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Keiichi Goto · Yasuhide Tanimoto · Takashi Tamura
Kaoru Mochida · Daisuke Arai · Mika Asahara
Masayuki Suzuki · Hidehiko Tanaka · Kenji Inagaki

Identification of thermoacidophilic bacteria and a new *Alicyclobacillus* genomic species isolated from acidic environments in Japan

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Abstract Sixty strains of thermoacidophilic bacteria have been isolated from soil and water samples obtained from various acidic environments in Japan. An initial comparative sequence analysis of the hypervariable regions of the 16S rDNA revealed that all strains could be assigned to the *Alicyclobacillus acidocaldarius–Alicyclobacillus* genomic species 1 group, which could be further subdivided into three clusters (Clusters I–III). On the basis of phenotypic characteristics, chemotaxonomic profiles, and phylogenetic data of six selected strains, five strains were identified as either *A. acidocaldarius* or *Alicyclobacillus* genomic species 1; however, one strain (MIH 332) could not be determined to belong to either of these species. 16S rDNA sequence homology values between strain MIH 332 and the reference strains of *A. acidocaldarius* (ATCC 27009^T) and *Alicyclobacillus* genomic species 1 (DSM 11984) were 98.8% and 99.1%, respectively, which were higher than the corresponding similarity between the reference strains (98.4%). On the other hand, DNA–DNA hybridization levels between strain MIH 332 and the reference strains were 39% and 44%, respectively, which were lower than the value between the reference strains (59% or 65%). However, the phenotype of strain MIH 332 was also similar to those of the reference strains, and a typical phenotype could not be found for the strain, thus indicating that the strain may be a new genomic species of *A. acidocaldarius*, for which the name *Alicyclobacillus* genomic species 2 is tentatively proposed. The results of this study suggest that *A. acidocaldarius* and its related species are widely distributed in acidic environments in Japan, with slight regional variations in morphological and genotypic characteristics.

Key words Identification · *Alicyclobacillus* · 16S rDNA · HV region · *Alicyclobacillus* genomic species 2 · Thermoacidophile · Distribution

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K. Goto (✉) · K. Mochida · D. Arai · M. Asahara
Microbiological and Analytical Group, Food Research Laboratories, Mitsui Norin Co. Ltd., 223-1 Miyahara, Fujieda, Shizuoka 426-0133, Japan
Tel. +81-54-6390080; Fax +81-54-6482001
e-mail: kgoto@mnk.co.jp

Y. Tanimoto · T. Tamura · H. Tanaka · K. Inagaki
Department of Bioresources Chemistry, Faculty of Agriculture, Okayama University, Okayama, Japan

M. Suzuki
Central Research Laboratories, Tokyo Food Techno Co. Ltd., Shizuoka, Japan

Introduction

The alicyclobacilli are thermoacidophilic, Gram-positive, rod-shaped, aerobic microorganisms that possess unique fatty acids (ω-cyclohexane or ω-cycloheptane fatty acids) as the major components of the cellular membrane. Prior to 1992, the three thermoacidophilic species were classified within the genus *Bacillus*, and named *B. acidocaldarius*, *B. acidoterrestris*, and *B. cycloheptanicus* (Darland and Brock 1971; Deinhard et al. 1987a, 1987b). However, comparative analysis of the 16S rRNA gene sequences and cellular fatty acid profiles led to the separation of these three species from the genus *Bacillus* and their reassignment to the new genus *Alicyclobacillus* (Wisotzkey et al. 1992). Recently, *A. hesperidum* and *Alicyclobacillus* genomic species 1 have been reported as new species of *Alicyclobacillus* (Albuquerque et al. 2000) along with the three species described previously: *A. acidocaldarius*, *A. acidoterrestris*, and *A. cycloheptanicus*. Also, novel thermoacidophilic bacteria have been isolated from soft drinks, or the raw material thereof, and have been reported as new species of *Alicyclobacillus*: *A. acidiphilus* (Matsubara et al. 2002) and *A. herbarius* (Goto et al. 2002a).

The organisms of genus *Alicyclobacillus* have been isolated from a range of habitats and substrates, including geothermal sites. The geothermal habitats are represented by water and sediments of thermal aquifers, sediments of creeks and small rivers, humid soil in fumarole zones, and submarine hot springs (Uchino and Doi 1967; Darland and Brock 1971; Nicolaus et al. 1998; Atkinson et al. 2000).

Nongeothermal substrates, such as soils, organic compost, manure, fruit, and heat-processed foods, are also natural sources of *Alicyclobacillus* (Deinhard et al. 1987a, b; Yamazaki et al. 1996; Pettipher et al. 1997; Albuquerque et al. 2000; Goto 2000; Jensen 2000; Walls and Chuyate 2000; Niwa 2001; Goto et al. 2002a; Matsubara et al. 2002). The presence of *Alicyclobacillus* in these habitats has been reported by many researchers in different parts of the world, including Australia, Brazil, Italy, Japan, Russia, and the United States.

Previously, 60 strains of thermoacidophilic bacteria were isolated from various acidic environments in Japan (Tanimoto et al. 1996), with three strains of these isolates characterized by molecular methods as new genomic species of the genus *Alicyclobacillus* (Hiraishi et al. 1997). As a continuation of this research, we report in detail on the properties of these 60 isolates on the basis of a polyphasic taxonomic analysis. The results suggest that the species *A. acidocaldarius* includes a more representative genomic species (*Alicyclobacillus* genomic species 2) than *Alicyclobacillus* genomic species 1, and that these species, which also show phenotypic variation among strains, are widely distributed in acidic environments in Japan.

Materials and methods

Strains and culture conditions

The 60 thermoacidophilic bacteria isolates (Tanimoto et al. 1996) and their sources are listed in Table 1. Reference strains were obtained from the American Type Culture Collection (ATCC, Manassas, Virginia, USA), the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM), (Braunschweig, Germany), and the Institute of Molecular and Cellular Biosciences (IAM, University of Tokyo, Tokyo, Japan). *A. acidiphilus* TA 67^T was kindly provided by Motohiro Niwa (Kirin Beverage Corporation, Samukawa-machi, Kanagawa, Japan).

Bacillus acidocaldarius medium (BAM medium) was used as the basal growth medium for isolates and reference strains (Deinhard et al. 1987a). For the fatty acid analysis, a semisynthetic medium was used for cultivation of these strains (Darland and Brock 1971). All organisms were grown aerobically at 50° or 60°C.

Morphological, physiological, and biochemical characteristics

Unless otherwise stated, all biochemical tests were performed as described previously (Darland and Brock 1971; Deinhard et al. 1987a, b; Albuquerque et al. 2000) in BAM liquid medium or on BAM agar. The growth temperature range was examined by measuring the turbidity at 578 nm. The pH range for growth was examined at 50° or 60°C in BAM medium with different pH values adjusted with 2 N H_2SO_4. Acidification was examined with API 50 CH test strips (bioMerieux) in BAM basal salts medium (Albuquerque et al. 2000) at the optimum growth temperature for each strain.

Chemotaxonomic characterization

The cultures used for quinone analysis were grown in 500-ml Erlenmeyer flasks containing 100 ml BAM medium at 60°C on a reciprocating shaker for 4 days. Quinones were extracted from freeze-dried cells as described previously (Sano et al. 1996) and analyzed by using an Alliance HPLC system equipped with a 996 photodiode array detector (Waters). A Mightysil RP-18 column (4.6 × 250 mm, Kanto Chemical) was used for separation and methanol:isopropanol (3:1) was used as the mobile phase. UV spectra of the peaks were used for the identification of quinones.

Table 1. Strains and their sources

Source	Location	Samples	Strains
Soil	Iozan, Hokkaido Pref.	1	
Soil, water	Lake Goshiki, Hokkaido Pref.	1	7 (FCS strain)
Soil, water	Norikura, Nagano Pref.	7	
Soil, water	Arashiyama, Kyoto Pref.	3	
Soil	Yubara, Okayama Pref.	1	
Soil, water	Yanahara, Okayama Pref.	10	
Soil	Okayama Univ., Okayama Pref.	20	
Soil	Yuba, Okayama Pref.	1	
Soil	Sauna bath, Okayama Pref.	1	
Activated sludge	Treatment plant, Okayama Pref.	3	6 (EMG strain)
Soil, water	Shionoe, Kagawa Pref.	5	
Soil, water	Unzen, Nagasaki Pref.	25	1 (UZ strain)
Soil, water	Minami Aso, Kumamoto Pref.	10	10 (SAS strain)
Soil, water	Mt. Aso, Kumamoto Pref.	3	
Soil, water	Amagase, Oita Pref.	7	
Soil, water	Sujiyu, Oita Pref.	29	27 (KH strain)
Soil, water	Ebino, Miyazaki Pref.	6	
Soil, water	Kirishima, Kagoshima Pref.	15	9 (MIH strain)

Cultures for fatty acid analysis were grown in 500-ml Erlenmeyer flasks containing 100 ml semisynthetic medium at 60°C on a reciprocating shaker for 4 days. Fatty acid methyl esters were obtained from fresh wet biomass by saponification, methylation, and extraction as described previously (Bligh and Dyer 1959; Metcalfe et al. 1996). The fatty acid methyl esters were analyzed by using an HP5890 GC (Hewlett Packard) equipped with a DX-303 (JEOL) mass spectrometer. A DB-1 fused silica capillary column (0.25 mm × 30 m, J and W) was used for separation. The temperature of the injection port was 250°C, and that of the oven was programmed to rise from 150° to 250°C at a rate of 5°C min^{-1}. Identification of the fatty acid methyl esters was performed by comparing mass spectra and retention time with fatty acid methyl ester standards (Supelco). The peak areas on the total ion chromatogram were used for quantification of the methyl esters.

DNA preparation, DNA base composition, and DNA–DNA hybridization

Genomic DNA for sequencing was extracted and purified by using a High Pure PCR Template Preparation Kit (Roche Molecular Biochemicals) following the protocols of the manufacturer.

The G + C content of the genomic DNA was determined by HPLC also as described previously (Tamakoka and Komagata 1984). For DNA–DNA hybridization, genomic DNA was extracted by using a QIAGEN Blood and Cell Culture DNA Maxi Kit according to the QIAGEN Genomic DNA Handbook (09/97) and purified by equilibrium centrifugation in a CsCl–ethidium bromide gradient (Treisman 1989) by using an Optima MAX ultracentrifuge and MLA-80 (Beckman Coulter) rotor. The desalting was performed with an Ultrafree-4 Centrifugal Filter Unit (Millipore). Levels of DNA relatedness were determined fluorometrically by the method of Ezaki et al. (1989) with photobiotin-labeled DNA probes and microplates. Each hybridization experiment was repeated at least three times.

Sequencing of the 16S rDNA and the hypervariable region

Sequencing of the hypervariable region (HV region; nucleotide positions 70–344 according to *B. subtilis* numbering) of the 16S rDNA was performed as reported previously (Sadaie et al. 1997; Goto et al. 2000, 2002b). Almost complete 16S rDNA sequences were determined by using a 16S rRNA gene kit following the protocols of the manufacturer (Applied Biosystems), and an ABI 310 automatic DNA sequencer (Applied Biosystems).

Computer analysis and construction of the phylogenetic tree

Sequence analysis was performed by using Gene Works software (version 2.0, IntelliGenetics) and the European Molecular Biology Laboratory (EMBL), GenBank, and DNA Data Bank of Japan (DDBJ) databases. Multiple sequence alignment, calculation of nucleotide substitution rates, construction of a neighbor-joining tree, and bootstrap analysis with 1,000 replicates for evaluation of phylogenetic tree topology were carried out using the Clustal W version 1.7 program (Kimura 1980; Saitou and Nei 1987; Thompson et al. 1994). Alignment gaps and unidentified base positions were not taken into account during these calculations.

Nucleotide sequence accession numbers

The 16S rDNA sequences determined in this study have been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under consecutive accession numbers from AB060161 to AB060166. The DDBJ accession numbers for other strains used in the phylogenetic analysis were as follows: *Alicyclobacillus* genomic species 1 DSM 11984 (AB059668), *A. acidocaldarius* ATCC 27009^T (AB042056), *A. acidoterrestris* ATCC 49025^T (AB042057), *A. acidiphilus* TA 67^T (AB059677), *A. hesperidum* DSM 12489^T (AB059678), *A. cycloheptanicus* DSM 4006^T (AB042059), and *A. herbarius* CP 1^T (AB042055).

Results and discussion

Grouping of the isolates

In order to select bacterial strains for further characterization, an initial comparative sequence analysis of the HV region was performed with 60 strains. The results showed that all strains belonged to the *A. acidocaldarius–Alicyclobacillus* genomic species 1 group (Fig. 1). Four of the 60 strains clustered with *A. acidocaldarius* (Cluster II), and 54 strains clustered with *Alicyclobacillus* genomic species 1 (Cluster I) at 99.6% and 100% sequence similarity, respectively. The remaining two strains, MIH 332 and MIH 342, had identical sequences but clustered neither with *Alicyclobacillus* genomic species 1, at 97.3% similarity, nor with *A. acidocaldarius*, at 99.2% similarity (Cluster III). Thus, we selected the three strains from Cluster I that had been previously characterized phylogenetically, KHA 31, MIH 2, and UZ 1 (Hiraishi et al. 1997), and one additional strain from each of the three clusters described above (KHC 3, MIH 321, and MIH 332) for further investigation.

Biochemical and physiological characteristics of the strains in the *A. acidocaldarius–Alicyclobacillus* genomic species 1 group

On BAM agar after 72 h of growth, all strains (ATCC 27009^T, DSM 11984, KHA 31, KHC 3, MIH 2, MIH 321, MIH 332, and UZ 1) formed round, slightly mucous, creamy-white colonies with a diameter of 1–4 mm and rod-shaped cells that were 2.0–4.5 μm long and 0.5–1.0 μm wide. Endospores were terminal or subterminal and sporangia

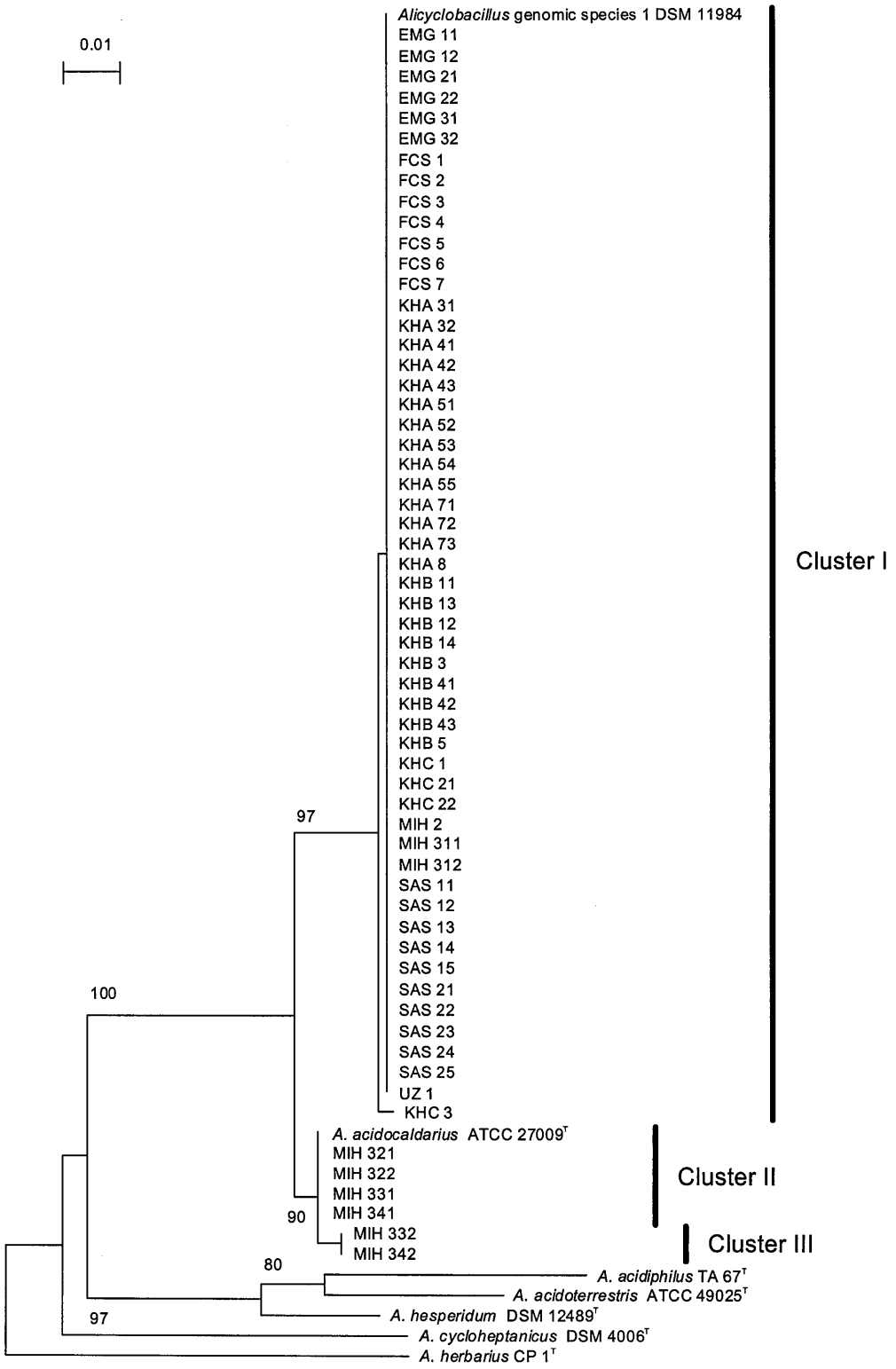

Fig. 1. Phylogenetic dendrogram showing the relationships among 60 isolates and the *Alicyclobacillus* reference strains, inferred from sequences of the hypervariable region. The dendrogram, constructed using the neighbor-joining method, is based on a comparison of 249 nucleotides (nt). *A. herbarius* CP 1^T was used as the out group. The *scale bar* represents 1 nt substitution per 100 nt. The *numbers* given on the branches indicate the percentage of 100 bootstrap replicates

were not swollen. Strains were very similar in their range of growth temperatures (35°–70°C) and pHs (2.5–6.0) as well as in their optimal temperature (55°–65°C) and pH (4.0–5.0). Cells of all strains were Gram-positive in young culture. None of the strains grew under anaerobic conditions or with 5% (w/v) NaCl in the medium.

All strains were oxidase negative. Catalase and Voges-Proskauer (VP) tests were positive or weakly positive. Gelatin and arbutin were hydrolyzed. Degradation of tyrosine and phenylalanine and formation of indole were not observed. All strains produced acid from glycerol, L-arabinose, ribose, D-xylose, D-galactose, D-glucose, D-

fructose, D-mannose, mannitol, cellobiose, maltose, sucrose, trehalose, glycogen, and D-tagatose. None of the strains produced acid from erythritol, D-arabinose, adonitol, methyl β-xyloside, dulcitol, sorbitol, *N*-acetyl-glucosamine, amygdalin, inulin, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-keto-gluconate, or 5-keto-gluconate. Differential biochemical and physiological characteristics are summarized in Table 2. Unlike the other strains of the *A. acidocaldarius–Alicyclobacillus* genomic species 1 group, strain MIH 332 reduced nitrate to nitrite. All strains produced acid from various carbon sources, and the acid formation patterns were very similar, yet distinctive.

DNA base, quinone, and fatty acid composition

The major respiratory quinone of all strains was menaquinone 7 (MK7), which reached levels as high as 77%–97% of the total quinines, with MK3 and MK4 also present at levels between 1% and 23%. The major fatty acid was ω-cyclohexyl C17:0 (47.6%–58.8%), and the relative proportions of ω-cyclohexane fatty acids (ω-cyclohexyl C17:0 and ω-cyclohexyl C19:0) reached 77.3%–90.2% (Table 3). The strain MIH 332^T had a higher relative proportion of ω-cyclohexyl C19:0 (42.6%) than the other strains (20.1%–34.2%). The remainder were mixtures of straight- and branched-chain fatty acids. All strains had DNA G + C contents in the range of 61.8–62.5 mol% (Table 4). These chemotaxonomical features were extremely similar among strains, which made it difficult to distinguish them solely on the basis of these parameters.

Phylogenetic analysis and DNA–DNA relatedness

Almost complete 16S rDNA sequences of the six strains in Clusters I–III (more than 1,500 nucleotides) were determined. A comparison of the 16S rDNA sequences showed that the sequences of the strains in Cluster I (KHA 31, KHC 3, MIH 2, and UZ 1) were >99.9% identical to that of *Alicyclobacillus* genomic species 1 DSM 11984. A sequence of the strain MIH 321 in Cluster II was 99.8% identical to that of *A. acidocaldarius* ATCC 27009^T. Sequence similarity values among strains in Clusters I and II ranged from 98.4% to 98.7%. Also, similarity values between strain MIH 332 and the reference strains of *A. acidocaldarius* (ATCC 27009^T) and *Alicyclobacillus* genomic species 1 (DSM 11984) were 98.8% and 99.1%, respectively, which were higher than the value between the reference strains (98.4%). Fig. 2 is a dendrogram of estimated phylogenetic relationships inferred from pairwise comparisons and evolutionary distance calculations derived from 1,499 nucleotide positions between positions 9 and 1,538 (*B. subtilis* numbering) from the 16S rDNA sequences of *Alicyclobacillus* strains. The inferred phylogenetic relationships indicated a clear delineation of the *A. acidocaldarius* cluster, MIH 332, and the *Alicyclobacillus* genomic species 1 cluster at bootstrap confidence levels of 97%–100%. Based on these results, we consider these strains to be closely related phylogenetically.

The results of the DNA–DNA hybridization are summarized in Table 4. DNA–DNA reassociation levels between *Alicyclobacillus* genomic species 1 DSM 11984 and other strains in Cluster I were 89%–93%. The level of genome

Table 2. Differential phenotypic characteristics of isolates and *Alicyclobacillus* reference strains

Characteristics	Cluster I					Cluster II		Cluster III
	Alicyclobacillus genomic species 1 DSM 11984	KHA 31	KHC 3	MIH 2	UZ 1	*A. acidocaldarius* ATCC 27009^T	MIH 321	MIH 332
Nitrate reduction	–	–	–	–	–	–	–	+
Acid production from:								
L-Xylose	–	–	–	–	+	–	–	–
L-Sorbose	–	+	+	+	+	+	+	+
Rhamnose	+	+	+	+	+	+	–	+
Inositol	–	+	–	–	–	–	–	–
Methyl α,D-mannoside	–	–	–	–	–	–	–	+
Methyl α,D-glucoside	–	+	+	+	+	+	–	+
Aesculin	–	+	+	+	+	+	+	+
Salicin	+	+	–	+	–	–	+	+
Lactose	+	+	+	–	+	+	+	+
Melibiose	–	–	–	–	–	+	+	+
Melezitose	+	–	–	+	–	–	+	+
D-Raffinose	+	+	–	+	–	–	+	+
Starch	–	–	–	–	–	–	–	+
Xylitol	–	–	+	–	–	–	+	+
β-Gentiobiose	+	–	–	+	–	–	–	+
D-Turanose	+	–	+	+	–	+	+	+
D-Lyxose	–	+	–	–	+	–	–	–

Table 3. Cellular fatty acids (%) of isolates and *Alicyclobacillus* reference strains

Cluster	Species	Strain	C15:0 I	C15:0 a	C16:0 i	C16:0 n	C17:0 i	C17:0 a	C18:0 n	ω C17:0	ω C19:0
I	*Alicyclobacillus*	DSM 11984	1.5	1.2	2.8	3.2	5.1	6.7	1.5	52.8	25.2
	genomic species 1	KHA 31	2.5	1.4	2.5	2.3	7.0	5.2	1.8	57.2	20.1
		MIH 2	0.9	0.6	1.9	2.5	5.0	4.9	2.7	52.3	29.2
		UZ 1	1.7	1.0	2.5	1.8	5.4	5.6	1.2	54.2	26.6
		KHC 3	1.4	1.1	1.5	1.8	5.2	6.0	2.0	58.8	22.2
II	*A. acidocaldarius*	ATCC 27009^T	1.7	0.7	1.9	2.0	3.6	1.1	2.5	52.3	34.2
		MIH 321	0.6	0.9	1.8	1.7	2.9	8.0	1.0	50.9	32.2
III		MIH 332	1.4	0.5	0.8	1.7	2.6	0.7	2.1	47.6	42.6

Table 4. DNA base compositions and levels of DNA–DNA hybridization relatedness

Cluster	Strain	GC content (mol%)	DNA–DNA hybridization value (%)	
			Alicyclobacillus genomic species 1 DSM 11984	*A. acidocaldarius* ATCC 27009^T
I	DSM 11984	62.0	100	59
	KHA 31	61.9	91	62
	MIH 2	62.3	92	56
	UZ 1	61.8	89	64
	KHC 3	62.4	93	66
II	ATCC 27009^T	61.9	65	100
	MIH 321	62.5	55	89
III	MIH 332	62.5	44	39

Values presented are the mean of three or more independent experiments

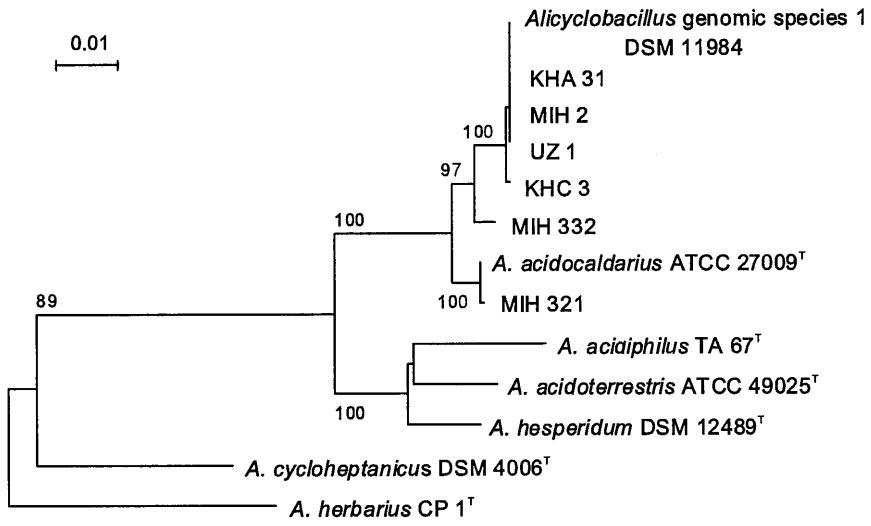

Fig. 2. Phylogenetic dendrogram showing the relationships among six isolates and the *Alicyclobacillus* reference strains, inferred from sequences of the 16S rDNA. The dendrogram, constructed using the neighbor-joining method, is based on a comparison of 1,499 nt. *A. herbarius* CP 1^T was used as the out group. The *scale bar* represents 1 nt substitution per 100 nt. The *numbers* given on branches indicate the percentage of 100 bootstrap replicates

identity between *A. acidocaldarius* ATCC 27009^T and strain MIH 321 (Cluster II) was calculated at 89%. Similarity between *Alicyclobacillus* genomic species 1 DSM 11984 and strain MIH 321 (Cluster II), and also between *A. acidocaldarius* ATCC 27009^T and strains in cluster I were intermediate (55%–66%). Therefore, on the basis of the phenotypic features and the phylogenetic relationships of the isolates in Clusters I and II, we identified strain MIH 321 as *A. acidocaldarius* and strains KHA 31, KHC 3, MIH 2, and UZ 1 as *Alicyclobacillus* genomic species 1, which is a genomic species of *A. acidocaldarius* (Albuquerque et al. 2000).

Strain MIH 332, on the other hand, showed DNA–DNA reassociation levels of 39% and 44% with the reference strains of *A. acidocaldarius* and *Alicyclobacillus* genomic species 1, respectively. The data revealed a significantly low

level of DNA relatedness between strain MIH 332 and the reference strains, which is, according to recognized criteria, indicative of strains of a different species or subspecies (Wayne et al. 1987). However, the close relationship between strain MIH 332 and the reference strains was confirmed by the high scores (98.8% and 99.1%) of sequence identity between the 16S rDNA of MIH 332 and that of the two reference strains. Furthermore, the typical phenotype was not observed for strain MIH 332 (Table 2, Table 3), thus indicating that strain MIH 332 may be a new genomic species of *A. acidocaldarius*, for which the name *Alicyclobacillus* genomic species 2 is tentatively proposed, on the basis of the description by Albuquerque et al. (2000).

In conclusion, our polyphasic data show that 60 isolates from various acidic environments belonged to three distinct clades of the genus *Alicyclobacillus*, which were identified as *A. acidocaldarius* and *Alicyclobacillus* genomic species 1 and 2. None of the other *Alicyclobacillus* and *Sulfobacillus* species, such as *A. acidoterrestris*, *A. cycloheptanicus*, or *S. acidophilus* were recognized in this research. Also, other strains of *A. acidocaldarius* have been previously isolated from Kusatsu hot spring (Gunma Pref.), Lake Tazawa hot spring, and Kuroyu hot spring (Akita Pref.) and characterized (Uchino and Doi 1967; Takayanagi et al. 1986; Sugimori et al. 1988). These microbial surveys, including ours, show that *A. acidocaldarius* and its genomic species are distributed widely in various acidic environments in Japan as the major population of thermoacidophiles. On the other hand, while phenotypes of these strains reveal highly similar morphologies, slight variations observed among strains indicate a diversity of these microorganisms at the strain level; possibly as a result of environmental heterogeneity characterized by gradients of temperature and acidity and variations in components present in the hot springs. In view of this species diversity, there is still room for further investigation into the validity of recognizing these genomic species as formal species.

Description of *Alicyclobacillus* genomic species 2

Cells are aerobic motile rods that are 2.0–4.5 μm long and 0.5–1.0 μm wide in 72 h culture on BAM agar. Ellipsoidal spores are terminal or subterminal and sporangia are not swollen. Gram-positive. Growth occurs at 35° and 70°C but not at 30° and 75°C. Optimum growth temperature is 55°–60°C. Optimum pH for growth is 4–4.5, and the growth is inhibited below pH 2.0 and above 6.5. No growth occurs in the presence of more than 5% (w/v) NaCl. Yeast extract or growth factors are not required for growth. Regarding the physiological characteristics, see the text and Table 2. The predominant menaquinone is MK7. The major fatty acids were ω-cyclohexyl C17:0 and ω-cyclohexyl C19:0. The G + C content is 62.5 mol% (determined by HPLC). Isolated from soil near a geyser in the Kirishima area of Kagoshima Prefecture. Strain MIH 332 has been deposited in the Institute of Molecular and Cellular Biosciences (IAM, University of Tokyo, Tokyo, Japan) as IAM 14934, and in the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany) as DSM 14672.

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References

Albuquerque L, Rainey FA, Chung AP, Sunna A, Nobre MF, Grote R, Antranikian G, da Costa MS (2000) *Alicyclobacillus hesperidum* sp. nov. and a related genomic species from solfataric soils of Sao Miguel in the Azores. Int J Syst Evol Microbiol 50:451–457

Atkinson T, Cairns S, Cowan DA, Danson MJ, Hough DW, Johnson DB, Norris PR, Raven N, Robinson C, Robson R, Sharp RJ (2000) A microbiological survey of Montserrat Island hydrothermal biotopes. Extremophiles 4:305–313

Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37:911–917

Darland G, Brock TG (1971) *Bacillus acidocaldarius* sp. nov., an acidophilic thermophilic spore-forming bacteria. J Gen Microbiol 67:9–15

Deinhard G, Blanz P, Poralla K, Altan E (1987a) *Bacillus acidoterrestris* sp. nov., a new thermotolerant acidophile isolated from different soils. Syst Appl Microbiol 10:47–53

Deinhard G, Saar J, Krischke W, Poralla K (1987b) *Bacillus cycloheptanicus* sp. nov., a new thermoacidophile containing ω-cycloheptane fatty acids. Syst Appl Microbiol 10:68–73

Ezaki T, Hashimoto Y, Yabuuchi E (1989) Fluorometric deoxyribonucleic acid–deoxyribonucleic acid hybridization in microdilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. Int J Syst Bacteriol 39:224–229

Goto K (2000) Thermoacidophilic bacteria: genus *Alicyclobacillus* (in Japanese). J Antibact Antifung Agents 28:499–508

Goto K, Omura T, Hara Y, Sadaie Y (2000) Application of the partial 16S rDNA sequence as an index for rapid identification of species in the genus *Bacillus*. J Gen Appl Microbiol 46:1–8

Goto K, Matsubara H, Mochida K, Hara Y, Niwa M, Yamasato K (2002a) *Alicyclobacillus herbarius* sp. nov., a novel bacterium with ω-cycloheptane fatty acids, isolated from herbal tea. Int J Syst Evol Microbiol

Goto K, Mochida K, Asahara M, Suzuki M, Yokota A (2002b) Application of hypervariable region of the 16S rDNA sequence as an index for the rapid identification of species in the genus *Alicyclobacillus*. J Gen Appl Microbiol (in press)

Hiraishi A, Inagaki K, Tanimoto Y, Iwasaki M, Kishimoto N, Tanaka H (1997) Phylogenetic characterization of a new thermoacidophilic bacterium isolated from hot springs in Japan. J Gen Appl Microbiol 43:295–304

Jensen N (2000) *Alicyclobacillus* in Australia. Food Aust 52:282–285

Kimura M (1980) A simple method for estimating evolutionary rates of base substitution through comparative studies of nucleotide sequences. J Mol Evol 16:111–120

Matsubara H, Goto K, Matsumura T, Mochida K, Iwaki M, Niwa M, Yamasato K (2002) *Alicyclobacillus acidiphilus* sp. nov., a new thermo-acidophilic, ω-alicyclic fatty acid-containing bacterium isolated from acidic beverages. Int J Syst Evol Microbiol (in press)

Metcalfe LD, Schmitz AA, Pelka JR (1996) Rapid preparation of fatty acid esters from lipids for gas chromatographic analysis. Anal Chem 38:514–515

Nicolaus B, Improta R, Manca MC, Lama L, Esposito E, Gambacorta A (1998) *Alicyclobacillus* from an unexplored geothermal soil in Antarctica: Mount Rittmann. Polar Biol 19:133–141

Niwa M (2001) Classification and various characteristics of new acidophilic-thermotolerant bacteria (in Japanese). Soft Drinks Tech 133:9–26

Pettipher GL, Osmundson ME, Murphy JM (1997) Methods for the detection and enumeration of *Alicyclobacillus acidoterrestris* and investigation of growth and production of taint in fruit juice and fruit-juice containing drinks. Lett Appl Microbiol 24:185–189

Sadaie Y, Yata K, Fujita M, Sagai H, Itaya M, Kasahara Y, Ogasawara N (1997) Nucleotide sequence and analysis of the *pho*B-*rrn*E-*gro*ESL region of the *Bacillus subtilis* chromosome. Microbiology 143:1861–1866

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Sano H, Sakai M, Nishijima M (1996) Application of MS to the search of the products from marine bacteria. J Mass Spectrom Soc Jpn 44:377–391

Sugimori K, Takayanagi S, Shirotani T (1988) Microorganisms living in extreme environments. (I) Acidophilic thermophilic bacteria living in Kuroyu hot spring, Akita Prefecture (in Japanese). J Med Soc Toho Univ 34:508–515

Takayanagi S, Sugimori K, Sendou M (1986) Identification and distribution of bacteria and algae living in acidic hot springs (in Japanese). Gen Educ Rev Toho Univ 18:33–43

Tamaoka J, Komagata K (1984) Determination of DNA base composition by reverse-phase high-performance liquid chromatography. FEMS Microbiol Lett 25:125–128

Tanimoto Y, Inagaki Y, Tanaka H (1996) Screening, isolation and characterization of thermophilic and acidophilic bacteria (in Japanese). Sci Rep Fac Agric Okayama Univ 85:15–21

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence weighing, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 76:4350–4354

Treisman R (1989) Purification of plasmid DNA. In: Sambrook J, Fritsch EF, Maniatis T (eds) Molecular cloning, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp 1.40–1.41

Uchino F, Doi S (1967) Acido-thermophilic bacteria from thermal waters. Agric Biol Chem 31:817–822

Walls I, Chuyate R (2000) Spoilage of fruit juices by *Alicyclobacillus acidoterrestris*. Food Aust 52:286–288

Wayne LG, Brenner DJ, Colwell RR, Grimont PAD, Kandler O, Krichevsky MI, Moore LH, Moore WEC, Murray RGE, Stackebrandt E, Staar MP, Trüper HG (1987) Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. Int J Syst Bacteriol 37:463–464

Wisotzkey JD, Jurtshuk P Jr, Fox GE, Deinhard G, Poralla K (1992) Comparative sequence analyses on the 16S rRNA (rDNA) of *Bacillus acidocaldarius*, *Bacillus acidoterrestris*, and *Bacillus cycloheptanicus* and proposal for creation of new genus, *Alicyclobacillus* gen. nov. Int J Syst Bacteriol 42:263–269

Yamazaki K, Tezuka H, Shinano H (1996) Isolation and identification of *Alicyclobacillus acidoterrestris* from acid beverages. Biosci Biotech Biochem 60:543–545