Alicyclobacillus pomorum sp. nov., a novel thermo-acidophilic, endospore-forming bacterium that does not possess ω -alicyclic fatty acids, and emended description of the genus *Alicyclobacillus*

Keiichi Goto,¹ Kaoru Mochida,¹ Mika Asahara,¹ Masayuki Suzuki,² Hiroaki Kasai³ and Akira Yokota⁴

Correspondence

Keiichi Goto
kgoto@mnk.co.jp

^{1,2}Microbiological and Analytical Group, Food Research Laboratories, Mitsui Norin Co. Ltd¹ and Central Research Laboratories, Tokyo Food Techno Co. Ltd², 223-1, Miyahara, Fujieda, Shizuoka 426-0133, Japan

³Marine Biotechnology Institute, 3-75-1, Heita, Kamaishi, Iwate 026-0001, Japan

⁴Institute of Molecular and Cellular Biosciences, University of Tokyo, 1-1, Yayoi 1-chome, Bunkyo-ku, Tokyo 113-0032, Japan

A thermo-acidophilic endospore-forming bacterium was isolated from a mixed fruit juice. The organism, strain 3A^T, was rod-shaped, grew aerobically at 30–60 °C (optimum 45–50 °C), pH 3.0–6.0 (optimum pH 4.0–4.5) and produced acid from various sugars. It contained menaquinone-7 as the major isoprenoid quinone. The G + C content of the DNA was 53.1 mol%. The predominant cellular fatty acids of the strain were iso-C_{15:0}, anteiso-C_{15:0}, iso-C_{16:0}, iso-C_{17:0} and anteiso-C_{17:0}, but ω -alicyclic fatty acids, which are characteristic of the genus *Alicyclobacillus*, were not found in the strain. Phylogenetic analyses based on both 16S rRNA and *gyrB* (DNA gyrase B subunit gene) gene sequences showed that strain 3A^T falls into the *Alicyclobacillus* cluster, validated by significant bootstrap values. However, strain 3A^T did not show a close relationship to the other species of the cluster. The level of 16S rDNA similarity between strain 3A^T and other strains of the cluster was between 92.5 and 95.5 %. The level of *gyrB* sequence similarity between strain 3A^T and other strains of the cluster was between 68.5 and 74.4 %. DNA–DNA hybridization values between strain 3A^T and phylogenetically related strains of the genera *Alicyclobacillus*, *Bacillus* and *Sulfobacillus* were under 13 %, indicating that strain 3A^T represents a distinct species. On the basis of these results, strain 3A^T should be classified as a novel *Alicyclobacillus* species. The name *Alicyclobacillus pomorum* is proposed for this organism. The type strain of *Alicyclobacillus pomorum* is strain 3A^T (= DSM 14955^T = IAM 14988^T).

The genus *Alicyclobacillus* (Wisotzkey *et al.*, 1992) consists of a group of thermo-acidophilic, strictly aerobic, heterotrophic, endospore-forming bacteria. The genus originally consisted of three species, *Alicyclobacillus acidocaldarius*, *Alicyclobacillus acidoterrestris* and *Alicyclobacillus cycloheptanicus*. In recent years, four novel species and one subspecies have been formally assigned to the genus; *Alicyclobacillus hesperidum* (Albuquerque *et al.*, 2000), *Alicyclobacillus herbarius* (Goto *et al.*, 2002a), *Alicyclobacillus acidiphilus* (Matsubara *et al.*, 2002), *Alicyclobacillus sendaiensis* (Tsuruoka *et al.* 2003) and *Alicyclobacillus acidocaldarius* subsp. *rittmannii* (Nicolaus *et al.*, 1998). In

addition, two genomic species, *Alicyclobacillus* genomic species 1 (Albuquerque *et al.*, 2000) and *Alicyclobacillus* genomic species 2 (Goto *et al.*, 2002b), have been reported as genomic species of *A. acidocaldarius*. All *Alicyclobacillus* species form one phylogenetic cluster (exclusive of the species *Sulfobacillus disulfidooxidans*) based on 16S rDNA sequence analysis and possess ω -alicyclic fatty acids (ω -cyclohexane or ω -cycloheptane fatty acids) as the major membrane lipid component. The *Alicyclobacillus* species have been isolated from natural sources such as hot springs and soil (Uchino & Doi, 1967; Darland & Brock, 1971; Hippchen *et al.*, 1981; Deinhard *et al.*, 1987a, b; Hiraishi *et al.*, 1997; Nicolaus *et al.*, 1998; Albuquerque *et al.*, 2000; Goto *et al.*, 2002b, Tsuruoka *et al.* 2003), as well as secondary spoiled fruit-based beverages (Yamazaki *et al.*, 1996; Goto *et al.*, 2002a; Matsubara *et al.*, 2002). During the course of a microbiological survey of various fruit juices, we

Published online ahead of print on 21 March 2003 as DOI 10.1099/ij.s.0.02546-0.

The DDBJ/GenBank/EMBL accession numbers for the 16S rDNA and *gyrB* gene sequences are AB089840–AB089859.

isolated an unusual *Alicyclobacillus*-like organism from a spoiled mixed fruit juice. Based on the results of a polyphasic taxonomic study, a novel and anomalous species of the genus *Alicyclobacillus*, *Alicyclobacillus pomorum* sp. nov., is described here.

Strain 3A^T was isolated from a spoiled mixed fruit juice (containing fresh orange, apple, mango, pineapple and raspberry juice imported into Japan from the USA) by using the dilution plating technique on a solid medium (YSG agar) containing (l⁻¹) 2 g yeast extract, 1 g glucose, 2 g soluble starch and 15 g agar (pH 3.7 with 1 M H₂SO₄). Other species isolated at the same time were *Bacillus subtilis*, *Lactobacillus plantarum*, *Lactobacillus brevis*, *Saccharomyces cerevisiae* and *Saccharomyces pastorianus*. Based on partial 16S rDNA sequence analyses (Goto *et al.*, 2000, 2002c), the strain was grouped into the *Alicyclobacillus* cluster. The strain, however, was found to be distinct from previously described species of the genus *Alicyclobacillus*.

To investigate the morphological and physiological characteristics of strain 3A^T, it was cultured in *Bacillus acidocaldarius* medium (BAM; Deinhard *et al.*, 1987a) at 45 °C. *Arthrobacter globiformis* JCM 1332^T, *Bacillus tusciae* IFO 15312^T, *Sulfobacillus acidophilus* DSM 10332^T, *Sulfobacillus disulfidooxidans* DSM 12064^T and *Sulfobacillus thermosulfidooxidans* DSM 9293^T were also cultivated according to the methods recommended in the corresponding DSM and IFO strain catalogues. *Alicyclobacillus acidiphilus* TA67^T was kindly provided by Motohiro Niwa (Kirin Beverage Corporation, Samukawa-machi, Kanagawa, Japan). Unless indicated otherwise, all morphological and physiological tests have been described in a previous study (Goto *et al.*, 2002a). Since the strain did not grow in the presence of indicators, the degree of acidification was determined by measuring the pH decrease (>0.4) of the culture using BAM basal salts medium without indicator to which a carbon source (2 g l⁻¹) was added. Acidification was observed every day for 7 days and the reading showing the strongest acidification was recorded. All tests were repeated in triplicate.

Cellular fatty acid analysis, menaquinone analysis and the preparation of DNA have been described in previous studies (Goto *et al.*, 2000, 2002a). The DNA G + C content was determined by the method of Tamaoka *et al.* (1984) using HPLC with a YMC-Pack ODS-AQ AQ302 column (4.6 × 150 mm; YMC) and 10 mM H₃PO₄/10 mM KH₂PO₄ (pH 3.5) as the mobile phase. Almost complete 16S rDNA sequences were determined using the 16S rRNA Gene Kit following the protocols of the manufacturer (Applied Biosystems). DNA–DNA hybridization experiments were performed by the method of Ezaki *et al.* (1989) using photobiotin-labelled DNA probes and microplates.

Nucleotide sequences of *gyrB* genes were determined directly from PCR fragments amplified by the methods described by Yamamoto & Harayama (1995) and Kasai *et al.* (2000). The primers used for *gyrB* gene amplification were

UP-1G (forward: 5'-GAAGTCATCATGACCGTTCTGCA-YGCNGGNGGNAARTTYGG-3') and UP-2r (reverse: 5'-AGCAGGGTACGGATGTGCGAGCCRTCNCACRTCNCR-RTCNGTCAT-3'). Primer UP-1G annealed at positions 274–314 and primer UP-2r annealed at positions 1486–1529 (relative to *Escherichia coli* K-12 gene numbering). The amplified products were purified by gel electrophoresis on 0.8% low-melting-point agarose (SeaPlaque GTG; FMC Bioproducts). The purified fragments were recovered from the agarose by a QIAquick gel extraction kit (Qiagen). Sequencing was carried out with an ABI PRISM Dye Terminator Cycle Sequencing Kit with sequencing primers UP-1S (forward: 5'-GAAGTCATCATGACCGTTCTGCA-3'; positions 274–296), *gyrS*-2F (reverse: 5'-GAACAAG-CSTTTTTRAATKCCGG-3'; positions 577–599), *gyr*-Int-R (reverse: 5'-CCGCGVACYTCRGTGTTGCC-3'; positions 1021–1040), *gyrS*-4R (reverse: 5'-GATTCCAGYACRCTTT-TTCGCCG-3'; positions 1177–1199) and UP-2rS (reverse: 5'-AGCAGGGTACGGATGTGCGAGCC-3'; positions 1507–1529). The products were analysed by an ABI PRISM 3100 Genetic Analyser (Applied Biosystems) according to the manufacturer's instructions.

Sequence analysis was performed using Gene Works (version 2.0; IntelliGenetics) and the GenBank/EMBL/DDBJ databases. Multiple sequence alignment was performed using CLUSTAL W version 1.8 (Thompson *et al.*, 1994). Also, the *gyrB* gene sequences and their deduced amino acid sequences were aligned by CLUSTAL W version 1.8 and the alignments were manually corrected. Alignment gaps and unidentified base positions were not taken into account for these calculations. A phylogenetic reconstruction was produced using MEGA2 (Kumar *et al.*, 2001) with the following settings: neighbour-joining (Saitou & Nei, 1987) based on Kimura's two-parameter model (Kimura, 1980) and maximum-parsimony (Lake, 1987) methods. The robustness of individual branches was estimated by bootstrapping with 1000 replicates (Felsenstein, 1985). The DDBJ accession numbers of 16S rDNA and *gyrB* gene sequences used for each phylogenetic analysis are shown in Fig. 1 and Fig. 2, respectively.

Cells of strain 3A^T were Gram-positive, but Gram-variable in old cultures, motile rods (2.0–4.0 × 0.8–1.0 µm) and formed oval spores subterminally in swollen sporangia. Colonies were flat, smooth and creamy white after 48 h culture on BAM agar. Growth of strain 3A^T occurred aerobically at 30 and 60 °C, but not at 25 or 65 °C. The optimal growth temperature was in the range of 45–50 °C. Strain 3A^T grew well at pH 4.0–4.5 with growth inhibited at pH 2.5 and 6.5. The strain failed to grow on BAM containing more than 2% (w/v) NaCl. No growth occurred under anaerobic conditions. Strain 3A^T could grow heterotrophically in an inorganic medium containing glucose or starch, without any growth factors. The generation time in BAM at 45 °C was approximately 1.5 h. Strain 3A^T had catalase and oxidase activities, but no nitrate reductase activity. Aesculin, starch and gelatin were hydrolysed, but

Fig. 1. Phylogenetic tree (constructed using the maximum-parsimony method) derived from an alignment comprising 16S rDNA sequences from phylogenetically related *Alicyclobacillus*, *Bacillus* and *Sulfobacillus* species. *Arthrobacter globiformis* JCM 1332^T served as outgroup. The final dataset included 1392 unambiguously aligned sites. Numbers represent percentages from 1000 replicate bootstrap samplings (frequencies of less than 50 % are not shown).

no hydrolysis of arbutin, phenylalanine or tyrosine was observed. Acid was produced from glycerol, ribose, D-glucose, D-fructose, D-mannose, L-sorbose, mannitol, methyl- α -D-glucoside, amygdalin, aesculin, salicin, maltose, sucrose, trehalose, D-turanose, D-tagatose and 5-ketogluconate. The differential phenotypic characteristics of strain 3A^T and *Alicyclobacillus* reference strains are shown in Table 1.

The major respiratory quinone of strain 3A^T was menaquinone-7 (MK7), which accounted for 97 % of the total, with MK3 as the vestigial quinone (3 %). The fatty acid profile of strain 3A^T consisted mainly of iso- and anteiso-branched acids combined with a small amount of straight-chain saturated (C_{16:0} and C_{18:0}) acids (Table 2). In the absence of ω -alicyclic fatty acids, the fatty acid profile of strain 3A^T is clearly different from that of *Alicyclobacillus* species and thus it is more similar to that of *Bacillus tusciae*. However, significant differences in the level of iso-C_{15:0}, anteiso-C_{15:0}, iso-C_{16:0} and iso-C_{18:0} distinguished strain 3A^T from *B. tusciae*. The G + C content of strain 3A^T was 53.1 mol%, which is in the range for known *Alicyclobacillus* species (53–63 %) and *Sulfobacillus* species (52–56 %).

The near complete 16S rDNA sequences between positions 37 and 1476 (*Escherichia coli* numbering; Brosius *et al.*, 1978) for strains 3A^T, *Alicyclobacillus acidocaldarius* subsp. *rittmannii* DSM 11297^T, *Arthrobacter globiformis* JCM 1332^T, *Sulfobacillus acidophilus* DSM 10332^T, *Sulfobacillus disulfidooxidans* DSM 12064^T and *Sulfobacillus thermosulfidooxidans* DSM 9293^T were determined. These sequences were aligned with published sequences available from the

GenBank/EMBL/DDBJ databases and the phylogenetic position of strain 3A^T was inferred by constructing a phylogenetic tree with either the neighbour-joining method (data not shown) or the maximum-parsimony method (Fig. 1). The phylogenetic analyses, based on 1392 unambiguous nucleotides, showed that strain 3A^T fell within the cluster composed of *Alicyclobacillus* species and *Sulfobacillus disulfidooxidans* DSM 12064^T in both trees and that the cluster was supported by high bootstrap values of 99 % (neighbour-joining tree) and 95 % (maximum-parsimony tree), respectively. However, strain 3A^T did not show a close relationship with any other species of the cluster. The same results were obtained when the phylogenetic tree was constructed based on a wider sample of species (available as supplementary material in IJSEM online at <http://ijs.sgmjournals.org/>). The sequence similarity values between strain 3A^T and other strains of the cluster exceeded 92 %, this being the lower limit for *Alicyclobacillus* species (Wisotzkey *et al.*, 1992). Similarity values between strain 3A^T and *Bacillus tusciae* IFO 15312^T, *Sulfobacillus acidophilus* DSM 10332^T or *S. thermosulfidooxidans* DSM 9293^T were all below 90 % (Table 3).

The partial *gyrB* nucleotide sequences, comprising at least 1164 bp, for strain 3A^T, nine *Alicyclobacillus* strains, *Bacillus tusciae* IFO 15312^T and three *Sulfobacillus* strains were determined. Phylogenetic analyses, based on the alignment of *gyrB* gene sequences (1146 nucleotides between *Escherichia coli* positions 316 and 1485; Adachi *et al.*, 1987), showed that strain 3A^T formed a monophyletic cluster with *Alicyclobacillus* species and *Sulfobacillus disulfidooxidans* DSM 12064^T in both the neighbour-joining tree (data not shown)

Fig. 2. Phylogenetic tree (constructed using the maximum-parsimony method) showing the phylogenetic position of strain 3A^T in relation to phylogenetically related *Alicyclobacillus*, *Bacillus* and *Sulfobacillus* species, based on *gyrB* gene sequences. *Sulfobacillus thermosulfidooxidans* DSM 9293^T served as outgroup. The final dataset included 1161 unambiguously aligned sites. Numbers represent percentages from 1000 replicate bootstrap samplings (frequencies of less than 50 % are not shown).

Table 1. Differential characteristics of *Alicyclobacillus pomorum* 3A^T and related *Alicyclobacillus* strains

Strains: 1, *Alicyclobacillus pomorum* 3A^T; 2, *Alicyclobacillus acidocaldarius* ATCC 27009^T; 3, *Alicyclobacillus* genomic species 1 DSM 11984; 4, *Alicyclobacillus* genomic species 2 MIH332; 5, *Alicyclobacillus acidoterrestris* ATCC 49025^T; 6, *Alicyclobacillus hesperidum* DSM 12489^T; 7, *Alicyclobacillus acidiphilus* TA67^T; 8, *Alicyclobacillus cycloheptanicus* DSM 4006; 9, *Alicyclobacillus herbarius* IAM 14883. +, Positive result; –, negative result; w, weakly positive result.

Characteristic	1	2	3*	4	5	6*	7†	8	9
Oxidase	+	–	–	–	–	–	–	+	–
Gelatin hydrolysis	+	+	+	+	+	+	–	–	–
Starch hydrolysis	+	+	+	+	–	+	–	–	–
Nitrate reduced to nitrite	–	–	–	+	–	–	–	–	+
Growth in 5 % NaCl	–	–	–	–	+	–	–	+	+
Acid production from:									
Glycerol	+	+	+	+	+	+	–	–	+
Erythritol	–	–	–	–	+	–	–	–	–
D-Arabinose	–	–	–	–	–	–	+	+	+
L-Arabinose	–	+	+	+	+	+	+	+	+
D-Xylose	–	+	+	+	–	–	+	+	+
L-Xylose	–	–	–	–	–	–	–	+	–
D-Galactose	–	+	+	+	+	+	+	–	+
L-Sorbose	+	–	–	+	–	–	+	+	–
Rhamnose	–	+	+	+	+	–	–	+	+
Inositol	–	–	–	–	+	–	–	–	–
Mannitol	+	+	+	+	+	+	–	+	+
Sorbitol	–	–	–	–	+	–	+	+	–
Methyl- α -D-mannoside	–	–	–	+	–	–	–	–	+
Methyl- α -D-glucoside	+	+	–	+	–	–	+	–	+
Amygdalin	+	–	–	–	–	–	–	–	+
Arbutin	–	+	+	+	–	–	+	–	+
Aesculin	+	+	–	+	–	–	+	+	+
Salicin	+	+	+	+	–	–	+	–	+
Cellobiose	–	+	+	+	+	+	+	–	+
Maltose	+	+	+	+	+	+	+	–	+
Lactose	–	+	+	+	+	+	+	–	+
Melibiose	–	+	–	+	w	–	–	–	+
Sucrose	+	+	+	+	+	+	+	–	+
Trehalose	+	+	+	+	+	+	+	–	+
Melezitose	–	–	+	+	–	–	+	–	+
D-Raffinose	–	+	+	+	–	–	+	–	+
Starch	–	+	–	+	–	w	–	–	–
Glycogen	–	+	+	+	–	+	–	–	–
Xylitol	–	–	–	+	+	–	+	–	–
β -Gentiobiose	–	–	+	+	–	w	+	–	+
D-Turanose	+	–	+	+	–	+	+	–	+
D-Lyxose	–	–	–	–	–	–	–	+	–
D-Tagatose	+	–	+	+	–	–	–	+	–
D-Fucose	–	–	–	–	–	–	–	–	+
5-Ketogluconate	+	–	–	–	–	–	–	+	+

*Data from Albuquerque *et al.* (2000).

†Data from Matsubara *et al.* (2002).

and the maximum-parsimony tree (Fig. 2) at bootstrap values of 81 and 87 %, respectively. Strain 3A^T clustered with *Alicyclobacillus cycloheptanicus* DSM 4006^T and *Alicyclobacillus herbarius* CP1^T in the trees, however, without

significant bootstrap values (21–68 %). *Sulfobacillus disulfidooxidans* DSM 12064^T branched off from the main *Alicyclobacillus* cluster; however, the relative branching order was not well supported by bootstrap values. The *gyrB*

Table 2. Cellular fatty acid composition of *Alicyclobacillus pomorum* 3A and related thermo-acidophilic endospore-forming bacteria

1, *Alicyclobacillus pomorum* 3A^T; 2, *Bacillus tusciae* IFO 15312^T; 3, *Alicyclobacillus acidocaldarius* cluster; 4, *Alicyclobacillus acidoterrestris* cluster; 5, *Alicyclobacillus cycloheptanicus* DSM 4006^T; 6, *Alicyclobacillus herbarius* IAM 14883^T; TR, trace.

Fatty acid	Composition (%)					
	1	2	3	4	5*	6*
C _{14:0}	0.5	TR		0–1.2	0.8	0.4
C _{15:0}	0.5	TR		0–0.7	0.6	TR
C _{16:0}	1.4	5.2	1.7–3.2	1.7–9.7	2.4	5.1
C _{17:0}	TR	1.5				0.5
C _{18:0}	1.3	4.9	1.5–2.5		1.3	2.4
iso-C _{15:0}	19.9	1.9	1.4–1.7	0.5–0.8	0.5	0.4
iso-C _{16:0}	18.3	27.5	0.8–2.8	0–0.5	1.0	3.8
iso-C _{17:0}	13.3	11.3	2.6–5.1	1.2–2.0	2.7	5.6
iso-C _{18:0}	TR	5.4				3.2
anteiso-C _{15:0}	9.9	4.2	0.5–1.2	0.5–1.1	TR	TR
anteiso-C _{17:0}	34.2	36.5	0.7–6.7	3.2–6.5	1.3	3.0
ω-Cyclohexane C _{17:0}			47.6–52.8	62.6–68.4		
ω-Cyclohexane C _{19:0}			25.2–42.6	18.9–23.9		
ω-Cycloheptane C _{18:0}					86.8	67.1
ω-Cycloheptane C _{18:0} 2OH					2.3	3.5
ω-Cycloheptane C _{20:0}					TR	4.5

*Data from Goto *et al.* (2002a).

gene sequence of strain 3A^T exhibited 68.5–74.4 % identity to other strains of *Alicyclobacillus* species and *Sulfobacillus disulfidooxidans* DSM 12064^T. Although the phylogenetic relationships, based on GyrB amino acid sequences (382 unambiguous amino acids), were also investigated using neighbour-joining and maximum-parsimony approaches

(data not shown), the topologies of the phylogenetic trees were almost identical between the trees and the accurate phylogenetic position of strain 3A^T in the cluster, especially in relation to *Alicyclobacillus cycloheptanicus*, *Alicyclobacillus herbarius* and *Sulfobacillus disulfidooxidans*, remained ambiguous as described for the nucleic acid data.

Table 3. DNA base compositions, DNA–DNA hybridization values and sequence similarities of 16S rDNA and *gyrB* between *Alicyclobacillus pomorum* 3A^T and related thermo-acidophilic endospore-forming bacteria

Species	Strain	G + C content (mol%)	Hybridization value (%) with 3A ^T	Similarity (%) (16S rDNA/ <i>gyrB</i>) with 3A ^T
<i>Alicyclobacillus pomorum</i>	3A ^T	53.1	100	100/100
<i>Alicyclobacillus</i> genomic species 1	DSM 11984	61.4	10	93.1/69.7
<i>Alicyclobacillus</i> genomic species 2	MIH-332	62.5	11	94.0/68.5
<i>Alicyclobacillus acidocaldarius</i> subsp. <i>acidocaldarius</i>	ATCC 27009 ^T	61.9	12	94.3/69.4
<i>Alicyclobacillus acidocaldarius</i> subsp. <i>rittmannii</i>	DSM 11297 ^T	62.7	10	94.3/68.9
<i>Alicyclobacillus acidoterrestris</i>	ATCC 49025 ^T	52.7	10	95.0/71.9
<i>Alicyclobacillus hesperidum</i>	DSM 12489 ^T	54.0	11	95.2/69.7
<i>Alicyclobacillus acidiphilus</i>	TA67 ^T	55.6	11	94.3/71.8
<i>Alicyclobacillus cycloheptanicus</i>	DSM 4006 ^T	57.2	13	95.5/73.8
<i>Alicyclobacillus herbarius</i>	DSM 13609 ^T	56.2	11	93.6/74.4
<i>Sulfobacillus disulfidooxidans</i>	DSM 12064 ^T	52.9	8	92.5/69.9
<i>Bacillus tusciae</i>	IFO 15312 ^T	58.6	7	89.1/68.3
<i>Sulfobacillus acidophilus</i>	DSM 10332 ^T	56.1	1	83.9/61.9
<i>Sulfobacillus thermosulfidooxidans</i>	DSM 9293 ^T	51.9	1	84.8/59.4

Determining the relationship between these organisms will require sequence data from additional isolates of *Alicyclobacillus* and related genera.

The DNA–DNA hybridization values for strain 3A^T and phylogenetically related strains of *Alicyclobacillus*, *Bacillus* and *Sulfobacillus* species were 1–13 %, which was well below the 70 % cut-off point recommended by Wayne *et al.* (1987) for the recognition of a genomic species.

According to the description of the genus *Alicyclobacillus* by Wisotzkey *et al.* (1992), the predominant membrane fatty acids of its members are ω -alicyclic acids that contain six- or seven-carbon rings. These fatty acids have been suggested to play an important role in the growth of *Alicyclobacillus* in thermal and acidic environments (De Rosa *et al.*, 1971; Kannenberg *et al.*, 1984; Poralla *et al.*, 1984; Krischke & Poralla, 1990; Moore *et al.*, 1997). Although the genus *Sulfobacillus* has also been reported to possess ω -alicyclic fatty acids (Golovacheva & Karavaiko, 1979; Dufresne *et al.*, 1996; Norris *et al.*, 1996), *Sulfobacillus disulfidooxidans* is meso-acidophilic, whereas *Sulfobacillus thermosulfidooxidans*, *Sulfobacillus acidophilus* and *Sulfobacillus disulfidooxidans* are thermo-acidophilic. Furthermore, *Curtobacterium pusillum* (Suzuki *et al.*, 1981) and *Propionibacterium cyclohexanicum* (Kusano *et al.*, 1997) also possess ω -alicyclic acids; however, these organisms are neither thermophilic nor acidophilic (*Propionibacterium cyclohexanicum* is an acidotolerant bacterium), thus the presence of ω -alicyclic acids does not necessarily relate to the physiological property of being thermo-acidophilic.

On the other hand, thermo-neutrophilic bacilli, such as *Aneurinibacillus thermoaerophilus* (Heyndrickx *et al.*, 1997), *Bacillus coagulans* (Nazina *et al.*, 2001), *Brevibacillus thermoruber* (Manachini *et al.*, 1985), *Geobacillus* species (Nazina *et al.*, 2001), *Thermobacillus* species (Touzel *et al.*, 2000) and *Ureibacillus* species (Fortina *et al.*, 2001), possess iso-C_{15:0}, anteiso-C_{15:0}, C_{16:0}, iso-C_{16:0}, iso-C_{17:0} and/or anteiso-C_{17:0} as major cellular fatty acids. Also, mesophilic and mildly acidophilic bacilli, such as *Bacillus naganoensis* (Tomimura *et al.*, 1990) and *Bacillus tusciae* IFO 15312^T (Table 2), possess iso-C_{14:0}, iso-C_{15:0}, anteiso-C_{15:0}, iso-C_{16:0}, iso-C_{17:0} and/or anteiso-C_{17:0} as major cellular fatty acids. The predominant cellular fatty acids of strain 3A^T are iso-C_{15:0}, anteiso-C_{15:0}, iso-C_{16:0}, iso-C_{17:0} and anteiso-C_{17:0} and the fatty acid composition is relatively similar to that of *Bacillus tusciae* IFO 15312^T. Within this list of species anteiso-C_{17:0} is consistently the most abundant fatty acid. However, strain 3A^T could be distinguished from *Bacillus tusciae* IFO 15312^T by the relative percentage of iso-C_{15:0}, anteiso-C_{15:0}, iso-C_{16:0} and iso-C_{18:0}. Moreover, values of DNA–DNA hybridization and sequence similarity in both 16S rDNA and *gyrB* genes between strain 3A^T and *Bacillus tusciae* IFO 15312^T were lower than for other *Alicyclobacillus* species.

Although strain 3A^T does not possess any ω -alicyclic fatty acids, the results of this polyphasic taxonomic analysis

clearly show that this thermo-acidophilic heterotrophic endospore-forming bacterium represents a hitherto unrecognized species within the genus *Alicyclobacillus*. Therefore, on the basis of all data obtained, we propose that the unknown bacterium should be classified within the genus *Alicyclobacillus*, as *Alicyclobacillus pomorum* sp. nov. In addition, the results of 16S rDNA and *gyrB* gene sequence analyses strongly suggested that *Sulfobacillus disulfidooxidans* is phylogenetically closely related to the genus *Alicyclobacillus* rather than the genus *Sulfobacillus*. However, *Alicyclobacillus* is heterotrophic while *Sulfobacillus* is mixotrophic, thus delineating these two genera. Further taxonomic studies are in progress to clarify phylogenetic heterogeneity within the genera *Alicyclobacillus* and *Sulfobacillus*.

Emended description of genus *Alicyclobacillus* Wisotzkey *et al.* 1992

The description of the genus *Alicyclobacillus* (Wisotzkey *et al.*, 1992) is emended to reflect the following chemotaxonomic characteristic. The cellular fatty acids profile consists of ω -alicyclic fatty acids and a small amount of straight- and branched-chain saturated fatty acids or only straight- and branched-chain saturated fatty acids instead of ω -alicyclic fatty acids.

Description of *Alicyclobacillus pomorum* sp. nov.

Alicyclobacillus pomorum (po.mo'rum. L. neut. N. *pomum* fruit; L. gen. pl. neut. n. *pomorum* of fruits).

Gram-positive, but Gram-variable in old cultures, strictly aerobic, motile, endospore-forming straight rods with rounded ends (2.0–4.0 × 0.8–1.0 µm). Endospores are oval and subterminal with swollen sporangia. Colonies on BAM agar are circular and 3–4 mm in diameter after 48 h and are not pigmented. Temperature range for growth is 30–60 °C; optimum growth temperature is 45–50 °C. pH optimum is 4.5–5.0; growth does not occur at pH 2.5 or 6.5. Growth factors are not required, but growth is further increased by adding yeast extract to the inorganic medium. The generation time is 1.5 h. Oxidase- and catalase-positive, but does not reduce nitrate to nitrite. Aesculin, gelatin and starch are hydrolysed, but arbutin, phenylalanine and tyrosine are not. Acid is produced from glycerol, ribose, D-glucose, D-fructose, D-mannose, L-sorbose, mannitol, methyl- α -D-glucoside, amygdalin, aesculin, salicin, maltose, sucrose, trehalose, D-turanose, D-tagatose and 5-ketogluconate. The major fatty acids are iso-C_{15:0}, anteiso-C_{15:0}, iso-C_{16:0}, iso-C_{17:0} and anteiso-C_{17:0}. The main quinone is menaquinone 7. The G + C content is 53.1 mol%. Type strain is strain 3A^T (=DSM 14955^T = IAM 14988^T).

Acknowledgements

We thank Professor Trüper for his valuable advice on nomenclature and Yuko Kato for her excellent technical assistance.

References

- Adachi, T., Mizuuchi, M., Robinson, E. A., Appella, E., O'Dea, M. H., Gellert, M. & Mizuuchi, K. (1987). DNA sequence of the *E. coli* *gyrB* gene: application of a new sequencing strategy. *Nucleic Acids Res* **15**, 771–784.
- Albuquerque, L., Rainey, F. A., Chung, A. P., Sunna, A., Nobre, M. F., Grote, R., Antranikian, G. & De Costa, M. S. (2000). *Alicyclobacillus hesperidum* sp. nov. and a related genomic species from solfataric soils of São Miguel in the Azores. *Int J Syst Evol Microbiol* **50**, 451–457.
- Brosius, J., Palmer, M. L., Kennedy, P. J. & Noller, H. F. (1978). Complete nucleotide sequence of the 16S ribosomal RNA gene from *Escherichia coli*. *Proc Natl Acad Sci U S A* **75**, 4801–4805.
- Darland, G. & Brock, T. D. (1971). *Bacillus acidocaldarius* sp. nov., an acidophilic thermophilic spore-forming bacterium. *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.
- De Rosa, M., Gambacorta, A., Minale, L. & Bu'Lock, J. D. (1971). Cyclohexane fatty acids from a thermophilic bacterium. *Chem Commun* **1019**, 1334.
- Dufresne, S., Bousquet, J., Boissinot, M. & Guay, R. (1996). *Sulfobacillus disulfidooxidans* sp. nov., a new acidophilic, disulfide-oxidizing, Gram-positive, spore-forming bacterium. *Int J Syst Bacteriol* **46**, 1056–1064.
- Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid–deoxyribonucleic acid hybridization in micro-dilution 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.
- Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **30**, 783–791.
- Fortina, M. G., Pukall, R., Schumann, P., Mora, D., Parini, C., Manachini, P. L. & Stackebrandt, E. (2001). *Ureibacillus* gen. nov., a new genus to accommodate *Bacillus thermosphaericus* (Anderson *et al.*, 1995), emendation of *Ureibacillus thermosphaericus* and description of *Ureibacillus terrenus* sp. nov. *Int J Syst Evol Microbiol* **51**, 447–455.
- Golovacheva, R. S. & Karavaiko, G. I. (1979). *Sulfobacillus* – a new genus of spore-forming thermophilic bacteria. Microbiology (English translation of *Mikrobiologiya*) **47**, 658–665.
- 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., Matsumura, T., Hara, Y., Niwa, M. & Yamasato, K. (2002a). *Alicyclobacillus herbarius* sp. nov., a novel bacterium containing ω -cycloheptane fatty acids, isolated from herbal tea. *Int J Syst Evol Microbiol* **52**, 109–113.
- Goto, K., Tanimoto, Y., Tamura, T., Mochida, K., Arai, D., Asahara, M., Suzuki, M., Tanaka, H. & Inagaki, K. (2002b). Identification of thermoacidophilic bacteria and a new *Alicyclobacillus* genomic species isolated from acidic environments in Japan. *Extremophiles* **6**, 333–340.
- Goto, K., Mochida, K., Asahara, M., Suzuki, M. & Yokota, A. (2002c). Application of the hypervariable region of the 16S rDNA sequence as an index for the rapid identification of species in the genus *Alicyclobacillus*. *J Gen Appl Microbiol* **48**, 243–250.
- Heyndrickx, M., Lebbe, L., Vancanneyt M. & 7 other authors (1997). A polyphasic reassessment of the genus *Aneurinibacillus*, reclassification of *Bacillus thermoaerophilus* (Meier-Stauffer *et al.*, 1996) as *Aneurinibacillus thermoaerophilus* comb. nov., and emended description of *A. aneurinilyticus* corrig., *A. migulanus*, and *A. thermoaerophilus*. *Int J Syst Bacteriol* **47**, 808–817.
- Hippchen, B., Roll, A. & Poralla, K. (1981). Occurrence in soil of thermo-acidophilic bacilli possessing ω -cyclohexane fatty acids and hopanoids. *Arch Microbiol* **129**, 53–55.
- Hiraishi, A., Inagaki, K., Tanimoto, Y., Iwasaki, M., Kishimoto, N. & Tanaka, H. (1997). Phylogenetic characterization of a new thermo-acidophilic bacterium isolated from hot spring in Japan. *J Gen Appl Microbiol* **43**, 295–304.
- Kannenbergh, E., Blume, A. & Poralla, K. (1984). Properties of ω -cyclohexane fatty acids in membranes. *FEBS* **172**, 331–334.
- Kasai, H., Tamura, T. & Harayama, S. (2000). Intrageneric relationship among *Micromonospora* species deduced from *gyrB*-based phylogeny and DNA relatedness. *Int J Syst Evol Microbiol* **50**, 127–134.
- Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitution through comparative studies of nucleotide sequence. *J Mol Evol* **16**, 111–120.
- Krischke, W. & Poralla, K. (1990). Properties of *Bacillus acidocaldarius* mutants deficient in ω -cyclohexyl fatty acid biosynthesis. *Arch Microbiol* **153**, 463–469.
- Kumar, S., Tamura, K., Jakobsen, I. B. & Nei, M. (2001). MEGA2: Molecular evolutionary genetics analysis software. *Bioinformatics* **17**, 1244–1245.
- Kusano, K., Yamada, H., Niwa, M. & Yamasato, K. (1997). *Propionibacterium cyclohexanicum* sp. nov., a new acid-tolerant ω -cyclohexyl fatty acid-containing propionibacterium isolated from spoiled orange juice. *Int J Syst Bacteriol* **47**, 825–831.
- Lake, J. A. (1987). A rate-independent technique for analysis of nucleic acid sequences: evolutionary parsimony. *Mol Biol Evol* **4**, 167–191.
- Manachini, P. L., Fortina, M. G., Parini, C. & Craveri, R. (1985). *Bacillus thermoruber* sp. nov. rev., a red-pigmented thermophilic bacterium. *Int J Syst Bacteriol* **35**, 493–496.
- 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* **52**, 1681–1685.
- Moore, B. S., Walker, K., Tornus, I., Handa, S., Poralla, K. & Floss, H. G. (1997). Biosynthetic studies of ω -cycloheptyl fatty acids in *Alicyclobacillus cycloheptanicus*. Formation of cycloheptanecarboxylic acid from phenylacetic acid. *J Org Chem* **62**, 2173–2185.
- Nazina, T. N., Tourova, T. P., Poltarau, A. B. & 8 other authors (2001). Taxonomic study of aerobic thermophilic bacilli: description of *Geobacillus subterraneus* gen. nov., sp. nov. and *Geobacillus uzenensis* sp. nov. from petroleum reservoirs and transfer of *Bacillus stearothermophilus*, *Bacillus thermocatenulatus*, *Bacillus thermoleovorans*, *Bacillus kaustophilus*, *Bacillus thermoglucosidasius* and *Bacillus thermodenitrificans* to *Geobacillus* as the new combinations *G. stearothermophilus*, *G. thermocatenulatus*, *G. thermoleovorans*, *G. kaustophilus*, *G. thermoglucosidasius* and *G. thermodenitrificans*. *Int J Syst Evol Microbiol* **51**, 433–446.
- Nicolaus, B., Improt, R., Manca, C. M., Lama, L., Esposito, E. & Gambacorta, A. (1998). Alicyclobacilli from an unexplored geothermal soil in Antarctica: Mount Rittmann. *Polar Biol* **19**, 133–141.
- Norris, P. R., Clark, D. A., Owen, J. P. & Waterhouse, S. (1996). Characteristics of *Sulfobacillus acidophilus* sp. nov. and other moderately thermophilic mineral-sulphide-oxidizing bacteria. *Microbiology* **142**, 775–783.

- Poralla, K., Härtner, T. & Kannenberg, E. (1984).** Effect of temperature and pH on the hopanoid content of *Bacillus acidocaldarius*. *FEBS Microbiol Lett* **23**, 253–256.
- Saitou, N. & Nei, M. (1987).** The neighbor-joining method: A new method for reconstruction of phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Suzuki, K., Saito, K., Kawaguchi, A., Okuda, S. & Komagata, K. (1981).** Occurrence of ω -cyclohexyl fatty acids in *Curtobacterium pusillum* strains. *J Gen Appl* **27**, 261–266.
- Tamaoka, J. & Komagata, K. (1984).** Determination of DNA base composition by reverse-phase high-performance liquid chromatography. *FEMS Microbiol Lett* **25**, 125–128.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994).** CLUSTAL W: Improving the sensitivity of progressive multiple sequence weighing, position-specific gap penalties and weight matrix choice. *Nucleic Acid Res* **22**, 4350–4354.
- Tomimura, E., Zeman, N. W., Frankiewicz, J. R. & Teague, W. M. (1990).** Description of *Bacillus naganoensis* sp. nov. *Int J Syst Bacteriol* **40**, 123–125.
- Touzel, J. P., O'Donohue, M., Debeire, P., Samain, E. & Breton, C. (2000).** *Thermobacillus xylanilyticus* gen. nov., sp. nov., a new aerobic thermophilic xylan-degrading bacterium isolated from farm soil. *Int J Syst Evol Microbiol* **50**, 315–320.
- Tsuruoka, N., Isono, Y., Shida, O., Hemmi, H., Nakayama, T. & Nishino, T. (2003).** *Alicyclobacillus sendaiensis* sp. nov., a novel acidophilic, slightly thermophilic species isolated from soil in Sendai, Japan. *Int J Syst Evol Microbiol* **53**, 1081–1084.
- Uchino, F. & Doi, S. (1967).** Acido-thermophilic bacteria from thermal waters. *Agric Biol Chem* **31**, 817–822.
- Wayne, L. G., Brenner, D. J., Colwell, R. R. & 9 other authors (1987).** International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.
- Wisotzkey, J. D., Jurtshuk, J. R. P., Fox, G. E., 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 a new genus, *Alicyclobacillus* gen. nov. *Int J Syst Bacteriol* **42**, 263–269.
- Yamamoto, S. & Harayama, S. (1995).** PCR amplification and direct sequencing of *gyrB* genes with universal primers and their application to the detection and taxonomic analysis of *Pseudomonas putida* strains. *Appl Environ Microbiol* **61**, 1104–1109.
- Yamazaki, K., Tezuka, H. & Shinano, H. (1996).** Isolation and identification of *Alicyclobacillus acidoterrestris* from acid beverages. *Biosci Biotechnol Biochem* **60**, 543–545.