Brevibacterium samyangense sp. nov., an actinomycete isolated from a beach sediment

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A novel actinomycete, strain SST-8^T, was isolated from sand sediment of Samyang Beach in Jeju, Korea, and subjected to a polyphasic taxonomic study. The organism, which produced opaque, circular, yellow colonies, with a coryneform morphology, showed the following chemotaxonomic characteristics: *meso*-diaminopimelic acid as the diamino acid in the peptidoglycan, MK-8(H₂) as the major menaquinone, phosphatidylglycerol as the only polar lipid, anteiso-C_{15:0} and anteiso-C_{17:0} as major fatty acids and a DNA G + C content of 70.7 mol%. The combination of morphological and chemotaxonomic features supported its classification in the genus *Brevibacterium*. Phylogenetic analyses, based on 16S rRNA gene sequence studies, showed that strain SST-8^T formed an intermediate branch between the *Brevibacterium luteolum*/*Brevibacterium otitidis* and *Brevibacterium mcbrellneri*/*Brevibacterium paucivorans* clusters. Sequence similarity calculations based on a neighbour-joining analysis revealed that the closest relatives of strain SST-8^T were the type strains of *B. paucivorans* (96.6%), *B. luteolum* (96.5%), *B. mcbrellneri* (96.3%), *Brevibacterium avium* (96.0%) and *B. otitidis* (95.9%). Based on a broad set of phenotypic and genetic data, it was evident that the strain represents a novel species of the genus *Brevibacterium*. The name *Brevibacterium samyangense* sp. nov. is proposed, with SST-8^T (=NRRL B-41420^T=KCCM 42316^T) as the type strain.

The genus *Brevibacterium*, originally proposed by Breed (1953), is clearly differentiated from actinomycete genera with coryneform morphology on the basis of chemotaxonomic characteristics (Collins *et al.*, 1983) and 16S rRNA gene sequence analysis (Heyrman *et al.*, 2004; Wauters *et al.*, 2004).

The genus contained, at the time of writing, 15 recognized species, mainly recovered from dairy products, clinical specimens, poultry and terrestrial environments, although a few, including *Brevibacterium celere* (Ivanova *et al.*, 2004) and *Brevibacterium stationis* (Jones & Keddle, 1986), were recovered from samples collected in marine environments. In the 16S rRNA gene sequence studies performed by Heyrman *et al.* (2004), *B. stationis* was shown to be related to members of the genus *Corynebacterium*. In the present study, a novel member of the genus *Brevibacterium*, recovered from beach sediment, was characterized taxonomically by use of a polyphasic approach. The resultant data supported that the organism should be classified as a novel species of the genus *Brevibacterium*.

During a study of marine actinomycetes, strain SST-8^T was isolated from beach sediment of the coast of Jeju Island,

Republic of Korea, and its taxonomic status was investigated. A sediment sample was taken at a depth of 30 cm below the beach surface. For bacterial isolation, 1 g sediment was placed into a sterile plastic tube containing 9 ml sterile distilled water and mixed in a tube rotator for 30 min. After aliquots (100 µl) of the sample were transferred into the isolation medium, the agar plates were incubated at 30 °C for 14 days. The isolation medium (SC-SW agar) consisted of 1% soluble starch, 0.03% casein, 0.2% KNO₃, 0.2% NaCl, 0.002% CaCO₃, 0.005% MgSO₄·7H₂O, 0.001% FeSO₄·7H₂O, 1.8% agar, 60% natural seawater and 40% distilled water (pH 7.2). Colonies were subcultivated on ISP2 medium (Shirling & Gottlieb, 1966) supplemented with 60% (v/v) sterilized natural seawater (YE-SW agar). Pure cultures were maintained on YE-SW agar plates and in 20% (v/v) glycerol solution supplemented with 60% (v/v) sterilized natural seawater at –20 and –80 °C.

Analysis of cellular fatty acids was performed according to the instructions of the Microbial Identification System (MIDI) (Sherlock Microbial Identification System; Hewlett Packard), with cells grown on trypticase soy agar for 48 h. Other chemotaxonomic characters were analysed as described by Lee *et al.* (2000b) as follows: the isomer of diaminopimelic acid (Staneck & Roberts, 1974), mycolic acids (Minnikin *et al.*, 1980), polar lipids (Minnikin *et al.*,

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain SST-8^T is DQ344485.

1977) and lipoquinones (Kroppenstedt, 1985). The fatty acid profile of strain SST-8^T was characterized by the predominance of anteiso-C_{17:0} (35.3%), anteiso-C_{15:0} (29.9%) and iso-C_{15:0} (15.5%), which is consistent with profiles for members of the genus *Brevibacterium* (Funke & Carlotti, 1994). Other fatty acids present at levels above 1% were iso-C_{17:0} (4.2%), C_{16:0} (4.2%), C_{18:0} (4.0%), iso-C_{16:0} (2.5%) and C_{18:1} (1.9%). Whole-cell hydrolysates contained *meso*-diaminopimelic acid; mycolic acids were not present and the predominant menaquinone was MK-8(H₂). All of these features are consistent with the chemotaxonomic description of the genus *Brevibacterium* (Collins *et al.*, 1983). The polar lipid profiles contained significant amounts of phosphatidylglycerol, but not diphosphatidylglycerol or phosphatidylinositol found in other members of the genus (Jones & Keddie, 1986).

Purification of chromosomal DNA was carried out by using the Wizard genomic DNA purification kit (Promega) according to the manufacturer's instructions. The 16S rRNA gene of strain SST-8^T was amplified by PCR as described by Lee *et al.* (2000a) and was subjected to direct sequence determination using an ABI PRISM BigDye Terminator cycle sequencing kit (Applied Biosystems) and an automatic DNA sequencer (model 3730xl; Applied Biosystems). The almost-complete 16S rRNA gene sequence of strain SST-8^T was determined (1426 nt), and the results of an initial BLAST search against GenBank sequence data indicated that the organism was related to the genus *Brevibacterium* of the family *Brevibacteriaceae*. Multiple alignment of the sequences was performed by using the CLUSTAL X program (Thompson *et al.*, 1997) and then manually optimized following the secondary structure of the bacterial 16S rRNA gene. Phylogenetic analyses were performed using three tree-making algorithms, namely the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) methods. A phylogenetic tree was reconstructed based on the neighbour-joining method from evolutionary distances calculated according to the method of Jukes & Cantor (1969). Confidence in the tree topology was evaluated by bootstrap analysis (Felsenstein, 1985) of 1000 replicated datasets. The neighbour-joining tree (Fig. 1) showed that strain SST-8^T formed an intermediate branch between the *Brevibacterium luteolum*/*Brevibacterium otitidis* and *Brevibacterium mcbrellneri*/*Brevibacterium paucivorans* clusters with 73% bootstrap support. This relationship was supported by all tree-making methods used in this study. Sequence similarity calculations after a neighbour-joining analysis revealed that the closest relatives of strain SST-8^T were the type strains of *B. paucivorans* (96.6%), *B. luteolum* (96.5%), *B. mcbrellneri* (96.3%), *Brevibacterium avium* (96.0%) and *B. otitidis* (95.9%). Levels of 16S rRNA gene sequence similarity between strain SST-8^T and the other recognized *Brevibacterium* species were in the range 95.0–95.5%. The results of these 16S rRNA gene sequence comparisons indicate that the isolate can be assigned to a separate genospecies without the need for further DNA–DNA

Fig. 1. A neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic position of strain SST-8^T within the radiation of the genus *Brevibacterium*. Evolutionary distances for tree construction were calculated using the Jukes–Cantor coefficient. *Micrococcus luteus* ATCC 381^T (M38242) was used as outgroup taxon (not shown). Asterisks indicate that the corresponding branches were also recovered in the maximum-likelihood and maximum-parsimony trees. Numbers at nodes indicate percentages of bootstrap support based on a neighbour-joining analysis of 1000 replicated datasets. Bar, 1 nucleotide substitution per 100 nucleotides.

hybridization experiments (Stackebrandt & Goebel, 1994). The DNA G + C content determined for strain SST-8^T was 70.7 mol%.

Colony pigmentation was observed visually and recorded after 5 days growth at 30 °C on YM-SW agar. Cell morphology was determined on cultures grown for 6, 12, 16, 24 and 72 h on YE-SW agar at 30 °C. Cell suspensions were made in sterile distilled water, stained negatively with India ink and observed by using a light microscope (magnification × 1000). Cell motility was observed under an Olympus light microscope equipped with phase-contrast optics (magnification × 400) and further confirmed by the presence of turbidity throughout the tube including semisolid medium. Temperature for growth was tested at 4, 10, 20, 30, 35, 42, 45 and 55 °C. NaCl tolerance was studied on ISP2 medium containing NaCl at final concentrations of 0–15% (w/v). Catalase activity was determined with a 3% (v/v) hydrogen peroxide solution. Oxidase activity was observed by oxidation of *N,N,N',N'*-tetramethyl-*p*-phenylenediamine. The ability of the organism to utilize a variety of substrates as sole carbon source was tested using GP2 microplates of the Microlog system (Biolog; 95 substrates). Cells were grown for 3 days at 30 °C on YE-SW agar and suspended in 2% (w/v) sea salts solution (Sigma). An aliquot of the suspension

(150 µl) was transferred to each well, and the plates were incubated for 48 h at 30 °C. Reduction of tetrazolium dye was determined by reading the microtitre plates at 595 nm using the microplate reader. API CORYNE and API ZYM strips (bioMérieux) were used according to the manufacturer's instructions. Degradation of casein, DL-tyrosine and xanthine was examined using the method described by Gordon *et al.* (1974). The results of the morphological, cultural and physiological characterization are given in the species description below.

Phenotypic features that can be used to differentiate between the new isolate and its phylogenetic neighbours within the genus *Brevibacterium* are given in Table 1. The combination of phenotypic and genotypic data supports that strain SST-8^T be classified as the type strain of a novel species of the genus *Brevibacterium*, *Brevibacterium samyangense* sp. nov.

Description of *Brevibacterium samyangense* sp. nov.

Brevibacterium samyangense (sam.yang'en.se. N.L. neut. adj. *samyangense* of Samyang Beach, Jeju, Republic of Korea, from where the type strain was isolated).

Cells are Gram-positive, catalase-positive, oxidase-negative, motile and non-spore-forming. On YE-SW agar, an apparent rod-coccus growth cycle is observed: after 6 h growth, cells are coccoid, occurring singly or in pairs, with the rare occurrence of short rods; after 12 and 16 h growth, most cells are irregular and slender rods, arranged in V-forms; after 24 and 72 h growth, cells are coccoid, occurring singly, in pairs or in chains. Colonies are opaque, convex, circular and bright yellow in colour with an entire edge. Temperature range for growth is 10–45 °C. No growth is observed at 4 or 55 °C. Growth occurs in the pH range 6.1–10.1, with better growth at higher pH. NaCl tolerance

for growth is observed, with good growth at 0–5 %, moderate growth at 6–9 % but only weak growth at 10 or 15 % NaCl. Degradation of DL-tyrosine is observed but not of casein or xanthine. The following substrates are utilized as sole carbon and energy sources for growth in Biolog GP2 tests: β -cyclodextrin, dextrin, glycogen, inulin, mannan, Tweens 40 and 80, *N*-acetyl-D-glucosamine, *N*-acetyl- β -D-mannosamine, amygdalin, L-arabinose, D-arabitol, arbutin, D-cellobiose, D-fructose, L-fucose, D-galactose, D-galacturonic acid, α -D-glucose, *myo*-inositol, α -D-lactose, lactulose, maltose, maltotriose, D-mannitol, D-mannose, D-melezitose, D-melibiose, methyl α -D-galactoside, methyl β -D-galactoside, 3-methyl D-glucoside, methyl α -D-glucoside, methyl β -D-glucoside, methyl α -D-mannoside, palatinose, D-psicose, D-raffinose, L-rhamnose, D-ribose, salicin, sedoheptulosan, D-sorbitol, stachyose, sucrose, D-tagatose, D-trehalose, turanose, xylitol, D-xylose, acetic acid, α -, β - and γ -hydroxybutyric acids, *p*-hydroxyphenylacetic acid, α -ketoglutaric acid, α -ketovaleric acid, lactamide, D-lactic acid methyl ester, L-lactic acid, L-malic acid, methylpyruvate, monomethylsuccinate, propionic acid, succinamic acid, succinic acid, *N*-acetyl-L-glutamic acid, L-alaninamide, D- and L-alanine, L-alanyl-glycine, L-asparagine, L-glutamic acid, glycyl L-glutamic acid, L-pyrogutamic acid, L-serine, putrescine, adenosine, 2'-deoxyadenosine, inosine, thymidine, uridine, adenosine 5'-monophosphate, thymidine 5'-monophosphate, uridine 5'-monophosphate, α -D-glucose 1-phosphate, D-glucose 6-phosphate and DL- α -glycerol phosphate. The following substrates in the Biolog GP2 system are not utilized: α -cyclodextrin, gentiobiose, D-gluconic acid, D-malic acid, 2,3-butanediol, glycerol and D-fructose 6-phosphate. In the API CORYNE system, pyrazinamidase, pyrrolidonyl arylamidase, alkaline phosphatase, β -galactosidase and α -glucosidase are positive. Nitrate reduction, β -glucuronidase, *N*-acetyl- β -glucosaminidase, β -glucosidase (aesculin hydrolysis), urease, gelatin

Table 1. Differential phenotypic characteristics of strain SST-8^T and its phylogenetic relatives in the genus *Brevibacterium*

Taxa: 1, strain SST-8^T; 2, *Brevibacterium avium* (data from Pascual & Collins, 1999); 3, *Brevibacterium luteolum* (Wauters *et al.*, 2003); 4, *Brevibacterium mcbrellneri* (McBride *et al.*, 1993); 5, *Brevibacterium otitidis* (Pascual *et al.*, 1996); 6, *Brevibacterium paucivorans* (Wauters *et al.*, 2001). Characteristics are recorded as: +, positive; W, weakly positive; –, negative; ND, no data available; ?, conflicting results between data given in species description and table of differential characteristics.

Characteristic	1	2	3	4	5	6
Growth at 20 °C	+	ND	+	–	–	–
Oxidase	–	+	ND	–	ND	ND
Growth on 10 % NaCl	+	ND	+	–	–	–
Nitrate reductase	–	+	–	–	–	–
Pyrazinamidase	+	ND	?	–	+	–
Pyrrolidonyl arylamidase	+	ND	+	–	+	–
Lipase (C14)	–	–	+	ND	–	–
Cystine arylamidase	W	+	–	ND	+	–
Origin of isolation	Beach sediment	Poultry	Clinical specimen	Clinical specimen	Clinical specimen	Clinical specimen
DNA G+C content (mol%)	70.7	ND	68.8	63.1	ND	55.8

hydrolysis and fermentation of D-ribose, D-xylose, D-mannitol, D-lactose and glycogen are negative. Acid production from D-glucose, D-maltose and sucrose are weakly positive. In additional tests with the API ZYM system, esterase (C4), esterase lipase (C8), leucine arylamidase, trypsin and naphthol-AS-BI-phosphohydrolase are positive. Valine arylamidase and cystine arylamidase are weakly positive. The following give negative responses: lipase (C14), α -chymotrypsin, acid phosphatase, α -galactosidase, β -glucuronidase, β -glucosidase, *N*-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase. The phospholipid profiles contain diphosphatidylglycerol. Mycolic acids are not present. The major cellular fatty acids are anteiso-C_{17:0}, anteiso-C_{15:0} and iso-C_{15:0}. The G + C content of the DNA is 70.7 mol%.

The type strain, SST-8^T (=NRRL B-41420^T=KCCM 42316^T), was isolated from sand sediment of Samyang Beach, Jeju Island, Republic of Korea.

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