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Construction by Site-Directed Mutagenesis of a 39-Kilodalton Mosquitocidal Protein Similar to the Larva-Processed Toxin of *Bacillus sphaericus* 2362

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After ingestion of the parasporal crystals of *Bacillus sphaericus*, mosquito larvae process the 42-kilodalton (kDa) toxin to a protein of 39 kDa, which has an increased toxicity (A. H. Broadwell and P. Baumann, Appl. Environ. Microbiol. 53:1333–1337, 1987). A similar activation is performed by trypsin and chymotrypsin. Using site-directed mutagenesis, we have constructed derivatives of the 42-kDa toxin with a deletion of 10 amino acids at the N terminus and deletions of 7, 17, or 20 amino acids at the C terminus. Toxicity for mosquito larvae was retained upon deletion of 7 or 17 amino acids but was lost upon deletion of 20 amino acids. Evidence is presented indicating that the protein containing deletions of 10 amino acids at the N terminus and 17 amino acids at the C terminus (corresponding to potential chymotrypsin cleavage sites) is similar to the 39-kDa protein produced in mosquito larvae or by digestion with chymotrypsin. Digestion with trypsin appears to generate a protein lacking 16 or 19 amino acids from the N terminus and 7 amino acids from the C terminus. As is the case with the recombinant-made 42-kDa protein, toxicity of its derivatives is dependent on the presence of a 51-kDa protein which is a component of the parasporal crystal of *B. sphaericus* 2362.

In the course of sporulation, *Bacillus sphaericus* 2362 makes a parasporal inclusion commonly designated as a crystal (20). Ingestion of this crystal by mosquito larvae is followed by its dissolution in the larval midgut and subsequent breakdown of the midgut epithelium of susceptible species (9, 10, 20). The crystal has been shown to consist of two proteins with molecular masses of 51 and 42 kilodaltons (kDa) (3, 6). Of the two proteins purified from the crystal, only the 42-kDa protein (or its derivative) was found to be toxic to mosquito larvae (4, 19). The genes coding for both of these proteins have been cloned and sequenced (2, 3, 13).

The guts of mosquito larvae contain chymotrypsin- and trypsinlike proteases (5, 16). Larvae which ingest the crystal rapidly degrade the 51-kDa protein with a transient accumulation of a 44-kDa intermediate and slowly degrade the 42-kDa protein to a protein of 39 kDa (1, 4, 7, 8). The latter conversion involves the removal of 10 amino acids at the N terminus (at a chymotrypsin cleavage site) and about 15 to 20 amino acids at the C terminus (Fig. 1) (7). Larval gut proteases, as well as trypsin and chymotrypsin, degrade the purified 42-kDa protein to a peptide of about 39 kDa. Using tissue culture-grown cells of *Culex quinquefasciatus*, it has been shown that the conversion of the 42-kDa protein to 39 kDa by larval gut proteases or by trypsin or chymotrypsin results in a 54-fold increase in toxicity (7; A. H. Broadwell, Ph.D. thesis, University of California, Davis, 1987).

Recently, we overproduced the 51- and the 42-kDa proteins (alone and in combination) in *Bacillus subtilis* (6) and confirmed past results which indicated that, singly, the recombinant-made proteins are not toxic for larvae but are toxic when combined (2, 3). The lack of toxicity of the 42-kDa recombinant-made protein is not due to the failure of larvae to process it to 39 kDa, as was shown by the accumulation of the latter protein in larvae which had ingested the 42-kDa protein-containing inclusion (8).

In the present study, using site-directed mutagenesis, we have deleted 10 amino acids from the N terminus of the 42-kDa protein and obtained a series of truncations at the C terminus (Fig. 1). Bioassays allowed delineation of the C-terminal region required for toxicity to mosquito larvae and the subsequent construction of a protein similar to the 39-kDa peptide which accumulates in their guts.

MATERIALS AND METHODS

Bacterial strains and plasmids. *B. subtilis* DB104, the host strain for the constructs, is deficient in extracellular alkaline and neutral proteases (14). The *B. subtilis* vector, pUB18, is a pUB110 derivative containing a M13mp18 multiple cloning site (21; T. H. Zaghloul, Ph.D. thesis, University of California, Davis, 1986).

Site-directed mutagenesis and expression in *B. subtilis*. Site-directed mutagenesis was performed by the gapped duplex method of Kramer and Fritz (15) as previously described (8). The starting material was construct 1-3a (8), which contains a 1,469-base-pair insert having an *Eco*RI site 3 base pairs before the ATG codon which initiates the 42-kDa protein. Following the region coding for the protein is a potential *B. sphaericus* terminator and a *Hin*dIII site. Our goal was the production of derivatives of the 42-kDa protein with deletions at the N and C termini corresponding to the chymotrypsin or trypsin sites which may be cleaved during the processing of the protein. A list of the oligonucleotides used, together with the relevant sequences, positions, and purposes of the site-directed mutagenesis, is presented in Table 1. The *Eco*RI-*Hin*dIII fragments were ligated to a 32-nucleotide *Bam*HI-*Eco*RI adaptor containing a *B. subtilis* ribosome-binding site (8). The resulting *Bam*HI-*Hin*dIII fragment was placed under the control of the *B. subtilis* subtilisin (*aprE*) promoter assembled in the vector pUB18 (18, 21). Details of these constructions have been previously described (8). The resulting plasmids were desig-

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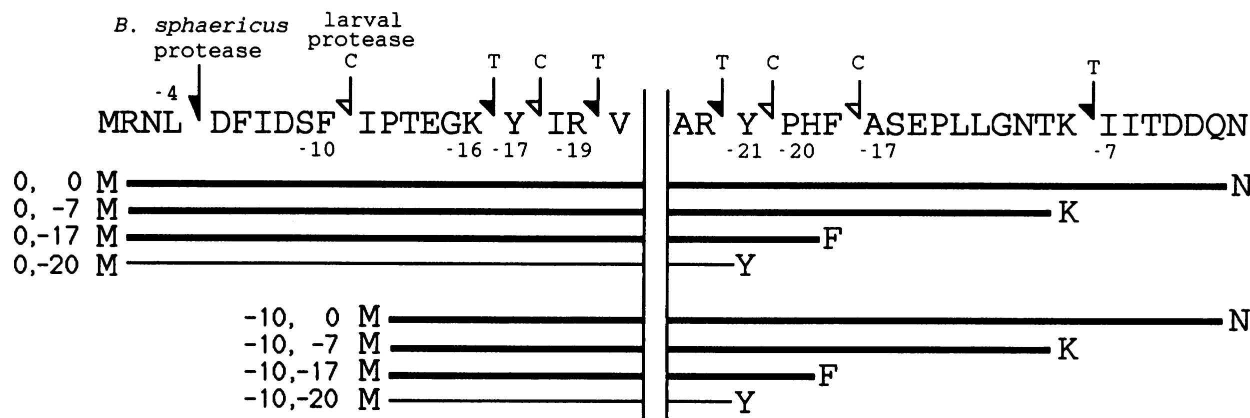

FIG. 1. Amino acid sequences at the N and C termini of the *B. sphaericus* 42-kDa toxin protein and its derivatives. C, Chymotrypsin site; T, trypsin site. Lines indicate the lengths of the proteins made by the constructs. Numbers indicate amino acid positions from the N and C termini. Thick lines indicate the constructs which retain toxicity in the presence of the 51-kDa protein, and thin lines indicate that the constructs are nontoxic.

nated pUE followed by a number (Table 2). In most cases, we will refer to these constructs by the number of amino acids deleted at the N terminus followed by a comma and the number of amino acids deleted at the C terminus (Fig. 1).

Other methods. Standard molecular biological methods were used (17). Application of these methods to *B. subtilis* has been previously described (8). Additional methods, such as sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% [wt/vol] polyacrylamide) and Western blots (immunoblots), have been described in our past publications (4, 7). In all cases, the antiserum used was directed against the 42-kDa protein (4). The preparation of *B. subtilis* cells containing the recombinant-made proteins and the determination of the amount necessary to kill 50% of the larvae of *Culex pipiens* (LC_{50}) were performed as previously described (8).

RESULTS

Properties of the constructs. The position of the open reading frame for the 42-kDa protein and the expected positions of the relevant restriction sites on the *Bam*HI-*Hin*dIII fragment which contained the constructs are shown in Fig. 2. The locations of the restriction sites were confirmed by digestion with *Bgl*II and *Hin*cII (for C-terminal deletions) and with *Bam*HI and *Xba*I (for N-terminal deletions) followed by electrophoresis of the DNA fragments in polyacrylamide gels (results not shown).

Expression in *B. subtilis*. A Western blot showing the highest-molecular-mass derivatives of the 42-kDa protein found in cell extracts of *B. subtilis* containing the recombinant plasmids is presented in Fig. 3. The calculated molecular masses of these proteins are presented in Table 2. A plot of the log of the mass of the proteins (0,0 to 0,−20 and −10,0 to −10,−20; lanes a through d and f through i) against the relative mobility gave a straight line with a correlation coefficient of 0.99 (results not shown). As with protein 0,0 (8), there was considerable degradation of the recombinant-made proteins to fragments smaller than 30 kDa (results not shown). A substantial decrease in the amount of protein detected was noted in extracts containing proteins lacking 20 amino acids at the C terminus (Fig. 3, lanes d and i).

Toxicity of *B. subtilis* containing the various constructs. *B.*

TABLE 1. Oligonucleotides used for site-directed mutagenesis

Position[a]	Length (base pairs)	Relevant sequence[b]	Purpose
1996	31	2008-GAATTCATG A GA	New *Eco*RI site 3 base pairs before the 41.9-kDa protein (mutagenesis performed as described in reference 8)
2025	36	2038-GAATTCATG TCT T A	Deletion of 10 amino acids from the N terminus of the 41.9-kDa protein, change of Ile to Met, new *Eco*RI site 3 base pairs before resulting 40.7-kDa protein
3083	54	NonNon 3103-TAATAAGATCT ATTATTACAGA	Deletion of seven amino acids from the C terminus of the 41.9-kDa protein and addition of two stop codons after amino acid 363 and a *Bgl*II site
3045	56	NonNon 3073-TAATAAGATCT GC AGT A C	Deletion of 17 amino acids from the C terminus of the 41.9-kDa protein and addition of two stop codons after amino acid 353 and a *Bgl*II site
3045	50	NonNon 3064-TAATAAGATCT CC C TTT GC	Deletion of 20 amino acids from the C terminus of the 41.9-kDa protein and addition of two stop codons after amino acid 350 and a *Bgl*II site

[a] Refers to the position of the first nucleotide of the oligonucleotides relative to the sequence of the 3,479-base-pair *Hin*dIII DNA fragment from *B. sphaericus* 2362 containing the genes coding for the 51.4- and 41.9-kDa proteins (3).

[b] Top line gives the sequence of the portion of the oligonucleotide of interest for this study; bottom line gives the original sequence of the *B. sphaericus* DNA. Underlining indicates a restriction site. ATG, Initiating methionine codon; Non, nonsense codon.

TABLE 2. Effect on toxicity to *C. pipiens* larvae of amino acid deletion at the N and C termini of the 41.9-kDa protein[a]

Amino acids deleted at:		Calculated mol mass (kDa)	*B. subtilis* LC_{50} (ng [dry wt]/ml)	Construct designation
N terminus	C terminus			
0	0	41.9	4.2	pUE1-3a[b]
0	−7	41.1	23	pUE387
0	−17	40.1	36	pUE393
0	−20	39.7	>5 × 10^4	pUE391
−10[c]	0	40.7	36	pUE38
−10[c]	−7	39.9	120	pUE388
−10[c]	−17	38.8	170	pUE392
−10[c]	−20	38.5	>1 × 10^5	pUE390

[a] Assayed in the presence of 0.5 μg (dry weight) of *B. subtilis* pUE381, which contains the 51-kDa protein (8).
[b] Construct described in reference 8.
[c] Amino acid 11, isoleucine, was changed to methionine.

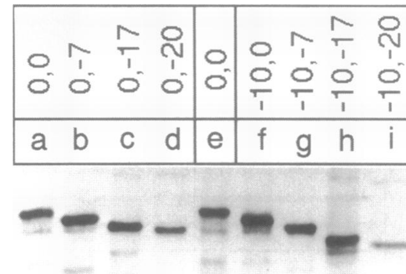

FIG. 3. A portion of a Western blot of extracts of *B. subtilis* showing the highest-molecular-mass bands made by the indicated constructs. (For the deduced molecular masses of the proteins, see Table 2.) The amount of sample used in milligrams (dry weight) of cells for each lane is as follows: a, 0.3; b, 0.3; c, 0.5; d, 1.8; e, 0.3; f, 1.5; g, 1.3; h, 1.8; i, 3.5.

subtilis strains producing the 42-kDa derivatives shown in Fig. 3 were not toxic to the larvae of *C. pipiens* (highest concentration tested, 420 μg/ml). The results of bioassays performed in the presence of 0.5 μg (dry weight) of *B. subtilis* containing pUE381 per ml, which produces only the 51-kDa protein (8), are presented in Table 2. Toxicity was retained with 10 amino acids deleted from the N terminus and the isoleucine at the eleventh amino acid exchanged for methionine. Proteins with deletions of 7 or 17 amino acids deleted from the C terminus retained toxicity, while a deletion of 20 amino acids resulted in a nontoxic protein. Although some of the difference in the LC_{50}s observed in Table 2 can be accounted for by the different amounts of the proteins synthesized by *B. subtilis*, it would appear that modifications of the proteins also have an effect. In the extreme case involving 0,0 and −10,−17 (Fig. 3, lanes a and h, respectively), the amount of the undegraded protein was approximately the same, while the amount of the sample in terms of dry weight of cells was about sixfold higher for −10,−17 than for 0,0. This difference is considerably less than the 40-fold difference between the LC_{50}s of these two preparations, suggesting that the modifications may have contributed to the reduction in the toxicity of the protein.

Comparisons of the −10,−17 protein with the products of protease digestion of the 42-kDa protein. A portion of a Western blot in which a comparison was made of the migration of the −10,−17 protein with that of crystal-derived, 42-kDa protein digested with chymotrypsin, larval gut protease, or trypsin is shown in Fig. 4. The migrations of the principal product of digestion with chymotrypsin or larval gut proteases and the lower band of the doublet resulting from incomplete trypsin digestion were similar or identical (lanes c through e). Unexpectedly, the molecular masses of these products appear to be slightly higher than that of the −10,−17 protein (compare lanes c through e with lanes b and f).

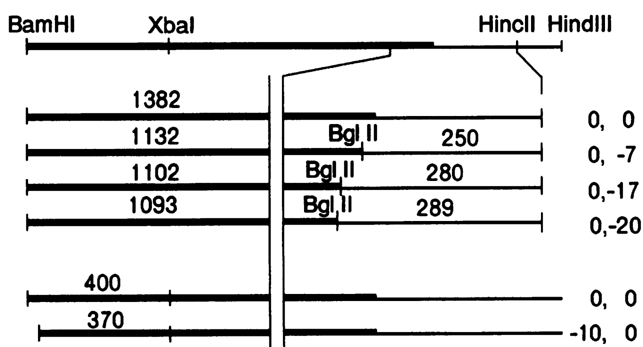

FIG. 2. Restriction map of the *Bam*HI-*Hin*dIII fragments containing the open reading frames (thick lines) of the various constructs, showing the position of the sites cut by the restriction enzymes. Numbers refer to the lengths of the fragments in base pairs. The column of pairs of numbers on the right refers to the number of amino acids deleted at the N and C termini, respectively.

DISCUSSION

A major goal of this work was the construction and overexpression of a protein corresponding to the larval gut protease-processed 39-kDa *B. sphaericus* crystal-derived protein. Previous studies indicated that the processing by gut enzymes involves the removal of 10 amino acids from the N terminus at a chymotrypsin cleavage site (3, 7) (Fig. 1) and approximately 15 to 20 amino acids at the C terminus. We have made proteins lacking 10 amino acids at the N terminus (substituting methionine for isoleucine) and proteins truncated at the chymotrypsin and trypsin cleavage sites at the C terminus (Fig. 1). Bioassays of *B. subtilis* cells containing these proteins indicated that a −10,−17 protein (deletions at chymotrypsin sites) was still active, while a deletion of three

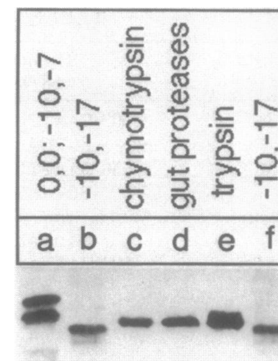

FIG. 4. A portion of a Western blot showing a comparison of selected recombinant-made proteins with the products of protease digestion of the 42-kDa toxin from the *B. sphaericus* crystal. The amount of sample used in milligrams (dry weight) of cells for each lane is as follows: a, 0.2 of 0,0 and 0.8 of −10,−7; b and f, 1.2 of −10,−17. Lanes c, d, and e contain products of digestion of 1.5 μg of the purified 42-kDa toxin.

additional amino acids from the C terminus resulted in a loss of activity (Fig. 1 and Table 2). Cleavage at a potential chymotrypsin site at a position other than −17 (C terminus) cannot occur, since −20 lacks activity and there are no other chymotrypsin sites following −17 (Fig. 1). Our results do not exclude the possibility that the −10,−20 protein may be nontoxic due to its enhanced susceptibility to proteolysis in the gut of mosquito larvae.

Treatment of the purified 42-kDa crystal-derived protein (which lacks the first four amino acids [7] [Fig. 1]) with larval gut proteases or chymotrypsin yielded bands with identical electrophoretic properties and an apparent molecular weight lower than that of the −10,−7 protein and higher than that of the −10,−17 protein (Fig. 4). The difference between the protease-treated proteins and the −10,−17 protein cannot be accounted for by the small difference in the molecular weight resulting from the substitution of methionine for isoleucine in the first amino acid of the −10,−17 protein. We do not have an explanation for these observations.

Treatment of the 42-kDa crystal-derived protein with trypsin yielded a product with a molecular mass of about 39 kDa (7) (Fig. 4). Since a deletion of 20 amino acids resulted in an inactive protein, a deletion of 21 amino acids following a potential trypsin site should also result in an inactive protein (Fig. 1). Deletion of seven amino acids at the only other available trypsin site resulted in a protein which retained activity, indicating that this could be a site of trypsin cleavage. With respect to the N terminus, potential trypsin sites are present at amino acids 16 and 19; the next trypsin site is at amino acid 49 (3). Cleavage at the latter site would give a protein with a calculated molecular mass of 35.4 kDa, which would be detectably smaller than the observed molecular mass of 39 kDa. From the similarity of the apparent molecular masses of trypsin- and chymotrypsin-processed toxin, it would appear that the former lacks 16 or 19 amino acids at the N terminus and seven amino acids at the C terminus.

It has been previously found that the recombinant 42-kDa protein produced in *E. coli* (2, 3, 12), *B. subtilis*, and *B. sphaericus* (strains 718 and SSII-1) (8) is not toxic to larvae of *C. pipiens* unless it is in the presence of the 51-kDa protein. The 42-kDa protein produced by *B. subtilis* is readily processed in the gut of mosquito larvae to a 39-kDa protein, so that the lack of toxicity cannot be explained by the absence of this conversion (8). Consistent with this finding are the results of the present study, in which toxicity of the various recombinant-made 42-kDa protein derivatives was detected only in the presence of the 51-kDa protein. Recently, we determined the ratio of the 42- and 51-kDa recombinant-made proteins required for maximal toxicity to larvae of *C. pipiens*. By using amorphous inclusions containing known amounts of these two proteins, it was found that approximately equimolar concentrations were required for maximal toxicity (L. Baumann, personal communication). We have also purified the 42-kDa protein from amorphous inclusions produced by *B. subtilis* which contain both the 51- and the 42-kDa proteins and have found that this protein is not toxic to mosquito larvae (tested at concentrations of up to 10 μg/ml); toxicity was observed in the presence of the 51-kDa protein. These results do not support our previous suggestion that a transient exposure of the 42-kDa protein to the 51-kDa protein is required for toxicity (8). The results do suggest that the *B. sphaericus* larvicide is a binary toxin in that both components are required for toxicity.

Several laboratories have obtained electrophoretically homogeneous preparations of the 42-kDa protein (or its derivatives) from the crystal of *B. sphaericus* and have found that these preparations were toxic for mosquito larvae and mosquito tissue culture-grown cells (4, 7, 10, 11, 19). The observation that both of the recombinant-made proteins are required for toxicity suggests that the preparations containing the 42-kDa protein purified from the *B. sphaericus* crystal are contaminated with small amounts of the 51-kDa protein or its derivatives. During our initial purification of the 42- and 51-kDa proteins from *B. sphaericus* crystals (4), we noted that considerable degradation of the 51-kDa protein was frequently observed in older cultures. Studies with larval gut proteases and whole larvae also indicated that this protein was readily degraded and that some of the products had molecular masses of 39 to 44 kDa (4, 8). It is possible that the toxicity of the 42-kDa protein preparations is due to contamination with small amounts of degradation products which were not detected in Western blots by antisera to the 51-kDa protein. That at least some degradation products of this size retain activity is indicated by our current studies designed to delineate the active fragment of the 51-kDa protein. Using site-directed mutagenesis, we constructed a 51-kDa protein derivative containing deletions at the N and C termini with a calculated molecular mass of 41.9 kDa which still retained activity (M. A. Clark, personal communication). Future studies with purified 51- and 42-kDa proteins as well as their derivatives will be required in order to understand their role in toxicity.

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