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

Yanfen Xue,1 Xinqi Zhang,1 Cheng Zhou,1 Yueju Zhao,1 Don A. Cowan,2
Shaun Heaphy,3 William D. Grant,3 Brian E. Jones,4 Antonio Ventosa5
and Yanhe Ma1

Correspondence
Yanhe Ma
mayanhe@sun.im.ac.cn

1State Key laboratory of Microbial Resources, Institute of Microbiology, Chinese Academy of
Sciences, Beijing 100080, People’s Republic of China
2Department of Biotechnology, University of the Western Cape, Bellville 7535, South Africa
3Department of Infection, Immunity and Inflammation, University of Leicester, Leicester
LE1 9HN, UK
4Genencor International BV, Archimedesweg 30, 2333 CN Leiden, The Netherlands
5Department of Microbiology and Parasitology, Faculty of Pharmacy, University of Sevilla,
41012 Sevilla, Spain

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 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).

Aerobic 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 industri-
ally 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; tryptone (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 (1-1) 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 treatment to determine the cell-wall structure, in parallel with KOH treatment (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 0–10% (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 using 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 C 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

Table 1. Differential characteristics of strain HA6T and phylogenetically closely related spore-forming species

<table>
<thead>
<tr>
<th>Characteristic</th>
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<td>37°C</td>
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<td>Starch</td>
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<td>–</td>
<td>–</td>
<td>d</td>
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<td>Isoprenoid quinones(s)</td>
<td>MK-7</td>
<td>MK-7</td>
<td>MK-7</td>
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<td>MK-7</td>
<td>NA</td>
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<tr>
<td>DNA G+C content (mol%)</td>
<td>45-2</td>
<td>40-41</td>
<td>38-41</td>
<td>42-44</td>
<td>49-52</td>
<td>42</td>
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</table>
The 16S rRNA gene sequence (1485 bp) of strain HA6 T 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 HA6 T was phylogenetically related to members of the family Bacillaceae. Pairwise similarity values for strain HA6 T with respect to taxa with validly published names were as follows: 93·1 % with thermophilic Bacillus smithii DSM 4216 T, 91·8 % with alkaliphilic Bacillus horti K13 T, 90·4 % with alkalithermophilic Bacillus thermocloacae DSM 5250 T 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 HA6 T 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 HA6 T clustered consistently with B. horti K13 T and branched at the periphery of the other related groups. Other thermophilic bacteria of Bacillus RNA group 5 and alkaliphilic bacteria of Bacillus RNA group 6 formed separate groups and were remotely related to strain HA6 T. These data indicate that strain HA6 T 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 HA6 T from the phylogenetically related spore-forming taxa (Table 1). For example, strain HA6 T is an obligate alkaliphile that does not grow at pH 7 and grows optimally at pH 8·5, whereas B. smithii DSM 4216 T and Geobacillus stearothermophilus DSM 22 T are neutrophilic, showing no growth above pH 9. Strain HA6 T is moderately thermophilic, being unable to grow at 37 °C and growing optimally at 60 °C, whereas B. horti K13 T, 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 HA6 T has only one predominant isoprenoid quinone of strain HA6 T was MK-7. The cell wall contained meso-diaminopimelic acid as the diagnostic diamino acid. The main cellular fatty acids of strain HA6 T were iso-C15:0 (33·8 %), anteiso-C15:0 (6·9 %), iso-C16:0 (7·6 %), C16:0 (2·9 %), iso-C17:0 (35·5 %) and anteiso-C17:0 (9·9 %). The genomic DNA G+C content was 45·2 mol%.

The chemotaxonomic features of strain HA6 T were typical of those of members of the family Bacillaceae. The major isoprenoid quinone of strain HA6 T was MK-7. The cell wall contained meso-diaminopimelic acid as the diagnostic diamino acid. The main cellular fatty acids of strain HA6 T were iso-C15:0 (33·8 %), anteiso-C15:0 (6·9 %), iso-C16:0 (7·6 %), C16:0 (2·9 %), iso-C17:0 (35·5 %) and anteiso-C17:0 (9·9 %). The genomic DNA G+C content was 45·2 mol%.

### Fig. 1.
Scanning electron micrograph of strain HA6 T, showing the rod-shaped morphology. Bar, 5 μm.

### Fig. 2.
Neighbour-joining phylogenetic tree of strain HA6 T and other related taxa, based on 16S rRNA gene sequences available from GenBank. Bootstrap values >50 (based on 100 replications) are shown at branching points. Bar, 0·05 expected changes per site.

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 HA6 T.
An alkalithermophilic genus, *Anoxybacillus*, has already been described (Pikuta et al., 2000). However, strain HA6T 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 HA6T. In addition, there are significant differences in the cellular fatty acid compositions of strain HA6T and species of the genus *Anoxybacillus*. The iso-C17:0 content of the novel isolate, as a percentage of the total fatty acid content, is significantly higher than that in *Anoxybacillus* species.

It is known that the complete oxidation of organic compounds is a feature of complex ecosystems. Strain HA6T 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 HA6T 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 HA6T, 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 alkaliphilic, 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 HA6T 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* 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 thermophilic and obligately alkaliphilic. 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 H₂S 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, D-melibiose, D-melezitose, inulin, erythritol, D-sorbitol, D-mannitol, glycerol, acetate, lactate, pyruvate, butyrate, citrate, succinate, galacturon acid and glucuronic acid. Shows weak growth on D-lactose, D-ribofuranose and D-salicin. Shows no growth on D-fructose, D-galactose, L-sorbose, L-arabinose, D-ribose, D-xyllose, maltose, glycogen, 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-C₁₅:₀ and iso-C₁₇:₀. The major 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% (Tₘ). The type strain, HA6T (=CGMCC 1.4242T = JCM 13486T), was isolated from an alkaline hot spring in Tengchong in China.

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