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# Larvicidal Properties of the 42 and 51 Kilodalton *Bacillus sphaericus* Proteins Expressed in Different Bacterial Hosts: Evidence for a Binary Toxin

Andrew H. Broadwell,[1] Linda Baumann, and Paul Baumann

Department of Microbiology, University of California, Davis, California, USA

**Abstract.** The 51 kilodalton (kDa) and the 42 kDa larvicidal proteins of *Bacillus sphaericus* 2362 appear to reside on a single transcriptional unit. Constructs containing the genes for both of these proteins or for only the 51 kDa or the 42 kDa protein were inserted into the appropriate expression vectors and the proteins over-produced in *Escherichia coli* JM105, and *Bacillus sphaericus* strain 718 and SSII-1. In all of these organisms the 51 kDa and 42 kDa proteins were not toxic alone but were toxic in combination. These results are interpreted to indicate that the *Bacillus sphaericus* 2362 larvicide is a binary toxin in that both the 51 and the 42 kDa proteins are required for toxicity.

Many strains of *Bacillus sphaericus* produce, in the course of sporulation, a parasporal crystal which contains proteins of 51 kilodaltons (kDa) and 42 kDa which are toxic for a variety of mosquito larvae [6, 11, 23]. The genes coding for these proteins have been cloned, and it appears that they reside in a single transcription unit [3, 5, 14]. Both the 51 kDa and the 42 kDa proteins, alone and in combination, were expressed in low levels in *Escherichia coli*, presumably using DNA regions with low promoter activity that were functional in this organism [7, 13]. Recently we have used a *Bacillus subtilis* expression vector (pUE) containing the *apr*E (subtilisin) promoter to over-produce the 51 kDa and 42 kDa proteins alone and in combination [9]. Our results indicated that, as in the case of previous studies with *E. coli* [5, 7, 13], in which the proteins were produced in low amounts, the 51 and 42 kDa proteins are alone not toxic for mosquito larvae but are toxic as a mixture. In the present study we have extended these observations by over-producing these proteins in *E. coli* and in two strains of *B. sphaericus*. Our results are interpreted to indicate that the *B. sphaericus* larvicide is a binary toxin in that both proteins are required for toxicity.

[1] Present address: DSIR, Plant Protection, Private Bag, Auckland, New Zealand.

## Materials and Methods

**Bacterial strains and plasmids.** The origins of *Bacillus sphaericus* SSII-1, *B. sphaericus* NSR 718, and *Escherichia coli* JM105 have been previously indicated [5–7]; *B. sphaericus* SSII-1 has a low toxicity for larvae of *Culex pipiens* and lacks the genes for the 51 and 42 kDa proteins [7, 23]. It has a high DNA homology (83%) to the highly toxic strains of *B. sphaericus* that have the genes for the 51 and 42 kDa proteins [16]. Strain 718 is a member of a distinct DNA homology group that is related (62% DNA homology) to the larvicidal strains of *B. sphaericus* [16]. Recently these groupings have been confirmed by a phenotypic characterization [1]. The construction and insertion of recombinants into the *B. subtilis* expression vector pUE (a derivative of pUB18) have been described [9]. pUE contains the *apr*E (subtilisin) promoter followed by a ribosome binding site and an Eco RI site [9]. By use of site-directed mutagenesis, an Eco RI site was placed 3 base pairs (bp) in front of the initiating methionine of the 51 or the 42 kDa proteins and the Eco RI-Hind III fragments inserted into pUE and used to transform *B. subtilis* [9]. The plasmids were purified from this organism and transformed by means of protoplast transformation [9] into *B. sphaericus* strains 718 and SSII-1. The Eco RI-Hind III fragments were also inserted into the *E. coli* expression vector pkk223-3 which is under the regulation of the *tac* promoter [2]. Plasmid C7a, which has a putative *B. sphaericus* promoter, has been described [4]. A diagram of these inserts is presented in Fig. 1.

Address reprint requests to: Dr. Paul Baumann, Department of Microbiology, University of California, Davis, CA 95616, USA.

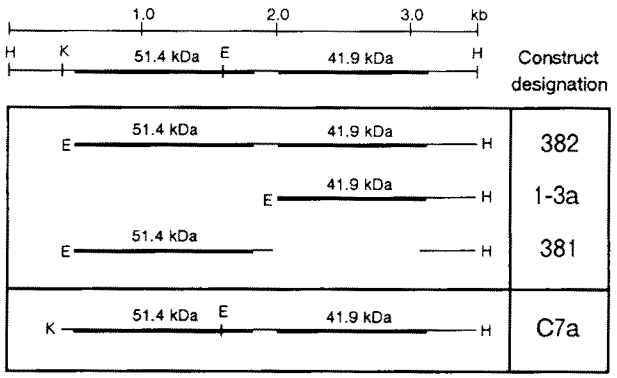


Fig. 1. Diagram of the constructs containing the genes coding for the 51 and/or 42 kDa proteins. Thick line indicates the regions coding for the toxin proteins. E = Eco RI, H = Hind III, K = Kpn I.

**General Methods.** Standard molecular biological methods were used [17], and their application to our work has been detailed in past publications [5, 9]. Sodium dodecyl sulfate polyacrylamide (12%) gel electrophoresis (SDS-PAGE) and Western immunoblots were performed as described [9]. The antiserum used in the Western immunoblots was a mixture of antisera to the 42 and the 51 kDa *B. sphaericus* 2362 proteins.

**Preparation of cells.** *Escherichia coli* JM105 containing the vector pkk223-3 with and without inserts was grown at 37°C in Luria broth [2, 17]. For the expression of the proteins isopropyl β-D-thiogalactoside (IPTG) was added during the exponential phase of growth and the incubation continued for an additional 30 min, at which time the cells were harvested by centrifugation. In the case of *B. sphaericus*, containing pUB18 or pUE with inserts, the cells were grown at 32°C in a complex medium suitable for sporulation, for 32–36 h, at which time they were harvested [9].

**Bioassays.** The concentration (in dry weight cells per ml) necessary to kill 50% of the larvae of *C. pipiens* ($LC_{50}$) was determined as previously described [9]. Dilutions were made of the toxin containing preparations such that the relative amounts of 1, 0.75, 0.5, and 0.25 were maintained at each 10-fold dilution. In total, 30 larvae were used for each dilution. After preliminary experiments, the effect on the $LC_{50}$ of the ratio cells of strain 718 containing the 51 kDa protein (pUE381) and cells containing 42 kDa protein (pUE1-3a) was determined. The following ratios were used (dry weight 42 kDa containing cells/dry weight 51 kDa containing cells): 1/0.15, 1/0.5, 1/1.7, 1/5, 1/15, 1/50. The $LC_{50}$ values are expressed as dry weight of the mixture of the cells with the 42 and 51 kDa proteins.

## Results

**Synthesis of the 42 and 51 kDa proteins in *Escherichia coli*.** The $LC_{50}$ values of IPTG-induced *E. coli* cells containing pkk223-3 with the inserts diagramed in Fig. 1, are presented in Table 1. Only *E. coli* pkk382, which contained the genes for both proteins, was toxic to larvae of *C. pipiens*. The $LC_{50}$ of 280 ng dry weight cells/ml was 20-fold more than that of a 36-h culture of *B. sphaericus* 2362 (14 ng dry weight/ml). *E. coli* pkk1-3a, which contains the gene for the 42 kDa protein, was toxic only in the presence of cells containing the 51 kDa protein (*E. coli* pkk381). Similarly, *E. coli* pkk381, which contains the gene for the 51 kDa protein, was not toxic unless assayed in the presence of *E. coli* pkk1-3a, which contains the 42 kDa protein. The $LC_{50}$ of *E. coli* pkk381 was about six times that of *E. coli* pkk1-3a (Table 1).

The results of the Western immunoblots were in general agreement with the results of the bioassays (Fig. 2, lanes a–e). The principal bands of the recombinant-made proteins (lanes b–d) migrated the same distance as the proteins from the crystal of *B. sphaericus* 2362 (lane a). *E. coli* pkk382 (lane b) produced considerably more of the 51 kDa protein (and a slightly lower molecular weight degradation product) than of the 42 kDa protein (the latter is indicated by an arrow). The relatively high $LC_{50}$ of 280 ng dry weight/ml was due to the low amount of the 42 kDa protein found in extracts of *E. coli* pkk382. This was indicated by the fact that addition of cells containing the 42 kDa protein but not of cells containing the 51 kDa protein led to a decrease in the $LC_{50}$. The highest amount of protein was produced by *E. coli* pkk1-3a (lane c) with the principal bands at 42 kDa and a degradation product at 27 kDa. *E. coli* pkk381 (lane d) produced primarily the 51 kDa protein and a slightly lesser molecular weight degradation product.

**Synthesis of the 42 and 51 kDa proteins in *B. sphaericus* 718 and SSII-1.** In these strains of *B. sphaericus* the genes in the inserts (Fig. 1) are under the control of the *apr*E (subtilisin) promoter of *B. subtilis*, which is expressed maximally during the early stages of sporulation [18]. As in the case of *E. coli*, only cells of *B. sphaericus* containing recombinants with genes for the 42 and 51 kDa proteins (pUE382) were toxic to larvae of *C. pipiens* (Table 1). Cells containing only the 42 kDa (pUE1-3a) or the 51 kDa (pUE381) protein were nontoxic. When these cells were combined, toxicity was observed. The $LC_{50}$ was in all cases lower with *B. sphaericus* 718 than with *B. sphaericus* SSII-1. As in the case of *E. coli*, the $LC_{50}$ values with pUE1-3a (42 kDa protein) were lower than those with pUE381 (51 kDa protein) (Table 1).

Western immunoblots of cell extracts from

Table 1. $LC_{50}$ values of different strains containing plasmids with the toxin genes of *Bacillus sphaericus* 2362[a]

| Construct | Toxin protein made (kDa) | Molecular weight of required protein (kDa) | | Host strain and plasmid | | |
|---|---|---|---|---|---|---|
| | | 51 | 42 | *E. coli* JM105 pkk | *B. sphaericus* 718 pUE | *B. sphaericus* SSII-1 pUE |
| Plasmid | None | | | $>5 \times 10^5$ | $>5 \times 10^5$ | $1.4 \times 10^4$ |
| 382 | 51 + 42 | − | + | 280 | 12[b] | 48 |
| 1-3a | 42 | + | − | 22[c] | 10[b,c] | 19[c] |
| 381 | 51 | − | + | 120[d] | 48[b,d] | 70[d] |
| C7a[e] | 51 + 42 | − | − | NT[f] | 13 | 110 |

[a] $LC_{50}$ values expressed as ng dry weight bacterial cells/ml.
[b] Values from reference [9].
*B. sphaericus* SSII-1 were not toxic at 2 μg/ml (highest concentrations tested).
[c] Assayed in the presence of 0.5 μg dry weight/ml of the same bacterial species containing the 51 kDa protein. Cells of *E. coli* JM105 or *B. sphaericus* 718 with only the 42 kDa protein were not toxic at 420 μg dry weight cells/ml whereas cells of *B. sphaericus* SSII-1 were not toxic at 2 μg/ml (highest concentrations tested).
[d] Assayed in the presence of 0.5 μg dry weight/ml of the same bacterial species containing the 42 kDa protein. Cells of *E. coli* JM 105 or *B. sphaericus* 718 with only the 51 kDa protein were not toxic at 420 μg dry weight cells/ml, whereas cells of *B. sphaericus* SSII-1 were not toxic at 2 μg/ml (highest concentrations tested).
[e] Under the regulation of a putative *B. sphaericus* promoter [4].
[f] NT = not tested.

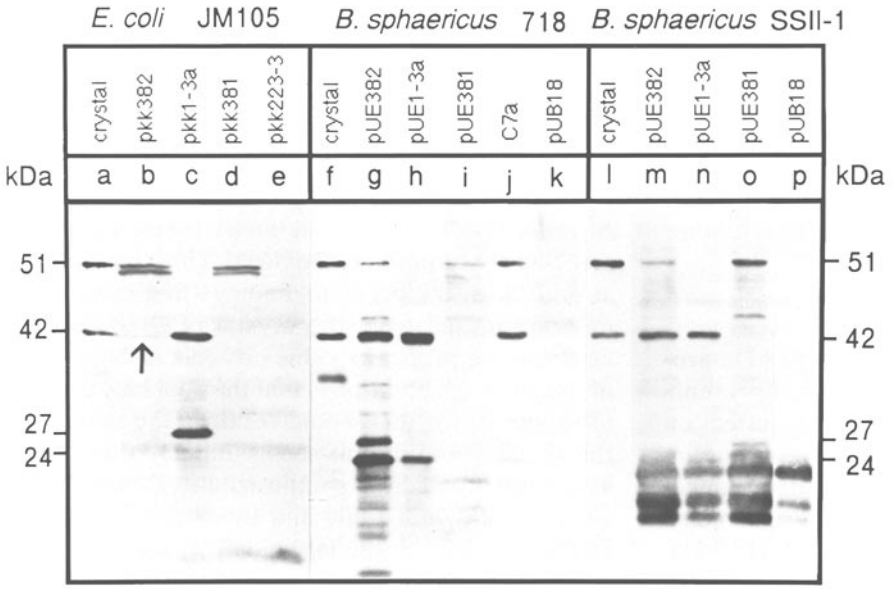


Fig. 2. Western immunoblot of extracts of different bacterial species containing the indicated recombinant plasmids. Bands were detected by antisera to the 42 and 51 kDa crystal proteins. For a diagram of the plasmids see Fig. 1. Crystal = purified crystal from *B. sphaericus* 2362. Arrow in lane (b) indicates the band corresponding to the 42 kDa protein. The amount of sample used (dry weight) is indicated following the lane designation. (a) 3 μg; (b) 1.2 mg; (c) 0.6 mg; (d,e) 1.2 mg; (f) 3 μg; (g,h,i) 0.8 mg; (j) 0.5 mg; (k) 0.8 mg; (l) 3 μg; (m) 0.6 mg; (n) 0.4 mg; (o) 1.2 mg; (p) 0.6 mg.

strains of *B. sphaericus* 718 and *B. sphaericus* SSII-1 containing the recombinant plasmids are presented in Fig. 2 (lanes f–p). In strain 718 considerable degradation of the recombinant-made protein was observed (lanes g–i). The principal degradation product of the 42 kDa protein had a molecular weight of about 24 kDa (lanes g, h). The results with SSII-1 were less satisfactory in that there was considerable background staining and all of the extracts, including extracts of cells with the vector alone, contained material below 26 kDa which reacted with antibody (lanes m–p). Bands migrating with the 51 and 42 kDa crystal proteins were, however, readily detected, and it would appear that there may be somewhat

less degradation of these proteins in this strain. In both strains 718 and SSII-1, the $LC_{50}$ determinations and the Western immunoblots indicated that less of the 51 kDa protein was produced than of the 42 kDa protein.

**Expression of the 42 and 51 kDa proteins from a putative *B. sphaericus* promoter.** Previously [4], we have inserted the Kpn I-Hind III fragment containing the genes for the 51 and 42 kDa proteins (Fig. 1) into pUB18, giving the plasmid C7a, and studied the kinetics of synthesis of these proteins in *B. subtilis*. In the present study, using protoplast transformation, we have placed this plasmid into *B. sphaericus* 718 and SSII-1. The $LC_{50}$ values of these two preparations for larvae of *C. pipiens* are presented in Table 1. There was an eight-fold difference in the $LC_{50}$ values; the lower value was observed with strain 718. Microscopic examination of the cultures at the time of harvesting indicated that 55% of the cells of *B. sphaericus* 718 had sporulated, while less than 0.1% of the cells of *B. sphaericus* SSII-1 had spores. Since the synthesis of the crystal proteins by *B. sphaericus* 2362 occurs midway in the sporulation cycle [8], it is possible that the higher $LC_{50}$ value obtained with SSII-1 was owing to the poor sporulation of this strain.

Examination of cell extracts in Western immunoblots indicated that approximately equal amounts of the 42 and 51 kDa proteins were made by strain 718 (Fig. 2, lane j) and SSII-1 (results not shown). This is in contrast to *B. subtilis* [4], in which more of the 42 kDa protein was made.

**Ratio of cells containing the 42 and the 51 kDa protein needed for maximal activity.** Figure 3 presents the results of an experiment in which the effect on the $LC_{50}$ of variation of the ratio of the cells containing the 42 and 51 kDa proteins was determined. Maximal toxicity ($LC_{50}$ of 75–82 ng dry weight cells/ml) was observed between a ratio of 1 (42 kDa-containing cells) to 1.5–5 (51 kDa-containing cells). The results of the Western immunoblots showed that approximately equal intensities of the 51 and 42kDa proteins were obtained when the ratio of the cells in the sample was about 4 to 1 (results not shown). These results indicate that stoichiometric amounts of both proteins are required for activity and that the optimal ratio of the 42 : 51 kDa protein may be close to 1 : 1.

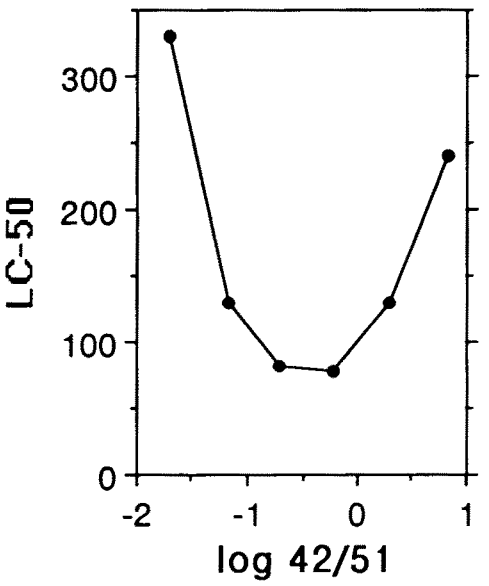


Fig. 3. Effect on the $LC_{50}$ of different ratios of the 42 and 51 kDa protein-containing cells of *B. sphaericus* 718. $LC_{50}$ expressed as ng/dry weight of the mixture of cells containing the 42 kDa and 51 kDa proteins.

## Discussion

The results of this and our previous study [9] indicate that, irrespective of the bacterial host (*Escherichia coli, Bacillus subtilis, or B. sphaericus*), the 51 and the 42 kDa proteins over-produced from the genes of *B. sphaericus* 2362 are not toxic alone but are toxic in combination. This finding indicates that the *B. sphaericus* larvicide is a binary toxin, since both proteins are required for toxicity. This conclusion is at odds with the previous findings that the 42 kDa protein purified from the crystal of *B. sphaericus* 2362 is toxic [6, 12, 19]. One possible interpretation of the latter observation is that the host may contribute some factor that would result in the toxicity of the 42 kDa protein. This seems unlikely since the 42 kDa protein which is synthesized in *B. sphaericus* SSII-1 is also not toxic and this strain has a high DNA homology to the larvicidal strains of this species [16]. A more likely explanation for this discrepancy is that the 42 kDa preparation obtained from the crystal of *B. sphaericus* 2362 was contaminated with small amounts of 51 kDa protein.

A number of observations are consistent with this interpretation. Subsequent to our initial purification of the 42 protein from the crystal of *B. sphaericus* 2362 [6], we obtained preparations that had a major reduction in toxicity; toxicity was enhanced

by addition of the 51 kDa protein. Using as starting material the amorphous inclusions from *B. subtilis* pUE382 containing both the 51 and 42 kDa proteins, we purified the 42 kDa protein and showed that it was not toxic for larvae (maximum concentration tested, 10 μg/ml). Toxicity was found upon addition of the 51 kDa protein from *B. subtilis* pUE381. Treatment of the 51 kDa protein with larval gut proteases led to formation of derivatives that migrated close to the 42 kDa proteins. Deletions at the N- and C-terminal regions of the 51 kDa protein by site-directed mutagenesis indicated that 51 kDa protein derivatives of about 41–43 kDa are toxic in the presence of the 42 kDa protein [M.A. Clark, personal communication]. These observations indicate that the 42 kDa protein is nontoxic and that the previously observed toxicity was probably owing to contamination with similar molecular weight derivatives of the 51 kDa protein.

Recently it has been claimed that the 42 kDa protein produced alone in *B. subtilis* is toxic for mosquito larvae [13]. Our observations indicating that this is not the case in three different host of *Bacillus*, of which one is highly related by DNA homology to the larvicidal strains of *B. sphaericus*, make this claim questionable. Furthermore, no direct evidence was provided indicating that the tested preparation lacked the 51 kDa protein or that the appropriate DNA fragment was inserted into the vector.

Examination of the effect of the ratio of cells containing the 42 and the 51 kDa protein on the $LC_{50}$ for *C. pipiens* has indicated that for maximal toxicity between 1.7 and 5-fold more of the 51 kDa protein-containing cells is required than of the 42 kDa protein-containing cells (Fig. 3). This is consistent with the results of the bioassays presented in Table 1, which showed that for the $LC_{50}$ four times more of the 51 kDa protein-containing cells was required than of the 42 kDa protein-containing cells. Quantitation of the amounts of the toxin proteins in cell-free extracts by means of western immunoblots indicated that the optimal ratio of the 42 : 51 kDa protein may be close to 1 : 1.

Recently we have determined the amount of 42 and 51 kDa proteins required for maximal toxicity, using particulate and solubilized amorphous inclusions purified from *B. subtilis* [10]. The concentration of the proteins was quantitated by densitometry. The results also indicated that approximately equimolar amounts were required for maximal toxicity.

These results and previous studies [5, 9, 13] indicate that, unlike the *B. thuringiensis* lepidoptera-, diptera-, or coleoptera-active toxins, which contain a single active toxin molecule [15], the *B. sphaericus* larvicide is a binary toxin. In this respect it resembles such well-studied binary toxins as the anthrax toxin [22], the $C_2$ botulinum toxin [20], and the *Clostridium perfringens* iota toxin [21]. In the case of *B. sphaericus* toxin, the role of the individual subunits is unclear since the mode of action is unknown. It is, however, possible that both subunits may act in concert, since there are regions of the 51 and the 42 kDa proteins that are conserved and may have a similar function.

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