APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 2009, p. 878–881 Vol. 75, No. 3
0099-2240/09/$08.00+0 doi:10.1128/AEM.01444-08
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A 1.1-Kilobase Region Downstream of the *bin* Operon in *Bacillus sphaericus* Strain 2362 Decreases Bin Yield and Crystal Size in Strain 2297[▽]

Hyun-Woo Park,[1]* Mujin Tang,[2] Yuko Sakano,[2] and Brian A. Federici[2,3]

John A. Mulrennan, Sr., Public Health Entomology Research & Education Center, CESTA, Florida A&M University, Panama City, Florida 32405[1]; Department of Entomology, University of California, Riverside, California 92521[2]; and Interdepartmental Graduate Programs in Cell, Molecular & Developmental Biology and Genetics, Genomics & Bioinformatics, University of California, Riverside, California 92521[3]

Received 27 June 2008/Accepted 25 November 2008

The 2297 strain of *Bacillus sphaericus* produces a crystal of the Bin (binary) toxin that is approximately fourfold larger than that of strain 2362, the strain currently used in VectoLex, a commercial mosquito larvicide. Comparison of the regions downstream from the *bin* operon in these two strains showed that strain 2362 contained a 1.6-kb region with four *orf* genes not found in strain 2297. Insertion of a 1.1-kb portion of this region from strain 2362 by homologous recombination downstream from the *bin* operon in strain 2297 reduced Bin toxin production by 50 to 70% and toxicity to fourth-instar larvae of *Culex quinquefasciatus* by 68%. These results suggest that the 1.6-kb region downstream from the *bin* operon in *B. sphaericus* 2362 is responsible for the lower Bin yield and smaller crystal size characteristic of this strain.

Mosquitocidal strains of *Bacillus sphaericus* produce several protein toxins. The most potent toxin is the so-called Bin (for binary) toxin produced during sporulation, whereas the less potent toxins, referred to as Mtx toxins (34 to 36 or 100 kDa), are produced during vegetative growth (6, 8). The Bin toxin forms a crystal on the inner surface of the exosporium membrane and remains associated with the spore after cell lysis, which contributes to the efficacy of *B. sphaericus* strains that produce this toxin. Alternatively, the Mtx proteins are soluble and degrade rapidly after synthesis, contributing little to the overall toxicity of *B. sphaericus*. More recently, another sporulation-associated toxin pair, related to the Cry toxins of *Bacillus thuringiensis*, has been found in *B. sphaericus* (13).

The Bin toxin is composed of two proteins, a 42-kDa toxic domain (BinA) and a 51-kDa binding domain (BinB), assembled in parasporal inclusions (6, 8, 14, 22). The operon encoding both proteins has been cloned and sequenced from several highly toxic strains (2, 3, 5, 10, 11). The sequences are extremely well conserved, so that amino acid sequence identities, typically 98% or more, are about the same among all the strains. Although the simultaneous presence of both proteins appears necessary for full toxicity, the differential activities toward various mosquito species displayed by different *B. sphaericus* strains depend on the origin of BinA, as demonstrated by in vitro mutagenesis analyses with variants of Bin (4). These observations suggest that BinA is the most important determinant of specificity and activity. The Bin toxin is generally highly active against larvae of *Anopheles*, *Culex*, and *Ochlerotatus* species (6).

Among highly mosquitocidal strains of *B. sphaericus*, strains 2297 and 2362 are the most widely studied (1–3, 9, 11, 14, 17, 21, 24). Strain 2297 demonstrates relatively low toxicity even though it produces a large parasporal crystal. In comparison, strain 2362, which produces a much smaller Bin crystal than strain 2297, has the highest toxicity against *Culex* species and therefore serves as the active ingredient of the microbial larvicide VectoLex (Valent BioSciences, Libertyville, IL). While amino acid sequence variations may explain differences in toxicity, factors governing Bin crystal size and yield differences between these two strains remain unknown.

As the first step in elucidating the mechanism of Bin crystal size regulation, we investigated the sequences flanking the *bin* operon on the chromosomes of *B. sphaericus* strains 2297 and 2362. The *bin* operon sequences of strains 2297 and 2362 and downstream sequences from the *bin* operon of strain 2297 are known (GenBank accession numbers AJ224478 for strain 2297 and M20390 for strain 2362). To obtain the upstream and downstream regions of the *bin* operon in strain 2362 and the upstream region in strain 2297, we sequenced these regions using chromosome walking techniques (23). The results showed that the upstream regions of these two *B. sphaericus* strains are well conserved. Both have the same potential coding sequence (CDS) located 449 bp upstream from the *binB* gene. The deduced amino acid sequence of this CDS (803 amino acids) showed 99, 55, and 51% identities with, respectively, a hypothetical protein, Bsph_3194 of *B. sphaericus* C3-41 (GenBank accession number ACA40703), ORF1 of *Paenibacillus popilliae* (GenBank accession number CAA67505), and a 19-kDa protein of *B. thuringiensis* subsp. *israelensis* (GenBank accession number CAA09344). The functions of these proteins are not known (7, 12, 16, 26).

With respect to the sequences downstream from the *bin* operon, we found a 1.6-kb fragment in strain 2362 that was not present in strain 2297 (Fig. 1). Within this region, four putative *orf* genes were identified, which we named *orfA*, *orfB*, *orfC*, and

* Corresponding author. Mailing address: Public Health Entomology Center, Florida A&M University, 4000 Frankford Avenue, Panama City, FL 32405. Phone: (850) 872-4184, ext. 35. Fax: (850) 872-4733. E-mail: hyun-woo.park@famu.edu.

▽ Published ahead of print on 5 December 2008.

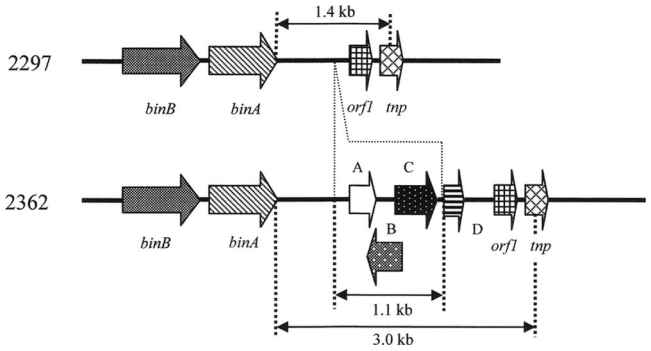

FIG. 1. Schematic illustration of genomic DNA of *Bacillus sphaericus* strains 2362 and 2297 in the region of the *bin* operon. Strain 2362 contains a 1.6-kb fragment downstream from the *bin* operon that is not found in strain 2297.

orfD. Among these, *orfA*, *orfC*, and *orfD* had the same orientation as the *bin* operon whereas *orfB* was in the opposite orientation. Furthermore, *orfA* and *orfC* were identified as CDSs, as previously described for *B. sphaericus* C3-41 (12). ORFA and ORFC showed 100 and 99% identities with Bsph_3191 (GenBank accession number ACA40700) and Bsph_3190 (GenBank accession number ACA40699). In addition, ORFA (156 amino acids) showed 40% identity with the nodulin-like protein of *Arabidopsis thaliana* (GenBank accession number BAB10904). ORFC was the longest (546 amino acids) and shared sequence homology with many different proteins, including the cytochrome *c* oxidase subunit II of *Agrobacterium tumefaciens* (26% identity; GenBank accession number AAL41783), NADH dehydrogenase subunit 5 of *Dirofilaria immitis* (25% identity; GenBank accession number CAD61200), and an envelope glycoprotein of human immunodeficiency virus 1 (28% identity; GenBank accession number AAL93397). ORFD was the shortest (153 amino acids) and did not show homology with any known proteins. Interestingly, ORFB (267 amino acids) showed 29% identity and 59% similarity with the multicopy suppressor (SUR7) of *rvs167* mutation in *Saccharomyces cerevisiae* (GenBank accession number NP_013660). The *rvs167* mutant shows a decrease in cell viability and an increased ratio of budded cells upon carbon or nitrogen exhaustion compared to the wild-type strain (25). Overexpression of *sur7* suppresses growth defects, actin cytoskeleton disorganization, and budding pattern alteration of mutant *S. cerevisiae* cells (25). ORFB showed 26% identity and 49% similarity with transposase OrfB of *Bacillus anthracis* strain Ames (GenBank accession number AAP26904).

To determine whether this additional region in strain 2362 had an effect on Bin crystal size and yield in strain 2297, the 1.1-kb fragment that contains *orfA*, *orfB*, and *orfC* of strain 2362 was inserted the same distance downstream from the *bin* operon of strain 2297 using homologous recombination (21, 24) (Fig. 2). The 1.4-kb downstream sequence from the *bin* operon of strain 2297 (Fig. 1) was amplified by using 2297DF (5′-TTTTCCTGTTAGATATACAGG-3′) as the forward primer and 2297DR (5′-AATTCGGTTTTAAATACCTTA-3′) as the reverse primer. The 1.1-kb extra region of strain 2362 was amplified by using 2362XF (5′-CTTTTGAATGAATTGA AAAAA-3′) as the forward primer and 2362XR (5′-TTTGT GGCTTTTAAGTGTGTT-3′) as the reverse primer. PCR was performed with Vent DNA polymerase (New England Biolabs, Ipswich, MA) for 30 cycles as follows: 94°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min. The PCR products were treated with T4 DNA polymerase to remove adenine residues and T4 polynucleotide kinase to add 5′ phosphates, and the 1.4-kb DNA fragment was inserted into the EcoRV site of the temperature-sensitive plasmid pRN5101 (21, 24) to generate pRN-2297. Then, pRN-2297 was digested with AsuII to insert the 1.1-kb PCR product containing most of the extra region of strain 2362. The resulting plasmid was named pRN-2297-INF. The orientation of the DNA fragments was confirmed by restriction enzyme digestion analysis. Due to the limitation of restriction enzymes that allows analysis of the orientation of the insertion into the 1.4-kb sequence downstream from the *bin* operon of strain 2297, *orfD* was excluded from the insertion. Chromosomal DNA of wild-type *B. sphaericus* strains 2297 and 2362 and that of the recombinant 2297 strain that has the 1.1-kb insertion of strain 2362 downstream from the *bin* operon (2297DSIN) were extracted using the NucleoBond plasmid DNA purification kit (BD Biosciences, San Jose, CA). PCR was performed to confirm the chromosomal insertion using primers 2297DF and 2362XR. The amplicon size from the chromosomal DNA of the recombinant 2297 strain was 2.5 kb, indicating that the 1.1-kb fragment was inserted into the target sequence (data not shown).

Wild-type *B. sphaericus* 2297 and 2362 and recombinant strain 2297 with the 1.1-kb 2362 insertion downstream from the *bin* operon (2297DSIN) were then grown in the sporulation medium MBS (14). To quantify Bin production per unit medium, the same amount of medium was taken from each culture and the proteins produced during fermentation were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (15) (Fig. 3). Bin production by strain 2297DSIN was markedly reduced, to a level corresponding to that of wild-type strain 2362, thereby demonstrating that the 1.1-kb insertion affected Bin yield. A recent study revealed that there are two copies of the *bin* operon in *B. sphaericus* C3-41—one on the chromosome and the other on a plasmid (12). Reduction of Bin yield by approximately 60% in strain 2297DSIN suggests that the effect of the 1.6-kb insert is *trans* acting and therefore reduces toxin yield from both copies of the gene. This in turn implies that the effect is due to an RNA or protein product from this region rather than a downstream effect in the DNA, such as secondary structure or binding of another factor, which would be expected to affect only the copy of the *bin* operon downstream of which it was inserted.

The number of spores produced per unit medium by each *B. sphaericus* strain was also measured to confirm that the observation was not simply due to the increase of the sporulated cell numbers (20). The results showed clearly that there was no statistical difference in the number of spores per milliliter between strains 2297 (5.8×10^8) and 2297DSIN (5.4×10^8), whereas production by strain 2362 was significantly higher than those of the other two (9.1×10^8). In addition, phase-contrast microscopy showed that the 1.1-kb insertion also reduced the size of the Bin crystal (data not shown). When *B. sphaericus*

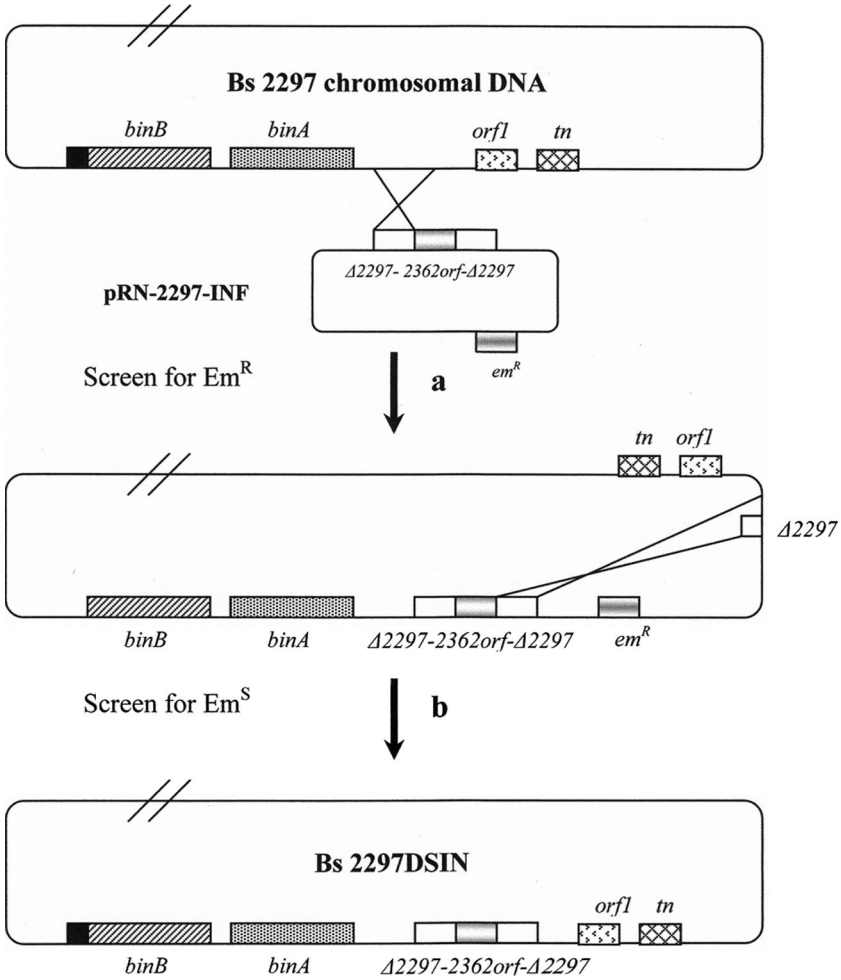

FIG. 2. Strategy for integration of the 1.1-kb downstream *bin* region of *Bacillus sphaericus* 2362 into the chromosome of *B. sphaericus* 2297 downstream from its *bin* operon. An erythromycin-resistant colony was selected after the first crossover (a), and one of the final erythromycin-sensitive colonies after the second crossover (b) was selected for further analysis.

strains were tested against fourth-instar larvae of *Culex quinquefasciatus* using established methods (18–20), the 50% and 95% lethal concentrations of recombinant strain 2297DSIN were significantly higher than those of wild-type strain 2297, supporting the results obtained with SDS-PAGE, spore count data, and phase-contrast microscopy (Table 1).

Despite many attempts, very little has been revealed regarding the molecular mechanisms that regulate Bin synthesis and crystallization in *B. sphaericus* (1, 6, 8, 24). In this regard, the present research demonstrated that the 1.1-kb fragment containing *orfA*, *orfB*, and *orfC* located downstream from the 2362 *bin* operon affects the size and yield of Bin crystal in strain 2297. The possibility of identification of the specific *orf* gene(s) within this fragment using additional knockout and/or insertion mutations and subsequent functional analysis of the *orf* gene(s) identified by these methods are currently under evaluation.

Nucleotide sequence accession numbers. The upstream and downstream regions of the *bin* operon in strain 2362 and the upstream region in strain 2297 have been submitted to

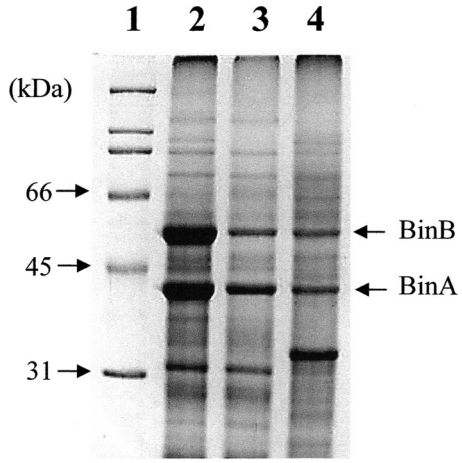

FIG. 3. SDS-PAGE analysis of three *Bacillus sphaericus* strains, wild-type strains 2362 and 2297 and recombinant strain 2297DSIN containing the 1.1-kb downstream region from strain 2362. Lane 1, molecular mass markers; lane 2, strain 2297; lane 3, strain 2297DSIN; lane 4, strain 2362. BinA and BinB are indicated by arrows.

TABLE 1. Toxicities of wild-type and recombinant *Bacillus sphaericus* strains to fourth-instar larvae of *Culex quinquefasciatus*

Strain	LC (fiducial limits)[a]		Slope
	50%	95%	
2362	9.5 (7.3–12.1)	40.8 (28.5–73.1)	2.6 ± 0.4
2297	72.6 (59.6–89.0)	234.6 (171.3–393.0)	3.2 ± 0.5
2297DSIN	121.6 (92.9–180.6)	754.8 (405.4–2,516.9)	2.1 ± 0.4

[a] Lethal concentrations (LCs) are based on 48-hour mortality and are expressed in ng/ml.

GenBank under accession numbers EU826482, EU826483, and EU826484, respectively.

We thank Jeffrey J. Johnson for his technical assistance during this study.

This research was partially supported by a grant to H.-W.P. from the U.S. Department of Agriculture (2007-38814-18497) and by grants to B.A.F. from the U.S. National Institutes of Health (AI145817 and AI054778).

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