Bacillus Strains Most Closely Related to Bacillus nealsonii Are Not Effectively Circumscribed within the Taxonomic Species Definition

1. Introduction

CBD 118 was one of the two first Bacillus strains not related to the B. cereus group reported to harbor the capsule genes carried on pXO2 by Bacillus anthracis (USF Center for Biological Defense (CBD)) [1, 2]. Luna et al. isolated and sequenced the capsule operon (capA, capB, capC, capD, and promoter), repA, capR, apcA, IS1627, ORF43, ORF48, and ORF61 on a large plasmid in CBD 118 [1]. Its status as a carrier of Bacillus anthracis capsule genes spurred research into determining its closest relatives, to aid in circumscribing the reservoir of genes essential for virulence in Bacillus. When near full length 16S rRNA gene sequences were compared, the most similar type strains to strain CBD 118 were Bacillus circulans ATCC 4513T (98.9%) and Bacillus nealsonii DSM 15077T (99.3%). Strain CBD 118 differed from B. circulans ATCC 4513T and B. nealsonii DSM 15077T for 10 and 12 of 100 phenotypic traits evaluated, respectively. The percentages of DNA:DNA binding in two pairings each of strain CBD 118 to B. circulans ATCC 4513T and B. nealsonii DSM 15077T were 12.5 and 10.2% and 10.8 and 8.3%, respectively. Thus, strain CBD 118 is differentiated by phenotypic and genome-based methods from the only validly named species with greater than 98.7% 16S rRNA gene sequence similarity [3–5]. Strain CBD 118 was the sole exemplar of a novel species. Prior to the proposal of novel species, studies of ten or more strains are recommended in order to detail intra-species diversity and to foster appropriate type strain assignment [6–8]. To identify the requisite closely related strains, the V1–V3 hypervariable regions of the 16S rRNA gene [9] from strain CBD 118 were compared to sequences available in GenBank. Eight potential sibling strains were obtained for study. Although the eight strains tested negative for capsule production and for the pXO2 genetic marker by PCR, the
2. Materials and Methods

2.1. Bacterial Strains. Nine Bacillus strains in this study were deposited in the Agricultural Research Service Culture Collection (ARSCC), U.S. Department of Agriculture, Peoria, III, USA. Accession numbers for the CBD collection and the ARSCC (NRRL) follow the original strain identifiers. Bacillus sp. CBD 118 = NRRL B-51264 was isolated from a powder initially suspected of harbouring B. anthracis [1]. Bacillus strains provided by colleagues: OSS 25 = CBD 1278 = NRRL B-59473 [21]; P307 = CBD 1284 = NRRL B-59474 and P308 = CBD 1285 = NRRL B-59475 (Harbor Branch Marine Microbial Culture Collection); C4TIF3B3 = CBD 1286 = NRRL B-59476 [22]; IAFILS6 = CBD 1287 = NRRL B-59477 [23]; AD5A = CBD 1288 = NRRL B-59478, U4A = CBD 1289 = NRRL B-59479 and ADP41I = CBD 1290 = NRRL B-59480 [24]. Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under accession numbers DQ374636, EU656111, EU683686, FJ554672, FJ943256–FJ943261. The type strains of B. circulans, ATCC 4513T and of B. nalearsonii, DSM 15077T were acquired from the American Type Culture Collection (ATCC) and the Deutsche Sammlung von Microorganismen und Zellkulturen (GmbH) (DSMZ), respectively.

2.2. Preservation and Authentication of Bacillus Strains. Upon receipt, each strain was subcultured by streaking to tryptic soy agar (TSA) and TSA with 5% sheep red blood cells (TSA-BA) and grown at 30°C. After 24, 48, and 72 h incubation, plated strains were examined for purity based on the presence of colonies of a single morphotype. A single, well-isolated and representative colony was designated as the progenitor colony and streaked for pure culture reisolation on plates of TSA, TSA-BA, and TSA with 5 mg L−1 MnSO4, incubated at 30°C for up to 72 h. Characteristic, well-isolated colonies on these plates served as first passage sources of inocula for initial phenotypic characterization as detailed below. Colony morphologies for each strain were observed at 24 and 48 h for consistency with the progenitor colony and were described for standard colony features including color, surface texture and degree of luster, relative transmittance of direct light through the colony, shape, margin configuration, elevation, diameter in mm and hemolysis reaction. Each of the Bacillus strains in this study including the type strains presented one or more differential colony features that were documented and subsequently monitored as evidence of purity and authenticity whenever strains were subcultured. Phenotypic tests and other procedures utilizing broths were routinely subcultured at the incubation end point to TSA-BA check plates. After 24 and 48 h incubation at 30°C, check plates were reviewed for the presence of colonies of a single, differential morphotype, characteristic of each strain.

The Bacillus including type strains were inoculated from the progenitor colony to aerated tryptic soy broth (TSB), grown to late log phase, subcultured to a TSA check plate, aseptically harvested by centrifugation, resuspended in TSB with 10% glycerol, aliquoted to multiple cryovials, and subjected to a controlled freeze prior to storage at −85°C. One week after cryostocking, a cryovial of each strain was thawed, subcultured on TSA and TSA-BA plates, enumerated for viability and again evaluated for the single, differential colony morphotype. Prior to retesting of phenotypic characters and other analyses, strains were subcultured from the cryostocks and endospore production was induced on TSA with
5 mg L⁻¹ MnSO₄ plates. Serial transfer of strains was restricted by the use of single, characteristic endospore-producing colonies as inoculation sources for subsequent testing.

Preservation of strain authenticity was evaluated at the end of the study. Four strains that formed an apparent genomic cluster were subcultured from cryopreserved stock, retested for seven differential phenotypic traits including colony morphotype and resequenced for the 16S rRNA gene. The resultant sequences were compared to the original sequences deposited in GenBank.

2.3. 16S rRNA Gene Sequence Analysis. Amplification of 16S rRNA gene sequences from Bacillus strains, sequencing of the approximately 1500 bp long products, fragment assemblies and alignment of 16S rRNA gene sequences from type strains of selected Bacillus species were performed as previously reported [2]; an additional primer, 534R, was employed in some amplifications. Identification of phylogenetic neighbors was carried out by BLAST 2.2.20+ [25] and megaBLAST (discontinuous option) [26] searches of GenBank [27]. Calculation of pairwise sequence similarity to nearest neighbors used the EzTaxon global alignment algorithm [28]. Alignments of 16S rDNA sequences were also made using the Infernal secondary structure based aligner and SeqMatch scores (S_ab) calculated with RDP10 at the Ribosomal Database Project website [29]. Nucleotide (nt) positions in the hypervariable regions V1–V3 were identified in the 16S sequence of strain CBD 118 by alignment with conserved regions at nt positions 48–70, 346–366, and 490–511 in rrnE of B. subtilis subsp. subtilis strain 168² (NC_000964.2, Locus tag: BSUr022, GenID: 2914197, updated 3/2010 to NC_000964.3, Locus tag: BSU_rRNA_30, GenID: 8303085) [9]. In the E. coli numbering system, regions V1–V3 correspond to nt positions 69–99, 137–242 and 433–497, respectively [30]. Using GenBank b2seg, pairwise alignments of 461 bp from strain CBD 118 (nt 29–461 corresponding to rrnE nt positions 48–511) were made to 16S rDNA sequences from closely related strains. Presumptive signature sequences (PSS) within the V1–V3 region were identified and compared to all GenBank sequences using BLASTN 2.2.20+ with parameters adjusted for short input sequences [25]. Dendograms were constructed from approximately 1390 bp or 448 bp using neighbor-joining, maximum parsimony and maximum likelihood algorithms (PHYLP v. 3.6.80) [31] with 1000 bootstrap replicates performed to estimate support for each branch.

2.4. DNA:DNA Hybridization Studies. Strains were subcultured from cryopreserved stock, grown to late log phase in aerated TSB, harvested by centrifugation and provided to the Deutsche Sammlung von Microorganismen und Zellkulturen (GmbH) (DSMZ) as ≥3 g wet weight biomass preserved at −85°C in TSB, 10% glycerol. Control strains of Bacillus and Paenibacillus included B. cereus ATCC 14579T, B. circulans ATCC 4513T, B. megaterium ATCC 14581T, B. nealsonii DSM 15077T, B. pumilus ATCC 7061T, B. thuringiensis ATCC 10792T, and P. polymyxa ATCC 43865T. Sporulation was induced on TSA with 5 mg L⁻¹ MnSO₄, grown for 40–48 h. Hemolysis reaction was determined on TSA with 5% sheep red blood cells (REMEL), grown for 48 h. Pigment production and mean colony diameter were evaluated on TSA, tryptone blood agar base and tryptone glucose yeast extract plates, 24, 48, 72 h and 1 week. Motility was determined by either stab inoculation of motility test medium (REMEL), observed at 24 and 48 h, or phase contrast observation of wet mounts made with aerated cells grown in TSB to log phase, 3 to 6 h. Cell morphology,

2.5. Cellular Fatty Acid Analysis. Cellular fatty acid (CFA) of Bacillus strains was determined by Microbial ID, Inc. (MIDI) at both study start and end point. Each strain was subcultured from cryopreserved stock, inoculated from a single, characteristic colony to a TSA slant and grown at 30°C for 48 h to foster endospore formation. Prior to shipment, each slant was subcultured on a TSA-BA check plate grown at 30°C and observed after 24 and 48 h incubation. The check plate for each strain was reviewed for colonies of a single, differential morphotype and the slant shipped to MIDI only after no evidence of contamination or mislabeling of strains was discerned.

Strains were grown under standardized conditions on tryptic soy broth agar quadrant streak plates at 28°C for 24 h. To reduce disparities in the effective physiological age of the cells, biomass was harvested from colonies growing in the third streaked quadrant. Fatty acid methyl esters were extracted by a four-step procedure of saponification, methylation, extraction and sample clean-up. Fatty acid peaks were analyzed by gas chromatography and named by comparing retention times to those in a known mixture. A dendrogram program used a multivariate clustering algorithm to produce unweighted pair matching based on similar CFA content between strains and generated a tree scaled to Euclidian distance (ED).

2.6. Phenotypic Characterization. All tests were incubated at 30°C unless otherwise noted [34]; incubation periods are specified. Differential tests were performed at minimum twice or as specified; prior to re-testing, strains were subcultured from cryostocks held at −85°C in TSB, 10% glycerol. Control strains of Bacillus and Paenibacillus included B. cereus ATCC 14579T, B. circulans ATCC 4513T, B. megaterium ATCC 14581T, B. nealsonii DSM 15077T, B. pumilus ATCC 7061T, B. thuringiensis ATCC 10792T, and P. polymyxa ATCC 43865T. Sporulation was induced on TSA with 5 mg L⁻¹ MnSO₄, grown for 40–48 h. Hemolysis reaction was determined on TSA with 5% sheep red blood cells (REMEL), grown for 48 h. Pigment production and mean colony diameter were evaluated on TSA, tryptone blood agar base and tryptone glucose yeast extract plates, 24, 48, 72 h and 1 week. Motility was determined by either stab inoculation of motility test medium (REMEL), observed at 24 and 48 h, or phase contrast observation of wet mounts made with aerated cells grown in TSB to log phase, 3 to 6 h. Cell morphology,
endospore characterization and swelling of the sporangium, presence of parasporal bodies and motility were observed in wet mounts using phase contrast microscopy at \( \times 1000 \) under oil. Anaerobic growth was evaluated after 1 week in the Mitsubishi Pack-Anaero anaerobic gas generating system with the following pre-reduced media: fluid thioglycollate medium with dextrose and indicator (REMEL), tryptone glucose yeast extract agar plates, and anaerobic agar [34], inoculated in the molten state. Oxidase reaction was tested with Kovács’ phenylenediamine redox dye reagent (Becton, Dickinson). Growth of cells at defined temperatures was tested in 3 mL of TSB in \( 13 \times 100 \text{mm} \) tubes for 48 h in water baths set to 30, 35, 40, 45, 50, 55, and 60 \( ^\circ \text{C} \) and examined for turbidity at 24 h intervals. Growth of cells at defined pH was tested in the same manner in TSB adjusted to pH 4.6, 5.6, 6.1, 6.5, 6.8, 7.3, 7.8, 8.1, and 8.5. Salt tolerance was tested on nutrient agar plates supplemented with 0, 1, 3, and 10% NaCl, incubated for 5 days. Physiological tests per-formed on cells grown in commercial media (REMEL) included casein and starch hydrolysis, incubated 14 days; growth on mannitol egg yolk polymyxin agar, incubated 48 h; growth in methyl red Voges-Proskauer (MRVP) broth for final pH and VP reaction, tested at 3, 5, and 7 days; nitrate reduction tested in nitrate broth at 3, 7, and 14 days and on nitrate agar slants, at 3 and 5 days; and growth on Sabouraud’s 4% glucose agar, pH 5.6, incubated 72 h. Gelatin hydrolysis was tested in 12% nutrient gelatin (REMEL) for 2 weeks. Hydrolysis of Tween-80 was tested on plates of a peptone-based medium [35], incubated for 4 weeks. Acid production from 49 carbohydrates or carbohydrate derivatives was tested using the API 50 CH panel and API CHB/E medium with mineral oil overlay, \( \geq 4 \) test panels per strain, in combination with eleven biochemical tests from the API 20E kit, \( \geq 2 \) test panels per strain, incubated for 48 and 24 h, respectively (bioMérieux). Acid production was read at 24 and 48 h in a semiquantitative way, where 0 was assigned to negative reactions of the same alkaline red as the no-carbohydrate control and 5 assigned to yellow indicator shifts of maximum intensity. Values of 1, 2, 3, or 4 were given to intermediate reactions with 3, 4, and 5 being considered positive. Differential phenotypic traits between paired strains were enumerated. Each differential character state was assigned a numerical value—that is, 1 = negative, 2 = variable, 3 = positive—and subjected to hierarchical cluster analysis (SPSS for Windows, Release 15.0.1.1, 2007). A dendrogram was generated using average linkage between groups, scaled in Euclidian distance units.

3. Results and Discussion

3.1. Bacterial Strains Obtained for Taxonomic Study. Eight Bacillus strains having \( \geq 99.7\% \) 16S rRNA gene sequence similarity [19, 36] and \( \geq 0.980 \) SeqMatch \( S_{ab} \) scores to 1495 bp of strain CBD 118 (DQ374636) were provided to the CBD for taxonomic comparisons: strain OSS 25 (EU683686), isolated from a metallurgic waste site in Italy [21]; strains P307 (FJ943260) and P308 (FJ54672), isolated from deep-water marine sponge (Discodorinia sp.), Bahamas; strain C4T1F3B3 (FJ943258), isolated from cultured flounder, USA [22]; strain IAFILS6 (FJ943259) isolated from a consortium degrading polyaromatic hydrocarbons, Canada [23]; strains AD5A (FJ943256), U4A (FJ943261), and ADP4II (FJ943257), isolated from plant thorns, Israel [24]. The most closely related type strains were Bacillus circulans ATCC 4513\(^T\) (AY724690) (98.9%; \( S_{ab} \) not ranked) and B. nealsonii DSM 15077\(^T\) (EU656111) (99.3%; \( S_{ab} \) 0.961).

3.2. Presumptive Signature Sequences. Hypervariable regions V1–V3 in the Bacillus 16S rRNA gene sequence had been reported to be discriminatory for most Bacillus species [9]. The eight Bacillus strains were identified as potential sibling strains when 461 bp spanning V1–V3 hypervariable regions of the 16S rRNA gene sequence of strain CBD 118 were compared to GenBank database sequences. Presumptive signature sequences (PSS) were identified in V1 at nt positions 71–92 (PSS\(_1A\) and PSS\(_1B\)) and in V2 at nt positions 183–223 (PSS\(_2\)) (Table 1). The eight Bacillus strains, strain CBD 118 and B. nealsonii DSM 15077\(^T\) differed from B. circulans ATCC 4513\(^T\) by two nucleotide changes in PSS\(_1\) and at five positions in PSS\(_2\). B. nealsonii DSM 15077\(^T\) also differed in PSS\(_2\) at four nucleotide positions and by insertion of two thymines. Since not all 16S rRNA sequences from B. circulans and B. nealsonii strains have been examined for these signatures, the PSS are termed presumptive. Strains CBD 118 and OSS 25 were identical for 461 bp spanning V1–V3 and differed from the other seven Bacillus strains by one bp in PSS\(_2\)a (Table 1). In nucleotide blast searches of the GenBank database, eight sequences—including that of strain CBD 118—and at five positions in PSS\(_2\) b was 99.8% similarity, suggesting all four were Bacillus strains more closely related to CBD 118 than to B. benzocevorans or B. nealsonii type strains. Searches coupling PSS\(_1\)B with PSS\(_2\) or PSS\(_2\)C identified other isolates with \( \geq 99\% \) sequence similarity to strains C4T1F3B3, P307 and P308 and to IAFILS6, AD5A, U4A, and ADP4II, respectively.

3.3. Phylogenetic Analysis. In a neighbor-joining (N-J) tree (Figure 1) based on approximately 1390 bp of 16S rRNA sequence, strains most closely related to B. circulans and B. nealsonii were divided into two well-supported sister clades. Strain CBD 118 and Bacillus strains with \( \geq 99.7\% \) sequence similarity to CBD 118 formed a complex clade with B. nealsonii DSM 15077\(^T\). In the subtree (Figure 1), the Bacillus strains grouped according to PSS\(_2\) type, but without strong bootstrap support. The B. circulans clade included B. circulans ATCC 4513\(^T\), one strain identified as B. circulans and two Bacillus spp. with \( \geq 99.5\% \) similarity to the type strain. The attribution of species-level identity based solely on 16S rRNA gene sequence similarity is known to be unreliable [6, 7] especially among Bacillus [36]. Keswani and Whitman [36] studied the relationship of 16S rRNA sequence similarity (\( S \)) to DNA:DNA hybridization (\( D \)). Among 40 Bacillus
Table 1: Presumptive signature sequences (PSS) conserved in 16S rRNA gene at nt positions 71–92 (PSS1) and 183–223 (PSS2) in respective V1 and V2 hypervariable regions of Bacillus nealsonii-related strains. “Numbering based on rrnE of Bacillus subtilis subsp. subtilis strain 168T (NC_000964.3); a gap at nt position 80 in PSS1 sequence alignments to rrnE is reflected in the numbering.

| PSS type | Bacillus Strain no. | GenBank Accession no. | Nucleotides of PSS |
|----------|---------------------|-----------------------|--------------------|
| PSS1A    | ATCC 4513<sup>T</sup> | AB215100              | GAC T T A A A A A G C T T T G C T T T T T A |
|          | B. circulans         |                       |                    |
|          | N3 B. circulans      |                       |                    |
|          | DSM 15077<sup>T</sup> | EU656111              | GAC T T A A A A A G C T T T G C T T T T T T |
| PSS1B    | B. nealsonii         |                       |                    |
|          | CBD 118              | DQ374636              | C C T T T T C T A C T A G T A G G A A A G — C T G A A A G C G G G T T T A — C G C |
|          | OSS 25               | EU683868              | C C T T T T C T A C T A G T A G G A A A G — C T G A A A G C G G G T T T A — C G C |
|          | P307                 | FJ943260              | C C T T T T C T A C T A G T A G G A A A G — C T G A A A G C G G G T T T A — C G C |
|          | P308                 | FJ554672              | C C T T T T C T A C T A G T A G G A A A G — C T G A A A G C G G G T T T A — C G C |
|          | C4T1F3B3             | FJ943258              | C C T T T T C T A C T A G T A G G A A A G — C T G A A A G C G G G T T T A — C G C |
|          | IAFILS6              | FJ943259              | C C T T T T C T A C T A G T A G G A A A G — C T G A A A G C G G G T T T A — C G C |
|          | AD5A                 | FJ943256              | C C T T T T C T A C T A G T A G G A A A G — C T G A A A G C G G G T T T A — C G C |
|          | U4A                  | FJ943261              | C C T T T T C T A C T A G T A G G A A A G — C T G A A A G C G G G T T T A — C G C |
|          | ADP4II               | FJ943257              | C C T T T T C T A C T A G T A G G A A A G — C T G A A A G C G G G T T T A — C G C |
| PSS2<sub>a</sub> | CBD 118              | DQ374636              | C C T T T T C T A C T A G T A G G A A A G — C T G A A A G C G G G T T T A — C G C |
|          | OSS 25               | EU683868              | C C T T T T C T A C T A G T A G G A A A G — C T G A A A G C G G G T T T A — C G C |
|          | CT18                 | EU606308              | C C T T T T C T A C T A G T A G G A A A G — C T G A A A G C G G G T T T A — C G C |
|          | Q2CJ3                | GU471201              | C C T T T T C T A C T A G T A G G A A A G — C T G A A A G C G G G T T T A — C G C |
| PSS2<sub>b</sub> | P307                 | FJ943260              | C C T T T T C T A C T A G T A G G A A A G — C T G A A A G C G G G T T T A — C G C |
|          | P308                 | FJ554672              | C C T T T T C T A C T A G T A G G A A A G — C T G A A A G C G G G T T T A — C G C |
|          | C4T1F3B3             | FJ943258              | C C T T T T C T A C T A G T A G G A A A G — C T G A A A G C G G G T T T A — C G C |
|          | IAFILS6              | FJ943259              | C C T T T T C T A C T A G T A G G A A A G — C T G A A A G C G G G T T T A — C G C |
|          | AD5A                 | FJ943256              | C C T T T T C T A C T A G T A G G A A A G — C T G A A A G C G G G T T T A — C G C |
|          | U4A                  | FJ943261              | C C T T T T C T A C T A G T A G G A A A G — C T G A A A G C G G G T T T A — C G C |
|          | ADP4II               | FJ943257              | C C T T T T C T A C T A G T A G G A A A G — C T G A A A G C G G G T T T A — C G C |
| PSS2<sub>c</sub> | DSM 15077<sup>T</sup> | EU656111              | C C T T T T C T A C T A G T A G G A A A G — C T G A A A G C G G G T T T A — C G C |
| PSS2<sub>b</sub> | DSM 15077<sup>T</sup> | EU656111              | C C T T T T C T A C T A G T A G G A A A G — C T G A A A G C G G G T T T A — C G C |

spp., for an S of 0.997, 0.993 or 0.991, D could be expected to be <0.70 about 50, 95 or 99% of the time, respectively. Strains OSS 25 and WZ12 in the B. nealsonii clade were identified in GenBank as B. circulans, however each had <99% sequence similarity to B. circulans ATCC 4513<sup>T</sup>, indicating the strains do not belong to that species [36]. Bacillus benzo- 

3.4. % DNA:DNA Similarity (%) S. DNA:DNA pairings were conducted between B. nealsonii DSM 15077<sup>T</sup> and two strains representing each PSS1 type—CBD 118, OSS 25 (PSS1a); P308, C4T1F3B3 (PSS1b); IAFILS6, AD5A (PSS1c) (Table 2). DNA:DNA% similarity (%) S data reveals clusters of closely-

related strains termed genomic groups or genomic species and remains the “gold standard” for species circumscription [3, 5, 19, 37, 38]. More distantly related groups are separated by discontinuities, commonly in the range of 50–70% S; usually only a few intermediate strains are found [37, 38]. The % S for each of the six Bacillus strains to B. nealsonii DSM 15077<sup>T</sup> was less than 40%, well below the recommend-
ed 70% threshold value to be circumscribed in that species [3, 5]. The % S between PSS1<sub>a</sub> strains CBD 118 and OSS 25 were 49.9% and 55.5%. In a previous set of pairings, % S of 63.8 and 61.6 suggest di

ferences in DNA quality bet-

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**Figure 1:** Phylogenetic relationship of strain CBD 118 with type strains of other *Bacillus* spp. and with strains from GenBank with highest similarities (accession numbers in parentheses). The Neighbor-Joining tree and subtree are based on approximately 1390 bp of the 16S rRNA gene. Numbers show the level of bootstrap support from 1000 repetitions with only those >750 shown. Bars, nucleotide substitutions per nucleotide.

**Table 2:** % DNA:DNA similarity between seven *Bacillus* species including *B. nealsonii* DSM 15077ᵀ with reference to presumptive signature sequence 2 (PSS₂) types. * Duplicate measurements from DNA:DNA hybridizations performed using spectrophotometric determinations.
Given the estimated 10% reproducibility of % S values (DSMZ), DNA:DNA pairings that have ≥80% S should meet or exceed the recommended 70% threshold to delineate taxa at the species level. Three of 21 strain pairings—P308 with C4T1F3B3; OSS 25 with C4T1F3B3; P308 with IAFILS6—tested at ≥80% S; therefore, these four strains appeared to represent a coherent genomic cluster. A diagram (Figure 2) in which the two measurements per pairing were averaged, illustrates varying degrees of genomic coherence among all 7 strains. Averaged % S of 93.5% strongly supports DNA relatedness between strains P308 (PSS₂b) and C4T1F3B3 (PSS₂b); % S of 83.4% also supports relatedness between P308 (PSS₂b) and IAFILS6 (PSS₂c). But relatedness between C4T1F3B3 (PSS₂b) and IAFILS6 (PSS₂c) is not similarly well supported at 70% S, thus the degree of relatedness of each strain to P308 was not reproduced in relation to each other. Also, within this apparent genomic cluster, averaged % S was 87.1% between OSS 25 (PSS₂a) and C4T1F3B3 (PSS₂b), 78.2% between OSS 25 (PSS₂a) and P308 (PSS₂b), but was 70.25% between OSS 25 (PSS₂a) and IAFILS6 (PSS₂c). A genomic cluster based on these four strains incorporates an ~24% range for % S and values for two pairings that may—given ~10% reproducibility—lie in the transitional range for species circumscription. Some strains of a species may show less than 70% S with the type strain or other strains of the same species, thus internal heterogeneity within genomic groupings and species is permitted [6, 7, 11, 16]. However, studies of the average nucleotide identity (ANI) of all conserved genes between any two genomes [13, 14, 39] support adoption of a higher rather than a relaxed threshold for species circumscription. The 70% threshold for species delineation based on DNA:DNA pairings corresponds to 95% ANI and 85% or 79% conserved protein coding genes between a pair of strains [39], thus substantial phenotypic differences were possible among two or more of these four strains.

### Table 3: Comparison of percent cellular fatty acid content between strain CBD 118, eight closely related strains, and most closely related strains. P308 (PSS 2b) and C4T1F3B3 strains. Averaged % S values for two pairings that may—given ~10% reproducibility—lie in the transitional range for species circumscription. Some strains of a species may show less than 70% S with the type strain or other strains of the same species, thus internal heterogeneity within genomic groupings and species is permitted [6, 7, 11, 16]. However, studies of the average nucleotide identity (ANI) of all conserved genes between any two genomes [13, 14, 39] support adoption of a higher rather than a relaxed threshold for species circumscription. The 70% threshold for species delineation based on DNA:DNA pairings corresponds to 95% ANI and 85% or 79% conserved protein coding genes between a pair of strains [39], thus substantial phenotypic differences were possible among two or more of these four strains.

| Strains | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 |
|---------|----|----|----|----|----|----|----|----|----|----|----|
| C₁₅₀ iso | 1.55 | 1.69 | 0.91 | 0.94 | 1.15 | 0.72 | 0.48 | 0.72 | 0.76 | 3.41 | 0.67 |
| C₁₅₀ anteiso | 0.24 | 0.53 | 0.21 | — | 0.22 | 0.24 | 0.18 | — | — | 0.79 | 0.17 |
| C₁₆₀ iso | 2.52 | 2.95 | 1.48 | 1.73 | 1.82 | 1.28 | 1.81 | 2.96 | 1.81 | 2.39 | 4.78 |
| C₁₆₀ anteiso | 9.93 | 3.97 | 6.37 | 7.90 | 7.04 | 4.25 | 4.71 | 3.82 | 3.58 | 13.67 | 11.66 |
| C₁₅₀ iso | 25.95 | 20.57 | 20.17 | 21.53 | 23.24 | 16.59 | 14.68 | 21.42 | 21.36 | 29.28 | 20.25 |
| C₁₅₀ anteiso | 42.78 | 60.69 | 49.74 | 43.94 | 44.73 | 46.86 | 51.51 | 45.18 | 44.28 | 35.09 | 40.64 |
| C₁₆₁ω7c alcohol | 0.23 | — | 0.20 | — | 0.21 | 0.18 | 0.73 | 0.60 | 0.50 | 0.59 | 1.02 |
| C₁₆₀ iso | 1.07 | 1.32 | 0.92 | 1.04 | 1.08 | 1.22 | 1.09 | 2.54 | 1.67 | 0.74 | 4.36 |
| C₁₆₁ω11c | 2.66 | 0.33 | 2.82 | 2.02 | 2.23 | 2.06 | 4.37 | 2.69 | 2.79 | 4.69 | 2.64 |
| C₁₆₀ | 8.84 | 4.22 | 7.59 | 12.55 | 9.58 | 5.68 | 3.47 | 3.73 | 3.02 | 4.60 | 5.58 |
| C₁₅₀ iso 3OH | — | 0.33 | — | — | — | — | — | — | 0.96 | 1.31 | — |
| C₁₅₀ 2OH | — | 0.61 | — | — | — | — | — | — | 0.83 | 1.42 | — |
| C₁₇₁ iso ω10c | — | — | — | — | — | — | — | — | 0.28 | 0.44 | 0.28 | 0.26 | 0.25 | 0.23 |
| Sum in feature 4† | — | — | 0.34 | — | — | 0.50 | 2.17 | 1.30 | 1.20 | 0.36 | 0.40 |
| C₁₇₀ iso | 0.80 | 0.36 | 1.22 | 1.25 | 1.21 | 3.09 | 1.21 | 1.61 | 1.54 | 0.87 | 1.27 |
| C₁₇₀ anteiso | 3.41 | 2.42 | 9.35 | 6.90 | 7.49 | 16.11 | 12.15 | 8.84 | 10.23 | 3.27 | 6.07 |
| C₁₆₀ iso 3OH | — | — | — | — | — | — | 0.42 | 0.34 | — | — |
| C₁₆₀ 2OH | — | — | — | — | — | — | 0.21 | 0.54 | — | — |
| C₁₇₀ iso 3OH | — | — | — | — | — | — | — | 0.30 | — | — |
| C₁₈₀ | — | — | 0.26 | 0.23 | — | — | 0.28 | 0.19 | — | — |
| C₁₇₀ 2OH | — | — | — | — | — | — | 0.16 | 0.59 | 1.46 | — | — |
| C₁₉₀ anteiso | — | — | 0.22 | — | — | 0.66 | 0.63 | 0.34 | 0.66 | — | — |

3.5. **Cellular Fatty Acid Analysis (CFA).** Cellular fatty acid compositions of the nine *Bacillus* strains, *B. circulans* ATCC 4513 and *B. nealsonii* DSM 15077 are compared in Table 3. Consistent with *Bacillus* [34], the major cellular fatty acids (CFA) measured in the strains were C₁₄₀, C₁₅₀-anteiso C₁₅₀-iso and C₁₆₀. Profiles from a second CFA analysis performed at the end of study (not shown) were consistent with those in Table 3. The second data set deviated in the absence of 1–7 very low % CFAs (most <0.5%; three <1.5%) from the profiles of each of the strains, suggesting differences between the two testing events in the effective physiological age of the strains. The ability to reproduce profiles for a single strain is dependent on standardized conditions for growth medium, incubation time and temperature, and effective physiological age of the cells ([34], MIDI technical literature). In the second analysis, slight changes in values of major and other CFAs for ≥9 strains followed a parallel pattern of elevation (C₁₅₀-iso, C₁₇₀-iso) or reduction (C₁₄₀, C₁₆₁ ω11c, C₁₆₀).
also suggesting differences in effective physiological age for those strains relative to the first testing event. However, salient differential CFAs were reproduced in the profiles of both data sets, supporting the authenticity of the strain set at both time points in our study. C15:0-anteiso was 60% in both data sets, supporting the authenticity of the strain set at both testing events. However, salient differential CFAs were reproduced in the profiles of both data sets, supporting the authenticity of the strain set at both testing events. However, salient differential CFAs were reproduced in the profiles of both data sets, supporting the authenticity of the strain set at both testing events. However, salient differential CFAs were reproduced in the profiles of both data sets, supporting the authenticity of the strain set at both testing events. However, salient differential CFAs were reproduced in the profiles of both data sets, supporting the authenticity of the strain set at both testing events. However, salient differential CFAs were reproduced in the profiles of both data sets, supporting the authenticity of the strain set at both testing events.

\[(\text{Figure 2: Diagram of averaged } \% \text{ DNA:DNA similarities (}\% \ S) \text{ between Bacillus strains DSM 15077}^\text{T}, \text{ CBD 118, OSS 25, P308, C4T1F3B3, IAFILS6, and AD5A with reference to the presumptive signature sequence 2 type (PSS}_2).\]

3.6. Differential Phenotypic Characterization. Twenty-five of 100 phenotypic traits differentiate among the nine Bacillus and the two most closely related type strains (Table 4). Type strains B. circulans ATCC 4513T and B. nealsonii DSM 15077T are distinguished from the other strains by lack of acetoin production and by acid from 2-ketogluconate. The numbers of characters that separate each pair of Bacillus strains were compiled in a matrix (Table 5) and presented in a dendrogram (Figure 4). The only consistency between the CFA-based (Figure 3) and phenotype-based dendrogram was the close linkage of two PSS2cs strains, U4A and ADP4II—one of only two instances in which strains of the same PSS2 type were directly linked in the phenotype-based dendrogram. Only 3 of 36 phenotypic pairings resulted in ≤5 character differences while 19 strain pairs had ≥10 differences (highest number = 13). As a group, strains IAFILS6, AD5A, U4A and ADP4II sharing PSS2c formed the most coherent group with between 3–8 character differences. But in the phenotype dendrogram, closely paired strains U4A and ADP4II shared PSS2c formed the most coherent group with between 3–8 character differences. But in the phenotype dendrogram, closely paired strains U4A and ADP4II shared PSS2c formed the most coherent group with between 3–8 character differences. But in the phenotype dendrogram, closely paired strains U4A and ADP4II shared PSS2c formed the most coherent group with between 3–8 character differences. But in the phenotype dendrogram, closely paired strains U4A and ADP4II shared PSS2c formed the most coherent group with between 3–8 character differences. But in the phenotype dendrogram, closely paired strains U4A and ADP4II shared PSS2c formed the most coherent group with between 3–8 character differences.

3.7. Incongruence of Character Sets and Application of a Bacterial Species Definition. The taxonomic species definition...
Table 4: Comparison of differential phenotypic traits for strain CBD 118, eight closely related strains, and most closely related type strains.

| Characteristic                              | Strain ID |
|---------------------------------------------|-----------|
|                                            | CBD 118   | OSS 25 | P307 | P308 | C4T1F3B3 | IAFILS6 | AD5A | U4A | ADP4II |
|                                            |           | PSS₂a   | PSS₂b | PSS₂b | PSS₂c   | PSS₂c   | PSS₂c | PSS₂c | PSS₂c |
|                                            | CBD 118   | 1       | 2     | 3     | 4       | 5       | 6       | 7     | 8     | 9     | 10    | 11    |
| Swelling of sporangium                     | Subtle    | --      | Subtle| Subtle| Overt   | Subtle  | Overt  | Overt | Overt | Overt | Overt |
| Colony diameter (mm)                       | 2         | 2       | 2     | 0.75  | 2       | 2       | 2      | 4     | 1.5   | 4     | 3     |
| Hemolysis reaction                         | --        | --      | alpha | --    | --      | --      | --     | --    | --    | --    | --    |
| Growth at 45°C                             | +         | Scant   | Scant | Scant | +       | Scant   | +      | --    | --    | --    | +     |
| Growth with 7% NaCl                        | +         | --      | +     | +     | +       | --      | +      | +     | +     | +     | +     |
| Tween-80 hydrolysis                        | +         | --      | --    | --    | Scant   | --      | --     | +     | +     | +     | +     |
| Acetoin production                         | --        | --      | +     | +     | +       | +       | +      | +     | +     | +     | +     |
| Nitrate reduction                          | --        | --      | +     | +     | --      | --      | +      | --    | --    | --    | +     |
| Acid from L-Arabinose                      | +         | +       | --    | --    | --      | --      | --     | --    | --    | --    | --    |
| D-Arabinol                                 | --        | v       | +     | +     | +       | +       | +      | --    | --    | +     | +     |
| Dulcitol                                   | --        | --      | --    | --    | --      | --      | +      | --    | --    | --    | --    |
| α-methyl-D-Glucoside                       | +         | +       | --    | +     | +       | v       | v      | +     | +     | +     | +     |
| Glycerol                                   | +         | +       | +     | --    | v       | --      | v      | --    | +     | +     | +     |
| Glycogen                                   | +         | --      | v     | --    | v       | +       | --     | --    | --    | --    | --    |
| Inulin                                     | +         | --      | --    | --    | --      | --      | --     | --    | --    | --    | --    |
| 2-Ketogluconate                            | +         | +       | --    | --    | --      | --      | --     | --    | --    | --    | --    |
| 5-Ketogluconate                            | --        | v       | +     | +     | +       | +       | +      | --    | --    | --    | --    |
| D-Lylose                                   | --        | +       | --    | --    | --      | --      | --     | --    | --    | --    | --    |
| α-methyl-D-Mannoside                       | +         | v       | v     | v     | --      | --      | --     | --    | --    | --    | --    |
| Sorbitol                                   | +         | +       | +     | +     | +       | +       | --     | +     | +     | +     | +     |
| D-Tagatose                                 | --        | +       | --    | --    | --      | --      | --     | --    | --    | --    | --    |
| Xylitol                                    | +         | --      | v     | --    | --      | --      | v      | --    | --    | --    | --    |
| D-Xylose                                   | +         | +       | --    | +     | --      | v       | v      | v     | v     | +     | +     |
| β-methyl-D-Xyloside                        | +         | v       | --    | v     | +       | +       | v      | --    | +     | +     | +     |

Table 5: Number of differential phenotypic traits between paired Bacillus strains among 100 traits tested with reference to the presumptive signature sequence 2 type (PSS₂).

| Strain ID | CBD 118 | OSS 25 | P307 | P308 | C4T1F3B3 | IAFILS6 | AD5A | U4A | ADP4II |
|-----------|---------|--------|------|------|----------|---------|------|-----|--------|
|           | PSS₂a   | PSS₂a  | PSS₂b | PSS₂b | PSS₂c   | PSS₂c   | PSS₂c | PSS₂c | PSS₂c |
| CBD 118   | 9       |        |       | 7    |          |         |      |      |        |
| OSS 25    | 8       | 12     |      |      |          |         |      |      |        |
| P307      | 8       | 11     | 7    |      |          |         |      |      |        |
| P308      | 11      | 9      | 10   | 13   |          |         |      |      |        |
| C4T1F3B3  | 12      | 12     | 8    | 12   | 9        |        |      |      |        |
| IAFILS6   | 13      | 12     | 10   | 13   | 10       | 7       |      |      |        |
| AD5A      | 10      | 11     | 6    | 10   | 10       | 6       | 5    |      |        |
| U4A       | 9       | 12     | 5    | 8    | 11       | 8       | 7    | 3    |        |

mandates that a species be a monophyletic group with a high degree of genomic similarity that also shares a high order of similarity in many independent phenotypic features [3, 5, 10, 11]. The eight strains collected for comparison to strain CBD 118 are monophyletic (Figure 1) when considering 16S rRNA gene similarity. While recognizing that 16S rRNA sequence lacks resolving power at the level of bacterial species [6, 7, 11, 36], we hypothesized that the PSS types might yet function as exclusionary thresholds, for example, species that shared a PSS₂ type might or might not be the same.
species, but strains with different PSS2 types would not be the same species. While the highest degree of DNA relatedness (93.5% S) was between PSS2b strains P308 and C4T1F3B3 (Figure 2), the hypothesis of an exclusionary threshold was contradicted by ≥70% S between strains of different PSS2 types. However, we suggest that the PSS2 types remain effective tools to search 16S rRNA sequence databases for more strains of >99.7% similarity.

No strain tested at greater than 70%–50% S in pairings to CBD 118 (Table 2) and the three strains most closely-related to CBD 118 (PSS2a) based on % S—P308 (PSS2b), C4T1F3B3 (PSS2b), and IAFILS6 (PSS2c)—can be differentiated by 8, 11 and 12 characters, respectively (Table 5). Strain OSS 25, most closely related to CBD 118 based on 16S rRNA sequence similarity and PSS2a, differs by 9 phenotypic characters as well as having only transitional range % S to CBD 118 and distant linkage based on CFA. It is recommended for *Bacillus* and related genera [6] that the 70% S threshold for species delineation not stand alone in delimiting species but should be supported by other characteristics that differentiate strains of the proposed species from other species. In the application of the taxonomic species definition, phenotype continues to have a salient role in the determination of break-points in genomic data for species circumscription and no single parameter—genomic properties or phenotypic traits—should be given undue prominence [3, 6, 37]. The classification that results from application of the taxonomic species definition should be predictive, establishing determinative properties and therefore cannot be based only on genomic characters [3, 7, 11]. The 70% threshold could be interpreted flexibly [6, 7, 11, 16, 37] and a more relaxed boundary used to circumscribe a genomic grouping of these four strains with CBD 118. The resultant grouping would, however, lack sufficient phenotypic cohesion to be of predictive value and therefore does not justify circumscription as a taxonomic species.

In polyphasic taxonomic studies when the strains and phenotypic characters tested were both sufficiently numerous, the resultant clustering pattern has generally reproduced the genomic grouping [7]. In this instance, the four strains with highest % S to support species circumscription are differentiated by multiple phenotypic, species level discriminators (Tables 2, 3, 5). Strain OSS 25 (PSS2a) paired with
In the phenotype dendrogram (Figure 4), OSS 25 was linked most closely with C4T1F3B3 but not with P308. Strains P308 (PSS2b) and IAFILS6 (PSS2c) share 83% S but are differentiated by CFA profiles and 12 traits. Strains P308 (PSS2b) and C4T1F3B3 (PSS2b) share the strongest DNA relatedness with 94% S and were closely linked based on CFA profiles but can be differentiated by 13 phenotypic characters and failure to be linked in the phenotype dendrogram. At the end of the study, these four strains were subcultured from cryopreserved stock, retested for six differential phenotypic traits and resequenced for the 16S rRNA gene. The resultant sequence for each strain was subjected to BLAST analysis and in each case resulted in a 100% match to the region of overlap with the ~1500 bp previously accessioned into GenBank for the strain. For each strain, the re-evaluation of six phenotypic characters—degree of endospore-driven swelling, colony diameter, hemolysis reaction, growth at 45°C, growth with 7% NaCl, and nitrate reduction—reproduced the results of previous testing shown in Table 4. These results indicate that the authenticity of these strains was maintained though the course of the study. The internal diversity of strains P308, C4T1F3B3, OSS 25 and IAFILS6 confounds delineation in a phenotypically coherent unit and their circumscription as one species accommodating multiple biovars or ecovars does not, in our minds, support a predictive taxonomy. No common ecological or disease state can be cited to justify the nomination of a pragmatic species epithet for these strains. The designation of genomovars, as originally proposed by Ursing et al. [38], applies to two or more genic strain clusters within a phenotypically coherent named species that cannot be phenotypically delimited from other strains of the nomenospecies. With these four strains, the converse is the case—one apparent genomic group of strains with four differential phenotypes. It is possible that each of these four strains is the sole exemplar of a novel species and that cohesive phenotypic clusters await the isolation and robust polyphasic characterization of more sibling strains. On the other hand, MLSA [20] on these and more sibling isolates are not new—see the taxonomic histories of Pseudomonas stutzeri [38] and Acinetobacter [40] to cite just two—whereas these nine Bacillus strains are demonstrably novel and their degrees of relatedness appear to confound the taxonomic species definition. Polyphasic data did not clarify relationships and illuminate coherent clusters among these strains—instead, potentially “transitional” forms were revealed. While acknowledging the current insufficiency of our data set, these strains are reminiscent of Model 9 of Istock et al. [12], “Highly variable partially recombining nonspecies”, in which clusters of strains may be discerned, but transitional strains erase any clear demarcation between clusters. Likewise, these strains may be an example of the “continuum of diversity” suggested to characterize groups in which forces promoting coherence dominate those promoting divergence of populations [13]. More data is required to clarify relationships among these strains—particularly sampling more strains in order to determine the range of variation and whether or not discrete phenotypic clusters exist. Indeed, it is hoped that researchers holding closely related strains recognizable by 100% identity to the PSS2 types will join in collaborating with labs having expertise in recommended methods of Bacillus identification [6] to characterize an expanded number of strains. To this end, the nine Bacillus strains in this study have been deposited in a publicly accessible culture collection.

Authors’ Contribution

K. K. Peak and K. E. Duncan contributed equally to this work.

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