

Reclassification of *Brevibacillus brevis* strains NCIMB 13288 and DSM 6472 (= NRRL NRS-887) as *Aneurinibacillus danicus* sp. nov. and *Brevibacillus limnophilus* sp. nov.

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Comparison of the hypervariable region (269–279 bases in length) at the 5' end of the 16S rDNA sequences of 29 bacterial strains that were identified previously as *Brevibacillus brevis* showed that 13 strains clustered with *Aneurinibacillus* species, eight strains clustered with *Bacillus* species and eight strains clustered with *Brevibacillus* species. Based on DNA–DNA hybridization results, 27 strains, not including [*Brevibacillus brevis*] NCIMB 13288 and [*Brevibacillus brevis*] DSM 6472, were reidentified as *Aneurinibacillus migulanus*, *Aneurinibacillus thermoaerophilus*, *Bacillus methanolicus*, *Bacillus oleronius*, *Brevibacillus agri*, *Brevibacillus brevis* and *Brevibacillus parabrevis*. [*Brevibacillus brevis*] NCIMB 13288, which was located in the *Aneurinibacillus* cluster, showed low DNA–DNA relatedness (< 14%) and low 16S rDNA sequence similarity (96.8–97.9%) to other *Aneurinibacillus* species. [*Brevibacillus brevis*] DSM 6472, which was located in the *Brevibacillus* cluster, also showed low DNA–DNA relatedness (< 12%) and low 16S rDNA sequence similarity (95.4–98.8%) to other *Brevibacillus* species. These genotypic and phylogenetic data, plus phenotypic and chemotaxonomic characteristics, suggest that [*Brevibacillus brevis*] NCIMB 13288 (= IAM 15048) and [*Brevibacillus brevis*] DSM 6472 (= NRRL NRS-887) represent novel species of the genera *Aneurinibacillus* and *Brevibacillus*, respectively, for which the names *Aneurinibacillus danicus* sp. nov. and *Brevibacillus limnophilus* sp. nov. are proposed.

Bacillus brevis was first described by Migula (1900). Subsequently, many strains, including some that were not in accordance with the criteria of *Bacillus brevis sensu stricto*, were classified as *Bacillus brevis*, according mainly to phenotype; as a result, *Bacillus brevis* has become a heterogeneous species, similar to *Bacillus circulans* (Nakamura & Swezey,

1983a, b). In the 1990s, based on polyphasic taxonomic studies of *Bacillus brevis* and its relatives, many species or strains were reclassified as novel species of the genera *Brevibacillus* and *Aneurinibacillus* (Nakamura, 1991, 1993; Takagi *et al.*, 1993; Shida *et al.*, 1994a, b, 1995, 1996; Meier-Stauffer *et al.*, 1996; Heyndrickx *et al.*, 1997). Recently, *Brevibacillus invocatus* was proposed as a novel species, according to the polyphasic identification of *Bacillus* and *Brevibacillus* strains from clinical, dairy and industrial specimens (Logan *et al.*, 2002). Thus, at the time of writing, 11 species are recognized to belong to the genus *Brevibacillus* and three species are recognized to belong to the genus *Aneurinibacillus*. However, with the exception of the thermophilic species *Brevibacillus thermoruber* and *Aneurinibacillus thermoaerophilus*, distinguishing between these species is extremely difficult, as most strains are unreactive or weak in many biochemical tests and few have useful differential phenotypic characteristics (Heyndrickx

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Abbreviation: HV, hypervariable.

The GenBank/EMBL/DDBJ accession numbers for the 16S rDNA sequences obtained in this study are AB112712–AB112731, as detailed in Fig. 2.

Tables showing differential characteristics between strain NCIMB 13288^T and related *Aneurinibacillus* strains and between strain DSM 6472^T and related *Brevibacillus* strains, and a figure showing consensus nucleotide sequences, are available as supplementary material in IJSEM Online.

et al., 1997; Logan *et al.*, 2002). Even with molecular analyses, such as amplified rDNA restriction analysis (ARDRA), identification of these species is not always simple (Logan *et al.*, 2002). In our previous studies, we demonstrated that a hypervariable (HV) region that corresponds to the 5' end of 16S rDNA (nucleotide positions 70–344; *Bacillus subtilis* numbering) is a useful marker for rapid identification and grouping of *Alicyclobacillus* species (Goto *et al.*, 2002a, b), *Bacillus* species (Goto *et al.*, 2000) and *Paenibacillus* species (Goto *et al.*, 2002c). In this study, to ascertain how well the region is conserved within *Brevibacillus* species, we investigated sequence similarities among 29 strains that were supplied as *Brevibacillus brevis*.

Fifty-two strains were obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany), IAM (Institute of Molecular and Cellular Biosciences, University of Tokyo, Tokyo, Japan), NBRC/IFO (NITE Biological Resource Center/Institute for Fermentation, Osaka, Japan) and NCIMB (National Collection of Industrial and Marine Bacteria, Aberdeen, UK). All strains were grown in nutrient broth and agar, or brain heart infusion broth and agar (Difco), at the optimum growth temperature (35 or 50 °C).

During phenotypic characterization of organisms, unless indicated otherwise, the methods described by Gordon *et al.* (1973) were used. Acidification was examined with API 50 CH test strips (bioMérieux) at the optimum growth temperature. Hydrolysis of DNA was determined by using DNase test agar (Difco). Oxidase activity was determined by using the bioMérieux oxidase test. Each test was repeated three times.

For quinone analysis, cells were grown in 100 ml nutrient broth at the optimum growth temperature in a reciprocal shaker for 24 h. Quinones were extracted from freeze-dried cells according to Sano *et al.* (1996) and analysed by using an Alliance HPLC system that was equipped with a Waters 996 Photodiode Array detector. A Mightysil RP-18 column (4.6 × 250 mm; Kanto Chemical) was used for separation and methanol/propan-2-ol (3:1, v/v) was used as the eluent. Quinones were identified by their UV spectra. For fatty acid analysis, cells were grown on trypticase soy agar (Difco) at the optimum growth temperature for 24 h, as recommended by the manufacturers of the Microbial Identification system (MIS; Microbial ID). Fatty acid methyl esters were extracted from fresh wet biomass by saponification and methylation, according to Bligh & Dyer (1959) and Metcalfe *et al.* (1996), and analysed by using MIS software (version 4.02).

Genomic DNA was extracted and purified by using a High Pure PCR Template Preparation kit (Roche). For DNA–DNA hybridization, genomic DNA was extracted by using a Qiagen Blood and Cell Culture DNA Maxi kit and purified by equilibrium centrifugation in CsCl/ethidium bromide gradients (Treisman, 1989) by using an Optima™ MAX Ultracentrifuge (Beckman Coulter). Samples were desalted

by using an Ultrafree-4 Centrifugal Filter unit (Millipore). Genetic relatedness was determined fluorometrically with photobiotin-labelled DNA probes and microplates, as described by Ezaki *et al.* (1989), by using a CytoFluor Series 4000 multiwell plate reader (PerSeptive Biosystems). Hybridization was carried out under stringent conditions (optimal renaturation temperature + 15 °C) for 3 h. Renaturation temperatures were based on the DNA G + C contents of the type strains of *Aneurinibacillus aneurinilyticus* (43.3%), *Bacillus methanolicus* (38.6%), *Bacillus oleroniensis* (34.9%) and *Brevibacillus brevis* (47.3%) (Ezaki *et al.*, 1989). Each hybridization experiment was repeated at least three times. DNA G + C contents were determined by HPLC using purified genomic DNA solution, as described previously (Tamaoka & Komagata, 1984), with genomic DNA of *Bacillus subtilis* IAM 12118^T as a control.

Almost-complete sequences were determined by using a 16S rRNA Gene kit (Applied Biosystems). Universal primers for amplification and sequencing of the HV region were: forward primer (F-1), 5'-GCYTAAYACATGCAAGTCGARCG-3' (corresponding to nucleotides 47–69 of *Bacillus subtilis* 16S rDNA; Y=C/T, R=A/G) and reverse primer (R-1), 5'-ACTGCTGCCTCCCGTAGGAGT-3' (corresponding to nucleotides 345–365 of *Bacillus subtilis* 16S rDNA). HV region DNA was amplified according to Sadaie *et al.* (1997) and sequenced by using a BigDye Terminator Cycle Sequencing kit (Applied Biosystems). An ABI Prism model 3100 genetic analyser (Applied Biosystems) was used for electrophoresis and data collection. Sequence analysis was performed by using GeneWorks (version 2.0; IntelliGenetics) and GenBank/EMBL/DBJ. Multiple sequence alignment was performed with CLUSTAL W version 1.7 (Thompson *et al.*, 1994). Phylogenetic trees were constructed from evolutionary data, which were calculated with Kimura's two-parameter model (Kimura, 1980), by using the neighbour-joining method of Saitou & Nei (1987). Robustness of individual branches was estimated by bootstrapping with 1000 replicates (Felsenstein, 1985).

To ascertain the conservation of the HV region within *Brevibacillus brevis* and its variation among different *Brevibacillus* species, we sequenced the HV regions of 29 strains that were identified previously as *Brevibacillus brevis*, as well as *Aneurinibacillus migulanus* DSM 8373, *A. thermoaerophilus* DSM 10155, *Brevibacillus agri* DSM 6366, *Brevibacillus borstelensis* DSM 6453, three strains of *Brevibacillus* sp. (DSM 4410, DSM 4411 and DSM 4412) and all type strains of *Aneurinibacillus* and *Brevibacillus* species. The results are summarized in Fig. 1 as a phylogenetic dendrogram, based on a comparison of 239 common nucleotide positions. Of the 29 *Brevibacillus brevis* strains, 13 clustered with *Aneurinibacillus* species, eight clustered with *Bacillus* species and eight clustered with *Brevibacillus* species. Of the eight that clustered with *Brevibacillus* species, two clustered with the type strain (DSM 30^T), along with *Brevibacillus choshinensis* DSM 8552^T and *Brevibacillus formosus* DSM 9885^T, all showing 100% sequence similarity.

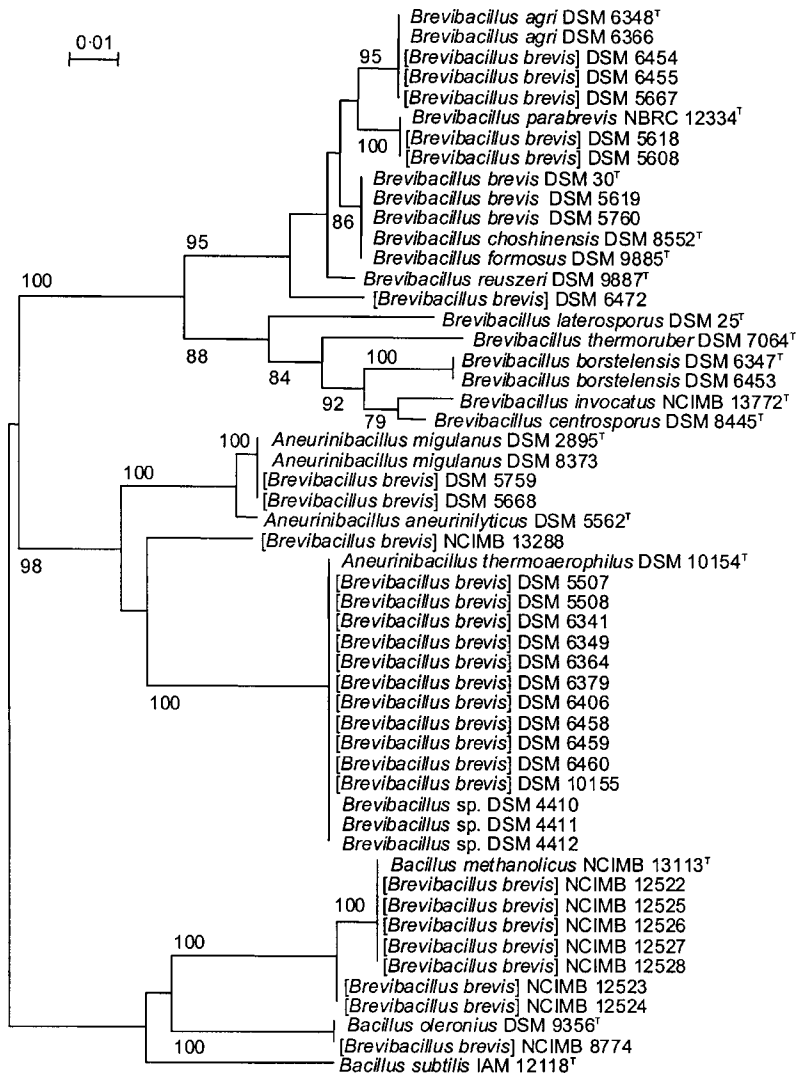


Fig. 1. Phylogenetic dendrogram based on the neighbour-joining method and derived from an alignment of 16S rDNA HV region sequences from *Brevibacillus brevis* strains and related taxa. The final dataset included 239 unambiguously aligned sites. Numbers represent percentages of 1000 replicate bootstrap samplings (frequencies < 70 % are not shown).

DSM 6472 and NCIMB 13288 did not cluster with any species of the genera *Aneurinibacillus* or *Brevibacillus*. Sequence similarity values within the clusters that contained *A. migulanus*, *A. thermoaerophilus*, *Bacillus oleronius*, *Brevibacillus agri* and *Brevibacillus parabrevis* were 100 %. In the *Bacillus methanolicus* cluster, NCIMB 12522, NCIMB 12525, NCIMB 12526, NCIMB 12527 and NCIMB 12528 all showed 100 % sequence similarity to *Bacillus methanolicus* NCIMB 13113^T. However, NCIMB 12523 and NCIMB 12524 differed at five nucleotide positions, including two polymorphic positions, with the closest relative being *Bacillus methanolicus* NCIMB 13113^T. NCIMB 12523 differed from NCIMB 12524 at the two polymorphic sites. On the other hand, *A. migulanus* DSM 8373, *A. thermoaerophilus* DSM 10155, *Brevibacillus agri* DSM 6366 and *Brevibacillus borstelensis* DSM 6453 clustered with their individual type strains with 100 % sequence similarity. Three strains of *Brevibacillus* sp. (DSM 4410, DSM 4411 and DSM 4412) also clustered with *A. thermoaerophilus* DSM 10154^T, with 100 % sequence similarity.

Nearly-complete 16S rDNA sequences (1486–1526 bp long) were determined for [*Brevibacillus brevis*] strains DSM 5619, DSM 5760, DSM 6472, NCIMB 12523, NCIMB 12524 and NCIMB 13288, which showed ambiguous relationships in the HV region dendrogram, and for the type strains of *Bacillus methanolicus*, *Aneurinibacillus* and *Brevibacillus* species. The sequences of NCIMB 12523 (GenBank accession no. AB112728) and NCIMB 12524 (AB112729) were 99.9 % similar. NCIMB 12523 and NCIMB 12524 both showed a close relationship to *Bacillus methanolicus* NCIMB 13113^T (AB112727), with 99.2 and 99.3 % similarity, respectively. Table 1 shows informative nucleotide positions among strains of the *Brevibacillus brevis*–*Brevibacillus choshinensis*–*Brevibacillus formosus* cluster. When polymorphic nucleotide positions were removed, the 16S rDNA sequences of *Brevibacillus brevis* DSM 30^T, [*Brevibacillus brevis*] DSM 5619 and *Brevibacillus formosus* DSM 9885^T were identical, but that of [*Brevibacillus brevis*] DSM 5760 differed from these three strains at nucleotide positions 612 and 620. By direct sequence comparison,

Table 1. Differential nucleotide positions among strains of the *Brevibacillus brevis*–*Brevibacillus formosus*–*Brevibacillus choshinensis* clusterNucleotide positions are given by reference to the 16S rDNA sequence of *Bacillus subtilis* (*rrE*). K, G/T; R, A/G; S, C/G; Y, C/T.

Strain	Nucleotide position													
	72	73	74	81	82	83	187	200	388	612	620	1055	1057	1266
<i>Brevibacillus brevis</i> :														
DSM 30 ^T (AB101593)	K	S	T	A	S	S	C	R	T	G	C	C	T	A
DSM 5619 (AB112730)	K	S	T	A	S	S	Y	G	T	G	C	C	T	A
DSM 5760 (AB112731)	G	G	T	A	C	C	C	G	T	A	T	C	T	R
<i>Brevibacillus formosus</i> DSM 9885 ^T (AB112712)														
	T	C	T	A	G	G	Y	G	T	G	C	C	T	A
<i>Brevibacillus choshinensis</i> DSM 8552 ^T (AB112713)														
	T	C	Y	R	G	G	C	G	C	A	T	T	C	G

Brevibacillus choshinensis DSM 8552^T differed from these four strains at six or seven reliable nucleotide positions (99.5–99.6% similarity). Thus, *Brevibacillus choshinensis* DSM 8552^T was an outlier of this cluster. However, nine informative nucleotide positions were removed as an alignment gap, making it difficult to describe the phylogeny of these five strains. Fig. 2 shows the phylogenetic tree of type strains of *Aneurinibacillus* and *Brevibacillus* species, [*Brevibacillus brevis*] DSM 6472 and [*Brevibacillus brevis*] NCIMB 13288, based on a comparison of 1430 common nucleotide positions. DSM 6472 and NCIMB 13288 were members of the genera *Brevibacillus* and *Aneurinibacillus*, respectively (these clusters were supported by high bootstrap values of 100%) but they differed clearly from species with validly published names. 16S rDNA sequence similarity of DSM 6472 to the type strains of *Brevibacillus* species ranged between 95.4 and 98.8% and sequence similarity of NCIMB 13288 to type strains of *Aneurinibacillus* species ranged between 96.8 and 97.9%. These results indicate that DSM 6472 and NCIMB 13288 are novel species of the genera *Brevibacillus* and *Aneurinibacillus*, respectively.

DNA G+C contents of the 52 strains were determined by HPLC and each cluster, grouped by HV region sequence, is summarized in Table 2. The DNA G+C contents of strains in the *Aneurinibacillus* cluster ranged from 42.9 to 46.7 mol%. The highest value, for [*Brevibacillus brevis*]

NCIMB 13288, was 46.7 mol%. DNA G+C contents of strains in the *Bacillus methanolicus* cluster ranged from 38.4 to 38.9 mol%, but these values were about 10 mol% lower than values that were estimated by the renaturation rate method (De Ley *et al.*, 1970; Arfman *et al.*, 1992). The DNA G+C content of *Bacillus methanolicus* NCIMB 13113^T, which was determined by the HPLC method at the TORAY Research Center (Tokyo, Japan), was 38.0 mol% (our data, 38.6 mol%); thus, we considered that the discrepancy was due to differences in the methods being used. The DNA G+C content of [*Brevibacillus brevis*] NCIMB 8774 (34.8 mol%) was in good accordance with the value from *Bacillus oleronius* DSM 9356^T. DNA G+C contents of strains in the *Brevibacillus* cluster ranged from 40.4 to 57.3 mol% and grouped well within each species cluster, respectively. The DNA G+C content of [*Brevibacillus brevis*] DSM 6472 was in the middle of the range, at 51.9 mol%.

Results of DNA–DNA hybridization experiments are shown in Table 2. In the clusters of *A. migulanus*, *A. thermoaerophilus*, *Bacillus oleronius*, *Brevibacillus agri*, *Brevibacillus borstelensis* and *Brevibacillus parabrevis*, there was at least 83% DNA–DNA relatedness between the type strain and other strains; thus, strains belonged to a single species within each cluster.

In the *Bacillus methanolicus* cluster, six strains with identical

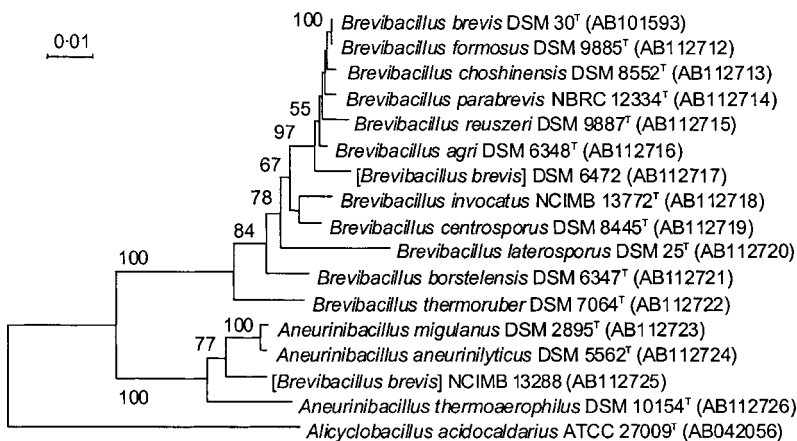
**Fig. 2.** Phylogenetic tree (constructed by using the neighbour-joining method) showing the phylogenetic position of strains DSM 6472 and NCIMB 13288 in relation to phylogenetically related *Aneurinibacillus* and *Brevibacillus* species, based on 16S rDNA sequences. *Alicyclobacillus acidocaldarius* ATCC 27009^T served as an outgroup. The final dataset included 1430 unambiguously aligned sites. Numbers represent percentages of 1000 replicate bootstrap samplings (frequencies < 50% are not shown).

Table 2. DNA G+C contents and DNA–DNA relatedness

Values are means of three or more repeated experiments.

Strain	DNA G+C content (mol%)	DNA–DNA hybridization (%) with:																							
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22		
1. <i>A. thermoaerophilus</i> DSM 1054 ^T	44.4	100	13	12	9																			6	
DSM 10155	44.6	92	11	11	10																				5
DSM 5507	45.1	95	14	11	10																				5
DSM 5508	44.9	100	14	11	10																				5
DSM 6341	44.6	100	13	11	11																				6
DSM 6349	44.7	100	14	11	11																				5
DSM 6364	44.6	97	10	13	9																				4
DSM 6379	44.8	96	11	10	10																				6
DSM 6406	45.1	94	12	12	10																				5
DSM 6458	44.3	99	12	10	12																				5
DSM 6459	44.6	100	12	9	8																				6
DSM 6460	45.6	90	11	8	10																				4
DSM 4410	44.5	92	12	8	10																				5
DSM 4411	45.0	94	13	10	10																				4
DSM 4412	44.8	96	14	9	12																				4
2. <i>A. thermoaerophilus</i> NCIMB 13288	46.7	13	100	12	13																				4
3. <i>A. migulans</i> DSM 2895 ^T	42.9	11	10	100	33																				5
DSM 8373	43.0	12	12	90	35																				6
DSM 5668	43.0	10	11	93	38																				6
DSM 5759	43.0	10	10	90	37																				5
4. <i>A. aneurinilyticus</i> DSM 5562 ^T	43.3	10	11	35	100																				5
5. <i>Bacillus methanolicus</i> ATCC 51375 ^T	38.6					100	59	59																	4
NCIMB 12528	38.6					95	53	57																	54
NCIMB 12527	38.6					95	56	58																	
NCIMB 12526	38.5					96	54	52																	5
NCIMB 12525	38.5					97	56	53																	5
NCIMB 12522	38.4					96	62	56																	5
6. NCIMB 12523	38.7					60	100	58																	5
7. NCIMB 12524	38.9					63	58	100																	5
8. <i>Bacillus oleronius</i> DSM 9356 ^T	34.9					8	10	8	100																5
NCIMB 8774	34.8								89																6
9. <i>Brevibacillus formosus</i> DSM 9885 ^T	47.0									100	46	56	50	13	11	8	9	9	10	9	11	11	9		9
10. <i>Brevibacillus brevis</i> DSM 30 ^T	47.3	6	5	6	4	5	6	6	4	53	100	55	55	14	13	11	11	12	10	11	12	10	9		9
11. DSM 5619	47.2									60	58	100	58	14	13	9	10	9	9	10	13	10	10		10
12. DSM 5760	47.3									61	51	58	100	13	14	10	12	12	11	9	12	10	9		9
13. <i>Brevibacillus chosinensis</i> DSM 8552 ^T	48.1									19	14	10	14	100	12	10	10	12	9	10	13	9	10		10
14. <i>Brevibacillus parabrevis</i> NBRC 12334 ^T	51.8									14	8	13	14	10	100	13	28	12	10	10	10	10	10		12

Table 2. cont.

Strain	DNA G+C content (mol%)	DNA-DNA hybridization (%) with:																					
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
DSM 5618	52.0	11	10	10	13	11	92	9	19	10	9	9	9	9	9	9	9	9	9	9	9	9	11
DSM 5608	51.9	12	9	10	10	9	83	10	20	9	10	10	11	11	11	11	11	11	11	11	11	11	9
15. <i>Brevibacillus reuszeri</i> DSM 9887 ^T	46.7	11	11	12	10	13	12	100	9	9	9	9	10	12	12	11							
16. <i>Brevibacillus agri</i> DSM 6348 ^T	53.4	13	12	12	10	13	35	9	100	9	12	10	12	13	9								
DSM 6366	53.3	13	11	11	10	11	30	9	95	10	7	10	9	9	9								
DSM 5667	53.8	13	11	15	9	13	32	9	86	8	7	9	10	11	9								
DSM 6454	53.2	12	10	13	10	14	28	8	90	10	10	12	11	8	10								
DSM 6455	53.4	11	12	10	9	12	31	10	87	10	9	13	9	10	11								
17. DSM 6472	51.9	12	9	9	10	11	11	10	11	100	10	12	9	12									
18. <i>Brevibacillus borstelensis</i> DSM 6347 ^T	51.4	12	8	9	11	12	10	9	10	9	100	13	12	10	10								
DSM 6453	50.8	10	10	10	9	11	11	10	9	9	83	22	10	12	9								
19. <i>Brevibacillus invocatus</i> NCIMB 13772 ^T	49.9	10	9	10	10	10	11	10	11	10	19	100	27	17	13								
20. <i>Brevibacillus centrosporus</i> DSM 8445 ^T	49.0	11	7	9	10	9	10	11	10	11	10	25	100	11	9								
21. <i>Brevibacillus thermoruber</i> DSM 7064 ^T	57.3	12	7	10	9	10	9	11	9	9	13	15	11	100	11								
22. <i>Brevibacillus laterosporus</i> DSM 25 ^T	40.4	12	7	11	9	9	8	9	8	9	11	10	10	9	100								

HV region sequences showed >95% DNA-DNA relatedness to the type strain of *Bacillus methanolicus* and were thus considered to belong to this species. On the other hand, strains NCIMB 12523 and NCIMB 12524 showed intermediate DNA-DNA relatedness values (52–63%) to other strains in the cluster, indicating some internal heterogeneity within *Bacillus methanolicus* (Arfman *et al.*, 1992). Although strains NCIMB 12523 and NCIMB 12524 could be subspecies of *Bacillus methanolicus*, we regarded strains NCIMB 12522–NCIMB 12528 as provisional members of *Bacillus methanolicus*, in agreement with Arfman *et al.* (1992).

Brevibacillus brevis DSM 30^T, DSM 5619, DSM 5760 and *Brevibacillus formosus* DSM 9885^T showed intermediate DNA-DNA relatedness (46–61%). A previous study (Shida *et al.*, 1995) reported that DNA-DNA relatedness between the type strains of *Brevibacillus brevis* and *Brevibacillus formosus* was 6%. In our study, DNA-DNA relatedness values were intermediate (46–61%), which is consistent with their highly similar 16S rDNA sequences (Amann *et al.*, 1992; Hiraishi & Ueda, 1994; Stackebrandt & Goebel, 1994) and almost-identical DNA G+C contents (47.0–47.3 mol%). Thus, *Brevibacillus brevis* and *Brevibacillus formosus* are closely related genetically and, furthermore, there are strains that are intermediate between the species. These observations are also supported by a previous polyphasic taxonomic study (Logan *et al.*, 2002). Although we tentatively regarded DSM 5619 and DSM 5760 as members of *Brevibacillus brevis*, further taxonomic studies are necessary to certify *Brevibacillus brevis* and *Brevibacillus formosus* as independent species. On the other hand, *Brevibacillus choshinensis* DSM 8552^T, an outlier of the cluster based on 16S rDNA sequence analysis, had an HV region sequence that was almost identical to those of these strains (Table 1) and showed low DNA-DNA relatedness (10–19%) to the other strains in the cluster. In API and other phenotypic analyses, *Brevibacillus choshinensis* could be distinguished from strains of *Brevibacillus brevis* and *Brevibacillus formosus*, whereas strains of *Brevibacillus brevis* and *Brevibacillus formosus* were indistinguishable (Logan *et al.*, 2002); thus, *Brevibacillus choshinensis* could be regarded as an independent species.

NCIMB 13288 had 8–38% DNA-DNA relatedness to strains within the *Aneurinibacillus* cluster. On the other hand, DSM 6472 had 8–12% DNA-DNA relatedness to strains within the *Brevibacillus* cluster. DNA-DNA hybridization data and 16S rDNA sequence analysis suggested that these strains were novel species of the genera *Aneurinibacillus* and *Brevibacillus*, respectively, and further taxonomic studies were carried out.

Differential phenotypic characteristics between DSM 6472 and closely related *Brevibacillus* reference strains, and between NCIMB 13288 and *Aneurinibacillus* type strains, are shown as Supplementary Tables A and B in IJSEM Online. Many biochemical characteristics of DSM 6472 were unreactive, but the strain could be distinguished from phylogenetically related species by a combination of

characteristics. However, distinguishing between strains remained difficult as currently available phenotypic tests produced either no results or unreliable results from replicates (Logan *et al.*, 2002).

NCIMB 13288 grew aerobically at 35 and 55 °C, but not at 30 or 60 °C; its optimal growth temperature was 45–50 °C. This thermophilic property clearly distinguished NCIMB 13288 from the mesophilic species *A. aneurinilyticus* and *A. migulanus*. Whilst NCIMB 13288 and *A. thermoaerophilus* are both thermophilic species, several characteristics distinguish them from each other (see Supplementary Table A, available in IJSEM Online).

The major respiratory quinone of DSM 6472 and NCIMB 13288 was menaquinone 7 (MK-7). The fatty acid profiles of DSM 6472, NCIMB 13288 and type strains of *Aneurinibacillus* species were investigated (Table 3). In DSM 6472, major fatty acids were iso-C_{15:0} (21.29%), anteiso-C_{15:0} (41.74%) and iso-C_{16:0} (12.40%). The profile of DSM 6472 was similar to those of other *Brevibacillus* species, but could be distinguished from them by a larger amount of iso-C_{16:0} (12.40%). In NCIMB 13288, major fatty acids were iso-C_{15:0} (57.67%), C_{16:0} (6.34%) and iso-C_{17:0} (7.99%); thus, this strain can be discriminated from other *Aneurinibacillus* species by lower amounts of anteiso-C_{15:0}, iso-C_{16:0} and C_{16:1}ω11cis. Whilst fatty acid analysis may

play a part in a polyphasic approach, we considered that it was not reliable on its own for identification of most taxa in the *Bacillus* group *sensu lato*, including *Aneurinibacillus* and *Brevibacillus*, as some other researchers have also suggested (Kämpfer, 1994; Logan *et al.*, 2002).

In this study, many strains that were identified previously as *Brevibacillus brevis* were reidentified as *A. migulanus*, *A. thermoaerophilus*, *Bacillus methanolicus*, *Bacillus oleronius*, *Brevibacillus agri* and *Brevibacillus parabrevis*, based on both HV region sequence analyses and DNA–DNA hybridization experiments. Misnaming in the past may have been due to difficulty in identifying unreactive spore-formers and the fact that there are few useful distinctive phenotypic characteristics among *Brevibacillus* species (Logan *et al.*, 2002). Whilst some strains show extremely close relationships between or within species, large-scale taxonomic studies that use many isolates and authentic strains are needed. Moreover, polyphasic taxonomic analyses clearly demonstrate that strains NCIMB 13288 and DSM 6472 are novel species of the genera *Aneurinibacillus* and *Brevibacillus*, respectively; therefore, the names *Aneurinibacillus danicus* sp. nov. and *Brevibacillus limnophilus* sp. nov. are proposed. The revived name *Brevibacillus limnophilus* is used for strain DSM 6472^T, which was originally deposited in the ARS Culture Collection as '*Bacillus limnophilus*' by Porter in 1940 and was later identified as *Bacillus brevis*.

In members of the genera *Aneurinibacillus* and *Brevibacillus*, the HV region sequence is conserved within species and has diverged between species, enabling identification and grouping, as we have already shown for *Alicyclobacillus*, *Bacillus* and *Paenibacillus*. Whilst there are still a few genera that have not yet been investigated, the HV region sequence is a powerful index for rapid and convenient identification and grouping of aerobic spore-formers. This method is of particular significance to the food industry and is gradually being used more widely. Consensual nucleotide sequences among species of the genera *Aneurinibacillus* and *Brevibacillus* are shown as a Supplementary Figure in IJSEM Online.

Description of *Aneurinibacillus danicus* sp. nov.

Aneurinibacillus danicus (da'ni.cus. N.L. adj. *danicus* Danish, pertaining to Denmark).

Gram-variable, strictly aerobic, motile, ellipsoidal spore-forming, straight rods (4.0–6.0 μm long and 0.8–1.0 μm wide). Ellipsoidal spores are oval and subterminal with swollen sporangia. Colonies on nutrient agar are circular, entire, smooth, flat, translucent and white (5–10 mm in diameter after 48 h). Temperature range for growth is 35–55 °C; optimum growth temperature is 45–50 °C. Optimum pH is 6.5–7.0; growth does not occur at pH 5.5 or 8.0. NCIMB 13288^T fails to grow in nutrient broth that contains >5% (w/v) NaCl and under anaerobic conditions. Positive for oxidase and catalase (weak), but negative for urease, utilization of citrate and nitrate

Table 3. Fatty acid profiles of strains DSM 6472, NCIMB 13288 and reference strains

Taxa: 1, DSM 6472; 2, Range of *Brevibacillus* species; 3, NCIMB 13288; 4, *A. thermoaerophilus* DSM 10154^T; 5, *A. migulanus* DSM 2895^T; 6, *A. aneurinilyticus* IAM 1077^T. Values are percentages of total fatty acids.

Fatty acid	1	2*	3	4	5	6
C _{14:0}		0.00–3.07	1.86		3.57	1.68
iso-C _{14:0}	4.51	1.72–12.45	1.19	5.71	4.65	1.16
iso-C _{14:1} ω9cis			3.75			
C _{15:0}	2.32	0.00–1.12	1.45	1.97	1.04	
iso-C _{15:0}	21.29	15.80–43.91	57.67	47.64	43.87	54.17
anteiso-C _{15:0}	41.74	20.55–59.80	0.90	3.34	7.32	4.35
iso-C _{15:1} F			2.08		1.04	
C _{16:0}	1.59	0.22–7.10	6.34	3.03	7.22	6.79
iso-C _{16:0}	12.4	1.50–6.51	3.55	19.64	3.87	1.68
iso-C _{16:1} H				1.94		
C _{16:1} ω5cis			1.11		1.31	
C _{16:1} ω7cis alcohol	3.7	0.00–5.49				
C _{16:1} ω11cis	1.14	0.00–2.78			7.59	10.92
iso-C _{17:0}	2.89	0.00–8.09	7.99	7.26	2.17	4.67
anteiso-C _{17:0}	5.36	0.28–3.38		1.18		
iso-C _{17:1} ω10cis	1.34	0.00–5.42			1.8	5.04
Summed feature 3			1.64		4.74	1.79
Summed feature 4	1.17	0.00–6.18	7.38	4.14	2.73	2.38

*Data from Logan *et al.* (2002).

reductase. Aesculin, casein, tyrosine (weak), gelatin and DNA are hydrolysed, but arbutin and starch are not. Acid is produced from glycerol, erythritol (weak), D-arabinose, L-arabinose (weak), D-xylose, L-xylose, ribose, adonitol, D-fructose, D-mannose, L-sorbose, dulcitol, sorbitol, xylitol, D-lyxose, D-tagatose and D-arabitol. Major fatty acids are iso-C_{15:0}, C_{16:0} and iso-C_{17:0}. Main quinone is MK-7. DNA G + C content is 46.7 mol%.

The type strain is NCIMB 13288^T (=IAM 15048^T).

Description of *Brevibacillus limnophilus* sp. nov.

Brevibacillus limnophilus (lim.no'phi.lus. Gr. n. *limnos* lake; L. adj. *philus* from Gr. adj. *philos* loving, friendly to; N.L. masc. adj. *limnophilus* lake-loving).

Gram-variable, strictly aerobic, motile, ellipsoidal spore-forming, straight rods (2.2–4.0 µm long and 0.5–0.6 µm wide). Ellipsoidal spores are oval and subterminal with swollen sporangia. Colonies on nutrient agar are circular, entire, smooth, convex, translucent and whitish-beige (3–4 mm in diameter after 48 h). Temperature range for growth is 20–45 °C; optimum growth temperature is 30–35 °C. Optimum pH is 7.0–7.5; growth does not occur at pH 6.0 or 8.5. DSM 6472^T fails to grow in nutrient broth that contains >2% (w/v) NaCl and under anaerobic conditions. Positive for catalase, but negative for oxidase and urease activities, utilization of citrate and nitrate reductase. Aesculin and DNA (weak) are hydrolysed, but arbutin, casein, gelatin, starch and tyrosine are not. Acid is produced from glycerol, L-arabinose, rhamnose, ribose and D-fructose. Major fatty acids are iso-C_{15:0}, anteiso-C_{15:0} and iso-C_{16:0}. Main quinone is MK-7. DNA G + C content is 51.9 mol%.

The type strain is DSM 6472^T (=NRRL NRS-887^T).

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