NOTE**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.**L. K. Nakamura,¹ Michael S. Roberts² and Frederick M. Cohan³Author for correspondence: L. K. Nakamura. Tel: +1 309 681 6395. Fax: +1 309 681 6672.
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Earlier phylogenetic studies based on the inferred DNA sequences of the *polC*, *rpoB* and *gyrA* genes suggested that strains of the species *Bacillus subtilis* formed two clusters, indicating the presence two closely related taxa; one contained the laboratory strain 168 and the other the laboratory strain W23. Significant sexual isolation was found between strain 168 and members of the group containing W23, but no sexual isolation was observed between strain 168 and other members of the 168 group. DNA reassociation between the two groups ranged from 58 to 69% and intragroup DNA relatedness ranged from 82 to 100%. Because group 168 strains were highly related to the *B. subtilis* type strain, they were considered to be bona fide members of the species. About 99.5% sequence identity was observed between the 16S rRNA genes of the 168 and W23 groups. Ribitol and anhydrosorbitol were principal cell wall constituents of the W23 but not of the 168 group. These observations revealed two closely related but genetically and phenotypically distinct groups within *B. subtilis* that correspond to two historically important strains. Subspecies distinction is proposed for the 168 and W23 groups, with the names *Bacillus subtilis* subsp. *subtilis* subsp. nov. and *Bacillus subtilis* subsp. *spizizenii* subsp. nov., respectively. The type strain of the former is NRRL NRS-744^T and the latter NRRL B-23049^T.

Keywords: *Bacillus subtilis* subsp. *subtilis* subsp. nov., *Bacillus subtilis* subsp. *spizizenii* subsp. nov.

Wild isolates closely related to *Bacillus subtilis* are phenotypically extremely similar, yet they fall into several distinct groups based on protein-coding gene sequences and sexual isolation (Cohan *et al.*, 1991; Roberts & Cohan, 1995). Two of these groups have been shown to represent previously undiscovered species *Bacillus mojavensis* (Roberts *et al.*, 1994) and *Bacillus vallismortis* (Roberts *et al.*, 1996). The third group (84 strains) is *B. subtilis*, but evidence suggests the presence of two sub-groups within the taxon (Roberts & Cohan, 1995). These two sub-groups show clear differences in their geographical distributions, suggesting that they are ecologically distinct (Roberts & Cohan, 1995). The present study has used DNA reassociation and 16S rRNA gene sequence analyses to clarify the taxonomic relationship of the *B. subtilis* sub-groups.

DNA relatedness was determined for 37 *B. subtilis*-like isolates from several different desert environments. These included 24 *B. subtilis*, five *B. vallismortis* and eight *B. mojavensis* strains. Derivatives of strains 168 (NRRL B-14819) and W23 (NRRL B-14820) were obtained from the *Bacillus* Genetic Stock Center. *B. subtilis* NRRL NRS-744^T, *Bacillus atrophaeus* NRRL NRS-213^T, *Bacillus licheniformis* NRRL NRS-1264^T and *Bacillus amyloliquefaciens* NRRL B-14393^T were provided by the ARS Culture Collection. DNA purification and measurement of reassociation by spectrophotometry were described previously (Nakamura & Swezey, 1983). The equation of De Ley *et al.* (1970) was used to calculate the levels of DNA reassociation. For 16S rRNA gene sequencing of NRRL B-23049^T, a DNA fragment of the gene from positions 27–1492 (all positional designations correspond to the *Escherichia*

coli sequence) was amplified by PCR using purified DNA and a primer combination of 27f and 1492r (Lane, 1991). Amplification products were purified with a GENECLAN II kit (Bio 101) and were sequenced with a Prism ABI dideoxy terminator cycle sequencing kit manufactured by Applied Biosystems. The protocols used were those recommended by the manufacturer. Sequences were determined with the automated Applied Biosystems model 377 DNA sequencer. The primers used for sequencing were described previously (Nakamura, 1996). A pairwise comparison was made of the aligned sequences of *B. subtilis* type strain (sequence X60646 obtained from GenBank) and NRRL B-23049^T, a W23-related strain from Tunisia. The 16S rRNA sequence identity between NRRL B-23049^T and *B. subtilis* type strain was 99.8%. Base substitutions of C for G, T for C and T for G occurred in the NRRL B-23049^T sequence at positions 183, 261 and 1128, respectively; C insertions were also noted at positions 856 and 929 and a CCC insertion at position 1022.

For cell wall analysis, organisms were grown in 100 ml TGY broth (Haynes *et al.*, 1955) for 18–20 h at 28 °C with shaking at 200 r.p.m. The cells were harvested by centrifugation at 4 °C and washed once with sterile physiological saline. The procedure of Cummins & Johnson (1971) was used to disrupt cells and to purify the cell walls obtained. Approximately 50–100 mg (wet weight) of cell wall preparation was hydrolysed with 0.75 ml 2 M trifluoroacetic acid by heating for 1 h at 120 °C. The hydrolysate was passed through an anion-exchange column (Bio-Rad AX-8, acetate form) and then dried under vacuum. Peracetylated aldononitrile derivatives of the hydrolysed cell walls were prepared by the method of Lance & Jones (1967). The products were analysed by capillary GC–MS. Sample components were resolved on a 25 m × 0.20 mm (internal diameter) column coated with cross-linked methyl-silicone (0.11 µm film thickness) in a Perkin Elmer Sigma 3b gas chromatograph equipped with a flame-ionization detector. The column temperature was held at 100 °C for 3 min and then raised at 7 °C min⁻¹ to 185 °C and held for 10 min. Mass-spectrometric analysis was carried out with a Hewlett Packard 5970 mass-selective detector. Ribitol and anhydrosorbitol (a hydrolysis product of the polyribitolphosphate moiety of ribitol teichoic acid) were identified by retention time and/or by mass-spectrophotometric analyses.

Roberts & Cohan (1995) studied the phylogenetic relationship of *B. subtilis* and related organisms on the basis of the inferred DNA sequences of protein-coding genes (*polC*, *rpoB* and *gyrA*). Their results showed that the *B. subtilis* strains clustered into a clade separate from those formed by *B. vallismortis*, *B. mojavensis*, *B. atrophaeus*, *B. amyloliquefaciens* and *B. licheniformis*. Moreover, the *B. subtilis* strains segregated into two subclades, one encompassing strain 168 and the other W23. These were designated the 168 group and the W23 group, respectively.

In several studies (Cohan *et al.*, 1991; Roberts &

Cohan, 1993; Roberts *et al.*, 1994, 1996), selected strains of the *B. subtilis* sub-groups, *B. vallismortis* and *B. mojavensis* were analysed for sexual isolation in transformation. Sexual isolation was quantified as the log₁₀ of the ratio of the homogamic transformation frequency (the frequency at which a recipient was transformed by its own mutant's DNA) to the heterogamic transformation frequency (the frequency at which the recipient was transformed by another strain's mutant). Strain 168 was sexually isolated (as recipient) from strains of *B. vallismortis* and *B. mojavensis*, with sexual isolation values of 0.20 ± 0.05 and 0.63 ± 0.03, respectively. Similarly, W23-group strains were mildly sexually isolated from strain 168, with an isolation value of 0.19 ± 0.03. In contrast, strains within the 168 group showed no sexual isolation from strain 168, with an isolation value of -0.01 ± 0.03. Sexual isolation is caused by sequence divergence between groups at the locus being transformed (Roberts & Cohan, 1993; Zawadzki *et al.*, 1995; Majewski & Cohan, 1998).

The high intragroup DNA relatedness of 82–100% for each group (Table 1) indicated the homogeneity within the 168 group and within the W23 group. It was noteworthy that the type strain of *B. subtilis*, NRRL NRS-744^T, was highly related to the 168 group. In contrast, intergroup relatedness ranging from 58 to 68% indicated reduced genetic relatedness, which was already suggested by the protein-coding-gene-based phylogeny and the sexual isolation analyses. The distant relationship of the *B. subtilis* sub-groups to *B. mojavensis*, *B. vallismortis*, *B. atrophaeus*, *B. amyloliquefaciens* and *B. licheniformis* was reaffirmed by low DNA relatedness values ranging from 13 to 39%. High identity (> 99.5%) was observed among 16S rRNA gene sequences of W23 group strain, NRRL B-23049^T (GenBank accession number AF074970) and those of *B. subtilis sensu stricto*, which includes strain 168. The virtually identical sequences indicated a close genetic relationship of these organisms.

The W23 and 168 group strains are identical for most phenotypic characteristics. However, several researchers have shown that the cell wall chemistry of the W23 strain and of the type and 168 strains were different; the cell wall of the former contained ribitol and glycerol teichoic acids and that of the latter only glycerol teichoic acid (Burger & Glaser, 1964; Chin *et al.*, 1966; Fox *et al.*, 1998). Fox *et al.* (1998) noted that the proportions of glycerol and ribitol plus anhydrosorbitol in W23 whole-cell hydrolysates fluctuated with the phosphate concentration of the growth medium. While virtually undetectable in the absence of phosphate, the ribitol plus anhydrosorbitol level increased with increases in phosphate concentration of the medium up to 10.5 mM. In the present study, because ribitol and anhydrosorbitol appeared to be the key differentiating factors between the strains, their presence, but not that of glycerol, were determined in the cell wall hydrolysates of the W23- and 168-related strains. The results showed the presence of ribitol and

Table 1. Level of DNA relatedness between *Bacillus subtilis* sub-groups

The reassociation values are the means of two determinations; the maximum difference found between determinations was 6%. The method of De Ley *et al.* (1970) was used to calculate reassociation values. The values in parentheses indicated that, by definition, the reassociation value was 100%.

Strain	% DNA reassociation with DNA from:			
	168 group		W23 group	
	B-14819	B-23074	B-14820	B-23049 ^T
168 Group				
B-14819 (1A2)	(100)	95	63	61
B-14823 (RO-NN-1)	94	97	69	60
B-23066 (RO-I-1)	100	99	59	65
B-23067 (T-89-18)	87	94	66	60
B-23068 (RO-M-1)	93	90	61	64
B-23069 (T-G-2-5)	96	91	59	63
B-23070 (RS-D-2)	92	94	60	62
B-23071 (T-3-A-18)	91	89	63	67
B-23073 (T-89-14)	88	84	63	64
B-23074 (RO-FF-1)	95	(100)	59	61
B-23075 (TF-32)	92	90	65	62
B-23076 (T-89-48)	93	97	67	60
W23 Group				
B-14697 (RO-SS-1)	65	67	82	90
B-14820 (2A2)	59	60	(100)	90
B-14822 (RO-E-2)	62	66	90	92
B-23049 ^T (TU-B-10 ^T)	61	61	99	(100)
B-23050 (DV1-E-2)	61	60	96	99
B-23051 (DV7-A-2)	67	69	87	100
B-23052 (DV7-B-4)	65	68	94	90
B-23053 (RS-B-1)	58	66	95	97
B-23054 (DV1-B-1)	61	62	87	98
B-23055 (RO-E-2)	63	64	93	90
B-23057 (DV6-C-4)	62	67	100	97
B-23060 (TU-E-6)	66	63	99	100
B-23064 (RO-PP-2)	60	62	97	90
B-23065 (RO-G-4)	65	68	100	95
<i>B. subtilis</i> NRS-744 ^T	92	98	59	63

anhydrosorbitol in the cell walls of nine W23-related strains (NRRL B-14820, NRRL B-23049^T, NRRL B-23050, NRRL B-23051, NRRL B-23052, NRRL B-23054, NRRL B-23055, NRRL B-23064 and NRRL B-23065). No ribitol was detected in cell walls of nine 168-related organisms (NRRL B-14819, NRRL B-23066, NRRL B-23067, NRRL B-14069, NRRL B-23070, NRRL B-23074, NRRL B-23075, NRRL B-23076 and NRRL NRS-744^T).

This and previous studies have revealed that the species *B. subtilis* includes two sub-groups. That these groups were close relatives was demonstrated by the virtually identical sequences of their 16S rRNA genes. Stackebrandt & Goebel (1994) have pointed out that high (> 99%) 16S sequence identity does not necessarily indicate conspecificity of organisms. Although

the separation of the two *B. subtilis* sub-groups on the basis of phylogenetic analyses of protein-coding genes was not strongly supported by bootstrap values, the sexual isolation and DNA-relatedness data stressed that these two groups were more than variant strains of the species and represented two genetically distinct entities. The 60–70% DNA relatedness between the two sub-groups suggested a subspecies relationship for the two groups (Johnson, 1973).

Interestingly, our study showed that the historical strain 168 clustered with the group containing the type strain, and W23 with the second group. Seki *et al.* (1978) had also noted moderate DNA relatedness between 168 and W23. Hence, the historically important genetic strains were more than species varieties but were strains of two genetically distinct entities. Fox

et al. (1998) and others have shown differences in the cell wall structures of W23 and 168; the former contains ribitol teichoic acid and the latter glycerol teichoic acid. In the present study, examination of 18 representative strains showed the presence of ribitol in the cell wall of the W23 group but not in the 168 group. On the basis of the DNA relatedness, differences in a key cell wall component and continuing interest in evolutionary genetic studies (Roberts & Cohan, 1993, 1995; Zawadzki *et al.*, 1995; Majewski & Cohan, 1998) engendered by the divergence between the 168 and W23 sub-groups, we propose that members of the W23 group are strains of a subspecies of *B. subtilis*, *Bacillus subtilis* subsp. *spizizenii* subsp. nov. This proposal automatically creates *Bacillus subtilis* subsp. *subtilis* subsp. nov.

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

Bacillus subtilis subsp. *subtilis* (sub'ti.lis. L. adj. *subtilis* slender).

Motile rods, measuring 0.5 µm by 2–3 µm and occurring singly or in short chains. Cylindrical spores are formed centrally or paracentrally in non-swollen sporangia. Strictly aerobic. Catalase formed. Growth occurs from 15–20 °C to 45–55 °C. Optimum growth temperature ranges from 28 to 30 °C. Grows in 7% (w/v) NaCl and at pH 5–6. Growth in the presence of 0.001% (w/v) lysozyme is variable. Produces acetyl-methylcarbinol (Voges–Proskauer test); pH in Voges–Proskauer broth 5.5–5.7. Indole is not produced. Hydrolyses starch and casein; tyrosine and phenylalanine are not decomposed. Nitrate is reduced to nitrite. Citrate is utilized; propionate is not utilized. D-Glucose, D-xylose, L-arabinose, mannitol and trehalose are fermented. Ribitol is not a cell wall constituent. Strains are not sexually isolated from strain 168. The type strain, NRRL NRS-744^T, is deposited at the ARS Culture Collection.

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

Bacillus subtilis subsp. *spizizenii* (spi.zi.ze'ni.i. L. gen. n. *spizizenii* named after the American bacteriologist J. Spizizen, whose seminal studies of *B. subtilis* made this species a model organism of bacterial genetics).

The morphological, physiological and biochemical characteristics are the same as those of *Bacillus subtilis* subsp. *subtilis*. DNA relatedness to *B. subtilis* subsp. *subtilis* ranges from 58 to 68%. Strains are moderately sexually isolated from strain 168. Phylogeny based on inferred sequences of *gyrA*, *polC* and *rpoB* separates this subspecies from *B. subtilis* subsp. *subtilis*. Ribitol is a principal cell wall constituent. The type strain, NRRL B-23049^T, was isolated from a Tunisian desert; it is deposited in the ARS Culture Collection.

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