Alkalibacillus silvisoli sp. nov., an alkaliphilic moderate halophile isolated from non-saline forest soil in Japan

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Two alkaliphilic, moderately halophilic bacteria, designated BM2^T and HN2, were isolated from non-saline forest soil in Japan. The cells of strain BM2^T were motile, aerobic, rod-shaped and Gram-positive. The peptidoglycan was of the A1_γ type, and the diamino acid was *meso*-diaminopimelic acid. Growth was observed at NaCl concentrations between 5.0 and 25.0 % (w/v) (the optimum being 10.0–15.0 %, w/v), at pH 7.0–10.0 (optimum, pH 9.0–9.5) and at 20–50 °C. The predominant isoprenoid quinone was MK-7. The major cellular fatty acids were iso-C_{15:0} and anteiso-C_{17:0}. The G + C content of total DNA of strain BM2^T was 37.0 mol%. Phylogenetic analysis of the 16S rRNA gene sequence showed that strain BM2^T was most closely related to *Alkalibacillus haloalkaliphilus* DSM 5271^T (98.0 % sequence similarity). DNA–DNA hybridization results indicated low levels of relatedness between strain BM2^T and *A. haloalkaliphilus* JCM 12303^T (23 and 16 % reciprocally), *Alkalibacillus filiformis* JCM 13893^T (25 and 21 %) and *Alkalibacillus salilacus* JCM 13894^T (27 and 19 %). On the basis of the phylogenetic and phenotypic characteristics, strain BM2^T represents a novel species, for which the name *Alkalibacillus silvisoli* sp. nov. is proposed. The type strain is BM2^T (=JCM 14193^T=DSM 18495^T).

Extremophiles are micro-organisms adapted to grow in conditions such as extreme pH, temperature, salinity, hydrostatic pressure and ultraviolet and ionizing radiation (Horikoshi & Grant, 1998; Rainey & Oren, 2006). In general, it has been believed that they survive in the extreme environments in which they had adapted for growth. Many extremophiles, however, have been isolated from places in which they would not have been expected to grow. For example, alkaliphilic micro-organisms have been isolated from acidic soil samples (pH 4.0) as well as from neutral and alkaline soils (Horikoshi, 1999). Halophilic micro-organisms are adapted to high levels of salinity and require a certain concentration of NaCl for optimum growth (Kushner & Kamekura, 1988; Oren, 2002). They have been isolated from various saline environments such as salt lakes (e.g. the Dead Sea, the Great Salt Lake), salterns, solar salts and subsurface salt formations. It has been tacitly believed that halophilic micro-organisms able to grow in media containing more than 20 % (w/v) (3.4 M) NaCl are restricted to habitats in saline environments, and no reports

have been published showing the isolation of halophilic micro-organisms from samples of ordinary garden soil. Recently, we isolated many moderate halophiles from non-saline soils in an area surrounding Tokyo, Japan, on agar plates of pH 9.5 containing 20.0 % NaCl (Echigo *et al.*, 2005). A couple of alkaliphilic moderate halophiles have been reported so far. *Bacillus oshimensis* was isolated from a soil sample obtained in Hokkaido, Japan (Yumoto *et al.*, 2005a), and *Oceanobacillus oncorhynchi* was isolated from the skin of a freshwater rainbow trout (Yumoto *et al.*, 2005b). Both strains grew in medium containing 0–20 % (w/v) NaCl, and optimal growth occurred at pH 9–10.

In this study, we isolated halophiles on agar plates containing 20.0 % NaCl (pH 9.5), and sequencing of the 16S rRNA genes led to the suggestion that some of the isolates were closely related to species of the genus *Alkalibacillus*, which currently comprises three species, *Alkalibacillus haloalkaliphilus* (Fritze, 1996; Jeon *et al.*, 2005), *Alkalibacillus filiformis* (Romano *et al.*, 2005) and *Alkalibacillus salilacus* (Jeon *et al.*, 2005). On the basis of the phylogenetic and phenotypic data obtained in this study, we propose that the moderately halophilic and alkaliphilic bacteria isolated from an ordinary non-saline forest soil sample represent a novel member of the genus *Alkalibacillus*.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains BM2^T and HN2 are AB264528 and AB267380, respectively.

Soil samples were taken from ordinary non-saline forests in many places in Saitama Prefecture and Chiba Prefecture, Japan; there are no highly saline environments such as salterns or salt lakes in the region. The NaCl contents, calculated from the Cl^- contents of soil extracts in water and determined using the method of Mohr (as described by Lenore *et al.*, 1998), were less than 0.1 % (w/v). The soil extracts were slightly acidic (pH 5.0–6.0).

A sample of soil (approx. 0.5 g) was placed on an agar plate and spread with a spatula; the plate was then incubated in a plastic bag (to prevent desiccation) at 37 °C for 3 weeks. The medium contained the following ingredients (l^{-1}): 5.0 g Casamino acids (Difco), 5.0 g yeast extract (Difco), 1.0 g sodium glutamate monohydrate, 3.0 g trisodium citrate dihydrate, 2.0 g KCl, 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 36 mg $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, 200.0 g (3.4 M) NaCl and 20 g Bacto-agar (Difco). After autoclaving, the pH was adjusted to 9.5 by the addition of precalculated amounts of sterile Na_2CO_3 solution. Colonies (0–8, at most, per plate) were picked up, transferred to fresh agar plates, and subjected to dilution plating and subculturing on agar plates of the same medium in order to obtain pure cultures. Two strains, BM2^T, which was isolated from a soil sample from Kawagoe, Saitama Prefecture, and strain HN2 (designated as no. 2 in Table 1 of Echigo *et al.*, 2005), which was isolated from a soil sample from Yachiyo, Chiba Prefecture, were selected for further characterization after partial sequencing (about 500 bp of the 5' end) of PCR-amplified 16S rRNA genes from 29 isolates. The level of gene sequence similarity between strain HN2 and strain BM2^T was 99.2 %. In addition, DNA–DNA hybridization (assessed by using the fluorometric method of Ezaki *et al.*, 1989) revealed high levels of relatedness (88 and 80 % reciprocally), suggesting that strains HN2 and BM2^T strains should be classified within the same species.

Growth was determined by inoculating precultures of the strains into 100 ml Erlenmeyer flasks containing 20 ml liquid medium at different NaCl concentrations [0, 5.0, 10.0, 15.0, 20.0, 25.0 and 30.0 % (w/v); pH 9.5, 37 °C], pH values [5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5 and 10.0; 10.0 % (w/v) NaCl, 37 °C] and temperatures [20, 30, 37, 40, 50, 60 and 70 °C; 10.0 % (w/v) NaCl, pH 9.5] and subjecting them to shaking at 120 r.p.m. Growth was monitored by taking 0.1 ml culture periodically and measuring the optical density at 660 nm.

Strain BM2^T grew at NaCl concentrations between 5.0 and 25.0 % (w/v), with optimal growth occurring at 10.0–15.0 % (w/v). Growth of strain BM2^T occurred at pH 7.0–10.0, with an optimal growth occurring at pH 9.0–9.5. The temperature range for growth extended from 20 to 50 °C, with an optimum at 30–37 °C. Growth of strain HN2 occurred between 0 and 25.0 % (w/v) NaCl, the optimum being at 5.0–10.0 % (w/v). The pH range for growth was 6.5–10.0, with an optimum at pH 8.5–9.5. On agar plates containing 10.0 % (w/v) NaCl, the colonies of both strains were cream in colour and opaque.

The following characterizations were performed on strain BM2^T only. The cells of this strain were rod-shaped, 0.3–0.5 µm wide and 4.0–7.0 µm long, and were motile by means of single polar flagella (when observed according to the method of Kodaka *et al.*, 1982). Endospore formation was investigated after spore-staining had been performed according to the method of Wirtz-Conklin (as described by Murray *et al.*, 1999). The cells of this strain were found to produce spherical terminal endospores within swollen sporangia on the isolation medium (containing NaCl at 10.0 %, w/v) at 37 °C. No gas vesicles were formed inside the cells. After fixation with acetic acid as described by Dussault (1955), cells from fresh cultures were Gram-positive but variable in old cultures. The KOH test and the L-alanine aminopeptidase test (Bactident aminopeptidase test strips; Merck) produced negative reactions, as was reported for Gram-positive bacteria (Gregersen, 1978). Anaerobic growth was not observed in an anaerobic jar (Echigo *et al.*, 2005) after incubation for 7 days at 37 °C.

Acid production from carbohydrates was tested in basal medium containing (l^{-1}) 1.0 g Casamino acids and 1.0 g yeast extract, supplemented with 10.0 g of the test carbohydrate; control cultures did not include the carbohydrates. The cultures were incubated at 37 °C under aerobic conditions for 3 days; growth was determined visually and the pH was measured with a pH electrode. Strain BM2^T produced acid from D-galactose, maltose, sucrose, D-trehalose and D-mannitol, but not from D-fructose, D-glucose or D-xylose.

Tests for catalase and oxidase activities and for the hydrolysis of starch, gelatin, casein, DNA, hippurate, aesculin, pullulan and Tween 80 were performed according to the procedures of Smibert & Krieg (1994), Oren *et al.* (1997) and Schlesner *et al.* (2001). Strain BM2^T produced a positive catalase reaction and a negative oxidase reaction. Casein and gelatin were hydrolysed, but starch, DNA, hippurate, aesculin, pullulan and Tween 80 were not. Nitrate reduction was not detected with the method involving sulfanilic acid and an α -naphthylamine reagent (Smibert & Krieg, 1981) and gas formation from nitrate was not detected using Durham tubes under anaerobic conditions.

Sensitivity to antimicrobial agents was tested using antibiotic-impregnated filter-paper discs placed on a pre-inoculated plate of isolation medium containing 10.0 % (w/v) NaCl. Inhibitory zones around the discs were recorded after 3 days incubation at 37 °C. Strain BM2^T was sensitive to (per disc) ampicillin (50 µg), bacitracin (25 µg), tetracycline (50 µg), streptomycin (100 µg), novobiocin (25 µg) and chloramphenicol (25 µg) and resistant to kanamycin (50 µg) and anisomycin (50 µg).

HPLC analysis of isoprenoid quinones and GC/MS analysis of fatty acid methyl esters were performed with cells grown in isolation medium containing 10.0 % (w/v) NaCl at 30 °C for 3 days according to the modified procedures of

Table 1. Differential characteristics of strain BM2^T and type strains of related *Alkalibacillus* species

Strains: 1, BM2^T; 2, *A. haloalkaliphilus* DSM 5271^T; 3, *A. filiformis* DSM 15448^T; 4, *A. salilacus* DSM 16460^T. Data are from Fritze (1996), Jeon *et al.* (2005), Romano *et al.* (2005) and the present study. +, Positive; –, negative; w, weak; DeMK, demethylmenaquinone.

Characteristic	1	2	3	4
Cell morphology	Long rods	Long rods	Long rods	Rods
Colony pigmentation	Cream	Creamy white (with 5–10% NaCl)	White to transparent	Cream
Motility	+	+	–	+
Gram stain	+	–	+	+
KOH test	–	–	+	–
NaCl concentration for growth (%)				
Range	5.0–25.0	>0 to 25.0	0–18.0	5.0–20.0
Optimum	10.0–15.0	5.0–10.0*	10.0	10.0–12.0
pH for growth				
Range	7.0–10.0	>7 to >9.7	7.0–10.0	7.0–9.0
Optimum	9.0–9.5	9.7	9.0	8.0
Temperature for growth (°C)				
Range	20–50	<50	15–45	15–40
Optimum	30–37	30–37*	30	30
Acid production from:				
D-Fructose	–	–*	–*	+
D-Galactose	+	–*	–*	–*
Maltose	+	–*	–*	–
D-Trehalose	+	–*	–*	–
D-Mannitol	+	–*	–*	–
Catalase	+	+	w	+
Oxidase	–	+	–	–
Hydrolysis of:				
Starch	–	w	–	–
Casein	+	–/w	–	–
Gelatin	+	+	+	–
Hippurate	–	+	–	–*
Aesculin	–	+	–*	+
Nitrate reduction	–	–	–	+
Sensitivity to streptomycin	+	–*	–	–*
DNA G+C content (mol%)	37.0	37.0–38.0†	39.5	41.0
Isoprenoid quinone(s)	MK-7	MK-7	MK-7, DeMK-6	MK-7
Major fatty acid(s)	iso-C _{15:0}	iso-C _{15:0}	iso-C _{15:0} , anteiso-C _{17:0}	iso-C _{15:0} , anteiso-C _{15:0}

*Determined in this study using *A. haloalkaliphilus* JCM 12303^T, *A. filiformis* JCM 13893^T or *A. salilacus* JCM 13894^T.

†Results from two different methods reported by Fritze (1996).

Tamaoka (1986) and Komagata & Suzuki (1987). The predominant isoprenoid quinone of strain BM2^T was MK-7. The cellular fatty acid profile of strain BM2^T was characterized by the presence of saturated branched fatty acids such as iso-C_{15:0} (42%), anteiso-C_{17:0} (20%), anteiso-C_{15:0} (6%), iso-C_{16:0} (6%), iso-C_{17:0} (6%) and anteiso-C_{16:0} (3%), which was in accord with reported profiles of *Alkalibacillus* species (Fritze, 1996; Jeon *et al.*, 2005; Romano *et al.*, 2005).

Preparation of the peptidoglycan and the determination of its structure were performed according to the modified

procedures of Schleifer & Kandler (1972), Schleifer (1985) and Schlesner *et al.* (2001). The purified peptidoglycan was hydrolysed in 4 M HCl at 100 °C for 16 h (total hydrolysate) or 45 min (partial hydrolysate). Diamino acids were identified from the total hydrolysate by one-dimensional TLC in methanol/pyridine/4 M HCl/water (80:10:4:26, by vol.). Amino acids and peptides were identified from the partial hydrolysate by using two-dimensional TLC. The first direction was developed in isopropanol/acetic acid/water (75:10:15, by vol.) and the second in α -dipicolin/25% ammonium hydrate/water (70:2:28, by vol.). The resulting fingerprints were compared with those from known

peptidoglycan structures. The peptidoglycan structure of strain BM2^T belonged to the A1 γ type, and the diamino acid was *meso*-diaminopimelic acid, as in all *Alkalibacillus* species (Fritze, 1996; Jeon *et al.*, 2005; Romano *et al.*, 2005).

Total DNAs of strain BM2^T were extracted by the method of Saito & Miura (1963). The 16S rRNA genes were amplified by PCRs with the following forward and reverse primers: 5'-AGAGTTTGTATCCTGGCTCAG-3' (positions 8–27 according to *Escherichia coli* numbering) and 5'-GGCTA-CCTTGTTACGACTT-3' (positions 1510–1492). The amplified DNAs were cloned by using the TA cloning kit (Invitrogen) and were sequenced using the ABI PRISM BigDye Terminator v3.1 cycle sequencing kits (Applied Biosystems) with the following primers: 5'-GGAAACA-GCTATGACCATG-3' (vector side), 5'-GACTACCAGGG-TATCTAATC-3' (positions 805–786), 5'-AGGGTTGCGC-TCGTTG-3' (positions 1115–1100) and 5'-GTAAACGA-CGGCCAGT-3' (vector side) on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). There was little heterogeneity (two to three bases) among the sequences (1492 bp) of several clones. The sequences determined and those of related strains retrieved from the DNA Database of Japan (Miyazaki *et al.*, 2003; Pearson & Lipman, 1988; Lipman & Pearson, 1985) were aligned using the CLUSTAL W multiple sequence alignment program (Thompson *et al.*, 1994). The phylogenetic tree was constructed using the neighbour-joining method (Saitou & Nei, 1987) and evaluated by bootstrap resampling using 1000 datasets (Felsenstein, 1985). The phylogenetic analysis showed that strain BM2^T was a member of the family *Bacillaceae*, being most closely related to the type strains of *A. haloalkaliphilus* (98.0 % sequence similarity), *A. filiformis* (97.8 % sequence similarity) and *A. salilacus* (95.9 % sequence similarity) (Fig. 1).

The G + C content of total DNA of strain BM2^T (determined using the HPLC method of Tamaoka & Komagata, 1984) was 37.0 mol%, which is similar to values reported for *A. haloalkaliphilus*, *A. filiformis* and *A. salilacus* (37.0–38.0, 39.5 and 41.0 mol%, respectively) (Fritze, 1996; Jeon *et al.*, 2005; Romano *et al.*, 2005).

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships of strain BM2^T and related strains. Bootstrap values, shown at branch nodes, are expressed as percentages of 1000 replicates. *Halobacillus halophilus* NCIMB 9251^T was used as an out-group. Bar, 0.005 changes per nucleotide position.

DNA–DNA hybridization between strain BM2^T and closely related species was assessed by using the fluorometric method of Ezaki *et al.* (1989). The results showed low levels of relatedness for strain BM2^T with respect to *A. haloalkaliphilus* JCM 12303^T (23 and 16 % reciprocally), *A. filiformis* JCM 13893^T (25 and 21 % reciprocally) and *A. salilacus* JCM 13894^T (27 and 19 % reciprocally).

These phenotypic and genotypic data suggest that the strain BM2^T belongs to the genus *Alkalibacillus* (Jeon *et al.*, 2005). In terms of phenotypic properties, there are differences between strain BM2^T and the three aforementioned species of the genus *Alkalibacillus*, as summarized in Table 1. Strain BM2^T was more halophilic than those three species, produced acid from many carbohydrates and showed sensitivity to streptomycin. Other differences between strain BM2^T and *A. haloalkaliphilus*, the most closely related species, include the pH range for growth, the temperature range for growth, Gram staining, the oxidase reaction and the results for starch hydrolysis and hippurate hydrolysis. Both the low levels of DNA–DNA relatedness and the phenotypic differences between strain BM2^T and the three recognized *Alkalibacillus* species indicated that the BM2^T represents a novel species of the genus *Alkalibacillus*, for which the name *Alkalibacillus silvisoli* sp. nov. is proposed.

Description of *Alkalibacillus silvisoli* sp. nov.

Alkalibacillus silvisoli (sil.vi.so'li. L. n. *silva* forest; L. n. *solum* soil; N.L. gen. n. *silvisoli* of forest soil, the source of isolation of the type strain).

Cells are rod-shaped, 0.3–0.5 × 4.0–7.0 µm in size and motile by means of single polar flagella. Spores are spherical and located terminally in swollen sporangia. Gram-positive in fresh culture but variable in old culture. Negative results are obtained in the KOH test and for L-alanine aminopeptidase. Peptidoglycan is of the A1 γ type, and the diamino acid is *meso*-diaminopimelic acid. Colonies on agar plates are cream in colour and opaque. Growth occurs at NaCl concentrations between 5.0 and 25.0 % (w/v), with optimal growth at 10.0–15.0 % (w/v). Growth occurs at pH 7.0–10.0, the optimal pH being 9.0–9.5. Growth is observed at temperatures in the range 20–50 °C, the optimum being at 30–37 °C. Anaerobic growth is not observed. Acid is produced from D-galactose, maltose, sucrose, D-trehalose and D-mannitol, but not from D-fructose, D-glucose or D-xylose. Catalase-positive and oxidase-negative. Hydrolysis of casein and gelatin is detected, but not for starch, DNA, hippurate, aesculin, pullulan or Tween 80. Reduction of nitrate and gas formation are not observed. Sensitive to ampicillin, bacitracin, tetracycline, streptomycin, novobiocin and chloramphenicol but resistant to kanamycin and anisomycin. The G + C content of total DNA is 37.0 mol%. The predominant isoprenoid quinone is MK-7. The major cellular fatty acids are iso-C_{15:0} (42 %), anteiso-C_{17:0} (20 %), anteiso-C_{15:0} (6 %), iso-C_{16:0} (6 %), iso-C_{17:0} (6 %) and anteiso-C_{16:0} (3 %).

The type strain, BM2^T (=JCM 14193^T=DSM 18495^T), was isolated from non-saline surface soil from a forest in Kawagoe, Saitama Prefecture, Japan. A reference strain, HN2, was isolated from non-saline surface soil from a forest in Yachiyo, Chiba Prefecture, Japan.

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