Brevibacterium marinum sp. nov., isolated from seawater

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A novel yellow-pigmented actinobacterium was isolated from seawater collected from Hwasun Beach in Jeju, Republic of Korea. A comparative analysis of the 16S rRNA gene sequence indicated that the organism, designated HFW-26^T, was closely related to members of the genus *Brevibacterium*. As found for other species of the genus *Brevibacterium*, strain HFW-26^T possessed meso-diaminopimelic acid as the diagnostic cell-wall diamino acid, contained MK-8(H₂) as the major menaquinone, contained polar lipids that included diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and an unknown phospholipid, and had anteiso-C_{15:0} and anteiso-C_{17:0} as the predominant fatty acids. The G + C content of the DNA was 71.4 mol%. The phylogenetically closest relative was *Brevibacterium picturae* DSM 16132^T (99.0% 16S rRNA gene sequence similarity). However, DNA–DNA hybridization of strain HFW-26^T showed 35.1–43.7% relatedness with respect to *B. picturae* DSM 16132^T. The novel isolate could be clearly distinguished from *B. picturae* DSM 16132^T on the basis of some cultural, physiological and biochemical characteristics. A battery of phenotypic and genetic data obtained in this study suggest that strain HFW-26^T represents a novel species of the genus *Brevibacterium*, for which the name *Brevibacterium marinum* sp. nov. is proposed. The type strain is HFW-26^T (=JBRI 2001^T=KCTC 19221^T=DSM 18964^T).

The genus *Brevibacterium*, which was originally proposed by Breed (1953), is classified within the family *Brevibacteriaceae*, suborder *Micrococcineae* (Stackebrandt *et al.*, 1997). At the time of writing, the following 15 species with validly published names, in addition to the type species *Brevibacterium linens*, remain within the genus: *Brevibacterium antiquum*, *B. aurantiacum*, *B. permense* (Gavrish *et al.*, 2004), *B. avium* (Pascual & Collins, 1999), *B. casei*, *B. epidermidis* (Collins *et al.*, 1983), *B. cereale* (Ivanova *et al.*, 2004), *B. iodinum* (Collins *et al.*, 1980), *B. luteolum* (Wauters *et al.*, 2003; Euzéby & Tindall, 2004), *B. mcbrellneri* (McBride *et al.*, 1993), *B. otitidis* (Pascual *et al.*, 1996), *B. paucivorans* (Wauters *et al.*, 2001), *B. picturae* (Heyrman *et al.*, 2004), *B. sanguinis* (Wauters *et al.*, 2004) and *B. samyangense* (Lee, 2006). While most of the species were isolated from dairy products, clinical specimens, poultry and soils, a few were recovered from marine environments, such as algae and beach sediment, as reported previously (Ivanova *et al.*, 2004; Lee, 2006).

In the present study, an actinomycete isolated from a seawater sample collected at Hwasun Beach, Jeju, Republic of Korea, was investigated by means of a polyphasic

taxonomic approach. For bacterial isolation, aliquots of a seawater sample were directly deposited on starch-casein agar (Küster & Williams, 1964) supplemented with 60% natural seawater instead of distilled water. A colony was further subcultured on marine agar 2216 (MA; Difco). The pure culture, designated strain HFW-26^T, was maintained at –20 and –80 °C as a glycerol solution that included 20% (v/v) distilled water and 60% (v/v) seawater. For phenotypic comparisons and DNA–DNA hybridizations, *B. picturae* DSM 16132^T was grown on trypticase soy agar (TSA; Difco) at 28 °C for 5 days.

Cell morphology and motility were observed by using phase-contrast and transmission electron microscopy, with cultures grown for 6, 15, 24, 48 and 72 h in trypticase soy broth (Difco) at 30 °C. Growth was tested on yeast extract-malt extract agar (ISP 2 medium; Shirling & Gottlieb, 1966), TSA and nutrient agar (Difco), with and without supplementation with 60% (v/v) natural seawater, incubated for 5 days at 28 °C. The growth temperature (4–40 °C) was tested on TSA. The initial pH for growth (determined on MA) was in the range 4.1–12.1. NaCl tolerance for growth was studied using ISP 2 medium as the basal medium. The Gram stain was determined using a Color Gram 2 F kit (bioMérieux). Degradation of casein, cellulose, hypoxanthine, DL-tyrosine and xanthine was tested on MA. Hydrolysis of DNA and starch was examined on DNase test agar (Difco) and starch agar (Difco),

The GenBank/EMBL/DBJ accession number for the 16S rRNA gene sequence of strain HFW-26^T is AM421807.

Transmission electron micrographs of cells of strain HFW-26^T are available as a supplementary figure with the online version of this paper.

respectively; after incubation for 5 days, the plates were flooded with 1 M HCl and iodine solution, respectively. Catalase and oxidase activities were checked as described previously (Lee, 2006). Other physiological and biochemical properties were determined using API Coryne and API ZYM strips (bioMérieux) according to the manufacturer's instructions. Strain HFW-26^T showed good growth on all media tested; the cells were non-motile rods (0.6–0.9 µm wide and 1.5–2.4 µm long) at exponential phase. In older cultures, the cells fragmented into short rods (0.5–0.6 µm wide and 0.7–1.0 µm long) that occasionally occurred singly or, more often, in pairs or in chains. Transmission electron micrographs of cells of strain HFW-26^T are shown in Supplementary Fig. S1 (available with the online version of this paper). The V-form arrangement was also observed when cells were studied using light and phase-contrast microscopy. Colonies were translucent, convex, smooth and circular with entire margins. The colony pigmentation was variable depending on the culture conditions: in the dark, colonies were white; in the light, colonies were bright yellow. Colonies of *B. picturae* DSM 16132^T were white in colour, irrespective of the culture conditions. Data for other physiological and biochemical properties of strain HFW-26^T are given in the species description and Table 1.

The 16S rRNA gene sequencing studies were performed as described elsewhere (Lee, 2006). An almost-complete 16S rRNA gene sequence (1455 nt) for strain HFW-26^T was determined in this study and compared with those of members of the genus *Brevibacterium*. A phylogenetic tree (Fig. 1) based on 16S rRNA gene sequences indicated that the organism belonged to the genus *Brevibacterium*: it formed a coherent cluster with *B. picturae*. This relationship was supported by a high bootstrap percentage (91 %) and was also found in the maximum-likelihood and maximum-parsimony trees. The phylogenetic neighbours

were *B. picturae* (99.0 % sequence similarity), *B. aurantiacum* (98.4 %) and *B. antiquum* (98.0 %). Strain HFW-26^T shared 16S rRNA gene sequence similarity values of 94.7–97.6 % with respect to the type strains of the other recognized species of the genus *Brevibacterium*.

DNA–DNA hybridization was carried out as described by De Ley *et al.* (1970), as modified by Huß *et al.* (1983), using a Cary 100 Bio UV/Vis-spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with an *in situ* temperature probe (Varian). Genomic DNA was isolated using a French pressure cell (Thermo Spectronic) and was purified by chromatography on hydroxyapatite as described by Cashion *et al.* (1977). Several species of the genus *Brevibacterium* have been found to have 99 % 16S rRNA gene sequence similarity with each other, but show DNA–DNA relatedness values that are below the 70 % threshold required for delineating a genospecies (Wauters *et al.*, 2001, 2003; Gavrish *et al.*, 2004). Therefore, DNA–DNA hybridization for strain HFW-26^T was performed only with respect to the type strain of the phylogenetically closest species. The levels of DNA–DNA relatedness for strain HFW-26^T and *B. picturae* DSM 16132^T were between 35.1 and 43.7 % when strain HFW-26^T was used as the probe. The DNA G + C content of strain HFW-26^T was 71.4 mol%, as determined by HPLC (Mesbah *et al.*, 1989).

Chemotaxonomic characteristics of strain HFW-26^T were investigated as described previously (Lee, 2006) to determine the type of diamino acid in the cell wall (Staneck & Roberts, 1974), the mycolic acids (Minnikin *et al.*, 1980), the polar lipids (Minnikin *et al.*, 1977) and the menaquinones (Kroppenstedt, 1985). Cells of strain HFW-26^T were grown in trypticase soy broth for 3 days at 30 °C, with shaking at 170 r.p.m. After harvesting, the biomass was washed in distilled water and freeze-dried. The results

Table 1. Differential characteristics for strain HFW-26^T and its phylogenetic neighbours

Strains: 1, HFW-26^T; 2, *B. antiquum* VKM Ac-2118^T (Gavrish *et al.*, 2004); 3, *B. aurantiacum* VKM Ac-2111^T (Gavrish *et al.*, 2004); 4, *B. picturae* DSM 16132^T (Heyrman *et al.*, 2004; this study). +, Positive; –, negative.

Characteristic	1	2	3	4
Colony colour	Yellow	White to orange	Orange	White
Growth at 10 °C	+	+	+	–
Growth in the presence of 15 % (w/v) NaCl	–	+	+	–*
Hydrolysis of casein	+	+	+	–
API Coryne tests				
Nitrate reduction	+	–	+	–
Pyrazinamidase	–	+	+	+
β-Galactosidase	+	–	–	–
α-Glucosidase	+	–	–	–
Urease	+	+	–	+
Gelatin hydrolysis	–	+	–	–*
Acid production from D-glucose	–	+	+	–*
Origin of isolation	Seawater	Permafrost sediment	Cheese	Mural painting

*Result differs from that reported previously (Heyrman *et al.*, 2004).

Fig. 1. Phylogenetic tree, based on 16S rRNA gene sequences, showing the position of strain HFW-26^T within the family *Brevibacteriaceae*. The tree was constructed using the Jukes–Cantor model (Jukes & Cantor, 1969) and the neighbour-joining method (Saitou & Nei, 1987). Asterisks indicate branches that were also found in the maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) trees. Numbers at the nodes indicate percentages of bootstrap support (based on a neighbour-joining analysis of 1000 resampled data). Bar, 1 substitution per 100 nucleotide positions.

of the chemotaxonomic analyses of strain HFW-26^T are given in the species description. All of the chemotaxonomic characteristics were in accordance with the genus description (Collins *et al.*, 1983). The cellular fatty acid methyl esters of the novel isolate and *B. picturae* DSM 16132^T were prepared from cells grown on TSA for 3 days at 30 °C and analysed according to the instructions for the Sherlock Microbial Identification System (version 6; MIDI). The cellular fatty acid content of strain HFW-26^T conformed to the characteristic profile for the genus *Brevibacterium* and consisted of saturated, anteiso- and iso-methyl branched acids. The major components were anteiso-C_{15:0} (41.2 %), anteiso-C_{17:0} (30.4 %), and C_{18:0} (12.0 %). The main difference between strain HFW-26^T and *B. picturae* DSM 16132^T was in relation to the proportion of C_{18:0} present (Table 2).

Although strain HFW-26^T shared a high level of 16S rRNA gene sequence similarity (99.0 %) with the type strain of *B.*

Table 2. Cellular fatty acid content (%) of strain HFW-26^T and its phylogenetically closest relative

Strains: 1, HFW-26^T; 2, *B. picturae* DSM 16132^T. Values that were less than 1 % of total fatty acids have been omitted.

Fatty acid	1	2
Straight-chain fatty acids		
C _{12:0}	2.9	1.3
C _{16:0}	5.8	3.4
C _{18:0}	12.0	3.0
Branched fatty acids		
iso-C _{15:0}	3.2	5.8
iso-C _{16:0}	3.0	3.4
iso-C _{17:0}	1.2	1.4
anteiso-C _{15:0}	41.2	49.7
anteiso-C _{17:0}	30.4	29.6

picturae, the relatively low level of DNA–DNA hybridization (35.1–43.7 %) revealed that the novel isolate does not belong to the species *B. picturae* (according to the 70 % DNA–DNA similarity threshold recommended for the definition of bacterial genospecies; Wayne *et al.*, 1987). Phenotypic features distinguishing strain HFW-26^T from its phylogenetic neighbours are given in Table 1. Strain HFW-26^T can also be readily differentiated from the other recognized species of the genus *Brevibacterium* with reference to some physiological and biochemical properties (Wauters *et al.*, 2003; Ivanova *et al.*, 2004; Lee, 2006).

On the basis of the phenotypic and genotypic data presented here, strain HFW-26^T represents a novel species of the genus *Brevibacterium*, for which the name *Brevibacterium marinum* sp. nov. is proposed.

Description of *Brevibacterium marinum* sp. nov.

Brevibacterium marinum (ma.ri'num. L. neut. adj. *marinum* of the sea, marine).

Cells are Gram-positive, catalase-positive, oxidase-negative, non-motile, non-spore-forming rods that are long (0.6–0.9 µm wide and 1.5–2.4 µm long) at exponential phase. As the culture ages, cells fragment into short rods (0.5–0.6 µm wide and 0.7–1.0 µm long) that occasionally occur singly and, more often, in pairs or in chains. V-form arrangements are also observed. Colonies are translucent, convex, smooth and circular with entire margins. Colonial pigmentation is variable depending on the culture conditions: in the dark, colonies are white; in the light, colonies are bright yellow. The temperature range for growth is 10–30 °C, with an optimum of 30 °C. No growth is observed at 4 or 37 °C. Growth occurs at initial pH values in the range 5.1–12.1. Growth occurs in the presence of up to 10 % NaCl. Casein and hypoxanthine are degraded, but cellulose, DL-tyrosine and xanthine are not degraded. Hydrolysis of DNA and starch is not observed. In the API Coryne system, the results for nitrate reduction, β-galactosidase, α-glucosidase, urease and catalase are positive. Negative for the following enzyme activities: pyrazinamidase, pyrrolidonyl arylamidase, alkaline phosphatase, β-glucuronidase, N-acetyl-β-glucosaminidase and β-glucosidase (aesculin hydrolysis). Hydrolysis of gelatin is not observed. Acid production from carbohydrates (D-glucose, maltose, D-ribose, sucrose, D-xylose, D-mannitol, D-lactose and glycogen) is negative. In the additional tests in the API ZYM system, esterase (C4) and esterase lipase (C8) are weakly positive, but alkaline phosphatase, lipase (C14), leucine arylamidase, valine arylamidase, cysteine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-glucosidase, α-mannosidase and α-fucosidase are negative. *meso*-Diaminopimelic acid is the diagnostic diamino acid in the cell wall. The polar lipids are diphosphatidylglycerol, phosphatidylglycerol and phosphatidylinositol, with an unknown phospholipid as a minor component. The major menaquinone is MK-8(H₂). Mycolic acids are absent. The

major cellular fatty acids are anteiso-C_{15:0}, anteiso-C_{17:0} and C_{18:0}. The DNA G + C content is 71.4 mol%.

The type strain, HFW-26^T (=J BRI 2001^T=KCTC 19221^T=DSM 18964^T), was isolated from seawater from Hwasun Beach in Jeju, Republic of Korea.

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References

- Breed, R. S. (1953). The families developed from *Bacteriaceae* Cohn with a description of the family *Brevibacteriaceae*. *Riass Commun VI Congr Int Microbiol Roma* 1, 10–15.
- Cashion, P., Holder-Franklin, M. A., McCully, J. & Franklin, M. (1977). A rapid method for base ratio determination of bacterial DNA. *Anal Biochem* 81, 461–466.
- Collins, M. D., Jones, D., Keddie, R. M. & Sneath, P. H. A. (1980). Reclassification of *Chromobacterium iodinum* (Davis) in a redefined genus *Brevibacterium* (Breed) as *Brevibacterium iodinum* nom. rev., comb. nov. *J Gen Microbiol* 120, 1–10.
- Collins, M. D., Farrow, J. A. E., Goodfellow, M. & Minnikin, D. E. (1983). *Brevibacterium casei* sp. nov. and *Brevibacterium epidermidis* sp. nov. *Syst Appl Microbiol* 4, 388–395.
- De Ley, J., Cattoir, H. & Reynaerts, A. (1970). The quantitative measurement of DNA hybridization from renaturation rates. *Eur J Biochem* 12, 133–142.
- Euzéby, J. P. & Tindall, B. J. (2004). Status of strains that contravene Rules 27(3) and 30 of the Bacteriological Code. Request for an Opinion. *Int J Syst Evol Microbiol* 54, 293–301.
- Felsenstein, J. (1981). Evolutionary trees from DNA sequences: a maximum likelihood approach. *J Mol Evol* 17, 368–376.
- Fitch, W. M. (1971). Toward defining the course of evolution: minimum change for a specific tree topology. *Syst Zool* 20, 406–416.
- Gavrich, E., Krauzova, V. I., Potekhina, N. V., Karasev, S. G., Plotnikova, E. G., Altyntseva, O. V., Korosteleva, L. A. & Evtushenko, L. I. (2004). Three new species of brevibacteria, *Brevibacterium antiquum* sp. nov., *Brevibacterium aurantiacum* sp. nov., and *Brevibacterium permense* sp. nov. *Microbiology* (English translation of *Mikrobiologiya*) 73, 176–183.
- Heyrman, J., Verbeeren, J., Schumann, P., Devos, J., Swings, J. & Vos, P. D. (2004). *Brevibacterium picturae* sp. nov., isolated from a damaged mural painting at the Saint-Catherine chapel (Castle Herberstein, Austria). *Int J Syst Evol Microbiol* 54, 1537–1541.
- Huß, V. A. R., Festl, H. & Schleifer, K. H. (1983). Studies on the spectrophotometric determination of DNA hybridization from renaturation rates. *Syst Appl Microbiol* 4, 184–192.
- Ivanova, E. P., Christen, R., Alexeeva, Y. V., Zhukova, N. V., Gorshkova, N. M., Lysenko, A. M., Mikhailov, V. V. & Nicolau, D. V. (2004). *Brevibacterium celere* sp. nov., isolated from degraded thallus of a brown alga. *Int J Syst Evol Microbiol* 54, 2107–2111.
- Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.

- Kroppenstedt, R. M. (1985).** Fatty acid and menaquinone analysis of actinomycetes and related organisms. In *Chemical Methods in Bacterial Systematics*, pp. 173–199. Edited by M. Goodfellow & D. E. Minnikin. London: Academic Press.
- Küster, E. & Williams, S. T. (1964).** Selection of media for isolation of streptomycetes. *Nature* **202**, 928–929.
- Lee, S. D. (2006).** *Brevibacterium samyangense* sp. nov., an actinomycete isolated from a beach sediment. *Int J Syst Evol Microbiol* **56**, 1889–1892.
- McBride, M. E., Ellner, K. M., Black, H. S., Clarridge, J. E. & Wolf, J. E. (1993).** A new *Brevibacterium* sp. isolated from infected genital hair of patients with white piedra. *J Med Microbiol* **39**, 255–261.
- 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.
- Minnikin, D. E., Alshamaony, L. & Goodfellow, M. (1977).** Differentiation of *Mycobacterium*, *Nocardia*, and related taxa by thin layer chromatographic analysis of whole-cell methanolsates. *J Gen Microbiol* **88**, 200–204.
- Minnikin, D. E., Hutchinson, I. G., Caldicott, A. B. & Goodfellow, M. (1980).** Thin layer chromatography of methanolsates of mycolic acid-containing bacteria. *J Chromatogr* **188**, 221–233.
- Pascual, C. & Collins, M. D. (1999).** *Brevibacterium avium* sp. nov., isolated from poultry. *Int J Syst Bacteriol* **49**, 1527–1530.
- Pascual, C., Collins, M. D., Funke, G. & Pitcher, D. G. (1996).** Phenotypic and genotypic characterization of two *Brevibacterium* strains from the human ear: description of *Brevibacterium otitidis* sp. nov. *Med Microbiol Lett* **5**, 113–123.
- Saitou, N. & Nei, M. (1987).** The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Shirling, E. B. & Gottlieb, D. (1966).** Methods for characterization of *Streptomyces* species. *Int J Syst Bacteriol* **16**, 313–340.
- Stackebrandt, E., Rainey, F. A. & Ward-Rainey, N. L. (1997).** Proposal for a new hierarchic classification system, *Actinobacteria* classis nov. *Int J Syst Bacteriol* **47**, 479–491.
- Staneck, J. L. & Roberts, G. D. (1974).** Simplified approach to identification of aerobic actinomycetes by thin-layer chromatography. *Appl Microbiol* **28**, 226–231.
- Wauters, G., Charlier, J., Janssens, M. & Delmée, M. (2001).** *Brevibacterium paucivorans* sp. nov., from human clinical specimens. *Int J Syst Evol Microbiol* **51**, 1703–1707.
- Wauters, G., Avesani, V., Laffineur, K., Charlier, J., Janssens, M., Van Bosterhaut, B. & Delmée, M. (2003).** *Brevibacterium lutescens* sp. nov., from human and environmental samples. *Int J Syst Evol Microbiol* **53**, 1321–1325.
- Wauters, G., Haase, G., Avesani, V., Charlier, J., Janssens, M., Broeck, J. V. & Delmée, M. (2004).** Identification of a novel *Brevibacterium* species isolated from humans and description of *Brevibacterium sanguinis* sp. nov. *J Clin Microbiol* **42**, 2829–2832.
- Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E. & other authors (1987).** International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.