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Phylogenetic relationships between *Bacillus* species and related genera inferred from comparison of 3′ end 16S rDNA and 5′ end 16S–23S ITS nucleotide sequences

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The nucleotide sequences of the 3′ end of the 16S rDNA and the 16S–23S internal transcribed spacer (ITS) of 40 *Bacillaceae* species were determined. These included 21 *Bacillus*, 9 *Paenibacillus*, 6 *Brevibacillus*, 2 *Geobacillus*, 1 *Marinibacillus* and 1 *Virgibacillus* species. Comparative sequence analysis of a 220 bp region covering a highly conserved 150 bp sequence located at the 3′ end of the 16S rRNA coding region and a conserved 70 bp sequence located at the 5′ end of the 16S–23S ITS of the 40 species and six sequences available in GenBank were used to infer the phylogenetic relationships between all 46 taxa. When a maximal distance ($D_{\max}$, where D refers to the number of nucleotide substitutions per site) of 0·31 was introduced as a threshold to determine groupings, 10 phylogenetically distinct clusters were revealed. Twenty-six *Bacillus* species were separated in seven groups (I, II, III, IV, V, VI and X), but *Bacillus circulans* remained ungrouped. All six *Brevibacillus* species under study were in Group VII. The nine *Paenibacillus* species fell into two distinct groups (VIII and IX). Species with $D_{\max}$ values within 0·05 were considered to be very closely related. These were *Bacillus psychrophilus* and *Bacillus psychrosaccharolyticus* in Group II; '*Bacillus maroccanus*' and *Bacillus simplex* in Group II; *Bacillus amyloliquefaciens*, *Bacillus atrophaeus*, *Bacillus mojavensis* and *Bacillus subtilis* in Group VI; *Bacillus fusiformis* and *Bacillus sphaericus* in Group VI; *Brevibacillus brevis* and *Brevibacillus formosus* in Group VII; *Paenibacillus gordonae* and *Paenibacillus validus* in Group VIII; and *Bacillus anthracis*, *Bacillus cereus*, *Bacillus mycoides* and *Bacillus thuringiensis* in Group X. The phylogenetic classification presented here is, in general, in agreement with current classifications based on phenotypic and molecular data. Our findings suggest, however, that in some cases, further divisions or, conversely, further groupings might be warranted. Should current classifications be re-examined in the light of our results, $D_{\max}$ values of 0·31 and 0·05, as exemplified here, may prove useful threshold values for the grouping of *Bacillaceae* into taxa akin to genera and species, respectively. These $D_{\max}$ thresholds may also reveal, in a different way, bacterial species for which further characterization might be warranted for proper classification and/or reassignment.

INTRODUCTION

As of 1986 (Claus & Berkeley, 1986), the genus *Bacillus* has encompassed a variety of phenotypically heterogeneous species exhibiting a wide range of nutritional requirements, physiological and metabolic diversity and DNA base composition. Numerical classification based on a series of phenetic characters has been used for the classification of 368 *Bacillus* strains into 79 clusters (Priest *et al.*, 1988). At about the same time, rRNA sequences were being established as a most useful molecular chronometer to infer phylogenetic relationships because they are present in all organisms and changes in the nucleotide sequences were deemed to occur in a clocklike manner (Woese, 1987). Soon, several *Bacillus* species were reclassified based on 16S rDNA sequence alignment. Rössler *et al.* (1991) grouped nine *Bacillus* species into four clusters. Ash *et al.* (1991) separated

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Abbreviations: $D_{\max}$, maximal distance, where distance D refers to the number of nucleotide substitutions per site; ITS, internal transcribed spacer.

The GenBank accession numbers for the 16S rDNA and 16S–23S ITS of different strains of *Bacillus* and related genera used in this work are AF478062–AF478111.

51 *Bacillus* species into five phylogenetically distinct clusters. Further characterizations at the genotypic and phenotypic levels of selected *Bacillus* species have led to the creation of several new genera: *Amphibacillus* (Niimura *et al.*, 1990), *Alicyclobacillus* (Wisotzkey *et al.*, 1992), *Paenibacillus* (Ash *et al.*, 1993), *Aneurinibacillus* and *Brevibacillus* (Shida *et al.*, 1996a), *Virgibacillus* (Heyndrickx *et al.*, 1998), *Gracilibacillus* and *Salibacillus* (Wainø *et al.*, 1999), *Filobacillus* (Schlesner *et al.*, 2001), *Geobacillus* (Nazina *et al.*, 2001), *Ureibacillus* (Fortina *et al.*, 2001), and *Jeotgalibacillus* and *Marinibacillus* (Yoon *et al.*, 2001). Recently, partial 16S rDNA sequence (Goto *et al.*, 2000) and rRNA gene restriction patterns (Joung & Côté, 2002) have been used for the rapid identification or classification of *Bacillus* species and related genera, respectively.

The 16S–23S internal transcribed spacer (ITS) region has been widely studied for the presence of functional motifs (Berg *et al.*, 1989; Nodwell & Greenblatt, 1993; Pfeiffer & Hartmann, 1997), specific processing sites (Apirion & Miczak, 1993) and secondary structures (Nour, 1998; Liiv *et al.*, 1998). Because the 16S–23S ITS region is hypervariable, as opposed to the more conserved 16S rRNA coding region, it has also been used in the study of prokaryotic diversity at the species and subspecies levels (Gürtler & Stanisich, 1996; García-Martínez *et al.*, 1999). The ITS-PCR fingerprints have been used to reveal length polymorphisms between *Bacillus* species (Daffonchio *et al.*, 1998a) and at the intra-specific level (Daffonchio *et al.*, 1998b). Part of the ITS has been amplified by PCR and used as a probe for the detection, identification and phylotyping of *Bacillus* species (de Silva *et al.*, 1998).

The current classification of species within the genus *Bacillus* and correlated genera is well established and is based on a combination of numerous experimental approaches. In the present study, we aimed to determine whether or not a combination of part of the 16S rRNA conserved sequence with part of the 16S–23S ITS hypervariable sequence could be informative enough to be used as a simple, useful marker for the classification of *Bacillus* species and related genera. A nucleotide sequence containing the last 150 bp located at the 3′ end of the 16S rRNA coding region and the first 70 bp located at the 5′ end of the 16S–23S ITS was used to infer the phylogenetic relationships between 46 *Bacillaceae* species and eight more distant bacterial species. The robustness of this classification will be assessed by comparison with the current *Bacillaceae* classifications.

METHODS

Bacterial strains and culture conditions. All the *Bacillaceae* species used in this study were obtained from the American Type Culture Collection (ATCC) (Table 1). They were grown following ATCC guidelines (Pienta *et al.*, 1996).

Escherichia coli strain TOP10 (Invitrogen) was used for cloning PCR fragments. Strain TOP10 was cultured on LB agar plates to select transformants or in LB broth, with shaking at 180–200 r.p.m. at 37 °C, overnight. When necessary, kanamycin was added to the medium at a final concentration of 50 μg ml^{-1}.

DNA extraction. For the total DNA isolation, bacterial cells were washed with TESS buffer [10 mM Tris/HCl, 1 mM $\mathrm{Na_2EDTA}$, 0·1 M NaCl and 0·1 % Sarkosyl (*N*-lauroylsarcosine)] and resuspended in TE buffer (10 mM Tris/HCl, 1 mM $\mathrm{Na_2EDTA}$). Cells were lysed with 50 mg lysozyme ml^{-1} and 0·1 % SDS. The subsequent phenol/chloroform extractions and ethanol precipitation were carried out as described by Sambrook *et al.* (1989).

Recombinant plasmid from *E. coli* strain TOP10 was isolated using the alkaline-lysis method (Stephen *et al.*, 1990) with some modifications. Sodium acetate (pH 5·2) was used to replace potassium acetate for renaturing DNA. After removing cell debris and chromosomal DNA by centrifugation, an equal volume of 7·5 M ammonium sulfate was added to precipitate RNA. The RNA was removed by centrifugation at 13 000 r.p.m. for 20 min. Plasmid DNA was precipitated with 2 vols ethanol, and the pellet was air-dried and resuspended in sterile water.

Amplification of the 3′ end 16S rDNA and the 16S–23S ITS region. The 3′ end of 16S rDNA, the 16S–23S ITS region and the 5′ end of 23S rDNA was amplified with a pair of primers: L516SF (5′-TCGCTAGTAATCGCGGATCAGC-3′) and L523SR (5′-GCATATCGGTGTTAGTCCCGTCC-3′). Amplification was performed in a Thermal Cycler 9600 (Perkin Elmer) in a total volume of 50 μl containing about 50 ng DNA, 0·25 μM each primer, 200 μM dNTP, 1·5 mM $\mathrm{MgCl_2}$ and 1·25 U *Taq* DNA polymerase (Qiagen). PCR was performed under the following conditions: 45 s at 95 °C and then 30 cycles of 15 s at 94 °C, 30 s at 53 °C and 90 s at 72 °C. Amplification products were visualized on agarose gels.

Cloning and sequencing methods. The amplified DNAs were cloned into a pCRII-TOPO cloning vector using the TOPO TA cloning kit (Invitrogen), following the manufacturer's instructions. Transformants were selected on LB agar plates containing kanamycin (50 μg ml^{-1}), X-Gal (40 μg ml^{-1}) and IPTG (0·5 mM). A single clone was selected for each *Bacillaceae* species. The recombinant plasmids were isolated using the alkaline-lysis method, digested with *Eco*RI and visualized on agarose gels to confirm the presence of an inserted fragment.

The dideoxynucleotide chain-termination method (Sanger *et al.*, 1977), using the near-infrared fluorescence automated DNA sequencer (LI-COR model 4200), was used to sequence the DNA fragments.

Sequence analysis. The 3′ end of the 16S rDNA and the 16S–23S ITS of the 40 *Bacillaceae* species sequenced in this study, as well as six other *Bacillus* sequences (Table 1), and eight other sequences from more distant species available in the GenBank database (*Clostridium perfringens*, AB045290; *Sarcina ventriculi*, AF110272; *Lactobacillus pantheris*, AF413523; *Desulfotomaculum kuznetsovii*, AY036903; *Thermoactinomyces vulgaris*, AF138739; *Streptococcus gallolyticus*, AF323911; *Deinococcus radiodurans*, NC_001263; '*Salmonella enterica*', NC_003198), were used for comparison. The sequences were aligned using the CLUSTAL W program (Thompson *et al.*, 1994) and the most parsimonious phylogenetic trees were constructed using the DNAPARS program of the PHYLIP package, version 3.6a2 (Felsenstein, 1989, 2001). The order of the input sequences was randomized by DNAPARS. Stability of the groupings was estimated by bootstrap analysis on 100 trees using SEQBOOT in the same package. Trees were visualized using TREEVIEW software, version 1.6.1 (Page, 1996).

RESULTS AND DISCUSSION

Two primers, one located about 200 nt upstream from the 3′ end of the 16S rRNA gene, the other about 80 nt

Table 1. *Bacillaceae* species used in this study, and GenBank accession numbers for 16S rDNA and 16S–23S ITS sequences

Species	Strain	GenBank accession no.*	$D_{\max}$ of 16S and 16S–23S ITS rDNA sequences within corresponding group	$D_{\max}$ of closely related clusters with $D_{\max} < 0{\cdot}05$
Group I			0·15	
Bacillus coagulans	ATCC 7050$^{\mathrm{T}}$	AF478062		
Geobacillus kaustophilus	ATCC 8005$^{\mathrm{T}}$	AF478063		
Geobacillus stearothermophilus	ATCC 12980$^{\mathrm{T}}$	AF478064		
Group II			0·23	
Bacillus azotoformans	ATCC 29788$^{\mathrm{T}}$	AF478065		
Bacillus megaterium	ATCC 14581$^{\mathrm{T}}$	AF478067		0·011
Bacillus pumilus	ATCC 7061$^{\mathrm{T}}$	AF478070		
'*Bacillus maroccanus*'	ATCC 25099	AF478066		0·011
Bacillus simplex	ATCC 49097$^{\mathrm{T}}$	AF478071		
Bacillus psychrophilus	ATCC 23304$^{\mathrm{T}}$	AF478068		0·035
Bacillus psychrosaccharolyticus	ATCC 23296$^{\mathrm{T}}$	AF478069		
Group III			0·17	
Bacillus laevolacticus	ATCC 23492$^{\mathrm{T}}$	AF478072		
Virgibacillus pantothenticus	ATCC 14576$^{\mathrm{T}}$	AF478073		
Group IV			0·16	
Bacillus badius	ATCC 14574$^{\mathrm{T}}$	AF478074		
Marinibacillus marinus	ATCC 29841$^{\mathrm{T}}$	AF478075		
Group V			0·16	
Bacillus flexus	ATCC 49095$^{\mathrm{T}}$	AF478076		
Bacillus halodurans		AB031215†		
Group VI			0·20	
Bacillus insolitus	ATCC 23299$^{\mathrm{T}}$	AF478084		
Bacillus licheniformis	ATCC 14580$^{\mathrm{T}}$	AF478085–AF478087		
'*Bacillus macroides*'	ATCC 12905	AF478088		
Bacillus amyloliquefaciens	ATCC 23350$^{\mathrm{T}}$	AF478077–AF478079		0·020
Bacillus atrophaeus	ATCC 49337$^{\mathrm{T}}$	AF478080		
Bacillus mojavensis	ATCC 51516$^{\mathrm{T}}$	AF478089		
Bacillus subtilis		NC_000964†		
Bacillus fusiformis	ATCC 7055$^{\mathrm{T}}$	AF478081–AF478083		0·007
Bacillus sphaericus	ATCC 14577$^{\mathrm{T}}$	AF478090		
Group VII			0·22	
Brevibacillus agri	ATCC 51360	AF478091		
Brevibacillus borstelensis	ATCC 51668$^{\mathrm{T}}$	AF478092, AF478093		
Brevibacillus choshinensis	ATCC 51359$^{\mathrm{T}}$	AF478095		
Brevibacillus parabrevis	ATCC 8186	AF478097		
Brevibacillus brevis	ATCC 8246$^{\mathrm{T}}$	AF478094		0·025
Brevibacillus formosus	ATCC 51669$^{\mathrm{T}}$	AF478096		
Group VIII			0·24	
Paenibacillus alvei	ATCC 6344$^{\mathrm{T}}$	AF478098		
Paenibacillus lautus	ATCC 43898$^{\mathrm{T}}$	AF478100		
Paenibacillus macerans	ATCC 8244$^{\mathrm{T}}$	AF478101		
Paenibacillus pabuli	ATCC 43899$^{\mathrm{T}}$	AF478102		
Paenibacillus gordonae	ATCC 29948$^{\mathrm{T}}$	AF478099		0·049
Paenibacillus validus	ATCC 43897$^{\mathrm{T}}$	AF478103		
Group IX			0·22	
Paenibacillus alginolyticus	ATCC 51185$^{\mathrm{T}}$	AF478104		
Paenibacillus chondroitinus	ATCC 51184$^{\mathrm{T}}$	AF478105		
Paenibacillus larvae	ATCC 9545$^{\mathrm{T}}$	AF478106		
Group X			0·13	
Bacillus lentus	ATCC 10840$^{\mathrm{T}}$	AF478107–AF478110		

Table 1. cont.

Species	Strain	GenBank accession no.*	$D_{\max}$ of 16S and 16S–23S ITS rDNA sequences within corresponding group	$D_{\max}$ of closely related clusters with $D_{\max} < 0{\cdot}05$
Bacillus anthracis		AY008265†		0·047
Bacillus cereus		Z84590†		
Bacillus mycoides		Z84591†		
Bacillus thuringiensis		Z84594†		
Ungrouped				
Bacillus circulans	ATCC 4513^{T}	AF478111		

*GenBank accession numbers AF478062–AF478111 were assigned to nucleotide sequences determined in this study.

†DNA sequences retrieved from GenBank.

downstream from the 5′ end of the 23S rRNA gene, were used to amplify the last 200 bp of the 16S rRNA gene and the entire 16S–23S ITS region from 40 *Bacillaceae* species. These included 21 *Bacillus*, 9 *Paenibacillus*, 6 *Brevibacillus*, 2 *Geobacillus*, 1 *Virgibacillus* and 1 *Marinibacillus* species. The amplified fragments varied in length from 450 to 850 bp. They were cloned and their nucleotide sequences determined. The homologous DNA sequences from six more *Bacillus* species, *Bacillus anthracis*, *Bacillus cereus*, *Bacillus mycoides*, *Bacillus halodurans*, *Bacillus subtilis* and *Bacillus thuringiensis*, available in GenBank, were added (Table 1). A multiple alignment of the nucleotide sequences from these 46 *Bacillaceae* species was performed.

Conservation of the 3′ end 16S rRNA coding region

Comparative analysis of the 3′ end 16S rRNA coding region reveals that at least the last 157 bp share extensive nucleotide identities with all 46 *Bacillaceae* species (alignment available as supplementary data in IJSEM Online at http://ijs.sgmjournals.org). This sequence encompasses the 16S rRNA gene highly conserved regions 2, 3 and 4 (Lane *et al.*, 1985; Weisburg *et al.*, 1991; Gürtler & Stanisich, 1996) located at nucleotide positions −157 to −140, −52 to −38, and −20 to −4, respectively. Region 2 is believed to be the most highly conserved sequence in eubacteria, archaea and eukaryotes (Lane *et al.*, 1985). Regions 3 and 4 are present in many eubacteria (Weisberg *et al.*, 1991). Region 2 was identical for all 46 *Bacillaceae* species analysed, except for *Bacillus anthracis* for which the corresponding sequence was not available in full. Only *Bacillus cereus* showed a nucleotide substitution in region 3, at position −38, where deoxyadenosine is replaced by deoxyguanosine. All six *Brevibacillus* species, *Brevibacillus agri*, *Brevibacillus borstelensis*, *Brevibacillus brevis*, *Brevibacillus choshinensis*, *Brevibacillus formosus* and *Brevibacillus parabrevis* shared the same nucleotide substitution: C to A in region 4, at nucleotide position −18.

The alignment of the 157 bp sequences of the 3′ end 16S rDNA of the 46 *Bacillaceae* species (Table 1) and species of five more Gram-positive genera, *Clostridium perfringens*, *Sarcina ventriculi*, *Lactobacillus pantheris*, *Desulfotomaculum kuznetsovii* and *Thermoactinomyces vulgaris*, two Gram-positive cocci, *Streptococcus gallolyticus* and *Deinococcus radiodurans*, and a Gram-negative, '*Salmonella enterica*', was used to construct a phylogenetic tree (Fig. 1). All 46 *Bacillaceae* species listed in Table 1 are present in the main Group A. *Lactobacillus pantheris*, *Sarcina ventriculi* and *Clostridium perfringens* formed the small Group B, whereas *Deinococcus radiodurans*, '*Salmonella enterica*', *Desulfotomaculum kuznetsovii*, *Thermoactinomyces vulgaris* and *Streptococcus gallolyticus* remained ungrouped. Within Group A, both *Geobacillus* species, all nine *Paenibacillus* species and all six *Brevibacillus* species can each be traced to respective common nodes, exclusive of other genera. Some closely related species, such as *Bacillus atrophaeus* and *Bacillus mojavensis*; *Bacillus lentus* and *Bacillus insolitus*; *Geobacillus kaustophilus* and *Geobacillus stearothermophilus*; *Bacillus halodurans* and *Bacillus anthracis*; *Bacillus psychrophilus* and *Bacillus psychrosaccharolyticus*; *Brevibacillus borstelensis*, *Brevibacillus brevis* and *Brevibacillus formosus*; *Brevibacillus parabrevis* and *Brevibacillus agri*; *Paenibacillus gordonae* and *Paenibacillus validus*; and *Paenibacillus alvei*, *Paenibacillus macerans* and *Paenibacillus larvae*, could not be distinguished from each other. Clearly, the 157 bp sequence at the 3′ end 16S rDNA was sufficient to distinguish the bacterial genera under study, separate genera closely related to *Bacillus* (Group A) from other more distant genera and cluster species within genera for *Geobacillus*, *Paenibacillus* and *Brevibacillus*. It could not, however, distinguish closely related species.

Conserved region of 16S–23S ITS between alleles

A total of three, two, three, three and four PCR fragments from different alleles of the 16S–23S ITS regions were cloned from *Bacillus amyloliquefaciens*, *Brevibacillus borstelensis*, *Bacillus fusiformis*, *Bacillus licheniformis* and *Bacillus lentus*, respectively (Fig. 2). The lengths of each 16S–23S ITS copy were 172, 230 and 348 bp for *Bacillus amyloliquefaciens*; 232, 303 and 344 bp for *Bacillus fusiformis*; 168, 172 and 334 bp

Fig. 1. Phylogenetic relationships of 46 *Bacillus*, *Brevibacillus*, *Paenibacillus*, *Virgibacillus* species, and eight distant species inferred from the alignment of the 157 bp 3′ end 16S rRNA coding region. Bootstrap values (expressed as percentages of 100 replications) are shown at branch points; values greater than 50 % were considered significant. The bar represents the unit length of the number of nucleotide substitutions per site. Abbreviations: *B.*, *Bacillus*; *Br.*, *Brevibacillus*; *G.*, *Geobacillus*; *M.*, *Marinibacillus*; *P.*, *Paenibacillus*; *V.*, *Virgibacillus*.

for *Bacillus licheniformis*; and 222, 273, 304 and 432 bp for *Bacillus lentus*. Two incomplete copies of 567 and 629 bp were obtained for *Brevibacillus borstelensis*. In each species, only the longest 16S–23S ITS copies carried two tRNA genes: $\mathrm{tRNA^{ile}}$ and $\mathrm{tRNA^{ala}}$, except *Brevibacillus borstelensis* for which both ITS copies (ITS 1 and ITS 2, Fig. 2c) harboured the two tRNA genes.

The nucleotide sequences of the 16S–23S ITS alleles were aligned for each species, respectively. The results of the alignments revealed how the homology was distributed within the 16S–23S ITS. It is interesting to note that the 5′ end 16S–23S ITS is conserved between alleles of the same strain. This holds true for the five species tested. It also proved true for the 10 16S–23S ITS allelic sequences of *Bacillus subtilis* (Fig. 2a) available from GenBank and the seven allelic sequences in *Bacillus halodurans* (data not shown). The conserved 5′ end 16S–23S ITS was at least 70 bp long in each bacterial strain studied. For *Brevibacillus borstelensis* this conserved region between both alleles was nearly 400 bp long (Fig. 2c).

In most cases, the central part of the 16S–23S ITS studied was less conserved. Deletions most often occurred in the region harbouring the $\mathrm{tRNA^{ile}}$ and $\mathrm{tRNA^{ala}}$ genes. Both tRNA genes were either coexistent or absent on the same allele. In no case was only either of the tRNA genes present. Whether or not the deletion of both tRNA genes on some alleles might be a consequence of gene regulation is debatable.

For some species, like *Bacillus subtilis* and *Bacillus amyloliquefaciens*, the 3′ end of the 16S–23S ITS region was also highly conserved between alleles. For the other species under study, this region was either less conserved or the conserved region was short.

To determine whether or not the conservation of the first 70 bp of the 5′ end of 16S–23S ITS allelic sequences within strains could be extended to other bacteria, copies of the 16S–23S ITS allelic sequences of seven Gram-positive bacteria available from GenBank were compared (alignment data not shown). These included *Streptococcus pyogenes* MGAS8232 (GenBank NC_003485), *Streptococcus pyogenes* M1 GAS (NC_002737), *Streptococcus pneumoniae* (NC_003098), *Staphylococcus aureus* (NC_003923), *Listeria*

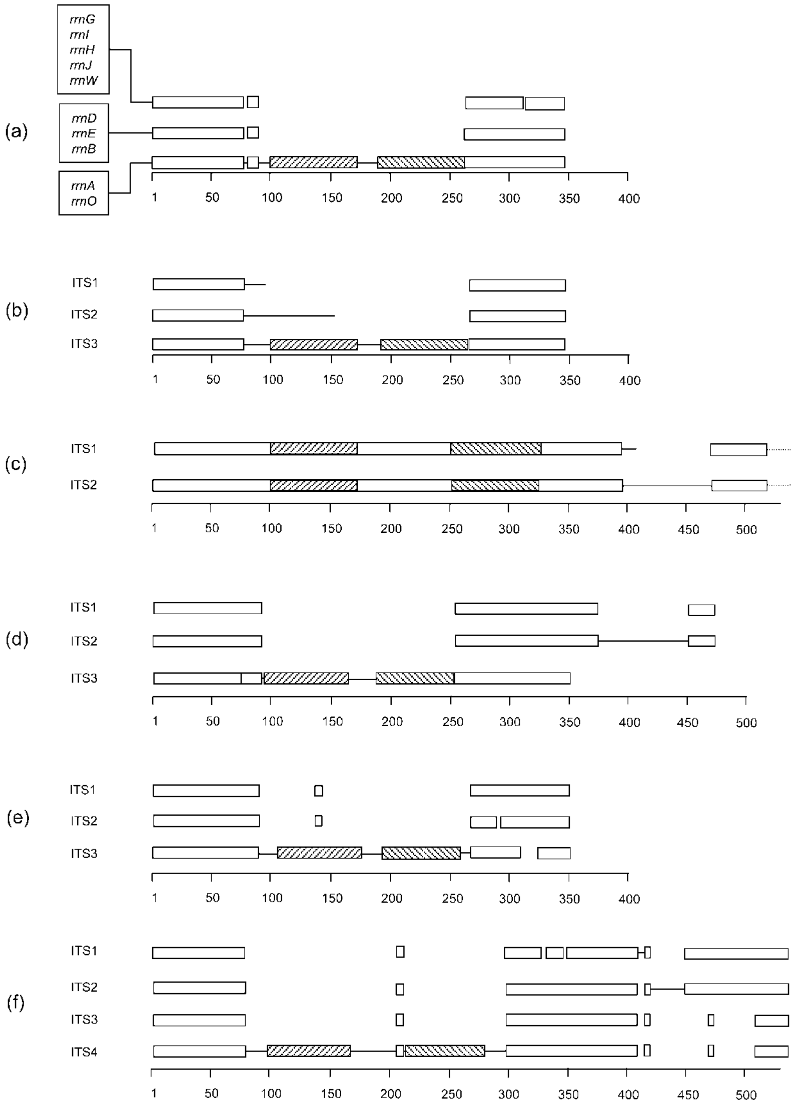

Fig. 2. Schematic representation of allelic 16S–23S rDNA ITS in (a) *Bacillus subtilis*, (b) *Bacillus amyloliquefaciens*, (c) *Brevibacillus borstelensis*, (d) *Bacillus fusiformis*, (e) *Bacillus licheniformis* and (f) *Bacillus lentus*. The open boxes represent regions of homologous nucleotide sequences between corresponding ITSs, within the same bacteria. Boxes with left and right diagonal lines represent $tRNA^{ile}$ and $tRNA^{ala}$, respectively. The solid lines represent sequences lacking conservation between allelic ITSs. The dotted lines in (c) refer to incomplete ITS sequences. The blank spaces between boxes and/or lines represent deletions. A scale in base pairs is placed under the ITS alleles of each species.

monocytogenes (NC_003210), *Clostridium acetobutylicum* (NC_003030) and *Clostridium perfringens* (NC_003366). They contained 6, 6, 4, 5, 6, 11 and 10 16S–23S ITS allelic sequences, respectively. The nucleotide sequence alignment of the 16S–23S ITS alleles showed that the first 70 bp were highly conserved between alleles of the same strain. They were also highly conserved between alleles of strains belonging to the same species, as exemplified by *Streptococcus pyogenes* MGAS8232 and *Streptococcus pyogenes* M1 GAS. The first 70 bp were not, however, conserved between alleles of different species of the same genus as exemplified by *Streptococcus pyogenes* and *Streptococcus pneumoniae* and by *Clostridium acetobutylicum* and *Clostridium perfringens*. This is in agreement with our results on selected species of *Bacillus* and related genera.

Bourque *et al.* (1995) have amplified, cloned and sequenced a single copy of the 16S–23S ITS of seven different *Bacillus thuringiensis* varieties and 18 *Bacillus thuringiensis* var. *kurstaki* strains. The length of the 16S–23S ITS for each *Bacillus thuringiensis* strain was around 144 bp and its nucleotide sequence was highly conserved throughout the strains. This suggests that the entire 16S–23S ITS region is conserved among alleles of any given *Bacillus thuringiensis* strains and between *Bacillus thuringiensis* varieties. Our results indicate that the 16S–23S ITS is not always conserved, even between allelic sequences within a bacterial strain, but the first 70 nt at the 5′ end of 16S–23S ITS are conserved between alleles within strains and within species.

Although the sequences of the first 70 nt at the 5′ 16S–23S ITS are conserved between the *rrn* alleles within a strain or within a species, the nucleotide identities between different species were quite variable (alignment data not shown). In some cases, no meaningful similarities in the 5′ 16S–23S ITS region were observed between species of the same genus. The 5′ end of the 16S–23S ITS nucleotide sequence could be species-specific and might provide a rapid, easy and useful marker for species discrimination and identification.

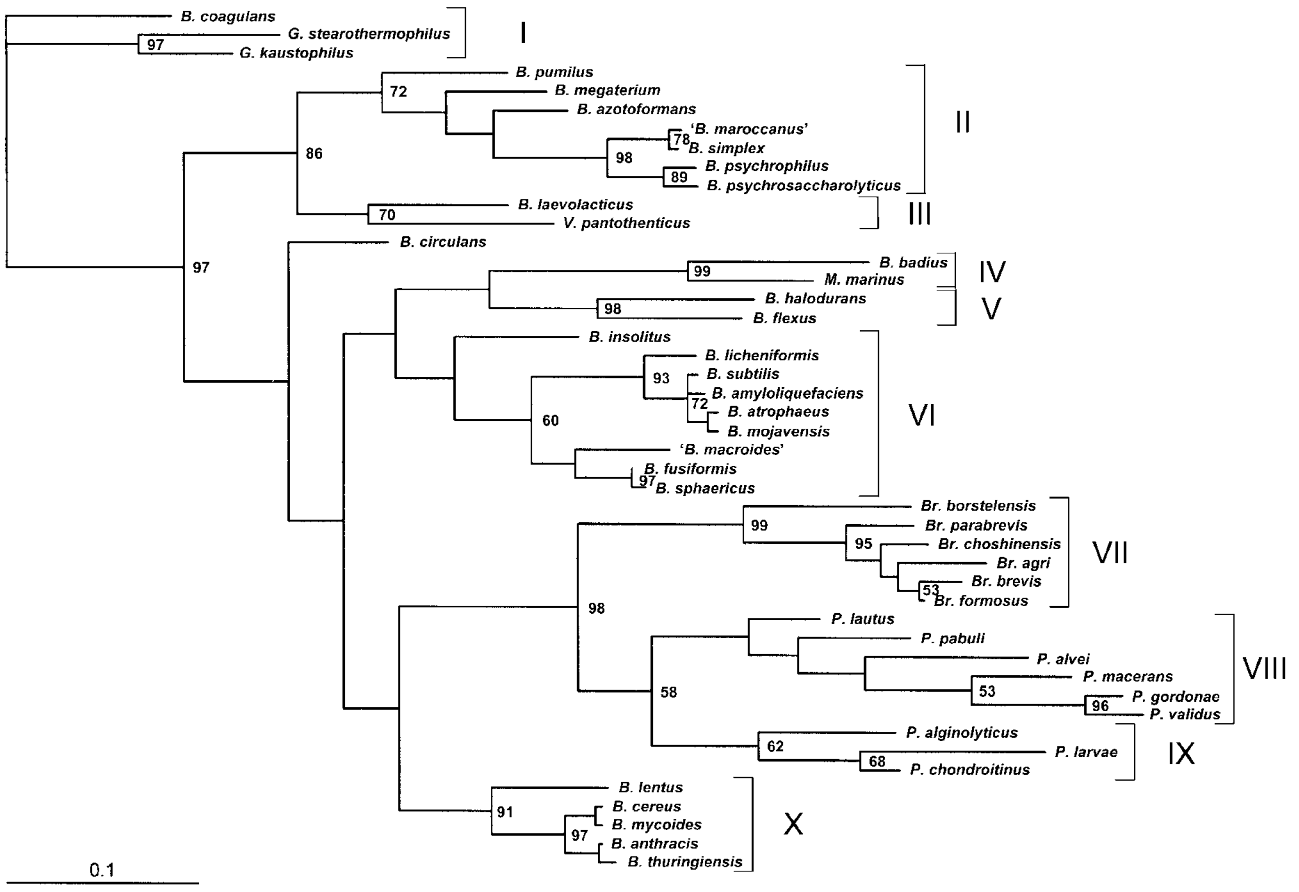

Fig. 3. Phylogenetic relationships of 46 *Bacillaceae* species inferred from the alignment of the 150 bp 3′ end 16S rDNA and the 70 bp 5′ 16S–23S ITS region. The *rrnH* allele of the 16S rRNA gene was used for *Bacillus subtilis* and *Bacillus halodurans*, respectively. The threshold value for groupings was $D_{\max} = 0{\cdot}31$. Bootstrap values (expressed as percentages of 100 replications) are shown at branch points; values greater than 50 % were considered significant. The bar represents the unit length of the number of nucleotide substitutions per site.

Phylogenetic analysis

The two highly conserved sequences identified above, the last 150 bp located at the 3′ 16S rDNA and the first 70 bp located at the 5′ 16S–23S ITS, were combined into a 220 bp sequence. A most parsimonious phylogenetic tree was constructed with the DNAPARS program of the PHYLIP package using the alignment of the 220 bp sequences of the 40 *Bacillaceae* species sequenced in this study and those of six *Bacillus* species available in GenBank (Fig. 3).

The resulting tree reveals 10 groups containing 45 *Bacillaceae* species and a single *Bacillus* species, *Bacillus circulans*, that remained ungrouped. These groups were defined as follows: when the maximal distance ($D_{\max}$; where distance D refers to the number of nucleotide substitutions per site) between species rooted at a common node was less than 0·31, these species were classified in the same group. The value of 0·31 was chosen as a threshold for grouping because it corresponds to the proportion of the length of the 16S–23S ITS used for sequence comparison to the total length of the DNA sequence used in the alignment (70 over 220 nt). With this threshold, species within a group could exhibit some nucleotide differences in their 5′ 16S–23S ITS, but would confine the identities of their nucleotide sequence in the 3′ end 16S rRNA coding region to almost perfect. $D_{\max}$ values between species within each group are given in Table 1. Although each group allowed for the two most distant species to share a $D_{\max}$ no greater than 0·31, the highest $D_{\max}$ observed here was 0·24 for species within Group VIII. Clearly each group could contain more distant *Bacillaceae* species, as long as they share a $D_{\max}$ up to 0·31. Presumably, other and new *Bacillaceae* species could be added on this phylogenetic tree and many could fall into one of these 10 existing groups.

Seven of the 10 groups were homogeneous. Groups II, V, VI and X contain seven, two, nine and five *Bacillus* species, respectively, exclusive of other genera. *Bacillus subtilis*, the *Bacillus* genus type species was found in Group VI. Group VII contains all six *Brevibacillus* species, exclusive of other genera. Likewise, Groups VIII and IX only contain six and three *Paenibacillus* species, respectively. The latter two groups originate from a single node and were later separated into two smaller branches using the grouping threshold value of 0·31. Both branches are within a $D_{\max}$ value of 0·464.

In addition to *Bacillus coagulans*, Group I contains two *Geobacillus* species, *Geobacillus stearothermophilus* and *Geobacillus kaustophilus*. Interestingly, Priest *et al.* (1988), using numerical phenetic data, also classified these three species in the same cluster. Two other clusters, Groups III and IV, each contained two *Bacillaceae* species from two different genera, *Bacillus laevolacticus* and *Virgibacillus pantothenticus* in Group III, and *Bacillus badius* and *Marinibacillus marinus* in Group IV. Whether both groups are heterogeneous as suggested by the different genera names is not clear. *Bacillus laevolacticus* in Group III and *Marinibacillus marinus* in Group IV were not included in the studies of Priest *et al.* (1988) and Ash *et al.* (1991). When the genus *Virgibacillus* was created (Heyndrickx *et al.*, 1998), *Bacillus laevolacticus* was not included in the study. Likewise, when the genus *Marinibacillus* was created (Yoon *et al.*, 2001), *Bacillus badius* was not included in the analysis. It appears that the relationship between *Bacillus laevolacticus* and *Virgibacillus pantothenticus* (Group III), and between *Bacillus badius* and *Marinibacillus marinus* (Group IV) could still be open to debate.

Whereas the analysis of nucleotide identities in the 3′ end 16S rDNA could distinguish *Bacillaceae* from other more distant bacterial taxa and distinguish between some *Bacillaceae* genera, additional analysis of nucleotide identities in the 5′ 16S–23S ITS has added complementary information essential for further clustering of more closely related species. The present groupings based only on nucleotide identities in the 3′ end 16S rDNA and 5′ 16S–23S ITS are, in general, in agreement with current classifications based on series of phenetic and molecular data.

In the dendrogram presented here, the genus *Bacillus* still appears heterogeneous. Twenty-six *Bacillus* species are distributed in seven distinct groups (I, II, III, IV, V, VI and X), and a 27th, *Bacillus circulans*, is ungrouped. In contrast, the newly formed genera *Brevibacillus* and *Paenibacillus* are, respectively, more homogeneous. In addition, some *Bacillus* groups are quite distant from others as exemplified by Groups II and V with a $D_{\max}$ value of 0·567. This is to be compared with a $D_{\max}$ value of 0·459 between two different *Bacillaceae* genera, the *Brevibacillus* Group VII and the *Paenibacillus* Group IX. Interestingly, $D_{\max}$ between both *Paenibacillus* Groups, VIII and IX, is 0·464. It would be worthwhile to further reassess the actual relatedness between the *Bacillus* species of the more distant groups to determine whether or not they are similar enough to be rightfully assigned to the same genus or whether the creation of novel genera would be warranted. If the latter holds true, a $D_{\max}$ of 0·31, as used here, will prove useful not only at clustering *Bacillaceae* species, but also at suggesting the creation of novel genera.

It is noteworthy that in certain groups, some species were closely related with $D_{\max}$ values less than 0·05. The species sharing a $D_{\max}$ of 0·05 are indicated in Table 1. These species share a high proportion of identical nucleotides, not only over their 3′ 16S rRNA coding region as expected, but also over their 5′ 16S–23S ITS. In Group II, *Bacillus psychrophilus* and *Bacillus psychrosaccharolyticus* were within a $D_{\max}$ of 0·035. The psychrophilic *Bacillus* species are all phenotypically related (Fox *et al.*, 1992) and their taxonomy is based on a few discriminating properties (Larkin & Stokes, 1967; Abd El-Rahman *et al.*, 2002). Although *Bacillus psychrophilus* has been unequivocally distinguished from *Bacillus globisporus* (Nakamura, 1984; Fox *et al.*, 1992), the distinction between *Bacillus psychrophilus* and *Bacillus psychrosaccharolyticus* could still be open to debate. Both species, however, were located in different groups based on 16S rRNA sequences (Ash *et al.*, 1991). In Group II, *Bacillus simplex* and '*Bacillus maroccanus*', were within a $D_{\max}$ of 0·011. Interestingly, *Bacillus simplex* and '*Bacillus maroccanus*' share an almost identical 16S rRNA gene nucleotide sequence (Ash *et al.*, 1991). '*Bacillus maroccanus*' is not a validly published name and whether *Bacillus simplex* and '*Bacillus maroccanus*' belong to the same species could also still be open to debate. In Group VI, *Bacillus amyloliquefaciens*, *Bacillus atrophaeus*, *Bacillus mojavensis* and *Bacillus subtilis* were within a $D_{\max}$ of 0·020. Likewise, these *Bacillus subtilis*-like bacteria are phenotypically very similar (Chun & Bae, 2000) and share almost identical 16S rRNA gene sequences (Ash *et al.*, 1991). *Bacillus fusiformis* and *Bacillus sphaericus* were highly similar with $D_{\max}$ values as low as 0·007. They are also very similar at the phenotypic level (Priest *et al.*, 1988) and their 16S rRNA gene sequence is very homologous (Ash *et al.*, 1991). In Group X, *Bacillus anthracis*, *Bacillus cereus*, *Bacillus mycoides* and *Bacillus thuringiensis* were also very similar with a $D_{\max}$ value of 0·047. The phenotypic and genotypic similarities between all four species has been well documented (Logan & Berkeley, 1984; Claus & Berkeley, 1986; Ash *et al.*, 1991). Recently, Helgason *et al.* (2000) proposed to regroup *Bacillus anthracis*, *Bacillus cereus* and *Bacillus thuringiensis* in a single species on the basis of genetic evidence. In Group VII, *Brevibacillus brevis* and *Brevibacillus formosus* were very close with a $D_{\max}$ value of 0·025. The similarities between both species is well documented (Shida *et al.*, 1995, 1996a, b). It is worth noting that *Brevibacillus parabrevis* isolates were originally classified as *Brevibacillus brevis.* They were later separated into two distinct species (Takagi *et al.*, 1993). Strain ATCC 8264$^{\mathrm{T}}$ was retained as the *Brevibacillus brevis* type strain. Strain ATCC 8186 was assigned to *Brevibacillus parabrevis*. In our study, the $D_{\max}$ between both strains was nearly 0·1, in agreement with their separation into two species. In Group VIII, *Paenibacillus gordonae* and *Paenibacillus validus* share a $D_{\max}$ value of 0·049, suggesting they might be the same species. Interestingly, both species have been proposed to be reclassified into a single species under the name *Paenibacillus validus* (Heyndrickx *et al.*, 1995). As seen here, species separated by $D_{\max}$ values within 0·05 are often phenotypically and genotypically very similar.

The D_{max} threshold of 0·05 may prove very useful in identifying species for which further analysis, including DNA–DNA reassociation, may be necessary to clarify whether or not their separation into different species was warranted.

The use of D_{max} thresholds of 0·31 and 0·05, as used here for *Bacillaceae* classification, based only on a combination of a 150 bp sequence located at the 3′ end of the 16S rRNA gene and a 70 bp sequence at the 5′ end of the ITS, is a simple, rapid approach, suited to larger screening programs and easily accessible to most laboratories. It may reveal, in a different way, *Bacillaceae* species for which further characterization, including thorough phenotypic comparison, 16S rRNA sequence data and DNA–DNA hybridization, might be warranted for proper classification and/or reassignment at the genus or species level, respectively. Whether these D_{max} thresholds may prove useful for other bacterial taxa as well remains to be assessed.

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REFERENCES

Abd El-Rahman, H. A., Fritze, D., Spröer, C. & Clauss, D. (2002). Two novel psychrotolerans species: *Bacillus psychrotolerans* sp. nov. and *Bacillus psychrodurans* sp. nov. which contain ornithine in their cell walls. *Int J Syst Evol Microbiol* **52**, 2127–2133.

Apirion, D. & Miczak, A. (1993). RNA processing in prokaryotic cells. *Bioessays* **15**, 113–120.

Ash, C., Farrow, A. E., Wallbanks, S. & Collins, M. D. (1991). Phylogenetic heterogeneity of the genus *Bacillus* revealed by comparative analysis of small-subunit-ribosomal RNA sequences. *Lett Appl Microbiol* **13**, 202–206.

Ash, C., Priest, F. G. & Collins, D. (1993). Molecular identification of rRNA group 3 bacilli (Ash, Farrow, Wallbanks and Collins) using a PCR probe test. Proposal for the creation of a new genus *Paenibacillus*. *Antonie van Leeuwenhoek* **64**, 253–260.

Berg, K. L., Squires, C. & Squires, C. L. (1989). Ribosomal RNA operon anti-termination. Function of leader and spacer region box B-box A sequences and their conservation in diverse micro-organisms. *J Mol Biol* **209**, 345–358.

Bourque, S. N., Valero, J. R., Lavoie, M. C. & Levesque, R. C. (1995). Comparative analysis of the 16S to 23S ribosomal intergenic spacer sequences of *Bacillus thuringiensis* strains and subspecies and of closely related species. *Appl Environ Microbiol* **61**, 1623–1626.

Chun, J. & Bae, K. S. (2000). Phylogenetic analysis of *Bacillus subtilis* and related taxa based on partial *gyrA* gene sequences. *Antonie van Leeuwenhoek* **78**, 123–127.

Claus, D. & Berkeley, R. C. W. (1986). Genus *Bacillus* Cohn 1872, 174^{AL}. In *Bergey's Manual of Systematic Bacteriology*, vol. 2, pp. 1105–1139. Edited by P. H. A. Sneath, N. S. Mair, M. E. Sharpe & J. G. Holt. Baltimore: Williams & Wilkins.

Daffonchio, D., Borin, S., Consolandi, A., Mora, D., Manachini, P. L. & Sorlini, C. (1998a). 16S–23S rRNA internal transcribed spacers as molecular markers for the species of the 16S rRNA group I of the genus *Bacillus*. *FEMS Microbiol Lett* **163**, 229–236.

Daffonchio, D., Borin, S., Frova, G., Manachini, P. L. & Sorlini, C. (1998b). PCR fingerprinting of whole genomes: the spacers between the 16S and 23S rRNA genes and of intergenic tRNA gene regions reveal a different intraspecific genomic variability of *Bacillus cereus* and *Bacillus licheniformis*. *Int J Syst Bacteriol* **48**, 107–116.

de Silva, S., Petterson, B., de Muro, M. A. & Priest, F. G. (1998). A DNA probe for the detection and identification of *Bacillus sporothermodurans* using the 16S–23S rDNA spacer region and phylogenetic analysis of some field isolates of *Bacillus* which form highly heat resistant spores. *Syst Appl Microbiol* **21**, 398–407.

Felsenstein, J. (1989). PHYLIP-phylogeny inference package (version 2). *Cladistics* **5**, 164–166.

Felsenstein, J. (2001). PHYLIP version 3.6a2. Distributed by the author. Department of Genetics, University of Washington, Seattle, WA, USA.

Fortina, M. G., Pukall, R., Schumann, P., Mora, D., Parini, C., Manachini, P. L. & Stackebrandt, E. (2001). *Ureibacillus* gen. nov., a new genus to accommodate *Bacillus thermosphaericus* (Andersson *et al.* 1995), emendation of *Ureibacillus thermosphaericus* and description of *Ureibacillus terrenus* sp. nov. *Int J Syst Evol Microbiol* **51**, 447–455.

Fox, G. E., Wisotzkey, J. D. & Jurtshuk, P., Jr (1992). How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. *Int J Syst Bacteriol* **42**, 166–170.

García-Martínez, J., Acinas, S. G., Antón, A. I. & Rodríguez-Valera, F. (1999). Use of the 16S–23S ribosomal genes spacer region in studies of prokaryotic diversity. *J Microbiol Methods* **36**, 55–64.

Goto, K., Omura, T., Hara, Y. & Sadaie, Y. (2000). Application of the partial 16S rDNA sequence as an index for rapid identification of species in the genus *Bacillus*. *J Gen Appl Microbiol* **46**, 1–8.

Gürtler, V. & Stanisich, V. A. (1996). New approaches to typing and identification of bacteria using the 16S–23S rDNA spacer region. *Microbiology* **142**, 3–16.

Helgason, E., Okstad, O. A., Caugant, D. A., Johansen, H. A., Fouet, A., Mock, M., Hegna, I. & Kolsto, A.-B. (2000). *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis* – one species on the basis of genetic evidence. *Appl Environ Microbiol* **66**, 2627–2630.

Heyndrickx, M., Vandemeulebroecke, K., Scheldeman, P. & 7 others authors (1995). *Paenibacillus* (formerly *Bacillus*) *gordonae* (Pichinoty *et al.* 1986) Ash *et al.* 1994 is a later subjective synonym of *Paenibacillus* (formerly *Bacillus) validus* (Nakamura 1984) Ash *et al.* 1994: emended description of *P. validus*. *Int J Syst Bacteriol* **45**, 661–669.

Heyndrickx, M., Lebbe, L., Kersters, K., De Vos, P., Forsyth, G. & Logan, N. A. (1998). *Virgibacillus*: a new genus to accommodate *Bacillus pantothenticus* (Proom and Knight 1950). Emended description of *Virgibacillus pantothenticus*. *Int J Syst Bacteriol* **48**, 99–106.

Joung, K.-B. & Côté, J.-C. (2002). Evaluation of ribosomal RNA gene restriction patterns for the classification of *Bacillus* species and related genera. *J Appl Microbiol* **92**, 97–108.

Lane, D. J., Pace, B., Olsen, G. J., Stahl, D. A., Sogin, M. J. & Pace, N. R. (1985). Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proc Natl Acad Sci U S A* **82**, 6955–6959.

Larkin, J. M. & Stokes, J. L. (1967). Taxonomy of psychrophylic strains of *Bacillus*. *J Bacteriol* **94**, 889–895.

Liiv, A., Tenson, T., Margus, T. & Remme, J. (1998). Multiple functions of the transcribed spacers in ribosomal RNA operons. *Biol Chem* **379**, 783–793.

Logan, N. A. & Berkeley, R. C. W. (1984). Identification of *Bacillus* strains using the API system. *J Gen Microbiol* **130**, 1871–1882.

Nakamura, L. K. (1984). *Bacillus psychrophilus* sp. nov., nom. rev. *Int J Syst Bacteriol* **34**, 121–123.

Nazina, T. N., Tourova, T. P., Poltaraus, A. B. & 8 other authors. (2001). Taxonomic study of aerobic thermophilic bacilli: descriptions of *Geobacillus subterraneus* gen. nov., sp. nov. and *Geobacillus uzenensis* sp. nov. from petroleum reservoirs and transfer of *Bacillus stearothermophilus*, *Bacillus thermocatenulatus*, *Bacillus thermoleovorans*, *Bacillus kaustophilus*, *Bacillus thermoglucosidasius* and *Bacillus thermodenitrificans* to *Geobacillus* as the new combinations *G. stearothermophilus*, *G. thermocatenulatus*, *G. thermoleovorans*, *G. kaustophilus*, *G. thermoglucosidasius* and *G. thermodenitrificans*. *Int J Syst Evol Microbiol* **51**, 433–446.

Niimura, Y., Koh, E., Yanagida, F., Suzuki, K.-I., Komagata, K. & Kozaki, M. (1990). *Amphibacillus xylanus* gen. nov., sp. nov., a facultatively anaerobic sporeforming xylan-digesting bacterium which lacks cytochrome, quinone, and catalase. *Int J Syst Bacteriol* **40**, 297–301.

Nodwell, J. R. & Greenblatt, J. (1993). Recognition of boxA antiterminator RNA by the *E. coli* antitermination factors NusB and ribosomal protein S10. *Cell* **72**, 261–268.

Nour, M. (1998). 16S–23S and 23S–5S intergenic spacer regions of lactobacilli: nucleotide sequence, secondary structure and comparative analysis. *Res Microbiol* **149**, 433–448.

Page, R. D. M. (1996). TREEVIEW: an application to display phylogenetic trees on personal computers. *Comput Appl Biosci* **12**, 357–358.

Pfeiffer, T. & Hartmann, R. K. (1997). Role of the spacer boxA of *Escherichia coli* ribosomal RNA operons in efficient 23S rRNA synthesis *in vivo*. *J Mol Biol* **265**, 385–393.

Pienta, P., Tang, J. & Cote, R. (1996). *ATCC Bacteria and Bacteriophages*, 19th edn. Rockville, MD, USA: American Type Culture Collection.

Priest, F. G., Goodfellow, M. & Todd, C. (1988). A numerical classification of the genus *Bacillus*. *J Gen Microbiol* **134**, 1847–1882.

Rössler, D., Ludwig, W., Schleifer, K. H., Lin, C., McGill, T. J., Wisotzkey, J. D., Jurtshuk, P., Jr & Fox, G. E. (1991). Phylogenetic diversity in the genus *Bacillus* as seen by 16S rRNA sequencing studies. *Syst Appl Microbiol* **14**, 266–269.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci U S A* **74**, 5463–5467.

Schlesner, H., Lawson, P. A., Collins, M. D., Weiss, N., Wehmeyer, U., Völker, H. & Thomm, M. (2001). *Filobacillus milensis* gen. nov., sp. nov., a new halophilic spore-forming bacterium with Orn-D-Glu-type peptidoglycan. *Int J Syst Evol Microbiol* **51**, 425–431.

Shida, O., Takagi, H., Kadowaki, K., Udaka, S., Nakamura, L. K. & Komagata, K. (1995). Proposal of *Bacillus reuszeri* sp. nov., *Bacillus formosus* sp. nov., nom. rev., and *Bacillus borstelensis* sp. nov., nom. rev. *Int J Syst Bacteriol* **45**, 93–100.

Shida, O., Takagi, H., Kadowaki, K. & Komagata, K. (1996a). Proposal for two new genera, *Brevibacillus* gen. nov. and *Aneurinibacillus* gen. nov. *Int J Syst Bacteriol* **46**, 939–946.

Shida, O., Takagi, H., Kadowaki, K., Yano, H. & Komagata, K. (1996b). Differentiation of species in the *Bacillus brevis* group and the *Bacillus aneurolyticus* group based on the electrophoretic whole-cell protein pattern. *Antonie van Leeuwenhoek* **70**, 31–39.

Stephen, D., Jones, C. & Schofield, J. P. (1990). A rapid method for isolating high quality plasmid DNA suitable for DNA sequencing. *Nucleic Acids Res* **18**, 7463–7464.

Takagi, H., Shida, O., Kadowaki, K., Komagata, K. & Udaka, S. (1993). Characterization of *Bacillus brevis* with descriptions of *Bacillus migulanus* sp. nov., *Bacillus choshinensis* sp. nov., *Bacillus parabrevis* sp. nov., and *Bacillus galactophilus* sp. nov. *Int J Syst Bacteriol* **43**, 221–231.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.

Wainø, M., Tindall, B. J., Schumann, P. & Ingvorsen, K. (1999). *Gracilibacillus* gen. nov., with description of *Gracilibacillus halotolerans* gen. nov., sp. nov.; transfer of *Bacillus dipsosauri* to *Gracilibacillus dipsosauri* comb. nov., and *Bacillus salexigens* to the genus *Salibacillus* gen. nov., as *Salibacillus salexigens* comb. nov. *Int J Syst Bacteriol* **49**, 821–831.

Weisburg, W. G., Barns, S. M., Pelletier, D. A. & Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* **173**, 697–703.

Wisotzkey, J. D., Jurtshuk, P., Jr, Fox, G. E., Deinhard, G. & Poralla, K. (1992). Comparative sequence analyses on the 16S rRNA (rDNA) of *Bacillus acidocaldarius*, *Bacillus acidoterrestris*, and *Bacillus cycloheptanicus* and proposal for creation of a new genus, *Alicyclobacillus* gen. nov. *Int J Syst Bacteriol* **42**, 263–269.

Woese, C. (1987). Bacterial evolution. *Microbiol Rev* **51**, 221–271.

Yoon, J.-H., Weiss, N., Lee, K.-C., Lee, I.-S., Kang, K. H. & Park, Y.-H. (2001). *Jeotgalibacillus alimentarius* gen. nov., sp. nov., a novel bacterium isolated from jeotgal with L-lysine in the cell wall, and reclassification of *Bacillus marinus* Rüger 1983 as *Marinibacillus marinus* gen. nov., comb. nov. *Int J Syst Evol Microbiol* **51**, 2087–2093.