Phylogeny and molecular taxonomy of the *Bacillus subtilis* species complex and description of *Bacillus subtilis* subsp. *inaquosorum* subsp. nov.

Alejandro P. Rooney,¹ Neil P. J. Price,² Christopher Ehrhardt,³
James L. Swezey¹ and Jason D. Bannan⁴

Correspondence

Alejandro P. Rooney

alejandro.rooney@ars.usda.gov

¹Microbial Genomics and Bioprocessing Research Unit, National Center for Agricultural Utilization Research, Agricultural Research Service, US Department of Agriculture, Peoria, IL 61604, USA

²Bioproducts and Biocatalysis Research Unit, National Center for Agricultural Utilization Research, Agricultural Research Service, US Department of Agriculture, Peoria, IL 61604, USA

³Counterterrorism and Forensic Science Research Unit, Federal Bureau of Investigation Academy, Quantico, VA 22135, USA

⁴Chemical-Biological Sciences Unit, Laboratory Division, Federal Bureau of Investigation, Quantico, VA 22135, USA

The *Bacillus subtilis* species complex is a tight assemblage of closely related species. For many years, it has been recognized that these species cannot be differentiated on the basis of phenotypic characteristics. Recently, it has been shown that phylogenetic analysis of the 16S rRNA gene also fails to differentiate species within the complex due to the highly conserved nature of the gene, yet DNA–DNA hybridization values fall well below 70 % for the same species comparisons. As a complementary approach, we propose that phylogenetic analysis of multiple protein-coding loci can be used as a means to detect and differentiate novel *Bacillus* taxa. Indeed, our phylogenetic analyses revealed the existence of a previously unknown group of strains closely related to, but distinct from, *Bacillus subtilis* subsp. *spizizenii*. Results of matrix-assisted laser desorption ionization-time of flight mass spectrometry analyses revealed that the group produces a novel surfactin-like lipopeptide with mass m/z 1120.8 that is not produced by the other currently recognized subspecies. In addition, the group displayed differences in the total cellular content of the fatty acids C_{16:0} and iso-C_{17:1}ω10c that distinguish it from the closely related *B. subtilis* subsp. *spizizenii*. Consequently, the correlation of these novel phenotypic traits with the phylogenetic distinctiveness of this previously unknown subspecies group showed that phylogenetic analysis of multiple protein-coding loci can be used as a means to detect and differentiate novel *Bacillus* taxa. Therefore, we propose that this new group should be recognized as representing a novel taxon, *Bacillus subtilis* subsp. *inaquosorum* subsp. nov., with the type strain NRRL B-23052^T (=KCTC 13429^T=BGSC 3A28^T).

INTRODUCTION

The genus *Bacillus* is composed of rod-shaped, endospore-forming bacteria that are members of the phylum *Firmicutes*. Owing largely to the fact that they are common inhabitants of soil and aquatic sediment, species within the genus are widespread in nature and are found in virtually every environment. While their main roles appear to involve carbon and nitrogen cycling, some species are well-known human and livestock pathogens (e.g. *Bacillus anthracis* and *Bacillus cereus*) and insect pathogens (e.g. *Bacillus thuringiensis*). However, the overwhelming majority of *Bacillus* species are non-pathogenic. Because of this fact, many have been exploited for biotechnological and

Abbreviations: FAME, fatty acid methyl ester; MALDI-TOF MS, matrix-assisted laser desorption ionization-time of flight mass spectrometry; NJ, neighbour-joining; ML, maximum-likelihood; MP, maximum-parsimony.

The GenBank/EMBL/DDBJ accession numbers for the sequences generated in this study are EU138452–EU138865.

A maximum-parsimony cladogram of the *Bacillus subtilis* species complex and tables showing the mean values of synonymous and non-synonymous substitutions per site among protein-coding genes and the geographical origins, isolation substrates and maximum growth temperatures for *B. subtilis* subsp. *inaquosorum* subsp. nov., *B. subtilis* subsp. *spizizenii* and *B. subtilis* subsp. *subtilis* are available as supplementary material with the online version of this paper.

industrial applications (Hou *et al.*, 2005; Ruiz-García *et al.*, 2005a, b; Bischoff *et al.*, 2006; Price *et al.*, 2007). One such species, which is also one of the more commonly encountered ones in the environment, is *Bacillus subtilis* (Cohn, 1872).

Over the last 20 years, several species very closely related to *B. subtilis* have been described. These species include *Bacillus amyloliquefaciens* (Priest *et al.*, 1987), *Bacillus atrophaeus* (Nakamura, 1989), *Bacillus axarquiensis* (Ruiz-García *et al.*, 2005b; but see Wang *et al.*, 2007), *Bacillus malacitensis* (Ruiz-García *et al.*, 2005b; but see Wang *et al.*, 2007), *Bacillus mojavensis* (Roberts *et al.*, 1994), *Bacillus sonorensis* (Palmisano *et al.*, 2001), *Bacillus tequilensis* (Gatson *et al.*, 2006), *Bacillus vallismortis* (Roberts *et al.*, 1996) and *Bacillus velezensis* (Ruiz-García *et al.*, 2005a; but see Wang *et al.*, 2008). Interestingly, most of the above species share a remarkably high level of 16S rRNA gene sequence similarity to *B. subtilis* (often 99% or greater) even though DNA–DNA hybridization values with the latter fall below 70%. Moreover, very few phenotypic or biochemical characteristics can be found that differentiate these species from *B. subtilis*. This high degree of phenotypic and biochemical similarity prompted Gordon *et al.* (1973) to characterize species closely related to *B. subtilis* as members of the ‘*subtilis*-group’ or ‘*subtilis*-spectrum’. Herein, we refer to this assemblage as the *B. subtilis* species complex.

The inability of phenotypic, biochemical and 16S rRNA gene phylogenetic analysis to identify species within the *B. subtilis* species complex and to resolve phylogenetic relationships among those species is an important problem to resolve for those interested in *Bacillus* systematics. Most importantly, it raises the question that, if one wishes to apply modern principles of phylogenetic systematics, where and how does one ‘draw the line’ in the attempt to delineate a novel species of *Bacillus*. Recently, it has been suggested that the use of multilocus sequence analysis should be investigated as a means to detect and define novel species of bacteria (Stackebrandt *et al.*, 2002; Gevers *et al.*, 2005). The purpose of this study was to investigate the use of multilocus phylogenetic analysis as a tool to detect novel taxa within the *B. subtilis* species complex, which has become a model system for the study of bacterial speciation (Cohan, 2001, 2002, 2006; Perry *et al.*, 2007).

METHODS

Strains and phenotypic analyses. All strains used in this study are permanently archived within the USA Department of Agriculture Agricultural Research Service (ARS) Culture Collection (commonly known by the acronym NRRL; <<http://nrrl.ncaur.usda.gov>>). Experiments involving temperature range for growth (4–58 °C), salt concentration tolerance (5–15%, w/v) and anaerobic growth were conducted using tryptone-glucose-yeast extract (TGY) media (5.0 g tryptone, 5.0 g yeast extract, 1.0 g glucose, 1.0 g K₂HPO₄, 1 l ddH₂O; pH 7.0). All other phenotypic analyses were conducted according to Gordon *et al.* (1973). Tests for the production of acid from various carbohydrates were conducted using the API CH 50 system

(bioMérieux), following the manufacturer’s suggested protocol. Lipopeptide biomarker profiles were determined by using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) using the method of Price *et al.* (2007) as follows. MALDI-TOF mass spectra were acquired on a Bruker-Daltonic Omnisflex instrument operating in reflectron mode. Ion source 1 was set to 19.0 kV and source 2 to 14.0 kV, with lens and reflector voltages of 9.20 and 20.00 kV, respectively. Aqueous samples were co-crystallized with 2,5-dihydrobenzoic acid matrix (10 mg ml^{−1} in acetonitrile) on a Bruker-Daltonics 49-place target. The laser output was at 337.1 nm, typically at 60% of 150 J maximum output, and 80 shots were accumulated. A 200 ns pulsed ion extraction was used with matrix suppression up to 200 Da. The instrument was calibrated externally on a dp series of malto-oligosaccharides.

Fatty acid methyl ester (FAME) analyses were conducted as follows. Lyophilized cultures maintained by the ARS Culture Collection were rehydrated in 3 ml trypticase soy broth (Difco) and incubated for 12 h at 30 °C with shaking at 200 r.p.m. Small (100 µl) aliquots of liquid culture were then spread onto trypticase soy agar (Difco) plates and incubated for 24 h at 30 °C. Following incubation, bacterial colonies (approx. 3 mg wet weight) were transferred to 2 ml screw cap vials and prepared for fatty acid extraction. Fatty acid extraction and analysis were performed according to the Instant FAME sample preparation method (MIDI, Inc.). Gas chromatography was performed on a Agilent 6890N analyzer using calibration standards (#1300-AA; MIDI, Inc.). The Sherlock Microbial Identification (MIDI, Inc.) software was used to assign GC peaks to individual fatty acid structures.

Gene sequence data collection. All strains were grown in 5 ml TGY broth and incubated aerobically at 28 °C for 24 h. DNA was extracted from each strain using a MasterPure Gram-positive DNA purification kit (Epicentre Biotechnologies) following the manufacturer’s protocol. For each strain, we obtained partial nucleotide sequences for the following genes: gyrase subunit A (*gyrA*), RNA polymerase subunit B (*rpoB*), phosphoribosylaminoimidazolecarboxamide formyltransferase (*purH*), DNA polymerase III subunit alpha (*polC*), 60 kDa heat-shock protein groEL (*groEL*) and 16S rRNA.

PCR amplifications were performed with 1 unit AmpliTaq DNA polymerase (Invitrogen Life Technologies), 2.5 mM MgCl₂, 200 µM dNTPs, 1.0 µM each of forward and reverse oligonucleotide primers, 1 × reaction buffer and 50–100 ng template DNA. The universal oligonucleotide primers 27f (Lane, 1991) and 1387r (Marchesi *et al.*, 1998) were used to amplify and sequence the 16S rRNA gene using an annealing temperature of 54 °C. The oligonucleotide primer sequences for all other genes are listed in Table 1. All PCRs were performed for 35 cycles, each consisting of a 30 s denaturation step at 94 °C, a 30 s annealing step between 45 and 55 °C (all amplifications were done using as high an annealing temperature as possible, with subsequent lowering in 2 °C increments until amplification occurred), and a 1 min extension step at 72 °C. Amplification products were purified using Montage PCR Cleanup Filter Plates (Millipore). Sequencing reactions were conducted using the ABI BigDye version 3.0 sequencing kit (Applied Biosystems) following the manufacturer’s suggested protocol but at one-fourth the recommended volume. Reaction products were purified via ethanol precipitation and run on an ABI3730 genetic analyzer (Applied Biosystems). DNA sequences were edited visually using Sequencher version 4.1.2 (Gene Codes) and aligned manually. The nucleotide sequence alignment of concatenated genes consisted of 5550 bp (928 bp from *gyrA*, 964 bp from *rpoB*, 875 bp from *purH*, 777 bp from *polC*, 835 bp from *groEL* and 1171 bp from 16S rRNA). We also included homologous nucleotide sequence data from the sequenced genomes of *B. subtilis* 168, *Bacillus licheniformis* DSM 13^T, and *B. cereus* ATCC 14579^T in the alignment. With the completed genome

Table 1. Primers used to amplify protein-coding loci

Primers are numbered according to the first nucleotide in their respective genes as per the *B. subtilis* 168 published genome sequence (GenBank accession no. NC_000964).

Primer name	Sequence (5'–3')
<i>gyrA</i> -42f*	CAGTCAGGAAATGCGTACGTCCTT
<i>gyrA</i> -1066r*	CAAGGTAATGCTCCAGGCATTGCT
<i>rpoB</i> -2292f	GACGTGGGATGGCTACAAC
<i>rpoB</i> -3354r	ATTGTGCGCTTTAACGATGG
<i>purH</i> -70f	ACAGAGCTTGGCGTTGAAGT
<i>purH</i> -1013r	GCTTCTTGGCTGAATGAAGG
<i>polC</i> -1505f	TTGTCGCTCAYAATGCAAGC
<i>polC</i> -2337r	YTCAAGCATTTCTCTGTCTG
<i>groEL</i> -550f	GAGCTTGAAGTKGTTGAAGG
<i>groEL</i> -1497r	TGAGCGTGTWACTTTTGTW

*The *gyrA* primer sequences were taken from Chun & Bae (2000); positional numbering was added in order to conform to the system in place for the above primers as well as the 16S rRNA gene primers.

data, the total number of strains analysed was 72. All sequences generated in this study have been deposited in GenBank and are retrievable by typing in their NRRL accession numbers in the Entrez retrieval system (<http://www.ncbi.nlm.nih.gov/sites/entrez>).

Phylogenetic analyses. Phylogenetic analyses of gene sequence data were conducted using neighbour-joining (NJ) (Saitou & Nei, 1987), maximum-likelihood (ML) and maximum-parsimony (MP) methods. The computer program MEGA 3.1 (Kumar *et al.*, 2004) was used to reconstruct NJ trees from Tamura & Nei (1993) gamma distances. The gamma shape parameter used was 0.223, which was estimated using the program DAMBE version 4.5.40 (Xia & Xie, 2001). The reliability of internal branches was assessed from 1500 bootstrap pseudoreplicates. The program DNAPARS in the software package PHYLIP (Felsenstein, 2006) was used to conduct MP analyses using 100 random-addition replicates. Gaps were considered an additional character state in the MP analysis. The reliability of internal branches was assessed from 1000 bootstrap pseudoreplicates. The program GARLI version 0.951 (Zwickl, 2006) was used to conduct ML analyses. A general time reversible model with gamma-distributed among-site rate variation and a proportion of invariant sites was used. The analysis was run for 5 million generations, with a threshold value of 10 000 for the termination condition. The reliability of internal branches was assessed from 500 bootstrap pseudoreplicates.

RESULTS AND DISCUSSION

The results from NJ, MP, ML and analyses of concatenated gene sequences are shown in Fig. 1 and Supplementary Fig. S1 (available in IJSEM Online). These figures represent the phylogeny reconstructed from the concatenated sequence of all five protein-coding loci and the 16S rRNA gene. The 16S rRNA gene is highly conserved and there is no phylogenetic information present in this gene for resolving closely related *B. subtilis* complex members (Roberts *et al.*, 1994, 1996; Chun & Bae, 2000; Palmisano *et al.*, 2001; Ruiz-García *et al.*, 2005a, b; Gatson *et al.*, 2006). However, there is some information present that

provides resolution regarding the deeper divergences in the complex (Ash *et al.*, 1991). Therefore, we included this gene in the concatenation. With respect to the protein-coding loci, the *gyrA* and *groEL* genes show some low levels of discordance in comparison to the other genes with respect to the phylogenetic placement of four strains of *B. subtilis*. The *gyrA* and *groEL* genes represent the fastest- and slowest-evolving loci, respectively, in our dataset as judged by levels of synonymous (d_s) and non-synonymous (d_n) site divergence (Supplementary Table S1, available in IJSEM Online). The phylogenetic discordance observed is discussed later in the text as it pertains to relationships among strains of *B. subtilis*.

Nevertheless, because the individual gene phylogenies are congruent to one another and to the concatenated gene phylogeny, we discuss relationships within the *B. subtilis* species complex in reference to the concatenated phylogenies only (Fig. 1 and Supplementary Fig. S1). We addressed the relationships from the most basal member of the complex to the most derived. Thus, we began with *Bacillus pumilus*. This species was found at the base of the *B. subtilis* species complex phylogeny. Interestingly, Gordon *et al.* (1973) speculated that it might one day be considered a 'variety of *B. subtilis* rather than a separate species' once more data were collected on a large number of *B. pumilus* strains. However, our results clearly indicate that *B. pumilus* forms a clade distinct from *B. subtilis* (Fig. 1 and Supplementary Fig. S1).

The next group of species is represented by *B. licheniformis* and *B. sonorensis*. These two species are reported to be differentiable on the basis of salt tolerance (Palmisano *et al.*, 2001). However, reliance on phenotypic characteristics for differentiating species of the *B. subtilis* species complex should be avoided, as certain traits that currently might be considered reliable might prove otherwise once more strains have been examined. For instance, it has been presumed for quite some time that *B. licheniformis* can be separated from *B. subtilis* on the basis of facultative anaerobic growth as well as growth at 56 °C (Kundrat, 1963; Gordon *et al.*, 1973). However, many strains of *B. subtilis* are capable of anaerobic growth in the presence of glucose (Rooney *et al.*, 2005) and a number of strains are capable of growth at or above 56 °C (Supplementary Table S2, available in IJSEM Online), which reinforces the observation that species within the *B. subtilis* complex cannot be reliably differentiated on the basis of morphological, physiological, biochemical or other phenotypic properties.

The species *B. amyloliquefaciens* is the next to branch off from the remainder of the *B. subtilis* species complex (Fig. 1 and Supplementary Fig. S1). *B. amyloliquefaciens* is an industrially important species that is used for a number of applications, most notably for the production of α -amylase. Recently, Ruiz-García *et al.* (2005a) described a group of strains closely related to *B. amyloliquefaciens* as representing a novel species, *B. velezensis*. The latter is also

Fig. 1. NJ phylogeny of the *B. subtilis* species complex. Numbers at branch points indicate bootstrap percentages from both NJ (before the slash '/') and ML (after the slash '/') analyses. Only values greater than 50% are shown. Because the ML tree was very similar to the NJ tree, only the latter is shown here. *B. cereus* was used as the outgroup taxon to root the tree.

potentially valuable to a number of industries, as certain strains were shown to produce high levels of surfactins (Ruiz-García *et al.*, 2005a). However, Wang *et al.* (2008) reclassified *B. velezensis* as a later heterotypic synonym of *B. amyloliquefaciens* on the basis of DNA–DNA hybridization data. In our study, *B. amyloliquefaciens* and *B. velezensis* (NRRL B-41580^T) were found to be very closely related and could not be reliably differentiated from one another or other *B. subtilis* complex species without the use of gene sequence data (Fig. 1 and Supplementary Fig. S1).

The next species to consider in the *B. subtilis* species complex phylogeny are *B. atrophaeus* and *B. mojavensis*. As

with the previously discussed two species, certain *B. atrophaeus* strains are potentially useful for industrial applications (Schmidt *et al.*, 1979). Strains of *B. atrophaeus* typically produce dark brown to black pigments but are otherwise indistinguishable from *B. subtilis* and related species on the basis of other phenotypic, morphological, physiological or biochemical characteristics. *B. mojavensis* is a desert-adapted species and is similarly difficult to distinguish from *B. subtilis*, except for minor differences in cell-wall fatty acid content (Roberts *et al.*, 1994). Two species described by Ruiz-García *et al.* (2005a), *B. malacitensis* and *B. axarquensis*, were found to be very closely related to *B. mojavensis*, but Wang *et al.* (2007)

reclassified these two species as later heterotypic synonyms of *B. mojavensis*. Similarly, the results of our phylogenetic analyses (Fig. 1 and Supplementary Fig. S1) show that strains NRRL B-41617^T and NRRL B-41618^T (the type strains of *B. axarquiensis* and *B. malacitensis*, respectively) cluster with the *B. mojavensis* type strain as well as another reference strain of that species.

The last group consists of *B. subtilis*, *B. tequilensis* and *B. vallismortis*. In Fig. 1 and Supplementary Fig. S1, three clades of *B. subtilis* can be recognized: (i) a clade corresponding to *B. subtilis* subsp. *subtilis* (Nakamura *et al.*, 1999); (ii) a clade corresponding to *B. subtilis* subsp. *spizizenii* (Nakamura *et al.*, 1999); and (iii) a third clade that we propose to be recognized as representing a novel subspecies, *Bacillus subtilis* subsp. *inaquosorum* subsp. nov. Each of the three groups was supported with high bootstrap values (99–100%; Fig. 1 and Supplementary Fig. S1). However, the bootstrap support for a monophyletic *B. subtilis* was weak (52–77%). In addition, the results of the MP analysis (Supplementary Fig. S1, in IJSEM Online) suggest that *B. subtilis*, as it is currently recognized (Nakamura *et al.*, 1999), is paraphyletic. In this case, *B. subtilis* subsp. *spizizenii* and the proposed subspecies *B. subtilis* subsp. *inaquosorum* subsp. nov. cluster apart from *B. subtilis* subsp. *subtilis*. This result needs further evaluation in follow-up studies, although it could be a simple case of systematic error in the MP tree. Regardless, the results of our analyses indicate that there is a third phylogenetic group of *B. subtilis* strains that cluster apart from the two currently recognized subspecies and that this group is closely related to *B. subtilis* subsp. *spizizenii*. Thus, to determine whether or not there were any phenotypic characteristics that could distinguish this new group from *B. subtilis* subsp. *spizizenii*, we conducted MALDI-TOF MS and FAME analyses.

MALDI-TOF MS biomarker profiles over the 300–1200 mass unit range were obtained. Abundant lipopeptide MS ions were observed in four subdivided mass ranges: (1) *m/z* 520–600, (2) *m/z* 710–770, (3) *m/z* 880–970 and (4) *m/z* 1050–1150. In mass range 1, ubiquitous lipopeptide ions were observed at *m/z* 562.8 and 590.8. In mass range 2, *m/z* 710.7 and 728.7 were present for ten strains, but were not species-specific. Mass range 3 contained four ions, *m/z* 886.9, 901.0, 914.0 and 930.9, previously assigned as probable kurstakins (Price *et al.*, 2007). These were generally present except for *B. vallismortis* NRRL B-14890^T. An ion (*m/z* 1057.8) in the high mass range was observed for all strains tested and is assigned as the [M+Na]⁺ molecular adduct ion for the surfactin lipopeptide Leu/Ile, C15 (Price *et al.*, 2007). The *B. subtilis* subsp. *inaquosorum* subsp. nov. strains were characterized by an additional major ion at *m/z* 1120.8 and a smaller ion at *m/z* 1106.8 (Fig. 2). These were observed for all *B. subtilis* subsp. *inaquosorum* subsp. nov. strains tested and often were the most intense ions observed. Similar markers (*m/z* 1121.8 and 1105.8) were seen for *B. tequilensis* NRRL B-41771^T, but spectral overlay showed these to be different

(Fig. 2). The smaller ion (*m/z* 1106.8) was also present in three *B. subtilis* subsp. *spizizenii* strains (NRRL B-23049^T, B-23055 and BD-576) but not the larger ion (*m/z* 1120.8), which was only observed among *B. subtilis* subsp. *inaquosorum* subsp. nov. strains. Hence, the biomarker at *m/z* 1120.8 is unique to *B. subtilis* subsp. *inaquosorum* subsp. nov. and can be used in addition to gene sequence analysis to differentiate these strains from other *B. subtilis* subspecies.

The results of the FAME analysis (Table 2) revealed that there were differences between *B. subtilis* subsp. *inaquosorum* subsp. nov. and *B. subtilis* subsp. *spizizenii* with respect to total cellular content of the fatty acids C_{16:0} and iso-C_{17:1}ω10c, which are minor components of the total complement. The values for these fatty acids are highlighted in bold in Table 2, which also includes data for the type strains of *B. subtilis* subsp. *subtilis*, *B. tequilensis* and *B. vallismortis* for comparative purposes. Under the growth conditions used, it was found that *B. subtilis* subsp. *inaquosorum* subsp. nov. made more C_{16:0} than *B. subtilis* subsp. *spizizenii*, whereas *B. subtilis* subsp. *spizizenii* made more iso-C_{17:1}ω10c than *B. subtilis* subsp. *inaquosorum* subsp. nov. In addition, *B. subtilis* subsp. *inaquosorum* strains made more C_{16:0} than iso-C_{17:1}ω10c, whereas the amounts were much closer in the case of the *B. subtilis* subsp. *spizizenii* strains.

Interestingly, strains from each of the three aforementioned subspecies clades were isolated from the same samples of desert soils from various locations around the world (Supplementary Table S2, in IJSEM Online) and in each case the strains display a pattern of reciprocal monophyly, in which strains from one subspecies cluster with other strains of the same subspecies regardless of where they originate geographically. Thus, for example, strains of all three *B. subtilis* subspecies that were isolated from the same Mojave Desert soil sample (Supplementary Table S2) do not cluster together; instead they cluster with other members of their respective subspecies (Fig. 1 and Supplementary Fig. S1). This pattern of reciprocal monophyly indicates that each subspecies is on its own evolutionary trajectory (Dykhuizen & Green, 1991; Avise, 2000; Cohan 2001, 2002) and also suggests that each subspecies fills an ecological niche distinct from the other subspecies (Cohan, 2001, 2002, 2006; Perry *et al.*, 2007; Koepfel *et al.*, 2008). This ecological distinction is most probably the explanation for the patterns of reciprocal monophyly and the production of unique lipopeptides and differences in total cellular fatty acid components. What is most important, however, is that groups are phylogenetically distinct and have embarked on separate evolutionary paths. Moreover, as we were able to correlate phylogenetic distinctiveness with novel phenotypic traits, our study shows that phylogenetic analysis of multiple protein-coding loci can be used to detect novel bacterial taxa, even those that are very closely related such as subspecies of *B. subtilis*. In that regard, we propose that the third subspecies clade of *B. subtilis* identified in this study should

Fig. 2. Representative MALDI-TOF MS ion profiles (m/z 1020–1160) of (a) *B. subtilis* subsp. *inaquosorum* subsp. nov. (B-23052^T); (b) *B. subtilis* subsp. *spizizenii* (B-23049^T); (c) *B. tequilensis* (B-41771^T); and (d) overlaid strains B-23052^T and B-41771^T. Of note are surfactin $[M+Na]^+$ ions (m/z 1044 and 1058) and a *B. subtilis* subsp. *inaquosorum* subsp. nov.-specific ion, m/z 1120.8.

Table 2. Cellular fatty acid contents of type and reference strains of *B. subtilis* subsp. *inaquosorum* subsp. nov. and *B. subtilis* subsp. *spizizenii*, as well as the type strains of *B. subtilis* subsp. *subtilis*, *B. tequilensis* and *B. vallismortis*

NRRL strain numbers: 1, B-23052^T; 2, B-23056; 3, B-23058; 4, B-14697; 5, BD-571; 6, BD-572; 7, B-23049^T; 8, B-23050; 9, B-23051; 10, B-23055; 11, BD-576. Values are percentages of the total.

Fatty acid	<i>B. subtilis</i> subsp. <i>inaquosorum</i>						<i>B. subtilis</i> subsp. <i>spizizenii</i>					<i>B. subtilis</i> subsp. <i>subtilis</i> NRS-744 ^T	<i>B. tequilensis</i> B-41771 ^T	<i>B. vallismortis</i> B-14890 ^T
	1	2	3	4	5	6	7	8	9	10	11			
iso-C _{13:0}	0.1	0.0	0.2	0.1	0.2	0.1	0.0	0.0	0.0	0.0	0.1	0.1	0.1	5.9
iso-C _{14:0}	0.6	1.2	0.9	0.9	0.7	0.7	0.6	0.6	0.6	0.6	0.6	0.8	0.7	6.2
C _{14:0}	0.2	0.3	0.3	0.2	0.2	0.2	0.1	0.0	0.1	0.0	0.1	0.2	0.2	3.3
iso-C _{15:0}	22.6	20.3	26.4	24.6	28.4	23.8	17.4	16.0	19.2	21.6	28.0	19.4	18.8	34.6
anteiso-C _{15:0}	39.2	42.1	39.7	37.5	39.5	40.4	39.5	39.5	41.0	39.2	36.8	38.2	47.1	3.7
C _{16:1} ω7c	0.2	0.2	0.3	0.3	0.2	0.4	0.5	0.3	0.4	0.4	0.3	1.2	0.4	0.6
C _{16:1} ω11c	0.4	0.4	0.6	0.6	0.3	0.6	0.8	0.5	0.7	0.5	0.5	1.6	1.1	0.1
iso-C _{16:0}	2.5	5.0	3.1	3.4	2.4	2.5	2.6	3.0	2.6	2.6	1.9	2.5	2.4	7.0
C _{16:0}	2.9	3.4	2.9	2.7	2.6	2.4	1.9	1.8	1.9	1.5	1.6	1.9	2.1	2.8
iso-C _{17:1} ω10c	1.0	0.4	0.8	0.9	0.6	1.3	2.1	1.3	1.7	1.8	1.7	5.2	1.4	1.4
iso-C _{17:1} ω5c	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.1
iso-C _{17:0}	14.3	10.8	12.1	15.1	12.6	12.5	15.3	15.8	13.4	15.1	15.0	11.2	7.0	8.9
anteiso-C _{17:0}	14.8	15.4	12.1	12.5	11.7	14.1	17.0	19.5	17.0	15.0	12.2	12.1	17.0	0.8
iso-C _{19:0}	0.0	0.0	0.0	0.1	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.6	0.0	1.2

be formally recognized as *Bacillus subtilis* subsp. *inaquosorum* subsp. nov.

Description of *Bacillus subtilis* subsp. *inaquosorum* subsp. nov.

Bacillus subtilis subsp. *inaquosorum* [in.a.quo.so'rum. L. adj. *inaquosus* poor of water, L. gen. pl. n. (*solium*) *inaquosorum* from (soils) poor of water (desert soils) from which this organism was isolated].

Vegetative cells are rods that measure $0.5 \times 3 \mu\text{m}$ and occur either singly or in chains. Motile. Cylindrical spores form centrally or paracentrally in non-swollen sporangia. Facultatively anaerobic on TGY agar or broth. Catalase-positive. Grows at 15–55 °C, with optimum growth at 28–30 °C. Growth in 10% (w/v) NaCl occurs after 72 h. Growth in the presence of 0.001% (w/v) lysozyme is variable. Produces acetyl-methylcarbinol (Voges-Proskauer test) at pH 5.5–5.7. Hydrolyses starch and casein; reduces nitrate to nitrite. Citrate is utilized. Acid is produced from amygdalin, glycogen, melibiose, D-glucose, cellobiose, D-mannitol, D-sorbitol and aesculin ferric citrate, as determined using the API CH 50 system. Produces a surfactin-like lipopeptide $[M + Na]^+$ molecular adduct ion product of mass m/z 1120.8 in MALDI-TOF MS analysis.

The type strain is NRRL B-23052^T (=KCTC 13429^T=BGSC 3A28^T) and is archived within the ARS Culture Collection, the Korean Collection for Type Cultures and the Bacillus Genetic Stock Center. Strains form distinct monophyletic clusters from other *B. subtilis* subspecies in concatenated gene sequence phylogenies.

ACKNOWLEDGEMENTS

We gratefully acknowledge H. N. Kline and T. Hartman for technical support. We thank C. P. Kurtzman, D. P. Labeda, K. O'Donnell and T. J. Ward for helpful comments. We thank M. Hart and E. Quesada for providing type strains of the species they described, as well as the many other individuals who deposited their strains into the ARS Culture Collection. The mention of firm names or trade products does not imply that they are endorsed or recommended by the US Department of Agriculture over other firms or similar products not mentioned.

REFERENCES

- Ash, C., Farrow, J. 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.
- Avise, J. C. (2000). *Phylogeography. The History and Formation of Species*. Cambridge, MA: Harvard University Press.
- Bischoff, K. M., Rooney, A. P., Li, X. L., Liu, S. & Hughes, S. R. (2006). Purification and characterization of a family 5 endoglucanase from a moderately thermophilic strain of *Bacillus licheniformis*. *Biotechnol Lett* **28**, 1761–1765.

- Chun, J. & Bae, K. S. (2000). Phylogenetic analysis of *Bacillus subtilis* and related taxa based on partial *gyrA* sequences. *Antonie Van Leeuwenhoek* **78**, 123–127.
- Cohan, F. M. (2001). Bacterial species and speciation. *Syst Biol* **50**, 513–524.
- Cohan, F. M. (2002). What are bacterial species? *Annu Rev Microbiol* **56**, 457–487.
- Cohan, F. M. (2006). Towards a conceptual and operational union of bacterial systematics, ecology, and evolution. *Philos Trans R Soc Lond B Biol Sci* **361**, 1985–1996.
- Cohn, F. (1872). Untersuchungen über bakterien. *Beitr Biol Pflanz* **1**, 127–244 (in German).
- Dykhuizen, D. E. & Green, L. (1991). Recombination in *Escherichia coli* and the definition of biological species. *J Bacteriol* **173**, 7257–7268.
- Felsenstein, J. (2006). PHYLIP (phylogeny inference package) version 3.66. Distributed by the author. Department of Genome Sciences, University of Washington, Seattle, USA. <http://evolution.gs.washington.edu/phylip.html>
- Gatson, J. W., Benz, B. F., Chandrasekaran, C., Satomi, M., Venkateswaran, K. & Hart, M. E. (2006). *Bacillus tequilensis* sp. nov., isolated from a 2000-year-old Mexican shaft-tomb, is closely related to *Bacillus subtilis*. *Int J Syst Evol Microbiol* **56**, 1475–1484.
- Gevers, D., Cohan, F. M., Lawrence, J. G., Spratt, B. G., Coenye, T., Feil, E. J., Stackebrandt, E., Van de Peer, Y., Vandamme, P. & other authors (2005). Re-evaluating prokaryotic species. *Nat Rev Microbiol* **3**, 733–739.
- Gordon, R. E., Haynes, W. C. & Pang, C. H.-N. (1973). *The Genus Bacillus*. US Department of Agriculture Handbook no. 427. Washington, DC: Agricultural Research Service.
- Hou, C. T., Labeda, D. P. & Rooney, A. P. (2005). Evaluation of microbial strains for linoleic acid hydroxylation and reclassification of strain ALA2. *Antonie Van Leeuwenhoek* **88**, 167–171.
- Koeppel, A., Perry, E. B., Sikorski, J., Krizanc, D., Warner, A., Ward, D. M., Rooney, A. P., Brambilla, E., Connor, N. & other authors (2008). Identifying the fundamental units of bacterial diversity: a paradigm shift to incorporate ecology into bacterial systematics. *Proc Natl Acad Sci U S A* **105**, 2504–2509.
- Kumar, S., Tamura, K. & Nei, M. (2004). MEGA3: integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Brief Bioinform* **5**, 150–163.
- Kundrat, W. (1963). Zur differenzierung aerober sporenbildner (Genus *Bacillus* Cohn). *Zentralbl F Veterinaarmed Reihe B* **10**, 418–426 (in German).
- Lane, D. J. (1991). 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*, pp. 115–175. Edited by E. Stackebrandt & M. Goodfellow. Chichester: Wiley.
- Marchesi, J. R., Sato, T., Weightman, A. J., Martin, T. A., Fry, J. C., Hiom, S. J., Dymock, D. & Wade, W. G. (1998). Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. *Appl Environ Microbiol* **64**, 795–799.
- Nakamura, L. K. (1989). Taxonomic relationship of black-pigmented *Bacillus subtilis* strains and a proposal for *Bacillus atrophaeus* sp. nov. *Int J Syst Bacteriol* **39**, 295–300.
- Nakamura, L. K., Roberts, M. S. & Cohan, F. M. (1999). Relationship of *Bacillus subtilis* clades associated with strains 168 and W23: a proposal for *Bacillus subtilis* subsp. *subtilis* subsp. nov. and *Bacillus subtilis* subsp. *spizizenii* subsp. nov. *Int J Syst Bacteriol* **49**, 1211–1215.
- Palmisano, M. M., Nakamura, L. K., Duncan, K. E., Istock, C. A. & Cohan, F. M. (2001). *Bacillus sonorensis* sp. nov., a close relative of

- Bacillus licheniformis*, isolated from soil in the Sonoran Desert, Arizona. *Int J Syst Evol Microbiol* **51**, 1671–1679.
- Perry, E. B., Krizan, D., Rooney, A. P., Sikorski, J., Nevo, E. & Cohan, F. M. (2007). Identifying the fundamental units of diversity among *Bacillus* isolates from “Evolution Canyon” III. *Isr J Ecol Evol* **52**, 543–552.
- Price, N. P. J., Rooney, A. P., Swezey, J. L., Perry, E. & Cohan, F. M. (2007). Mass spectroscopic analysis of lipopeptide for *Bacillus* strains isolated from diverse geographical locations. *FEMS Microbiol Lett* **271**, 83–89.
- Priest, F. G., Goodfellow, M., Shute, L. A. & Berkeley, R. C. W. (1987). *Bacillus amyloliquefaciens* sp. nov., nom. rev. *Int J Syst Bacteriol* **37**, 69–71.
- Roberts, M. S., Nakamura, L. K. & Cohan, F. M. (1994). *Bacillus mojavenis* sp. nov., distinguishable from *Bacillus subtilis* by sexual isolation, divergence in DNA sequence, and differences in fatty acid composition. *Int J Syst Bacteriol* **44**, 256–264.
- Roberts, M. S., Nakamura, L. K. & Cohan, F. M. (1996). *Bacillus vallismortis* sp. nov., a close relative of *Bacillus subtilis*, isolated from soil in Death Valley, California. *Int J Syst Bacteriol* **46**, 470–475.
- Rooney, A. P., Swezey, J. L., Wicklow, D. T. & McAtee, M. J. (2005). Bacterial species diversity in cigarettes linked to an investigation of severe pneumonitis in U.S. Military personnel deployed in Operation Iraqi Freedom. *Curr Microbiol* **51**, 46–52.
- Ruiz-García, C., Béjar, V., Martínez-Checa, F., Llamas, I. & Quesada, E. (2005a). *Bacillus velezensis* sp. nov., a surfactant-producing bacterium isolated from the river Velez in Malaga, southern Spain. *Int J Syst Evol Microbiol* **55**, 191–195.
- Ruiz-García, C., Quesada, E., Martínez-Checa, F., Llamas, I., Urdaci, M. C. & Béjar, V. (2005b). *Bacillus axarquiensis* sp. nov. and *Bacillus malacitensis* sp. nov., isolated from river-mouth sediments in southern Spain. *Int J Syst Evol Microbiol* **55**, 1279–1285.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Schmidt, D. D., Frommer, W., Miller, L. & Truscheit, E. (1979). Glucosidase-inhibitoren aus Bazillen. *Naturwissenschaften* **66**, 584–585 (in German).
- Stackebrandt, E., Frederiksen, W., Garrity, G. M., Grimont, P. A. D., Kämpfer, P., Maiden, M. C. J., Nesme, X., Rossello-Mora, R., Swings, J. & other authors (2002). Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. *Int J Syst Evol Microbiol* **52**, 1043–1052.
- Tamura, K. & Nei, M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol Biol Evol* **10**, 512–526.
- Wang, L. T., Lee, F. L., Tai, C. J., Yokota, A. & Kuo, H. P. (2007). Reclassification of *Bacillus axarquiensis* Ruiz-García *et al.* 2005 and *Bacillus malacitensis* Ruiz-García *et al.* 2005 as later heterotypic synonyms of *Bacillus mojavenis* Roberts *et al.* 1994. *Int J Syst Evol Microbiol* **57**, 1663–1667.
- Wang, L. T., Lee, F. L., Tai, C. J. & Kuo, H. P. (2008). *Bacillus velezensis* is a later heterotypic synonym of *Bacillus amyloliquefaciens*. *Int J Syst Evol Microbiol* **58**, 671–675.
- Xia, X. & Xie, Z. (2001). DAMBE: data analysis in molecular biology and evolution. *J Hered* **92**, 371–373.
- Zwickl, D. J. (2006). Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion. The University of Texas at Austin. www.bio.utexas.edu/faculty/antisense/garli/Garli.html