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Cloning of the Gene for the Larvicidal Toxin of *Bacillus sphaericus* 2362: Evidence for a Family of Related Sequences

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During sporulation, *Bacillus sphaericus* 2362 produces a parasporal crystalline protein which is toxic for the larvae of a number of mosquito species. Using the *Escherichia coli* cloning vector λgt11, in which gene products of the inserts may be fused to β-galactosidase, we isolated 29 bacteriophages which produced peptides reacting with antiserum to crystal protein. On the basis of restriction enzyme analyses of the recombinants and Ouchterlony immunodiffusion experiments with induced lysogens as a source of antigens, the recombinants were assigned to three groups, designated A, B, and C. Group A consisted of three clones which appeared to express all or part of the *B. sphaericus* toxin gene from their own promoters and one clone producing a β-galactosidase-toxin fusion protein. The host cells of two induced recombinant lysogens of this group were toxic to larvae of *Culex pipiens*. A cell suspension containing 174 ng (dry weight) of the more toxic recombinant per ml killed 50% of the larvae. Both recombinants formed peptides with molecular sizes of 27, 43, and 63 kilodaltons (kDa). The antigenically related 27- and 43-kDa peptides were distinct from the 63-kDa peptide, which resembled crystals from sporulating cells of *B. sphaericus* in which antigenically distinct 43- and 63-kDa proteins are derived from a 125-kDa precursor. A 3.5-kilobase *Hin*dIII fragment from recombinants having toxic activity against larvae was subcloned into pGEM-3-blue. *E. coli* cells harboring this fragment were toxic to mosquito larvae and produced peptides of 27, 43, and 63 kDa. The distribution of the *A* gene among strains of *B. sphaericus* of different toxicities suggested that it is the sole or principal gene encoding the larvicidal crystal protein. The two recombinants of group B and the 23 of group C were all β-galactosidase fusion proteins, suggesting that in *E. coli* these genes were not readily expressed from their own promoters. The distribution of these two genes in different strains of *B. sphaericus* suggested that they do not have a role in the toxicity of this species to mosquito larvae.

Bacillus sphaericus is a species which includes isolates pathogenic to the larvae of a number of mosquito species that are important vectors in the transmission of certain human and animal diseases (18, 30). Larvicidal activity is not present in all strains, and those which are effective against larvae can be subdivided according to their degree of toxicity (30). All highly toxic strains contain a parasporal crystalline inclusion composed of a protein which is solubilized under alkaline conditions (10, 11, 24, 30). Antisera to the proteins from this inclusion completely eliminate toxicity, indicating that in these strains, the crystal protein is the principal or sole larvicidal toxin (6, 9).

Several studies have suggested that the undegraded crystal protein has a molecular size of about 125 kilodaltons (kDa) (6, 7). Analysis of the kinetics of synthesis of the larvicide in strain 2362 has shown that once exponential growth is completed, the 125-kDa protein begins to accumulate. Degradation to lower-molecular-weight peptides occurs during the course of sporulation (7). The crystal from a 48-h culture of this strain consists primarily of proteins of 43, 63, and 110 kDa. These proteins have been purified, and it has been established that the 110- and 43-kDa proteins have toxic activity against larvae of *Culex pipiens*, whereas the 63-kDa peptide does not. Only the 43-kDa peptide is toxic for tissue culture-grown cells of *Culex quinquefasciatus* (7, 8). These results suggest that the sporulation-associated degradation of the high-molecular-weight protein results in the conversion of a protoxin (125, 110 kDa) to a toxin (43 kDa).

After ingestion by larvae, the 63-, 110-, and 125-kDa peptides in purified crystal from a 48-h culture of *B. sphaericus* 2362 are rapidly degraded, while the 43-kDa peptide is converted to a molecule of 40 kDa (8). In vivo studies have shown that the conversion of the 43-kDa peptide to the 40-kDa peptide is performed by larval gut proteases from several mosquito species and that the resulting peptide has a 54-fold increase in toxicity against tissue culture-grown cells of *C. quinquefasciatus* (8). Thus, the pathway of activation of the 125-kDa protoxin to a 40-kDa toxin appears to involve sequential activation reactions associated with sporulation and proteolysis in the larval gut.

In this communication we describe the cloning of the larvicidal crystal gene of *B. sphaericus* 2362 into the *Escherichia coli* vector λgt11 (16). This vector has a number of useful properties in that recombinant genes which cannot be expressed by *E. coli* can be detected by immunological methods as fusion proteins with β-galactosidase. The expression of these fusion proteins is greatly enhanced by the addition of isophenylthio-D-β-galactoside (IPTG), an inducer of β-galactosidase, at the time of induction of the lysogens. Genes which can be expressed by their own promoters may also be detected as β-galactosidase fusions or as separate peptides. In the latter case, the addition of IPTG to induced lysogens should have little or no effect on expression since these genes are not transcribed from the β-galactosidase promoter. In our past studies we have obtained antisera to the purified, non-cross-reacting 43- and

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63-kDa peptides. These antisera as well as an antiserum to a mixture of the 43-, 63-, and 110-kDa peptides have been used for the detection of proteins in recombinant λgt11 plaques. Our results indicate that the gene encoding the crystal larvicide of this species belongs to a family of at least three related genes.

MATERIALS AND METHODS

Bacterial strains. The strains of *B. sphaericus* used in this study were the gift of A. A. Yousten. *E. coli* TB-1 was purchased from Bethesda Research Laboratories (Gaithersburg, Md.). *E. coli* Y1090r$^-$ and Y1089r$^-$ were included in the Protoclone λgt11 System kit obtained from Promega Biotech (Madison, Wis.). *E. coli* JM107 (28) was the host strain for M13mp19.

General procedures. Most of the methods used in this study have been described by Maniatis et al. (22). These include restriction endonuclease analysis of DNA, agarose gel electrophoresis, and mini-preparations of λ bacteriophage and plasmid DNA. Transformation of competent cells by plasmid DNA was performed by the procedure of Hanahan (14). The enzymes and substrates purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.), New England Biolabs (Beverly, Mass.), and Promega Biotech were used according to the directions provided by the manufacturers. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as described previously (6). The proteins were electroblotted onto nitrocellulose paper and detected with antiserum to the 43-kDa crystal peptide (anti-43), antiserum to the 63-kDa peptide (anti-63), or a mixture of both antisera with the Promega Biotech Protoblot Immunoscreen System (Western blot, immunoblot) as described previously (6). Bioassays were performed as detailed by Baumann et al. (6) with either *E. coli* Y1089r$^-$ containing induced lysogens of the λgt11 recombinants or, in the case of subclones in pGEM-3-blue, early-stationary-phase cells of *E. coli* TB-1. Protein content was determined by the method of Lowry et al. (21), with bovine serum albumin as the standard.

Construction of genomic library. The Protoclone λgt11 System (Promega Biotech) was used in these studies. The methods are described in detail by Huynh et al. (16) and the manufacturer. Only an outline of the methods and the minor modifications introduced in our study will be given. Cells of *B. sphaericus* 2362 were grown and lysed as detailed by Krych et al. (17), and the DNA was purified by the method of Ballard et al. (3). The DNA was diluted to 50 μg/ml and sheared by 500 passages through a 25-gauge needle. The DNA fragments in this preparation had a size range of 5 to 8 kilobases (kb). From the results of our initial experiments performed by the procedures of Huynh et al. (16), it appeared that there were difficulties associated with methylation of three adjacent *Eco*RI sites in one of the cloned genes (see Results and Discussion). We therefore subjected 5 μg of DNA to sequential treatment with 2-μg quantities of *Eco*RI methylase, followed with precipitation of the DNA with acetate-ethanol to remove glycerol introduced with the enzyme (22). This procedure was repeated five times. After extraction with phenol, the ends of the DNA were filled in with T4 DNA polymerase. Following another phenol extraction, phosphorylated *Eco*RI linkers (New England Biolabs) were blunt-end ligated, and the preparation was treated with *Eco*RI. Free linkers were removed by passage through a G-50 (Pharmacia, Uppsala, Sweden) spun column (22), and the DNA was ligated into *Eco*RI-cut and phosphatase-treated λgt11. With the Packagene Lambda DNA System (Promega Biotech), the DNA was packaged and plated onto the indicator strain (*E. coli* Y1090r$^-$). Five parallel preparations had titers ranging from 0.3×10^6 to 4.8×10^6 PFU/ml; 57 to 92% of the phage had inserts.

Immunological detection of recombinants. We used the Protoblot λgt11 immunoscreening system (Promega Biotech) according to the directions of the manufacturer and Huynh et al. (16). Approximately 2×10^4 bacteriophage particles were plated, with *E. coli* Y1090r$^-$ as the host. After an incubation of 3.5 h at 42°C, the developing plaques were overlaid with a nitrocellulose filter disk impregnated with 10 mM IPTG and incubated for another 3.5 h at 37°C. The filters were removed, and the proteins present in the plaques were exposed to antiserum to the 110-, 63-, and 43-kDa proteins from *B. sphaericus* 2362 (anti-cry; 1/500 dilution). Plaques containing proteins reacting with anti-cry were visualized by adding anti-rabbit immunoglobulin G (IgG) conjugated to alkaline phosphatase to the filters and then treating with a chromogenic alkaline phosphatase substrate (Promega Biotech). The phages were plaque purified at least three times. During the last purification of some of the clones, a mixture of anti-43 and anti-63 (1/500 dilution of each) was substituted for anti-cry, with no difference in the intensity of the reaction noted. Of a total of 6.3×10^5 recombinants screened, we obtained 29 bacteriophages which made proteins with antigenic determinants of the crystal peptides.

Preparation of cell extracts and Ouchterlony immunodiffusion experiments. *E. coli* Y1089r$^-$ was lysogenized as described (16). Before cell extracts were prepared (4), both the lysogen and β-galactosidase were induced by the method of Huynh et al. (16). Ouchterlony immunodiffusion experiments were performed as previously detailed (4), with anti-cry at a dilution of 1:8.

Southern blot hybridization with M13 probes. The appropriate *Eco*RI fragments from the recombinants were subcloned into M13mp19 by the procedure of Messing (23). The replicative form of the recombinant M13mp19 phage was purified by $CsCl_2$-ethidium bromide density gradient centrifugation (22). DNAs from different strains of *B. sphaericus* were digested with the appropriate enzymes, electrophoresed in agarose gels, and electroblotted onto Zeta-Probe membranes (Bio-Rad Laboratories, Richmond, Calif.) by the procedures recommended by the manufacturer. Radiolabeled hybridization probes of M13mp19 recombinants containing the appropriate inserts were obtained with a nick translation kit and [^{35}S]dATP (Amersham Corp., Arlington Heights, Ill.) in accordance with the instructions of the manufacturer. Hybridization of the probes to DNA immobilized on Zeta-Probe membranes was carried out as described by Bio-Rad. X-ray film (AR) (Eastman Kodak Co., Rochester, N.Y.) was exposed to the membrane for 12 to 24 h at 22°C. The film was then processed as outlined by the manufacturer.

Subcloning into GEM-3-blue. The pGEM-3-blue vector was purchased from Promega Biotech and used according to their instructions, with *E. coli* TB-1 as the host.

RESULTS AND DISCUSSION

A total of 29 recombinants were characterized by a variety of procedures. A schematic representation of the results of Ouchterlony immunodiffusion experiments is shown in Fig. 1. The significance of spurring patterns among antigens is discussed in Baumann et al. (4). Restriction maps of the λgt11 recombinants are presented in Fig. 2, 4, and 6. On the

basis of these results and the molecular weights of the proteins detected in Western immunoblots (see Fig. 3 and 5), the recombinants were assigned to three groups, which will be considered in turn.

Group A. In Ouchterlony immunodiffusion experiments, the induced lysogens of gt84, gt91-2, and gt168-2 formed single precipitin bands which gave reactions of identity among themselves (Fig. 1). All formed a spur over gt91-1, yet its reactions of partial identity with group B and nonidentity with group C placed it in group A. Figure 2 presents the restriction maps derived for the four recombinants of this group, and Fig. 3 shows a Western immunoblot in which the proteins were detected with antiserum specific to the 43-kDa peptide (anti-43; panel A) and antiserum specific to the 63-kDa peptide (anti-63; panel B) of *B. sphaericus* 2362 (6). One recombinant (gt84) contained three bands of 150, 43, and 27 kDa. The 150-kDa molecule reacted only with anti-63, and the 43-kDa band only with anti-43, while the band(s) at 27 kDa reacted strongly with anti-43 and weakly with anti-63. All the bands were greatly reduced when IPTG was omitted from the medium (results not shown). These findings suggest that a 34-kDa fragment of the 63-kDa crystal protein (140 kDa minus the molecular weight of β-galactosidase), in tandem with the entire 43-kDa protein, is fused to the β-galactosidase of *E. coli* and that in this strain, as in *B. sphaericus* the 43-kDa moiety is released by proteolysis (7). The location of the fusion protein, relative to restriction sites in the insert of gt84, indicates that transcription of the gene must proceed from left to right (Fig. 2). Retention of antigenic determinants of the 63-kDa peptide in the fusion protein and release of the 43-kDa peptide provide evidence that the latter is in the C-terminal portion of the 125-kDa larvicidal protein while the 63-kDa peptide resides at the N-terminal region.

The protein levels in gt91-1, gt91-2, and gt168-2 were not significantly affected by IPTG (results not shown), suggesting that the gene was being transcribed from its own promoter. The principal peptides produced by recombinants gt91-2 and gt168-2 were 43 and 27 kDa, with a minor peptide at 24 kDa; all reacted with anti-43 (Fig. 3). Both clones also contained a peptide of 63 kDa and a faint peptide at 27 kDa which reacted with anti-63. Western immunoblots of induced lysogens of gt91-2 and gt168-2 resembled the crystal from a 48-h culture of *B. sphaericus* 2362 in that both contained 43- and 63-kDa peptides. They differed in containing a 27-kDa protein and in lacking 110- and 125-kDa peptides.

Recombinant gt91-1 (Fig. 2) made a single protein of about 63 kDa with antigenic determinants of the 63-kDa crystal protein and no detectable proteins with antigenic determinants of the 43-kDa crystal peptide (Fig. 3). This observation, together with the position of the end of the insert (Fig.

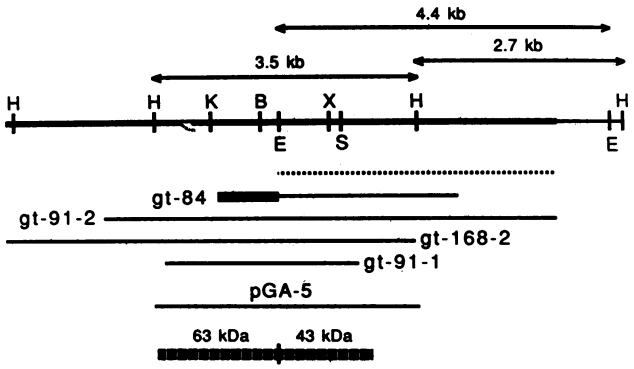

FIG. 2. Restriction analysis of group A recombinants. Letters indicate restriction sites: B, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; S, *Sac*I; X, *Xba*I. The locations of the *Eco*RI and *Hin*dIII sites on the thin line at the right end of the map are based on a map obtained by using the M13mp19 hybridization probe. The dotted line indicates the fragment used as a hybridization probe in the experiments presented in Fig. 7 and 8 as well as Table 2. The thick bar at the left end of gt84 represents the portion of the 63-kDa protein (expressed as base pair equivalents) which was fused to β-galactosidase. For each recombinant, the side bearing the designation indicates the position of the λgt11 β-galactosidase gene. The thick dashed line at the bottom of the figure gives the approximate length of the 63- and the 43-kDa proteins, as base pair equivalents, as well as their tentative position on the map.

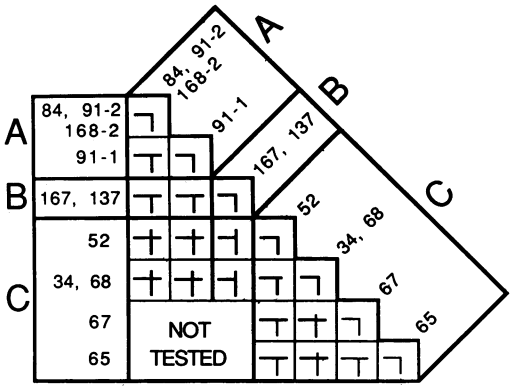

FIG. 1. Schematic representation of the results from Ouchterlony immunodiffusion experiments with cell extracts of induced lysogens reacting with anti-cry. For group C, only results for selected recombinants are shown. In each square, the lines represent the precipitin bands formed between antibody diffusing from a well positioned in the bottom left corner and antigens diffusing from wells at the top and right sides. Recombinants listed diagonally were in the top wells and those listed vertically were in the right wells. The width of the lines gives a rough indication of the intensity of the band. Fused lines indicate a reaction of antigenic identity or near identity, a single spur a reaction of partial identity, and a double spur a reaction of nonidentity. Letters designate the groups to which the lysogens were assigned.

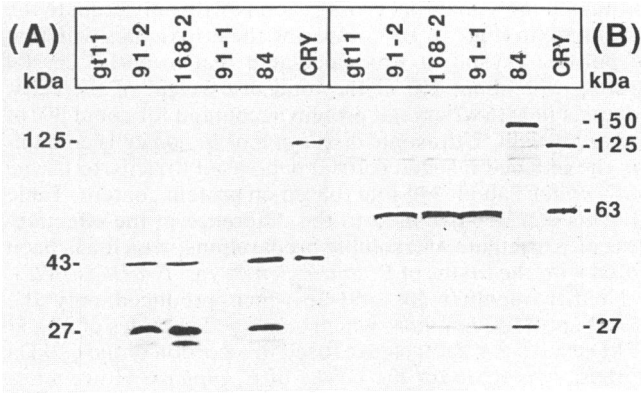

FIG. 3. Western immunoblots of cell extracts of induced lysogens of the λgt11 recombinants of group A, developed with anti-43 (A) and anti-63 (B) antisera. The amount of sample used is indicated in parentheses after the designation: λgt11 (150 μg), gt91-2 (150 μg), gt168-2 (100 μg), gt91-1 (100 μg), gt84 (50 μg), crystal (3 μg). Figure shows a 12% (vol/vol) polyacrylamide gel.

TABLE 1. Larvicidal activities of recombinants containing inserts from *B. sphaericus* 2362

Prepn	Amount (ng/ml) of prepn required		95% Confidence limits for LC_{50} (ng/ml)	
	LC_{50}	LC_{90}	Lowest	Highest
B. sphaericus 2362 crystal	5.3	12.5	4.9	5.7
λgt91-2	556	1,410	530	584
λgt168-2	174	480	166	183
λgt168-2 soluble protein[a]	32,300	87,100	29,360	35,500
λgt91-1	—[b]			
λgt84	—			
λgt91-1 and λgt84[c]	430	1,580	398	464
λgt11	—			
pGA-5	567	1,260	540	595
pGEM-3-blue	—			

[a] Cell extract; protein determined by method of Lowry et al. (21).
[b] —, Maximum amount tested was 100 μg (dry weight) of cells per ml.
[c] Equal amounts (dry weight) of λgt91-1 and λgt84.

2), provides additional evidence that the 63-kDa peptide is located at the N-terminal region of the crystal protein while the 43-kDa peptide is in the C-terminal region and again indicates that the direction of transcription (Fig. 2) is from left to right. The absence of antigenic determinants of the 43-kDa peptide in extracts of gt91-1 explains the spur formed over this recombinant by all the remaining strains of group A (Fig. 1).

The nature of the 27-kDa bands observed in Fig. 2 is not clear. In gt91-1 a minor band at 27 kDa reacted with anti-63 but was not detected by anti-43, whereas in two of the other recombinants the 27-kDa bands reacted with both antisera. This suggests the presence of two peptides of about 27 kDa with different antigenic determinants. From the relative intensities of the 43- and 63-kDa bands, it appears that the anti-43-reacting 27-kDa protein is a degradation product of the 43-kDa molecule. The origin of the anti-63-reacting 27-kDa band is unknown.

E. coli cells containing induced lysogens of gt91-2 and gt168-2 were toxic to second to third instar larvae of *C. pipiens* (Table 1). The amount of cells (dry weight) needed to kill 50% of the larvae (LC_{50}) was higher for gt91-2 than for gt168-2. This is consistent with the observation that gt168-2 contained more material cross-reacting with antiserum to the crystal toxin (Fig. 3). By comparing these toxicities with that of purified crystal, it was calculated that in gt91-2 crystal protein was about 1% of the total dry weight of the cells, whereas in gt168-2 crystal protein accounted for about 3% of the dry weight. Ultrasonic disruption of *E. coli* cells containing the induced lysogen gt168-2 decreased toxicity to larvae of *C. pipiens* about 370-fold (based on protein content; Table 1). This can be attributed to the difference in the effectiveness of particulate and soluble preparations, which has been noted with the toxins of *B. thuringiensis* var. *israelensis* (25).

Neither recombinant gt91-1, which produced only the 63-kDa protein, nor gt84, which contained peptides of 27 and 43 kDa and a β-galactosidase fused to a portion of the 63-kDa protein, was toxic for the larvae of *C. pipiens*. However, a mixture of equal amounts of these preparations did exhibit toxicity (Table 1). This finding appears to contradict our previous results showing that the purified 43-kDa peptide alone (derived from the crystal of *B. sphaericus* 2362) is toxic for larvae of *C. pipiens*. We have no adequate explanation for this observation. In addition, the toxicity of the combined extracts suggests a possible role for the 63-kDa protein in the potentiation of toxicity.

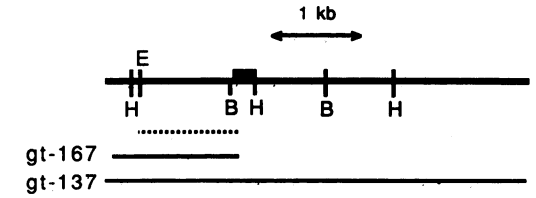

FIG. 4. Results of the restriction analysis of group B recombinants. For abbreviations, see the legend to Fig. 2. The thick bar on the map indicates the calculated position of the C terminus of the fusion protein. The dotted line indicates the hybridization probe used in the experiments presented in Fig. 7 and 8 and in Table 2.

From an inspection of the overlapping regions of recombinants gt91-2 and gt168-2 (Fig. 2), as well as the results of the Western immunoblots (Fig. 3) and the bioassays (Table 1), it appeared that the 3.5-kb *Hin*dIII fragment would contain the complete gene for the 125-kDa protoxin, including the promoter region. This possibility was consistent with the fact that about 3.3 kb is necessary to code for a protein of 125 kDa. The 3.5-kb *Hin*dIII fragment was therefore subcloned into the plasmid vector pGEM-3-blue to produce pGA-5. *E. coli* TB-1 cells harboring this fragment and harvested in the early stationary phase of growth were toxic to larvae of *C. pipiens* (Table 1). Western blots revealed the presence of peptides of 27, 43, and 63 kDa, as previously observed with gt91-2 and gt168-2 (Fig. 3).

Group B. The two recombinants of this group made fusion proteins which gave a reaction of identity in immunodiffusion plates (Fig. 1). The restriction map of the recombinants is presented in Fig. 4. Western immunoblots showed evidence for degradation of the gt137 protein in *E. coli* Y1089r$^-$ (Fig. 5). The highest-molecular-weight fusion protein bands contained *B. sphaericus* protein fragments of about 69 and 62 kDa for gt137 and gt167, respectively. Although cells containing these fusion proteins were found to be nontoxic to larvae of *C. pipiens* (up to 100 μg [dry weight] of cells per ml tested), it should be noted that such proteins might be inherently nontoxic due to the absence or masking of active sites (26).

Group C. Of the 29 recombinants reacting with anti-cry, 23 were found to belong to group C. Despite extensive treat-

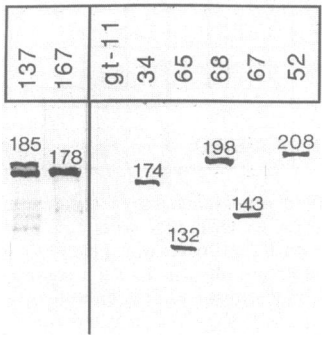

FIG. 5. Western immunoblots of cell extracts of induced lysogens of the λgt11 recombinants containing fusion proteins of groups B (gt137 and gt167) and C (gt34, gt65, gt68, gt67, and gt52), developed with a mixture of anti-43 and anti-63. The sizes of the fusion proteins (in kilodaltons) are indicated above each band. Each lane contained 12 μg of protein; 5% (wt/vol) polyacrylamide gel.

ment of the *B. sphaericus* DNA with *Eco*RI methylase (see Materials and Methods), 20 of these 23 recombinants had inserts which began at one of three adjacent *Eco*RI sites (Fig. 6). Furthermore, five of the seven inserts started at the first (left) *Eco*RI site (Fig. 6) and ended at the second or third *Eco*RI site. All three sites were in phase with the β-galactosidase gene of λgt11, allowing the production of fusion proteins and favoring the detection of this group of recombinants. Although methylation of these *Eco*RI sites was not uniform (perhaps due to their proximity), it was evident that this DNA could be methylated, since 10 recombinants extended over the third *Eco*RI site, four recombinants spanned the fourth *Eco*RI site, and the tail ends of the majority of the recombinants ended randomly (Fig. 6).

In addition to establishing the members of group C as a distinct group, the immunodiffusion data were useful in differentiating some of the inserts on the basis of size (Fig. 1). For example, gt52, which had the largest portion of the *B. sphaericus* protein (Fig. 5 and 6), formed a spur over all the remaining group C recombinants (Fig. 1). While the difference between gt34 and gt68 (57 and 82 kDa, respectively) was not sufficient to be distinguishable by Ouchterlony immunodiffusion (producing a reaction of identity), both proteins formed a double spur over gt67 and gt65 (Fig. 1). The latter two formed light precipitin bands due to the small size of the fusion protein (Fig. 5 and 6) but were nevertheless distinguishable from one another by their spurring patterns (Fig. 1).

Fusion proteins from representative members of this group were not toxic to larvae of *C. pipiens* (up to 100 μg [dry weight] of cells per ml tested).

Distribution of the A, B, and C sequences in other *B. sphaericus* strains. Table 2 lists some of the properties of the *B. sphaericus* strains screened in this survey. *Eco*RI fragments from representatives of each of the recombinant groups (dotted lines in Fig. 2, 4, and 6) were subcloned into M13mp19 and then used as probes in Southern hybridization experiments. Figure 7 presents the results of probing *Eco*RI-digested DNA from low-toxicity strains SSII-1 and Kellen K and highly toxic isolates 1593, 2362, and 2297. The results for *Hin*dIII digests are presented in Fig. 8, and a summary of all these results is shown in Table 2. The results for strain 1691 are included in Table 2.

In the highly toxic strains 1593, 1691, and 2362, the group A probe hybridized to fragments of identical size (4.4 kb in the *Eco*RI-digested DNA and 3.5 and 2.7 kb in the *Hin*dIII digests) (Fig. 7A and 8A, Table 2). Strain 2297, which is also highly toxic, differed from the others in having a 2.1-kb *Eco*RI fragment and a faint 0.94-kb *Eco*RI piece as well as a single 4.6-kb *Hin*dIII fragment. There was no detectable hybridization between the A probe and DNA from the low-toxicity strains (SSII-1 and Kellen K). Hybridization experiments with a probe from the B group detected similar sequences in strains 1593, 1691, and 2362 (a 5.2-kb *Eco*RI fragment and a 1.8-kb *Hin*dIII piece) which were absent from strains 2297 and SSII-1. Strain Kellen K was unique in binding the B probe to a 6.7-kb *Eco*RI piece and a 3.9-kb *Hin*dIII fragment. Sequences complementary to probe C were present only in strains 1593, 1691, and 2362.

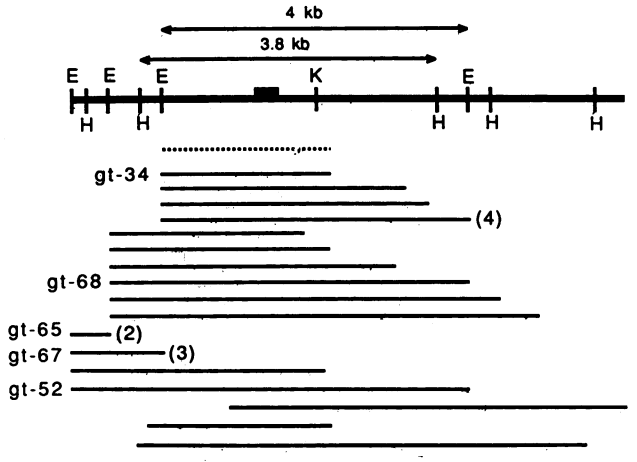

FIG. 6. Restriction analysis of recombinants of group C. See the legend to Fig. 2 for details. Numbers in parentheses indicate the number of times a particular insert was isolated.

These results are in agreement with the phage groupings of Yousten (29) and the flagellar serotypes of De Barjac et al. (12). They also imply that only the protein encoded by recombinants of group A is necessary for high toxicity, since strain 2297, which lacks all but the group A gene, had a level of toxicity comparable to that of strains 1593, 1691, and 2362. Minimal or no involvement of the group B gene in toxicity is indicated by the fact that the Kellen K strain, which is mildly toxic, contained a sequence complementary to the B probe but was not significantly more toxic than SSII-1, which lacked all three sequences.

In the experiments presented in Fig. 7 and 8, prolonged exposure of the X-ray film to the membranes revealed additional faint bands. In Fig. 7B and 8B, light bands corresponding to those in Fig. 7C and 8C were detected, and the converse was also observed (results not shown). These findings provide evidence of nucleotide sequence similarity

TABLE 2. Summary of some properties of the *B. sphaericus* strains used

Strain	LC_{50} (CFU/ml)[a]	Phage group[b]	Flagellar serotype[c]	Size of fragments (kb) of *B. sphaericus* DNA that hybridized to probes					
				*Eco*RI			*Hin*dIII		
				A	B	C	A	B	C
SSII-1	2.5×10^8	2	2						
Kellen K	1×10^8	1	1a		6.7			3.9	
1593	2.5×10^2	3	5a, 5b	4.4	5.2	4.0	3.5, 2.7	1.8	3.8
1691	2.4×10^2	3	5a, 5b	4.4	5.2	4.0	3.5, 2.7	1.8	3.8
2362	1.1×10^2	3	5a, 5b	4.4	5.2	4.0	3.5, 2.7	1.8	3.8
2297	6.5×10^1	4	25	2.1, 0.94			4.6		

[a] Toxicity for *C. pipiens* expressed as heat-resistant CFU. Data for 2362 from Baumann et al. (6) and Broadwell and Baumann (7), for Kellen K from Yousten (30); all remaining values from Davidson and Myers (10).

[b] From Yousten (29).

[c] From De Barjac et al. (12).

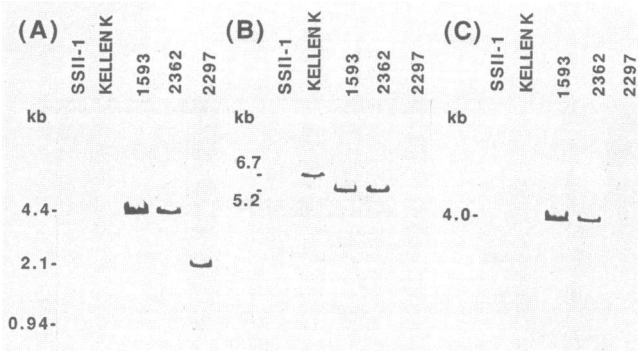

FIG. 7. Southern blots of *Eco*RI-digested DNA from various strains of *B. sphaericus* hybridized with ^{35}S-radiolabeled M13mp19 probes containing inserts from group A (A), group B (B), and group C (C). Each lane contained 8 μg of DNA; 0.8% (wt/vol) agarose gel.

between regions of DNA corresponding to probes B and C. No hybridization was detected between group A and the remaining groups under these conditions despite the considerable antigenic similarity among these gene products. This discrepancy is explained by the different sensitivities of the methods used to measure nucleic acid and amino acid sequence homologies in this study. Antigenic similarities were detected with an antiserum obtained after a relatively lengthy immunization period so that it was able to produce precipitin bands with proteins having as little as 70% amino acid sequence similarity (5). The stringent conditions of our in vitro DNA-DNA hybridization experiments (22) required over 96% nucleotide sequence complementarity for the formation of a stable duplex, precluding DNA hybridization between less closely related nucleic acid sequences.

Relationship to past studies. Ganesan et al. (13) have presented evidence for the cloning of a *B. sphaericus* 1593 larvicide into *E. coli* by using the plasmid vector pHV33. The clone, consisting of a 3.7-kb *Sau*3A insert, was obtained among the first 100 recombinants screened for toxic activity against mosquito larvae. Toxicity was greatly increased in late-stationary-phase cells but only after ultrasonic disruption in the presence of Triton X-100. Probes prepared from the insert hybridized with six *Eco*RI fragments from different strains of *B. sphaericus* (20). Maxicell experiments revealed four peptide products of 12, 15, 19, and 21 kDa (20). The properties of this toxin and its gene are totally different from those of our group A recombinants. The differences include

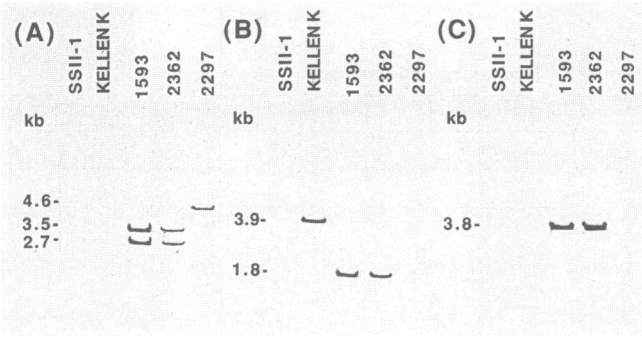

FIG. 8. Southern blots of *Hin*dIII-digested DNA from various strains of *B. sphaericus* hybridized with ^{35}S-radiolabeled M13mp19 probes containing inserts from group A (A), group B (B), and group C (C). Each lane contained 8 μg of DNA; 0.8% (wt/vol) agarose gel.

the sizes of the *Eco*RI fragments hybridizing with the recombinants and the molecular weights of the gene products. The restriction map of our insert bears no relation to the restriction map of their insert despite use of the same restriction enzymes: *Eco*RI, *Hin*dIII, *Bgl*II, *Xba*I, and *Kpn*I (13). Our 3.5-kb *Hin*dIII subclone in pGEM-3-blue was expressed in the exponential as well as the stationary phase of growth (Table 1; results of bioassays and Western immunoblots not shown). Ultrasonic disruption of *E. coli* cells containing the toxin greatly reduced toxicity, as would be expected from the fact that mosquito larvae ingest particulate matter better than soluble material (Table 1) (25). The converse was true for the clone of Ganesan et al. (13); cells disrupted in the presence of Triton X-100 exhibited greatly enhanced toxicity. These major differences in properties clearly indicate that the insert cloned by Ganesan et al. (13) is not the same as the *B. sphaericus* toxin gene isolated in our laboratory.

Summary and conclusions. We have cloned a 3.5-kb *Hin*dIII fragment containing the gene for the larvicidal crystal protein of *B. sphaericus* 2362. The gene is expressed in *E. coli*, and, as in *B. sphaericus* the gene product appears to be cleaved into two antigenically distinct fragments of 63 and 43 kDa. In *E. coli* an additional 27-kDa fragment is observed which contains antigenic determinants of the 43-kDa protein. Two additional protein products derived from *B. sphaericus* DNA cloned into *E. coli* were found to be antigenically related to the crystal toxin. Since these were isolated only as fusion proteins, it is possible that they cannot be expressed in *E. coli*, as is the case for many sporulation genes (19). Despite their antigenic similarity to the crystal toxin, these genes do not appear to contribute to the larvicidal activity of *B. sphaericus* (Table 2). By analogy to *B. thuringiensis* strains which produce a *Lepidoptera*-active crystal protein, it is possible that these genes either are cryptic or, if expressed, make products which are constituents of the *B. sphaericus* spore coat (2). We have not performed studies to localize these genes on either the chromosome or any of the previously described plasmids of *B. sphaericus* (1, 15). The evidence from our studies that the toxic portion of the *B. sphaericus* protein resides in the C-terminal portion of the molecule is in marked contrast to the lepidopteran-specific protoxin of *B. thuringiensis* (2, 27), in which the toxin occupies the N-terminal portion of the protoxin.

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