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Distribution and Characterization of Mosquitocidal Toxin Genes in Some Strains of *Bacillus sphaericus*

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The binary toxin of *Bacillus sphaericus* strains forms a crystal in sporulating cells, while the mosquitocidal toxin is located in the cytoplasm of vegetative cells. The distribution of binary toxin (*btx*) and mosquitocidal toxin (*mtx*) genes in 53 strains of *B. sphaericus* was determined by hybridization of specific gene probes to chromosomal DNA in Southern blots. *btx* genes were found in all strains of serotype 5a5b examined and in some strains of serotypes 1a, 3, 6, 25, and 48, while *mtx* genes were detected in strains of serotypes 1a, 2a2b, 5a5b, 6, 9a9c, 25, and 48. Serotype 26a26b strains lacked both toxin genes, as did some strains of serotypes 2a2b, 3, 6, and 48. Partial DNA sequences of *btx* genes from five strains, together with published sequences, revealed four types of toxin among mosquitocidal *B. sphaericus* strains. Most of the 42-kDa toxin gene of *btx* was identical in strains from serotypes 1a, 3, 6, and 48, and the gene is here classified as a type 1 *btx* gene. A serotype 3 strain isolated in Singapore possessed a unique 42-kDa toxin gene, here designated type 4; while the *btx* genes from strains of serotypes 5a5b and 25 are referred to as types 2 and 3, respectively.

Some strains of *Bacillus sphaericus* are toxic toward mosquito larvae and can be used as biological control agents of these important vectors of disease (for reviews, see references 8 and 9). These entomocidal *B. sphaericus* strains synthesize two types of toxin. The binary toxin (Btx) accumulates during the early stages of sporulation and forms a small crystal in the mother cell. The crystal comprises equimolar amounts of two proteins of 41.9 and 51.4 kDa, often referred to as the 42- and 51-kDa toxins, respectively (5). Both proteins are needed for larval toxicity. The larger acts as a binding protein, enabling the entry of the 42-kDa protein into the midgut cells of the larval gut (14). Strains which synthesize the binary toxin are referred to as high-toxicity strains because of the acute toxicity conferred by the large amounts of protein in the crystal. Mosquitocidal toxin (Mtx) is unrelated to Btx. It is synthesized during exponential-phase growth and is proteolytically degraded as the cells enter the stationary phase. Mtx has significant homology to ADP-ribosylating-type toxins (16). Many high-toxicity strains synthesize both Mtx and the binary toxin, while others synthesize only the binary toxin (12). Low-toxicity strains synthesize only Mtx or neither toxin (8, 11).

Given the variation in the types of toxins synthesized by mosquitocidal *B. sphaericus* strains, it was important to develop typing systems for strain identification. Flagellar (H) serotyping, similar to that for *B. thuringiensis* strains, has been used to allocate entomocidal strains to nine different serotypes (10), which, because of early confusion about *B. sphaericus* classification, are not in strict numerical order (15). In early studies, toxicity and serotype appeared to correlate; high-toxicity strains were classified largely in serotypes 5a5b, 6, and 25, and low-toxicity strains were classified in serotypes 1a, 2a2b, and 9a9c (8, 10). However, the discovery of high-toxicity strains from Ghana belonging to serotypes 3, 6, and 48 (19) confused the supposed correlation between serotype and toxicity and suggested that the relationships may not be so straightforward. Here we show that with the exception of serotype 5a5b strains, there is little correlation between the serotype and the presence of toxin genes and that the 42-kDa Btx protein of a serotype 3 strain isolated in Singapore (12) has a unique amino acid sequence.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains used in this study are listed in Table 1. Bacteria were grown at 37°C on NYSM, which consisted of nutrient agar or broth supplemented with yeast extract (0.05%) and salts (7×10^{-4} M $CaCl_2$, 5×10^{-5} M $MnCl_2$, 10^{-3} M $MgCl_2$).

DNA manipulations and bioassays. Chromosomal DNA was prepared, digested with *Hin*dIII (for *btx* hybridizations) or *Cla*I (for *mtx* hybridizations), transferred to nylon membranes, and hybridized to probes for the binary and mosquitocidal toxin genes as described previously (3, 11). For sequencing, *btx* DNA was amplified from chromosomal DNA of strains LP1-G, 9002, IAB 881, IAB 872, and PR-1 with primers 1711 forward (5′-CCTATAACTAATCCACT TACGC) and 2710 reverse (5′-CAGTTTTTGTCTCTTTAGAGCC), which are complementary to regions 1690 to 1711 and 2731 to 2710, respectively, of the *btx* gene sequence (4). The PCR conditions involved denaturation at 95°C for 3 min followed by 28 cycles of 93°C for 1 min, 48°C for 1 min, and 72°C for 2 min with *Taq* polymerase (Promega). The amplification product was purified on QIAquick spin columns (Qiagen) and sequenced in an Applied Biosystems 377A DNA-sequencing system with PRISM Ready Reaction DyeDeoxy Terminator cycle-sequencing kits (Applied Biosystems) and the PCR primers.

Strains were bioassayed for toxicity against second-instar larvae of *Anopheles stephensi* as described previously (1).

RESULTS

Distribution of toxin genes. Binary toxin genes were detected in chromosomal DNA from strains from serotypes 1a, 3, 5a5b, 6, 25, and 48 but not in strains from serotypes 2a2b, 9a9c, or 26a26b (Table 1). The genes were located either on 3.5-kb *Hin*dIII fragments (serotype 1a, 5a5b, 6, and 48 strains) or on 4.2-kb *Hin*dIII fragments (serotype 25 strains) as described previously (2, 12). Interestingly, the location of *btx* varied in serotype 3 strains. The gene was found on a 4.2-kb *Hin*dIII fragment in the strains from Singapore and on a 3.5-kb fragment in strain IAB 881 from Ghana.

Mosquitocidal toxin genes were detected in strains from serotypes 1a, 2a2b, 5a5b, 6, 9a9c, 25, and 48 (for examples, see

TABLE 1. Distribution of toxin genes in some strains of *B. sphaericus*

Strain	Origin	Serotype	*btx* gene	*mtx* gene	Source[a]
K	United States	1a	−	+	1
Q	United States	1a	−	+	1
9002	Indonesia	1a	+	+	2
9201	Indonesia	1a	+	+	2
9301	Indonesia	1a	+	+	2
BS-197	Indonesia	1a	+	+	2
SSII-1	India	2a2b	−	+	1
1889	Israel	2a2b	−	+	2
1883	Israel	2a2b	−	+	2
4b 1	Nicaragua	2a2b	−	−	2
LP24-4	Singapore	2a2b	−	−	3
LP35-6	Singapore	2a2b	−	−	3
17N	Caledonia	2a2b	−	ND[b]	2
COK 1	United States	2a2b	−	−	2
K 8908	Indonesia	2a2b	−	−	2
BDG2	France	3	−	−	2
SL 42	United States	3	−	−	2
IAB 881	Ghana	3	+	−	3
LP1-G	Singapore	3	+	−	3
LP7-A	Singapore	3	+	−	3
LP12-AS	Singapore	3	+	−	3
LP14-8	Singapore	3	+	−	3
LP20-E	Singapore	3	+	−	3
1593	India	5a5b	+	+	1
1691	El Salvador	5a5b	+	+	2
2013.6	Romania	5a5b	+	+	2
2362	Nigeria	5a5b	+	+	1
2317.3	Thailand	5a5b	+	+	1
2500	Thailand	5a5b	+	+	1
BSE18	Scotland	5a5b	+	+	3
BM1	United States	6	+	+	2
IAB 59	Ghana	6	+	+	1
S06 015	Iraq	6	−	−	2
IAB 481	Ghana	6	+	+	2
IAB 620.1	Ghana	6	+	+	2
IAB 460	Ghana	6	+	+	2
B55	Indonesia	6	−	−	2
COK 31	Turkey	9a9c	−	+	2
COK 34	Turkey	9a9c	−	+	2
2297	Sri Lanka	25	+	+	1
2627	Israel	25	+	−	2
IMR 6	Malaysia	25	+	+	2
1602	Canada	25	+	+	2
2173	India	26a26b	−	−	1
2315	Thailand	26a26b	−	−	1
2377	Indonesia	26a26b	−	−	2
LB 29	Czech Republic	26a26b	−	−	2
BM2	United States	26a26b	−	−	2
S26 009	United States	26a26b	−	−	2
18W1.2	Iraq	26a26b	−	−	2
IMR 66.1	Malaysia	48	−	−	2
IAB 872	Ghana	48	+	+	2
Pr-1	Scotland	48[c]	+	+	3

[a] Sources of strains; 1, A. A. Yousten, Department of Biology, Virginia State Polytechnic Institute and University, Blacksburg, Va.; 2, Collection of *B. thuringiensis* and *B. sphaericus*, Institut Pasteur, Paris; 3, this laboratory.

[b] ND, not done.

[c] Allocation based on pulsed-field gel electrophoresis of *Sma*I-digested chromosomal DNA (unpublished results).

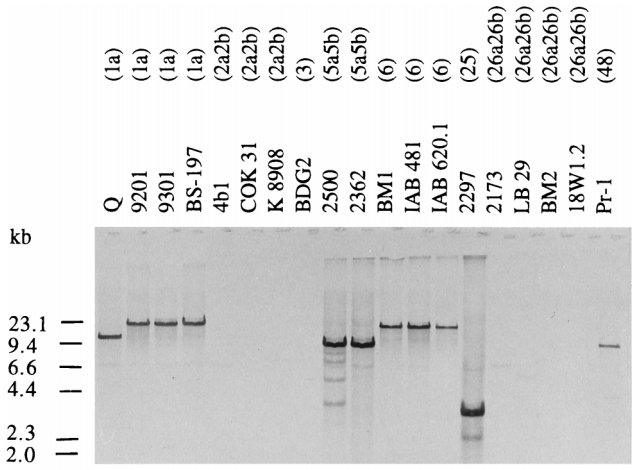

FIG. 1. Southern blot hybridization of *Cla*I-digested chromosomal DNA from representative strains of *B. sphaericus* (H serotypes in brackets) with a probe amplified from the *mtx* gene of *B. sphaericus* SSII-1.

Fig. 1). These were located on variably sized *Cla*I DNA fragments, whose sizes can be roughly estimated in conjunction with the earlier studies of Thanabalu et al. (16). In serotype 1a strains, *mtx* was located either on fragments of about 11 kb (strains K and Q from the United States) or on a larger one (strains from Indonesia) which migrates to the same extent as the 13-kb *mtx* fragments from serotype 6 strains (16). *mtx* was located on fragments of about 10 kb in strains from serotypes 5a5b and 48 and a smaller, 3-kb fragment in strain 2297 (serotype 25). The more weakly hybridizing bands in Fig. 1, particularly evident in lane 9 (strain 2500), may originate from a slightly impure probe, as is often found with PCR-generated probes, or may represent hybridization to other *mtx* genes (13, 17). The genome in *B. sphaericus* is obviously more heterogeneous in the area of the *mtx* genes than in the *btx* regions, and there is some variation within serotypes, suggesting that the serotype is not a clear indicator of the genomic structure of this species.

Overall, there was little correlation between the serotype and toxin gene distribution except for serotype 5a5b strains, which invariably contained both toxin genes (Table 1). However, two serotype 1a strains from the United States contained the *mtx* gene only, while four from Indonesia contained both the *btx* and *mtx* genes. Serotype 2a2b strains contained either *mtx* without the binary toxin gene or neither gene. Serotype 3 strains from Singapore contained only the *btx* gene, as did IAB 881 from Ghana, but other strains had neither gene. Similarly, serotype 6 strains from Ghana contained both toxin genes, but those from Indonesia, Iraq, and Brazil contained neither. An atypical strain of serotype 25 from Israel (strain 2627) contained only *btx*, but all other strains examined from serotype 25 contained both toxin genes. Serotype 26a26b strains did not contain either of the toxin genes. The three strains of serotype 48 varied and contained either both of the genes or none.

Partial sequences of binary toxin genes. The sequences of the binary toxin gene operons from serotype 5a5b strains differ from those of the operons from serotypes 6 and 25 largely in the distal region of the 51-kDa gene, in the spacer between the genes, and in the proximal region of the 42-kDa gene (7). The region around position 100 of the 42-kDa protein is particularly important for toxicity and target range (6). We therefore sequenced this portion of the DNA (from nucleotides 1726 to 2709) of strains from serotypes that had not been previously

TABLE 2. Comparison of partial binary toxin gene sequences from some strains of *B. sphaericus*

Position[c]		Binary toxin type[a]							
		Type 1 (9002 [1a], IAB 881 [3], IAB 59 [6],[b] Pr-1 [48] IAB 872 [48])		Type 2 (1593 [5a5b], 2317.3 [5a5b], BSE 18 [5a5b], 2362 [5a5b])[b]		Type 3 (2297 [25])[b]		Type 4 (LP1-G [3])	
N[d]	AA[d]	N	AA	N	AA	N	AA	N	AA
1844		–		–		CT	CT		
1851		C		C		A		A	
1909		T		T		A		A	
1994		T		T		A		A	
2139		C		C		T		T	
2169		T		T		C		C	
2253		C		C		T		T	
2291	93	T	Leu	T	Leu	T	Leu	C	Ser
2308	99	G	Val	G	Val	T	Phe	G	Val
2323 2324	104	G A	Glu	G C	Ala	T C	Ser	T C	Ser
2386	125	C	His	C	His	A	Asn	A	Asn
2412		T		T		C		C	
2417	135	A	Tyr	A	Tyr	T	Phe	T	Phe
2490		A		A		T		T	
2643		C		C		G		G	

[a] Strain (serotype) allocation to toxin types is shown in the respective columns.
[b] Data for these strains from complete published (4, 7) or unpublished sequences.
[c] Based on the published sequences for binary toxin genes (4, 7).
[d] N, nucleotide; AA, amino acid.

examined: 9002 (serotype 1a), LP1-G and IAB 881 (serotype 3), and IAB 872 and Pr-1 (serotype 48). The comparison of these DNA sequences with those of strains from serotypes 5a5b, 6, and 25, which had been determined previously (7), is shown in Table 2. The partial sequences of *btx* genes from strains of serotypes 1a, 6, and 48 were identical. Moreover, one of the serotype 3 strains (IAB 881) shared this sequence. We have labeled this type 1 in Table 2. Four serotype 5a5b strains share a unique sequence (type 2), which differed from type 1 in only one residue in the area sequenced, an A-to-C transversion at position 2324, resulting in a Glu-to-Ala change at position 104 of the 42-kDa protein. The *btx* gene of the strain from Singapore resembled a hybrid of the serotype 5a5b (type 2) and serotype 25a25b (type 3) genes. It contained the 2-bp insertion in the gap between the two genes of the binary toxin typical of strain 2297 (serotype 25a25b) and most of the variable positions favored by strain 2297 except the G at position 2308, which is typical of a type 2 sequence and results in Val rather than Phe at position 99 in the 42-kDa protein. A unique cytosine at position 2291 in the *btx* gene of strain LP1-G (type 4) results in an altered protein sequence with a serine rather than a leucine residue at position 93.

Pathogenicity of toxic strains from serotypes 1a, 3, and 48. Representative bacteria from serotypes 1a, 3, and 48 produced small crystals which were visible under phase-contrast microscopy (not shown), indicating that the *btx* genes in these bacteria were expressed normally. The toxicity against *A. stephensi* larvae was assessed over 2 days with strains 2362 (serotype 5a5b), IAB 59 (serotype 6), and 2297 (serotype 25) as controls, and the results are compared with published data for *Culex quinquefasciatus* (12, 18) in Table 3. All toxin types were less effective against *Anopheles* than *Culex* larvae, and the types 3 and 4 toxins were generally less active than those of types 1 and 2. The only consistent differences between the two pairs of toxins are the residues at positions 125 and 135, indicating that these may be relevant to toxicity.

DISCUSSION

High toxicity to mosquito larvae is generally associated with *B. sphaericus* strains of serotype 5a5b, which are by far the most common mosquitocidal *B. sphaericus* strains isolated (15). However, we show here that high toxicity is by no means limited to this serotype and that binary toxin genes can be detected in six of the nine described serotypes of mosquitocidal *B. sphaericus*, including serotype 1a, which was previously thought to contain only weakly toxic strains. Similarly, the *mtx* gene is not resident in all strains and is absent from strains of several serotypes, notably all those of serotype 3 examined and most of serotype 2a2b. This variation in toxin gene distribution within serotypes casts doubt on the value of the serotyping scheme for predicting toxicity. Strains within serotypes 1a, 2a2b, 3, 6, 25, and 48 all showed heterogeneity of the toxicity genotype to various degrees. Only strains of serotypes 5a5b (highly toxic) and 26a26b (weakly toxic) were consistent in their toxicity patterns. Thus, although serotyping provides a useful framework for strain identification, its reliability as a predictive tool must be treated cautiously.

In characterizing the *btx* genes from several strains, we took account of the considerable conservation among these genes

TABLE 3. Relative toxicities of some strains of *B. sphaericus* to mosquito larvae

Strain	Serotype	*btx* type	LC_{50}[a] (10^3 spores/ml) for:	
			A. stephensi	*C. quinquefasciatus*[b]
IAB 59	6	1	10	3
2362	5a5b	2	10	2
2297	25	3	30	5
LP1-G	3	4	45	14

[a] LC_{50}, 50% lethal concentration.
[b] Data for *C. quinquefasciatus* are taken from references 12 and 16.

(7) and sequenced only the spacer region between the two genes and the proximal region of the 42-kDa toxin gene. From these data, we could initially allocate *btx* genes from strains 9002, IAB 881, Pr-1 and IAB 872, to type 1. Indeed, the full sequence of *btx* from strain IAB 881 has recently been determined (10a) and is identical to that of strain IAB 59, thus confirming this allocation. The *btx* genes were assigned to four individual types, which represent two lineages. One lineage contains types 1 and 2 genes which differ in only seven positions over the entire 3.5 kb encompassing the operon and flanking DNA, while types 3 and 4 are similarly closely related and differ in only two positions over the region sequenced in this study. However, the differences between the lineages (i.e., between types 1 and 3) are more numerous, with 26 differences over the full sequence (7). Moreover, type 1 and 2 genes are located on 3.5-kb fragments of chromosomal DNA, while type 3 and 4 genes reside on larger, 4.2-kb DNA loci (2, 12; also see above). From the activity perspective, type 1 and 2 toxins are more toxic against both *Anopheles* and *Culex* larvae than are type 3 and 4 toxins (Table 3). It seems likely that the *btx* genes diverged into two major lines of descent and that evolutionary change is now proceeding in the two lineages.

The variation in *btx* distribution prompts further evolutionary speculations. Type 1 toxin genes are widely distributed and occur in backgrounds which differ in serotype and, from random amplified polymorphic DNA analysis (20) and multilocus enzyme electrophoresis (21), in genomic composition. Although *btx* is located on the chromosome (2), it can presumably be lost (e.g., serotype 26a26b and others) or gained as a discrete DNA fragment defined by the 3.5- and 4.2-kb *Hin*dIII bands seen in Southern blot hybridizations (2, 12). The likely explanation for the occurrence of the identical type 1 gene in different backgrounds is that lateral transfer of these loci has occurred, and it is relevant that type 1 genes are common in strains of diverse serotype but originating in Ghana (the IAB series of strains in Table 2). Such lateral transfer could also explain the generation of hybrid genes such as the new type 4 *btx*, which could have arisen through recombination between types 1 or 2 and 3 or, alternatively, by convergent evolutionary change. Strains of serotype 5a5b, on the other hand, illustrate an interesting ecological adaption in that the several hundred known strains of this serotype probably represent a successful clone of identical strains that has become established in the absence of significant genetic interchange (15). Future screening programs could usefully concentrate on excluding serotype 5a5b strains and attempt to isolate rare or novel serotypes. The large differences in toxicity associated with small changes in binary toxin gene structure suggest that further variants of the *B. sphaericus* crystal protein with novel mosquito target ranges should be extant.

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