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Sequence Analysis of the Mosquitocidal Toxin Genes Encoding 51.4- and 41.9-Kilodalton Proteins from *Bacillus sphaericus* 2362 and 2297

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The nucleotide sequences of a 3,479-base-pair *Hin*dIII DNA fragment from *Bacillus sphaericus* 2362 and a 2,940-base-pair fragment from strain 2297 were determined; only minor differences were detected between them. Each contained two open reading frames coding for proteins of 51.4 and 41.9 kilodaltons. Both proteins were required for toxicity to larvae of the mosquito *Culex pipiens*.

The parasporal crystal synthesized by sporulating cultures of *Bacillus sphaericus* is toxic to larvae of a number of disease-transmitting mosquito species (27). The predominant proteins in crystals obtained from 48-h cultures of *B. sphaericus* 2297 and 2362 have molecular masses of 125, 110, 63, and 43 kilodaltons (kDa) as estimated from their migrations in sodium dodecyl sulfate-polyacrylamide gels (3, 5). The 110-, 63-, and 43-kDa proteins from strain 2362 have been purified, and only the 110- and 43-kDa proteins are toxic to larvae of *Culex pipiens* (3, 5). Immunological analyses with antisera prepared against the 63- and 43-kDa proteins indicated that these molecules are antigenically distinct, since they have no detectable cross-reactivity (3). From the observation that both antisera reacted with the 110- and 125-kDa proteins, we inferred that the 125-kDa protein was the precursor of the 110-kDa peptide which, in turn, was degraded to the 63- and 43-kDa peptides. Our data on the kinetics of synthesis of crystal proteins during sporulation (5), as well as the cloning and expression in *Escherichia coli* of the genes coding for the crystal proteins (2), were interpreted in line with these initial observations. Sequencing of a 3.5-kilobase DNA fragment coding for the 63- and 43-kDa proteins of *B. sphaericus* 2362 showed that this interpretation is untenable, since the two are on separate genes. Recently, the DNAs coding for the 43-kDa proteins from *B. sphaericus* 1593 and 2362 have been cloned into *E. coli* (4, 12) by using oligonucleotide probes based on the N-terminal sequence data for the 43-kDa protein of strain 2362 (3) to detect the appropriate recombinants. From their DNA sequences it was concluded that the 43-kDa peptide does not arise from a higher-molecular-weight precursor (4, 12).

In the present communication, we present the sequences of the DNAs coding for the 63- and 43-kDa proteins from *B. sphaericus* 2297 and 2362. Since the deduced molecular masses of these two proteins are 51.4 and 41.9 kDa, respectively, we have designated them accordingly. Strain 2297 was chosen for comparison since it had a restriction pattern different from that of strains 1593, 1691, and 2362 (2).

MATERIALS AND METHODS

Cloning and DNA sequencing procedures. The procedures previously described (2) were used to clone the genes coding for the 51.4- and 41.9-kDa proteins of *B. sphaericus* 2297 into *E. coli*, with λgt11 as the vector (15). Two recombinants were obtained which were designated gt1 and gt20 (Fig. 1). Their characteristics were similar to those of the λgt11 recombinants containing the corresponding genes from *B. sphaericus* 2362 (3). *E. coli* containing gt20 made a protein which, in Western immunoblots, comigrated with the 51.4-kDa protein from the crystal of *B. sphaericus* 2362 and reacted only with antiserum to this protein. Similarly, *E. coli* containing gt1 made a protein which had the antigenic and electrophoretic properties of the 41.9-kDa protein (results not shown). A 3.5-kilobase *Hin*dIII DNA fragment containing both genes from *B. sphaericus* 2362 was previously cloned into the vector pGEM-blue and designated pGA-5 (2).

For sequencing, the DNA fragments indicated in Fig. 1 were subcloned into M13mp18-M13mp19 by standard methods (18, 19). A series of overlapping deletions was obtained by using the IBI Cyclone System (International Biotechnologies, Inc., New Haven, Conn.), which is based on the procedure of Dale et al. (8). For the 3,479-base pair (bp) *Hin*dIII DNA fragment from *B. sphaericus* 2362, both strands of the segments consisting of nucleotides (nt) 1 to 2698 and 3185 to 3479 were sequenced by the dideoxy chain termination method (20) (Fig. 2). Only one strand of the region, comprising nt 2699 to 3184, was sequenced, since it was identical to the DNA sequence found by Berry and Hindley (4) (spanning bp 1689 to 3368 of our sequence). For *B. sphaericus* 2297, the DNA corresponding to nt 399 to 3336 in Fig. 2 was sequenced.

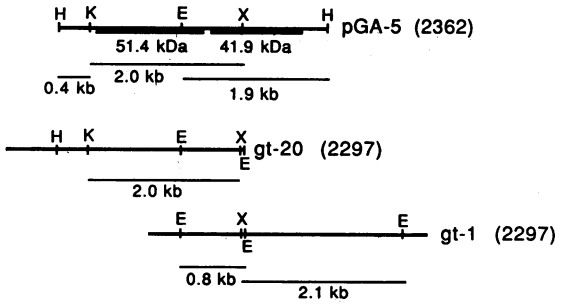

FIG. 1. Restriction map of the *B. sphaericus* DNA used for sequence analysis. Fine lines delineate DNA fragments which were subcloned into M13mp18-M13mp19 and used to generate a series of overlapping deletions for sequencing. The positions of the 51.4- and 41.9-kDa proteins are indicated. Numbers in parentheses indicate the strain of *B. sphaericus* from which the DNA was obtained. Abbreviations: H, *Hin*dIII; K, *Kpn*I; E, *Eco*RI; X, *Xba*I.

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CTTGTCAACATGTGAAGATTAAAGGTAACTTTCAGTTTCTTTCTGTGTAACAATACACGAAGTAATATATGTATTTCTATAGAAATTAAT      90

CAAAAAAAGACCTAGTGAACTGCACCCTGTCAAGTAGATAGTGGAAATAATAAAAATGTATTAAGCGGCTTGTGCTCTTAATTCTAAGAA     180

GGTGATGAAAAACTTGCACTCTAGGGCGTACCAAGCAATCAAAGGAGAAACGTTGTATCTCCGGATTAATTCGAACTACAACGTCTATTG     270

TAGATTAAACCGTAAGAGATTATATAATTATTATAACAATACTTTAATTAATTCAATTGAAATTAAACAAACAACTTTTTCGGTATATAC     360

TATCTAACATATCGGTTTCAGTCCCATCGTTCTAAAGGTACCTTCTTTTTGGTTAACGTTATTTAATGAACTTTTTAGGTTTTAAATAAT     450

ATAATGAGAAGTATTTTTTATCAATGATAAGGAGATGAAGAAAGCATGTGCGATTCAAAAGACAATTCTGGCGTTTCAGAAAAATGCGGA     540
                                                  M  C  D  S  K  D  N  S  G  V  S  E  K  C  G

AAGAAATTTACTAATTACCCGCTAAATACTACTCCTACAAGCCTAAATTATAACCTTCCAGAAATATCAAAAAAATTTTATAACCTTAAG     630
K  K  F  T  N  Y  P  L  N  T  T  P  T  S  L  N  Y  N  L  P  E  I  S  K  K  F  Y  N  L  K

AATAAATATTCACGGAATGGTTATGGTTTATCAAAAACCGAATTTCCTTCAAGTATCGAAAATTGCCCATCTAACGAATATTCAATAATG     720
N  K  Y  S  A  N  G  Y  G  L  S  K  T  E  F  P  S  S  I  E  N  C  P  S  N  E  Y  S  I  M

TATGATAATAAAGATCCTCGATTCTTGATTCGGTTTTTATTAGATGATGGTAGATATATTATTGCAGATAGAGACGATGGAGAAGTTTTT     810
Y  D  N  K  D  P  R  F  L  I  R  F  L  L  D  D  G  R  Y  I  I  A  D  R  D  D  G  E  V  F

GATGAAGCACCTACTTATTTGGATAATAACAATCACCCTATCATAAGTAGACATTATACCGGAGAAGAGAGACAAAAGTTTGAGCAGGTA     900
D  E  A  P  T  Y  L  D  N  N  N  H  P  I  I  S  R  H  Y  T  G  E  E  R  Q  K  F  E  Q  V

GGTAGTGGAGATTATATTACGGGAGAGCAATTTTTTCAATTCTATACACAAAACAAAACACGTGTATTGTCAAATTGTAGGGCGCTTGAC     990
G  S  G  D  Y  I  T  G  E  Q  F  F  Q  F  Y  T  Q  N  K  T  R  V  L  S  N  C  R  A  L  D

AGTAGGACAATATTACTATCTACTGCAAAAATCTTCCCAATTTACCCTCCAGCTTCTGAAACTCAACTAACAGCTTTCGTTAATAGTTCA    1080
S  R  T  I  L  L  S  T  A  K  I  F  P  I  Y  P  P  A  S  E  T  Q  L  T  A  F  V  N  S  S

TTTTATGCTGCGGCAATTCCTCAATTACCCCAAACATCCTTACTTGAGAATATTCCTGAGCCTACTAGTCTCGATGATTCTGGAGTATTA    1170
F  Y  A  A  A  I  P  Q  L  P  Q  T  S  L  L  E  N  I  P  E  P  T  S  L  D  D  S  G  V  L

CCAAAAGATGCAGTAAGAGCAGTTAAAGGAAGTGCGCTATTACCTTGTATAATAGTACATGATCCTAATTTAAACAATTCCGATAAAATG    1260
P  K  D  A  V  R  A  V  K  G  S  A  L  L  P  C  I  I  V  H  D  P  N  L  N  N  S  D  K  M

AAATTTAATACCTACTATCTTTTAGAATATAAAGAATACTGGCATCAATTATGGTCACAAATTATACCTGCTCATCAAACTGTAAAAATA    1350
K  F  N  T  Y  Y  L  L  E  Y  K  E  Y  W  H  Q  L  W  S  Q  I  I  P  A  H  Q  T  V  K  I

CAGGAACGAACAGGAATATCTGAAGTTGTACAAAATAGCATGATTGAAGATTTAAATATGTATATTGGAGCAGATTTTGGCATGCTTTTT    1440
Q  E  R  T  G  I  S  E  V  V  Q  N  S  M  I  E  D  L  N  M  Y  I  G  A  D  F  G  M  L  F

TATTTTAGATCTAGTGGATTTAAGGAACAAATAACAAGGGGGCTAAATAGGCCTTTATCCCAAACGACCACTCAGTTAGGAGAAAGAGTA    1530
Y  F  R  S  S  G  F  K  E  Q  I  T  R  G  L  N  R  P  L  S  Q  T  T  T  Q  L  G  E  R  V

GAAGAAATGGAGTATTATAATTCTAATGATTTGGATGTTAGATATGTGAAATACGCATTGGCTAGAGAATTCACACTAAAACGCGTTAAT    1620
E  E  M  E  Y  Y  N  S  N  D  L  D  V  R  Y  V  K  Y  A  L  A  R  E  F  T  L  K  R  V  N

GGTGAAATTGTAAAAAATTGGGTTGCTGTAGATTATCGATTGGCAGGTATACAATCGTATCCTAATGCACCTATAACTAATCCACTTACG    1710
G  E  I  V  K  N  W  V  A  V  D  Y  R  L  A  G  I  Q  S  Y  P  N  A  P  I  T  N  P  L  T

CTAACAAAACATACAATTATTCGATGTGAAAATAGTTACGATGGACACATATTTAAAACACCTTTAATCTTTAAAAATGGTGAAGTTATT    1800
L  T  K  H  T  I  I  R  C  E  N  S  Y  D  G  H  I  F  K  T  P  L  I  F  K  N  G  E  V  I

GTAAAAACGAATGAAGAATTAATACCTAAAATTAACCAGTGATACTTTAACTTCAAATATTCATTACCATGTTATTTAAAATAGTAGATA    1890
V  K  T  N  E  E  L  I  P  K  I  N  Q

GATGAAATAAATAGTATATATTAAGACAACAACTTAATTTTGACACATAAGAATAATTTTTAAATGTATAAATAGTATTTAGAGTGTTAT    1980
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RESULTS

Deductions from the sequence of *B. sphaericus* 2362. Two large open reading frames (ORFs) were found in the DNA sequence (Fig. 2). The first ORF extends from the methionine at nt 496 to nt 1839 and codes for a protein of 448 amino acids with a deduced molecular weight of 51,373. This protein migrates in sodium dodecyl sulfate-polyacrylamide gels at a rate corresponding to a protein of about 63 kDa (3). After six stop codons in the same reading frame, the second ORF begins at nt 2014 and terminates at nt 3123. The deduced molecular weight of the protein coded by this region is 41,873, representing 370 amino acids. In sodium dodecyl sulfate-polyacrylamide gels its migration corresponds to about 43 kDa (3). The stop codon at nt 3124 to 3126 is followed by seven additional stop codons within the next 100 nt. At nt 481 a putative Shine-Dalgarno (S-D) sequence is found (GGAGA); the spacing between the middle A and the ATG initiation codon (12 bp) is typical of that found in

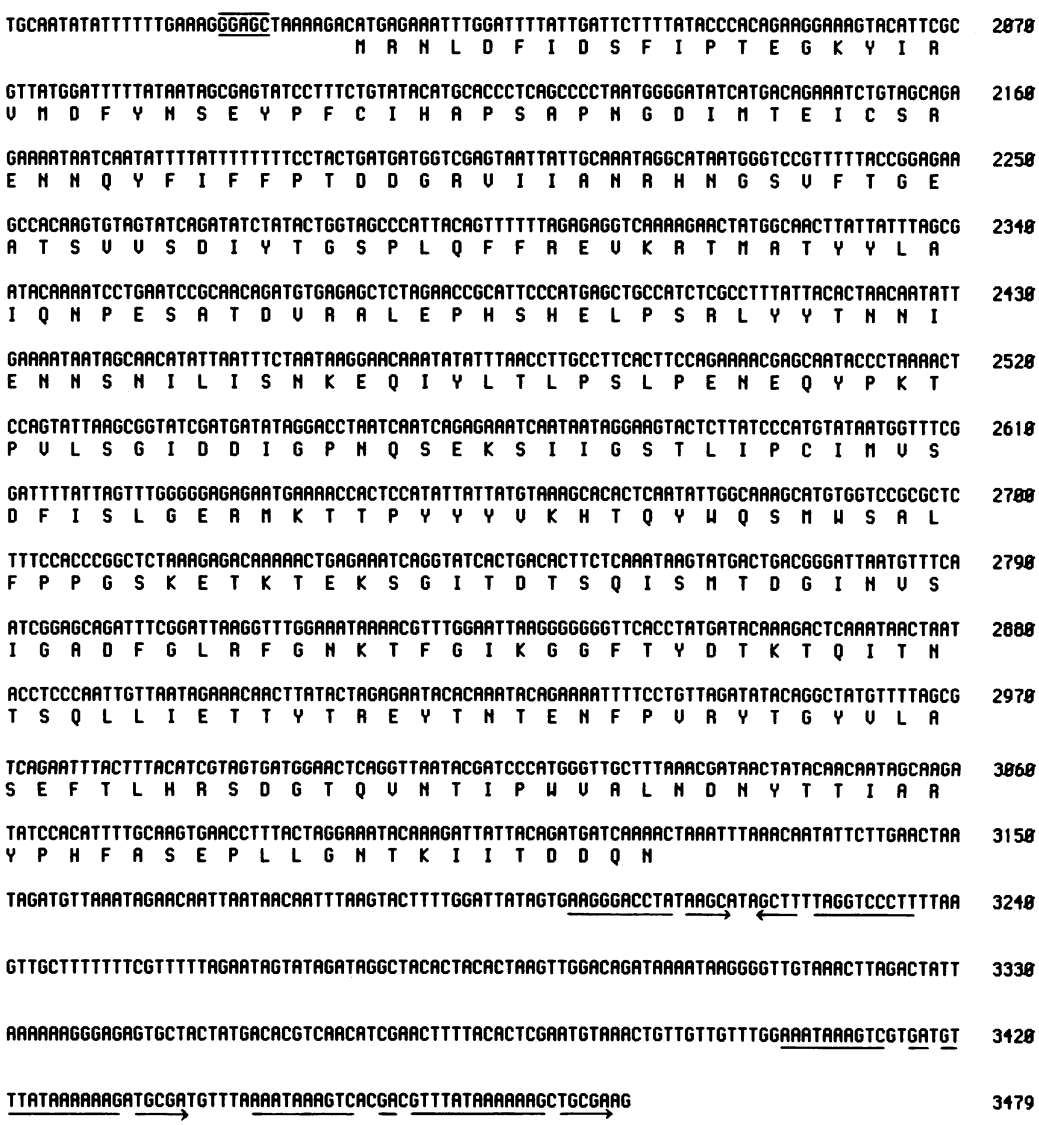

FIG. 2. Nucleotide sequence of the 3,479-bp *Hin*dIII DNA fragment from *B. sphaericus* 2362 which contains the structural genes for the 51.4- and 41.9-kDa proteins. The predicted amino acid sequence is given in the single-letter code. Double lines designate potential S-D sequences. Arrows designate a potential hairpin structure and two direct repeats.

gram-positive organisms (11). The sequence AAGGAG ATGA, which begins at position 479, matches, in 9 of 10 nt, the corresponding sequence at the 3′ end of the 16S rRNA of *Bacillus subtilis*. The free energy of binding between this region of the *B. sphaericus* mRNA and its counterpart on the *B. subtilis* 16S rRNA (calculated by the method of Tinoco et al. [23]) is −15.2 kcal/mol (−63.6 kJ/mol), which is well within the range found in gram-positive organisms (11). As noted by Berry and Hindley (4), another potential S-D sequence (GGAGC) begins at nt 2001. The middle A in this sequence is 10 bp away from the ATG codon which initiates the 41.9-kDa protein. The sequence AAGGGAGCTAA which begins at nt 1998 matches 8 of 11 corresponding positions at the 3′ end of the *B. subtilis* 16S rRNA. The free energy of binding was calculated to be −10.6 kcal/mol (−44.4 kJ/mol), which is considerably lower than the average (−16.7 kcal/mol [−69.9 kJ/mol]) found in gram-positive organisms (11). Since the 3′ sequence of *B. sphaericus* 16S rRNA is not known, the significance of this difference cannot be assessed.

The fragment consisting of nt 3204 to 3236 contains an inverted repeat which is complementary in 14 of 15 nucleotides. This G+C-rich hairpin loop, which is followed by a row of T's, suggests a transcription termination signal. No comparable hairpin structures were found in any other part of the sequence, including the DNA region between the sequences coding for the 51.4- and 41.9-kDa proteins (nt 1840 to 2013), suggesting that these crystal protein genes reside within a single transcriptional operon. This is consistent with our past observation that these two molecules are synthesized at about the same time in sporulating cells (5). Furthermore, in a recombinant in which the genes for β-galactosidase and the 51.4-kDa protein were fused, expression of both the fusion product and the 41.9-kDa protein was under the control of β-galactosidase inducer (2).

We made a search for DNA sequences similar to known promoters in *B. subtilis* (10) and *Bacillus thuringiensis* subsp. *kurstaki* (26) and *israelensis* (24) and found a number of potential candidates. However, since promoter sequences are relatively A+T rich and the A+T content of *B. sphae-*

ricus DNA is high (about 65 mol%) (27) and since known sporulation promoters exhibit considerable differences (10), a conclusion regarding their significance cannot be made. At the end of the *Hin*dIII fragment are two direct repeats (nt 3404 to 3437 and 3444 to 3477) which match in 30 of the 34 nt. The row of A's bounded by regions of G's and C's is reminiscent of a sequence found in the inverted repeats of Tn*4430*, a transposon of *B. thuringiensis* (17).

Two additional short ORFs of 180 and 96 nt were detected in the 3.5-kb *Hin*dIII fragment. The first begins at nt 185 and ends at nt 364. Since it is not preceded by a region resembling a S-D sequence, it is probably not expressed. The second region begins at nt 3351 and ends at nt 3446. It is preceded by a potential S-D sequence which has a high similarity to the S-D sequence preceding the structural gene for the 41.9-kDa protein. Hindley and Berry (12) also detected this short ORF in *B. sphaericus* 1593.

Properties of the 41.9- and 51.4-kDa proteins deduced from their amino acid sequences. A comparison of the N-terminal portion of the deduced sequence of the 41.9-kDa protein with that of the protein purified from the crystal of *B. sphaericus* 2362 (3) indicates that the latter molecule is missing the first four amino acids. Our past work has shown that toxicity of the crystal-derived 41.9-kDa molecule is greatly enhanced by at least two other processing steps. When the crystal is ingested by mosquito larvae, it is modified by the larval gut proteases (consisting of chymotrypsinlike and trypsinlike enzymes [6]), which remove six additional amino acids from the N terminus and approximately 20 amino acids from the C terminus (Fig. 3), resulting in a 54-fold increase in the toxicity of the protein for tissue culture-grown cells of *Culex quinquefasciatus* (3, 6, 9). In the deduced amino acid sequence, residue 349 is an arginine. This may provide a site for cleavage by trypsin, thereby reducing the C terminus by 21 amino acids. A summary of the possible steps involved in the processing of the 41.9-kDa molecule is shown in Fig. 3.

Using the MacGene Plus program (Applied Genetic Technology, Inc., Fairview Park, Ohio), we have searched for sequence similarity between the 41.9- and 51.4-kDa proteins and the Lepidoptera- or Diptera-active proteins from *B. thuringiensis* subsp. *kurstaki* (1, 21), *berliner* (13), and *israelensis* (22, 24, 25). No similarity was detected. However, significant sequence similarity was found between the 41.9- and the 51.4-kDa proteins. Starting with amino acid 85 of the 51.4-kDa protein and amino acid 56 of the 41.9-kDa protein, the two molecules could be aligned so that 87 of 333 amino acids were identical. A summary of these matches, together with the necessary adjustments (which involved the introduction of gaps or, in one case, the removal of an amino acid) is illustrated in Fig. 4. It is noteworthy that the identical amino acids contained within the last 163 amino acids common to both proteins aligned without any adjustments.

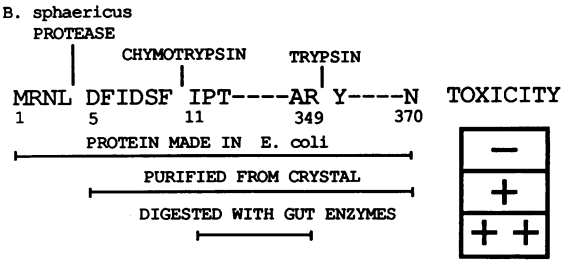

FIG. 3. Possible steps involved in the activation of the 41.9-kDa larvicide. Vertical lines indicate positions at which the protein is cleaved. Horizontal lines indicate the length of the protein. Symbols: −, no toxicity for larvae (2); +, toxicity for mosquito larvae and tissue culture-grown cells (3, 5); ++, increased toxicity for tissue culture-grown mosquito cells (6).

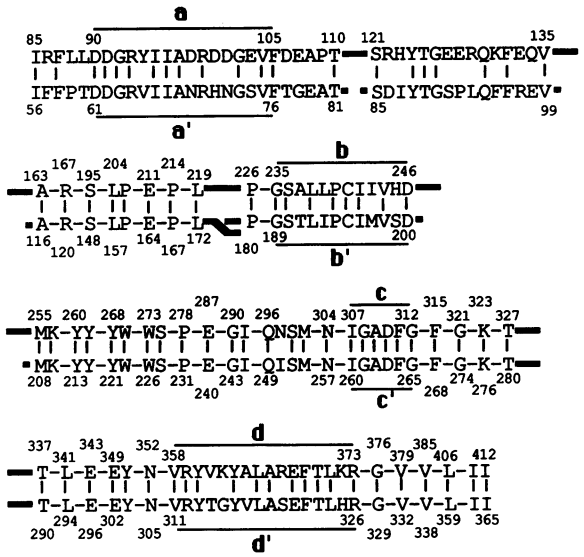

FIG. 4. Amino acid sequence similarity of the 51.4- and 41.9-kDa proteins. The top sequence is from the 51.4-kDa protein; the bottom sequence is from the 41.9-kDa protein. Numbers designate the positions of the amino acids in the sequence. Vertical lines indicate matching amino acids. The thick bars at the ends of the clusters represent the adjustments necessary for alignment. Bars of equal length represent an equal number of nonmatching amino acids; those of unequal lengths represent gaps; the overlapping bar indicates the removal of one amino acid. Clusters of amino acids bounded by lines and designated by letters indicate the major regions of sequence similarity.

We have analyzed the hydropathy of the deduced amino acid sequences of the 51.4- and 41.9-kDa proteins by using the computerized method of Kyte and Doolittle (16). The results of this analysis (with a window of 9 amino acids), as well as the location of the regions showing the greatest amino acid sequence similarity, are presented in Fig. 5. Several features emerge from this comparison. The most highly conserved amino acid sequences, b,b′ and c,c′, are also the major hydrophobic regions of these two proteins. Sequences a,a′ and d,d′ are similar in that they are primarily hydrophilic. The hydrophobic regions are presumably internal to the protein and may have the potential to interact with the cell membrane (14).

Comparison of the DNA sequence from *B. sphaericus* 2362 with sequences from other strains of this species. The differences between the sequence of the 2,940-nt DNA fragment from *B. sphaericus* 2297 and the analogous sequence of strain 2362 (corresponding to positions 399 to 3336 in Fig. 2) are summarized in Table 1. The substitution of an A for a C at nt 2386 created an *Eco*RI site in strain 2297, which distinguishes it from strains 1593, 1691, and 2362 (2). Most of the changes were in the DNA sequence coding for the 41.9-kDa protein; 7 of the 12 changes were silent, and the remaining 5 involved amino acid substitutions. Two of six changes in the 51.4-kDa protein were silent. The three changes spanning nt 1435 to 1446 involved two nearly adjacent substitutions: a leucine was replaced by a tyrosine, and a nearby phenylalanine was changed to a leucine. Hindley and Berry (12) have indicated that in comparisons of

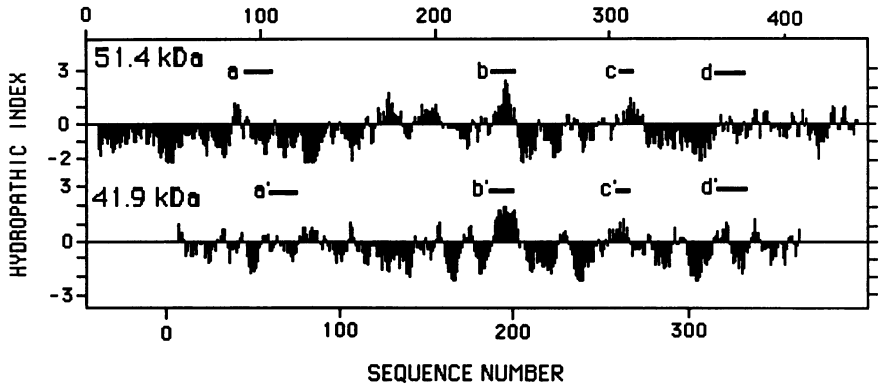

FIG. 5. Hydropathic analysis of the deduced amino acid sequences of the 51.4- and 41.9-kDa proteins; plots are aligned by the identical sequences in b and b'. The letters designate the highly conserved regions shown in Fig. 4.

B. sphaericus 1593 and 2362, the positions corresponding to nt 1615 to 3451 in Fig. 2 are identical. Our data for strains 2297 and 2362 showed an additional G at nt 1613, following nt 166 of the sequence obtained for strain 1593 by Hindley and Berry (12). Inclusion of this G in their sequence creates an ORF of 392 nt corresponding to the latter portion of the 51.4-kDa protein gene. This is consistent with our past work, which established that the 51.4-kDa protein is made by strain 1593 (3).

TABLE 1. Sequence differences between the DNAs of *B. sphaericus* strains

Sequence region	Strain 2362		Nucleotide in strain:		Amino acid change
	Position	Nucleotide	2297[a]	1593[b]	
51.4 kDa	1435, 1436	CT	TA		L to Y
	1446	T	G		F to L
	1455	T	C		None
	1660	T	A		L to M
	1677	G	A		None
Space	after 1844		insert CT		Noncoding
	1851	C	A		Noncoding
	1909	T	A		Noncoding
	1994	T	A		Noncoding
41.9 kDa	2139	C	T		None
	2169	T	C		None
	2253	C	T		None
	2308	G	T		V to F
	2323	G	T		A to S
	2386	C	A		H to N
	2412	T	C		None
	2417	A	T		Y to F
	2490	A	T		None
	2643	C	G		None
	2745	C	T		None
	2813	G	A		R to K
51.4 kDa[c]	1506	G		C	None
	1507	A		C	T to P
	1582	T		C	Y to H

[a] Comparisons involved nt 399 to 3336 in Fig. 2.
[b] Comparisons involve nt 1448 to 3451 in Fig. 2 (12).
[c] The sequence compared began at the latter part of the DNA coding for the 51.4-kDa protein (12).

DISCUSSION

From the data presented in this study and the reinterpretation of our past results, it is apparent that the crystal of *B. sphaericus* contains at least two larvicidal proteins. The initial protein made in the course of sporulation is a 125-kDa molecule; its decrease is paralleled by the appearance and accumulation of a 110-kDa peptide (5). Since the toxicity of the latter protein can no longer be explained by its degradation to a toxic 41.9-kDa peptide, it is clear that it, together with its presumed 125-kDa precursor, constitutes another toxin. The possibility that two of our other categories of clones (2) might code for a 110- and/or 125-kDa toxin is presently under investigation.

In our previous studies we found that the crystal-derived 41.9-kDa protein, but not the 51.4-kDa peptide, was toxic to mosquito larvae (3). Two findings which remain to be explained are the absence of toxicity of the recombinant-produced 41.9-kDa protein and the observation that a mixture of the recombinant-produced 41.9- and 51.4-kDa proteins is toxic (2). When mixed in a 1:1 ratio, recombinants gt1 and gt20 from strain 2297 killed 50% of the larvae of *C. pipiens* at 0.49 μg (dry weight)/ml, a concentration comparable to that observed with combinations of recombinants from strain 2362 (2). Although the mode of action of the *B. sphaericus* crystal toxins is not understood, electron-microscopic evidence indicates that the target is the gut epithelial cells of the insect (7, 27). It is possible that the pathway of sequential activation of the 41.9-kDa protein proposed in Fig. 3 is initiated by a *B. sphaericus*-mediated removal of the four N-terminal amino acids, making the protein susceptible to further activation by larval gut proteases. We have no explanation for the requirement of both the 41.9- and the 51.4-kDa recombinant-produced proteins for toxicity. Their structural similarity (Fig. 4 and 5) suggests that the 51.4-kDa peptide may provide a function which is masked in the nonactivated 41.9-kDa protein. The difference in the charges of these two proteins (3) indicates that they may be held together in solution by electrostatic bonds and function as a single unit. These speculations offer several lines of inquiry which we are currently pursuing.

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