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# *Scopulibacillus darangshiensis* gen. nov., sp. nov., Isolated from Rock

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**A novel, Gram-positive bacterium, designated DLS-06$^T$, was isolated from scoria (volcanic ash) under rock on the peak of small mountain (300 m above the sea level; known as Darangshi Oreum) in Jeju, Republic of Korea. The cells of the isolate were aerobic, oxidase-negative, catalase-positive, endospore-forming, non-motile rods. The organism grew at 25~30°C and initial pH 6.1~9.1. A neighbour-joining tree based on 16S rRNA gene sequences showed that the organism was related to members of the family "*Sporolactobacillaceae*" and related taxa. The phylogenetic neighbours were *Pullulanibacillus naganoensis* (95.2% 16S rRNA gene sequence similarity), *Tuberibacillus calidus* (95.0%) and *Sporolactobacillus* (91.8~94.2%). Levels of 16S rRNA gene sequence similarity of the isolate to representatives of other genera were in the range of 87.2~93.7%. The organism contained *meso*-diaminopimelic acid as the diagnostic diamino acid in the cell-wall peptidoglycan. The predominant menaquinone was MK-7. The polar lipid profile contained diphosphatidylglycerol, phosphatidylglycerol, an unknown ninhydrin-positive phospholipid, three unknown phospholipids and an unknown lipid. The major fatty acids were anteiso-$C_{15:0}$ and anteiso-$C_{17:0}$. The G+C content of the DNA was 50.8 mol%. On the basis of the phenotypic and phylogenetic data presented in this study, this organism represents a novel genus and species in the order *Bacillales*, for which the name *Scopulibacillus darangshiensis* gen. nov., sp. nov. is proposed. The type strain is DLS-06$^T$ (=DSM 19377$^T$ =KCTC 13161$^T$).**

***Keywords*****:** *Scopulibacillus darangshiensis* gen. nov., sp. nov., Darangshi Oreum, 16S rRNA gene sequence

The family "*Sporolactobacillaceae*" (Garrity and Holt, 2001) of the order *Bacillales* contains the genera *Marinococcus* (Hao *et al*., 1984) and *Sporolactobacillus* (Kitahara and Suzuki, 1963). Two other genera phylogenetically related to this family, *Tuberibacillus* and *Pullulanibacillus*, were recently described by Hatayama *et al.* (2006). *Tuberibacillus calidus* was isolated from compost pile, while *Pullulanibacillus naganoensis* was erected on the basis of reclassification of *Bacillus naganoensis*. In this paper, we described the classification and identification of a Gram-positive, spore-forming, rod-shaped bacterium from a rock by a polyphasic approach based on morphological, physiological, chemotaxonomic, and phylogenetic analyses.

## Materials and Methods

### Isolation and maintenance of microorganisms

Strain DLS-06$^T$ was isolated from scoria (volcanic ash) under rock on the peak of Darangshi Oreum (Small Mountain 300 m above the sea), in Jeju, Republic of Korea. Scoria sample was collected from the surface of rock with spatula. Bacterial isolation was performed by the dilution-plating technique on starch-casein agar that was generally used for the isolation of mycelium-forming actinomycetes. The ingredients of the medium contained 1% soluble starch, 0.03% casein, 0.2% $KNO_3$, 0.2% NaCl, 0.002% $CaCO_3$, 0.005% $MgSO_4 \cdot 7H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, and 1.8% agar (pH 7.2). The isolate was maintained on YMG agar (0.4% yeast extract, 1% malt extract, 0.4% glucose, and 1.8% agar; pH 7.2) at 4°C and as 20% (v/v) glycerol suspension at -20°C and -80°C. For chemotaxonomic comparison, *Tuberibacillus calidus* DSM 17572$^T$ was grown on CYC medium [48.0 g Czapek Dox agar (Merck, USA), 2.0 g BBL yeast extract (BBL, USA), 6.1 g Bacto casamino acids (BBL), 0.02 g tryptophan, 10 mg $MgSO_4 \cdot 7H_2O$, 1,000 ml distilled water, pH 6.0] for 3 days at 55°C, while *Pullulanibacillus naganoensis* DSM 10191$^T$ was grown for 3 days at 30°C on CYC medium (pH 5.0).

### Morphological and cultural characterization

Growth was tested on CYC medium, YMG agar, trypticase soy agar (TSA; BBL), and nutrient agar (NA; BBL). Cell morphology and endospores were examined by Olympus light microscope equipped with phase-contrast optics (magnification ×400) with the cells grown on YMG agar for 48 h at 30°C. The presence of flagella was checked by using a model JEM-1010 transmission electron microscope. Colony morphology was investigated by using a dissecting microscope (Olympus, Japan) and its color was observed after incubation for 3 days on YMG agar.

### Physiological and biochemical characterization

Gram stain was performed using COLOR GRAM-2 kit (bioMérieux, France) according to the instructions of the

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manufacturer. The following biochemical features were determined as described by MacFaddin (1980): catalase and oxidase activities, nitrate reduction and hydrolysis of aesculin, casein, gelatin, and urea. Degradation of hypoxanthine, DL-tyrosine, and xanthine was determined as described by Gordon *et al.* (1974). Hydrolysis of starch and DNA was tested using starch agar (BBL) and DNase test agar (BBL), respectively. Hydrolysis of chitin (0.5%, w/v), CM-cellulose (0.5%, w/v), and elastin (0.4%, w/v) was examined on YMG agar. Growth was checked on YMG agar at temperatures 4, 10, 20, 25, 30, and 37°C, and in the presence of 0~9% (w/v) NaCl. The initial pH for growth was tested in the range of 4.1~12.1. Acid production from various carbohydrates was tested by using OF basal medium (BBL) supplemented with filter-sterilized carbon source at the final concentration of 1% (w/v). Carbon sources tested were the following: D-arabinose, L-arabinose, D-cellobiose, dextran, D-fructose, D-galactose, D-glucose, inulin, D-lactose, maltose, D-mannose, D-melezitose, melibiose, α-methyl-D-glucoside, α-methyl-D-mannoside, D-raffinose, L-rhamnose, L-ribose, salicin, L-sorbose, sucrose, D-trehalose, D-xylose, adonitol, dulcitol, *meso*-erythritol, glycerol, *meso*-inositol, D-mannitol, D-sorbitol, and D-xylitol. After harvesting on YMG agar, the cells were washed twice with sterile distilled water before inoculation. The results were recorded after incubation for 7 days at 30°C under aerobic condition.

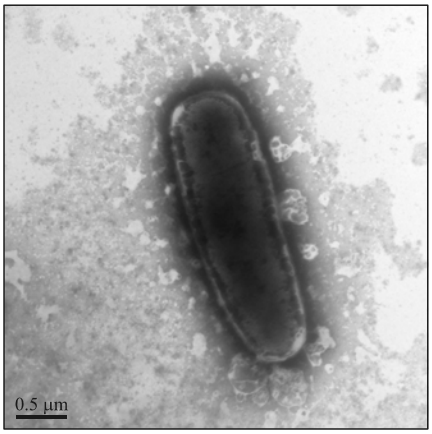


**Fig. 1.** Cell morphology of strain DLS-06$^T$ as determined by a transmission electron microscope. Strain DLS-06$^T$ was grown on YMG agar for 48 h at 30°C.

## 16S rRNA gene sequence analyses

Chromosomal DNA was isolated using Wizard DNA purification kit (Promega, USA). Amplification of the 16S rRNA gene by PCR was performed using universal primers (Lane, 1991) as described previously (Lee *et al.*, 2000). The reaction mixture for the PCR contained 50 ng DNA, 2 mM dNTPs, 1 U DyNAzyme$^{TM}$ DNA polymerase (Finnzymes), 1× DyNAzyme$^{TM}$ buffer, and 2 μM each primer. The resultant PCR product was purified using Wizard PCR preps DNA purification kit (Promega) and directly sequenced us-

**Table 1.** Differential characteristics of strains DLS-06$^T$ from the type strains of phylogenetic neighbors
Taxa: 1, Strain DLS-06$^T$; 2, *Marinococcus* (data from Hao *et al.*, 1984; Li *et al.*, 2005); 3, *P. naganoensis* (data from Tomimura *et al*., 1990; Hatayama *et al.*, 2006, except for major cellular fatty acids); 4, *Sinobaca* (data from Li *et al*., 2006, 2008); 5, *Sporolactobacillus* (data from Andersch *et al*., 1994; Yanagida *et al.*, 1997; Hatayama *et al.*, 2006; Chang *et al.*, 2008); 6, *T. calidus* (data from Hatayama *et al.*, 2006, except for major cellular fatty acids) +, Positive; -, negative; w, weakly positive; v, variable; ND, not determined or no data available; i, iso; ai, anteiso.

| Characteristic | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Oxidase activity | - | v | - | - | - | + |
| Temperature range for growth (°C) | 25~30 | 28 | 28~33 | 28 | 15~45 | 40~60 |
| pH range for growth | 6.1~9.1 | 6.0~10.0 | 4.0~6.0 | 8.0~9.5 | 5.0~8.0 | 5.0~7.0 |
| NaCl tolerance (%) | 0~4 | 0~25 | 0~5 | 1~25 | 0~7 | 0~4 |
| Growth on TSA | + | ND | + | ND | ND | - |
| Hydrolysis of: | | | | | | |
| Starch | - | - | + | + | v | - |
| Casein | - | v | - | + | - | + |
| Nitrate reduction | - | v | - | - | - | + |
| Acid production from: | | | | | | |
| Arabinose | - | - | + | ND | - | + |
| Lactose | - | ND | w | ND | - | - |
| Xylose | - | + | + | ND | - | + |
| Cell-wall sugars: | | | | | | |
| Galactose | - | ND | + | + | + | - |
| Glucose | + | ND | + | - | v | - |
| Mannose | - | ND | - | - | v | - |
| Rhamnose | - | ND | + | - | v | - |
| Ribose | - | ND | - | + | - | - |
| Major fatty acids (>10%) | ai-$C_{15:0}$, ai-$C_{17:0}$ | ai-$C_{15:0}$, ai-$C_{17:0}$, i-$C_{16:0}$ | i-$C_{15:0}$, i-$C_{16:0}$, ai-$C_{15:0}$, ai-$C_{17:0}$ | ai-$C_{15:0}$, ai-$C_{17:0}$, | ai-$C_{17:0}$, ai-$C_{15:0}$, i-$C_{15:0}$ | ai-$C_{17:0}$, i-$C_{16:0}$, i-$C_{17:0}$, |
| DNA G+C content (mol%) | 50.8 | 44.9~48.5 | 45±2 | 47 | 43~50.6 | 47.3 |

ing ABI PRISM BigDye Terminator cycle sequencing kit (Applied Biosystems, USA) and automatic DNA sequencer (model 3730×l; Applied Biosystems). Multiple alignment of the 16S rRNA gene sequences were performed using CLUSTAL_X software package (Thompson *et al.*, 1997). Evolutionary distance values were calculated using the method of Jukes and Cantor (1969). A phylogenetic tree was drawn by using neighbour-joining (Saitou and Nei, 1987) method, with the results of bootstrap analysis (Felsenstein, 1985) based on 1,000 replicated datasets. Maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) analyses were performed using DNAML and DNAPARS programs of the PHYLIP package (Felsenstein, 1993).

## Chemotaxonomic analyses

The isomer of diamino acid in the cell-wall peptidoglycan

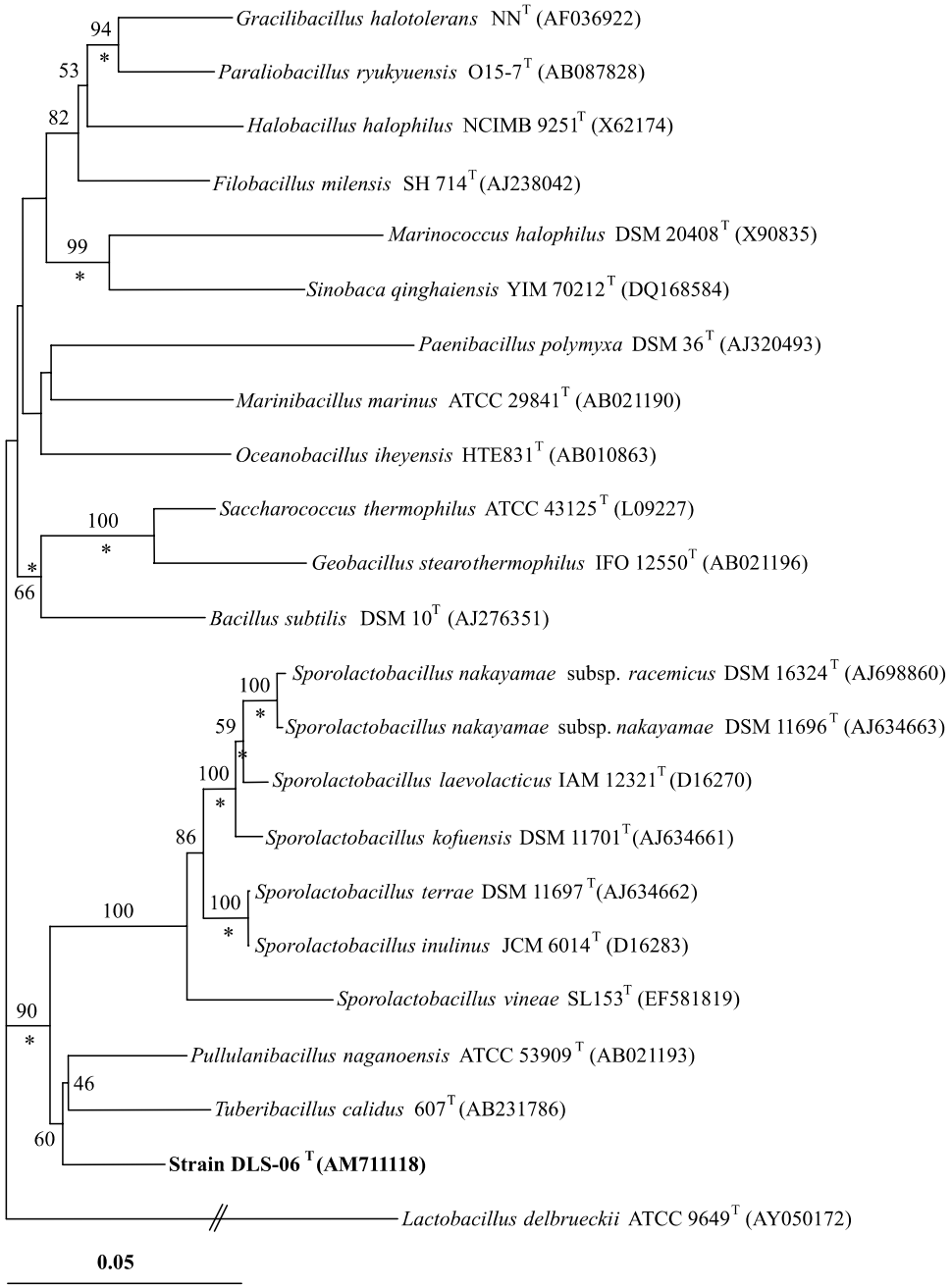


**Fig. 2.** A neighbor-joining tree showing phylogenetic relationship between strain DLS-06$^T$ and related taxa, based on the analysis of 16S rRNA gene sequences. Asterisk represents the corresponding branches found in maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) methods. Numbers at the nodes indicate bootstrap values (>45%). Scale bar, 0.05 substitutions per nucleotide position.

was determined by the method of Staneck and Roberts (1974). Whole-cell sugars were analyzed by gas chromatography as described by Saddler *et al.* (1991). The extraction and analysis of polar lipids were performed as described previously (Minnikin *et al.*, 1977, 1984). Analysis of menaquinones by HPLC was performed as described previously (Kroppenstedt, 1985). Quantitative analysis of cellular fatty acid was performed according to the instructions of the Microbial Identification System (version 6; MIDI). For determining cellular fatty acid profile, strain DLS-06$^T$ was grown for 3 days at 30°C on TSA (pH was adjusted at 7.0) and CYC medium (pH 7.0), respectively. *P. naganoensis* DSM 10191$^T$ was grown for 3 days at 30°C on CYC medium (pH 5.0), while *Tuberibacillus calidus* DSM 17572$^T$ was cultured on CYC medium (pH 6.0) for 3 days at 55°C. DNA G+C content was determined by the method of Mesbah *et al.* (1989), with genomic DNA isolated as described previously (Hopwood *et al*., 1985).

## Results and Discussion

Strain DLS-06$^T$ showed good growth on all of the media tested. The cells of strain DLS-06$^T$ were aerobic, oxidase-negative, catalase-positive, non-motile rods (Fig. 1). Oval-shaped, subterminal endospores were formed. The results of the other cultural, physiological, and biochemical characterization are given in Table 1 and the species description.

An almost-complete 16S rRNA gene sequence (1446 nt) of strain DLS-06$^T$ was subjected to a similarity search using BLASTN (Altschul *et al.*, 1997), revealing that the isolate showed 16S rRNA gene sequence similarity values 96% or less than to *Bacillus racemilacticus*, *T. calidus*, and members of the genus *Sporolactobacillus*. The 16S rRNA gene sequence of strain DLS-06$^T$ was aligned with the corresponding sequences retrieved from GenBank databases using the CLUSTAL_X program (Thompson *et al.*, 1997). After gaps and alignment uncertainties being eliminated, a total of 1,386 nucleotide positions present in all of the sequences were used for phylogenetic analyses. A neighbor-joining tree (Fig. 2) based on 16S rRNA gene sequences revealed that strain DLS-06$^T$ formed a distinct sublineage within the radiation encompassing members of the genera *Pullulanibacillus*, *Tuberibacillus*, and *Sporolactobacillus*, albeit with no support by a high bootstrap value and by the trees of two other treeing algorithms. The 16S rRNA gene sequence similarities of strain DLS-06$^T$ to its phylogenetic neighbours were *P. naganoensis* (95.2% sequence similarity), *T. calidus* (95.0%), and *Sporolactobacillus* (91.8~94.2%). Strain DLS-06$^T$ showed

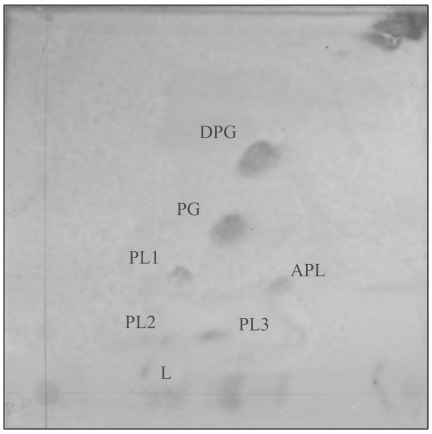


**Fig. 3.** Polar lipid profile of strain DLS-06$^T$ as analyzed by thin-layer chromatography. The running solvents were used as follow: first running solvent A (chloroform:methanol:water=65:25:4, v/v/v) and second running solvent B (chloroform:acetic acid:methanol:water=80:15:12:4, v/v/v/v). The following reagents for detecting polar lipids were used: 0.2% (w/v) ninhydrin reagent for amino lipids, zinzadze reagent for phospholipids, 5% (w/v) molybdophosphoric acid for all lipids and α-naphthol reagent for glycolipids. DPG, Diphosphatidylglycerol; PG, phosphatidylglycerol; APL, an unknown ninhydrin-positive phospholipid; PL, an known phospholipid; L, an unknown lipid.

**Table 2.** Cellular fatty acid compositions of strain DLS-06$^T$ and the type strains of phylogenetic neighbors
Taxa: 1, Strain DLS-06$^T$ (grown on TSA, pH 7.0); 2, strain DLS-06$^T$ (grown on CYC, pH 7.0); 3, *P. naganoensis* DSM 10191$^T$ (grown on CYC, pH 5.0); 4, *T. calidus* DSM 17572$^T$ (grown on CYC, pH 6.0). Fatty acids less than 1.0% were omitted. -, Not detected or not described. *T. calidus* was cultured for 3 days at 55°C, while the remaining strains were incubated for 3 days at 30°C.

| Fatty acid | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| Saturated fatty acids: | | | | |
| $C_{12:0}$ | - | 1.1 | 1.8 | - |
| $C_{16:0}$ | 3.5 | 4.8 | 4.4 | 4.6 |
| $C_{18:0}$ | 2.4 | 4.0 | 2.0 | 4.0 |
| Unsaturated fatty acid: | | | | |
| $C_{16:1}$ 2OH | - | - | 1.4 | 1.0 |
| Branched fatty acids: | | | | |
| iso-$C_{15:0}$ | 6.3 | 2.1 | 10.4 | 3.1 |
| iso-$C_{16:0}$ | 3.8 | 4.2 | 12.5 | 10.0 |
| iso-$C_{17:0}$ | 3.9 | 1.1 | 3.9 | 14.5 |
| iso-$C_{17:0}$ 3OH | - | - | - | 2.3 |
| anteiso-$C_{15:0}$ | 40.5 | 40.0 | 29.5 | 2.8 |
| anteiso-$C_{17:0}$ | 33.8 | 39.1 | 31.8 | 54.2 |
| anteiso-$C_{19:0}$ | 1.1 | - | - | - |

16S rRNA gene sequence similarity values of 87.2~93.7% to representatives of the other genera. In the previously study (Garrity and Holt, 2001), the genus *Marinococcus* (Hao *et al*., 1984) was reported to be a member of the family "*Sporolactobacillaceae*" of the order *Bacillales*, together with the genus *Sporolactobacillus*. Recently, the genus *Sinobaca* (Li *et al.*, 2006, 2008) was described to be a phylogenetically closest neighbor to members of the genus *Marinococcus*. However, these genera were remotely related to the genus *Sporolactobacillus* and related genera in our analysis (Fig. 2) and it is suggested that they need to be excluded from the family "*Sporolactobacillaceae*".

Strain DLS-06$^T$ contained *meso*-diaminopimelic acid (DAP) as the diamino acid in the cell wall peptidoglycan and glucose as whole-cell sugar, respectively. The polar lipid profile of strain DLS-06$^T$ contained diphosphatidylglycerol, phosphatidylglycerol, an unknown ninhydrin-positive phospholipid and an unknown phospholipid PL1 as major amounts. Additionally, minor amounts of two unknown phospholipids (PL2 and PL3) and an unknown lipid were also detected, but phosphatidylethanolamine, phosphatidylserine, phosphatidylcholine or a glycolipid was not present (Fig. 3). Menaquinones (MK)-7 was the major menaquinone. MK-5 or MK-6 was not detected in strain DLS-06$^T$, in contrast to the genera *Tuberibacillus*, *Pullulanibacillus* (Hatayama *et al.*, 2006), and *Sporolactobacillus* (Andersch *et al.*, 1994; Yanagida *et al.*, 1997) that contained minor amounts of MK-5 and MK-6. The G+C content of the DNA was 50.8 mol%. The cellular fatty acid profile of strain DLS-06$^T$ consisted of saturated, iso- and anteiso-branched components. The major fatty acids were anteiso-$C_{15:0}$ (40.0~40.5%) and anteiso-$C_{17:0}$ (33.8~39.1%), irrespective of culture media used. Of the reference strains, the major fatty acids (>10%) of *P. naganoensis* DSM 10191$^T$ showed considerable difference from those reported previously (Hatayama *et al.*, 2006) in proportion of iso-$C_{16:0}$ and in the absence/presence of anteiso-$C_{17:0}$, anteiso-$C_{15:0}$, iso-$C_{15:0}$, and $C_{16:0}$. The cellular fatty acid compositions of strain DLS-06$^T$ and the type strains of related taxa are given in Table 2.

In addition to low 16S rRNA sequence similarity values (<95.0%), strain DLS-06$^T$ significantly differs from the phylogenetic neighbors, the genera *Tuberibacillus*, *Pullulanibacillus*, and *Sporolactobacillus*, by temperature and pH range for growth, cellular fatty acids, DNA G+C content and the other chemotaxonomic features (Table 1). In our phylogenetic analysis, the genus *Marinococcus* of the family "*Sporolactobacillaceae*" (Garrity and Holt, 2001) and the genus *Sinobaca* (Li *et al.*, 2006, 2008) were remotely related to strain DLS-06$^T$ and above genera (Fig. 2). 16S rRNA gene similarity values of strain DLS-06$^T$ to *Marinococcus halophilus* DSM 20408$^T$ and *Sinobaca qinghaiensis* YIM 70212$^T$ were 98.9% and 91.2%, respectively. The novel isolate can be readily distinguished from members of the genera *Marinococcus* and *Sinobaca* by NaCl tolerance, fatty acid compositions and DNA G+C content (Table 1).

On the basis of the phenotypic and phylogenetic data presented here, strain DLS-06$^T$ represents a novel genus and species in the order *Bacillales*, for which the name *Scopulibacillus darangshiensis* gen. nov., sp. nov. is proposed.

**Description of *Scopulibacillus* gen. nov.**

*Scopulibacillus* (Sco.pu.li.ba.cil'lus. L. masc. n. *scopulus* rock; L. dim. n. *bacillus* a small rod; N.L. masc. n. *Scopulibacillus* a rod isolated from rock).

Cells are aerobic, non-motile, Gram-positive rods (0.9~2.8 μm). Oxidase-negative. Catalase-positive. Oval-shaped, subterminal endospores are formed. The major menaquinone is MK-7. The polar lipid profile consists of diphosphatidylglycerol, phosphatidylglycerol, an unknown ninhydrin-positive phospholipid and an unknown phospholipid as major amounts. The major fatty acids are anteiso-$C_{15:0}$ and anteiso-$C_{17:0}$. The G+C content of the DNA is 50.8 mol%. The genus phylogenetically related to the genera *P. naganoensis*, *T. calidus*, and *Sporolactobacillus* of the family "*Sporolactobacillaceae*" in the order *Bacillales*. The type species is *Scopulibacillus darangshiensis*.

**Description of *Scopulibacillus darangshiensis* sp. nov.**

*Scopulibacillus darangshiensis* (da.rang.shi'en.sis. N.L. masc. adj. *darangshiensis* pertaining to Darangshi Oreum in Jeju, Republic of Korea, where the type strain was isolated).

On YMG agar, colonies of the cells are convex, smooth, circular, cream-coloured, and reach 1~3 mm diameter after incubation for 3 days. The cells grow well on CYC medium, YMG agar, TSA, and NA. Growth occurs at 25~30°C, initial pH 6.1~9.1 and in the presence of 0~4% NaCl. Growth does not occur at 20 or 33°C. Good growth is observed at 30°C, initial pH 7.1~9.1 and in the range of 0~3% NaCl. Degradation of chitin, CM-cellulose, DNA, elastin, hypoxanthine, tyrosine, urea, and xanthine is negative. Hydrolysis of aesculin is positive. Gelatin liquefaction is positive. Nitrate reduction is not observed. Acid production only occurs from D-glucose. Acid production does not occur from D-arabinose, L-arabinose, D-cellobiose, dextrin, D-fructose, D-galactose, inulin, maltose, D-mannose, D-melezitose, melibiose, α-methyl-D-glucoside, α-methyl-D-mannoside, D-raffinose, L-rhamnose, L-ribose, salicin, L-sorbose, sucrose, D-trehalose, adonitol, D-dulcitol, *meso*-erythritol, glycerol, *meso*-inositol, D-mannitol, D-sorbitol, and D-xylitol. The major fatty acids are anteiso-$C_{15:0}$ (40.0~40.5%) and anteiso-$C_{17:0}$ (33.8~ 39.1%). The G+C content of the DNA is 50.8 mol%.

The type strain, DLS-06$^T$ (=DSM 19377$^T$ =KCTC 13161$^T$), was isolated from a rock sample collected from the peak of Darangshi Oreum in Jeju, Republic of Korea.

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