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Cloning, Sequencing, and Expression of a Gene Encoding a 100-Kilodalton Mosquitocidal Toxin from *Bacillus sphaericus* SSII-1

THIRUMARAN THANABALU, JOHN HINDLEY, JEANNETTE JACKSON-YAP, AND COLIN BERRY*

Institute of Molecular and Cell Biology, National University of Singapore, 10 Kent Ridge Crescent, Singapore 0511, Republic of Singapore

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A cosmid library was prepared from a partial *Bam*HI digest of total DNA from *Bacillus sphaericus* SSII-1. Two hundred fifty *Escherichia coli* clones were screened for toxicity against larvae of the mosquito *Culex quinquefasciatus*. One toxic clone, designated pKF2, was chosen for further study. Two toxic subclones, designated pXP33 and pXP34, obtained by ligating *Pst*I-derived fragments of pKF2 into pUC18, contained the same 3.8-kb fragment, but in opposite orientations. Sequence analysis revealed the presence of an open reading frame corresponding to a 100-kDa protein and the 3′ end of a further open reading frame having significant homology to open reading frames of transposons Tn*501* and Tn*21*. The sequence of the SSII-1 toxin was compared with those of known toxins and was found to show regional homology to those of ADP-ribosyltransferase toxins. The distribution of the toxin gene among other *B. sphaericus* strains was examined.

Bacillus sphaericus is an aerobic spore-forming *Bacillus* species, several strains of which are pathogenic for mosquito larvae (13). *B. sphaericus* SSII-1 was isolated in 1973 from infected mosquito larvae collected in India (36). This strain was considerably more toxic than the other *B. sphaericus* strains known at that time. Early studies indicated that the toxin was retained within or attached to the bacterial cells and that the toxic activity was markedly unstable. Storage of cells under refrigeration or heating at 80°C resulted in the loss of activity. Toxin production occurred predominantly in the vegetative phase of growth before the onset of sporulation (27, 29).

Further studies on *B. sphaericus* SSII-1 declined rapidly with the discovery of strains, such as 1593 (36a), 2362 (40), and 2297 (41), which had higher toxicities. In contrast to strain SSII-1, these strains develop relatively stable, high toxicity at the onset of sporulation (7). To date, two types of toxin have been characterized in the highly toxic strains: a pair of proteins of 51.4 and 41.9 kDa associated with the parasporal crystal (3, 5, 19) and a toxin of 110 kDa related to a 125-kDa surface layer protein (5, 6). Genes encoding the 51.4- and 41.9-kDa proteins were shown to be absent from strain SSII-1 (4). In the same study, Baumann et al. showed that sequences inserted in their group C clones were also absent from strain SSII-1. The group C clones were later shown to contain sequences corresponding to the gene encoding the 125-kDa protein (6). Davidson (12) described a toxin of 100 kDa isolated from the cytoplasm of sporulating *B. sphaericus* 1593 cells. The distribution of this toxin among other *B. sphaericus* strains has not been studied. Its relationship, if any, to the 110-kDa toxin of Baumann et al. (5) has not been established.

In the present study, toxicity against second-instar larvae of the mosquito *Culex quinquefasciatus* was used to identify a clone carrying a mosquitocidal toxin gene from *B. sphaericus* SSII-1 in a cosmid library. A 3.8-kb *Pst*I fragment was inserted into pUC18, and the recombinant was shown to express toxicity after transformation into *Escherichia coli* HB101. The 3.8-kb fragment was sequenced, and the distribution of the SSII-1 toxin gene among other strains of *B. sphaericus* was examined.

MATERIALS AND METHODS

Bacterial strains and vectors. *B. sphaericus* SSII-1, ATCC 7054, and ATCC 7055 were obtained from E. W. Davidson; strains Kellen Q, 2315, and 31 were obtained from A. A. Yousten; strain BSE18 was obtained from F. Priest; strain 1593M was obtained from J. Szulmajster; and all other *Bacillus* strains were obtained from H. de Barjac.

Construction of the cosmid library. To prepare total genomic DNA from *B. sphaericus* SSII-1, we lysed cells in the presence of 1% sodium dodecyl sulfate and 0.1 mg of proteinase K per ml. DNA was purified from the lysate by the method of Ausubel et al. (2). In brief, hexadecyltrimethyl ammonium bromide was added, and the mixture was incubated at 65°C for 20 min prior to extraction with chloroform-isoamyl alcohol. DNA was precipitated with ethanol from the aqueous phase and purified by banding on a cesium chloride gradient containing ethidium bromide. DNA was partially digested with *Bam*HI under conditions found to yield a predominance of fragments in the range of 20 to 40 kb. Typically, 0.12 U of enzyme was used for each microgram of DNA in a 15-min incubation at 37°C. Fragments from the partial digest were ligated into *Bam*HI-cut, phosphatase-treated cosmid pHC79 (20). The cosmids were packaged with the Packagene in vitro packaging system (Promega) in accordance with the manufacturer's instructions, transfected into *E. coli* HB101, and plated onto LB agar containing 60 μg of ampicillin per ml.

Isolation of toxic clones. Two hundred fifty colonies were individually picked and stored. Pooled groups of 10 colonies each were grown overnight and assayed for toxicity. Ten second-instar *C. quinquefasciatus* larvae were placed in 5 ml of water, to which the cells for assay were added. Mortality was assessed after 24 h of incubation at 30°C. One pool exhibited toxicity. The individual clones in this pool were separately assayed against *C. quinquefasciatus* larvae. A

* Corresponding author.

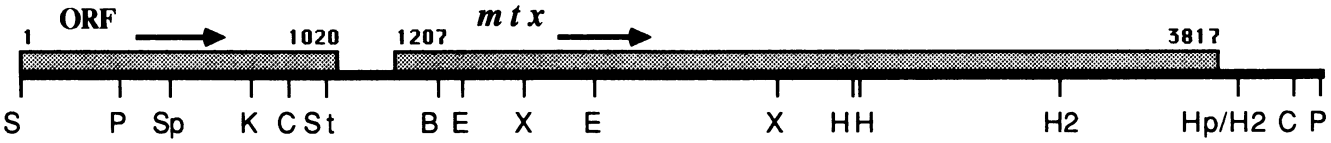

FIG. 1. Restriction map of the sequence presented in Fig. 2. Shaded boxes represent the *mtx* (mosquitocidal toxin) gene and upstream open reading frame (ORF). Arrows show gene orientation. Nucleotide numbers correspond to those shown in Fig. 2, beginning at the first nucleotide of the *Sau*3AI site. Restriction enzymes: S, *Sau*3AI (not unique); P, *Pst*I; Sp, *Sph*I; K, *Kpn*I; C, *Cla*I; St, *Stu*I; B, *Bam*HI; E, *Eco*RI; X, *Xmn*I; H, *Hin*dIII; H2, *Hin*dII; Hp, *Hpa*I.

single toxin-producing clone, designated pKF2, was identified and used as the parent clone for the experiments.

Subcloning of the toxin-encoding fragment. Subclones were produced from cosmid clone pKF2 to focus on the region of the inserted DNA encoding toxicity. At each step, subclones were assayed for larvicidal activity, and those exhibiting toxicity were chosen for further manipulation.

Cosmid clone pKF2 was subjected to partial digestion with *Sau*3AI (0.2 μg of cosmid DNA, 0.1 U of *Sau*3AI, 15 min, 37°C), and the resulting fragments were ligated into *Bam*HI-cut, phosphatase-treated pUC18. Four toxic clones were produced, and restriction mapping revealed that they contained overlapping inserts. The plasmid designated pKG19 contained the shortest inserted sequence (approximately 9 kb) and was chosen for further subcloning. This plasmid was digested with *Xba*I to remove an approximately 4.5-kb fragment. Religation of the remaining insert yielded clone pKG19X, which retained full toxicity. A *Pst*I fragment of 3.8 kb derived from pKG19X was removed from pKG19X and recloned into *Pst*I-cut, phosphatase-treated pUC18. Two toxic clones, pXP33 and pXP34, which contained the same *Pst*I fragment in opposite orientations relative to the pUC18 vector were produced.

DNA sequencing. Double-stranded DNA sequencing was carried out by the method of Heinrich (18) with plasmid DNA as the template. Subclones from plasmids pXP33 and pXP34 were made in pUC18 with the restriction sites shown in Fig. 1. A sequence further upstream was obtained from clone pKG19 (nucleotides [nt] 1 to 312). DNA was sequenced with the pUC-M13 forward and reverse primers. In addition, further oligonucleotide primers were synthesized to ensure complete sequencing of both strands.

Sequence analyses. Sequence analyses and manipulations were performed with the following software: DNASTAR package (DNASTAR Inc.), PC/GENE package (IntelliGenetics), and UWGCG package (University of Wisconsin). Initial protein sequence alignments done with the DNASTAR package were based on the method of Lipman and Pearson (22). Multiple alignments were created with the Gapzero shell (31a), which uses the programs of the UWGCG package (14) run on the SEQNET central computer at the SERC Laboratory, Daresbury, United Kingdom. Hydropathy predictions were made with the PC/GENE package by the method of Kyte and Doolittle (21).

Distribution of the toxin gene. Total DNA was prepared from 16 strains of *B. sphaericus* as described above. Approximately 5 μg of each preparation was digested to completion with *Cla*I and subjected to electrophoresis in a Tris-borate-EDTA (TBE)-buffered 1% agarose gel (24). DNA was blotted onto a Hybond-N membrane (Amersham International) by the method of Southern (37). The *Eco*RI fragment (nt 1395 to 1815; Fig. 2) derived from the SSII-1 toxin gene was radiolabeled by the nick translation method of Rigby et al. (34) and used as a probe for the toxin-encoding sequence.

Nucleotide sequence accession number. The nucleotide sequence shown in Fig. 2 was submitted to the GenBank database (nucleotide sequence accession number M60446).

RESULTS

Features of the sequence. Figure 2 shows a sequence of 4,133 nt containing two open reading frames along with the deduced amino acid sequence. There are similarities between the restriction site pattern of this sequence and that published by Souza et al. (38) for their clone pAS377PT. Their clone was derived from DNA isolated from *B. sphaericus* 1593M and may represent the equivalent toxin from this strain.

Sequence of the toxin. The toxicity of recombinants containing sequences corresponding to nt 960 to 4133 (*Stu*I-*Pst*I) and nt 307 to 3864 (*Pst*I-*Hpa*I) indicates that the open reading frame commencing at nt 1207 and terminating at nt 3817 represents the toxin gene. We have designated this gene the *mtx* (mosquitocidal toxin) gene. This gene encodes a protein of 100.557 kDa and 870 amino acids in length.

The deduced protein has features characteristic of signal peptides of gram-positive bacteria, namely, (i) positively charged residues in the N terminus followed by (ii) a stretch of residues with a neutral or hydrophobic character and (iii) a cleavage site (often after an alanine residue) at the C terminus of the signal sequence (10). The N terminus of the deduced protein contains three lysine residues, an isoleucine-rich stretch of neutral and hydrophobic residues, and a potential cleavage site following the alanine residue at position 30, forming a potential signal sequence. However, the toxic activity of our SSII-1 cultures was found to be associated with the cell pellet and not with the medium.

Figure 3 shows the hydropathy profile for the deduced toxin sequence. Two interesting regions of hydrophobicity can be seen. The first is the potential signal sequence described above. The second, from positions 43 to 60 (-AA SLTWLMDMSSLLYQLI-), was predicted to be a potential transmembrane helix by the method of Rao and Argos (33). As the mechanism of action of this toxin is unknown, we cannot assess whether this region plays an important role in toxicity.

The deduced amino acid sequence of the 100-kDa toxin was compared with protein sequences in the NBRF-PIR database with the algorithm of Lipman and Pearson (22). Whereas the overall homology of the toxin with proteins in the database was low, a strong regional homology was found between the 100-kDa toxin and the S1 subunit of pertussis toxin (31). The homologous sequence corresponds in part to a region of the pertussis toxin (residues 8 to 15 of the S1 subunit) found to be essential for activity (11) and previously observed to be related to the A subunits of both cholera toxin and *E. coli* heat-labile toxin (23, 31). These three toxins act by ADP-ribosylation of G proteins. Figure 4 shows the

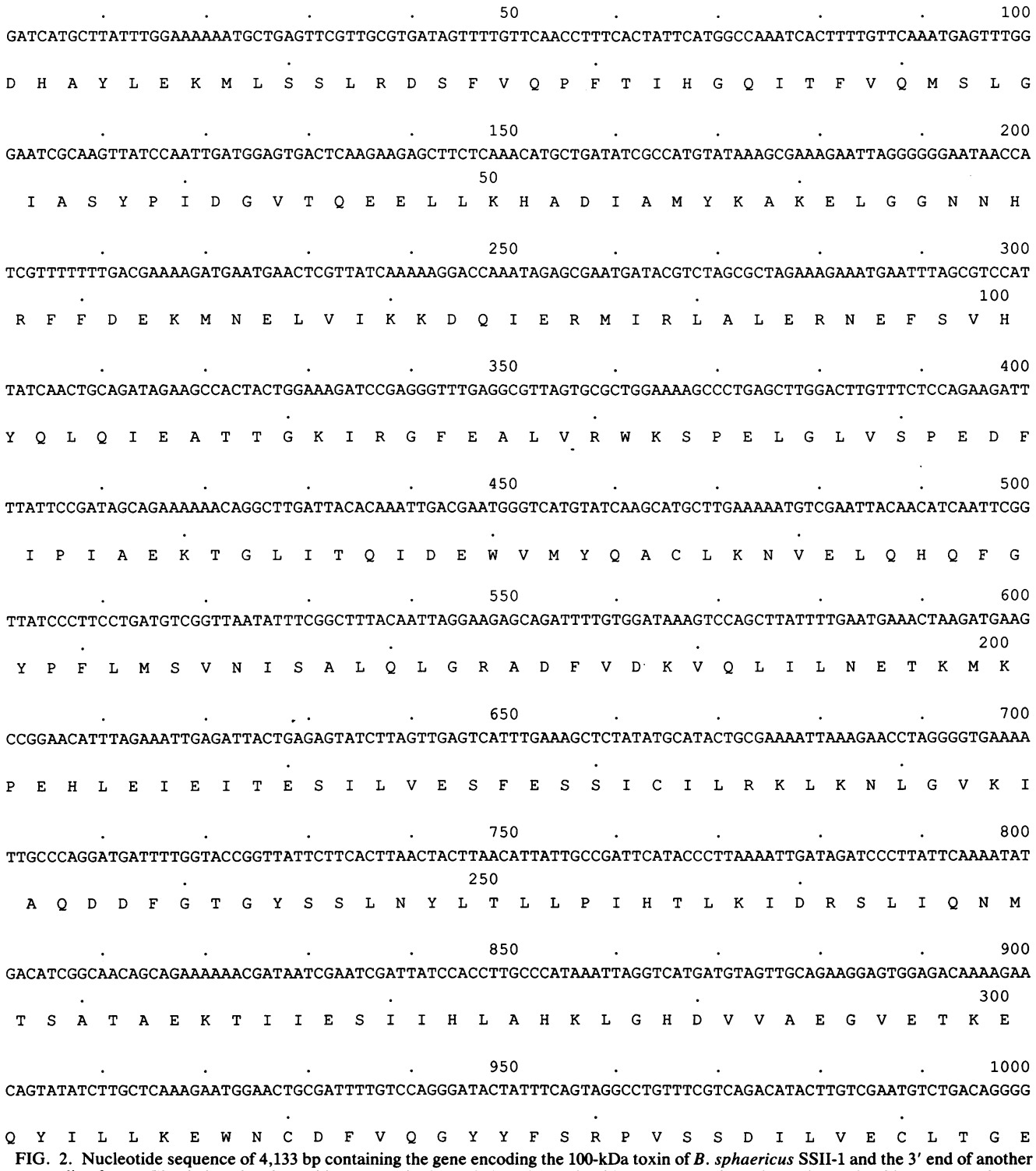

FIG. 2. Nucleotide sequence of 4,133 bp containing the gene encoding the 100-kDa toxin of *B. sphaericus* SSII-1 and the 3′ end of another open reading frame. The deduced amino acid sequence is shown below the nucleotide sequence. Lines above the nucleotide sequence denote inverted repeats; single underlining indicates putative promoter sequences; and double underlining indicates the ribosome-binding site.

homologies between these toxins and the 100-kDa toxin from *B. sphaericus* SSII-1. A further sequence homology between the 100-kDa toxin and ADP-ribosyltransferase toxins was found in the sequence Glu-Ile-Val-Arg-Ile-Trp (amino acids 219 to 224). The sequence Glu-X-X-X-X-Trp has been found in exotoxin A from *Pseudomonas aeruginosa* and in the A subunit of diphtheria toxin (9). These Glu and Trp residues of diphtheria toxin have been shown to be important in binding to the nicotinamide moiety of NAD (25). No significant homology was found between the 100-kDa toxin and

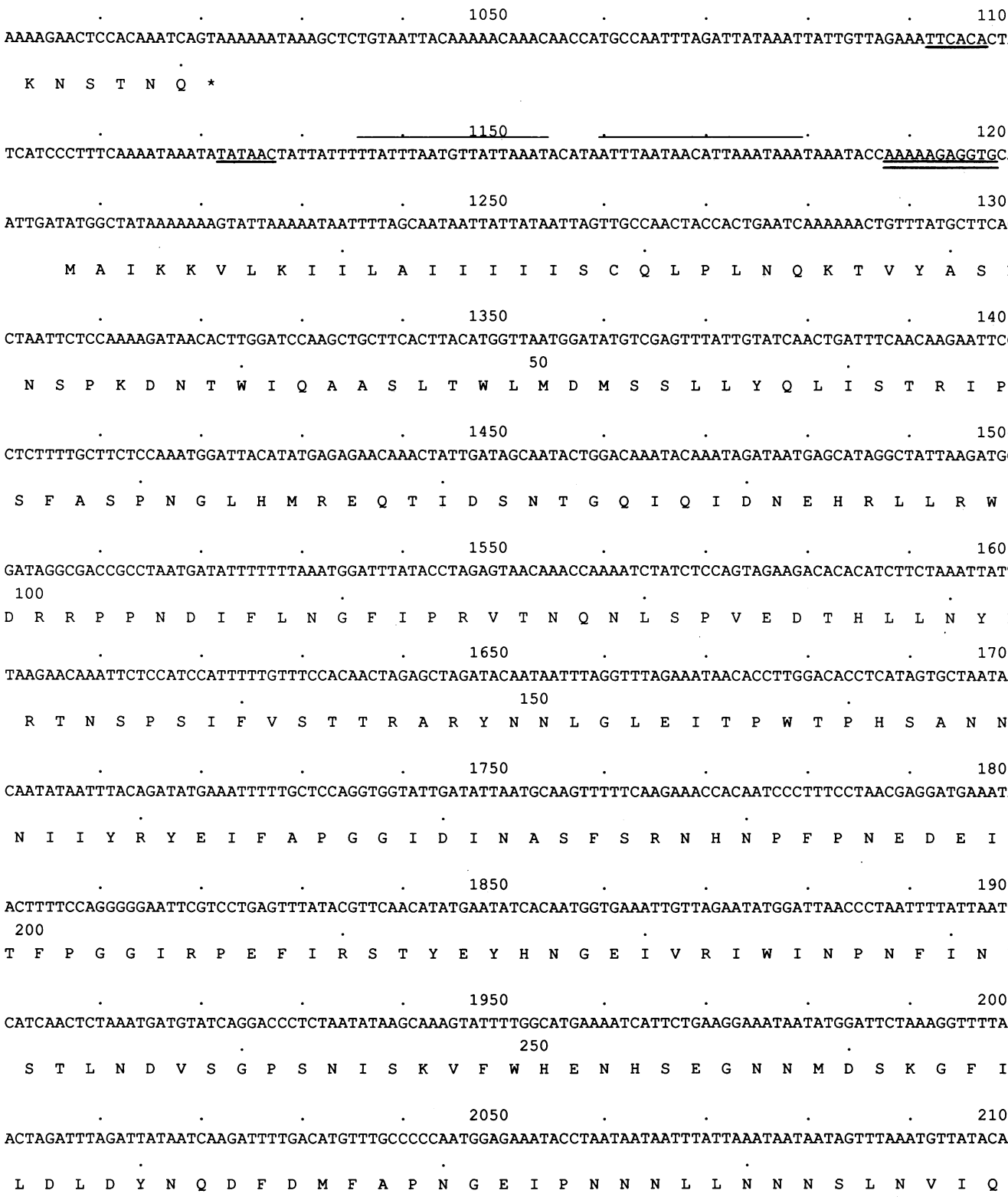

FIG. 2.—*Continued.*

other *B. sphaericus* toxins, namely, the 51.4- and 41.9-kDa toxins (3, 19) and the 125-kDa surface layer protein, from which the 110-kDa toxin is derived (6).

Upstream elements. The putative ribosome-binding site for the toxin gene, AAAAAGAGGTG (nt 1188 to 1198), shows a 10-base homology to the 3′ terminus of *Bacillus subtilis* 16S rRNA (26). The free energy of binding for these two sequences, calculated by the method of Tinoco et al. (39), is

```
        .         .         .         .      2150       .         .         .         .      2200
AACTCAGAATATCAAATCAAAAATAAGAAGGATAGGAATATTGTTGTAACGTTAGATTCTGATTATGGAGGAAGTCCAGTAGAGTCGTATAAGAATTTTG
 300                                 .                   .                   .
N  S  E  Y  Q  I  K  N  K  K  D  R  N  I  V  V  T  L  D  S  D  Y  G  G  S  P  V  E  S  Y  K  N  F  G

        .         .         .         .      2250       .         .         .         .      2300
GTTTTGAAAATCAAAAATGGAACATCAAATACGATAGCAAAAAAAATGCTTATAAAATCTACAATAGAGAAACCCCTACTTTACTATTAAGTTGGAATAG
                            .                  350                  .
  F  E  N  Q  K  W  N  I  K  Y  D  S  K  K  N  A  Y  K  I  Y  N  R  E  T  P  T  L  L  L  S  W  N  S

        .         .         .         .      2350       .         .         .         .      2400
TAACTCGTCTAATGGTGAACAAGTGATTAGAGGTTATACTGAAAGTGGTTCAAATAATCAATATTGGACAATAGAAAAAAATGTAAATGGTTTTTATAAA
                  .                   .                   .
 N  S  S  N  G  E  Q  V  I  R  G  Y  T  E  S  G  S  N  N  Q  Y  W  T  I  E  K  N  V  N  G  F  Y  K

        .         .         .         .      2450       .         .         .         .      2500
TTTAGAAATCTTTCTGACCCTAGCAAAATATTAGACTTAAAAGATGGTAACACTCTAAATAAAACTCCTTTAGTCGTTTCAAGTGAGAACAGTAGCTCAT
 400                                 .                   .                   .
F  R  N  L  S  D  P  S  K  I  L  D  L  K  D  G  N  T  L  N  K  T  P  L  V  V  S  S  E  N  S  S  S  S

        .         .         .         .      2550       .         .         .         .      2600
CACAAGAGTGGTTAATTGAAAAAACTAATTATCAAACTGTTAAAGATGGTACTTATCAAGTATCTTCAAAATTAAATGAAAATAAAGTTATTGAACAAAT
                            .                  450                  .
  Q  E  W  L  I  E  K  T  N  Y  Q  T  V  K  D  G  T  Y  Q  V  S  S  K  L  N  E  N  K  V  I  E  Q  I

        .         .         .         .      2650       .         .         .         .      2700
TTCTACTAACAAAGTTCATATTTTCTCAAACTCGGATAAAGAAAATCAAGTTTGGAATCTTATCTACAACCCTATCCTCAAAGCTTATAAAATTAAAAGC
                  .                   .                   .
 S  T  N  K  V  H  I  F  S  N  S  D  K  E  N  Q  V  W  N  L  I  Y  N  P  I  L  K  A  Y  K  I  K  S

        .         .         .         .      2750       .         .         .         .      2800
TTAAAGTATCCTAACTATTCATTGGCTTGGGATAGTAATAATACTAGAACAATTGTAGCAGCTACAGGGGATTATAATGATCAATATTGGTTAATAGAAA
 500                                 .                   .                   .
L  K  Y  P  N  Y  S  L  A  W  D  S  N  N  T  R  T  I  V  A  A  T  G  D  Y  N  D  Q  Y  W  L  I  E  R

        .         .         .         .      2850       .         .         .         .      2900
GAAACGAGGATAATACTTATATAATTAGAAATTATGAAAATAGAAAAATTGTTTTAGATTTATCAAATGGTAGTACTACTGATGGCAATGGATTATTAGG
                            .                  550                  .
  N  E  D  N  T  Y  I  I  R  N  Y  E  N  R  K  I  V  L  D  L  S  N  G  S  T  T  D  G  N  G  L  L  G

        .         .         .         .      2950       .         .         .         .      3000
ATTTGAATTTCATGGGGGAATAAATCAAAGATGGATCATTAAGCCTTTTTCATTTAATTCTATTCAAGATGGGATTTATCAATTTATGACCGTAATTAAT
                  .                   .                   .
 F  E  F  H  G  G  I  N  Q  R  W  I  I  K  P  F  S  F  N  S  I  Q  D  G  I  Y  Q  F  M  T  V  I  N

        .         .         .         .      3050       .         .         .         .      3100
CAGGATTTAATTGCTGACTTGACGACTAATAATTATACAATTGCTACGAAAACAAATAACTATTCTAGTAATCAAAAATGGACAGTAACTTATAATGATA
 600                                 .                   .                   .
Q  D  L  I  A  D  L  T  T  N  N  Y  T  I  A  T  K  T  N  N  Y  S  S  N  Q  K  W  T  V  T  Y  N  D  K
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FIG. 2.—*Continued.*

−15.4 kcal (ca. −64.4 kJ) per mol. Similar ribosome-binding sites have been proposed for other *B. sphaericus* genes, for example, GAAAGGGG for the *Bsp*RI modification methylase gene of strain R (32) and GGAGG for the 125-kDa surface layer protein gene of strain 2362 (6).

It is possible to identify several sequences in the region upstream of the toxin gene which resemble promoters. The putative promoter sequences −10 TATAAC (nt 1122 to 1127) and −35 TTCACA (nt 1092 to 1097) show homology to the consensus sequences for the σ^{55} vegetative promoter of *B. subtilis* (−10 TATAAA and −35 TTGACA).

Located between the −10 region and the ribosome-binding site for the toxin gene is an A+T-rich inverted repeat sequence (nt 1135 to 1179) capable of forming a hairpin loop

```
        .         .         .         .      3150       .         .         .         .      3200
AAAAACGTGCCTATAAAATTAGGAATTTACAACATGCTCATCTCAGTTTAGCATGGGATAGCAATCACTCAGATAAAATTTTTGGCGCTACCGGAGATTA
                            .                650                  .
  K  R  A  Y  K  I  R  N  L  Q  H  A  H  L  S  L  A  W  D  S  N  H  S  D  K  I  F  G  A  T  G  D  Y

        .         .         .         .      3250       .         .         .         .      3300
TGATGATCAATATTGGATTCCTATTTTGCAAACAGATGGATCATTTATTTTTAGGAATTATAAAAATCCAAATAAGATATTTGGAACAAATGGTCAACCC
          .                   .                   .
  D  D  Q  Y  W  I  P  I  L  Q  T  D  G  S  F  I  F  R  N  Y  K  N  P  N  K  I  F  G  T  N  G  Q  P

        .         .         .         .      3350       .         .         .         .      3400
ATTAATGATATTCCGTTAAAGGCTCAAGATGTAACTGGACAAAATAATCAAAAGTGGTATTTAAGACATTTAAATTCTTCCAATAATTTTACTGGATACT
 700                          .                   .                   .
  I  N  D  I  P  L  K  A  Q  D  V  T  G  Q  N  N  Q  K  W  Y  L  R  H  L  N  S  S  N  N  F  T  G  Y  F

        .         .         .         .      3450       .         .         .         .      3500
TTAACATATCCAGTAAAAAGAATTTTAATAAAATTATTACTATGAACTCAAATAAAACTCAAGCTGTTATTTTTGACAATATTGGAATTAACAATCAGTC
                            .                750                  .
  N  I  S  S  K  K  N  F  N  K  I  I  T  M  N  S  N  K  T  Q  A  V  I  F  D  N  I  G  I  N  N  Q  S

        .         .         .         .      3550       .         .         .         .      3600
ATGGAAACTGAAATATAATGACAATAAAAATGCATATCAGATTCACATTTTAGATAACTTTTTATACTTTCAAGGCGGCCATAATATTGTTGCTACCATG
          .                   .                   .
  W  K  L  K  Y  N  D  N  K  N  A  Y  Q  I  H  I  L  D  N  F  L  Y  F  Q  G  G  H  N  I  V  A  T  M

        .         .         .         .      3650       .         .         .         .      3700
CGAAATGTTACTAATGATGATTTAAGAAGTTATTGGTATGTAGAATATAACTTTAATAAAGATGGGTTTATAATAAGAAACGCATTTGATACAAGCTACG
 800                          .                   .                   .
  R  N  V  T  N  D  D  L  R  S  Y  W  Y  V  E  Y  N  F  N  K  D  G  F  I  I  R  N  A  F  D  T  S  Y  V

        .         .         .         .      3750       .         .         .         .      3800
TACTTGATGTATTTCAAGGAAATTTTGCTAACAATACACCTATCATCACTTATCAAAACTATTTAAACGACAACCAGTTATGGAATTTTATACCTTCATT
                            .                850                  .
  L  D  V  F  Q  G  N  F  A  N  N  T  P  I  I  T  Y  Q  N  Y  L  N  D  N  Q  L  W  N  F  I  P  S  L

        .         .         .         .      3850       .         .         .         .      3900
AGGTGTAGAACCTAGATAGTAATGACTGTTTTATGCATAGAAGACAAGCAAAAAAGAGGTTAACAGAGATATAATGAAATTATAATAACATCTAAATTGT
          .
  G  V  E  P  R  *  *  *

   ____.________.   ____.________.      3950       .         .         .         .      4000
AAATAAAGGGTGATACATATCGTAATATGTATCACCCTTTTTACTTTTGTACAACAATCACTACCGATTGTAGGTTAAAATAAAGTTAGATTTTTTTCTG

        .         .         .         .      4050       .         .         .         .      4100
TGATCTAATCTCCAATAAAAACCTTTCCCTTTTCCTCTCTTAAAATGCAGGTATCGATTTAGTAGCCTTCTTAAATTTTTAAAAAAGATGAATTATCAAT

          .         .      4030
TTGATTTACCGTTAAAATTTATTTATACTGCAG
```

FIG. 2.—*Continued.*

structure with a free energy of −22.6 kcal (ca. −94.6 kJ) per mol. It appears unlikely that this sequence represents the transcriptional termination signal for the upstream open reading frame, as it does not show the usual features of such a sequence (a G+C-rich hairpin loop followed by a T-rich region [16, 35]). Procaryotes are known to utilize posttranscriptional mechanisms for the control of gene expression; many of these procaryotes use sequences located around the translational initiation site (17). For example, T4 DNA polymerase acts as an autogenous translational repressor by binding to an mRNA sequence which includes the ribosome-binding site and a hairpin loop structure located 6 nt upstream (1). It is possible that the inverted repeat sequence upstream of the toxin gene is involved in regulating its expression.

Downstream elements. A G+C-rich inverted repeat se-

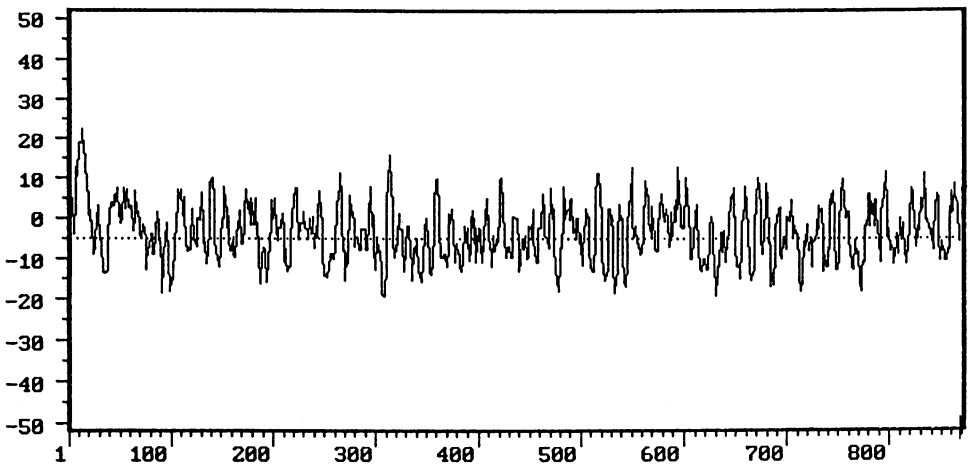

FIG. 3. Hydropathy profile for the amino acid sequence of the 100-kDa toxin. Amino acid numbers are marked on the horizontal axis, and units of hydropathicity (calculated by the algorithm of Kyte and Doolittle [21]) are marked on the vertical axis. The dotted line at −5 divides hydrophobic regions above from hydrophilic regions below.

quence capable of forming a hairpin loop structure with a free energy of −27.6 kcal (ca. −115.5 kJ) per mol begins 86 nt downstream of the translational stop codon of the toxin gene. This inverted repeat sequence is followed by a T-rich sequence and thus has the characteristics of a transcriptional termination signal.

Upstream open reading frame. The sequence presented in Fig. 2 also contains the 3′ end of another open reading frame (nt 1 to 1020). Beginning after methionine residue 74 in our sequence there was significant homology to open reading frames ORF2 from Tn*501* and ORF2a from Tn*21* (8). The Tn*21* homology continues after residue 290 in our sequence in ORF2b (15) (Fig. 5). The function of these open reading frames of the mercury resistance-conferring transposons Tn*501* and Tn*21* is not known. *B. sphaericus* SSII-1 was tested for mercury resistance by two methods. The ability of the bacteria to grow on LB agar containing 10 μg of mercuric acetate per ml and the ability of the bacteria to volatilize mercuric chloride were tested by the method of Nakamura and Nakahara (30). No colonies were formed on mercuric acetate plates after incubation at 30°C for 72 h, and no evidence of mercuric chloride volatilization was seen. These results indicate that the upstream open reading frame is not associated with mercury resistance in *B. sphaericus* SSII-1.

Distribution of the toxin sequence. Figure 6 shows the results of Southern hybridization of a toxin gene probe to *Cla*I-digested DNA from various *B. sphaericus* strains. The *mtx* gene is widely distributed among both high- and low-toxicity strains. The sizes of the major hybridizing bands appeared to vary among serotypes, with estimated sizes of approximately 11 kb in the H1a strain, 3.2 kb in H2a,2b strains, 10 kb in H5a,5b strains, 13 kb in the H6 strain, 8.4 kb in the H9a,9c strain, and 2.9 kb in the H25 strain. Additional weakly hybridizing bands were also seen in some strains. These bands are currently under investigation. In addition to DNA from the strains shown in Fig. 6, *Cla*I-digested DNA from the weakly toxic strains 2173 and 2315 (serotype H26a,26b) and the nontoxic strains BM2 (serotype H26a,26b) and ATCC 7054 and ATCC 7055 (serotype H9) was used in similar hybridization experiments. No hybridization of the toxin gene probe was observed (results not shown).

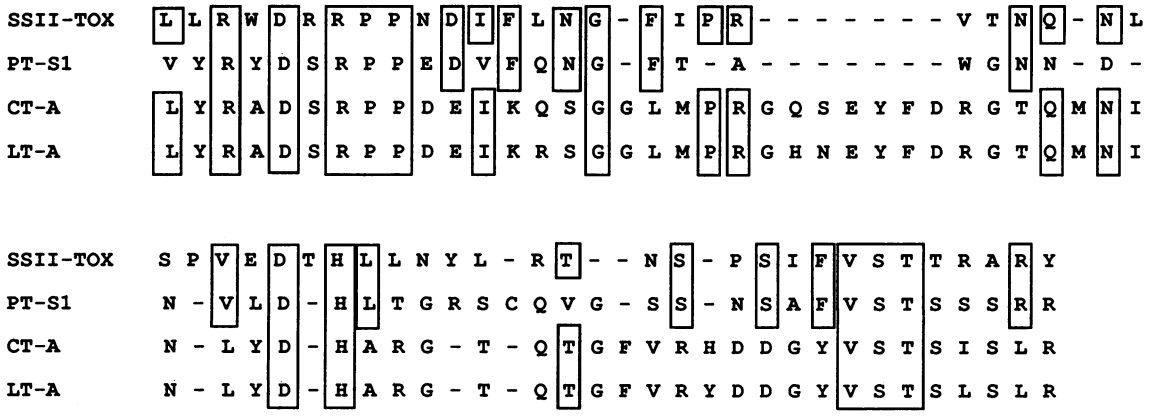

FIG. 4. Sequence comparisons between the 100-kDa toxin and other bacterial toxins. The deduced amino acid sequence from amino acids 95 to 148 of the 100-kDa toxin from *B. sphaericus* SSII-1 (SSII-TOX) is aligned with amino acids 7 to 58 of the S1 subunit of pertussis toxin (PT-S1) (31) and amino acids 5 to 67 of the A subunit of cholera toxin (CT-A) and the A subunit of *E. coli* heat-labile toxin (LT-A) (42). Boxes indicate regions of identity between the sequence of the 100-kDa toxin and the sequence(s) of one or more of the other toxins.

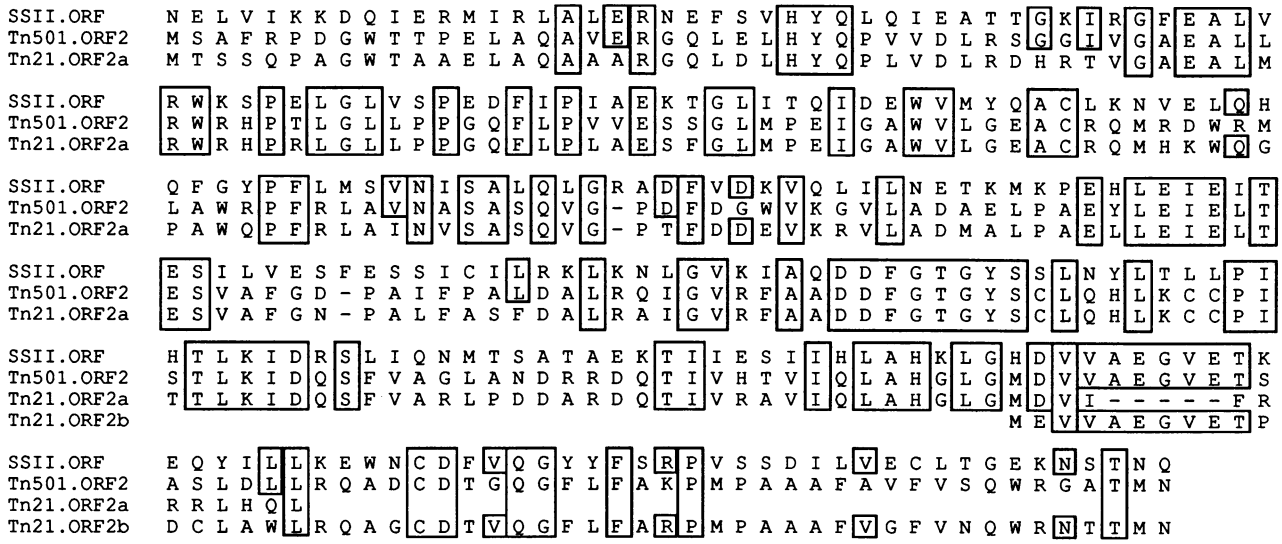

FIG. 5. Sequence comparisons between the SSII-1 upstream open reading frame (SSII.ORF) (amino acids 75 to 340) and amino acids 1 to 264 of ORF2 from Tn*501*, 1 to 224 of ORF2a from Tn*21* (8), and 1 to 52 of ORF2b from Tn*21* (15). Boxes indicate regions of identity between the deduced sequence of the upstream open reading frame and the sequence(s) of one or more of the other open reading frames.

Toxicity of the *E. coli* clones. Serial 10-fold dilutions of *E. coli* containing clones pXP33 and pXP34 were assayed for toxicity for second-instar larvae of the mosquitoes *C. quinquefasciatus* and *Aedes aegypti*. Both clones were toxic for both species of mosquitoes, causing 100% mortality in *C. quinquefasciatus* at minimum cell concentrations of 2×10^6 cells per ml at 24 h and 2×10^5 cells per ml at 48 h and in *A. aegypti* at minimum cell concentrations of 2×10^7 cells per ml at 24 h and 2×10^6 cells per ml at 48 h. The minimum concentrations of *B. sphaericus* SSII-1 cells necessary to produce 100% mortality were found to be 1/10 the concentrations of the recombinants in every case, possibly because of lower expression in the recombinants or the presence of other, unidentified toxins in strain SSII-1.

The toxicity of *B. sphaericus* SSII-1 declined rapidly upon storage at 4°C. The same loss of toxicity was observed with *E. coli* transformed with our initial cosmid clone, pKF2. However, greater stability was observed in toxins produced by later subclones derived from pKF2. The toxicity of these subclones was maintained for at least 3 weeks at 4°C.

DISCUSSION

The discovery and characterization of further toxins from *B. sphaericus* is important in the understanding of the entomocidal activity of the organism. In this paper, we have described the isolation and characterization of a gene from *B. sphaericus* SSII-1 encoding a 100-kDa toxin active against both *C. quinquefasciatus* and *A. aegypti* mosquitoes. We have also shown the homology of an upstream open reading frame with open reading frames of transposons Tn*501* and Tn*21* having unknown function.

Analysis of the deduced amino acid sequence of the 100-kDa protein shows that its N terminus resembles a gram-positive signal sequence which may act as a point of attachment to the bacterial cell membrane. The sequence of hydrophobic amino acids from positions 43 to 60 is a potential membrane-spanning region. Myers and Yousten (29) and Davidson (12) have described toxins predominantly associated with the cell membrane fractions of broken *B. sphaericus* 1593 cells, indicating that some toxins may be present in a membrane-bound form. The 100-kDa toxin has some homology to bacterial toxins which act by ADP-ribosylation of G proteins. We are currently investigating the possibility that the 100-kDa toxin may have a similar mechanism of action.

Putative promoters for the toxin gene closely resemble the vegetative promoters recognized by the *B. subtilis* RNA polymerase associated with σ^{55}, consistent with reports that toxicity in *B. sphaericus* SSII-1 is produced during vegetative growth (27, 28).

The *mtx* gene encoding the 100-kDa toxin was found to be widely distributed among toxic *B. sphaericus* strains, with the exception of those of serotype H26a,26b. The finding that highly toxic strains also contain this gene may be important in determining the target range of the strains. All of the highly toxic strains tested in this study contained the *mtx* gene in addition to the genes for the 51.4- and 41.9-kDa toxins. The similarities between the restriction map presented in Fig. 1 and that presented by Souza et al. (38) for their clone pAS377PT suggest that these authors have cloned the gene encoding the 100-kDa toxin from strain 1593M. They reported that the product of clone pAS377PT in *E. coli* maxicells was a protein of 29 kDa. This protein may represent a proteolytic product of the 100-kDa protein that we have described. It is possible that in the highly toxic strains, the 100-kDa toxin or a peptide derived from it may act synergistically with the 51.4- and 41.9-kDa toxins.

Our results show that *E. coli* transformed with subclones carrying the toxin gene exhibits more stable toxic activity than does *B. sphaericus* SSII-1. Our initial cosmid clone also showed low toxin stability, similar to that of SSII-1. This result suggests that a factor responsible for this instability may be encoded within the region cloned in cosmid pKF2 but deleted in later subclones. This factor may be, for example, a specific proteolytic enzyme involved in the breakdown of the 100-kDa toxin. The instability of the toxin in *B. sphaericus* SSII-1 may explain the low toxicity observed in this strain. It is not known whether the toxicity of the 100-kDa toxin in highly toxic strains is subject to rapid

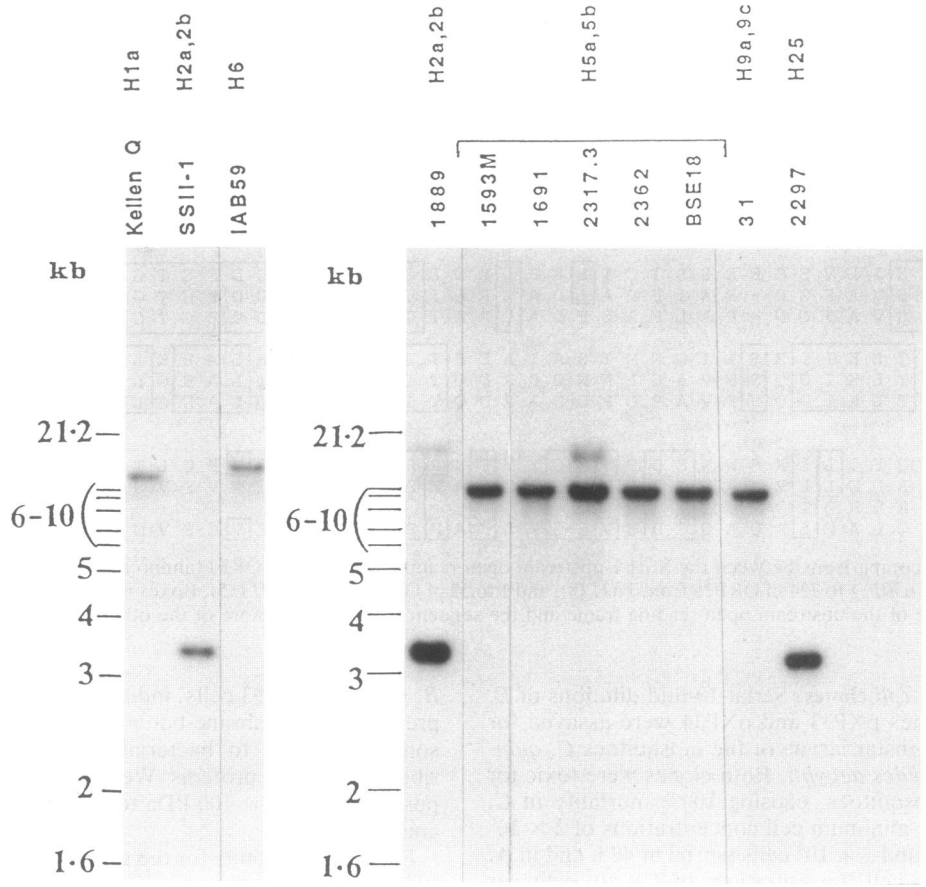

FIG. 6. Southern blots of *Cla*I-digested DNA from various high- and low-toxicity strains of *B. sphaericus* hybridized with a 420-bp radiolabeled probe derived from the 100-kDa toxin gene. High-toxicity strains: IAB59, 1593M, 1691, 2317.3, 2362, BSE18, and 2297. Low-toxicity strains: Kellen Q, SSII-1, 1889, and 31.

degradation during storage, as the activity of this toxin is likely to be masked by the activity of the 51.4- and 41.9-kDa toxins found in these strains.

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