Brachybacterium squillarum sp. nov., isolated from salt-fermented seafood

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A Gram-positive bacterium, strain M-6-3^T, was isolated from salt-fermented seafood in Korea. The organism grew in 0–10 % (w/v) NaCl and at 25–37 °C, with optimal growth occurring in 5 % NaCl and at 28–30 °C. The peptidoglycan type was variation A4γ with *meso*-diaminopimelic acid as the diagnostic cell-wall diamino acid. The polar lipid profile of strain M-6-3^T consisted of diphosphatidylglycerol, phosphatidylglycerol, an unidentified phospholipid and an unknown glycolipid. Strain M-6-3^T contained MK-7 as the major component of the quinone system and anteiso-$C_{15:0}$ (62.1 %) as the predominant fatty acid. Based on 16S rRNA gene sequence similarity studies, strain M-6-3^T was most closely related to *Brachybacterium rhamnosum* LMG 19848^T (98.5 %). The G+C content of the genomic DNA was 71.5 mol% and the mean DNA–DNA hybridization value with reference strains was 14.32 ± 2.0 %. Based on phenotypic, genotypic and phylogenetic analyses, it is proposed that strain M-6-3^T represents a novel species for which the name *Brachybacterium squillarum* sp. nov. is proposed; the type strain is M-6-3^T (=KACC 14221^T =JCM 16464^T).

The genus *Brachybacterium* belongs to the family *Dermabacteraceae*, class *Actinobacteria*, and was first proposed by Collins *et al.* (1988) to accommodate *Brachybacterium faecium*. Cells of *B. faecium*, isolated from poultry deep litter, were Gram-positive, coccoid-shaped and grew under aerobic conditions (Collins *et al.*, 1988). Since this genus was established in 1988, twelve species have been described based on phenotypic, biochemical and chemotaxonomic characteristics: *Brachybacterium alimentarium*, *B. conglomeratum*, *B. faecium*, *B. fresconis*, *B. muris*, *B. nesterenkovii*, *B. paraconglomeratum*, *B. phenoliresistens*, *B. rhamnosum*, *B. sacelli*, *B. tyrofermentans* and *B. zhongshanense* (Buczolits *et al.*, 2003; Chou *et al.*, 2007; Collins *et al.*, 1988; Gvozdyak *et al.*, 1992; Heyrman *et al.*, 2002; Schubert *et al.*, 1996; Takeuchi *et al.*, 1995; Zhang *et al.*, 2007). While carrying out a study examining the microbial diversity in salt-fermented food, a novel species, M-6-3^T, was isolated from a salt-fermented food made of tiny shrimp.

A pure culture of strain M-6-3^T was isolated by using several rounds of dilution streaking on marine agar (MA). To identify the 16S rRNA gene sequence, PCR was performed using a PCR Pre-Mix (SolGent) and two previously described, bacteria-specific primers (8F, 1492R) (Baker *et al.*, 2003). Sequences were obtained with a PRISM 3730XL DNA analyser (Applied Biosystems). Based on sequence similarities, four primers (8F, 968F, 518R and 1492R) were used to obtain the majority of the M-6-3^T 16S rRNA gene sequence and the fragments were assembled using SeqMan software (DNASTAR). The 16S rRNA sequences of the isolate and its relatives were aligned using CLUSTAL X (1.83) (Thompson *et al.*, 1997) and a phylogenetic tree was reconstructed using MEGA4 (Tamura *et al.*, 2007). In addition, neighbour-joining was used to reconstruct a phylogenetic tree (Saitou & Nei, 1987). One thousand randomly chosen bootstrap replications were set up for the phylogenetic tree, as shown in Fig. 1. Analysis of 16S rRNA gene sequences based on the EzTaxon server (Chun *et al.*, 2007) clearly showed that strain M-6-3^T was associated with members of the genus *Brachybacterium* and closely related to *B. rhamnosum* LMG 19848^T (98.5 %), *B. muris* C3H-21^T (98.3 %), *B. nesterenkovii* DSM 9573^T (98.1 %), *B. sacelli* LMG 20345^T (97.9 %), *B. fresconis* LMG 20336^T (97.6 %), *B. zhongshanense* JB^T (97.5 %), *B. paraconglomeratum* LMG 19861^T (97.5 %), *B. faecium* DSM 4810^T (97.4 %), *B. alimentarium* CNRZ 925^T (97.4 %), *B. phenoliresistens* phenol-A^T (97.3 %), *B. conglomeratum* IFO 15472^T (97.2 %) and *B. tyrofermentans* CNRZ 926^T (96.9 %). Based on neighbour-joining, maximum-parsimony and maximum-likelihood algorithms including all described members of the genus *Brachybacterium* and *Devriesea agamarum* IMP2^T as an outgroup, strain M-6-3^T was most closely related to *B. rhamnosum* DSM 10240^T, *B. muris* DSM

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain M-6-3^T is GQ339911.

A supplementary table and a supplementary figure are available with the online version of this paper.

15460^T and *B. nesterenkovii* DSM 9573^T. Thus, for further analyses, *B. rhamnosum*, *B. muris* and *B. nesterenkovii* were used as reference strains.

To determine the optimal growth conditions for M-6-3^T, cultures were grown in marine broth (MB; BBL) at 4, 15, 25, 30, 35 and 45 °C and pH 4.0–13.0 (at unit intervals). NaCl tolerance was determined in MB modified to contain 0, 0.5, 1, 3, 5, 7, 10, 15, 20, 25 and 30 % (w/v) NaCl. Growth was determined by measuring the turbidity of cultures (optical density) at 600 nm (OD_{600}). Growth on MA under anaerobic conditions was checked in an anaerobic chamber filled with a mixture of gases (N_2 : H_2 : CO_2 at 90 : 5 : 5) for 1 week. Results showed that M-6-3^T grew at 30 °C and pH 7.0 ± 0.2 on MA. Unless otherwise stated, all further tests used to characterize the isolate were performed under these conditions.

Colony and cell morphologies were observed using a light microscope (ECLIPSE 50*i*, Nikon). Motility tests were performed using BBL motility test medium (BBL). For Gram reactions, a Gram Staining kit (bioMérieux) was used according to the manufacturer's instructions. Oxidase activity was assessed with 1 % (v/v) *p*-tetramethyl phenylenediamine (bioMérieux). Catalase activity was determined by observing bubble production after 3 % hydrogen peroxide was added to a sample of M-6-3^T cells. Media containing 0.01 % (w/v) $CaCl_2$ and 1 % (v/v) Tweens 20, 40, 60 or 80 (Holding & Collee, 1971) were used to perform Tween decomposition tests. Enzymic activity and acid production from various carbohydrates were determined using API 20E (bioMérieux). Substrate utilization tests were performed using Biolog GP2 Microplates according to the manufacturer's instructions. Differential phenotypic characteristics of strain M-6-3^T and reference strains are given in Table 1.

Purified cell-wall preparations were obtained by the method of Schleifer & Kandler (1972) and the amino acids and peptides in peptidoglycan hydrolysates were analysed as described by Groth *et al.* (1997). The peptidoglycan of strain M-6-3^T contained *meso*-diaminopimelic acid, alanine, glycine, aspartic acid and glutamic acid, as observed in *B. muris* DSM 15460^T. The peptidoglycan type of strain M-6-3^T was A4γ [*meso*-diaminopimelic acid(D-Glu)$_2$ type] (Schleifer & Kandler, 1972). These results show that strain M-6-3^T was a coherent member of the genus *Brachybacterium*. To assess cellular fatty acids in strain M-6-3^T, *B. rhamnosum* DSM 10240^T, *B. muris* DSM 15460^T and *B. nesterenkovii* DSM 9573^T, cells were cultured for 72 h on MA at 30 °C and pH 7.0 ± 0.2. Cellular fatty acids were extracted as described by the Sherlock Microbial Identification System (MIDI, 1999) and then analysed by GC. The fatty acid profile of strain M-6-3^T contained a large amount of anteiso-$C_{15:0}$ (62.1 %) and a significant amount of anteiso-$C_{17:0}$ (12.3 %), fatty acids which are common in the profiles of other known species of the genus *Brachybacterium* (Buczolits *et al.*, 2003; Collins *et al.*, 1988; Gvozdyak *et al.*, 1992; Takeuchi *et al.*, 1995). Additionally, significant amounts of iso-$C_{16:0}$ (13.2 %), iso-$C_{14:0}$ (7.0 %) and iso-$C_{15:0}$ (5.4 %) were also found. The detailed compositions are shown in Table 2. Polar lipids were extracted by using the method of Xin *et al.* (2000) and detected by using two-dimensional TLC sprayed with detection reagents, according to Tindall (1990). The major lipids found in strain M-6-3^T included diphosphatidylglycerol, phosphatidylglycerol, an unidentified phospholipid and an unknown glycolipid (see Supplementary Fig. S1 available in IJSEM Online). Menaquinones were extracted, purified and analysed by HPLC as described by Komagata & Suzuki (1987). As with other species of the genus *Brachybacterium*, the isolate contained MK-7 as the major

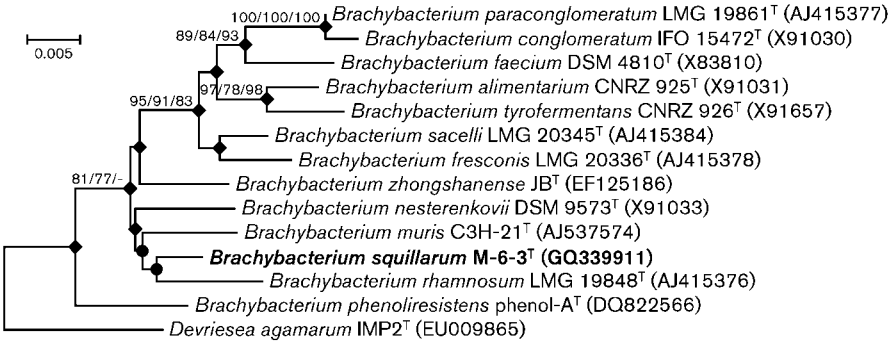

Fig. 1. A phylogenetic consensus tree based on 16S rRNA gene sequences. Filled diamonds indicate corresponding nodes (groupings) that were recovered in phylogenetic consensus trees reconstructed by the neighbour-joining, maximum-parsimony or maximum-likelihood algorithms. Filled circles indicate collective branches that were present in phylogenetic consensus trees reconstructed by both the neighbour-joining and maximum-likelihood algorithms. Numbers at nodes indicate bootstrap values (percentages of 1000 replications) for neighbour-joining, maximum-parsimony and maximum-likelihood trees, respectively. Values lower than 70 % are not indicated. *Devriesea agamarum* IMP2^T was included as an outgroup. Bar, 5 substitutions per 1000 nt positions.

Table 1. Differential characteristics of strain M-6-3^T and members of the genus *Brachybacterium*

Strains: 1, *B. squillarum* sp. nov. M-6-3^T (data from this study); 2, *B. rhamnosum* DSM 10240^T (data from this study except where marked); 3, *B. muris* DSM 15460^T (data from this study); 4, *B. nesterenkovii* DSM 9573^T (data from this study except where marked); 5, *B. sacelli* LMG 20345^T (data from Heyrman *et al.*, 2002); 6, *B. fresconis* LMG 20336^T (data from Heyrman *et al.*, 2002); 7, *B. zhongshanense* JB^T (data from Zhang *et al.*, 2007); 8, *B. paraconglomeratum* LMG 19861^T (data from Takeuchi *et al.*, 1995); 9, *B. faecium* DSM 4810^T (data from Collins *et al.*, 1988); 10, *B. alimentarium* CNRZ 925^T (data from Schubert *et al.*, 1996); 11, *B. phenoliresistens* phenol-A^T (data from Chou *et al.*, 2007); 12, *B. conglomeratum* IFO 15472^T (data from Takeuchi *et al.*, 1995). +, Positive; –, negative; (+), weakly positive; ND, not determined.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12
Oxidase activity	−	+	+	+	−	−	−	−	−	+	−	−
Growth with:												
5 % NaCl	+	+	+	−	+	+	+	+	+	+	+	+
10 % NaCl	+	(+)	+	−	+	+	+	(+)	(+)	+	−	+
Urease activity	−	+	+	+	−	+	+	+	−	+	−	(+)
Gelatin hydrolysis	+	+	−	−	+	+	+	−	−	+	−	(+)
Acid production from:												
D-Glucose	−	+	−	−	+	+	+	+	+	+	+	+
D-Mannitol	+	+	−	−	ND	ND	ND	−	−	ND	+	−
Inositol	+	+	−	−	ND	ND	ND	+	−	ND	+	−
D-Sorbitol	−	+	−	−	−	−	+	−	−	−	−	−
L-Rhamnose	+	+	−	−	(+)	(+)	+	−	−	+	+	−
Sucrose	+	+	−	+	−	−	+	+	−	−	+	+
Melibiose	−	+	−	−	−	+	+	−	ND	+	−	ND
Amygdalin	−	+	−	+	ND	ND	+	+	ND	ND	+	ND
L-Arabinose	−	+	−	+	−	−	+	+	(+)	ND	+	+
DNA G+C content (mol%)	71.5	71.5*	ND	72.0*	70.3	70.4	71.2	68.6	69.4	73.0	70.8	70.6

*Data from Buczolits *et al.* (2003).

component of the quinone system (Buczolits *et al.*, 2003; Chou *et al.*, 2007; Collins *et al.*, 1988; Gvozdyak *et al.*, 1992; Heyrman *et al.*, 2002; Schubert *et al.*, 1996; Takeuchi *et al.*, 1995; Zhang *et al.*, 2007).

Genomic DNA from M-6-3^T, *B. rhamnosum* DSM 10240^T, *B. muris* DSM 15460^T and *B. nesterenkovii* DSM 9573^T was isolated using an UltraClean Microbial DNA Isolation kit (MOBIO Laboratories). The DNA G+C content of strain M-6-3^T, analysed fluorimetrically using SYBR Green I and real-time PCR (Gonzalez & Saiz-Jimenez, 2002), was determined as 71.5 mol%, using genomic DNA of *Escherichia coli* K-12 as a calibration reference (Gonzalez & Saiz-Jimenez, 2002). Cy5-labelled DNA probes and genome-spotted microarrays (Bae *et al.*, 2005; Chang *et al.*, 2008) were used to measure DNA–DNA hybridization. Cy5-dUTP-labelled target DNA (1 μg) was mixed with hybridization solution containing 50 % formamide, 3× SSC, 1.25 μg unlabelled herring sperm DNA and 0.3 % SDS; 7 μl of the mixture was hybridized with probe DNAs on a microarray slide. The microarray slide was placed into a hybridization chamber, boiled for 5 min to denature the hybridization solution and plunged immediately into a 37 °C water bath for overnight hybridization. The microarray slide was scanned with a genepix 400A (Axon instruments) microarray scanner and the signal-to-noise ratio of each probe was calculated using a previously reported formula (Loy *et al.*, 2005). DNA–DNA hybridization data are shown in detail in Supplementary Table S1. The mean DNA–DNA hybridization value with reference strains was 14.32±2.0 %.

Table 2. Fatty acid compositions of strain M-6-3^T and reference members of the genus *Brachybacterium*

Strains: 1, *B. squillarum* sp. nov. M-6-3^T; 2, *B. rhamnosum* DSM 10240^T; 3, *B. muris* DSM 15460^T; 4, *B. nesterenkovii* DSM 9573^T. All data shown are from the present study. Values are percentages of total fatty acids. −, Not detected.

Fatty acid	1	2	3	4
i-$C_{14:0}$	7.0	5.9	4.1	5.0
i-$C_{15:1}$	−	−	5.7	−
i-$C_{15:0}$	5.4	7.5	4.8	5.4
ai-$C_{15:0}$	62.1	65.3	73.1	89.6
i-$C_{16:0}$	13.2	7.7	5.8	−
ai-$C_{17:0}$	12.3	5.6	6.5	−

Based on phenotypic, genotypic (G+C content and DNA–DNA hybridization) and chemotaxonomic data, as well as phylogenetic analyses, it is suggested that strain M-6-3^T represents a novel species of the genus *Brachybacterium*, for which the name *Brachybacterium squillarum* sp. nov. is proposed.

Description of *Brachybacterium squillarum* sp. nov.

Brachybacterium squillarum (squil.la′rum. L. gen. pl. n. *squillarum* of/from shrimp).

Cells are 1.0–1.5 μm in diameter, coccoid-shaped, non-motile and Gram-positive. Endospores are not formed. Colonies on MA plates are camel yellow-coloured, circular in form and convex with regular edges. Grows at 25–37 °C; no growth occurs at 45 °C. Grows at pH 6.0–9.0, with optimal growth at pH 7.0, under aerobic conditions. Growth occurs in 0–10 % (w/v) NaCl, with optimal growth in 5 % NaCl (w/v). Does not grow under anaerobic conditions. Negative for catalase and oxidase activities. Able to hydrolyse Tween 60, but not Tweens 20, 40 or 80. Analysis using the API 20E system revealed that cells are positive for gelatinase and acid production from D-mannitol, inositol, L-rhamnose and sucrose, but negative for β-galactosidase (ONPG hydrolysis), L-arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, citrate utilization, H_2S production, urease, tryptophan deaminase, indole production and acetoin production (Voges–Proskauer). They are also negative for acid production from D-glucose, D-sorbitol, melibiose, amygdalin and L-arabinose. In the Biolog GP2 test, dextrin, glycogen, D-arabitol, L-fucose, D-galactose, D-gluconic acid, α-D-glucose, maltose, maltotriose, D-mannitol, D-mannose, melezitose, melibiose, D-psicose, raffinose, D-sorbitol, stachyose, sucrose, turanose, pyruvic acid, adenosine, inosine and uridine are oxidized, but not α-cyclodextrin, β-cyclodextrin, inulin, mannan, *N*-acetyl-D-glucosamine, *N*-acetyl-β-D-mannosamine, amygdalin, L-arabinose, arbutin, cellobiose, D-fructose, D-galacturonic acid, gentiobiose, *myo*-inositol, lactose, lactulose, methyl α-D-galactoside, methyl β-D-galactoside, 3-methyl glucose, methyl α-D-glucoside, methyl β-D-glucoside, methyl α-D-mannoside, palatinose, L-rhamnose, D-ribose, salicin, sedoheptulosan, D-tagatose, trehalose, xylitol, D-xylose, acetic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, *p*-hydroxyphenylacetic acid, α-ketoglutaric acid, α-ketovaleric acid, lactamide, D-lactic acid methyl ester, L-lactic acid, D-malic acid, L-malic acid, pyruvic acid methyl ester, succinic acid monomethyl ester, propionic acid, succinamic acid, succinic acid, *N*-acetyl-L-glutamic acid, L-alaninamide, D-alanine, L-alanine, L-alanyl glycine, L-asparagine, L-glutamic acid, glycyl L-glutamic acid, L-pyroglutamic acid, L-serine, putrescine, 2,3-butanediol, glycerol, 2′-deoxy adenosine, thymidine, AMP, TMP, UMP, D-fructose 6-phosphate, α-D-glucose 1-phosphate, D-glucose 6-phosphate or DL-α-glycerol phosphate. The diagnostic cell-wall diamino acid is *meso*-diaminopimelic acid and the peptidoglycan type is A4γ, containing amino acids *meso*-diaminopimelic acid, alanine, glycine, aspartic acid and glutamic acid. The polar lipid profile consists of diphosphatidylglycerol, phosphatidylglycerol, and phospho- and glycolipids. The major menaquinone is MK-7. The major fatty acids are anteiso-$C_{15:0}$, anteiso-$C_{17:0}$, iso-$C_{14:0}$, iso-$C_{15:0}$ and iso-$C_{16:0}$.

The type strain is M-6-3^T (=KACC 14221^T =JCM 16464^T), isolated from a salt-fermented seafood. The DNA G+C content of the type strain is 71.5 mol%.

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