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Full Paper

Application of the hypervariable region of the 16S rDNA sequence as an index for the rapid identification of species in the genus *Alicyclobacillus*

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A comparison of the 16S rRNA gene (rDNA) sequences of seven type strains belonging to different *Alicyclobacillus* species (i.e., five validated species, one proposed species and one genomic species) suggested that the 5′ end hypervariable region (259–273 bases in length) of 16S rDNA was specific for the respective type strains. Further phylogenetic analysis based on DNA sequences of the hypervariable region using 24 *Alicyclobacillus* strains revealed that the strains could be categorized into five species and the *A. acidocaldarius-Alicyclobacillus* genomic species 1 group. The hypervariable region was highly conserved among the five species: *A. acidiphilus*, *A. acidoterrestris*, *A. cycloheptanicus*, *A. herbarius*, and *A. hesperidum.* The strains in the *A. acidocaldarius-Alicyclobacillus* genomic species 1 group were subdivided into two clusters (Clusters I and II) based on DNA sequences in the hypervariable region. On the basis of phenotypic characteristics, chemotaxonomic and phylogenetic analyses, and DNA-DNA hybridization data, strains in Cluster I were grouped as *Alicyclobacillus* genomic species 1 and strains in Cluster II were re-identified as *A. acidocaldarius*, thereby demonstrating that the hypervariable regions were also highly conserved within these two species. These results suggest that as is the case with *Bacillus*, the hypervariable region is significantly species-specific in the genus *Alicyclobacillus* to distinguish *Alicyclobacillus* species by DNA sequence comparison of the hypervariable region.

Key Words——*Alicyclobacillus*; *Bacillus*; grouping; hypervariable region; identification; 16S rRNA gene

Introduction

The genus *Alicyclobacillus* is comprised of aerobic, Gram-positive, endospore-forming bacilli. The bacteria are acidophilic, thermophilic, and possess a unique type of lipid (ω-alicyclic fatty acid) as the major membrane fatty acid component in their cells.

Until 1992, the three thermoacidophilic species were classified in the genus *Bacillus* (Darland and Brock, 1971; Deinhard et al., 1987a, b). However, phylogenetic analysis based on a sequence comparison of the 16S rDNA and cellular membrane fatty acid profiles led to the separation of these three species from the genus *Bacillus*, and their assignment as new genus, *Alicyclobacillus* (Wisotzkey et al., 1992). Recently, *A.*

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acidiphilus (Matsubara et al., 2002), *A. herbarius* (Goto et al., 2002), *A. hesperidum* and *Alicyclobacillus* genomic species 1 (Albuquerque et al., 2000) have been reported as new species of *Alicyclobacillus*, along with three species described previously: *A. acidocaldarius*, *A. acidoterrestris* and *A. cycloheptanicus.*

The identification of *Alicyclobacillus* species is usually performed by analysis of morphological characteristics and traditional biochemical assays. Additionally, chemotaxonomic tests, especially the analysis of cellular fatty acids, which provide important information for the differentiation of *Alicyclobacillus* species, are necessary. However, the differentiation between *Alicyclobacillus* species is not conclusive in many cases, leading to the necessity of using molecular taxonomic techniques. Recently, development of the randomly amplified polymorphic DNA (RAPD) (Yamazaki et al., 1997) and ribotyping (Jensen et al., 1993) methods have allowed for the rapid identification of *A. acidoterrestris.* However, these methods are not suitable for the identification of other *Alicyclobacillus* species.

We demonstrated previously that the 5′ end hypervariable region (HV region) of the 16S rDNA is a useful index for the identification or grouping of *Bacillus* species (Goto et al., 2000). In this study, 24 strains of the genus *Alicyclobacillus* were subjected to a sequence comparison of the HV regions, for the phylogenetic analysis. The results showed that the HV region is a very efficient index for identifying or grouping *Alicyclobacillus* species.

Materials and Methods

Strains and culture conditions. The 25 strains used in this study were obtained from ATCC (American Type Culture Collection, Manassas, VA, USA), DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany), IAM (Institute of Molecular and Cellular Biosciences, The University of Tokyo, Tokyo, Japan) and IFO (Institute for Fermentation, Osaka, Japan). *Alicyclobacillus acidiphilus* TA 67^T was kindly provided by Motohiro Niwa (Kirin Beverage Corporation, Samukawa-machi, Kanagawa, Japan). The *Alicyclobacillus* strains were grown in *Bacillus acidocaldarius* medium (BAM medium; Deinhard et al., 1987a) at 50 or 60°C. *B. subtilis* IAM 12118^T and *B. tusciae* IFO 15312^T were cultivated by the methods recommended in the IAM and IFO strain catalogues, respectively.

Morphological, physiological and biochemical characteristics. All biochemical tests were performed according to the methods of Darland and Brock (1971), Deinhard et al. (1987a, b) and Albuquerque et al. (2000), either in BAM liquid or in BAM agar medium. The cell growth was estimated by measuring the turbidity at 578 nm. The pH range for growth was examined at 60°C in BAM medium, the pH of which was 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. The cultures used for quinone analysis were grown in 500 ml Erlenmeyer flasks containing 100 ml BAM medium at 60°C in a reciprocal shaker for 4 days. Quinones were extracted from freeze-dried cells according to the method of Sano et al. (1996) and were analyzed using an Alliance HPLC system equipped with a 996 photo diodearray detector (Waters Corp., Milford, MA, USA). A Mightysil RP-18 column (4.6×250 mm, Kanto Chemical, Tokyo, Japan) was used for separation, and methanol-*iso*-propanol (3 : 1, v/v) was used as an eluent. UV spectra of the peaks were used for identification of quinones.

The cells for fatty acid analysis were grown in 500 ml Erlenmeyer flasks containing 100 ml semi-synthetic basal medium (Hippchen et al., 1981) at 60°C on a reciprocal shaker for 4 days. Fatty acid methyl esters were extracted from fresh wet biomass through saponification, methylation and extraction according to the method of Bligh and Dyer (1959) and Metcalfe et al. (1996). The fatty acid methyl esters were analyzed using a HP5890 gas chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with a DX-303 mass spectrometer (JEOL, Tokyo, Japan). A DB-1 fused silica capillary column (0.25 mm×30 m, J&W Scientific, Folsom, CA, USA) was used for separation. The temperature of the injection port was 250°C, and that of the oven was programmed at 150–250°C at a rate of 5°C min^{-1}. Identification of the fatty acid methyl esters was estimated by comparing retention time and gas chromatography mass spectrometry using fatty acid methyl ester standards (Supelco, Milwaukee, WI, USA). The peak areas on the total ion chromatogram were used for quantification of the fatty acid methyl esters.

Genomic DNA extraction and DNA-DNA hybridization. The genomic DNA was extracted and purified

using the High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany) following the protocols of the manufacturer.

For 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 (Treisman, 1989) using the Optima™ MAX Ultracentrifuge (Beckman Coulter, Inc., Fullerton, CA, USA). Desalting was performed using Ultrafree-4 Centrifugal Filter Unit (Millipore, Bedford, MA, USA). Genetic relatedness was determined fluorometrically by the method of Ezaki et al. (1989) using photobiotin-labelled DNA probes and microplates. Each hybridization experiment was repeated at least three times.

Sequencing of 16S rDNA and hypervariable region. Almost complete 16S rDNA sequences were determined using the 16S rRNA Gene Kit following the protocols of the manufacturer (Applied Biosystems, Foster City, CA, USA).

The universal primers for amplification and sequencing of the HV region were as follows: a forward primer (F-1): 5′-GCY TAA YAC ATG CAA GTC GAR CG-3′ (corresponding to nucleotide positions 47 to 69 of *B. subtilis* 16S rDNA; Y=C and T, R=A and G) and a reverse primer (R-1): 5′-ACT GCT GCC TCC CGT AGG AGT-3′ (corresponding to nucleotide positions 345 to 365 of *B. subtilis* 16S rDNA). These primers were modified from the previously reported primer set for *Bacillus* species (Goto et al., 2000) and 16S rDNA bacterial sequences available on the GenBank/EMBL/DDBJ databases. The DNA corresponding to HV region was amplified with the primer set according to the method of Sadaie et al. (1997). The PCR product was purified using AutoSeq™ G-50 (Amersham Pharmacia Biotech, Inc., Uppsala, Sweden). Sequencing reactions were run using a Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems) and the primers, then sequence reaction products, purified with Centri-Sep Spin Columns (Applied Biosystems). A Model ABI 310 automatic DNA sequencer (Applied Biosystems) was used for sample electrophoresis and data collection.

Computer analysis and construction of phylogenetic dendrogram. Sequence analysis was performed using Gene Works (version 2.0, IntelliGenetics, Inc., Mountain View, CA, USA) and the GenBank/EMBL/DDBJ databases. Multiple sequence alignment was performed with CLUSTAL W version 1.7 (Thompson et al., 1994). The phylogenetic trees were constructed from the evolutionary data calculated with Kimura's two-parameter model (Kimura, 1980), by using neighbor-joining method of Saitou and Nei (1987). The robustness for individual branches was estimated by bootstrapping with 1,000 replicates (Felsenstein, 1985). Alignment gaps and unidentified base positions were not taken into account for the calculations.

Nucleotide sequence accession numbers. The DDBJ accession numbers used for phylogenetic analysis are presented in Fig. 1. The accession numbers used for modification of the primer set were as follows: *Alcaligenes faecalis* (M22508), *Agrobacterium tumefaciens* (D12784), *Clostridium tetani* (X74770), *Cytophaga fermentans* (M58766), *Erwinia carotovora* (M59149), *Escherichia coli* (J01859, K02555, M24828, M24833-M24837, M24911, M24996), *Hydrogenobacter acidophilus* (D16296), *Klebsiella pneumoniae* (AF130983), *Pseudomonas fluorescens* (AJ278814), *Salmonella typhimurium* (U90316), *Serratia marcescens* (M59160) and *Thermoactinomyces vulgaris* (M77491).

Results and Discussion

Selection of the 16S rDNA variable region in Alicyclobacillus species

Multiple sequence alignment was carried out using the 16S rDNA sequences of the type strains of *A. acidiphilus*, *A. acidocaldarius*, *A. acidoterrestris*, *A. cycloheptanicus*, *A. herbarius*, *A. hesperidum* and *Alicyclobacillus* genomic species 1 in order to find the most informative region of the 16S rDNA. The results showed that the 5′ end region of *Alicyclobacillus* species corresponding to the HV region of *Bacillus* (nucleotide positions 70–344; *B. subtilis* numbering) was the most variable region among the seven type strains, being specific for individual type strains (Fig. 2). Sequence similarities of the HV region among the type strains ranged from 82.3 to 98.1% (average: 89.6%), which were lower than those of the complete 16S rDNA (91.5–98.5%, average: 94.5%). Comparisons of HV region sequences obtained from the type strains of *Alicyclobacillus* species enabled the distinction of individual strains, indicating that the HV region was a useful index for the identification or grouping of *Alicyclobacillus* species.

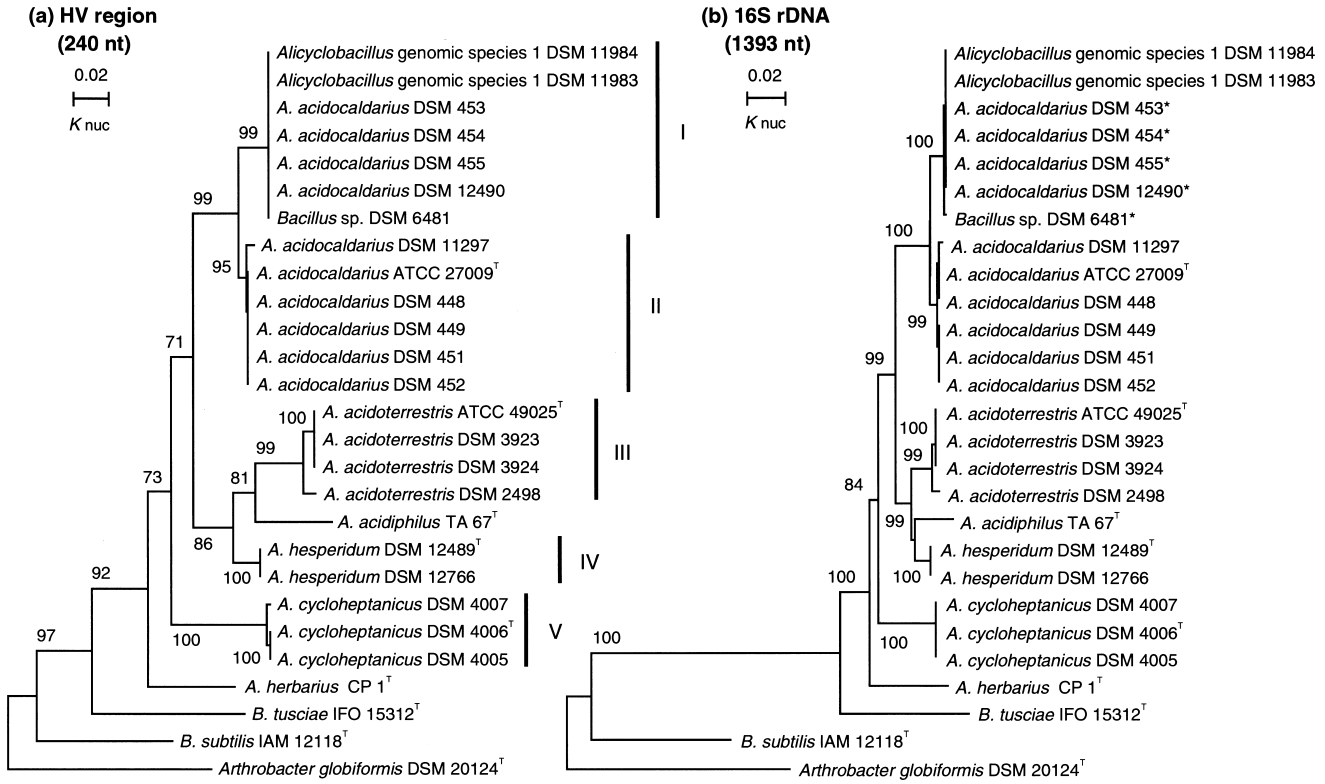

Fig. 1. Phylogenetic trees of *Alicyclobacillus* strains and related *Bacillus* strains, inferred from sequences of the HV region (a) and 16S rDNA (b).

The trees, constructed using the neighbor-joining method, were based on a comparison of 240 nt (a) and 1,393 nt (b). *Arthrobacter globiformis* DSM 20124^T was used at the out group. The numbers given on branches indicate the percentage of 1,000 bootstrap replicates. I, *Alicyclobacillus* genomic species 1 cluster; II, *A. acidocaldarius* cluster; III, *A. acidoterrestris* cluster; IV, *A. hesperidum* cluster; V, *A. cycloheptanicus* cluster. Accession numbers of the 16S rDNA sequence are as follows: *Alicyclobacillus* genomic species 1 DSM 453 (AB059663), DSM 454 (AB059664), DSM 455 (AB059665), DSM 6481 (AB059666), DSM 11983 (AB059667), DSM 11984 (AB059668), and DSM 12490 (AB059669); *A. acidocaldarius* ATCC 27009^T (AB042056), DSM 448 (AB059670), DSM 449 (AB059671), DSM 451 (AB059672), DSM 452 (AB059673), and DSM 11297 (AB059674); *A. acidoterrestris* ATCC 49025^T (AB042057), DSM 2498 (AB059675), DSM 3923 (AB042058), and DSM 3924 (AB059676); *A. acidiphilus* TA 67^T (AB059677); *A. hesperidum* DSM 12489^T (AB059678), and DSM 12766 (AB059679); *A. cycloheptanicus* DSM 4005 (AB059680), DSM 4006^T (AB042059), and DSM 4007 (AB059681); *A. herbarius* CP 1^T (AB042055); *B. tusciae* IFO 15312^T (AB042062); *B. subtilis* IAM 12118^T (AB042061); *Arthrobacter globiformis* DSM 20124^T (X80736). * Strains which grouped to *Alicyclobacillus* genomic species 1.

Comparative sequence analyses of HV region and 16S rDNA

To examine the conservation of the HV region within species, we compared the DNA sequence of the HV region derived from 17 *Alicyclobacillus* strains. Comparative sequence analysis of the HV region revealed that these strains could be classified into five clusters. Clusters I and II are distinguishable from each other at a similarity level of less than 98.8%. A phylogenetic tree of the strains is shown in Fig. 1(a). Strains of *A. acidoterrestris*, *A. cycloheptanicus* and *A. hesperidum* possessed more than 99.1% HV region sequence similarity to each type strain (Clusters III–V), and relatedness between these strains was further supported by high DNA-DNA homology values (Table 1). On the other hand, strains of *A. acidocaldarius* were divided into two clusters (Clusters I and II). Cluster II was found to be composed of only *A. acidocaldarius* strains, including the type strain of the species (>98.8% similarity), whereas Cluster I exhibited a complicated species composition, including the reference strain of *Alicyclobacillus* genomic species 1 (>99.6% similarity), and these clusters were well supported by high bootstrap values.

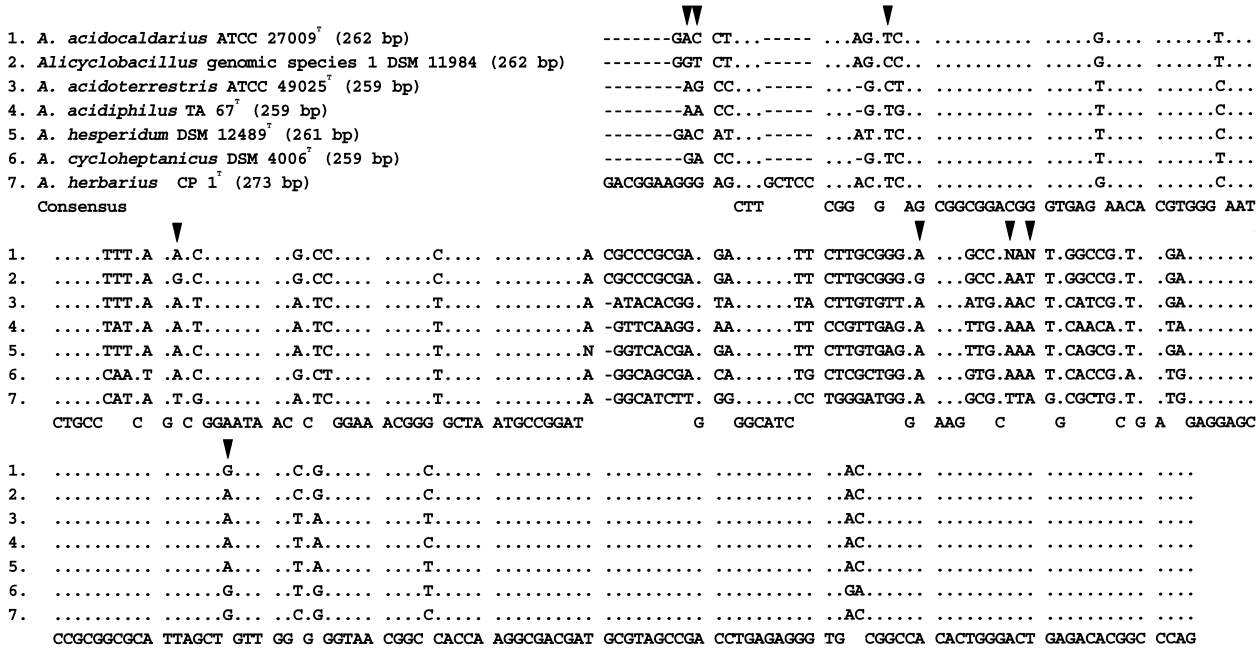

Fig. 2. Multiple sequence alignment of the HV region sequences from seven strains of *Alicyclobacillus* species. Nucleotide numbers of each HV region are presented in parentheses. Dots indicate conserved nucleotides, dashes indicate gaps. Arrows indicate distinctive nucleotide sites between *A. acidocaldarius* and *Alicyclobacillus* genomic species 1.

On the other hand, a comparison of the almost complete 16S rDNA sequences of Clusters I and II showed that the sequences in the former cluster were more than 99.5% identical with that of *Alicyclobacillus* genomic species 1 DSM 11984, and the sequences in the latter cluster were more than 99.5% identical with that of *A. acidocaldarius* ATCC 27009^T. Sequence similarities between clusters were from 98.2 to 98.5%. Sequence similarities lower than 95.4% (minimum: 91.3%) were found with other *Alicyclobacillus* species. The phylogenetic tree is shown in Fig. 1(b). The tree is similar to that of the HV region, and the general topologies of the trees obtained from the HV region and from the complete 16S rDNA sequences are basically the same. However, genetic distance was more emphasized in the HV region tree. 16S rDNA sequence analysis showed that these strains in Clusters I and II were closely related to each other, but shared an almost identical sequence (>99.5%) to two clusters at high bootstrap values. Accordingly, in order to certify the conservation of the HV region within the grouped species, it was necessary to clarify the identity of the strains in the two clusters.

Re-identification of Alicyclobacillus spp.

To ascertain the identity of species in Clusters I and II, strains in the clusters (Fig. 1) were subjected to physiological and biochemical tests, chemotaxonomic and phylogenetic analyses, and DNA-DNA hybridization experiments.

Differential phenotypic characteristics are summarized in Table 2. Although several differences were observed in the acid formation pattern among the strains, they were not distinguishable from each other based on their physiological and biochemical characteristics.

The major respiratory quinone of all strains was menaquinone 7 (MK7), which reached 71–96% of the total. MK3 was also present at 4–29%. The major fatty acid was ω-cyclohexyl 17:0 (47.1–68.7%) and the relative proportions of ω-cyclohexane fatty acid (ω-cyclohexyl 17:0 and ω-cyclohexyl 19:0) reached 74.0–93.2%. The remainder was a mixture of straight- and branched-chain fatty acids. All strains had DNA G+C contents in the range 61.4–62.7 mol% (Table 1). These chemotaxonomical features were extremely similar among the strains.

The results of DNA-DNA hybridization are summarized in Table 1. DNA-DNA binding levels between *Alicyclobacillus* genomic species 1 DSM 11984 and other strains in Cluster I were found to be from 79 to 99%. In addition, the binding levels between *A. acidocaldarius* ATCC 27009^T and the other strains in Cluster II were

Table 1. DNA base compositions and levels of DNA-DNA relatedness.

Strain	Cluster	G+C content (mol%)	DNA-DNA reassociation with (%)							
			1	2	3	4	5	6	7	8
Alicyclobacillus genomic species 1 DSM 11984	I	61.4	100	66	16	21	20	15	13	8
Alicyclobacillus genomic species 1 DSM 11983	I	61.7	99	57						
A. acidocaldarius DSM 453	I	61.7	93	65						
A. acidocaldarius DSM 454	I	61.4	89	61						
A. acidocaldarius DSM 455	I	61.5	86	57						
A. acidocaldarius DSM 12490	I	61.9	90	63						
Bacillus sp. DSM 6481	I	61.5	79	59						
A. acidocaldarius DSM 11297	II	62.7	58	81						
A. acidocaldarius ATCC 27009^T	II	61.9	57	100	20	16	29	13	10	10
A. acidocaldarius DSM 448	II	62.6	61	92						
A. acidocaldarius DSM 449	II	62.1	60	99						
A. acidocaldarius DSM 451	II	62.7	51	95						
A. acidocaldarius DSM 452	II	62.4	52	93						
A. acidoterrestris ATCC 49025^T	III	52.7	12	11	100	23	24	15	12	11
A. acidoterrestris DSM 3923	III	52.0			86	20	26			
A. acidoterrestris DSM 3924	III	52.5			89	19	26			
A. acidoterrestris DSM 2498	III	51.1			75	27	23			
A. acidiphilus TA 67^T		55.6	14	15	20	100	31	22	12	9
A. hesperidum DSM 12489^T	IV	54.0	23	24	30	25	100	13	10	12
A. hesperidum DSM 12766	IV	54.5			32	21	92			
A. cycloheptanicus DSM 4006^T	V	57.2	12	11	13	21	19	100	12	8
A. cycloheptanicus DSM 4005	V	57.0						86		
A. cycloheptanicus DSM 4007	V	56.6						79		
A. herbarius CP 1^T		57.0	9	10	9	14	13	16	100	11
Bacillus tusciae IFO 15312^T		58.6	7	9	6	5	7	5	4	100

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

1, *Alicyclobacillus* genomic species 1 DSM 11984; 2, *A. acidocaldarius* ATCC 27009^T; 3, *A. acidoterrestris* ATCC 49025^T; 4, *A. acidiphilus* TA 67^T; 5, *A. hespericum* DSM 12489^T; 6, *A. cycloheptanicus* DSM 4006^T; 7, *A. herbarius* CP 1^T; 8, *B. tusciae* IFO 15312^T.

from 81 to 99%. On the other hand, the binding levels between *Alicyclobacillus* genomic species DSM 11984 and the strains in Cluster II, and between *A. acidocaldarius* ATCC 27009^T and the strains in Cluster I were intermediate (51–66%). The data revealed an intermediate DNA relatedness between Clusters I and II indicative of strains of a subspecies or genomic species (Albuquerque et al., 2000; Wayne et al., 1987). Although the strains in Cluster I were sufficiently distinguishable from *A. acidocaldarius* by means of their genotypic discrepancies, the strains could not be differentiated phenotypically from *A. acidocaldarius.* Therefore it is considered appropriate that strains in Cluster I are classified as belonging to *Alicyclobacillus* genomic species 1, which is closely related to *A. acidocaldarius*, as described by Albuquerque et al. (2000).

Thus, based on their phenotypic properties, cellular fatty acid profiles, and their phylogenetic inference, strains DSM 448, DSM 449, DSM 451, DSM 452, and DSM 11297 were re-identified as *A. acidocaldarius* and strains DSM 453, DSM 454, DSM 455, DSM 6481, DSM 11983, and DSM 12490 were grouped as *Alicyclobacillus* genomic species 1 (reference strain: DSM 11984). The results from re-identification revealed that the two clusters determined from analysis of the HV region each comprised the one species, and therefore the HV region was also highly conserved in these two species.

This study has demonstrated that the HV region sequence of the genus *Alicyclobacillus* is sufficiently different among species (82.3–98.5% sequence similar-

Table 2. Differential phenotypic characteristics of *Alicyclobacillus* strains in Clusters I and II.

Characteristics	Cluster I							Cluster II					
	1	2	3	4	5	6	7	8	9	10	11	12	13
V-P test	+	+	−	−	−	+	+	+	−	+	+	+	+
Degradation of starch	+	−	+	+	+	+	+	+	+	+	+	+	+
Acid production from													
D-arabinose	−	−	−	−	−	−	+	−	−	−	−	−	−
L-arabinose	+	+	+	+	+	+	+	−	+	+	+	+	+
ribose	+	+	+	+	+	+	+	−	+	+	+	+	+
L-xylose	−	−	−	−	−	−	+	−	−	−	−	−	−
methyl β-xyloside	−	−	−	−	−	+	+	−	−	−	−	+	−
L-sorbose	−	+	+	−	+	+	+	−	+	−	−	−	−
rhamnose	+	+	+	+	+	+	−	−	+	−	−	−	−
inositol	−	+	−	−	+	−	+	−	−	−	−	+	+
sorbitol	−	−	−	−	−	+	+	−	−	−	−	−	−
methyl α,D-mannoside	−	−	+	+	+	+	+	−	−	−	−	+	+
amygdalin	−	−	−	+	−	−	−	−	−	−	−	−	−
arbutin	+	−	+	+	+	−	+	+	+	+	+	+	+
aesculin	−	−	−	+	+	+	−	+	+	+	+	+	+
salicin	+	−	−	+	+	+	+	+	−	−	−	−	+
melibiose	−	+	+	+	+	+	+	+	+	+	+	+	+
inulin	−	−	−	−	−	−	+	−	−	−	+	−	+
melezitose	+	+	+	+	+	+	+	−	−	+	+	+	+
D-raffinose	+	+	+	+	+	+	+	+	−	+	+	+	+
glycogen	+	−	+	+	+	+	+	+	+	+	−	+	+
xylitol	−	−	−	−	−	+	+	−	−	−	−	−	−
β-gentiobiose	+	+	+	+	+	+	+	+	−	+	+	+	+
D-lyxose	−	−	+	−	−	−	−	−	−	−	−	−	−
D-tagatose	+	+	+	−	+	+	+	+	+	−	+	+	+
D-fucose	−	−	+	−	−	−	−	−	−	−	−	−	+
L-fucose	−	−	+	−	−	−	+	−	−	−	−	−	−
D-arabitol	−	−	−	−	−	−	+	−	−	−	−	−	−

1, *Alicyclobacillus* genomic species 1 DSM 11984; 2, *Alicyclobacillus* genomic species 1 DSM 11983; 3, *A. acidocaldarius* DSM 453; 4, *A. acidocaldarius* DSM 454; 5, *A. acidocaldarius* DSM 455; 6, *Alicyclobacillus* sp. DSM 12490; 7, *Bacillus* sp. DSM 6481; 8, *A. acidocaldarius* DSM 11297; 9, *A. acidocaldarius* ATCC 27009^T; 10, *A. acidocaldarius* DSM 448; 11, *A. acidocaldarius* DSM 449; 12, *A. acidocaldarius* DSM 451; 13, *A. acidocaldarius* DSM 452. −, negative result; +, positive result. Other morphological, physiological and biochemical characteristics were identical with those of *A. acidocaldarius* (Albuquerque et al., 2000; Goto et al., 2002).

ity), and is highly conserved within species (>98.8% sequence similarity) as to be able to identify or group *Alicyclobacillus* species by sequence comparison of the HV region, as can be done for the genus *Bacillus.* Moreover, this identification method allows the obvious discrimination between *A. acidocaldarius* and *Alicyclobacillus* genomic species 1 according to differential nucleotides (Fig. 2), which cannot be performed through the comparison of phenotypic characterizations. Also, the HV region is relatively short (approximately 250–300 bp in length) and can be easily sequenced using a commercial kit, so that use of the HV region as an index for rapid identification can be widely applied for various purposes, such as the investigation of species composition or distribution, inspection of strains, and the recognition of a new species.

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Note Added in Manuscript

Recently, *Alicyclobacillus acidocaldarius* subsp. *rittmannii* (Nicolaus et al., 1998) was validated in *Int. J. Syst. Evol. Microbiol.*, **52**: 3 (2002) as a subspecies of *A. acidocaldarius.* In this study, the type strain of this subspecies, DSM 11297^T, which was designated as *A. acidocaldarius* at that time, clustered tightly with *A. acidocaldarius* in both the 16S rDNA and the HV region trees. Accordingly, *A. acidocaldarius* subsp. *rittmannii* is identified or grouped as *A. acidocaldarius* (formally *A. acidocaldarius* subsp. *acidocaldarius*) by sequence comparisons of the HV region.