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NOTES

New High-Toxicity Mosquitocidal Strains of *Bacillus sphaericus* Lacking a 100-Kilodalton-Toxin Gene

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Five new high-toxicity mosquitocidal strains of *Bacillus sphaericus* were isolated in Singapore. They all belong to phage group 8 and have binary toxin (51.4- plus 41.9-kDa) genes located on the chromosome but lack a 100-kDa-toxin gene. These strains of *B. sphaericus* constitute a new subgroup, as only two weakly toxic strains in phage group 8 have previously been described and all the known high-toxicity strains have both binary toxin and 100-kDa-toxin genes.

Bacillus sphaericus is a common gram-positive aerobic bacterium with round spores found in soil and water environments (2). Strains toxic to mosquito larvae have been isolated from all over the world, for example, SSII-1 from India, 1593 from Indonesia, 2362 from Nigeria, 2297 from Sri Lanka, and IAB59 from Ghana (2, 7, 15). More than 380 *B. sphaericus* strains which are toxic to the larvae of mosquitoes in three genera (*Culex*, *Aedes*, and *Anopheles*) have been collected in the World Health Organization Collaborating Centre for Entomopathogenic *Bacillus* at the Institut Pasteur, Paris, France (14). *B. sphaericus* strains have been placed in bacteriophage groups 1, 2, 3, 4, and 8 and in flagellar serotypes H1, H2a2b, H3, H5a5b, H6, H25, and H48 (2, 15). On the basis of DNA homologies between 62 strains, *B. sphaericus* strains have been divided into six groups and all the toxic strains fall into the DNA homology subgroup IIA (1, 6, 8).

Toxin genes of *B. sphaericus* encoding a crystal or binary toxin (comprising 51- and 42-kDa proteins) and a 100-kDa toxin have been characterized (2, 13). The analysis of 51- and 42-kDa-toxin genes from several strains of *B. sphaericus* revealed the existence of a family of related sequences, but it is unclear whether these genes are located on the chromosome or on plasmids (2). The binary toxin genes show a very high degree of nucleotide sequence conservation between five highly toxic strains, with little or no variation in the encoded amino acid sequences (3). The weakly expressed 100-kDa-toxin gene of strain SSII-1 was cloned and sequenced, and a homologous gene has been found in strains with high and low toxicities, but the extent of the sequence variation of this gene type is not yet known (13).

Tropical countries are the main breeding places of different mosquito species which transmit serious human diseases such as malaria, filariasis, dengue hemorrhagic fever, and yellow fever. Strains of *B. sphaericus* have been found in Indonesia, Malaysia, and Thailand (11, 12), but there are no reports of the isolation of *B. sphaericus* in Singapore. In this paper, we describe the isolation in Singapore of five novel strains of *B. sphaericus* which are highly toxic to *Culex quinquefasciatus* larvae.

Reference strains. The *B. sphaericus* strains 2297 (phage type 4, serotype H25), 2362 (phage type 3, serotype H5a5b), ATCC 13805, ATCC 7055, and ATCC 14577 were a generous gift from A. A. Yousten, Department of Biology, Virginia Polytechnic Institute and State University. Strain SSII-1 was kindly provided by E. W. Davidson, Department of Zoology, Arizona State University. The nontoxic strain ATCC 13805 was used as a negative control in Southern blot analysis and as food to adjust the cell concentrations in the 50% lethal concentration (LC_{50}) tests.

Soil sample treatment and isolation of strains. Soil and mud samples (1 g) were suspended in sterile water (5 ml) and incubated at 80°C for 15 min (7). Aliquots were plated onto BATS agar plates (17) which were incubated at 30°C for up to 5 days. Water samples (5 ml) were incubated directly at 80°C for 15 min and centrifuged at 7,000 rpm (Sorvall RT6000B centrifuge) for 10 min. The pellets were resuspended in sterile water (0.5 ml) and plated onto BATS agar. Single colonies were picked and purified for further identification.

Characterization of strains. Colonies on BATS agar were examined by phase-contrast microscopy for spore morphology. Strains with round spores were tested further for their toxicity to the second- or third-instar larvae of *C. quinquefasciatus* by using a rough toxicity test: single colonies were streaked onto fresh BATS agar and incubated for 48 h at 30°C; bacteria were taken from the plate and resuspended in 1 ml of water (A_{550} of 1.0 and cell concentration of about 10^{10} per ml). After one washing with water, the cell pellet was resuspended in 1 ml of water and fed to five larvae in a 1.5-ml plastic well tray (ICN Biomedicals). The strain ATCC 13805 was used as a control under the same conditions.

The phage-typing of the isolated strains was performed as described previously (16), and the phages used were kindly provided by A. A. Yousten. Bacteria were grown on NYSM agar (17) at 30°C. Phages 1A, 2, 3, 63, and 64 were propagated on strain SSII-1; phages 12 and SST were propagated on strain Kellen K; and phages 4, 5, and 14 were propagated on strain 1593 (16). For typing, bacteria were seeded into

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TABLE 1. Sporulation rates, toxin gene sizes, and LC_{50}s of Singapore strains and reference strains

Strain	Sporulation (%)	DNA homology group	Toxin gene size(s) (kDa)	LC_{50} at 48 h[a] (cells/ml)
LP1-G	70	IIA	51, 42	1.4×10^4
LP7-A	50	IIA	51, 42	3.2×10^4
LP12-AS	95	IIA	51, 42	9.1×10^3
LP14-8	95	IIA	51, 42	7.7×10^3
LP20-E	70	IIA	51, 42	1.0×10^4
2297[b]	95	IIA	51, 42, 100	5.1×10^3
2362[b]	95	IIA	51, 42, 100	2.0×10^3

[a] Means of three experiments.
[b] Reference strains.

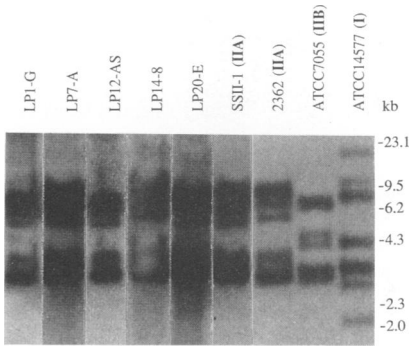

FIG. 1. Autoradiographs of *Hin*dIII digests of DNA from new Singapore strains and reference strains of *B. sphaericus* hybridized with a [α-^{32}P]dCTP-labeled 16S rRNA gene probe. The names of the strains and the DNA homology groups of the reference strains (in parentheses) are shown at the top. DNA markers are indicated on the right.

NYSM soft agar overlays and 20 μl of diluted phage suspension was spotted onto the surface. Plates were incubated at 30°C for 18 h. The production of individual plaques at the same dilution or at a 10-fold more concentrated dilution than that producing individual plaques on the normal phage-propagating strain was noted as a positive result. Patterns of test results were compared with those for standard strains 1593 (phage group 3), SSII-1 (phage group 2), and Kellen K (phage group 1). Typing by phage P4 was done by A. A. Yousten. DNA grouping was performed by examining 16S rRNA gene restriction enzyme digestion patterns (6) with an [α-^{32}P]dCTP-labeled probe.

Bioassay. Individual colonies from BATS agar were inoculated into 100-ml NYSM medium in a 1-liter flask and incubated at 30°C and 150 rpm for 48 h. Sporulation rates of the strains (Table 1) were estimated by plating heated (80°C for 15 min) and unheated cells on Luria broth (with 15% agar) plates and comparing the numbers of colonies. The cells were harvested by centrifugation (6,000 rpm for 10 min) (Sorvall RT6000B centrifuge), washed once with 0.85% saline, and resuspended in water to a final A_{550} of 0.5 (equivalent to approximately 10^8 cells/ml). Further dilutions in triplicate were made to determine the cell concentrations causing 100 and 0% mortality to 10 second- or third-instar larvae of *C. quinquefasciatus* in plastic cups containing 10 ml of cell suspension at 30°C for 48 h. Between 100 and 0% mortality, a further 5 dilutions were made. The final cell concentration in all assays was maintained at 5×10^8 cells/ml by adding cells of nontoxic strain ATCC 13805. After correction for mortality of the controls, the LC_{50} values were calculated by using the log-probit analysis method (9).

Southern blot hybridization. Chromosomal DNA of *B. sphaericus* was isolated as described previously (5). The *Hpa*I-*Xmn*I fragment (2.5 kb) within the 3.5-kb *Hin*dIII fragment of the binary toxin genes of strain 2297 cloned in pUC12 and the *Hpa*I-*Bam*HI fragment (2.6 kb) within the 100-kDa gene of strain SSII-1 cloned in pUC18 were labeled with [^{32}P]dATP by using the nick translation kit of Amersham. The chromosomal DNAs of the strains were digested either with *Hin*dIII (for the binary toxin genes) or *Cla*I (for the 100-kDa-toxin gene). After 1% agarose gel electrophoresis, DNA was blotted onto Hybond-N (Amersham). The hybridization in 10% (wt/vol) dextran sulfate–1 M NaCl–1% (wt/vol) sodium dodecyl sulfate (SDS)–40 mM Tris-HCl (pH 8.0) at 65°C and washing conditions were as described previously (10).

Dot blot hybridization. Overnight bacterial culture (0.1 ml) in L broth was loaded onto Hybond-N which had been soaked in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) before being loaded into a Minifold SRC-96 vacuum blot machine under vacuum (Schleicher & Schuell). The membrane was transferred to a series of Whatman 3MM filter papers saturated with different solutions: 2 mg of lysozyme per ml in Tris-EDTA buffer for 30 min; 10% SDS for 5 min; denaturation buffer (0.5 N NaOH–1.5 M NaCl) for 5 min; neutralization buffer (1.5 M NaCl–0.5 M Tris-HCl, pH 7.4) for 5 min; and 2× SSC for 5 to 10 min. Then, the half-dried membrane was UV illuminated for 2.5 min. The hybridization conditions were the same as for the Southern blots described above.

Five *B. sphaericus* strains were isolated from mud and water samples collected from various locations in Singapore, including drains and reservoirs. Chemical insecticides have rarely been used, and no *B. sphaericus* formulations have ever been used in these locations. The five strains all showed toxicity to the second- and third-instar larvae of *C. quinquefasciatus* in the rough toxicity test. Bacteriophage typing (16) revealed that the five strains served as hosts for phages 1A, 2, 3, 4, 5, 14, 63, and 64 but not for phages 12, P4, and SST (4). Although phage P4 produced a slightly positive reaction with the five strains at a dilution 100- to 1,000-fold more concentrated than that producing plaques on the propagating reference strain 2297, this was judged to be a negative reaction by the guidelines used for phage typing. The slightly positive reaction suggests that the five strains should be considered atypical members of phage group 8, since the known strains in this phage group are completely negative for phage P4 (4, 16).

The 16S rRNA genes of *Escherichia coli* hybridize with restriction enzyme-digested *B. sphaericus* DNA, giving characteristic band patterns which correspond to the DNA homology groups of *B. sphaericus* (6). Four reference strains of *B. sphaericus* were used: ATCC 14577 for DNA group I, 2362 and SSII-1 for DNA group IIA, and ATCC 7055 for DNA group IIB. The *Hin*dIII-digested DNAs were separated by agarose gel electrophoresis, and the hybridization was performed by using an [α-^{32}P]dCTP-labeled rRNA gene probe (6). All five strains showed the typical band patterns of DNA homology group IIA (Fig. 1).

Two classes of toxin genes of *B. sphaericus* have previously been characterized: the binary toxin (51- plus 42-kDa) genes and the 100-kDa-toxin gene (2, 13). In order to determine whether either of these two classes of genes was

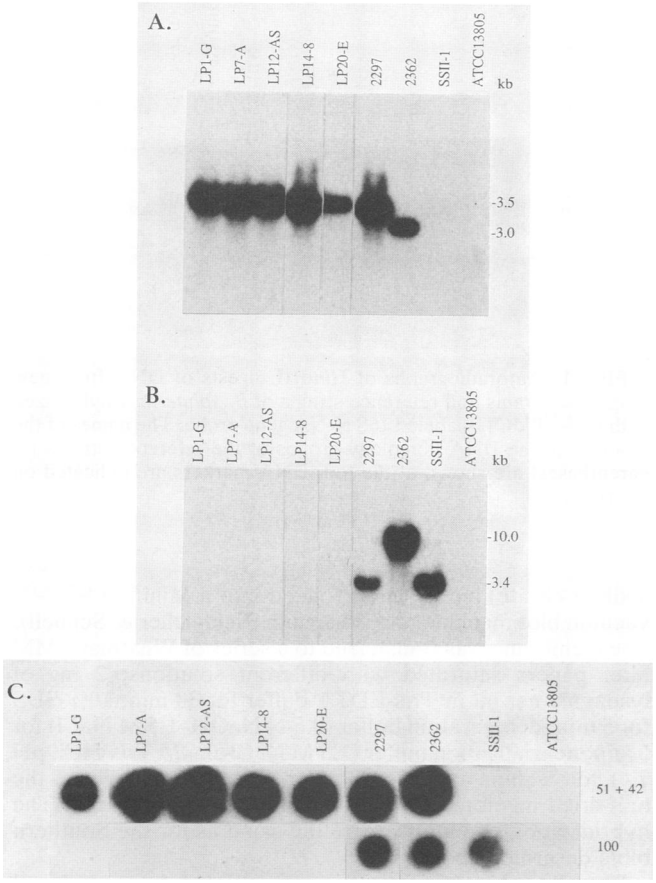

FIG. 2. Hybridization of DNA from five new Singapore strains and reference strains of *B. sphaericus* with probes for known toxin genes. (A) Southern blot analysis of *Hin*dIII-digested chromosomal DNAs hybridized with a ^{32}P-labeled *Hpa*I-*Xmn*I fragment (2.5 kb) within the open reading frame of the binary toxin genes of strain 2297. (B) Southern blot analysis of *Cla*I-digested chromosomal DNA hybridized with a ^{32}P-labeled *Hpa*I-*Bam*HI fragment (2.6 kb) within the 100-kDa-toxin gene of strain SSII-1. The reference strains (A and B) are 2297, 2362, SSII-1, and ATCC 13805. (C) Dot blot analysis. Total DNAs of strains hybridized with the binary toxin gene probe (upper line) and the 100-kDa-toxin gene probe (lower line) are shown.

present in the Singapore strains of *B. sphaericus*, a [^{32}P]dATP-labeled *Hpa*I-*Xmn*I fragment (2.5 kb) within the open reading frame of the binary toxin genes of strain 2297 and a [^{32}P]dATP-labeled *Hpa*I-*Bam*HI fragment (2.6 kb) within the open reading frame of the 100-kDa-toxin gene of strain SSII-1 were hybridized with the chromosomal DNAs of the five new strains. The two reference strains, 2297 and 2362, were used as positive controls for the binary toxin genes, and strains 2297, 2362, and SSII-1 were used as positive controls for the 100-kDa-toxin gene. Strain ATCC 13805 was used as a negative control. From the Southern blot (Fig. 2A), it is clear that all five strains harbor chromosomal binary toxin genes and the size of the *Hin*dIII fragments (3.5 kb) is similar to the size of the binary toxin gene of strain 2297 but is different from that of strain 2362 (3.0 kb). The labeled binary toxin gene probe failed to hybridize with purified plasmid DNA from the five strains in similar Southern blot experiments, in accord with the observation that the location of the binary toxin genes is chromosomal (data not shown). None of the Singapore strains appear to have a 100-kDa-toxin gene (Fig. 2B), as confirmed with dot blots using total cellular DNA (Fig. 2C).

The five strains of *B. sphaericus* did not sporulate in Luria broth medium, and bacteria grown in this medium were nontoxic to *C. quinquefasciatus* larvae. In contrast, they sporulated in NYSM medium at 30°C for 48 h and were toxic to *C. quinquefasciatus* larvae. The sporulation rate of LP7-A was relatively low (50%), the rates for LP1-G and LP20-E were higher (70%), and the highest sporulation rates were observed in strains LP12-AS and LP14-8 (Table 1). For second- or third-instar larvae of *C. quinquefasciatus*, the LC_{50} values of the five new strains grown in NYSM medium varied between 7.7×10^3 and 3.2×10^4 cells/ml, which are close to the values for the most toxic strains, 2297 and 2362 (Table 1). LP14-8 was the most toxic of the new strains, only 1.5 times less toxic than 2297 and 3.8 times less toxic than 2362.

In summary, five new mosquitocidal strains of *B. sphaericus* were isolated from soil and water samples from several locations in Singapore, and they have been characterized in terms of the presence of known toxin genes, location of toxin genes, phage group, DNA homology group, sporulation rate, and toxicity to mosquito larvae. The five strains were similar to each other in these respects. However, there was some variation in both the sporulation rates (50 to 90%) and toxicities to *C. quinquefasciatus* larvae (LC_{50} 7.7×10^3 to 32×10^3 cells/ml), with a correlation between the extent of sporulation and toxicity (Table 1). As with the known high-toxicity strains of *B. sphaericus*, the larvicidal activity of the five new strains was totally dependent on sporulation. Therefore, it is a reasonable supposition that mutations in one or more sporulation genes which lowered the sporulation rate had also reduced the toxicity.

In some respects, the five Singapore strains resemble the known high-toxicity strains (2): they all belong to DNA homology group IIA (2, 6, 8), they are only 1.5 to 6.4 times less toxic to *C. quinquefasciatus* larvae than reference strain 2297 and 3.8 to 16 times less toxic than reference strain 2362, their toxicity is sporulation dependent, and they possess genes which hybridize with a binary toxin gene probe. They appear to be more closely related to strain 2297 than to strain 2362 on the basis of the size of the DNA fragment hybridizing with the binary toxin gene probe (Fig. 2A), the degree of toxicity to *Culex* larvae, and the complete lack of toxicity to *Aedes aegypti* larvae. On the other hand, the five Singapore strains are distinct from all known high-toxicity strains of *B. sphaericus*, since they are classified as atypical members of phage group 8 and they lack a 100-kDa-toxin gene. The only known strains in phage group 8 are in the low-toxicity class (4) since they are 10^3 to 10^4 times less toxic than strains 1593 and 2362. The toxin gene constitution of the five new strains is unique because they have binary toxin genes and they lack a 100-kDa-toxin gene, whereas all the tested high-toxicity strains of *B. sphaericus* (e.g., 2297, 2362, 1593, and IAB59) have both binary and 100-kDa-toxin genes (13).

Finally, it was not previously known whether the binary toxin genes of *B. sphaericus* strains are borne on a plasmid or on the chromosome (2). Here, we have provided evidence that the binary toxin genes in the five new Singapore strains are chromosomally located.

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