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Allelic Diversity and Population Structure of *Bacillus sphaericus* as Revealed by Multilocus Sequence Typing▽†

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The genetic diversity of 35 *Bacillus sphaericus* strains was analyzed by a newly developed multilocus sequence typing (MLST) scheme, toxin gene pool survey, and mosquito bioassay. The results demonstrated that strains assigned to the same sequence type (ST) had the same occurrence of toxin genes. Further sequence analysis revealed that toxic strains presented a nearly clonal population structure, whereas nontoxic strains had a high level of heterogeneity and were significantly distinct from toxic strains.

Bacillus sphaericus is an aerobic, mesophilic, spore-forming, and Gram-positive bacterium that is commonly isolated from soil. Some strains are toxic toward mosquito larvae and have been widely used as biocontrol agents for disease-transmitting mosquitoes (18). The larvicidal activity of *B. sphaericus* mainly originates from the binary toxins (Bin proteins), which are produced during sporulation and form the major toxic components in commercial *B. sphaericus* strains. Other identified toxin proteins, such as Mtx1, Mtx2, and Mtx3, are produced during vegetative growth but play a minor role in toxicity because of their low production and quick degradation by *B. sphaericus* proteinases (23). Recently, a new two-component set of Cry toxins, Cry48Aa and Cry49Aa, were characterized in *B. sphaericus* strain IAB59, and a purified equimolar mixture of both proteins exhibited high target specificity against *Culex quinquefasciatus* (10, 11).

The evolutionary model and systematic classification of *B. sphaericus* is a continual source of debate. Several typing methods, including DNA homology analysis (13), serotyping (2), multilocus enzyme electrophoresis (MLEE) (25), 16S rRNA gene analysis (16, 17), and mosquitocidal toxin gene detection by PCR (8), have been used for the identification of toxic *B. sphaericus*. Based on the results of DNA-DNA hybridization experiments, isolates of *B. sphaericus* can be divided into five DNA groups, and mosquitocidal *B. sphaericus* isolates were all found within group IIA (13). Serotyping revealed that pathogenic *B. sphaericus* strains can be grouped into 9 (H_1, H_2, H_3, H_5, H_6, H_9, H_{25}, H_{26}, and H_{48}) of 49 serotypes (2). Furthermore, the 16S rRNA gene sequences of toxic strains were essentially identical. Likewise, a PCR-based method for classification according to the toxin genes provides very limited information and is insufficient to distinguish *B. sphaericus* as a single species. Thus, the evolutionary relationship between toxic and nontoxic strains has yet to be clearly established. In order to characterize the isolates and explore the population structure of *B. sphaericus*, a multilocus sequence typing (MLST) scheme was employed for the analysis of 35 *B. sphaericus* strains for the first time (Table 1) by sequencing six chromosomally encoded housekeeping genes, *adk* (adenylate kinase), *ccpA* (catabolite control protein A), *pycA* (pyruvate carboxylase), *glyA* (serine hydroxylmethyl transferase), *glcK* (glucose 6-phosphate kinase), and *glpF* (glycerol uptake facilitator protein).

The six MLST gene fragments of 35 strains were amplified and sequenced using primers designed based on the nucleotide sequences of these loci in *B. sphaericus* strain C3-41 (GenBank accession number NC_010382) (see Table S1 in the supplemental material). All the nucleotide sequences will be submitted to the MLST database (http://www.mlst.net/). Data are available from the authors on request. The sequences obtained for each gene were aligned without indels. Each unique sequence was assigned an arbitrary allele number, and the combination of allele numbers for all six loci of a given isolate was then assigned an arbitrary sequence type (ST). Descriptive analysis of the genetic variability at MLST loci was performed with the DNAsp package, version 5.10.00 (19). As shown in Table 2, the MLST gene fragments varied in length from 414 bp (*glpF*) to 654 bp (*glcK*), and the average GC contents of different gene fragments ranged from 37.4% (*glcK*) to 42.0% (*pycA*). The ratio of nonsynonymous to synonymous mutations (K_a/K_s) with a Jukes-Cantor correction was much less than one for all loci, from 0.0185 (*ccpA*) to 0.1196 (*glcK*), indicating the absence of a strong positive selection pressure and the suitability of these loci for genetic population studies. The genetic diversity of the six loci ranged from $\pi = 0.0249$ (*adk*) to $\pi = 0.0715$ (*glcK*), with the number of alleles ranging from 8 (*adk*) to 12 (*glcK*). The average number of alleles per locus (9.5) was higher than that observed by MLEE (4.0) (25). Altogether, 17 unique allelic profiles, or sequence types (STs), which were numbered sequentially, were observed in the 35 isolates (Table 1). All toxic strains belonging to the DNA IIA group and involving the nine serotypes could be assigned to 11 STs, indicating that the MLST method possessed a better discriminatory power.

For a better understanding of the relationship of allele diversity between toxic and nontoxic strains, the occurrence of

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TABLE 1. *B. sphaericus* strains used in this study and their characteristics

Strain	Origin	ST	Allelic profile[a]	PCR result for[b]:							Serotype[c]	Toxicity[d]
				binA	*binB*	*mtx1*	*Mtx2*	*Mtx3*	*cry48Aa*	*cry49Aa*		
C3-41	China	1	1, 2, 6, 6, 5, 1	+	+	+	+	+	−	−	H_5	H
2362	USA	1	1, 2, 6, 6, 5, 1	+	+	+	+	+	−	−	H_5	H
1593	Indonesia	1	1, 2, 6, 6, 5, 1	+	+	+	+	+	−	−	H_5	H
2317-2	USA	1	1, 2, 6, 6, 5, 1	+	+	+	+	+	−	−	H_5	H
Bs44-4	China	1	1, 2, 6, 6, 5, 1	+	+	+	+	+	−	−	H_5	H
Bs77-5	China	1	1, 2, 6, 6, 5, 1	+	+	+	+	+	−	−	H_5	H
Bs90-2	China	1	1, 2, 6, 6, 5, 1	+	+	+	+	+	−	−	H_5	M
Bs91-5	China	1	1, 2, 6, 6, 5, 1	+	+	+	+	+	−	−	H_5	H
Bs150-3	China	1	1, 2, 6, 6, 5, 1	+	+	+	+	+	−	−	H_5	M
Bs356-11	China	1	1, 2, 6, 6, 5, 1	+	+	+	+	+	−	−	H_5	H
Bs436-8	China	1	1, 2, 6, 6, 5, 1	+	+	+	+	+	−	−	H_5	H
S-35	China	1	1, 2, 6, 6, 5, 1	+	+	+	+	+	−	−	H_5	H
S-70	China	1	1, 2, 6, 6, 5, 1	+	+	+	+	+	−	−	H_5	H
S-128	China	1	1, 2, 6, 6, 5, 1	+	+	+	+	+	−	−	H_5	H
47-6b	China	2	1, 6, 3, 7, 10, 4	+	+	+	+	+	+	+	H_6	H
IAB59	Ghana	2	1, 6, 3, 7, 10, 4	+	+	+	+	+	+	+	H_6	H
IAB763	Ghana	2	1, 6, 3, 7, 10, 4	+	+	+	+	+	+	+	H_6	H
IAB769	Ghana	2	1, 6, 3, 7, 10, 4	+	+	+	+	+	+	+	H_6	H
Bs208-6	China	3	4, 8, 9, 1, 8, 7	−	−	−	−	−	−	−	ND	N
Bs225-2	China	3	4, 8, 9, 1, 8, 7	−	−	−	−	−	−	−	ND	N
2297	USA	4	6, 6, 6, 7, 2, 11	+	+	+	+	+	−	−	H_{25}	H
IAB872	Ghana	5	1, 2, 6, 7, 4, 2	+	+	+	+	+	−	−	H_{48}	H
IAB881	Ghana	6	1, 6, 6, 6, 11, 1	+	+	−	−	+	−	−	H_3	H
Bs117-3	China	6	1, 6, 6, 6, 11, 1	+	+	−	−	+	−	−	H_5	M
NRS1693	USA	7	7, 4, 2, 2, 7, 6	−	−	−	+	−	−	−	H_2	N
2314-2	Thailand	8	2, 5, 4, 4, 3, 5	−	−	−	−	−	−	−	H_9	N
LP1-G	Singapore	9	1, 2, 6, 3, 6, 8	+	+	−	−	+	+	+	H_3	H
Bs-197	India	10	1, 6, 1, 7, 11, 4	+	+	+	+	+	−	−	H_1	M
2173	USA	11	1, 2, 6, 7, 11, 3	−	−	−	−	+	+	+	H_{26}	M
Cok31	Turkey	12	6, 2, 6, 7, 11, 1	−	−	+	+	+	−	−	H_9	L
SSII-1	India	13	6, 6, 6, 7, 11, 4	−	−	+	+	+	−	−	H_2	L
Kellen Q	USA	14	3, 2, 6, 3, 10, 2	−	−	+	+	+	−	−	H_1	L
Dak614	France	15	8, 3, 7, 9, 9, 8	−	−	−	−	−	−	−	H_4	N
SO7001	France	16	7, 1, 5, 8, 12, 9	−	−	−	+	−	−	−	ND	N
Bs227-3	China	17	5, 7, 8, 5, 1, 10	−	−	−	−	−	−	−	ND	N

[a] The number refers to the allele number, and allelic profile is given in the following order: *adk*, *ccpA*, *pycA*, *glyA*, *glcK*, *glpF*.
[b] +, present; −, absent.
[c] Serotype is as determined by de Barjac et al. (2). ND, not determined.
[d] Effect on larvae of *Culex quinquefasciatus*. H, high toxicity (50% lethal concentration [LC_{50}] ≤ 1 ng of fermentation broth per ml); M, moderate toxicity (LC_{50} ≈ 100 ng of fermentation broth per ml); N, nontoxic; L, low toxicity (LC_{50} ≥ 1 μg of fermentation broth per ml). Note that the number of nontoxic strains used for analysis is much less than the number of toxic strains. This is because although different degenerate PCR strategies have been tried (data not shown), for most of the nontoxic strains, the products of the six loci for MLST are not available simultaneously due to the genetic diversity.

toxin genes and mosquitocidal activities against *C. quinquefasciatus* larvae were surveyed. PCR assays with seven toxin genes, *binA*, *binB*, *mtx1*, *mtx2*, *mtx3*, *cry48Aa*, and *cry49Aa*, were performed for the 35 strains (see primers in Table S1 in the supplemental material). The bioassays were carried out with the method proposed by the World Health Organization (24), and four toxicity levels, high, moderate, low, and nontoxicity, were determined. It was observed that highly or moderately

TABLE 2. Sequence variation at six loci

Locus	Target[a]	Fragment (gene)[b] size (bp)	No. of alleles	Avg GC content (%)	K_s[c]	K_a[d]	K_a/K_s	π[e]	No. (%) of polymorphic sites		
									Total	Toxic	Nontoxic
adk	Bsph_4593	531 (654)	8	39.5	0.1013	0.0075	0.0740	0.0249	77 (14.5)	2 (0.4)	72 (13.6)
ccpA	Bsph_4200	579 (1,005)	8	40.4	0.3192	0.0059	0.0185	0.0428	132 (22.8)	1 (0.2)	128 (21.6)
pycA	Bsph_1392	534 (3,435)	9	42.0	0.3608	0.0086	0.0238	0.0480	133 (24.9)	2 (0.4)	128 (24.0)
glyA	Bsph_1005	552 (1,242)	9	40.2	0.2419	0.0054	0.0223	0.0414	117 (21.2)	3 (0.6)	105 (19.0)
glcK	Bsph_1259	654 (909)	12	37.4	0.3872	0.0463	0.1196	0.0715	250 (38.2)	6 (0.9)	235 (36.0)
glpF	Bsph_0241	414 (804)	11	39.0	0.2488	0.0164	0.0659	0.0496	107 (25.8)	7 (1.7)[f]	104 (25.1)

[a] Based on *B. sphaericus* C3-41 genome (GenBank accession number NC_010382; Hu et al. [6]).
[b] Values in parentheses represent the total length of the gene.
[c] K_s, number of synonymous changes per synonymous site.
[d] K_a, number of nonsynonymous changes per nonsynonymous site.
[e] π, nucleotide diversity per site.
[f] Toxic strain LP1-G shared an identical *glpF* gene fragment with nontoxic strain Dak614 and, therefore, was not included in the analysis.

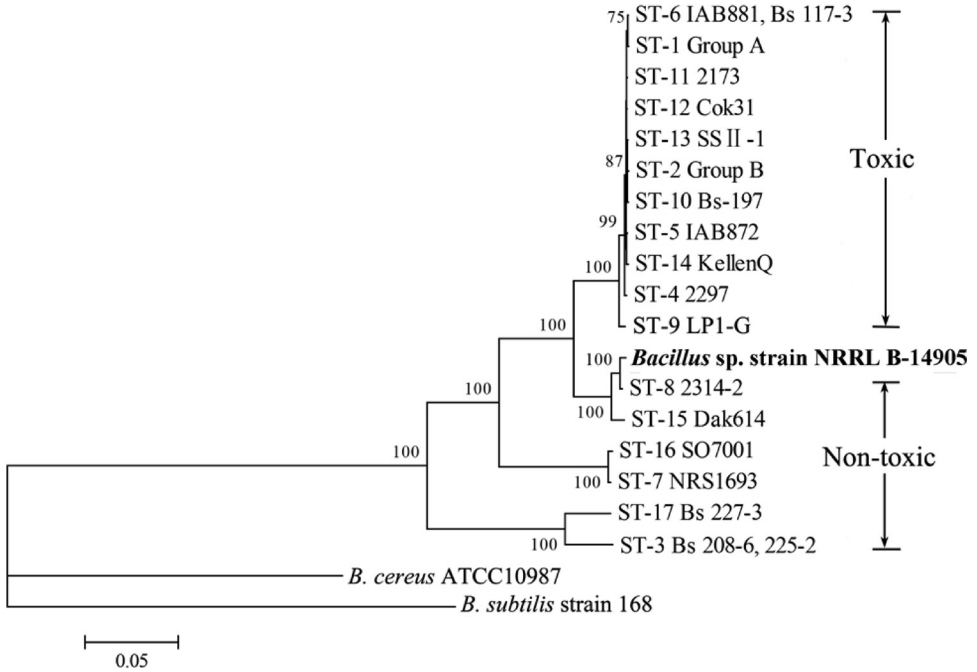

FIG. 1. NJ phylogenetic tree constructed from the concatenated sequences of six loci of *B. sphaericus* and three related *Bacillus* species. One definite toxic-specific cluster (Toxic) was identified. Group A and Group B refer to strains of the indicated ST listed in Table 1. *B. cereus* ATCC 10987 and *B. subtilis* strain 168 were used as outgroups. Bootstrap values above 70% are indicated. All branch lengths are drawn to scale.

toxic *B. sphaericus* strains contained at least one of the two pairs of genes *binA-binB* and *cry48Aa-cry49Aa*, whereas low-toxicity strains possessed only three *mtx* genes and nontoxic strains contained only the *mtx2* gene or no toxin genes at all. Remarkably, the strains assigned to the same ST harbored the same toxin genes (Table 1).

Using MEGA version 4.0 software (22), a neighbor-joining (NJ) phylogenetic tree based on the Kimura two-parameter model was constructed for the 3,264-bp concatenated sequences of six loci of *B. sphaericus* isolates (Fig. 1). The nucleotide sequences of the equivalent loci of *B. cereus* ATCC 10987, *B. subtilis* strain 168, and *Bacillus* sp. strain NRRL B-14905 (GenBank accession numbers NC_003909, NC_000964, and NZ_AAXV00000000) were included for comparison. The phylogenetic tree revealed that all toxic *B. sphaericus* strains were most closely related and fell into one distinct tight cluster despite their worldwide distribution, whereas nontoxic strains were more diverse and were grouped into several other clusters. NJ trees were also constructed individually for all six loci to examine the degrees of congruence between trees (see Fig. S1 in the supplemental material). Five trees based on *adk*, *ccpA*, *pycA*, *glyA*, and *glcK* showed similar topologies, while the tree derived from *glpF* showed minor differences. Specifically, ST9 (strain LP1-G) shared an identical *glpF* gene fragment with ST15 (strain Dak614). Previous studies showed that *B. sphaericus* and *Bacillus* sp. strain NRRL B-14905 have similar genotypic and phenotypic characteristics, including the inability to metabolize polysaccharides, suggesting that they should be reclassified into one genus (1, 20). In this study, the phylogenetic analysis revealed that *B. sphaericus* strains were closer to *Bacillus* sp. strain NRRL B-14905 than to *B. cereus* ATCC 10987 and *Bacillus subtilis* strain 168, which were positioned as outgroups on the phylogenetic tree (Fig. 1; also see Fig. S1 in the supplemental material).

To examine the effect of recombination on diversification of *B. sphaericus* population, split decomposition analysis with SplitsTree version 4.10 software (7) was performed. When the concatenated sequences were investigated, the split graph showed a networklike structure (see Fig. S2 in the supplemental material), and evidence of significant recombination was found ($P = 0.044$). However, the evidence of recombination disappeared when ST9 (strain LP1-G) was removed from the analysis. This could be explained by the investigation of horizontal gene transfer (HGT) for the concatenated sequences using the RDP3Beta40 package (15), which revealed only one HGT event ($P \leq 1.518 \times 10^{-5}$). Specifically, the *glpF* gene fragment of ST9 (strain LP1-G) was involved in the HGT event, consistent with the incongruence between the *glpF* gene tree and the other five gene trees. Evidence for recombination was also tested by determining the standardized index of association, I^{S_A} (5), with the use of SRART version 2.0 software (9). The I^{S_A} values were significantly higher than 0 ($I^{S_A} = 0.526$, $P < 0.001$) for 35 *B. sphaericus* strains, indicating that alleles were in strong linkage disequilibrium. However, when taking STs as the unit, which excluded the pandemic isolates from the datasets, a lower value was observed ($I^{S_A} = 0.241$, $P < 0.001$), indicating the presence of a limited amount of recombination based on the nucleotide differences which did not completely destroy the linkage between alleles. This observation was in accordance with the results of RDP analysis, although the latter detected the HGT event caused by the migration of nucleotide fragments. Nevertheless, the split graphs should be interpreted carefully because of the low number of alleles

obtained. Furthermore, the ClonalFrame version 1.1 package (4) was employed to evaluate the influences of recombination relative to those of mutation on sequence diversification: two complementary measures of recombination rate were calculated, ρ/θ (the relative frequencies of occurrence of recombination and mutation) and r/m (the relative effects of recombination and mutation). Five independent ClonalFrame runs were performed for 17 unique STs, with each consisting of 100,000 burn-in iterations and 200,000 sampling iterations, and the samples from five independent runs were then concatenated for further analysis. We found a mean value for ρ (the recombination rate times two) of 1.152. The frequency of occurrence of recombination relative to that of mutation (ρ/θ) was 0.007, and the effect of recombination relative to that of mutation (r/m) was 0.113 (see Table S2 in the supplemental material). This estimate of the frequency and effect of recombination indicated that recombination was relatively rare compared to the rates for most species, such as the *B. cereus* group (21), *Campylobacter coli* (14), and *Listeria monocytogenes* (3), and that mutations were largely responsible for the generation of sequence diversity in *B. sphaericus*. Overall, only a limited amount of recombination in the evolution of *B. sphaericus* might happen based on the analysis by several methods.

In conclusion, although the presence of a certain degree of recombination cannot be excluded, our results demonstrated that there was a nearly clonal population structure in toxic strains, as opposed to the otherwise broad diversity among the nontoxic *B. sphaericus* isolates. One question is, why is there relatively little sequence diversity in toxic strains compared to that in nontoxic *B. sphaericus* strains? The results of previous studies indicate that *Bacillus anthracis* is a recently emerging pathogen compared to *Bacillus cereus*. It has been suggested that *B. anthracis* has evolved as a monomorphic lineage because of strict coevolution involving both the chromosome and the two large plasmids pXO1 and pXO2 following acquisition of the virulence plasmids by HGT (12). Similarly, compared to mosquitocidal *B. sphaericus*, nontoxic *B. sphaericus* is likely to be a more ancient lineage and has undergone an evolutionary radiation in response to different selection pressures, in which sequence variation is enhanced by limited recombination. Until the latter period of evolution, some strains have acquired the toxic genes by coincident HGT events, such as *binA-binB*, which seemed to be spread by a transposon according to the bilateral sequence analysis from Hu et al. (6). Furthermore, mosquito-bacterium interactions or the coevolution of *B. sphaericus* with other bacteria (e.g., *Bacillus thuringiensis*) in mosquito breeding habitats may provide more clues for the evolution or acquisition of toxin genes. The combination of a *B. sphaericus* Bin-like protein (Cry49Aa) and a *B. thuringiensis* 3-domain Cry protein (Cry48Aa) in some *B. sphaericus* strains may provide an important argument for this speculation. Once they obtain the ability for toxicity to mosquitoes, these toxic strains expand quickly and form an independent population structure, in which sequence variation is mainly introduced by point mutations. However, more extensive studies are necessary to accurately assess the population structure of *B. sphaericus*, especially for nontoxic strains.

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