Proposal of six species of moderately thermophilic, acidophilic, endospore-forming bacteria: *Alicyclobacillus contaminans* sp. nov., *Alicyclobacillus fastidiosus* sp. nov., *Alicyclobacillus kakegawensis* sp. nov., *Alicyclobacillus macrosporangiidus* sp. nov., *Alicyclobacillus sacchari* sp. nov. and *Alicyclobacillus shizuokensis* sp. nov.

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Moderately thermophilic, acidophilic, spore-forming bacteria (146 strains) were isolated from various beverages and environments. Based on the results of sequence analysis of the hypervariable region of the 16S rRNA gene, eight of the strains represent novel species of the genus *Alicyclobacillus*. These strains were designated 3-A191^T, 4-A336^T, 5-A83J^T, 5-A167N, 5-A239-2O-A^T, E-8, RB718^T and S-TAB^T. Phylogenetic analyses of 16S rRNA and DNA gyrase B subunit (*gyrB*) nucleotide sequences confirmed that the eight strains belonged to the *Alicyclobacillus* clade. Cells of the eight strains were Gram-positive or Gram-variable, strictly aerobic and rod-shaped. The strains grew well under acidic and moderately thermal conditions, produced acid from various sugars, contained menaquinone 7 as the major isoprenoid quinone and did not produce guaiacol. ω -Alicyclic fatty acids were the predominant lipid component of strains 4-A336^T, 5-A83J^T, 5-A167N, RB718^T and S-TAB^T. No ω -alicyclic fatty acids were detected in strains 3-A191^T, 5-A239-2O-A^T or E-8, but iso- and anteiso-branched fatty acids and small amounts of straight-chain saturated fatty acids were detected instead. According to the DNA–DNA hybridization data and distinct morphological, physiological, chemotaxonomical and genetic traits, the eight strains represent six novel species within the genus *Alicyclobacillus*, for which the following names are proposed: *Alicyclobacillus contaminans* sp. nov. (type strain 3-A191^T = DSM 17975^T = IAM 15224^T), *Alicyclobacillus fastidiosus* sp. nov. (type strain S-TAB^T = DSM 17978^T = IAM 15229^T), *Alicyclobacillus kakegawensis* sp. nov. (type strain 5-A83J^T = DSM 17979^T = IAM 15227^T), *Alicyclobacillus macrosporangiidus* sp. nov. (type strain 5-A239-2O-A^T = DSM 17980^T = IAM 15370^T), *Alicyclobacillus sacchari* sp. nov. (type strain RB718^T = DSM 17974^T = IAM 15230^T) and *Alicyclobacillus shizuokensis* sp. nov. (type strain 4-A336^T = DSM 17981^T = IAM 15226^T).

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *gyrB* gene sequences are AB264020–AB264027 and AB264028–AB264035, respectively.

A table detailing the DNA base compositions and DNA–DNA hybridization values between the novel isolates and type strains of related *Alicyclobacillus* species is available as supplementary material with the online version of this paper.

At the end of 2005, the Gram-positive genus *Alicyclobacillus* comprised 11 species (Darland & Brock, 1971; Deinhard *et al.*, 1987a, b; Wisotzkey *et al.*, 1992; Dufresne *et al.*, 1996; Albuquerque *et al.*, 2000; Goto *et al.*, 2002a, 2003; Matsubara *et al.*, 2002; Tsuruoka *et al.*, 2003; Simbahan *et al.*, 2004; Karavaiko *et al.*, 2005), two subspecies (Nicolaus *et al.*, 1998) and two genomic species (Albuquerque *et al.*, 2000; Goto *et al.*, 2002b). The type species is *Alicyclobacillus acidocaldarius* (Darland & Brock, 1971; Wisotzkey *et al.*, 1992). The genus is characterized by moderately thermophilic, acidophilic, strictly aerobic, endospore-forming bacilli with ω -alicyclic fatty acids (ω -cyclohexane or ω -cycloheptane) as the major membrane lipid component. Exceptions are *Alicyclobacillus disulfidooxidans*, which grows under mesophilic conditions, and *Alicyclobacillus pomorum*, which does not contain any ω -alicyclic fatty acids. The bacteria mainly inhabit hot springs and soil, but can also infest fruit and crops (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). Some species, in particular *Alicyclobacillus acidoterrestris*, can cause deterioration of beverages by the production of guaiacol, which has a creosote-like odour (Cerny *et al.*, 1984; Sprittstoesser *et al.*, 1994; Borlinghaus & Engel, 1997; Wisse & Parish, 1998; Eiroa *et al.*, 1999; Duong & Jensen, 2000; Jensen, 2000). Accordingly, detection of contaminating *Alicyclobacillus* is an important quality control measure for the beverage industry.

We isolated 146 moderately thermophilic, acidophilic, spore-forming bacteria during the course of a microbiological survey of various beverages (including their raw materials) and environments. According to sequence analysis results for the hypervariable region of the 16S rRNA gene (Goto *et al.*, 2002c; Kato *et al.*, 2005), all of the strains belonged to the *Alicyclobacillus* clade but eight of the 146 strains were found to be distinct from recognized *Alicyclobacillus* species. The objective of the present study was to establish the taxonomic positions of the eight strains, by using a combination of polyphasic taxonomic approaches.

Type/reference strains of *Alicyclobacillus* species were obtained from the ATCC, DSMZ and IAM. *Alicyclobacillus* strains were grown on YSG agar [I^{-1} : 2 g yeast extract, 1 g glucose, 2 g soluble starch and 15 g agar (pH 3.7 with 0.5 M H_2SO_4)] at the optimum growth temperature for each. *Bacillus subtilis* IAM 12118^T was cultivated using the method recommended in the IAM strain catalogue.

Unless otherwise indicated, morphological observations and biochemical tests were performed using the methods of Darland & Brock (1971), Deinhard *et al.* (1987a, b), Albuquerque *et al.* (2000) and Goto *et al.* (2002c). Cultures were grown in either BAM liquid or 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 °C (for strain S-TAB^T), 50 °C (for

strains 4-A336^T, 5-A167N, 5-A239-2O-A^T, E-8 and RB718^T) and 55 °C (for strains 3-A191^T and 5-A83J^T) in BAM medium, with the pH adjusted using 1 M H_2SO_4 . Acidification was examined with API 50 CH test strips (bioMérieux) in BAM basal salts medium (Albuquerque *et al.*, 2000), at the optimum growth temperature. Guaiacol production was tested using a peroxidase kit (Kyokuto Pharmaceutical Industrial). All tests were conducted in triplicate.

Cellular fatty acids and menaquinones were analysed as described previously (Goto *et al.*, 2002c, 2003). The DNA G + C content (mol%) 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.

Genomic DNA was extracted using a Qiagen Blood & Cell Culture DNA maxi kit (Qiagen) and purified by equilibrium centrifugation in CsCl/ethidium bromide gradients (Treisman, 1989) using an Optima MAX ultracentrifuge (80 000 r.p.m. × 18 h; Beckman Coulter). Desalting was performed using an Ultrafree-4 centrifugal filter unit (Millipore).

Sequencing of the hypervariable region located at the 5'-end region of the 16S rRNA gene (about 270 bp) has been described previously (Goto *et al.*, 2002c). Nearly complete 16S rRNA gene sequences were determined using a 16S rRNA gene kit (Applied Biosystems). Nucleotide sequences of *gyrB* were determined directly from PCR fragments using methods described by Yamamoto & Harayama (1995), Kasai *et al.* (2000) and Goto *et al.* (2003).

Sequence analysis was performed using Gene Works (version 2.0; IntelliGenetics) and the GenBank/EMBL/DDBJ databases. Multiple sequence alignments were performed using CLUSTAL W version 1.8 (Thompson *et al.*, 1994). The *gyrB* nucleotide and deduced amino acid sequences were aligned using CLUSTAL W version 1.8, and then corrected manually. Alignment gaps and unidentified base positions were not taken into account for these calculations. Phylogenetic reconstructions were produced using MEGA3.1 (Kumar *et al.*, 2004) with neighbour-joining (Saitou & Nei, 1987) based on the Kimura two-parameter model (Kimura, 1980) and maximum-parsimony (Lake, 1987). The robustness of individual branches was estimated by bootstrap analysis based on 1000 replicates (Felsenstein, 1985). The GenBank/EMBL/DDBJ accession numbers of 16S rRNA and *gyrB* gene sequences used for the phylogenetic analysis are shown in Figs 1 and 2, respectively.

DNA–DNA hybridization experiments were performed according to the method of Ezaki *et al.* (1989) using photobiotin-labelled DNA probes and microplates. Hybridization was carried out under stringent conditions (optimal renaturation temperature, +15 °C) for 3 h. Based on the DNA G + C contents of the type strains of *Alicyclobacillus*

Fig. 1. Neighbour-joining phylogenetic tree of the eight isolates and *Alicyclobacillus* reference species, based on 16S rRNA gene sequence comparisons. *B. subtilis* IAM 12118^T was used as the outgroup. In the final dataset, 1468 unambiguous bases were aligned. Bootstrap percentages based on 1000 replicates are shown; only values greater than 70% are shown. Bar, 0.01 substitutions per nucleotide position.

acidocaldarius subsp. *acidocaldarius* (61.9 mol%) and *A. acidoterrestris* (52.7 mol%), the renaturation temperatures were calculated to be 65 °C for strains 3-A191^T, 4-A336^T, 5-A83J^T, 5-A167N, 5-A239-20-A^T and E-8, and 45 °C for strains RB718^T and S-TAB^T. Each hybridization experiment was conducted at least three times.

Strains E-8, RB718^T and S-TAB^T were isolated from orange juice, liquid sugar and apple juice, respectively, using the standard dilution plating technique on YSG agar. Growth was at 45 °C. Strains RB718^T and S-TAB^T were kindly provided by Ms Rie Yamamoto (Coca Cola Tokyo Research & Development Company Limited) and Ms Mikiko Yamanaka (Suntory Limited), respectively. Strains 3-A191^T, 4-A336^T, 5-A83J^T, 5-A167N and 5-A239-20-A^T

were isolated from soil samples collected from crop fields in four cities (Fuji, Fujieda, Kakegawa and Shizuoka) in the Shizuoka prefecture of Japan, using the standard dilution plating technique. Growth was at 50 °C.

Sequence analyses of the hypervariable region of the 16S rRNA gene (Goto *et al.*, 2002c; Kato *et al.*, 2005) showed that the eight strains (3-A191^T, 4-A336^T, 5-A83J^T, 5-A167N, 5-A239-20-A^T, E-8, RB718^T and S-TAB^T) belonged to the *Alicyclobacillus* clade. The strains were distinct from recognized *Alicyclobacillus* species in a hypervariable region-based tree (data not shown). Strains 3-A191^T and 5-A83J^T formed clusters with strains E-8 and 5-A167N, respectively, and had sequence similarities of 98.9 and 98.8%, respectively. The remaining strains clustered with *A. acidocaldarius* and

Fig. 2. Neighbour-joining phylogenetic tree of the eight isolates and *Alicyclobacillus* reference species, based on *gyrB* gene sequence comparisons. *B. subtilis* IAM 12118^T was used as the outgroup. In the final dataset, 1142 unambiguous bases were aligned. Bootstrap percentages based on 1000 replicates are shown; only values greater than 70% are shown. Bar, 0.02 substitutions per nucleotide position.

Alicyclobacillus genomic species 1 (Goto *et al.*, 2006). All eight strains were further characterized using polyphasic taxonomic methods.

Basic differential phenotypic characteristics of the novel isolates and related *Alicyclobacillus* reference strains are shown in Table 1. Characteristics of strains 3-A191^T and 5-A83J^T were similar to those of strains E-8 and 5-A167N, respectively. All isolates were Gram-positive, but were Gram-variable after 5 days growth. Sporangia swelling was observed. After growth for 48 h on BAM agar, colonies were circular and were not pigmented. No growth occurred under anaerobic conditions. The strains grew well under acidic and moderately thermal conditions. Growth factors were not required, but growth was further increased by the addition of yeast extract to the inorganic medium. Oxidase and nitrate reductase activities were negative. Gas formation from glucose was not observed. Indole was not produced. Voges–Proskauer reaction was negative. Guaiacol was not produced. All strains grew on BAM agar containing 2% (w/v) NaCl. Phenylalanine and tyrosine were not hydrolysed. Acid was produced from L-arabinose, ribose, D-xylitol, D-galactose, D-glucose, D-fructose, D-mannose and trehalose, but not from ribitol, sorbitol, N-acetylglucosamine, L-arabitol, gluconate, 2-ketogluconate and 5-ketogluconate. All strains produced spores within several days of growth (BAM agar), with the exception of strain S-TAB^T, which produced only small amounts of spores after 10 days. In addition, many vegetative cells of strain S-TAB^T on YSG agar died within 7 days and therefore repeated subculturing was difficult using the most popular YSG medium for cultivation of *Alicyclobacillus*. The diameter of swollen sporangia from strain 5-A239-2O-A^T was more than three times that of vegetative cells (Fig. 3).

Menaquinone 7 and menaquinone 3 were the major and minor respiratory quinones, respectively, of the isolated strains. The DNA G + C contents of the strains ranged from 53.9 to 62.5 mol% (see Supplementary Table S1 available in IJSEM Online). These values were within the range 48.7–62.7 mol%, which has been described previously for the genus *Alicyclobacillus* (Karavaiko *et al.*, 2005).

Cellular fatty acid compositions are shown in Table 2. The predominant lipids were the fatty acids ω -cyclohexane, ω -cyclohexyl C_{17:0} and ω -cyclohexyl C_{19:0}, for strains RB718^T and S-TAB^T, whereas the most abundant fatty acids (75.0–91.3%) for strains 4-A336^T, 5-A83J^T and 5-A167N were ω -cycloheptane, ω -cycloheptyl C_{18:0}, ω -cycloheptyl C_{18:0} 2-OH and ω -cycloheptyl C_{20:0}.

The fatty acids present in strains 3-A191^T, 5-A239-2O-A^T and E-8 were predominantly iso- and anteiso-branched acids, together with small amounts of straight-chain saturated acids. Strain 5-A239-2O-A^T had the highest relative proportion of iso-C_{16:0}, with 44.2% of the total fatty acids comprising this lipid. Strains 3-A191^T and E-8 had very similar fatty acid compositions. Their predominant lipid, anteiso-C_{17:0}, comprised 43.8 and 53.2%,

respectively, of the total lipid content. This proportion was similar to that of *A. pomorum*, where anteiso-C_{17:0} comprised 44.9% of the total lipid content. By comparison with *A. pomorum*, strains 3-A191^T and E-8 had lower amounts of iso-C_{15:0} and anteiso-C_{15:0}, but higher amounts of iso-C_{17:0}; thus these strains could be distinguished according to their fatty acid profiles.

The nearly complete nucleotide sequence of the 16S rRNA gene was determined for the eight isolates and for *Alicyclobacillus tolerans* DSM 16297^T and *Alicyclobacillus vulcanalis* DSM 16176^T. The sequences ranged in length from 1488 to 1526 bp, and 1468 bp were unambiguous for all isolates. The 16S rRNA gene sequence fragments were aligned with sequences available in the GenBank/EMBL/DDBJ databases and the phylogenetic positions of the strains were inferred by constructing a phylogenetic tree using either the neighbour-joining (Fig. 1) or the maximum-parsimony (data not shown) methods. Phylogenetic analyses showed that all strains fell within the cluster composed of *Alicyclobacillus* species. In detail, strains 3-A191^T and E-8 clustered with *A. pomorum*, strains 4-A336^T, 5-A83J^T and 5-A167N clustered with *Alicyclobacillus herbarius*, strain RB718^T clustered with *Alicyclobacillus hesperidum* and strain S-TAB^T clustered with *A. acidoterrestris* with high bootstrap values. Strain 5-A239-2O-A^T formed a deep lineage within the genus *Alicyclobacillus* (similarity values to the other species of 92.3–94.6%). The sequence similarities among the isolates and the other type/reference strains of *Alicyclobacillus* species ranged from 90.2 to 99.7%. Wisotzkey *et al.* (1992) proposed that 16S rRNA gene sequences must be at least 92% similar to belong to the genus *Alicyclobacillus*, but several sequence matchings were below 92% in this study. Similarity values between the isolates and either *Bacillus tusciae* NBRC 15312^T, *Sulfobacillus acidophilus* DSM 10332^T or *Sulfobacillus thermosulfidooxidans* DSM 9293^T were all below 90% (data not shown). The sequence similarity values between strains 3-A191^T and E-8 and between strains 5-A83J^T and 5-A167N were 99.8 and 99.3%, respectively.

The nucleotide sequence of a fragment of the *gyrB* gene was determined for the eight isolates and for *A. tolerans* DSM 16297^T and *A. vulcanalis* DSM 16176^T. Of the fragments sequenced (sized 1167–1170 bp), 1142 bp were unambiguous and similar to positions 316–1485 in the *Escherichia coli gyrB* gene (Adachi *et al.*, 1987). The *gyrB* sequence fragments were aligned with sequences available in the GenBank/EMBL/DDBJ databases and the phylogenetic relationships were determined as described for the 16S rRNA gene. The positions of strains in the *gyrB*-based tree were the same as in the 16S rRNA gene-based tree; however, relationships among the strains were more distinct in the *gyrB* tree (Figs 1 and 2). The position of strain 5-A239-2O-A^T was still ambiguous, but the strain appeared to have some relationship to *Alicyclobacillus cycloheptanicus* and *A. herbarius* in the *gyrB* gene-based tree (Fig. 2). The *gyrB* gene sequences of the isolates exhibited 64.9–95.1%

Table 1. Differential characteristics of the type strains of the novel species and related *Alicyclobacillus* species

Strains: 1, *A. acidocaldarius* subsp. *acidocaldarius* ATCC 27009^T; 2, *A. sacchari* sp. nov. RB718^T; 3, *A. hesperidum* DSM 12489^T; 4, *A. acidiphilus* TA-67^T; 5, *A. fastidiosus* sp. nov. S-TAB^T; 6, *A. acidoterrestris* ATCC 49025^T; 7, *A. kakegawensis* sp. nov. 5-A83J^T and 5-A167N; 8, *A. shizuokensis* sp. nov. 4-A336^T; 9, *A. herbarius* IAM 14883^T; 10, *A. macrosporangiidus* sp. nov. 5-A239-2O-A^T; 11, *A. contaminans* sp. nov. 3-A191^T and E-8; 12, *A. pomorum* 3A^T; 13, *A. cycloheptanicus* DSM 4006^T. +, Positive; –, negative; w, weakly positive; v, variable between strains.

Characteristic	1	2	3*	4†	5	6	7	8	9	10	11	12	13
Motility	+	+	+	+	–	+	+	+	+	+	+	+	+
Oxidase	–	–	–	–	–	–	–	–	–	–	–	+	+
Catalase	+	–	+	+	+	+	w	+	+	w	–	+	+
Hydrolysis of:													
Gelatin	+	+	+	–	+	+	–	–	–	–	+	+	–
Starch	+	+	+	–	–	–	–	–	–	–	–	+	–
Nitrate reduced to nitrite	–	–	–	–	–	–	–	–	+	–	–	–	–
Growth in 5% NaCl	–	–	–	–	–	+	–	+	+	+	–	–	+
Acid production from:													
Glycerol	+	+	+	–	–	+	–	–	+	+	+	+	–
Erythritol	–	–	–	–	–	+	v	–	–	–	–	–	–
D-Arabinose	–	–	–	+	+	–	+	–	+	+	–	–	+
L-Arabinose	+	+	+	+	+	+	+	+	+	+	+	–	+
D-Xylose	+	+	–	+	+	–	+	+	+	+	+	–	+
L-Xylose	–	–	–	–	–	–	+	+	–	+	–	–	+
Methyl β -xyloside	–	+	–	–	+	–	–	–	–	–	–	–	–
D-Galactose	+	+	+	+	+	+	+	+	+	+	+	–	–
L-Sorbose	–	–	–	+	–	–	+	–	–	+	v	+	+
Rhamnose	+	+	–	–	+	+	+	–	+	+	v	–	+
Inositol	–	–	–	–	+	+	v	–	–	–	–	–	–
Mannitol	+	+	+	–	+	+	+	+	+	–	+	+	+
Sorbitol	–	–	–	+	–	+	+	–	–	+	–	–	+
Methyl α -D-mannoside	–	–	–	–	–	–	+	–	+	+	–	–	–
Methyl α -D-glucoside	+	+	–	+	–	–	+	–	+	–	–	+	–
Amygdalin	–	–	–	–	–	–	+	–	+	–	–	+	–
Arbutin	+	+	–	+	–	–	+	+	+	–	+	–	–
Aesculin	+	–	–	+	–	–	+	+	+	+	+	+	+
Salicin	+	+	–	+	–	–	+	+	+	+	v	+	–
Cellobiose	+	+	+	+	–	+	+	+	+	–	+	–	–
Maltose	+	+	+	+	–	+	+	+	+	+	+	+	–
Lactose	+	+	+	+	–	+	+	–	+	+	v	–	–
Melibiose	+	+	–	–	+	w	–	+	+	–	–	–	–
Sucrose	+	+	+	+	–	+	+	+	+	+	+	+	–
Trehalose	+	+	+	+	+	+	+	+	+	+	+	+	–
Inulin	–	+	–	–	–	–	–	–	–	–	–	–	–
Melezitose	–	+	–	+	–	–	v	–	+	–	–	–	–
D-Raffinose	+	+	–	+	+	–	–	–	+	–	–	–	–
Starch	+	+	w	–	–	–	–	–	–	–	–	–	–
Glycogen	+	+	+	–	–	–	–	–	–	–	–	–	–
Xylitol	–	–	–	+	–	+	+	–	–	+	–	–	–
β -Gentiobiose	–	+	w	+	–	–	+	+	+	+	v	–	–
D-Turanose	–	+	+	+	–	–	+	–	+	–	–	+	–
D-Lyxose	–	–	–	–	+	–	+	–	–	+	–	–	+
D-Tagatose	–	–	–	–	+	–	v	–	–	–	v	+	+
D-Fucose	–	–	–	–	+	–	–	–	+	–	–	–	–
L-Fucose	–	–	–	–	+	–	–	–	–	–	–	–	–
D-Arabitol	–	–	–	–	–	–	+	–	–	+	–	–	–
5-Ketogluconate	–	–	–	–	–	–	–	–	+	–	–	+	+

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

†Data from Matsubara *et al.* (2002).

Fig. 3. Phase-contrast micrograph of cells of *A. macrosporangiidus* sp. nov. strain 5-A239-2O-A^T from 3-day-old cultures grown on YSG agar. Bar, 5 µm.

similarity (mean 72.7 %) to the other type/reference strains of *Alicyclobacillus* species. By comparison, the sequence similarity values between strains 3-A191^T and E-8 and between strains 5-A83J^T and 5-A167N were 99.5 and 99.3 %, respectively.

DNA–DNA hybridization values between strains 5-A83J^T and 5-A167N were 74 and 71 %, respectively, confirming that the two strains represent the same species (Wayne *et al.*, 1987) (see Supplementary Table S1 available in IJSEM Online). Strains 3-A191^T and E-8 were also assigned as representing a single species with values of 75 and 80 %, respectively. The DNA–DNA hybridization values of the isolates with type/reference strains of *Alicyclobacillus* species ranged from 0 to 37 %, which were well below the 70 % cut-off value recommended by Wayne *et al.* (1987) for the delineation of separate species.

In conclusion, based on the results of the polyphasic taxonomic analysis, the eight thermo-acidophilic, endospore-forming bacteria represent six novel species within the genus *Alicyclobacillus*. Here, we propose that the novel bacteria should be classified within the genus *Alicyclobacillus*, as *Alicyclobacillus contaminans* sp. nov. (strains 3-A191^T and E-8), *Alicyclobacillus fastidiosus* sp. nov. (strain S-TAB^T), *Alicyclobacillus kakegawensis* sp. nov. (strains 5-A83J^T and 5-A167N), *Alicyclobacillus macrosporangiidus* sp. nov. (strain 5-A239-2O-A^T), *Alicyclobacillus sacchari* sp. nov. (strain RB718^T) and *Alicyclobacillus shizuokensis* sp. nov. (strain 4-A336^T).

Description of *Alicyclobacillus contaminans* sp. nov.

Alicyclobacillus contaminans (con.ta'mi.nans. L. part. adj. *contaminans* contaminating, referring to contamination of fruit juice).

Gram-positive but Gram-variable in old cultures, strictly aerobic, motile, endospore-forming straight rods with

rounded ends (4.0–5.0 µm long and 0.8–0.9 µm wide). Endospores are ellipsoidal and subterminal with swollen sporangia. Colonies on BAM agar are non-pigmented (creamy white), circular, opaque, entire, umbonate and 3–5 mm in diameter after 48 h. Temperature range for growth is 35–60 °C; optimum growth temperature is 50–55 °C. The pH optimum is 4.0–4.5; growth does not occur at pH 3.0 or 6.0. Growth occurs in the presence of 0–2 % (w/v) NaCl but not 5 % (w/v) NaCl. Oxidase- and catalase-negative. Nitrate reduction, Voges–Proskauer test and indole production are negative. Gelatin and aesculin are hydrolysed, but phenylalanine, starch and tyrosine are not, and arbutin is variable. Acid is produced from glycerol, L-arabinose, ribose, D-xylose, D-galactose, D-glucose, D-fructose, D-mannose, mannitol, cellobiose, maltose, sucrose and trehalose. Variable from L-sorbose, rhamnose, salicin, lactose, β-gentiobiose and D-tagatose. Major fatty acids are iso-C_{16:0} (13.7–16.9 %), iso-C_{17:0} (22.8–29.3 %) and ante-iso-C_{17:0} (43.8–53.2 %). The main quinone is menaquinone 7. The DNA G + C content is 60.1–60.6 mol%.

The type strain, 3-A191^T (= DSM 17975^T = IAM 15224^T), was isolated from soil of a crop field in Fuji city. Strain E-8 (= IAM 15228) was isolated from orange juice.

Description of *Alicyclobacillus fastidiosus* sp. nov.

Alicyclobacillus fastidiosus (fas.ti.di.o'sus. L. masc. adj. *fastidiosus* fastidious, referring to its fastidious character).

Gram-positive but Gram-variable in old cultures, strictly aerobic, non-motile, endospore-forming straight rods with rounded ends (4.0–5.0 µm long and 0.9–1.0 µm wide). Endospores are ellipsoidal and subterminal with swollen sporangia. Colonies on BAM agar are non-pigmented (creamy white), circular, opaque, entire, flat and 3–4 mm in diameter after 48 h. Temperature range for growth is 20–55 °C; optimum growth temperature is 40–45 °C. Optimum pH is 4.0–4.5; growth does not occur at pH 2.0 or 5.5. Growth occurs in the presence of 0–2 % (w/v) NaCl, but not at 5 % (w/v) NaCl. Oxidase-negative. Catalase-positive. Nitrate reduction, Voges–Proskauer test and indole production are negative. Gelatin is hydrolysed, but aesculin, arbutin, phenylalanine, starch and tyrosine are not. Acid is produced from D-arabinose, L-arabinose, ribose, D-xylose, methyl β-xyloside, D-galactose, D-glucose, D-fructose, D-mannose, rhamnose, inositol, mannitol, melibiose, trehalose, D-raffinose, D-lyxose, D-tagatose, D-fucose and L-fucose. Major fatty acids are ω-cyclohexyl C_{17:0} and ω-cyclohexyl C_{19:0}. The following fatty acids are present in smaller amounts: iso-C_{16:0} (8.3 %), iso-C_{17:0} (5.3 %) and anteiso-C_{17:0} (5.4 %). The main quinone is menaquinone 7. The DNA G + C content of the type strain is 53.9 mol%.

The type strain, S-TAB^T (= DSM 17978^T = IAM 15229^T), was isolated from apple juice.

Table 2. Cellular fatty acid compositions (%) of type/reference strains of the novel species and related *Alicyclobacillus* species

Strains: 1, *A. sendaiensis* JCM 11817^T; 2, *Alicyclobacillus* genomic species 2 MIH332; 3, *Alicyclobacillus* genomic species 1 DSM 11984; 4, *A. acidocaldarius* subsp. *rittmannii* DSM 11297^T; 5, *A. acidocaldarius* subsp. *acidocaldarius* ATCC 27009^T; 6, *A. vulcanalis* DSM 16176^T; 7, *A. sacchari* sp. nov. RB718^T; 8, *A. hesperidum* DSM 12489^T; 9, *A. acidiphilus* TA-67^T; 10, *A. fastidiosus* sp. nov. S-TAB^T; 11, *A. acidoterrestris* ATCC 49025^T; 12, *A. kakegawensis* sp. nov. 5-A83J^T; 13, *A. shizuokensis* sp. nov. 4-A336^T; 14, *A. herbarius* IAM 14883^T; 15, *A. macrosporangii* sp. nov. 5-A239-2O-A^T; 16, *A. contaminans* sp. nov. 3-A191^T; 17, *A. pomorum* 3A^T; 18, *A. cycloheptanicus* DSM 4006^T; 19, *A. disulfidooxidans* DSM 12064^T; 20, *A. tolerans* DSM 16297^T. tr, Trace amount (<0.5%); —, not detected; ND, no data.

Fatty acid	1*	2	3	4	5	6†	7	8	9	10	11	12	13	14	15	16	17	18	19	20‡
C _{15:0}	tr	—	—	—	—	—	—	—	—	—	—	1.2	0.5	—	—	—	—	0.6	—	ND
C _{16:0}	4.9	1.7	3.2	1.9	0.5	7.8	0.5	1.7	2.2	1.3	0.8	8.8	6.1	3.5	6.1	0.9	0.7	2.4	0.8	ND
C _{17:0}	0.6	—	—	—	—	—	—	—	—	—	—	1.2	1.6	0.6	—	0.6	—	—	—	ND
C _{18:0}	0.9	2.1	1.5	2.1	—	—	—	—	—	—	—	—	—	1.0	—	0.6	—	1.3	—	ND
iso-C _{15:0}	1.1	1.4	1.5	2.4	2.6	1.5	0.8	0.5	0.6	2.5	tr	tr	0.7	tr	2.1	1.8	10.6	0.5	0.7	ND
iso-C _{16:0}	1.6	0.8	2.8	1.8	2.3	4.2	—	0.5	—	8.3	—	0.9	1.9	4.7	44.2	16.9	20.3	1.0	11.3	ND
iso-C _{17:0}	4.5	2.6	5.1	5.1	10.1	8.3	2.4	2.0	1.2	5.3	1.2	0.5	4.0	5.5	16.7	29.3	11.1	2.7	0.8	ND
iso-C _{18:0}	—	—	—	—	—	—	—	—	—	tr	—	—	—	2.9	4.5	4.7	0.5	—	—	ND
iso-C _{19:0}	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.9	—	—	—	ND
anteiso-C _{15:0}	1.5	0.5	1.2	0.9	tr	0.7	0.5	1.1	0.5	1.9	tr	—	—	—	1.2	0.5	11.9	tr	1.9	ND
anteiso-C _{17:0}	10.5	0.7	6.7	2	1.8	8.5	4.6	6.5	3.2	5.4	3.7	—	—	2.3	25.2	43.8	44.9	1.3	17.4	ND
ω-Cyclohexane C _{17:0}	44.1	47.6	52.8	52.4	51.1	46.2	64.4	67.8	68.4	61.1	61.7	—	—	—	—	—	—	—	42.7	27.5–60.0
ω-Cyclohexane C _{17:0} 2-OH	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	8.2–11.3
ω-Cyclohexane C _{19:0}	30.2	42.6	25.2	31.4	31.3	22.8	26.9	19.9	23.9	13.9	31.7	—	—	—	—	—	—	—	7.2	2.1–7.9
ω-Cycloheptane C _{18:0}	—	—	—	—	—	—	—	—	—	—	—	65.6	65.4	55.3	—	—	—	86.8	—	—
ω-Cycloheptane C _{18:0} 2-OH	—	—	—	—	—	—	—	—	—	—	—	15.0	12.4	9.7	—	—	—	2.3	—	—
ω-Cycloheptane C _{20:0}	—	—	—	—	—	—	—	—	—	—	—	6.4	7.4	14.2	—	—	—	tr	—	—

*Data from Tsuruoka *et al.* (2003).

†Data from Simbahan *et al.* (2004).

‡Data from Karavaiko *et al.* (2005).

Description of *Alicyclobacillus kakegawensis* sp. nov.

Alicyclobacillus kakegawensis (ka.ke.ga.wa.en'sis. N.L. masc. adj. *kakegawensis* pertaining to Kakegawa, a city in Shizuoka Prefecture, Japan, where the type strain was isolated).

Gram-positive but Gram-variable in old cultures, strictly aerobic, motile, endospore-forming straight rods with rounded ends (4.0–5.0 µm long and 0.6–0.7 µm wide). Endospores are oval and subterminal with swollen sporangia. Colonies on BAM agar are non-pigmented (creamy white), circular, opaque, entire, flat and 2–3 mm in diameter after 48 h. Temperature range for growth is 40–60 °C; optimum growth temperature is 50–55 °C. pH optimum is 4.0–4.5; growth does not occur at pH 3.0 or 6.5. Growth occurs in the presence of 0–2 % (w/v) NaCl but not at 5 % (w/v) NaCl. Oxidase-negative. Catalase weakly positive. Nitrate reduction, Voges–Proskauer test and indole production are negative. Aesculin and arbutin are hydrolysed, but gelatin, phenylalanine, starch and tyrosine are not. Acid is produced from D-arabinose, L-arabinose, ribose, D-xylose, L-xylose, D-galactose, D-glucose, D-fructose, D-mannose, L-sorbose, rhamnose, mannitol, sorbitol, methyl α-D-mannoside, methyl α-D-glucoside, amygdalin, salicin, cellobiose, maltose, lactose, sucrose, trehalose, xylitol, β-gentiobiose, D-turanose, D-lyxose and D-arabitol. Variable acid production from erythritol, inositol, melezitose and D-tagatose. Major fatty acids are ω-cyclohexyl C_{18:0}, ω-cyclohexyl C_{18:0} 2-OH and ω-cyclohexyl C_{20:0}. The following fatty acids are present in smaller amounts: C_{16:0} (4.2–8.8 %) and iso-C_{17:0} (0.5–4.7 %). The main quinone is menaquinone 7. The DNA G + C content is 61.3–61.7 mol%.

The type strain, 5-A83J^T (= DSM 17979^T = IAM 15227^T), and strain 5-A167N (= IAM 15225) were isolated from soil of a crop field in Kakegawa city.

Description of *Alicyclobacillus macrosporangiidus* sp. nov.

Alicyclobacillus macrosporangiidus (ma.cro.spo.ran'gi.i.dus. Gr. adj. *macro* big; N.L. n. *sporangium* sporangia; L. suff. *-idus* suffix expressing a quality or tendency; N.L. masc. adj. *macrosporangiidus* having large sporangia).

Gram-positive but Gram-variable in old cultures, strictly aerobic, motile, endospore-forming straight rods with rounded ends (5.0–6.0 µm long and 0.7–0.8 µm wide). Endospores are oval and terminal with swollen sporangia. Colonies on BAM agar are non-pigmented (creamy white), circular, opaque, entire, convex and 2–4 mm in diameter after 48 h. Temperature range for growth is 35–60 °C; optimum growth temperature is 50–55 °C. pH optimum is 4.0–4.5; growth does not occur at pH 3.0 or 6.5. Growth occurs in the presence of 0–5 % (w/v) NaCl but not at 7 % (w/v) NaCl. Oxidase-negative. Catalase weakly positive. Nitrate reduction, Voges–Proskauer test and indole production are negative. Aesculin is hydrolysed, but gelatin, arbutin, phenylalanine, starch and tyrosine are not. Acid is

produced from glycerol, D-arabinose, L-arabinose, ribose, D-xylose, L-xylose, D-galactose, D-glucose, D-fructose, D-mannose, L-sorbose, rhamnose, sorbitol, methyl α-D-mannoside, salicin, maltose, lactose, sucrose, trehalose, xylitol, β-gentiobiose, D-lyxose and D-arabitol. Major fatty acids are iso-C_{16:0} (44.2 %), iso-C_{17:0} (16.7 %) and anteiso-C_{17:0} (25.2 %). The main quinone is menaquinone 7. The DNA G + C content of the type strain is 62.5 mol%.

The type strain, 5-A239-20-A^T (= DSM 17980^T = IAM 15370^T), was isolated from soil of a crop field in Fujieda city.

Description of *Alicyclobacillus sacchari* sp. nov.

Alicyclobacillus sacchari (sac'cha.ri. N.L. gen. n. *sacchari* of sugar, referring to the source of isolation).

Gram-positive but Gram-variable in old cultures, strictly aerobic, motile, endospore-forming straight rods with rounded ends (4.0–5.0 µm long and 0.6–0.7 µm wide). Endospores are ellipsoidal and subterminal with swollen sporangia. Colonies on BAM agar are non-pigmented (creamy white), circular, opaque, entire, umbonate and 3–5 mm in diameter after 48 h. Temperature range for growth is 30–55 °C; optimum growth temperature is 45–50 °C. pH optimum is 4.0–4.5; growth does not occur at pH 2.0 or 6.0. Growth occurs in the presence of 0–2 % (w/v) NaCl, but not at 5 % (w/v) NaCl. Oxidase, catalase and nitrate reduction are negative. Voges–Proskauer test and indole production are negative. Arbutin, gelatin and starch are hydrolysed, but aesculin, phenylalanine and tyrosine are not. Acid is produced from glycerol, L-arabinose, ribose, D-xylose, methyl β-xyloside, D-galactose, D-glucose, D-fructose, D-mannose, rhamnose, mannitol, methyl α-D-glucoside, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, melezitose, D-raffinose, glycogen, β-gentiobiose and D-turanose. Major fatty acids are ω-cyclohexyl C_{17:0} and ω-cyclohexyl C_{19:0}. The following fatty acids are present in smaller amounts: iso-C_{17:0} (2.4 %) and anteiso-C_{17:0} (4.6 %). The main quinone is menaquinone 7. The DNA G + C content of the type strain is 56.6 mol%.

The type strain, RB718^T (= DSM 17974^T = IAM 15230^T), was isolated from liquid sugar.

Description of *Alicyclobacillus shizuokensis* sp. nov.

Alicyclobacillus shizuokensis (shi.zu.o.ken'sis. N.L. masc. adj. *shizuokensis* pertaining to Shizuoka, a city in Shizuoka Prefecture, Japan, where the type strain was isolated).

Gram-positive but Gram-variable in old cultures, strictly aerobic, motile, endospore-forming straight rods with rounded ends (4.0–5.0 µm long and 0.7–0.8 µm wide). Endospores are oval and subterminal with swollen sporangia. Colonies on BAM agar are non-pigmented (creamy white), circular, opaque, entire, convex and 1–2 mm in diameter after 48 h. Temperature range for growth is 35–60 °C; optimum growth temperature is 45–50 °C. pH

optimum is 4.0–4.5; growth does not occur at pH 3.0 or 6.5. Growth occurs in the presence of 0–5 % (w/v) NaCl, but not 7 % (w/v) NaCl. Catalase-positive. Oxidase and nitrate reduction are negative. Voges–Proskauer test and indole production are negative. Aesculin and arbutin are hydrolysed, but gelatin, phenylalanine, starch and tyrosine are not. Acid is produced from L-arabinose, ribose, D-xylose, L-xylose, D-galactose, D-glucose, D-fructose, D-mannose, mannitol, salicin, cellobiose, maltose, melibiose, sucrose, trehalose and β -gentiobiose. Major fatty acids are ω -cyclohexyl C_{18:0}, ω -cyclohexyl C_{18:0} 2-OH and ω -cyclohexyl C_{20:0}. The following fatty acids are present in smaller amounts: C_{16:0} (6.1 %) and iso-C_{17:0} (4.0 %). The main quinone is menaquinone 7. The DNA G + C content of the type strain is 60.5 mol%.

The type strain, 4-A336^T (= DSM 17981^T = IAM 15226^T), was isolated from soil of a crop field in Shizuoka city.

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