Alicyclobacillus sendaiensis sp. nov., a novel acidophilic, slightly thermophilic species isolated from soil in Sendai, Japan

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An acidophilic, slightly thermophilic bacterium, designated strain NTAP-1^T, that produces a thermostable extracellular acid collagenase activity with potential industrial applications was isolated from soil of Aoba-yama Park, Sendai, Japan. The temperature range for growth was 40–65 °C, with an optimum at 55 °C, and the pH range for growth was 2.5–6.5, with an optimum at pH 5.5. Analysis of the 16S rDNA sequence of strain NTAP-1^T showed that it is most closely related to strains of the genus *Alicyclobacillus*. Consistently, the major constituents of the cell-membrane lipid of strain NTAP-1^T were ω -alicyclic fatty acids. However, DNA–DNA reassociation studies showed only low similarities (less than 33 %) to any type strain of *Alicyclobacillus*. On the basis of the phenotypic and genotypic properties, a novel species is proposed, *Alicyclobacillus sendaiensis* sp. nov., represented by strain NTAP-1^T (=JCM 11817^T = ATCC BAA-609^T).

Three thermoacidophilic species of the genus *Bacillus*, *Bacillus acidocaldarius*, *Bacillus acidoterrestris* and *Bacillus cycloheptanicus*, were reclassified into the genus *Alicyclobacillus* on the basis of the distinctive phylogenetic position of the organisms in relation to those of other *Bacillus* species, as well as their unique chemotaxonomic properties, in particular the occurrence of ω -alicyclic fatty acids as major fatty acid constituents of their cell-membrane lipids (Wisotzkey *et al.*, 1992). Recently, novel species of the genus *Alicyclobacillus* have also been proposed: *Alicyclobacillus hesperidum* (Albuquerque *et al.*, 2000) and *Alicyclobacillus herbarius* (Goto *et al.*, 2002).

During the course of our screening programme for thermostable ‘acid collagenase’ (which has potential applications in biotechnology), we isolated, from soil at Aoba-yama Park, Sendai, Miyagi, Japan, an acidophilic bacterium that produces an extracellular thermostable collagenase with an optimum pH for catalytic activity at 3.9 (Nakayama *et al.*, 2000). This bacterium, strain NTAP-1^T, was an aerobic, endospore-forming, rod-shaped bacterium that stained Gram-negative and was tentatively assigned as a strain of the genus *Bacillus*. In this study, we have carried out

extensive physiological, chemotaxonomic and phylogenetic analyses to show that strain NTAP-1^T represents a novel species of the genus *Alicyclobacillus*, which we have named *Alicyclobacillus sendaiensis* sp. nov.

Strain NTAP-1^T was isolated from soil using an agar medium (pH 4.8) containing 1.5 % gelatin, 0.01 % yeast extract, 0.85 % NaCl, 0.5 % KH₂PO₄ and 0.001 % MgSO₄·7H₂O at 60–70 °C (Nakayama *et al.*, 2000). The type strains *Alicyclobacillus acidocaldarius* DSM 446^T, *Alicyclobacillus acidoterrestris* DSM 3922^T, *Alicyclobacillus cycloheptanicus* DSM 4006^T and *A. hesperidum* DSM 12489^T were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany. Cells of strain NTAP-1^T were stored at –80 °C in broth cultures supplemented with 15 % (w/v) glycerol.

Bacterial growth was monitored for up to 7 days after inoculation by measuring the turbidity (600 nm) of cultures in 5 ml liquid *Bacillus acidocaldarius* medium (BAM; Deinhard *et al.*, 1987) at specified pH values (see below) in 25 ml metal-capped test tubes incubated in a water-bath incubator. For the measurement of turbidity, an uninoculated control was used as a blank. The growth temperature range of the organisms was examined at pH 4.0 between 23 and 75 °C in 5 °C steps. To determine the pH range for growth, the organisms were grown at 55 °C as described above except that the pH of the medium was adjusted to different values with 1.0 M H₂SO₄; all pH measurements

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The DDBJ accession number for the 16S rDNA sequence of strain NTAP-1^T is AB084128.

were performed at room temperature. The effects of NaCl (0, 2, 3, 4, 5, 7 and 9%, w/v) on growth were examined at pH 5.5 and 55 °C. Anaerobic growth was tested using incubation at 55 °C in 10 ml rubber-sealed screw-cap tubes containing liquid BAM (pH 5.5, 9 ml) covered with liquid paraffin.

Cell morphology was determined using light microscopy during the exponential growth phase in BAM (pH 5.5). Sporulation was observed by using phase-contrast microscopy with cells grown to stationary phase. Gram staining was performed using exponentially growing cells according to Hucker's modification (Cowan & Steel, 1965), with reagents produced by Nacalai Tesque. Flagellation was examined using Leifson's method (Cowan & Steel, 1965). The following physiological tests were carried out as described previously (Albuquerque *et al.*, 2000): catalase and oxidase reactions, the Voges-Proskauer reaction, the oxidation/fermentation test and tests for H₂S production, nitrate reduction, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase, β -galactosidase, gelatinase and urease. Acid production from carbon sources (see Table 2) was examined using API 50 CH test strips (bioMérieux) and BAM (pH 4.0) without glucose. Cells were resuspended in BAM without glucose at a cell density corresponding to tube no. 2 of the McFarland series of standard opacities (Cowan & Steel, 1965). Cell suspensions (200 μ l) were added to API 50 CH test-strip wells as recommended by the manufacturer and incubated at 55 °C. Acidification was observed every day for 7 days by measuring the pH of cultures, using a compact pH meter (model B-212; Horiba). Medium showing a pH drop of more than 0.5 pH units, relative to a control, was regarded as positive with respect to acid production. Cultures for analysis of cellular fatty acids, isoprenoid quinones and cell-wall diamino acids were grown in 2 l Erlenmeyer flasks containing 1 l BAM at pH 5.5 and 55 °C in a reciprocal shaker until the exponential phase of growth. Analyses for cellular fatty acids, isoprenoid quinones and cell-wall diamino acids were carried out as described by Komagata & Suzuki (1987).

Isolation and purification of chromosomal DNA and estimation of DNA base composition by HPLC were performed according to Tamaoka & Komagata (1984). DNA reassociation values were determined as described by Ezaki *et al.* (1989). Briefly, probes for DNA hybridization were prepared from cells of *A. acidocaldarius* DSM 446^T, *A. acidoterrestris* DSM 3922^T, *A. cycloheptanicus* DSM 4006^T and *A. hesperidum* DSM 12489^T. Probe DNAs were biotinylated with photobiotin and hybridized with single-stranded unlabelled chromosomal DNA fragments of strain NTAP-1^T. Hybridized DNA fragments were visualized using alkaline phosphatase colour-detection methods (Ezaki *et al.*, 1989). 16S rDNA was amplified by a PCR (Weisburg *et al.*, 1991) with the universal bacterial primers 10F (5'-AGTTTGSTCCTGGCTC-3') and 1500R (5'-GGTACCTTGTTACGA-3'). PCR products were purified with

Fig. 1. Phase-contrast micrograph of sporulating cells of *Alicyclobacillus sendaiensis* sp. nov. Cells were cultured on liquid BAM for 3 days at 55 °C.

MicroSpin columns S-400 HR (Amersham Biosciences) and sequenced using a CEQ2000XL DNA Analysis system (Beckman Coulter). Previously published 16S rDNA sequences were obtained from the GenBank/EMBL/DDBJ databases (see Fig. 2). Multiple alignment of sequences, calculation of nucleotide substitution rates (K_{nuc} values; Kimura, 1980), construction of a neighbour-joining phylogenetic tree (Saitou & Nei, 1987) and bootstrap analysis with 1000 replicates for the evaluation of phylogenetic tree topology (Felsenstein, 1985) were carried out using CLUSTAL W version 1.81.

Cells of strain NTAP-1^T are rod-shaped, 2–3 μ m long and 0.8 μ m in diameter. Cells stained Gram-negative and were non-motile. Ellipsoid endospores were produced (Fig. 1). Colonies of strain NTAP-1^T formed on BAM were circular,

Fig. 2. Phylogenetic relationships of *Alicyclobacillus* species and some aerobic, rod-shaped, endospore-forming bacteria, based on 16 rRNA gene sequences. GenBank/EMBL/DDBJ accession numbers are shown in parentheses. The branching pattern was generated by the neighbour-joining method. Numbers indicate bootstrap percentages greater than 90%. Bar, 0.01 nucleotide substitution per site.

convex, white and semi-transparent, 1 mm in diameter after 30 h growth at 55 °C.

A 1438-nt stretch of the 16S rDNA sequence, representing 93 % of the *Escherichia coli* 16S rDNA sequence, was determined and compared with available 16S rDNA sequences to construct a phylogenetic tree (Fig. 2). The results clearly showed that strain NTAP-1^T falls within the radiation of the genus *Alicyclobacillus*. Similarity values between the 16S rDNA sequence of strain NTAP-1^T and those of *A. acidoterrestris* DSM 3923^T, *A. acidocaldarius* DSM 11297^T, *A. hesperidum* DSM 12766^T, *A. cycloheptanicus* DSM 4006^T, *A. herbarius* DSM 13609^T and *Bacillus tusciae* DSM 2912^T were respectively 98.2, 97.9, 95.1, 93.5, 92.4 and 89.2 %. The G + C content of strain NTAP-1^T was determined, by HPLC, to be 62.3 mol%, which is higher than the values determined in this laboratory for *A. acidoterrestris* DSM 3922^T (51.5 mol%), *A. cycloheptanicus* DSM 4006^T (57.9 mol%) and *A. acidocaldarius* DSM 446^T (60.8 mol%). DNA–DNA reassociation studies of strain NTAP-1^T were performed using type strains whose 16S rDNA sequences showed more than 95 % similarity to that of strain NTAP-1^T. Strain NTAP-1^T showed 33, 26 and 5 % DNA–DNA reassociation, respectively, with *A. acidocaldarius* DSM 446^T, *A. hesperidum* DSM 12489^T and *A. acidoterrestris* DSM 3922^T. These values are significantly lower than the threshold value (70 %) recommended for the delineation of a novel species. Thus, strain NTAP-1^T can be distinguished from representatives of all described species of *Alicyclobacillus*.

Table 1. Cellular fatty acid composition of strain NTAP-1^T and *Alicyclobacillus* strains

Strains: 1, NTAP-1^T; 2, *A. acidocaldarius* ATCC 27009^T; 3, *A. acidoterrestris* DSM 3923^T; 4, *A. cycloheptanicus* DAM 4006^T; 5, *A. herbarius* DSM 13609^T (data in columns 2–5 from Goto *et al.*, 2002); 6, *A. hesperidum* DSM 12489^T (data from Albuquerque *et al.*, 2000). Values are percentages of total fatty acids. TR, Trace.

Fatty acid	1	2	3	4	5	6
14:0				0.8	0.4	
15:0	0.2			0.6	TR	
15:0 iso	1.1	1.4	TR	0.5	0.4	5.4
15:0 anteiso	1.5		TR	TR	TR	6.6
16:0	4.9		2.5	2.4	5.1	2.1
16:0 iso	1.6	1.4		1.0	3.8	0.9
17:0	0.6				0.5	
17:0 iso	4.5	1.9	1.2	2.7	5.6	4.9
17:0 anteiso	10.5	2.3	4.2	1.3	3.0	10.3
18:0	0.9			1.3	2.4	
18:0 iso					3.2	
ω -Cyclohexane C _{17:0}	44.1	78.0	67.4			56.8
ω -Cyclohexane C _{19:0}	30.2	16.0	24.6			13.3
ω -Cycloheptane C _{18:0}				86.8	67.1	
ω -Cycloheptane (2-OH) C _{18:0}				2.3	3.5	
ω -Cycloheptane C _{20:0}				TR	4.5	

The fatty acid composition of strain NTAP-1^T is shown in Table 1. ω -Alicyclic fatty acids were the major cellular fatty acids, as is the case for strains of the genus *Alicyclobacillus*. Examination of the respiratory lipoquinone content of strain NTAP-1^T showed that menaquinones were the only respiratory lipoquinones detected, menaquinone-7 being the predominant type. Strain NTAP-1^T contained *meso*-diaminopimelic acid as the wall diamino acid.

Strain NTAP-1^T was able to grow in BAM at pH 2.5–6.5

Table 2. Phenotypic characteristics that differentiate species of the genus *Alicyclobacillus*

Strains: 1, NTAP-1^T; 2, *A. acidocaldarius* DSM 446^T; 3, *A. acidoterrestris* DSM 3922^T; 4, *A. hesperidum* DSM 12489^T; 5, *A. cycloheptanicus* DSM 4006^T (data in columns 1–5 from this study); 6, *A. herbarius* DSM 13609^T (data from Goto *et al.*, 2002). +, Positive; w, weakly positive; –, negative; NR, not reported. All strains were positive for acid production from D-ribose, D-xylose, D-glucose, D-fructose, D-mannose and mannitol. All were negative for acid production from L-xylose, adonitol, methyl β -xyloside, L-sorbose, dulcitol, N-acetyl-D-glucosamine, inulin, D-lyxose, D-tagatose, L-fucose, L-arabitol, gluconate and 2-ketogluconate.

Characteristic	1	2	3	4	5	6
Voges–Proskauer reaction	+	–	–	–	NR	–
Catalase activity	–	w	w	–	+	+
Acid production from:						
Glycerol	+	+	+	–	+	+
Erythritol	–	–	+	–	–	–
D-Arabinose	–	–	–	–	–	+
L-Arabinose	+	+	+	+	–	+
D-Galactose	+	+	+	+	–	+
Rhamnose	–	+	+	–	+	+
Inositol	–	–	+	–	+	–
D-Sorbitol	–	–	+	–	–	–
Methyl α -D-mannoside	–	–	–	–	–	+
Methyl α -D-glucoside	+	–	–	–	–	+
Amygdalin	–	–	–	–	–	+
Arbutin	+	–	+	–	–	+
Aesculin	–	–	–	–	–	+
Salicin	+	–	+	+	–	+
Cellobiose	+	+	+	+	–	+
Maltose	+	+	+	+	–	+
Lactose	+	+	+	–	–	+
Melibiose	–	+	–	–	–	+
Sucrose	+	+	–	+	–	+
Trehalose	+	+	–	+	–	+
Melezitose	–	–	–	–	–	+
D-Raffinose	+	+	–	–	–	+
Glycogen	+	–	–	+	–	–
Xylitol	–	–	+	–	+	–
Gentiobiose	–	–	+	–	+	+
D-Turanose	+	+	–	–	–	+
D-Arabitol	–	–	+	–	–	–
D-Fucose	–	–	–	–	–	+
5-Ketogluconate	–	–	–	–	+	+

(at 55 °C) or at 40–65 °C (at pH 4.0). The optimum pH and temperature values for growth were pH 5.5 and 55 °C. Anaerobic growth did not occur. The organism grew in the presence of 4 % (w/v) NaCl; however, no growth was observed at NaCl concentrations of 5 % or higher. NTAP-1^T was positive for the Voges–Proskauer reaction, nitrate reduction and gelatinase activity, but negative for the oxidation/fermentation test and tests for H₂S production, catalase, oxidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase, β -galactosidase and urease. Acid production from various carbon sources is summarized in Table 2. This acid-production pattern also distinguishes strain NTAP-1^T from known species of *Alicyclobacillus*.

Description of *Alicyclobacillus sendaiensis* sp. nov.

Alicyclobacillus sendaiensis (sen.dai.en'sis. N.L. masc. adj. *sendaiensis* of Sendai, a city in Miyagi Prefecture, Japan, where the type strain was isolated).

Rod-shaped, endospore-forming, strictly aerobic organism. Cells stain Gram-negative. Round spores lie terminally in swollen sporangia. The rods measure 2–3 $\times$ 0.8 μ m. Cell wall contains *meso*-diaminopimelic acid. The predominant isoprenoid quinone is menaquinone-7. Major fatty acid components are ω -alicyclic acids (of the ω -cyclohexyl type). The temperature range for growth is 40–65 °C (optimum 55 °C). The pH range for growth is 2.5–6.5 (optimum pH 5.5). Grows in the presence of 4 % (w/v) NaCl in BAM. Positive in the Voges–Proskauer reaction and the nitrate-reduction test, but negative in the oxidation/fermentation test and for H₂S production, catalase, oxidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase, β -galactosidase and urease. The pattern of acid production is shown in Table 2. The G + C content of the DNA of the type strain is 62.3 mol%.

The type strain, strain NTAP-1^T (=JCM 11817^T=ATCC BAA-609^T), was isolated from soil of Aoba-yama Park, Sendai, Japan.

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