–21]. The genome data of strain WHS-Z9^T and its related genus *Brevibacterium* were uploaded to the Type Strain Genome Server (https://tygs.dsmz.de/) [22] to reconstruct a phylogenomic tree. The single-copy orthologous cluster protein sequences with the command '- e=1e-5 - cov=50 - identity=50' were extracted by Proteinortho version 6. After multiple comparisons of single-copy orthologous cluster protein sequences by the MUSCLE program [23], phylogenetic analysis was conducted using the neighbour-joining tree-making algorithm using MEGA version X. The average nucleotide identity (ANI) values were calculated using EzBioCloud web service and digital DNA–DNA hybridization (dDDH) values were calculated by the Genome-to-Genome Distance Calculator 3.0 (GGDC 3.0) [24, 25]. The percentage of conserved proteins (POCP) between the genomes of strain WHS-Z9^T and selected type strains of the genus *Brevibacterium* were calculated [26]. Average amino acid identity (AAI) values were also calculated as a mean similarity of orthologous genes [27].

The whole genome sequence of strain WHS-Z9^T was a total stretch of 4217721 bp, which was uploaded to NCBI GenBank with accession number CP093443. The genome annotation was carried out by the NCBI Prokaryotic Genome Annotation Pipeline. The WHS-Z9^T genome contained 3715 CDSs, 12 rRNA genes, 10 sRNA genes and 48 tRNA genes. The DNA G+C content of WHS-Z9^T genome was 65.2%, which was in accordance with the fact that the DNA G+C contents of the genus of *Brevibacterium* are between 64.2 and 65.8% (Table 1). As predicted by the antiSMASH 6.0 program [28], the genome of strain WHS-Z9^T harboured eight secondary metabolite biosynthetic gene clusters (Table S1). Other strains of the genus *Brevibacterium*, with the exception of strain *B. limosum* o2^T, had the potential to produce endophenazine A1, endophenazine F and endophenazine G. Natural phenazine compounds showed antitumour and antibacterial activities and were potential cancer therapeutic agents [29, 30].

Different from the phylogenetic tree of 16S rRNA sequences, WHS-Z9^T forms a separate branch based on genome sequences (Fig. 2) and single-copy orthologous cluster protein sequences (Fig. 3) phylogenetic analysis. As shown in Table 2, among the genomes of strain WHS-Z9^T and 10 related type species, the ANIb values are 83.3–85.5% and the ANIm values are 86.4–87.9%,

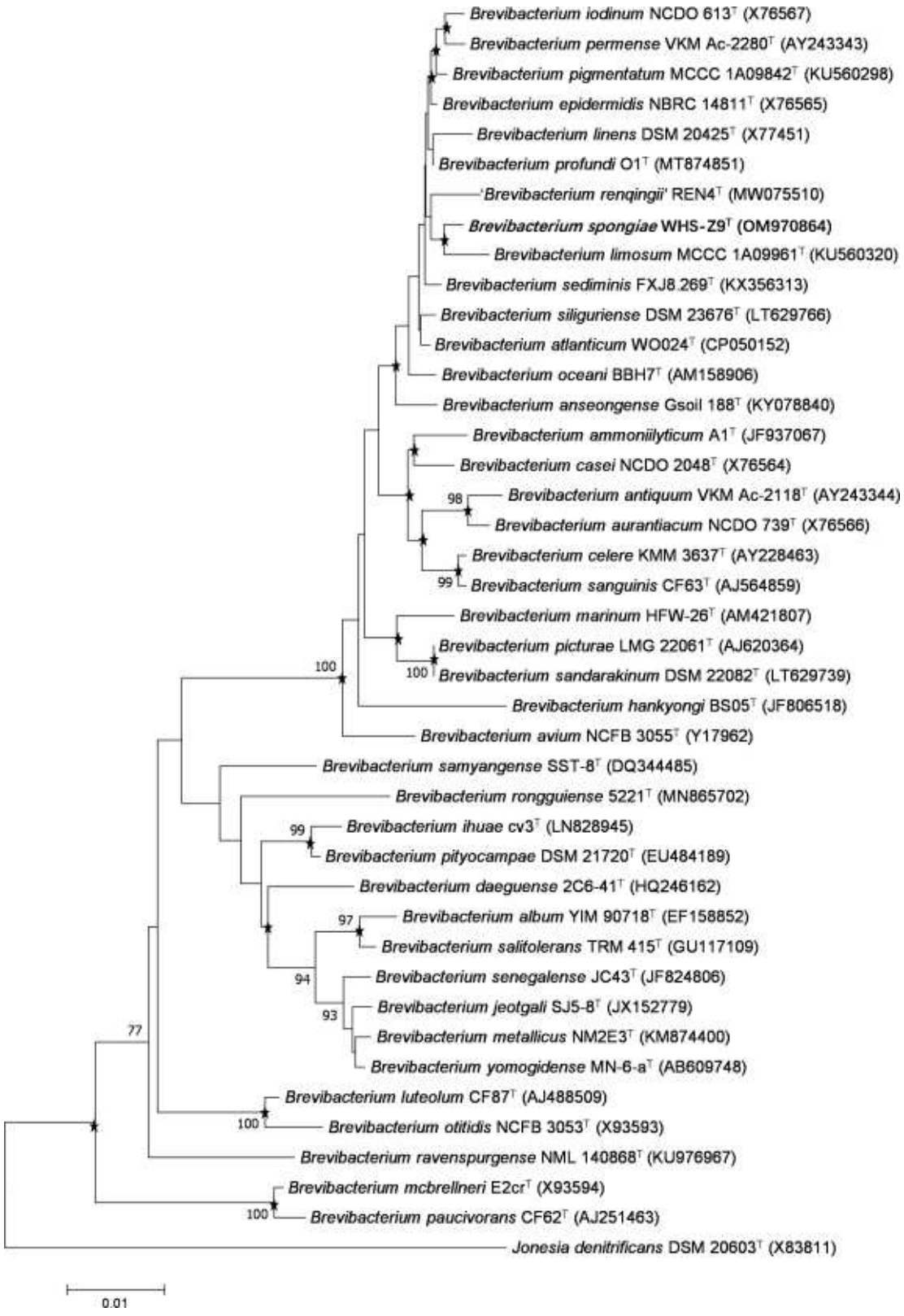

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of strain WHS-Z9^T within the genus *Brevibacterium*. Values above each branch indicate bootstrap values (>70%) from the neighbour-joining method as percentages of 1000 replicates. *Jonesia denitrificans* DSM 20603^T (X83811) was used as an outgroup. Nodes that are present in all trees are labelled by stars. Bar, 0.01 substitutions per nucleotide position.

Table 1. Differential characteristics of strain WHS-Z9^T with respect to the known type strains of genus *Brevibacterium*

Strains: 1, WHS-Z9^T; 2, *B. limosum* o2^T; 3, *B. linens* CNRZ 918^T; 4, *B. siliguriense* MB18^T; 5, *B. permense* VKM Ac-2280^T; 6, '*B. renqingii*' REN4^T; 7, *B. epidermidis* NBRC 14811^T; 8, *B. sediminis* FXJ8.269^T. Data are from this study, except where indicated. +, Positive; –, negative; w, weak positive.

Characteristic	1	2	3	4	5	6	7	8
Growth temperature (°C):								
Range	10–40	4–45	5–37	20–37	7–37	28–37	10–37	8–37
Optimum	20	30	20–25	28	24	30	30	30
Growth pH:								
Range	4.5–9.5	4.5–10.0	4.5–9.0	4.5–9.5	4.5–8.5	6.0–10.0	4.5–10.0	4.5–11.0
Optimum	8.5	8.5	6.0	7.0	7.0	8.0	8.0	7.0
NaCl concentration for growth (%):								
Range	2–14	0–14	0–16	0–15	0–16	0–11	0–16	0–16
Optimum	4	2	4	2	11	3	4	4
Nitrate reduction	–	+	+	+	–	+	–	+
Assimilation of:								
Glucose	+	+	w	w	+	w	+	w
Mannitol	w	w	w	w	–	–	+	–
Potassium gluconate	w	w	w	+	–	+	–	w
Capric acid	–	–	–	w	–	–	–	–
Adipic acid	w	w	+	+	w	w	–	w
Hydrolysis of:								
Urease	–	+	–	+	–	–	–	–
Enzyme activity:								
Trypsin	w	–	–	–	w	–	–	–
α-Chymotrypsin	–	–	–	w	–	–	–	–
Carbon source utilization:								
N-Acetyl neuraminic acid	+	+	+	+	–	–	–	–
L-Fucose	–	–	w	+	–	w	–	–
myo-Inositol	+	–	–	–	–	–	–	+
Serine	–	w	–	w	–	–	–	–
Gelatin	w	–	w	+	–	–	–	+
Gluconic acid	+	+	–	+	–	+	–	–
Glucuronic acid	–	–	w	w	w	+	–	–
Quinic acid	+	+	–	+	–	+	–	–
Saccharic acid	+	+	–	–	–	+	–	–
Methyl pyruvate	+	+	–	–	w	w	–	+
Bromo-succinic acid	+	w	–	w	w	+	–	–
Formic acid	+	+	–	+	+	+	–	+
DNA G+C content (%)	65.23	64.8*	64.8†	64.2‡	64.5§	65.8¶	66.2–67.2#	64.0–64.2**

*Data from Pei *et al.* [3].
†Data from Collins *et al.* [6].
‡Data from Kumar *et al.* [8].
§Data from Gavrish *et al.* [4].
¶Data from Yan *et al.* [9].
#Data from Collins *et al.* [7].
**Data from Chen *et al.* [5].

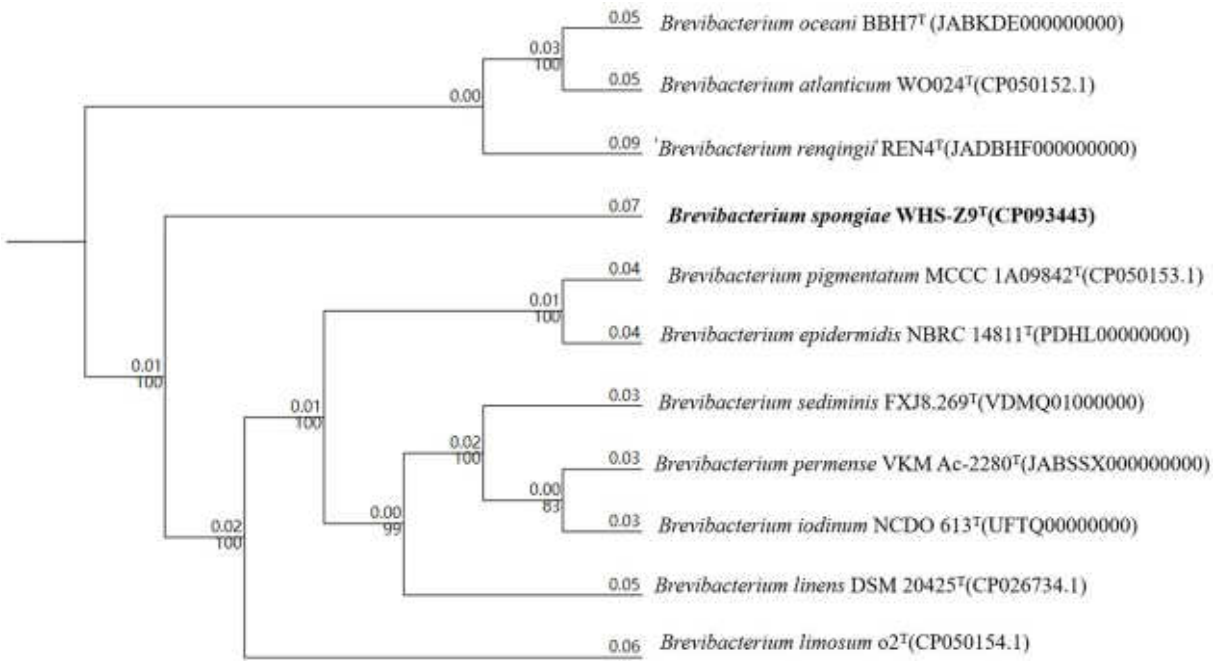

Fig. 2. Phylogenomic tree based on genome sequences of strain WHS-Z9^T and the related type species using the genome BLAST distance phylogeny (GBDP) method. The numbers above branches are GBDP pseudo-bootstrap support values>60% from 100 replications, with an average branch support of 95.5%. GenBank accession numbers are included in parentheses.

which are lower than the recommended threshold values for species delineation [31]. The dDDH values between strain WHS-Z9^T and its neighbouring type strains range from 41.9 to 57.5%, which are far below the 70% threshold for species delineation [31]. POCP values between the genomes of strain WHS-Z9^T and members of genera *Brevibacterium* are 76.8–82.9%, while the AAI values are 83.7–87.0%. Although there is no generally recognized genus boundary, recent studies suggested AAI (60–80%) and

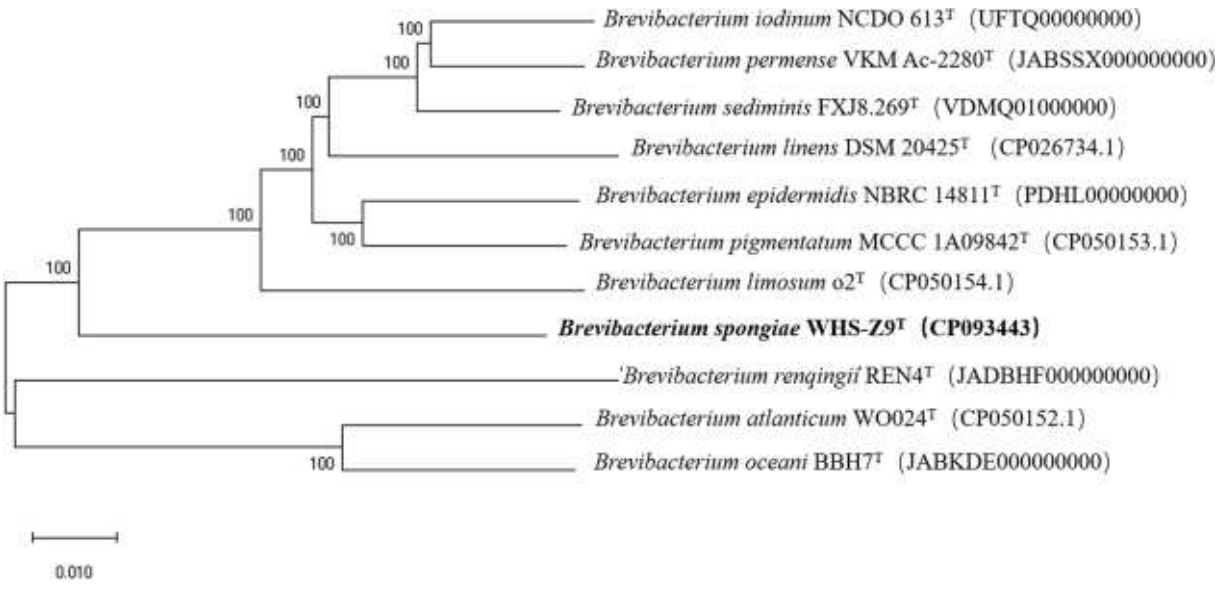

Fig. 3. Neighbour-joining tree based on the partition of 1984 single-copy orthologous cluster protein sequences showing the phylogenetic relationship of strain WHS-Z9^T. Bootstrap values are based on 1000 replicates. Bar., 0.01 substitutions per nucleotide position. GenBank accession numbers are given in parentheses.

Table 2. The percentage of conserved protein (POCP), average amino acid identity (AAI), average nucleotide identity (ANI) and digital DNA–DNA hybridization (dDDH) values between strain WHS-Z9^T and related type species of the genus *Brevibacterium*

ANIb, ANI based on BLAST; ANIm, ANI based on MUMmer; CI, confidence interval; GGDC, Genome-to-Genome Distance Calculator.

WHS-Z9^T vs	Accession no.	POCP (%)	AAI (%)	ANI (%)		GGDC	
				ANI (%)	ANIm	dDDH (%)*	Model CI (%)
B. sediminis FXJ8.269^T	VDMQ01000000	81.9	86.4	84.7	87.4	55.7	52.2–59.2
B. limosum o2^T	CP050154.1	81.3	86.2	85.0	87.5	52.8	49.4–56.3
'*B. renqingii*' REN4^T	JADBHF000000000	76.8	83.7	83.3	86.4	41.9	38.5–45.4
B. linens DSM 20425^T	CP026734.1	81.1	86.5	84.9	87.4	51.9	48.4–55.3
B. epidermidis NBRC 14811^T	PDHL00000000	82.8	86.9	85.1	87.6	56.9	53.4–60.5
B. pigmentatum MCCC 1A09842^T	CP050153.1	82.9	87.0	85.5	87.9	57.5	53.9–61.0
B. permense VKM Ac-2280^T	JABSSX000000000	78.8	86.1	84.8	87.5	50.3	46.8–53.7
B. iodinum NCDO 613^T	UFTQ00000000	79.4	86.2	84.9	87.5	49.6	46.2–53.1
B. atlanticum WO024^T	CP050152.1	78.0	84.0	83.4	86.7	43.8	40.5–47.3
B. oceani BBH7^T	JABKDE000000000	79.7	84.4	83.4	87.0	44.4	41.0–47.8

*Formula 2 based on a generalized linear model (identities/high-scoring segment pair) was used for dDDH analysis.

POCP (50%) could be thresholds for distinguishing genera [26, 32]. The results of the phylogenetic analyses of the 16S rRNA gene and whole genomes provide strong evidence for recognizing WHS-Z9^T as a novel species in the genus *Brevibacterium*.

The Gram-staining was performed using a Gram stain kit (Hangzhou Tianhe Microorganism Reagent Co.) according to the manufacturer's instructions. Growth at various temperatures (4, 10, 15, 20, 28, 37, 40 and 50 °C) was measured on the MA medium after up to 7 days of incubation. The requirement and tolerance of NaCl for growth were tested in MB medium without NaCl. The final NaCl concentrations supplemented in the above broth were 1–16% (w/v; intervals of 1%). Growth at different salt concentrations and various pH values (pH 4–10 at intervals of 0.5 pH units) were assessed after incubation for 3 days by measuring the OD_{600} (Multifunctional enzyme marker-Spark; Tecan). The following buffers (final concentration, 50 mM) were used to adjust the pH of MB. Acetate buffer was used for pH 4.0–5.5, phosphate buffer was used for pH 6.0–8.0 and Tris buffer was used for pH 8.5–10.0. According to the manufacturer's instructions; anaerobic growth was determined on an MA medium at 20 °C after 3 days of incubation using AnaeroPack-Anaero (MGC) [33]. Cell morphology was observed by a scanning electron microscope (Sirion 200, FEI) after 3 days of incubation in MB medium at 20 °C. Colony appearance was observed on MA medium after 3 days of incubation at 20 °C.

Strain WHS-Z9^T was Gram-stain-positive and strictly aerobic. Under a scanning electron microscope, cells of WHS-Z9^T were about 0.6–0.8 µm long and 0.3–0.5 µm wide, appearing to be rod-shaped without flagella (Fig. S3). Colonies were yellow, circular, convex with entire margins and 1 mm in diameter after incubation for 3 days on MA at 20 °C. Strain WHS-Z9^T grew at 10–40 °C and showed fast growth at 20 °C. It was able to grow at pH 4.5–9.5 with the highest cell concentration at pH 8.5 and in the presence of 2–14% (w/v) NaCl with the highest cell concentration at 4% (w/v) NaCl.

Catalase activity was determined by assessing the bubble production by adding a drop of 3% (v/v) H_2O_2 on the bacterial culture cultured on MA plates at 20 °C for 3 days. Oxidase activity was determined based on the oxidation of tetramethyl-*p*-phenylenediamine [34] after 3 days incubation on MA plates at 20 °C, meanwhile the acid production and enzyme activity for 49 carbohydrates were determined by API 50 CH and API ZYM systems (bioMérieux). According to the manufacturers' instructions, the API 20 NE system (bioMérieux) and Biolog GEN III microporous plates were used to determine nitrate reduction, assimilation reaction, hydrolysis reaction and carbon source utilization. All these were carried out in duplicate and repeated twice to ensure reproducibility. The susceptibility of strain WHS-Z9^T to 22 different types of antibiotics was tested. Discs (Bio-Rad) containing the following antibiotics were used: 2 µg (clindamycin), 5 µg (ciprofloxacin, levofloxacin and rifampicin), 10 µg (ampicillin, gentamicin, tobramycin, amoxicillin and streptomycin), 10 U (penicillin), 15 µg (clarithromycin and erythromycin), 30 µg (aztreonam, ceftriaxone, cefotaxime, ceftazidime, chloramphenicol, kanamycin, tetracycline and vancomycin), 75 µg (mezlocillin) and 100 µg (piperacillin). The cell suspensions and the discs were placed on MA plates which were incubated at 20 °C for 3 days and the radius of the growth-inhibition zones was measured. The sensitivity measurement of bacteria to antibacterial agents was based on the diameter of the inhibition ring around the paper. The larger the inhibition ring, the more sensitive the bacteria are to the

Table 3. Differential chemotaxonomic characteristics of strain WHS-Z9^T and its closely related species of the genus *Brevibacterium*

Strains: 1, WHS-Z9^T; 2, *B. limosum* o2^T; 3, *B. linens* CNRZ 918^T; 4, *B. siliguriense* MB18^T; 5, *B. permense* VKM Ac-2280^T; 6, '*B. renqingii*' REN4^T; 7, *B. epidermidis* NBRC 14811^T; 8, *B. sediminis* FXJ8.269^T. Data are from this study, except where indicated. TR, Trace (<1.0%); –, not detected; NA, no data available; MK, menaquinone; DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PL, unidentified phospholipid; GL, unidentified glycolipid; DMDG, dimannosyl diacylglycerol; PGL, phosphatidylglycerollipids. The MIDI system groups these fatty acids together as one feature with a single percentage of the total [38]. Major fatty acid components are marked in bold.

Component	1	2	3	4	5	6	7	8
Major Menaquinones	MK-8(H_2)	MK-7(H_2), MK-8(H_2)*	MK-8(H_2)†	MK-8(H_2)‡	MK-8(H_2)§	MK-8(H_2)¶	MK-8(H_2)	MK-8(H_2)
Saturated fatty acids:								
$C_{16:0}$	TR	2.0	3.4	TR	TR	–	0.7	0.24
$C_{18:0}$	TR	1.5	2.8	TR	TR	–	–	–
Branched fatty acids:								
iso-$C_{17:0}$	TR	1.3	–	–	–	–	0.8	0.55
iso-$C_{15:0}$	3.6	5.5	4.5	5.9	4.6	–	6.8	7.39
iso-$C_{16:0}$	4.2	3.0	3.7	–	–	–	**20.3**	2.78
anteiso-$C_{15:0}$	**44.6**	**41.8**	**39.2**	**43.4**	**42.1**	**51.2**	**43.5**	**61.6**
anteiso-$C_{17:1}$	2.5	TR	TR	TR		–	–	1.74
anteiso-$C_{17:0}$	**39.0**	**36.8**	**34.2**	**37.6**	**41.6**	**31.3**	**24.0**	**18.52**
Unsaturated fatty acids:								
$C_{18:1}$ *ω9c*	TR	1.5	1.7	TR	TR	–	–	–
Major Polar lipids:	DPG, GL	DPG, PG, GL*	DPG, PG, DMDG†	DPG, PG‡	NA	DPG, PG, PGL, PL¶	DPG, PG#	DPG, PG, GL**

*Data from Pei *et al.* [3].
†Data from Collins *et al.* [6].
‡Data from Kumar *et al.* [8].
§Data from Gavrish *et al.* [4].
¶Data from Yan *et al.* [9].
#Data from Collins *et al.* [7].
**Data from Chen *et al.* [5].

antibacterial agents: the diameter of the inhibition ring >20 mm is extremely sensitive, >15–20 mm is highly sensitive, 10–15 mm is moderately sensitive and <10 mm is resistant [35].

Strain WHS-Z9^T was positive for catalase, which was consistent with *B. limosum* o2^T, *B. siliguriense* MB18^T, *B. linens* CNRZ 918^T, *B. sediminis* FXJ8.269^T, *B. epidermidis* NBRC 14811^T and *B. permense* VKM Ac-2280^T. Strain WHS-Z9^T was negative for oxidase, which was consistent with *B. limosum* o2^T, *B. siliguriense* MB18^T, *B. linens* CNRZ 918^T, *B. epidermidis* NBRC 14811^T and *B. permense* VKM Ac-2280^T. No reduction of nitrate to nitrite was found in WHS-Z9^T. Strain WHS-Z9^T was positive for the hydrolysis of gelatin but negative for the hydrolysis of aesculin, urease and arginine. Results were positive for the assimilation of glucose, mannose (weak), potassium gluconate (weak), adipic acid (weak), malic acid, trisodium citrate, phenylacetic acid and mannitol (weak), but negative for the assimilation of L-arabinose, maltose, capric acid and *N*-acetyl-glucosamine. The Biolog GEN III microporous plates analysis showed that strain WHS-Z9^T was negative for maltose, trehalose, cellobiose, gentiobiose, sucrose, turanose, stachyose, lactose, melibiose, methyl *β*-D-glucoside, salicin, *N*-acetyl-D-glucosamine, *N*-acetyl-*β*-dmannosamine, 3-methyl glucose, fucose, L-fucose, L-rhamnose, sorbitol, glucose-6-PO_4, aspartic acid, serine, L-galactonic acid lactone, glucuronic acid, mucic acid and lactic acid methyl ester. Other phenotypic characteristics, compared with those of the closest phylogenetic relatives, are shown in Table 1. Strain WHS-Z9^T was resistant to (μg per disc unless otherwise stated) gentamicin (10), kanamycin (30), clindamycin (2), levofloxacin (5), aztreonam (30) and ciprofloxacin (5), but susceptible to streptomycin (10), chloramphenicol (30), ceftazidime (30), rifampicin (5), erythromycin

(15), clarithromycin (15), tetracycline (30), tobramycin (10), mezlocillin (5), vancomycin (30), amoxicillin (10), penicillin (10U), cefotaxime (30), ceftriaxone (30) and ampicillin (10).

After incubation for 72 h at 20 °C on MA medium, well-grown cells were harvested and fatty acid methyl esters were prepared as described previously [36]. The fatty acids were analysed by gas chromatography (Agilent Technologies 6850) and identified using the TSBA6.0 database of the Microbial Identification System [37, 38]. Isoprenoid quinones were extracted from freeze-dried cells (100 mg) with chloroform–methanol (2:1, v/v) and analysed using previously described methods [39, 40]. Polar lipids were extracted and separated on silica gel 60 aluminum-backed thin-layer plates according to Minnikin *et al.* [41]. *α*-Naphthol, ninhydrin and molybdatophosphoric acid were used to detect other polar lipids according to Tindall [42]. The amino acids of the cell wall were determined as described previously with TLC cellulose 50 glass plates (Merck) [40].

As summarized in Table 3, the main fatty acid components of strain WHS-Z9^T (>10%) were anteiso-$C_{15:0}$ (44.6%) and anteiso-$C_{17:0}$ (39.0%). The main components were generally identical to those of *B. limosum* o2^T, *B. linens* CNRZ 918^T, *B. siliguriense* MB18^T, *B. permense* VKM Ac-2280^T, *B. sediminis* FXJ8.269^T, *B. epidermidis* NBRC 14811^T and '*B. renqingii*' REN4^T, but differences were observed in the relative proportions of individual components among these strains. The predominant respiratory quinone of WHS-Z9^T was MK-8(H_2), which was typical of *Brevibacterium*. The diagnostic polar lipids of the isolate were diphosphatidylglycerol and glycolipid. The polar lipids of strain WHS-Z9^T included glycolipid, which was consistent with *B. limosum* o2^T and *B. sediminis* FXJ8.269^T. The polar lipid profile of strain WHS-Z9^T had diphosphatidylglycerol, which was consistent with *B. limosum* o2^T, *B. linens* CNRZ 918^T, *B. permense* VKM Ac-2280^T, *B. sediminis* FXJ8.269^T, *B. epidermidis* NBRC 14811^T and '*B. renqingii*' REN4^T (Fig. S4). The diagnostic cell-wall diamino acid of strain WHS-Z9^T was *meso*-diaminopimelic acid (Fig. S5).

Thus, based on the above results, strain WHS-Z9^T represents a novel species of the genus *Brevibacterium*, for which the name *Brevibacterium spongiae* sp. nov. is proposed.

DESCRIPTION OF *BREVIBACTERIUM SPONGIAE* SP. NOV.

Brevibacterium spongiae (spon'gi.ae. L. gen. n. *spongiae* of a sponge, the source of the type strain).

Cells are Gram-stain-positive, non-spore-forming, non-motile, short rods (0.6–0.8 μm long and 0.3–0.5 μm wide). Colonies are yellow, circular, convex with entire margins and 1 mm in diameter after incubation for 3 days on MA at 20 °C. Growth occurs at 10–40 °C, at pH 4.5–9.5 and salinity (NaCl equivalent) up to 14% (w/v). Catalase activity is positive. Oxidase activity is negative. Positive for the hydrolysis of gelatin, assimilation of glucose, mannose (weak), mannitol (weak), potassium gluconate (weak), adipic acid (weak), malic acid, trisodium citrate and phenylacetic acid. Negative for nitrite reduction, indole production, glucose fermentation, arginine dihydrolase, urease, *β*-glucosidase, *β*-galactosidase and assimilation of L-arabinose, *N*-acetyl-glucosamine, maltose and capric acid. Alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, lipase (C14), valine arylamidase and trypsin are present. Dextrin, *N*-acetyl neuraminic acid, α-D-glucose, mannose, fructose, galactose, inosine, trehalose, mannitol, arabitol, *myo*-inositol, glycerol, fructose-6-PO_4, gelatin (weak), glycyl-L-proline, L-alanine, L-arginine, L-aspartic acid, L-glutamic acid, L-histidine, L-pyroglutamic acid, L-serine, pectin (weak), D-galacturonic acid (weak), gluconic acid, glucuronamide (weak), quinic acid, saccharic acid, *p*-hydroxy-phenylacetic acid, methyl pyruvate, L-lactic acid, citric acid, *α*-keto-glutaric acid, D-malic acid, L-malic acid, bromo-succinic acid, Tween 40, *γ*-amino-butryric acid, *α*-hydroxy-butyric acid, *β*-hydroxy-DL-butyric acid, *α*-keto-butyric acid, acetoacetic acid(weak), propionic acid, acetic acid and formic acid can be utilized. The major fatty acids (>10%) are anteiso-$C_{15:0}$ and anteiso-$C_{17:0}$. The predominant respiratory quinone is MK-8(H_2), and the diagnostic polar lipids are diphosphatidylglycerol and glycolipid. The diagnostic cell-wall diamino acid of strain WHS-Z9^T is *meso*-diaminopimelic acid.

The type strain, WHS-Z9^T (=MCCC 1K07845^T=KCTC 49848^T), was isolated from a marine sponge *Hymeniacidon* sp. collected from Weihai, Shandong Province, PR China. The DNA G+C content of the type strain is 65.2%.

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Author contributions
Z.L. designed the research and project outline. M.Z. and J.S. performed isolation and polyphasic taxonomy. M.Z. performed genome analysis. M.Z. and Z.L. drafted the manuscript. All authors read and approved the final manuscript.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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