Bacillus alkalidiazotrophicus sp. nov., a diazotrophic, low salt-tolerant alkaliphile isolated from Mongolian soda soil

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Strain MS 6^T was obtained from a microoxic enrichment with a soda soil sample from north-eastern Mongolia in nitrogen-free alkaline medium at pH 10. The isolate had clostridia-like motile cells and formed ellipsoid endospores. It was able to fix dinitrogen gas growing on nitrogen-free alkaline medium. Strain MS 6^T was a strictly fermentative bacterium without a respiratory chain, although it had a high catalase activity and tolerated aerobic conditions. It was an obligate alkaliphile with a pH range for growth between 7.5 and 10.6 (optimum at 9.0–9.5). Growth and nitrogen fixation at pH 10 were possible at a total salt content of up to 1.2 M Na⁺ (optimum at 0.2–0.3 M). The dominant cellular fatty acids included C_{16:0}, C_{16:1ω7}, anteiso-C_{15:0} and C_{14:0}. The dominant isoprenoid quinone was MK-7. The cell-wall peptidoglycan contained meso-diaminopimelic acid as the diagnostic diamino acid. 16S rRNA gene sequencing identified strain MS 6^T as a member of the genus *Bacillus*. Its closest relative was *Bacillus arseniciselenatis* E1H^T. The key functional nitrogenase gene *nifH* was detected in both strain MS 6^T and its close relative and these strains formed a novel lineage in the *nifH* gene family. On the basis of these phenotypic and genetic comparisons, strain MS 6^T is proposed to represent a novel species of the genus *Bacillus*, *Bacillus alkalidiazotrophicus* sp. nov. with the type strain MS 6^T (=NCCB 100213^T=UNIQEM U377^T).

Soils containing substantial concentrations of sodium carbonates among the dominant soluble salts in their upper profile (*soda* or *sodic* soils, *soda solonchaks*) are a unique extreme habitat that is characterized by high salinity and alkalinity. These soils are distributed in dry steppe and semidesert areas such as south-western Siberia, north-eastern Mongolia and north China in Central Asia, Egypt in Africa, India, Hungary in Europe and North American Steppes (Basilevich, 1965; Kondorskaya, 1965). In contrast to well-studied soda lakes (Jones *et al.*, 1998; Zavarzin *et al.*, 1999; Oren, 2002; Sorokin & Kuenen,

2005), very little is known about the microbial diversity and functioning of soda solonchaks. Their major differences when compared with soda lakes, which include higher aeration and long desiccation periods, might result in the domination of different haloalkaliphilic taxa, such as Gram-positive bacteria, for example.

So far, a few studies on nitrogen fixation (NF) in soda lakes have identified cyanobacteria as the only active diazotrophic group (Oremland, 1990; Herbst, 1998; Zavarzin *et al.*, 1999). Quite recently, this potential ability was also discovered in a few haloalkaliphilic bacteria isolated from soda lake sediments, such as the aerobic gammaproteobacteria *Thiالكاليسپرا microaerophila* and *Alkalilimnicola halodurans* (Tourova *et al.*, 2007) and the obligate anaerobes *Clostridium alkalicellulosi* (Zhilina *et al.*, 2005) and *Geoalkalibacter ferrihydriticus* (Zavarzina *et al.*, 2006). A survey of functional nitrogenase genes in the alkaline saline Mono Lake has also shown the possible presence of a diverse diazotrophic bacterial population, with a domination of members of the class *Deltaproteobacteria*, but it appeared that no nitrogen fixation was taking place *in situ* (Steward *et al.*, 2004).

Abbreviation: NF, nitrogen fixation.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *nifH* gene sequences for *Bacillus alkalidiazotrophicus* sp. nov. MS 6^T are EU143680 and EU204959, respectively. The GenBank/EMBL/DDBJ accession number for the *nifH* gene sequence of *Bacillus arseniciselenatis* E1H^T determined in this study is EU204960.

Supplementary figures showing the cell morphology of strain MS 6^T grown anaerobically at 0.6 M Na⁺ and pH 10 with glucose and two additional phylogenetic trees are available with the online version of this paper.

In this paper, we describe a novel diazotrophic member of the RNA group 6 of the genus *Bacillus* that was isolated from a sample of soda soil from north-eastern Mongolia with high potential NF activity. NF potential has never before been recognized in this cluster of low G+C Gram-positive bacteria.

The soda soil sample used for the enrichment (top 5 cm) was taken from the north-eastern part of the Mongolian dry steppe (Choibalsan province) in 1999 and was kept in sterile plastic Petri dish at 4 °C before use. The sample had a pH of 10.35 in a 1:5 water extract with a total soluble salt content of 4.8 % and a total soluble carbonate alkalinity of 50 mM (analysed according to Mineev *et al.*, 1989). Potential NF activity was measured by a standard acetylene reduction assay (Hardy *et al.*, 1968) using a 5 g soil sample flooded with 3 ml water in 15 ml serum bottles at 0, 2 and 20 % O₂ in the gas phase. After 2 days preincubation with water, 10 % acetylene was added to the gas phase and 5 mg glucose was added. Ethylene production, as measured daily by GC, was taken as an indication of NF potential. The same method was used to measure NF activity in pure cultures, except that acetylene was added after the replacement of the gas phase with argon.

An enrichment culture was started from the activated sample using 10 % inoculum at microoxic conditions (10 ml medium in 15 ml flasks with rubber stoppers) at 25 °C on nitrogen-free soda medium with the following composition (l⁻¹): 22 g Na₂CO₃, 8 g NaHCO₃, 6 g NaCl and 0.5 g K₂HPO₄. The pH after sterilization was 10.0. After sterilization, the medium was supplemented with 1 mM MgSO₄, 1 ml l⁻¹ of a trace metal solution and a vitamin mixture (Pfennig & Lippert, 1966) and 2 g l⁻¹ filter-sterilized glucose solution. After the appearance of visible turbidity, NF was measured and, in the case of a positive result, the culture was transferred into fresh medium at a 1:100 dilution several times for stabilization. Finally, the cultures were serially diluted and the maximum active dilution (6) was plated onto solid medium with the same composition prepared by 1:1 mixing of double-strength liquid soda medium and 4 % agar at 50 °C. The plates were incubated in gas-tight jars (Oxoid) under a 95 % argon/5 % N₂ atmosphere for one week. Colonies with different morphologies were placed into the liquid medium and the resultant cultures were checked for NF potential.

The potential NF activity in the soda soil sample from Mongolia was maximal at 2 % O₂ [1.4–2.1 µmol N (g soil d⁻¹)] and was only slightly inhibited in a 100 % air atmosphere. However, the best growth in the subsequent enrichment cultures at pH 10 was observed under micro-oxic/anoxic conditions. The isolation of a pure culture was complicated by a rapid lawn-type growth of bacilli which did not fix nitrogen. The use of dry plates with a short incubation time eventually enabled the selection of 12 different types of colonies from which only one resulted in an active diazotrophic culture. The strain was designated MS 6^T.

The phenotypic characterization of the strain included light and electron microscopy, growth experiments to determine the substrate utilization profile (at pH 10 and 0.6 M total Na⁺; the medium composition was the same as for the enrichment) and the influence of pH/salt on growth (Sorokin, 2005) and NF activity. Routine cultivation of pure culture was performed in 30 ml serum bottles completely filled with medium, sealed with butyl rubber stoppers and incubated statically at 30 °C. Growth was estimated by OD₆₀₀ measurements. For the latter, the cells were grown in standard nitrogen-free medium at pH 10, centrifuged and washed in 0.5 M sodium bicarbonate, pH 8, at a protein concentration of 20 mg ml⁻¹. To determine the pH range, cells were resuspended at a concentration of 0.2 mg protein ml⁻¹ in a series of buffers containing 0.6 M total Na⁺ (Sorokin, 2005). For the salt range test, NaHCO₃/Na₂CO₃ buffer at pH 10 was used at a concentration 0.1–2 M Na⁺. The tests were performed in triplicate in 7 ml serum flasks with 3 ml cell suspension under anoxic conditions. Before the flasks were closed, sodium sulfide (1 mM) and glucose (1 mg ml⁻¹) were added. Ethylene production was measured every 4 h. All activity experiments were performed in duplicate, the data represent mean values with a standard deviation not exceeding 15 %. The final products of glucose fermentation were detected by HPLC-anionic chromatography with a HPX-87-H column (Bio-Rad) at 60 °C, a UV/RI detector and 5 mM H₂SO₄ at 0.6 ml min⁻¹ as a carrier. The samples were neutralized to pH 7 before analysis. Cell protein was determined by the Lowry method (Lowry *et al.*, 1951). Catalase activity in washed cells was quantified by an iodimetric method (Sumner & Dounce, 1955). For electron microscopy, the cells were prefixed in glutaraldehyde (final concentration 3 % v/v) at pH 7.0 in the presence of 0.5 M NaCl, post-fixed in 1 % (w/v) OsO₄/0.5 M NaCl for 12 h at room temperature and then washed and stained overnight with 1 % (w/v) uranyl acetate. Finally, the cells were dehydrated using an ethanol series and embedded in Epon resin. Thin sections were contrasted with 1 % (w/v) lead citrate.

Cells of strain MS 6^T were large rods with narrow edges and were highly motile with peritrichous flagella. The cell wall was of the Gram-positive type (as revealed by thin sectioning and tested with 3 % KOH). Cells formed ellipsoidal thermostable endospores (see Supplementary Fig. S1 in IJSEM Online). Strain MS 6^T was found to be a strictly fermentative bacterium that used various simple carbohydrates but was able to tolerate fully aerobic conditions, probably due to a relatively high catalase activity [0.4 U (mg protein)⁻¹]. It was also remarkably sulfide-tolerant, being capable of growth in the presence of up to 50 mM sulfide at pH 10. No respiration was observed in washed cells grown in the presence of air and no anaerobic growth was noted with lactate and nitrate, arsenate or selenate/selenite as electron acceptors. The final fermentation products from glucose were ethanol, acetate and formate. Strain MS 6^T was an obligate alkaliphile with

a relatively low salt tolerance. Nitrogenase activity in washed cells showed a similar pH/salt dependence to that observed in growing cells, except that a lower salt tolerance was found (Fig. 1).

Genomic DNA was isolated according to the method of Marmur (1961). Determination of the G + C content of the DNA and DNA–DNA hybridization experiments were performed with the thermal denaturation/reassociation technique (Marmur & Doty, 1962; De Ley *et al.*, 1970). The 16S rRNA genes were amplified using the general bacterial primers 11F–1492R (Lane, 1991). The 470 kb fragment of the *nifH* gene was amplified with a specially designed and previously tested primer pair F1/R6 (5'-TAYGGIAARGGIGGIATIGGIAARTC-3')/5'-GCCATCAT YTCICC-IGA-3') (Marusina *et al.*, 2001). The PCR products were purified from low-melting point agarose using the Wizard PCR-Prep kit (Promega). Sequencing was performed using Big Dye Terminator v.3.1 sequencing

reaction kit with an ABI 3730 DNA automatic sequencer (Applied Biosystems). Preliminary phylogenetic analysis of the new sequences was performed with the NCBI BLAST server (<http://www.ncbi.nlm.nih.gov/BLAST/>). The nucleotide and inferred amino acid sequences were aligned with sequences from the GenBank using CLUSTAL W. Phylogenetic trees were reconstructed with four different algorithms using the TREECON W (Van de Peer & De Wachter, 1994) and PHYLIP 3.5c (Felsenstein, 1993) software packages. Pairwise evolutionary distances (expressed as estimated changes per 100 nucleotides) were computed by using the Jukes & Cantor method. The tree topology was very similar in all types of tree, one of the final results was represented by a phylogenetic tree constructed by the neighbour-joining method. An extended version of the phylogenetic tree is available as Supplementary Fig. S2 in IJSEM Online. Bootstrap analysis (1000 replications) was used to validate the reproducibility of the branching patterns of the trees.

Phylogenetic analysis based on sequencing of the 16S rRNA gene placed strain MS 6^T in the genus *Bacillus*, in RNA group 6, a subgroup separated from RNA group 1 to accommodate mainly alkaliphilic *Bacillus* species (Nielsen *et al.*, 1994; Yumoto, 2002). The closest culturable relative, with 97 % gene sequence similarity, was *Bacillus arseniciselenatis* (Fig. 2a). *B. arseniciselenatis* was described as an obligately anaerobic haloalkaliphile from Mono Lake that respire arsenate and selenate (Switzer Blum *et al.*, 1998). In addition to a clearly distinct physiology, DNA–DNA hybridization between the novel strain and *B. arseniciselenatis* confirmed that strain MS 6^T was a separate species (55 % relatedness). Furthermore, all diazotrophic members of the bacilli are currently accommodated within the genus *Paenibacillus* (Achouak *et al.*, 1999). Therefore, it is concluded that strain MS 6^T represents a novel lineage of diazotrophs within the Gram-positive bacteria with a low G + C content. This was further substantiated by the analysis of the *nifH* gene, which was detected in both strain MS 6^T and *B. arseniciselenatis*. The sequences obtained were clearly different from the known *nifH* gene sequences available in GenBank and they formed an independent lineage within the *nifH* gene family cluster I related to the genus *Paenibacillus* (Fig. 2b). Whether *B. arseniciselenatis* is actually able to fix N₂ remains to be tested.

Chemotaxonomic analyses of cells of the novel strain, grown at pH 10, 0.6 M Na⁺ and 30 °C, included determination of the cellular fatty acid profile (Zhilina *et al.*, 1997) and analysis of isoprenoid lipoquinones (Tindall, 1996) and the cell-wall peptidoglycan (Streshinskaia *et al.*, 1979). The dominant fatty acids included C_{16:0} (30.7 %), C_{16:1ω7} (29 %), anteiso-C_{15:0} (12.6 %) and C_{14:0} (10.3 %). A relatively high content of unsaturated C_{16:1ω} is certainly unusual for bacilli (Kämpfer, 2002). However, the polar lipid composition might be significantly influenced by the extreme conditions, particularly by the high pH and salt. Therefore, a direct comparison with non-extremophilic relatives is not always possible. MK-7 was detected as the

Fig. 1. Influence of pH at 0.6 M Na⁺ (a) and Na⁺ at pH 10 (b) on fermentative growth (open circles) and nitrogenase activity of washed cells (closed circles) of strain MS 6^T with glucose as substrate. The values are expressed as a percentage of the maximal (100 %) growth rate (for growth experiments) or of maximal nitrogenase activity (for washed cells experiments).

Fig. 2. Phylogenetic position among the closest neighbour taxa based on 16S rRNA gene sequence analysis (a) and inferred amino acid sequence analysis of the *nifH* gene (b) of the diazotrophic haloalkaliphilic isolate MS 6^T and *Bacillus arseniciselenatis* E1H^T (nucleotide sequence determined in this study). Tree topology and evolutionary distances were determined by the neighbour-joining method with Jukes & Cantor corrections. Numbers at the nodes indicate the percentage of bootstrap values for the clade of this group in 1000 replications. Bars, substitutions per 100 nucleotides.

dominant isoprenoid quinone and MK-8 was found as a minor component (8%). *meso*-Diaminopimelic acid was the diagnostic diamino acid present in the cell-wall peptidoglycan.

Based on phenotypic and genetic comparisons, the new haloalkaliphilic diazotrophic member of the *Bacillus* RNA group 6 isolated from soda soil is proposed to represent a novel species, *Bacillus alkalidiazotrophicus* sp. nov. A

Table 1. Comparative characteristics of strain MS 6^T and related species of the genus *Bacillus*

Strains: 1, MS 6^T; 2, *B. arseniciselenatis* E1H^T (data from Switzer-Blum *et al.*, 1998); 3, *B. macyae* DSM 16346^T (Santini *et al.*, 2004); 4, *B. pseudofirmus* DSM 8715^T (Nielsen *et al.*, 1995). +, Positive; –, negative; ND, no data.

Characteristic	1	2	3	4
Motility	+	–	+	+
Aerobic growth	–	–	–	+
Fermentative growth	+	+	–	–
Growth at:				
pH 7	–	+	+	+
pH 10	+	+	–	+
43 °C	+	ND	–	+
10 % NaCl	–	+	–	+
Anaerobic growth with:				
Arsenate	–	+	+	ND
Catalase	+	+	+	+
Diazotrophy	+	ND (<i>nifH</i> present)	ND	ND
Nitrate	–	+	+	ND
Oxidase	–	+	–	+
Selenate	–	+	–	ND
Hydrolysis of polymers	Starch, glycogen	Starch	Starch, gelatin, casein	Starch, gelatin
Utilization of pentoses	–	–	–	+
DNA G+C content (mol%)	37.1 (<i>T_m</i>)	ND	37.0 (<i>T_m</i>)	39.0 (<i>T_m</i>)
Habitat	Soda soil	Soda lake	Gold mine	Soil

comparison of the phenotypic characteristics of the new strain with its closest phylogenetic relatives is presented in Table 1.

Description of *Bacillus alkalidiazotrophicus* sp. nov.

Bacillus alkalidiazotrophicus [al.ka.li.di.a.zo.tro'phi.cus. N.L. n. *alkali* (from Arabic *al qaliy* soda ash); N.L. masc. adj. *diazotrophicus* feeding on dinitrogen; N.L. adj. *alkalidiazotrophicus* alkaliphile fixing dinitrogen].

Cells are large Gram-positive rods with tapered edges, 0.6–1.2 × 3–8 µm, and are highly motile with peritrichous flagella. Strictly fermentative, aerotolerant anaerobe. The following carbohydrates can be fermented: glucose, fructose, maltose, sucrose, melibiose, melezitose, cellobiose, raffinose, trehalose, mannitol and lactose (weakly). Also capable of hydrolysis and utilization of starch and glycogen. Diazotrophic. Catalase-positive, oxidase-negative, cytochromes are absent. Nitrate is not reduced. Obligate alkaliphile with a pH range for growth between 7.8 and 10.6 (optimum is 9.5) and a salt range from 0.1 to 1.2 M Na⁺ (optimum is 0.2–0.4 M). Mesophilic with a temperature range for growth between 15 and 43 °C (optimum at 33–35 °C). The dominant fatty acids include C_{16:0}, C_{16:1ω7}, anteiso-C_{15:0} and C_{14:0}. The dominant isoprenoid quinone is MK-7. The cell-wall peptidoglycan contains meso-diaminopimelic acid as the diagnostic diamino acid.

The type strain, MS 6^T (=NCCB 100213^T=UNIQEM U377^T), was isolated from soda solonchak soil in

Mongolia. The DNA G+C content of the type strain is 37.1 mol% (*T_m*).

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