Caldalkalibacillus thermarum gen. nov., sp. nov., a novel alkalithermophilic bacterium from a hot spring in China

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A thermophilic, alkaliphilic and catalase-positive bacterium, designated strain HA6ᵀ, was isolated from a hot spring in China. The strain was aerobic and chemo-organotrophic and grew optimally at 60 °C, pH 8.5–9 and 1·5 % (w/v) NaCl. The cells were Gram-positive rods, forming single terminal endospores. The predominant cellular fatty acids were iso-C₁₅ : 0 and iso-C₁₇ : 0. The cell-wall peptidoglycan contained meso-diaminopimelic acid. The genomic DNA G+C content was 45·2 mol%. Phylogenetic analysis based on the 16S rRNA gene sequence revealed that strain HA6ᵀ formed a distinct lineage within the family Bacillaceae and was most closely related to Bacillus horti K13ᵀ and Bacillus smithii DSM 4216ᵀ, with sequence similarities of 91·8 and 93·1 %, respectively. On the basis of its physiological and molecular properties, strain HA6ᵀ should be placed in a novel genus and species, for which the name Caldalkalibacillus thermarum gen. nov., sp. nov. is proposed. The type strain of Caldalkalibacillus thermarum is strain HA6ᵀ (= CGMCC 1.4242ᵀ = JCM 13486ᵀ).

Alkalithermophiles are extremophiles that are adapted to two extreme conditions – a combination of alkaline and thermophilic growth conditions. These organisms are of interest for both fundamental and applied sciences, since they are potential sources of industrially valuable enzymes and represent interesting models for physiological studies (Wiegel, 1998; Wiegel & Kevbrin, 2004). Alkaliphilic and thermophilic catalases, for example, have become industrially important enzymes because of their extensive use in industrial processes in the food, dairy, textile and pulp and paper industries (Gudelj et al., 2001; Thompson et al., 2003). Aerobic alkalithermophiles are potentially good sources of alkaliphilic and thermophilic catalases.

Alkalithermophilic bacteria have been isolated from a variety of environments, including some mesophilic ones. The majority of aerobic alkaliphilic and thermophilic species described belong to the genus Bacillus and related genera arising from the splitting of the genus Bacillus (Demharter & Hensel, 1989; Pikuta et al., 2000; Wiegel & Kevbrin, 2004). During the course of our search for aerobic alkaliphilic and thermophilic bacteria, strain HA6ᵀ was isolated from an alkaline hot spring. This strain is able to produce relatively high levels of catalase activity, optimally at pH 10 and 60–65 °C. Here we present the characterization and taxonomy of strain HA6ᵀ, and its assignment to a new genus.

Strain HA6ᵀ was isolated from water samples taken from an alkaline hot spring (80 °C, pH 9·0), Drumbeat Spring in Rehai Park in the Tengchong area of China. Enrichment and isolation were done at 60 °C on medium A containing the following (g l⁻¹): NH₄NO₃, 1·3; K₂HPO₄, 0·28; MgSO₄·7H₂O, 0·25; CaCl₂·2H₂O, 0·075; NaCl, 5·0; yeast extract (Oxoid), 1; yeast extract (Oxoid), 1; Na₂CO₃, 5·0. The concentrated carbonate stock solution was sterilized separately and added to the medium prior to incubation. The final pH (measured at 25 °C) was 9·0 (pH 8·5 at 60 °C). Solid media contained

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain HA6ᵀ is AY753654.
2.0% (w/v) agar. The isolate was routinely grown aerobically at 60 °C on modified medium A (MMA) containing (1 g) 5.0 g yeast extract, 5.0 g tryptone and 15 g NaCl. On MMA at 60 °C, strain HA6T formed yellow–white, circular, translucent colonies.

Cell morphology was observed by using light microscopy and scanning electron microscopy. Gram staining was used to determine the cell-wall structure, in parallel with KOH testing (Gregersen, 1978). General physiological and biochemical tests (including the Voges–Proskauer reaction, the methyl red test, tests for H2S production, indole production, nitrate reduction and biopolymer hydrolysis and tests for catalase, oxidase, urease and phosphatase activity) were performed as described previously (Smibert & Krieg, 1981). Unless otherwise indicated, all tests were performed in triplicate in media containing 1 g tryptone l−1 and 1 g yeast extract l−1. Substrate utilization was tested in MMA containing 0.2% (w/v) Na2CO3 and incubated at 60 °C. The NaCl concentrations in the range 0–10% (w/v) (0.5% increments) were tested in medium at 60 °C and pH 8.5. The pH values tested were in the range 7.0–10.5 (at 60 °C, with increments of 0.5 pH units). Temperatures ranging from 35 to 75 °C were tested (at pH 8.5, with 5 °C increments). The pH of the MMA was adjusted prior to inoculation with concentrated Na2CO3 and was measured at 60 °C with a model PB-10 pH meter (Sartorius) equipped with a combination pH electrode and temperature probe. Growth was monitored by assessing the turbidity as OD600. Substrate utilization was tested in MMA containing 1 g tryptone l−1 and 1 g yeast extract l−1. Substrates in sterile stock solutions were each added to the medium at a final concentration of 20 mM, except for organic acids (0.2%, w/v). Control cultures were grown without any substrate additions. Growth of the third subculture was determined, after two transfers of culture material under the same conditions.

Isoprenoid quinones, extracted and purified from freeze-dried cells using the method of Collins (1985), were analysed by HPLC. The peptidoglycan composition was analysed by one-dimensional chromatography as described by Schleifer (1985), using cellulose thin-layer plates. Cellular fatty acids were determined at the DSMZ (German Collection of Micro-organisms, Braunschweig, Germany). Genomic DNA was extracted by a method described previously (Pitcher et al., 1989). The G + C content of the genomic DNA was determined by the thermal denaturation method, according to Marmur & Doty (1962). The methods used for PCR amplification of the 16S rRNA gene, sequencing of the PCR products and determination of the phylogenetic position were described by Zhang et al. (2002).

The physiological, biochemical and morphological characteristics of strain HA6T are given in the genus and species descriptions and in Table 1. Strain HA6T was found to be a moderately thermophilic, spore-forming bacillus (Fig. 1). Growth was observed at temperatures of 45–65 °C. The strain grew in liquid MMA at pH 7.5–9.5 at 60 °C, but did not grow without Na2CO3 (pH60 7.2), which means that the novel isolate is obligately alkaliphilic. The strain grew with NaCl at concentrations in the range 0–6% (w/v); only

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<td>DNA G+C content (mol%)</td>
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<td>40–41</td>
<td>38–41</td>
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<td>49–52</td>
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</table>
weak growth was observed on MMA agar with 0.5 % (w/v) K₂CO₃ in the absence of NaCl or Na₂CO₃. In the test for glucose oxidation, no gas was visualized in inverted Durham tubes, but weak acidification of glucose was detected. The pH of cultures after growth decreased by 0.1-0.2 pH units relative to the control. This pH change may have resulted from the absorption by the bicarbonate buffer of CO₂ produced by the culture. Further studies will be necessary to clarify the products of glucose oxidation by strain HA6ᵀ.

The chemotaxonomic features of strain HA6ᵀ were typical of those of members of the family *Bacillaceae*. The major isoprenoid quinone of strain HA6ᵀ was MK-7. The cell wall contained *meso*-diaminopimelic acid as the diagnostic diamino acid. The main cellular fatty acids of strain HA6ᵀ were iso-C₁₅:₀ (33·8 %), anteiso-C₁₅:₀ (6·9 %), iso-C₁₆:₀ (7·6 %), C₁₆:₀ (2·95 %), iso-C₁₇:₀ (35·5 %) and anteiso-C₁₇:₀ (9·9 %). The genomic DNA G+C content was 45·2 mol%.

The 16S rRNA gene sequence (1485 bp) of strain HA6ᵀ was determined and compared with sequences available in the EMBL database by using the FASTA3 program (Pearson & Lipman, 1988). The 16S rRNA gene sequence similarity, calculated using FASTA3 at the EBI, and the phylogenetic tree, constructed using the neighbour-joining method in the TREECONW software, showed that strain HA6ᵀ was phylogenetically related to members of the family *Bacillaceae*. Pairwise similarity values for strain HA6ᵀ with respect to taxa with validly published names were as follows: 93·1 % with thermophilic *Bacillus smithii* DSM 4216ᵀ, 91·8 % with alkaliphilic *Bacillus horti* K13ᵀ, 90·4 % with alkalithermo-philic *Bacillus thermoclaoae* DSM 5250ᵀ and 91·3-91·7 % with members of the genus *Geobacillus*. Sequence similarities of less than 91·0 % were found with respect to other species of the *Bacillaceae* with validly published names. On the phylogenetic tree (Fig. 2), strain HA6ᵀ is located close to the species of the genus *Bacillus* and other related genera, namely *Geobacillus* and *Anoxybacillus*. Its position occurs on a separate lineage within the family *Bacillaceae*. Despite the presence of the short branch of *B. smithii* giving the higher sequence-similarity value, there is no bootstrap support for this relationship. *B. smithii* belongs to a different cluster within the family *Bacillaceae*. However, strain HA6ᵀ clustered consistently with *B. horti* K13ᵀ and branched at the periphery of the other related groups. Other therophilic bacteria of *Bacillus* rRNA group 5 and alkaliphilic bacteria of *Bacillus* rRNA group 6 formed separate groups and were remotely related to strain HA6ᵀ. These data indicate that strain HA6ᵀ is phylogenetically distinct from the known species and genera of the family *Bacillaceae* and represents a novel genus and species.

This phylogenetic conclusion is further supported by phenotypic features. The differences in some features, such as optimal temperature and pH for growth, reduction of nitrate, hydrolysis of starch, acid production from glucose, the major menaquinone, fatty acid profiles and the genomic DNA G+C content, clearly differentiate strain HA6ᵀ from the phylogenetically related spore-forming taxa (Table 1). For example, strain HA6ᵀ is an obligate alkaliphile that does not grow at pH 7 and grows optimally at pH 8·5, whereas *B. smithii* DSM 4216ᵀ and *Geobacillus* *thermoasterophilus* DSM 22ᵀ are neutrophilic, showing no growth above pH 9. Strain HA6ᵀ is moderately therophilic, being unable to grow at 37 °C and growing optimally at 60 °C, whereas *B. horti* K13ᵀ, the closest species (on the basis of the 16S rRNA gene sequence), shows good growth at 37 °C and does not grow at 60 °C. Strain HA6ᵀ has only one predominant isoprenoid
An alkalithermophilic genus, *Anoxybacillus*, has already been described (Pikuta et al., 2000). However, strain HA6^T^ is clearly distinguishable from species of the genus *Anoxy-

bacillus*, as this genus was defined as being able to grow anaerobically and being able to produce acid from glucose fermentation, unlike strain HA6^T^.

An alkali-thermophilic genus, *Anoxybacillus*, has already been described (Pikuta et al., 2000). However, strain HA6^T^ is clearly distinguishable from species of the genus *Anoxybacillus*, as this genus was defined as being able to grow anaerobically and being able to produce acid from glucose fermentation, unlike strain HA6^T^.

It is known that the complete oxidation of organic compounds is a feature of complex ecosystems. Strain HA6^T^ was isolated from a hot spring, a habitat in which nutrients are limited and the substrate-mineralization roles of the various members of the microbial community are not well understood. The ability of the novel strain to grow on acetate suggests that this micro-organism may play a significant role in the complete oxidation of organic compounds within the hot-spring microbial community.

On the basis of its phenotypic and genotypic characteristics, strain HA6^T^ cannot be confidently assigned to any of the currently known spore-forming genera of the family Bacillaceae, and therefore represents a novel genus and species. The 16S rRNA gene sequence of strain HA6^T^, when compared with those available in the EMBL database, showed a very high level of similarity (99.5 %) with respect to strain TA1.A2, a bacterium isolated from a thermal spring in New Zealand and which has not been assigned to any existing species or given a validly published species name. Peddie et al. (1999) studied the bioenergetics of strain TA1.A2: it is also thermophilic and alkaliophilic, showing optimal growth at pH 9-2 and 70 °C. The different optima for pH and temperature for growth and the high sequence similarity may indicate that the two strains belong to different species of the same genus. Therefore, strain HA6^T^ should be placed in a novel genus and species, for which we propose the name *Caldalkalibacillus thermarum* gen. nov., sp. nov.

**Description of *Caldalkalibacillus thermarum* gen. nov.**

*Caldalkalibacillus* (Cal.dal.ka.bi.ba.ci.lus. L. adj. caldus hot; N.L. n. alkali alkali; L. n. bacillus small rod; N.L. masc. n. *Caldalkalibacillus* bacillus living under hot and alkaline conditions).

Cells are Gram-positive, non-motile, rod-shaped and produce terminal spherical endospores. Obligately aerobic, alkali-philic and thermophilic. Chemo-organotrophic. Catalase-positive. Cell-wall peptidoglycan contains meso-diaminopimelic acid. Major isoprenoid quinone is MK-7. The G+C content of the genomic DNA of the type species is 45.2 mol% (T_m). Predominant cellular fatty acids are iso-C_{15:0} and iso-C_{17:0}. Phylogenetically, the genus belongs to the family Bacillaceae. The type species is *Caldalkalibacillus thermarum*.

**Description of *Caldalkalibacillus thermarum* sp. nov.**

*Caldalkalibacillus thermarum* (ther.ma’rum. L. gen. pl. n. thermarum of warm springs).

Cells are non-motile and rod-shaped (width, 0-5 μm; length, 3-0-6-5 μm). Spherical endospores are formed terminally in swollen sporangia. Gram-positive and KOH-test-negative. Colonies are yellow–white, translucent, circular, smooth, low convex and entire. Strictly aerobic, moderately thermo-philic and obligately alkaliophilic. The temperature for growth is 45–65 °C, with an optimum at 60 °C. The pH range for growth is 7.5–10, with an optimum at pH 8.5. Growth occurs in the presence of NaCl at 0–6 % (w/v) and optimally at 1.5 % NaCl. Oxidase- and catalase-positive. Negative for nitrate reduction, nitrite reduction, the Voges–Proskauer test, the methyl red reaction and H_2S production. Hydrolysis of starch is weak. Aesculin, cellulose, pectin, chitin, casein, gelatin and Tween 80 are not hydrolysed. Phosphatase- and urease-negative. Positive for the production of indole and ammonia from tryptone. Grows on D-glucose, D-mannose, L-rhamnose, sucrose, D-trehalose, cellobiose, melibiose, D-melezitose, inulin, erthyritol, D-sorbitol, D-mannitol, glycerol, acetate, pyruvate, butyrate, citrate, succinate, galacturonic acid and glucuronic acid. Shows weak growth on D-lactose, D-raffinose and D-salicin. Shows no growth on D-fructose, D-galactose, L-sorbose, L-arabinose, D-ribose, D-xylose, maltose, glycopycen, adonitol, inositol, gluconic acid, formate, oxalate, propionate, malonate, isocitrate, ketoglutarate or malate. Cells are resistant to novobiocin and bacitracin, but susceptible to ampicillin, erythromycin, norfloxacin, neomycin, rifampicin, tetracycline, streptomycin, chloramphenicol, kanamycin and ciprofloxacin. The major cellular fatty acids are iso-C_{15:0} and iso-C_{17:0}. The main menaquinone type is MK-7. Cell-wall peptidoglycan contains meso-diaminopimelic acid. The G+C content of the genomic DNA of the type strain is 45.2 mol% (T_m).

The type strain, HA6^T^ (=CGMCC 1.4242^T^ =JCM 13486^T^), was isolated from an alkaline hot spring in Tengchong in China.

**Acknowledgements**

This work was supported by grants from the Ministry of Science and Technology of China (973 programs, 2004CB719605 and
2003CB716001), the Chinese Academy of Sciences (Knowledge Innovation Program, KSCX2-SW-33) and the European Commission (‘Multigenome Access Technology for Industrial Catalysts’, contract no. QLK3-CT-2002-01972).

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