Diversity of *Alicyclobacillus* isolated from fruit juices and their raw materials, and emended description of *Alicyclobacillus acidocaldarius*

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In the course of a microbiological survey of various beverages and their raw materials, 180 moderately thermophilic, acidophilic, spore-forming bacteria were isolated from several samples. Seven representative strains, selected from 180 isolates based on morphology and the hyper-variable region of the 16S rRNA gene, were identified as *Alicyclobacillus acidocaldarius, A. acidoterrestris* and *Alicyclobacillus* genomic species 1, according to their phenotypic, chemotaxonomic, genotypic and phylogenetic features. Cellular fatty acid composition, quinone species, and DNA base composition of the seven strains were similar to those of the type/reference strains. However, physiological and biochemical characteristics were variable within each species. Moreover, sequence analyses of the 16S rRNA and partial *gyrB* genes revealed that, in a few strains, the phylogeny derived from both molecules was not consistent. Results of a ribotype analysis also supported this genetic heterogeneity. These results indicate a high level of diversity within species in the genus *Alicyclobacillus* with regards to both phenotype and genotype. In addition, an emended description of *A. acidocaldarius* is given on the basis of obtained polyphasic taxonomic data.

Key words: *Alicyclobacillus acidocaldarius, A. acidoterrestris, Alicyclobacillus* genomic species 1, *gyrB*

INTRODUCTION

Members of the genus *Alicyclobacillus* are Gram-positive, moderately thermophilic (optimum temperature range for growth: 45-60℃), acidophilic (optimum pH range for growth: 3.5-5.0), aerobic, spore-forming bacilli. They had previously been assigned to the genus *Bacillus* (Darland & Brock, 1971; Deinhard *et al.*, 1987a, 1987b) and were reclassified as the novel genus *Alicyclobacillus* (Wisotzkey *et al.*, 1992) based on sequences of the 16S rRNA gene and the presence of unique ω-alicyclic fatty acids in the cellular membrane. Up until 2005, this genus comprised ten species, one subspecies and two genomic species, with the type species being *Alicyclobacillus acidocaldarius* (Albuquerque *et al.*, 2000; Dufresne *et al.*, 1996; Goto *et al.*, 2002a, 2002b, 2003; Karavaiko *et al.*, 2005; Matsubara *et al.*, 2002; Nicolaus *et al.*, 1998; Simbahan *et al.*, 2004; Tsuruoka *et al.*, 2003).

The bacteria inhabit natural environments, including hot springs and soils (Albuquerque *et al.*, 2000; Darland & Brock, 1971; Deinhard *et al.*, 1987b; Dufresne *et al.*, 1996; Goto *et al.*, 2002b; Hiraishi *et al.*, 1997; Karavaiko *et al.*, 2005; Nicolaus *et al.*, 1998; Simbahan *et al.*, 2004; Tsuruoka *et al.*, 2003; Uchino & Doi, 1967); however, they also contaminate various fruits, fruit juices, sugar syrups and other food stuffs (Borlinghaus & Engel, 1997; Cerny *et al.*, 1984; Deinhard *et al.*, 1987a; Duong & Jensen, 2000; Eiroa *et al.*, 1999; Goto *et al.*, 2002a, 2003; Jensen, 2000; Komitopoulou *et al.*, 1999; Matsubara *et al.*, 2002; Silva & Gibbs, 2000; Splittstoesser & Churey, 1996; Splittstoesser *et al.*, 1994; Walls & Chuyate, 2000; Wisse & Parish, 1998; Yamazaki *et al.*, 1996). Though spoilage of food does not always occur, contamination, mainly by *A. acidoterrestris*, causes the spoilage of beverages resulting in a strong, medicinal-

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like flavour (Borlinghaus & Engel, 1997; Cerny *et al.*, 1984; Duong & Jensen, 2000; Eiroa *et al.*, 1999; Jensen, 2000; Jensen & Whitfield, 2003; Komitopoulou *et al.*, 1999; Orr *et al.*, 2000; Pettipher *et al.*, 1997; Silva & Gibbs, 2000; Splittstoesser & Churey, 1996; Splittstoesser *et al.*, 1994; Walls & Chuyate, 2000; Wisse & Parish, 1998; Yamazaki *et al.*, 1996). Spoilage potential is influenced by the soluble oxygen concentration, type of packaging, storage temperature, and juice type, as well as the bacterial species and strains (Cerny *et al.*, 2000; Eiroa *et al.*, 1999; Orr *et al.*, 2000; Splittstoesser *et al.*, 1994, 1998). In addition to these factors, elimination from the processing environment and sterilization by conventional heat processes are not effective (Eiroa *et al.*, 1999; Silva & Gibbs, 2000; Splittstoesser *et al.*, 1998) and control of these bacteria is very difficult. Although *Alicyclobacillus* is not pathogenic (Walls & Chuyate, 1998), it is recognized as a nuisance bacterium in the food industry world-wide.

During the course of a microbiological survey of various beverages and their raw materials, we have isolated 181 moderately thermophilic acidophilic spore-forming bacteria. We have previously proposed a novel *Alicyclobacillus* species, *A. pomorum*, for an isolate from a mixed fruit juice (Goto *et al.*, 2003). In this report, we describe the polyphasic taxonomic characterisation of seven strains, selected from 180 isolates by morphological observation and analysis of the hyper-variable region of the 16S rRNA gene (HV region) (Goto *et al.*, 2000, 2002c, 2002d; Kato *et al.*, 2005), along with an emendation of the species *A. acidocaldarius*.

MATERIALS AND METHODS

Strains and culture conditions

The 180 strains used in this study were isolated from orange and lemon juices, concentrated orange, apple and watermelon juices, hyssop and striped bamboo leaves and various soft drinks (Table 1) 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 H_2SO_4). Type/reference strains of *Alicyclobacillus* species were obtained from the ATCC (American Type Culture Collection, Manassas, Virginia, USA), DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, German Collection of Microorganisms and Cell Cultures, Germany), IAM (Institute of Molecular and Cellular Biosciences, The University of Tokyo, Tokyo, Japan) and IFO (Institute for Fermentation, Osaka, Japan). *A. acidiphilus* TA 67^T was kindly provided by Motohiro Niwa (Kirin Beverage Corporation, Samukawa-machi, Kanagawa, Japan). *Alicyclobacillus* strains were grown in YSG medium at 50 or 60℃. *B. subtilis* IAM 12118^T was cultivated by the method recommended in the IAM strain catalogue of strains, 3rd edition (2004).

Morphological, physiological and biochemical characteristics

Unless otherwise indicated, morphological observations and biochemical tests were performed using the methods of Albuquerque *et al.* (2000), Darland & Brock (1971) and Deinhard *et al.* (1987a, b), either in BAM liquid or in BAM agar medium (Deinhard *et al.*, 1987a, b). Cell growth was estimated by measuring turbidity at 578 nm. The pH range for growth was determined at 45℃ (for *A. acidoterrestris*) and 60℃ (for *A. acidocaldarius* and *Alicyclobacillus* genomic species 1) in BAM medium, with the pH adjusted with 2 N H_2SO_4. Acidification was examined with API 50 CH test strips (bioMérieux, Marcy-l' Etoile, France) in BAM basal salts medium (Albuquerque *et al.*, 2000), at the optimum growth temperature.

Chemotaxonomic characterization

Cellular fatty acid and menaquinones have been analysed as described previously (Goto *et al.*, 2000, 2003). The DNA G+C content was determined by using the method of Tamaoka & Komagata (1984) using HPLC with a YMC-Pack ODS-AQ AQ302 column (4.6×150 mm; YMC) and 10 mM H_3PO_4–10 mM KH_2PO_4 (pH 3.5) as the mobile phase.

DNA extraction, sequencings, phylogenetic analysis and DNA-DNA hybridization

Genomic DNA was extracted using the QIAGEN Blood & Cell Culture DNA Maxi Kit (QIAGEN GmbH, Hilden, Germany), according to the QIAGEN Genomic DNA Handbook 09/97, and purified by equilibrium centrifugation in CsCl-ethidium bromide gradients (Treismen, 1989) using the Optima^{TM} MAX Ultracentrifuge (Beckman Coulter Inc., CA, USA). Desalting was performed using the Ultrafree-4 Centrifugal Filter Unit (Millipore, MA, USA).

Sequencing of the hyper-variable region of the 16S rRNA gene (HV region) and phylogenetic analyses were described previously (Goto *et al.*, 2002c). Nearly

Table 1. Strains and their sources

Source		Samples	Strains	Tested strains
Orange juice	Japan, Brazil, U.S.A.	13	46	7[a]
Lemon juice	Italy, U.S.A., Argentina, etc.	4	14	1
Conc. Orange juice	Brazil, U.S.A.	7	61	9[b]
Conc. Apple juice	Austria	4	16	2
Conc. Water melon juice	Thailand	2	10	2
Hyssop leaf	Hungary	1	6	1[c]
Striped bamboo leaf	China	1	4	2
Soft drink	Japan, U.S.A.	5	23	3[d]
Total			180	27

[a] including strains OJ5 (subgroup 5) and OR3 (*A. acidoterrestris*).
[b] including strains P2 (*Alicyclobacillus* genomic species 1) and SO-6 (subgroup 6).
[c] including strain HP2 (subgroup 4).
[d] including strains 3B (subgroup 2) and 3W (subgroup 3).

complete 16S rRNA gene sequences were determined using the 16S rRNA Gene Kit following the protocols of the manufacturer (Applied Biosystems, CA, USA). Nucleotide sequences of *gyrB* genes were determined directly from PCR fragments using the methods described by Goto *et al.* (2003), Kasai *et al.* (2000) and Yamamoto & Harayama (1995). The topologies of the phylogenetic trees constructed by the neighbor-joining (Saitou & Nei, 1987), maximum likelihood (Sneath & Sokal, 1973) and unweighted pair-group methods (Felsenstein, 1981) were almost identical, accordingly only the phylogenetic trees constructed by the neighbor-joining method are presented.

Nucleotide accession numbers of 16S rRNA gene sequences determined or used in this study were as follows: (type/reference strains) *A. acidiphilus* TA67^T, AB059677; *A. acidocaldarius* subsp. *acidocaldarius* ATCC 27009^T, AB042056; *A. acidocaldarius* subsp. *rittmannii* DSM 11297^T, AB089859; *A. acidoterrestris* ATCC 49025^T, AB042057; AB222247; *A. cycloheptanicus* DSM 4006^T, AB042059; *A. disulfidooxidans* DSM 12064^T, AB089843; *Alicyclobacillus* genomic species 1 DSM 11984, AB059668; *Alicyclobacillus* genomic species 2 MIH332, AB060165; *A. herbarius* CP1^T, AB042055; *A. hesperidum* DSM 12489^T, AB059678; *A. pomorum* 3A^T, AB089840; *A. sendaiensis* JCM 11817^T, *A. tolerans* DSM 16297^T, AB222265; *A. vulcanalis* DSM 16176^T, AB222267 and *B. subtilis* IAM 12118^T, AB042061; (isolates) 3B, AB222253; 3W, AB222255; HP2, AB222261; OJ5, AB222257; OR3, AB222263; P2, AB222251 and SO-6, AB222259.

Nucleotide accession numbers of *gyrB* gene sequences either determined or used in this study were as follows: (type/reference strains) *A. acidiphilus* TA67^T, AB089850; *A. acidocaldarius* subsp. *acidocaldarius* ATCC 27009^T, AB089846; *A. acidocaldarius* subsp. *rittmannii* DSM 11297^T, AB089847; *A. acidoterrestris* ATCC 49025^T, AB089852; *Alicyclobacillus* genomic species 1 DSM 11984, AB089849; *Alicyclobacillus* genomic species 2 MIH332, AB222250; *A. cycloheptanicus* DSM 4006^T, AB089854; *A. disulfidooxidans* DSM 12064^T, AB089855; *A. herbarius* CP1^T, AB089853; *A. hesperidum* DSM 12489^T, AB089851; *A. pomorum* 3A^T, AB089845; *A. sendaiensis* JCM 11817^T, AB222248; *A. tolerans* DSM 16297^T, AB222266; *A. vulcanalis* DSM 16176^T, AB222268 and *B. subtilis* IAM 12118^T, AB099104; (isolates) 3B, AB222254; 3W, AB222256; HP2, AB222262; OJ5, AB222258; OR3, AB222264; P2, AB222252 and SO-6, AB222260.

DNA-DNA hybridization experiments were performed using the method of Ezaki *et al.* (1989) and employed photobiotin-labelled DNA probes and microplates. Hybridization was carried out under stringent conditions (optimal re-naturation temperature+15℃) for 3 hours. Based on the DNA G+C contents of type strains of *A. acidocaldarius* subsp. *acidocaldarius* (61.9%) and *A. acidoterrestris* (52.7%), the re-naturation temperatures were calculated at 65℃ for *A. acidocaldarius* group and 46℃ for *A. acidoterrestris*.

Ribotyping analysis

Strains were grown on YSG agar at 45 or 60℃ for 48 h. Automated ribotyping was performed using the instruments and conditions described for the RiboPrinter microbial characterization system (DuPont-Qualicon, Wilmington, DE, USA). The banding patterns for *Eco*RI ribotyping were read using GelConvert software (DuPont-Qualicon). Cluster analysis of the ribotype patterns was based on the UPGMA method (Sethi, 1997; Sokal & Michener, 1958) using the software BioNumerics 3.0 (Applied Maths, Sint-Martens-Latem, Belgium). According to the guidelines for the system, greater than 85% similarity among strains is regarded as similar species. *A.acidoterrestris* DSM 3923 and DSM 3924, which showed more than 85% DNA-DNA similarity values to *A. acidoterrestris* ATCC 49025^T, were used as positive controls (Goto *et al*., 2002c). The experiments were repeated in triplicate at least.

RESULTS

Grouping of isolates

A total of 180 moderately thermophilic, acidophilic, aerobic, spore-forming bacilli have been isolated from fruit juices, herb teas and soft drinks (Table 1). On the basis of the morphology and origin of the isolates, 27 strains were chosen for HV region analysis (Goto *et al*., 2000, 2002c). Colony morphology was typically flat, smooth, and creamy white, however, strain 3W from a soft drink had irregular convex, undulant and translucent white colonies. Based on the results of the HV region analysis (Goto *et al*., 2002c), the strains were tentatively identified as *A. acidocaldarius* (25 strains, including 3W), *A. acidoterrestris* (one strain: OR3), and *Alicyclobacillus* genomic species 1 (one strain: P2). The *A. acidocaldarius* isolates were further divided into six subgroups, 1 (eight strains, represented by ATCC 27009^T), 2 (one strain, *ibid*. strain 3B), 3 (one strain, *ibid*. strain 3W), 4 (six strains, *ibid*. strain P2), 5 (five strains, *ibid*. strain OJ5), and 6 (four strains, *ibid*. strain SO-6) based on HV region sequence differences (Table 2). Finally, one strain of *A. acidoterrestris*, one strain of *Alicyclobacillus* genomic species 1, and five strains of *A. acidocaldarius* which were representatives of each of subgroups 2 to 6 were used for further taxonomic studies.

Phenotypic characteristics

All strains were Gram-positive but Gram-variable in old cultures, motile rods (3.0-5.0 μm long and 0.8-1.0 μm wide) and formed oval-ellipsoidal spores subterminally in swollen sporangia. Growth of strains 3B, 3W, HP2, OJ 5, SO-6 and P2 occurred at 35 and 70℃, but not at 30 or 75℃. Optimal growth temperature for the strains was in the range of 60-65℃. Growth of strain OR3 occurred at 20 and 55℃, but not at 15 or 60℃. The optimal growth temperature for this strain was in the range of 45-50℃. All strains were very similar in the range of pH that enabled growth (2.5-6.0) as well as the optimal pH (3.5-4.5). None of the strains grew under anaerobic conditions. All strains grew on BAM containing 2% (w/v) NaCl. All strains were catalase positive, but oxidase and nitrate reductase negative. Gelatin was hydrolysed, but no hydrolysis of phenylalanine or tyrosine was observed. All strains produced acid from D-glucose, D-mannose, maltose and cellobiose, but not from D-arabinose, dulcitol, *N*-acetyl-glucosamine, inulin, D-fucose, D-lyxose, gluconate and 2-ketogluconate. The differential phenotypic characteristics of the strains as well as the *Alicyclobacillus* type/reference strains are shown in Table 3.

DNA base, menaquinone and fatty acid compositions

The DNA G+C base composition of the strains ranged from 51.5-62.9 mol% (Table 4). All strains possessed MK-7 as the major menaquinone, reaching levels as high as 80-97% of the total. MK-3 was also present at levels between 3 and 20% (data not shown). The major fatty acid was ω-cyclohexyl 17 : 0 (44.9-68.7%) and the relative proportions of ω-cyclohexane fatty acids (ω-cyclohexyl 17 : 0 and ω-cyclohexyl 19 : 0) ranged between 76.4 and 95.1%. The remainder was a mixture of straight- and branched-chain fatty acids (data not shown). These chemotaxonomic features were consistent within each species.

Sequence comparison and phylogenetic analysis

Nearly complete 16S rRNA gene sequences (1,489-1,521 bp) were determined for seven selected isolates (3B, 3W, HP2, OJ5, OR3, P2 and SO-6), *A. tolerans* DSM 16297^T and *A. vulcanalis* DSM 16176^T. Sequence data were compared and phylogenetic analyses were performed. Strain OR3

Table 2. Mismatch, gap and polymorphic position numbers among *A. acidocaldarius* strains in HV region of the 16S rRNA gene

		Strain	1		2		3		4		5		6	
			M	N	M	N	M	N	M	N	M	N	M	N
1		subsp. *acidocaldarius* ATCC 27009^T	–	–										
2		3B	0	8	–	–								
3		3W	1	8	0	9	–	–						
4	*A. acidocaldarius*	HP2	1	2	2	7	2	6	–	–				
5		OJ5	0	5	0	7	0	8	2	4	–	–		
6		SO-6	0	4	0	7	2	6	3	2	0	4	–	–
7		subsp. *rittmannii* DSM 11297^T	0	2	0	7	0	6	0	0	0	4	0	2

Abbreviations: M: mismatch position number; N, gap and polymorphic position number.

clustered with *A. acidoterrestris* ATCC 49025^T with a sequence similarity of 99.1%. Strain P2 clustered with *Alicyclobacillus* genomic species 1 DSM 11984 and had 100% sequence similarity. These values were calculated excluding 31 gaps and 14 polymorphic positions. Strains 3B, 3W, HP2, OJ5 and SO-6 clustered with *A. acidocaldarius* ATCC 27009^T showed sequence similarities between 99.2 and 100%. Gaps (0-3 positions) and polymorphic positions (1-19 positions) were also excluded from these calculations. These five strains also showing 99.2-99.9% sequence similarity to *A. acidocaldarius* subsp. *rittmannii* DSM 11297^T. Gaps (0-2 positions) and polymorphic positions (2-15 positions) were excluded. In the 16S rRNA gene-based tree (Fig. 1), the seven selected isolates formed a cluster with *A. acidocaldarius*, *A. acidoterrestris* and *Alicyclobacillus* genomic species 1 at high bootstrap values.

In order to clarify relationships in detail, partial *gyrB* gene sequences were determined for the seven isolates (3B, 3W, HP2, OJ5, OR3, P2 and SO-6; 1,167 bp), *A. tolerans* DSM 16297^T (1,170 bp) and *A. vulcanalis* DSM 16176^T (1,167 bp), and the sequences were compared to infer the phylogeny. Sequence similarities among HP2, OJ5, P2 and several species of the *A. acidocaldarius* group are summarized in Table 5. Clustering patterns were similar between the nucleotide sequence-based tree (Fig. 2) and amino acid-based tree (data not shown). The tree derived from nucleotide sequences showed higher bootstrap values and greater divergence compared to the amino acid-based tree. Strain OR3 clustered with *A. acidoterrestris* ATCC 49025^T having identical sequences, while the 16S rRNA gene sequence of strain OR3 differed from that of the type strain. Strain P2 clustered with *Alicyclobacillus* genomic species 1 DSM 11984, however, it was more closely related to strain OJ5 that formed a cluster with *A. acidocaldarius* in the 16S rRNA gene-based tree. The *gyrB* gene sequence of strain OJ5 was more similar to that of *Alicyclobacillus* genomic species 1 than *A. acidocaldarius*. Strains 3B, 3W and SO-6 clustered with *A. acidocaldarius* ATCC 27009^T and the nucleotide sequence similarities among these strains ranged from 98.0 to 98.8%. Amino acid sequences for strains ATCC 27009^T, 3B and 3W were identical, and differed from that of strain SO-6 at only one position. On the other hand, strain HP2 was located with the *A. acidocaldarius* group in the *gyrB* gene-based tree but did not cluster with the species or subspecies. The *gyrB* gene-derived phylogenies were more informative than the 16S rRNA gene-based tree, however, their topologies were not congruent in all cases.

DNA-DNA relatedness

The results of DNA-DNA hybridization experiments are summarized in Table 4. DNA-DNA similarities between isolates and the type/reference strains of *Alicyclobacillus* were more than 70% within each cluster of the 16S rRNA gene-based tree (Fig. 1). DNA-DNA similarity between *A. acidocaldarius* subsp. *rittmannii* DSM 11297^T and *A. acidocaldarius* ATCC 27009^T was reported to be 69.7% in a previous paper (Nicolaus *et al*., 1998), however, in this study, strain DSM 11297^T showed 75 and 81% similarities to strain ATCC 27009^T. On the other hand, strains of *A. acidocaldarius* and *Alicyclobacillus* genomic species 1 showed intermediate DNA-DNA similarities (52-68%) to each other.

Table 3. Biochemical characteristics cf isolates and *Alicyclobacillus* type/reference strains

Characteristics	*Alicyclobacillus* genomic species 1		*A. acidocaldarius*							*A. acidoterrestris*	
	DSM 11984	P2	subsp. *acidocaldarius* ATCC 27009^{T}	3B	3W	OJ5	SO-6	HP2	subsp. *rittmannii* DSM 11297^{T}	ATCC 49025^{T}	OR3
Hydrolysis of starch	+	+	+	−	−	−	−	−	+	−	−
Growth in 5% NaCl	−	−	−	−	−	−	−	−	−	+	+
Acid from											
glycerol	+	+	+	−	−	−	+	+	+	+	+
erythritol	−	−	−	−	−	−	−	+	−	+	+
L-arabinose	+	+	+	+	+	+	+	+	−	+	+
ribose	+	+	+	+	+	+	+	+	−	+	+
D-xylose	+	+	+	+	−	+	+	+	+	+	+
L-xylose	−	−	−	−	−	−	−	+	−	−	−
adonitol	−	−	−	−	−	−	−	−	−	+	+
methyl *β*-xyloside	−	−	−	−	−	−	−	+	−	−	−
D-galactose	+	+	+	+	+	−	+	+	+	+	+
D-fructose	+	+	+	+	−	+	+	+	+	+	+
L-sorbose	−	+	+	+	+	+	+	−	−	−	−
rhamnose	+	+	+	−	−	−	−	+	−	+	+
inositol	−	−	−	−	−	−	−	+	−	+	−
mannitol	+	+	+	+	−	+	−	+	+	+	+
sorbitol	−	−	−	−	−	−	−	−	−	+	+
methyl *α*, D-mannoside	−	+	−	+	−	−	+	+	−	+	−
methyl *α*, D-glucoside	−	+	+	+	+	+	+	+	+	+	+
amygdalin	−	−	−	+	−	−	−	+	−	+	−
arbutin	+	+	+	+	+	−	+	+	+	+	+
aesculin	−	+	+	−	−	+	+	−	+	+	+
salicin	+	−	−	+	+	−	−	+	+	+	+
lactose	+	−	+	+	+	−	+	+	+	+	+
melibiose	−	+	+	−	−	−	+	−	+	−	−
sucrose	+	+	+	+	+	+	+	+	+	+	−
trehalose	+	+	+	−	+	+	+	+	+	+	−
melezitose	+	+	−	+	−	+	+	+	−	+	−
D-raffinose	+	+	−	−	−	−	+	−	+	−	−
starch	−	−	−	+	−	−	−	−	−	−	−
glycogen	+	−	+	+	−	−	−	−	+	−	−
xylitol	−	−	−	−	−	−	+	−	−	+	+
β-gentiobiose	+	+	−	+	+	+	+	+	+	+	+
D-turanose	+	+	+	+	+	+	+	−	+	+	−
D-tagatose	+	+	+	+	+	+	+	−	+	−	−
L-fucose	−	−	−	−	+	−	−	−	−	−	−
D-arabitol	−	−	−	−	−	−	−	−	−	+	+
L-arabitol	−	−	−	−	−	−	−	−	−	−	+
5-keto-gluconate	−	−	−	+	−	−	−	−	−	−	−

Abbreviations: +; positive, −; negative
Boxes indicate the identical features in the species.

Table 4. DNA base composition, DNA-DNA similarity (%) of isolates and several *Alicyclobacillus* type/reference species

	Strain	G+C content (mol%)	ATCC 27009^T	DSM 11297^T	DSM 11984	ATCC 49025^T
A. acidocaldarius	subsp. *acidocaldarius* ATCC 27009^T	61.9	**100**	75	68	20
	3B	62.7	93		56	
	3W	62.9	89		65	
	OJ5	62.5	75		54	
	SO-6	62.4	92		65	
	HP2	62.2	70		57	
	subsp. *rittmannii* DSM 11297^T	62.7	81	**100**	63	
Alicyclobacillus genomic species 1	DSM 11984	61.4	66	60	**100**	12
	P2	62.1	52		91	
A. acidoterrestris	ATCC 49025^T	52.7	11	8	11	**100**
	OR3	51.5				72

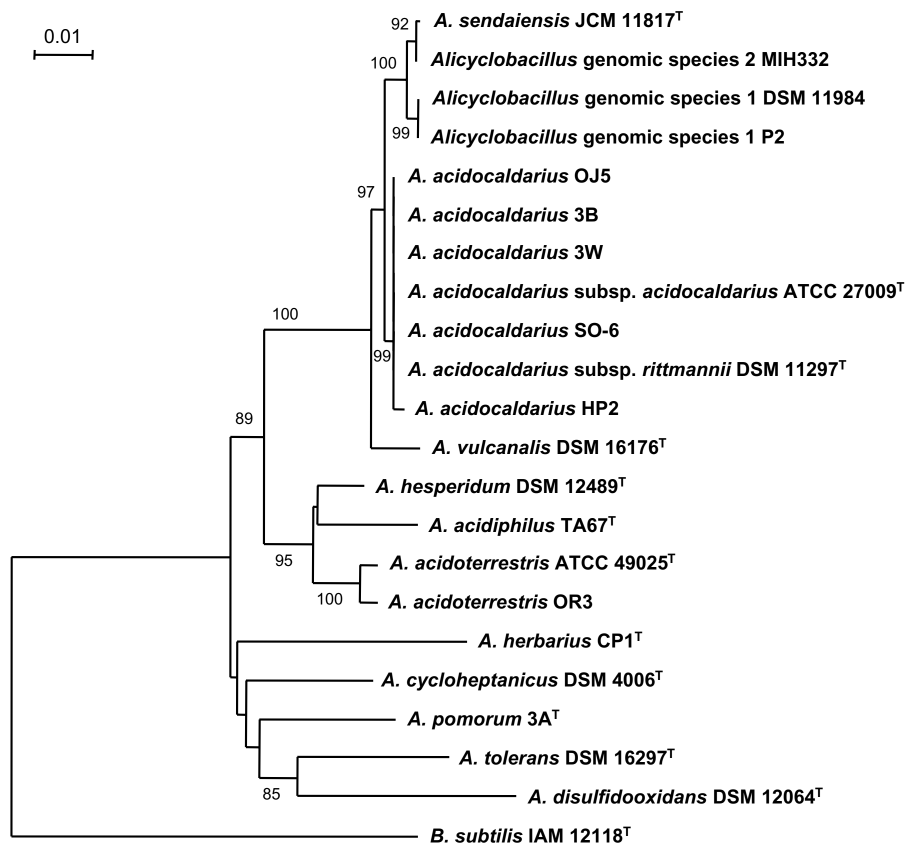

Fig. 1 Phylogenetic tree of *Alicyclobacillus* strains, based on neighbour-joining, derived from an alignment comprising 16S rRNA gene sequences

B. subtilis served as the outgroup. The dendrograms, constructed using the neighbor-joining method, were based on a comparison of 1,428 nt. The dataset was resampled 1,000 times by using the bootstrap option and the percentage values are given at the nodes. Scale bar indicates the number of substitutions per nucleotide position.

Table 5. *gyrB* gene nucleotide sequence and the amino acid sequence similarity (%) matrix for the strains of *A. acidocaldarius* and *Alicyclobacillus* genomic species 1

		Strain	*A. acidocaldarius*				*Alicyclobacillus* genomic species 1	
			1	2	3	4	5	6
1	*A. acidocaldarius*	subsp. *acidocaldarius* ATCC 27009^T	**100**	91.3	90.3	94.4	91.1	91.3
2		OJ5	97.2	**100**	87.5	89.9	94.9	99.0
3		HP2	98.2	96.7	**100**	91.4	87.8	87.7
4		subsp. *rittmannii* DSM 11297^T	99.2	96.7	98.2	**100**	90.1	89.9
5	*Alicyclobacillus* genomic species 1	DSM 11984	96.7	99.0	96.7	96.1	**100**	98.7
6		P2	97.4	99.7	96.9	96.9	94.5	**100**

Lower-left diagonal corner of the table indicates nucleotide sequence similarities and upper-right diagonal corner indicates amino acid sequence similarities.

Ribotyping analysis

In order to investigate genotypic heterogeneity within each species in more detail, strains of *A. acidocaldarius*, *Alicyclobacillus* genomic species 1 and *A. acidoterrestris* were subjected to ribotyping analysis. The results are summarized in Table 6. According to the results, *A. acidoterrestris* DSM 3923 and DSM 3924 showed more than 90% similarity to *A. acidoterrestris* ATCC 49025^T. None of the isolates, however, showed more than 85% similarity to the type strains, which is the index for regarding species as being the same (Sethi, 1997; Sokal & Michener, 1958).

Based on all obtained results, strains 3B, 3W, HP2, OJ5 and SO-6 were identified as *A. acidocaldarius*, strain P2 was identified as *Alicyclobacillus* genomic species 1, and strain OR3 was identified as *A. acidoterrestris*, according to the scheme of Wayne *et al.* (1987). These results also indicated that *A. acidocaldarius* is indistinguishable at the subspecies level, thus *A. acidocaldarius* subsp. *rittmannii* DSM 11297^T should be incorporated in *A. acidocaldarius*.

DISCUSSION

This paper describes an investigation into the distribution of *Alicyclobacillus* in beverages and their raw materials. While actual sources of *Alicyclobacillus* are not limited to those in this study (Goto *et al.*, 2002b; Hiraishi *et al.*, 1997; Uchino & Doi, 1967), the results of this survey revealed that a large number of raw materials for beverage production and the final products were contaminated by *Alicyclobacillus* (Table 1). These strains were roughly grouped as *A. acidocaldarius*, *A. acidoterrestris* and *Alicyclobacillus* genomic species 1, with the majority of isolates being *A. acidocaldarius*, which does not normally grow in these products. In the present survey, it was also noted that *A. acidoterrestris,* which can often grow and produce a medicine-like odor in these products, was only a minor contaminant. These results indicated that there is little diversity of the genus *Alicyclobacillus* at the species level in these sources, though *A. acidiphilus* (Matsubara *et al.*, 2002), *A. herbarius* (Goto *et al.*, 2002a) and *A. pomorum* (Goto *et al.*, 2003) have been isolated only rarely.

Chemotaxonomic features (fatty acids composition, quinone type and DNA G+C content) of the isolates were very similar within each species. On the other hand, physiological and biochemical characteristics of *A. acidocaldarius* varied within the species. Strains of *A. acidoterrestris* and *Alicyclobacillus* genomic species 1 seemed to have relatively similar characteristics within the species (Table 3), however, these similarities may have been due to lack of test strains compared to similar studies (Goto *et al.*, 2002b, 2002c; Yamazaki *et al.*, 1996). The physiological and biochemical characteristics of these species are typically rich in diversity making it difficult to find common characteristics to define or identify *Alicyclobacillus* species (Goto *et al.*, 2002b, 2002c). *Alicyclobacillus* strains have also been isolated from spoiled beverages and the causative strains used in challenge tests to evaluate the potential for spoilage. However, these strains do not always show the same growth patterns in the same food and bever-

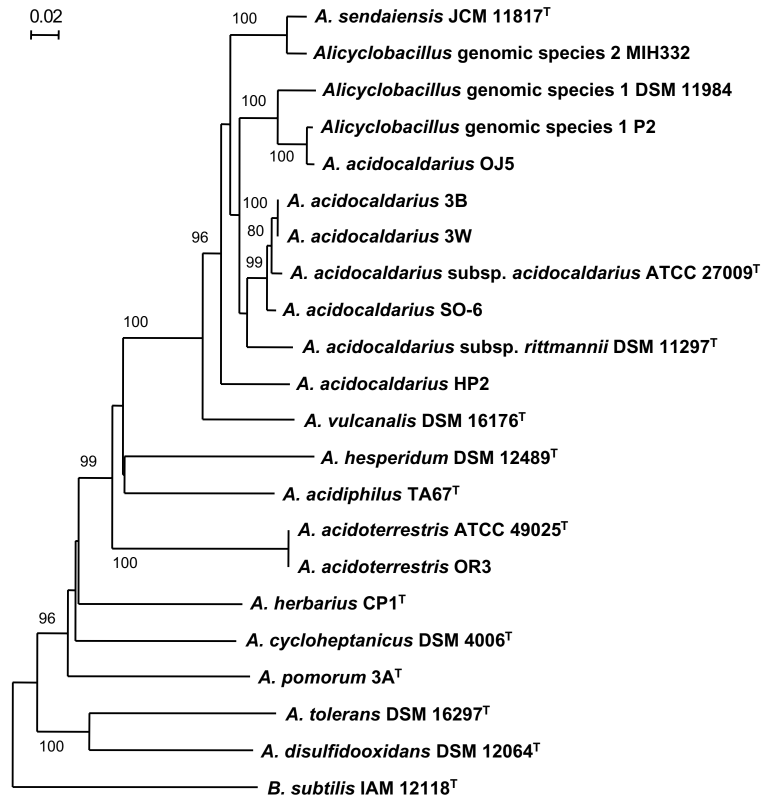

Fig. 2 Phylogenetic tree of *Alicyclobacillus* strains, based on neighbour-joining, derived from an alignment comprising *gyrB* gene sequences

B. subtilis served as the outgroup. The dendrograms, constructed using the neighbor-joining method, were based on a comparison of 1,151 nt. The dataset was resampled 1,000 times by using the bootstrap option and the percentage values are given at the nodes. Scale bar indicates the number of substitutions per nucleotide position.

age products. This phenomenon may be due to the original diversity of phenotypes in *Alicyclobacillus*. Alternatively, diversity and adaptation may have arisen from the strains having survived and grown with the products or raw materials acting as an enrichment medium.

This diversity was also observed in the genotypes. DNA-DNA hybridization results supported the results of the phylogenetic analysis based on 16S rRNA gene sequences. Several strains, however, revealed minimal DNA-DNA similarity for being the same species (70–75%) as a type/reference strain (Table 4). A large number of polymorphisms also existed in the HV region (Table 2) as compared with other bacteria (Goto *et al.*, 2000, 2002c, d; Kato *et al.*, 2005). Moreover, all ribotype similarities were under 85% (Table 6), indicating that this genotype was specific for each tested strain. Ribotyping analysis within *A. acidocaldarius* subgroups was not examined, however, these data suggest a certain genotypic heterogeneity in *Alicyclobacillus*. On the other hand, strains of the *A. acidocaldarius* group and *Alicyclobacillus* genomic species 1 showed intermediate DNA-DNA similarities of 52–68% to each other, though *A. acidocaldarius* subsp. *rittmannii* DSM 11297^T showed high similarities (75 and 81%) to the type strain of *A. acidocaldarius* subsp. *acidocaldarius*. These results indicate that *Alicyclobacillus* genomic species 1, instead of *A. acidocaldarius* subsp. *rittmannii*, is a subspecies of *A. acidocaldarius*. Investigation into the validity of recognizing *Alicyclobacillus* genomic species 1 as a formal species is in progress.

Relationships between *Alicyclobacillus* strains

Table 6. Ribotype similarities (%) among isolates and the related *Alicyclobacillus* type/reference strains

		Strain	1	2	3	4	5	6	7	8	9	10	11
1		subsp. *acidocaldarius* ATCC 27009^T	100										
2		3 B	52	100									
3		3 W	45	38	100								
4	*A. acidocaldarius*	OJ 5	31	41	55	100							
5		SO 6	37	64	58	70	100						
6		HP 2	28	21	42	51	38	100					
7		subsp. *rittmannii* DSM 11297^T	39	53	32	45	51	21	100				
8	*Alicyclobacillus*	DSM 11984	27	35	34	47	39	27	45	100			
9	genomic species 1	P 2	28	30	23	47	30	31	59	68	100		
10		ATCC 49025^T										100	
11	*A. acidoterrestris*	OR 3										44	100
		DSM 3923										92	46
		DSM 3924										99	47

Values presented are the means of three or more independent experiments.

were more clearly resolved using the *gyrB* gene analysis, however, the results differed slightly compared to the 16S rRNA gene phylogeny (Fig. 1 and Fig. 2). The phylogenetic relationships inferred from 16S rRNA gene analysis were supported by DNA-DNA hybridization data, but not by the *gyrB* gene analysis. On the contrary, in *Acinetobacter*, both of the data from DNA-DNA hybridization and phenotypic characterization supported the relationships proposed by *gyrB* gene analysis, but not the 16S rRNA gene analysis (Yamamoto & Harayama, 1996). These results suggested that an appropriate marker gene for the classification and identification of bacterial species varies depending on the taxon being investigated. The recommendation that phylogenetic analyses from more than three housekeeping genes are necessary in order to use phylogenetic relationships as a basis for taxonomic assignment may be reasonable (Stackebrandt *et al.*, 2002).

High diversity in phenotype and genotype is not restricted to *Alicyclobacillus* but is also seen in *Lactobacillus* (Gatti *et al.*, 1999; Giraffa *et al.*, 2000) and acetic acid bacteria (Lisdiyanti *et al.*, 2003). The contrary has been noted for the genus *Methylobacterium* (Kato *et al.*, 2005). It is interesting that strain diversity levels vary by taxon; the heterogeneity seen within *Alicyclobacillus* species, not only with regards to phenotype but also genotype, requires further work to accurately characterize these variations. We consider that the *Alicyclobacillus* strains, which have been isolated from various beverages and their raw materials, are of much value as microbial resources, necessitating their deposition in an appropriate culture collection for further studies by academia and industry.

Based on several characteristics in comparison with *A. acidocaldarius* ATCC 27009^T, strain MR1^T (= DSM 11297^T) was named as *A. acidocaldarius* subsp. *rittmannii* in 1998 (Nicolaus *et al.*, 1998) and this was validated in 2002 (Validation List No. 84). However, according to our data, the differential characteristics are thought to be a part of the diversity within *A. acidocaldarius*, thus *A. acidocaldarius* subsp. *rittmannii* should be included in *A. acidocaldarius*.

Emendation of the species *Alicyclobacillus acidocaldarius* (Darland and Brock 1971) Wisotzkey *et al.*, 1992

Basonym: *Alicyclobacillus acidocaldarius* subspecies *rittmannii* Nicolaus *et al.* 1998. *Alicyclobacillus acidocaldarius* subspecies *acidocaldarius* which was automatically created according to the International Code of Nomenclature of Bacteria (1990 Revision) Rule 46.

The description of *Alicyclobacillus acidocaldarius* is as given previously (Darland & Brock, 1971; Wisotzkey *et al.*, 1992), with the following amendments. No growth occurs in the presence of 5%

NaCl. Catalase is positive. Negative for oxidase and nitrite reduction. Gelatinase-positive, but variable for starch hydrolysis. The 16S rRNA gene sequence exhibits more than 99.2% similarities within species. The DNA G+C content is 61.9–62.5 mol% (as determined by the HPLC method).

Differential characteristics of *A. acidocaldarius* subsp. *rittmannii* as described previously (Nicolaus *et al.*, 1998) are covered by the above description of *A. acidocaldarius*.

Type strain: ATCC 27009=DSM 446=JCM 5260 =NBRC 15652=KCTC 1825=BCRC 14685=CCUG 28 521=CIP 106131=HAMBI 2071=HAMBI 2073=LMG 7119=NCCB 89167=NCIMB 11725=NRRL B-14509.

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飲料およびその原料より分離された *Alicyclobacillus* 属細菌の多様性，および *A. acidocaldarius* の記載修正

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様々な清涼飲料およびその原料に関する微生物調査の一環として，総計180菌株の中度好熱性，好酸性有胞子細菌を幾つかの検体より分離した．これらの菌株から形態および16S rRNA遺伝子の高度多様性領域の差違に基づき7菌株を選抜し，多相分類学的な試験を行った結果，それぞれ *A. acidocaldarius*，*A. acidoterrestris* および *Alicyclobacillus* genomic species 1と同定された．これら7菌株の菌体脂肪酸組成，キノン分子種およびDNAの塩基組成はそれぞれの基準・標準株と類似する一方，生理・生化学的性状には多くの相違（多様性）が認められた．さらに，16S rRNAおよび *gyrB* 遺伝子塩基配列の分子系統解析の結果，菌株によっては両分子系統樹間で系統の不一致が認められた．リボタイプ解析の結果もまた遺伝的な不均質性を支持した．以上の結果，*Alicyclobacillus* 属細菌における表現形質および遺伝子型は種内で高い多様性を示すことが示唆された．併せて，得られた試験結果に基づき，*A. acidocaldarius* の定義修正に関する記載を行った．