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Genetic Determinants of Host Ranges of *Bacillus sphaericus* Mosquito Larvicidal Toxins

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The 51.4-kDa–41.9-kDa binary toxin produced by different strains of *Bacillus sphaericus* shows differential activity toward *Culex quinquefasciatus*, *Aedes atropalpus*, and *Aedes aegypti* mosquito larvae. The patterns of larvicidal activity toward all three mosquito species and growth retardation in *A. aegypti* have been shown to be due to the 41.9-kDa protein. By using mutant toxins expressed in *Escherichia coli*, insecticidal activity and growth retardation correlated with amino acids centered around position 100 of the 41.9-kDa protein. In its response to these toxins, *A. atropalpus* resembled *C. quinquefasciatus* rather than its congener, *A. aegypti*.

Bacillus sphaericus is a widespread aerobic spore-forming bacterium, some strains of which produce protein toxins which are lethal to mosquito larvae. Highly toxic strains of *B. sphaericus* contain a pair of proteins of 51.4 and 41.9 kDa which are both required for toxicity to larvae (5, 6, 13, 18), while the 41.9-kDa protein alone may be sufficient for toxicity to cultured mosquito cells (1).

The highly toxic strains of *B. sphaericus* have different relative toxicities to larvae of various mosquito species. Strains in the H serotype H5a5b, including 2362 and 1593, are generally very toxic to *Culex pipiens*, *Culex quinquefasciatus*, *Anopheles stephensi*, and *Mansonia uniformis* and are somewhat less toxic to *Toxorhynchites rutilis rutilis* and *Aedes aegypti*. Strains in H serotypes H6 (strain IAB59) and H25 (strain 2297) are also very toxic to *C. pipiens*, *C. quinquefasciatus*, and *A. stephensi* but have very low toxicity or are nontoxic to *A. aegypti*, *M. uniformis*, or *T. rutilis rutilis* (16, 22, 23). Minor variations in toxicity among strains within serotype H5a5b (such as 1593 and 2362) are likely to be due to the presence of other toxins, such as the 100-kDa protein cloned by Thanabalu et al. (21), since the sequences of the 51.4- and 41.9-kDa proteins along with their transcriptional and translational control sequences are identical in these strains (3). However, strains 2362, IAB59, and 2297 differ in amino acid sequences in both the 41.9- and 51.4-kDa proteins (2, 4). In this study we compare the toxicities of *Escherichia coli* containing cloned genes encoding the 51.4- and 41.9-kDa proteins from these three *B. sphaericus* strains and of spore-toxin preparations from the original *B. sphaericus* strains against *C. quinquefasciatus*, *A. aegypti*, and *Aedes atropalpus* larvae. Differential toxicity was found not only in the overall toxicity of the strains but also in the temporal pattern of mortality and in sublethal effects of the toxins. Our results indicate that the 41.9-kDa toxin protein is responsible for host range, though overall toxicity is the function of both proteins. Furthermore, we have constructed and assayed hybrid and mutant toxin proteins in an attempt to identify amino acids responsible for differential toxicity. Host range specificity appears to be centered around a single short region in the 41.9-kDa protein.

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MATERIALS AND METHODS

Toxin gene clones in *E. coli*. The structures of the constructs carrying one or both of the toxin genes (gene 51, coding for the 51.4-kDa toxin protein, and gene 42, coding for the 41.9-kDa toxin protein) from *B. sphaericus* 2362, 2297, and IAB59 used in this study are shown in Fig. 1. The origins of these plasmids are as follows.

(i) *B. sphaericus* 2297. The construction of plasmids containing both gene 51 and gene 42 in pUC12 (pCC2297-M), gene 51 alone in pUC12 (pCC2297-R), and gene 42 alone in pKK223-3 (pMK31) has previously been described (13, 18).

(ii) *B. sphaericus* IAB59. The clone pIAB59.B containing both gene 51 and gene 42 on a 3.5-kb *Hin*dIII fragment in pUC18 has been described elsewhere (4). To clone gene 42 alone, the *Eco*RI-*Hin*dIII fragment (nucleotides [nt] 1597 to 3477) was excised from pIAB59.B and was ligated into *Eco*RI-*Hin*dIII-cut, phosphatase-treated pUC18 to yield the clone pIAB59.4. In this construct, expression of the 41.9-kDa protein is driven from the *lac* promoter of the pUC18 vector.

(iii) *B. sphaericus* 2362. The clone encoding gene 42 (pJC2362-1) described previously (4) was found to produce the 41.9-kDa protein under control of the *lac* promoter of the pUC12 vector. The plasmid containing both gene 51 and gene 42 from strain 2362 was constructed as follows. Total DNA from this strain was digested with *Hin*dIII (Promega), and fragments were separated by electrophoresis through a 1.0% (wt/vol) agarose Tris-acetate-EDTA gel. Fragments of approximately 3.5 kb were isolated and ligated into *Hin*dIII-cut, phosphatase-treated pUC18. The resulting transformed *E. coli* cells were screened for the presence of toxin genes by using the labelled insert from pJC2362-1 as a probe. One

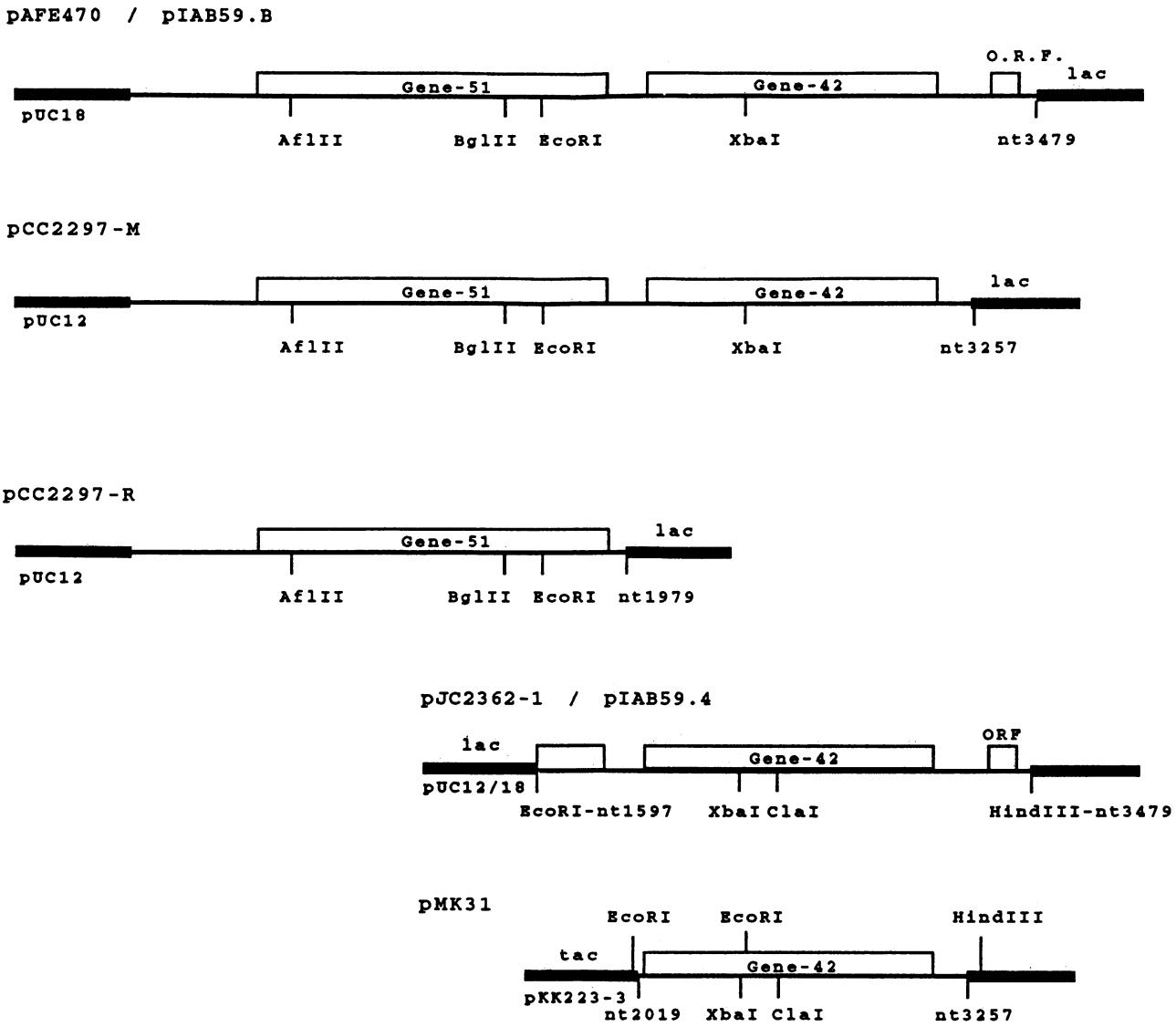

FIG. 1. Plasmid constructions used in this study. pAFE470, pIAB59, and pCC2297-M encode both the 51.4- and 41.9-kDa proteins from *B. sphaericus* 2362, IAB59, and 2297, respectively (4, 13, 18). Plasmid pCC2297-R encodes the 51.4-kDa protein from strain 2297 (13, 18). Plasmids pJC2362-1, pIAB59.4, and pMK31 encode the 41.9-kDa proteins from strains 2362, IAB59, and 2297, respectively (4, 13, 18). ORF, open reading frame.

clone, designated pAFE470, was chosen as it is equivalent to clone pIAB59.B.

Construction of plasmids expressing mutant proteins. (i) Mutation of amino acids 314 and 317 in the 51.4-kDa protein of *B. sphaericus* 2297. A double mutation—Tyr to Leu at amino acid 314 and Leu to Phe at amino acid 317 (corresponding to a replacement of the amino acids at these positions in strain 2297 with those of strain 2362 [Table 1])—was made as follows. Plasmid pCC2297-R was digested with *Afl*II and *Bgl*II, and the larger of the resulting fragments was purified. Plasmid pAFE470 was digested with the same enzymes, and the smaller, 823-bp fragment was gel purified and ligated into the *Afl*II-*Bgl*II-digested pCC2297-R vector described above. The resulting plasmid, designated p51Y-L.314./L-F.317, was sequenced to confirm that the intended mutations had been made.

(ii) Mutation of amino acid 99 in the 41.9-kDa protein of *B. sphaericus* 2297. The mutation of Phe at position 99 in the 41.9-kDa protein to Val was produced by site-directed mutagenesis. An approximately 400-bp *Eco*RI fragment was excised from plasmid pMK31 and was ligated into *Eco*RI-cut, phosphatase-treated M13mp18. After transfection into *E. coli* MV1190, clear plaques were picked and single-stranded M13 DNA was prepared. Clones containing the toxin gene fragment in the desired orientation were selected following sequencing of the inserted DNA by using the M13 universal primer. Site-directed mutagenesis was performed with the Muta-Gene M13 in vitro mutagenesis kit (Bio-Rad). Annealing of the mutagenic oligonucleotide to single-stranded template DNA and complimentary-strand synthesis were performed according to the manufacturer's instructions. The mutagenic oligonucleotide was a 30-mer (nt 2293 to 2322) (2) bearing a T→G mutation in the first position of codon 99 of the 41.9-kDa toxin gene (nt 2308) (2), producing

TABLE 1. Amino acid differences among binary toxin proteins in three *B. sphaericus* strains (2, 3, 4)

Protein	Plasmid	Amino acid:			
		Position	In 2362	In 2297	In IAB59
51.4 kDa		69	Ser	Ser	Ala
		70	Asn	Asn	Lys
		110	Thr	Thr	Ile
	p51Y-L.314/L-F.317	314	Leu	Tyr[a]	His
		317	Phe	Leu[a]	Leu
		389	Leu	Met	Leu
41.9 kDa	p42F-V.99	99	Val	Phe[a,b]	Val
	p42S-A.104	104	Ala	Ser[a]	
	p42S-E.104	104		Ser[b]	Glu
	p42S-G.104	104		Ser[c]	
		125	His	Asn	His
		135	Tyr	Phe	Tyr
	p42K-R.267	267	Arg	Lys[a,b]	Arg
Double mutant	p42S-A.104/K-R.267	104	Ala	Ser[a]	
		267	Arg	Lys[a,b]	Arg

[a] The 2297 sequence was changed to that of 2362.
[b] The 2297 sequence was changed to that of IAB59.
[c] The 2297 sequence was changed to a unique sequence (Gly).

a Phe→Val substitution in amino acid 99 (Table 1). The resulting double-stranded phage DNA was transfected into *E. coli* MV1190. Plaques containing mutant phage were identified by plaque hybridization to end-labelled mutagenic oligonucleotide followed by washing at increasing stringency to dissociate probe from nonmutant phage showing mismatched binding of the probe. Double-stranded DNA was isolated from mutant-phage-infected cells, and the *Eco*RI fragment containing the mutated sequence was isolated and recloned into the large fragment of *Eco*RI-digested pMK31. The recombinant plasmid was sequenced in order to check orientation and that the desired mutation had been made. The plasmid encoding the Phe→Val mutation was designated p42F-V.99 (Table 1).

(iii) Mutation of amino acid 104 in the 41.9-kDa protein of *B. sphaericus* 2297. Three mutations of amino acid 104 were constructed as described above for the p42F-V.99 mutant. The mutations produced were Ser to Ala (corresponding to a replacement of the amino acid in strain 2297 with that of strain 2362); Ser to Glu (corresponding to the replacement of the amino acid in strain 2297 with that of strain IAB59); and Ser to Gly (corresponding to a change to a novel amino acid which like Ala in strain 2362 is a nonpolar residue but has not been found in this position in any *B. sphaericus* toxin). The mutagenic oligonucleotides used were 36-mers corresponding to nt 2305 to 2340 in the sequence of Baumann et al. (2), bearing mutation TCA→GCA (S→A), TCA→GAA (S→E), or TCA→GGA (S→G) in codon 104 of the 41.9-kDa toxin gene (nt 2323 to 2325). Recombinant plasmids were sequenced in order to check orientation and that the desired mutations had been made. Plasmids encoding the Ser→Ala, Ser→Glu, and Ser→Gly mutations were designated p42S-A.104, p42S-E.104, and p42S-G.104, respectively (Table 1).

(iv) Mutation of amino acid 267 in the 41.9-kDa protein of *B. sphaericus* 2297. A single mutation of amino acid 267 from Lys to Arg (corresponding to a replacement of the amino acid in strain 2297 with that of strain 2362) was produced as follows. Plasmid pMK31 was digested with *Cla*I and *Hin*dIII, and the larger of the resulting fragments was purified. Plasmid pJC2362-1 was digested with the same restriction enzymes, and the resulting 941-bp fragment was isolated and ligated into the *Cla*I-*Hin*dIII-digested pMK31 vector described above. The resulting plasmid, designated p42K-R.267 (Table 1), was sequenced to confirm that the intended mutation had been made.

(v) Production of a double mutation in positions 104 and 267 in the 41.9-kDa protein of *B. sphaericus* 2297. A double mutation—Ser to Ala at amino acid 104 and Lys to Arg at amino acid 267—was produced by following the same protocol as for the construction of plasmid p42K-R.267 described above, except that the mutant plasmid p42S-A.104 was used in place of the nonmutant pMK31 vector. The resulting plasmid, designated p42S-A.104/K-R.267 (Table 1), was sequenced to check the orientation of the insert and to confirm that the desired mutation had been made.

Mosquito larval assays. (i) *B. sphaericus* assays. *B. sphaericus* 2362, 2297, and IAB59 were cultured on MBS medium (15) for 72 h, centrifuged from the medium (30,000 × *g*, 15 min), and washed twice with water. Spores with associated toxin were plated for viable-cell counts and were bioassayed against second-instar *C. quinquefasciatus*, *A. aegypti*, and *A. atropalpus* larvae. The bacteria were diluted into 20 ml of water containing 10 larvae and 100 μl of 1% yeast suspension. Surviving larvae were counted at 24, 48, and 72 h. The 50% lethal concentrations (LC_{50}s) were calculated from average mortality observed at 48 h, by using log/probit paper.

(ii) *E. coli* assays. All plasmids were transformed in *E. coli* JM105 for assay. *E. coli* cells were grown on Luria agar plates containing 50 μg of ampicillin per ml for 24 h. Suspensions of *E. coli* cells for each comparative assay were counted by using a hemocytometer and adjusted to the desired cell numbers. For assays in which the 51.4- and 41.9-kDa proteins were cloned separately, equal concentrations of *E. coli* cells containing each protein were used. Five first-instar *C. quinquefasciatus*, *A. aegypti*, or *A. atropalpus* larvae were introduced into each well of a 96-well plastic spot plate (ICI-Lux), and 0.5 ml of bacterial suspension was added along with 50 μl of a 1% yeast suspension. The plates were covered with plastic wrap, and surviving larvae were

TABLE 2. Results of bioassays of *B. sphaericus* spores against *C. quinquefasciatus* second-instar larvae[a]

Strain	LC_{50} (spores/ml) at:		% Change[b]
	24 h	48 h	
2362	1.0×10^2	8.0×10^1	25
2297	6.5×10^3	3.5×10^2	176
IAB59	1.5×10^3	2.0×10^2	650

[a] Data are averages of results of three assays.
[b] The increase in mortality between 24 and 48 h of bioassay is indicated as the percent change in LC_{50}: (LC_{50} at 24 h − LC_{50} at 48 h)/(LC_{50} at 48 h) × 100.

TABLE 3. Results of bioassays of *E. coli* expressing both 51.4- and 41.9-kDa *B. sphaericus* toxin proteins against first-instar *C. quinquefasciatus* larvae[a]

Source of toxin proteins	LC_{50} (cells/ml) at:		% Change[b]
	24 h	48 h	
2362	6.5×10^6	4.5×10^6	44
2297	2.0×10^8	2.0×10^7	900
IAB59	2.0×10^8	3.0×10^7	566

[a] Strain 2362 protein data are averages of results of four assays; other data are averages of results of three assays.
[b] The increase in mortality between 24 and 48 h of bioassay is indicated as the percent change in LC_{50}: (LC_{50} at 24 h − LC_{50} at 48 h)/(LC_{50} at 48 h) × 100.

counted at 24, 48, and 72 h. Four wells (20 larvae) were used at each dilution.

RESULTS

Mortality of control larvae, fed with yeast alone or *E. coli* transformed with vector alone and not expressing *B. sphaericus* proteins, was less than 10% in all assays. *E. coli* cells expressing the 51.4-kDa or 41.9-kDa toxin protein cloned separately were not toxic to larvae unless mixed with cells expressing the complimentary protein.

Assay of *B. sphaericus* spores. Second-instar *C. quinquefasciatus* larvae were highly sensitive to spores of *B. sphaericus* 2362, 2297, and IAB59 (Table 2). Nearly all mortality in *C. quinquefasciatus* fed 2362 spores was expressed by 24 h; little additional mortality occurred during the next 48 h. Much lower mortality was observed at 24 h in assays of 2297 or IAB59 spores, and a large increase in mortality was observed between 24 and 48 h (Table 2). Strain 2362 spores were ca. two to four times as toxic as spores of 2297 or IAB59 to *C. quinquefasciatus*, as determined from the concentration of spores estimated to cause 50% mortality at 48 h. *A. atropalpus* larvae exhibited sensitivity (LC_{50} at 48 h: 2362, 3.5×10^1 spores per ml; 2297, 2×10^2 spores per ml; IAB59, 8×10^2 spores per ml) and a pattern of expression of mortality very similar to that observed with *C. quinquefasciatus*.

A. aegypti larvae were most sensitive to spores of strain 2362, although these larvae were ca. 300-fold less sensitive to these spores than were *C. quinquefasciatus* larvae (Fig. 2). As with *C. quinquefasciatus*, mortality of second-instar *A. aegypti* larvae exposed to strain 2362 spores increased little between 24 and 72 h (not shown). *A. aegypti* larvae were much less sensitive to spores of strains 2297 and IAB59 than to strain 2362 spores, and larvae fed 2297 and IAB59 spores continued to die in small numbers over the entire 72-h bioassay period. The mortality produced by strain 2297 and IAB59 spores was not predictably related to the concentration of spores presented to *A. aegypti* larvae (Fig. 2) and did not exceed 65% even at a concentration of 3×10^8 spores per ml (not shown).

Assay of *E. coli* clones. In assays of *E. coli* cells transformed with pAFE470, pCC2297-M, or pIAB59.B expressing both the 51.4- and 41.9-kDa proteins from strains 2362, 2297, and IAB59, respectively, results reflected those obtained with the original host strain from which the clones

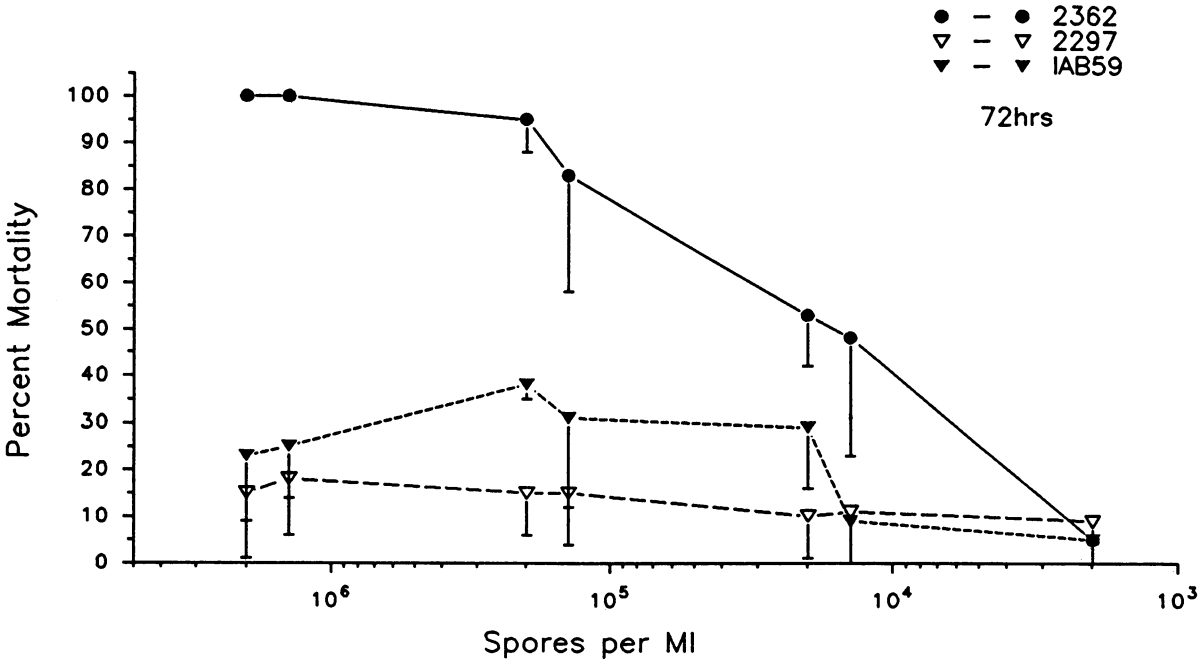

FIG. 2. Assays of *B. sphaericus* spores against second-instar *A. aegypti* larvae, 72-h data. Mean from three assays.

TABLE 4. Results of bioassays with *E. coli* expressing native 51.4-kDa protein from strain 2297 in equal concentrations with cells expressing native 41.9-kDa protein[a] against first-instar *C. quinquefasciatus* larvae

Source of 41.9-kDa toxin protein	LC_{50} (cells of each type/ml) at:		% Change[b]
	24 h	48 h	
2362	2.5×10^5	1.6×10^5	56
2297	1.2×10^6	1.3×10^5	650
IAB59[c]	4.0×10^5	1.5×10^5	167

[a] Data are averages of results from three assays.
[b] The increase in mortality between 24 and 48 h of bioassay is indicated as the percent change in LC_{50}: (LC_{50} at 24 h − LC_{50} at 48 h)/(LC_{50} at 48 h) × 100.
[c] Cells expressing the IAB59 41.9-kDa protein produced average mortality less than 100% at 24 h at concentrations up to and including 10^9 cells per ml.

were derived. In assays against *C. quinquefasciatus*, *E. coli* expressing the toxins from strain 2362 produced most mortality in the first 24 h of bioassay (Table 3) whereas *E. coli* expressing the toxins from strain 2297 or IAB59 produced a marked increase in mortality between 24 and 48 h (Table 3).

E. coli cells were transformed with plasmids pMK31 and pCC2297-R, expressing, respectively, the native 41.9- and 51.4-kDa proteins from strain 2297. Cells containing these plasmids were used in a 1:1 ratio in assays against *C. quinquefasciatus*, *A. aegypti*, and *A. atropalpus* larvae. The toxicity of this combination of cells to *C. quinquefasciatus* ($LC_{50} = 1.3 \times 10^5$) (Table 4) was found to be approximately 150 times greater than that of *E. coli* transformed with pCC2297-M ($LC_{50} = 2.0 \times 10^7$; Table 3) in which both proteins are expressed in the same cell. The above combination of cells containing pMK31 and pCC2297-R was used as the standard against which the activities of all other combinations were assessed.

E. coli cells containing pCC2297-R expressing the 51.4-kDa protein from strain 2297 were assayed in combination with cells containing pJC2362-1, pMK31, or pIAB59.4 expressing the 41.9-kDa protein from strain 2362, 2297, or IAB59, respectively. In assays against *C. quinquefasciatus*, the combination in which the 2362 41.9-kDa protein was expressed produced greatest mortality in the first 24 h of bioassay, the pattern of temporal mortality associated with the parent strain 2362 (Table 4). In contrast, the combinations in which the 2297 or IAB59 41.9-kDa protein was expressed produced a delayed onset of mortality (Table 4). This indicates that the temporal pattern of mortality in assays against *C. quinquefasciatus* larvae is linked to the origin of the 41.9-kDa protein.

A. atropalpus larvae exhibited similar sensitivity and patterns of mortality in response to these combinations of *E. coli* cells, as did *C. quinquefasciatus* larvae.

In assays against *A. aegypti*, *E. coli* expressing the 41.9-kDa toxin from strain 2362 in combination with the 51.4-kDa protein from 2297 produced both mortality and growth retardation at high concentrations (Fig. 3A and 4A). Many of the surviving larvae in these assays remained in the first or early second instar for the entire 72-h bioassay period (Fig. 4A), whereas control larvae fed with *E. coli* not expressing *B. sphaericus* proteins or 51.4- or 41.9-kDa protein without the complimentary protein developed normally to third or fourth instar within 72 h. *A. aegypti* larvae exhibiting growth retardation were removed from the bioassay containers after 72 h and were placed in clean water with fish food and yeast. Approximately 50% of these larvae died within the next 4 days, while the remainder developed normally to fourth-instar larvae or pupae during this period. *E. coli* expressing the toxins from strain 2297 produced very little mortality or growth retardation in *A. aegypti* (Fig. 3B and 4B), while *E. coli* expressing the IAB59 toxins produced no toxicity or growth retardation up to a concentration of 10^{10} cells per ml (not shown). Growth retardation was not observed in surviving *C. quinquefasciatus* or *A. atropalpus* larvae.

Assay of mutant toxin clones. Suitable amino acids for mutagenesis were chosen by comparing amino acid sequences of the 51.4- and 41.9-kDa proteins between strains of *B. sphaericus* (Table 1). Those amino acids which varied among all three strains (amino acid 314 in the 51.4-kDa protein and amino acid 104 in the 41.9-kDa protein) were the first targets for mutagenesis. Further mutations in the 41.9-kDa protein were made at amino acid 99, which varies between strains 2297 and 2362 and is close to residue 104 in the primary amino acid sequence, and at amino acid 267, which lies in the same position relative to homology region C as amino acid 314 in the 51.4-kDa protein. Homology region C is a region of complete homology between the 51.4- and 41.9-kDa proteins (2).

E. coli cells containing p51Y-L.314/L-F.317 were assayed in combination with cells containing pMK31. In assays against *C. quinquefasciatus*, there was no change in the temporal pattern of mortality compared with that caused by the standard combination but the final toxicity was slightly enhanced (Table 5). In assays against *A. aegypti* the cells expressing the mutant 51.4-kDa protein produced enhanced toxicity and slightly enhanced the growth retardation effects (Fig. 3, open triangles; Fig. 4, hatched bars).

Mutation of the 2297 41.9-kDa protein in amino acid 99 to be identical to the 2362 protein (F→V) resulted in toxicity toward *C. quinquefasciatus* in the first 24 h of assay nearly identical to that of pJC2362-1 and slightly enhanced overall toxicity and growth retardation of *A. aegypti* larvae (Table 6; Fig. 3C and 4C).

Experiments with *E. coli* containing plasmids encoding mutations in amino acid 104 of the 41.9-kDa protein showed important effects on toxicity when assayed in combination with cells containing pCC2297-R. Assays using *E. coli* containing p42S-G.104 or p42S-E.104 showed a decrease in toxicity toward *C. quinquefasciatus* of, respectively, 10-fold (Table 6) or 250-fold (not shown) compared with that of the standard combination. These mutants were completely inactive against *A. aegypti*. In assays using cells containing p42S-A.104 against *C. quinquefasciatus*, mortality was approximately equal to that of the standard combination (Table 6) while toxicity to *A. aegypti* was slightly increased (Fig. 3D). In addition, a greater percentage of surviving *A. aegypti* larvae exhibited growth retardation than was normally observed in larvae fed toxins derived from strain 2297 (Fig. 4D).

E. coli cells containing p42K-R.267 were assayed in combination with cells containing pCC2297-R. In assays against *C. quinquefasciatus* total mortality was similar to that produced by the standard combination (Table 6). No effect on the temporal pattern of mortality of *C. quinquefasciatus* larvae was observed, total mortality at 72 h in assays of *A. aegypti* was similar to that produced by the standard combination (not shown), and growth retardation of *A. aegypti* was not observed.

E. coli cells containing the double mutant p42S-A.104/K-R.267 were assayed in combination with cells containing pCC2297-R. This mutant was not as toxic toward *C. quinquefasciatus* as p42S-A.104 (Table 6); however, the percent change in LC_{50} between 24 and 48 h was reduced, and the

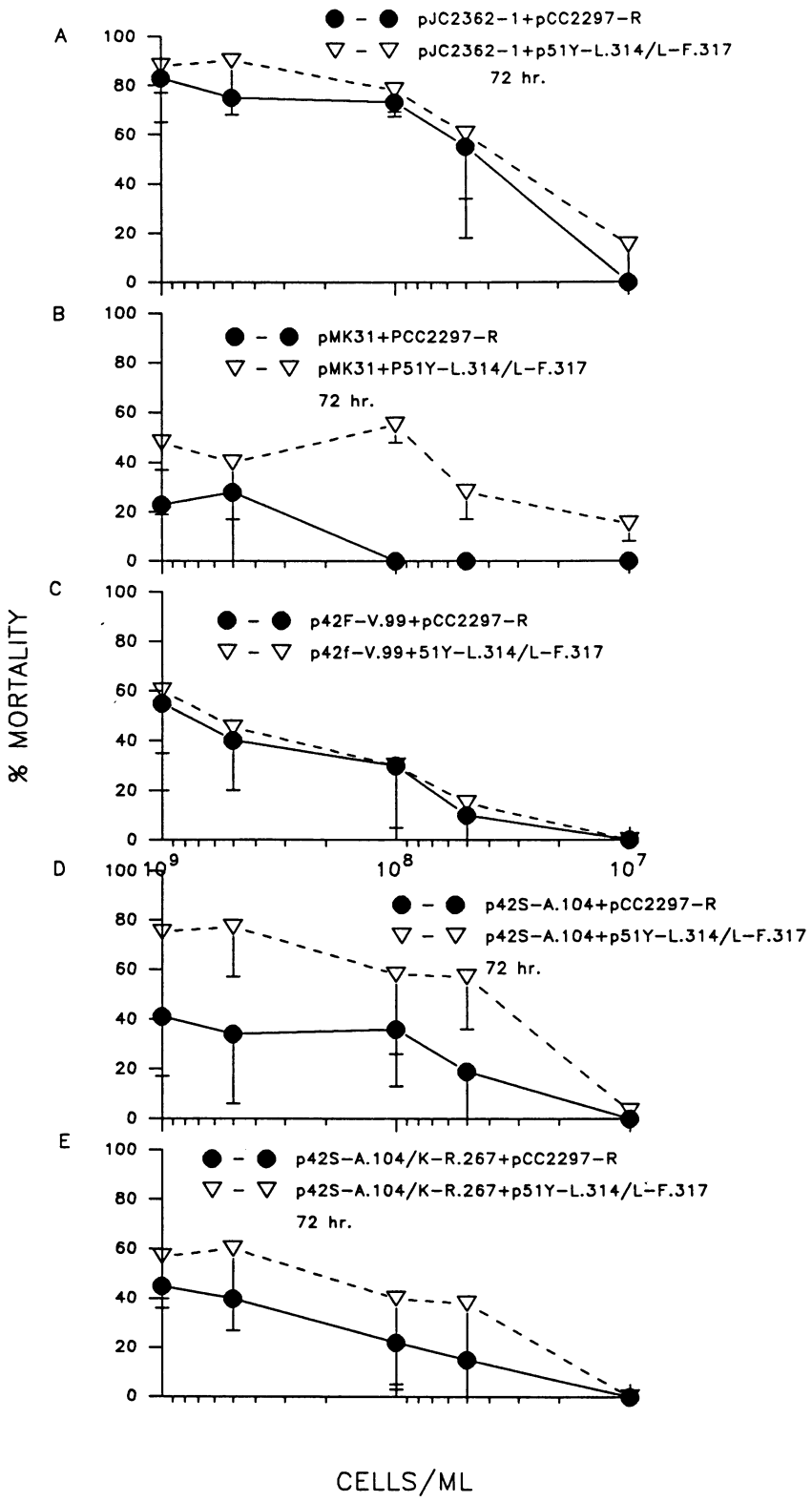

FIG. 3. Assays of *E. coli* expressing native 51.4-kDa protein from strain 2297 (pCC2297-R) or mutant 51.4-kDa protein (p51Y-L.314/L-F.317) in equal concentrations with cells expressing 41.9-kDa protein, against first-instar *A. aegypti* larvae; 72-h data. (A) Native protein from 2362 (pJC2362-1), mean from two assays; (B) native protein from 2297 (pMK31), mean from two assays; (C) mutation in amino acid 99 (p42F-V.99), mean from three assays; (D) mutation in amino acid 104 (p42S-A.104), mean from three assays; (E) double mutation in amino acids 104 and 267 (p42S-A.104/K-R267), mean from three assays.

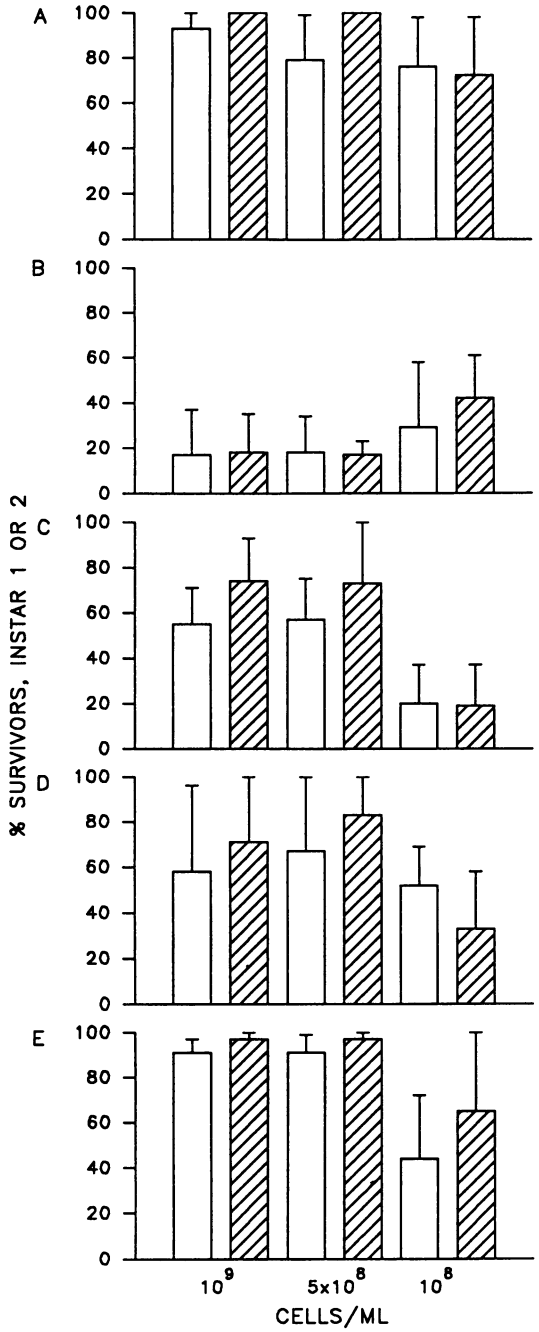

FIG. 4. Percentage of surviving *A. aegypti* larvae remaining in the first or second instar in assays of *E. coli* expressing native 51.4-kDa protein from 2297 (pCC2297-R; open bars) or mutant 51.4-kDa protein (p51Y-L.314/L-F.317; hatched bars) in equal concentrations with cells expressing 41.9-kDa protein, against first-instar *A. aegypti* larvae; 72-h data. (A) Native protein from 2362 (pJC2362-1), mean from four assays; (B) native protein from 2297 (pMK31), mean from four assays; (C) mutation in amino acid 99 (p42F-V.99), mean from three assays; (D) mutation in amino acid 104 (p42S-A.104), mean from four assays; (E) double mutation in amino acids 104 and 267 (p42S-A.104/K-R267), mean from three assays.

TABLE 5. Results of bioassays with *E. coli* expressing 51.4-kDa protein from mutant plasmid p51Y-L.314/L-F.317 in equal concentrations with cells expressing native 41.9-kDa protein against first-instar *C. quinquefasciatus* larvae[a]

Source of 41.9-kDa toxin protein	LC_{50} (cells of each type/ml) at:		% Change[b]
	24 h	48 h	
2362	1.2×10^5	6.8×10^4	11
2297	2.1×10^5	9.0×10^4	133
IAB59	3.0×10^6	9.0×10^5	233

[a] Data for strain 2297 protein are averages of results of three assays; other data are averages of results of two assays.

[b] The increase in mortality between 24 and 48 h of bioassay is indicated as the percent change in LC_{50}: (LC_{50} at 24 h − LC_{50} at 48 h)/(LC_{50} at 48 h) × 100.

double mutant produced growth retardation in a higher proportion of surviving *A. aegypti* larvae (Fig. 4E).

E. coli cells transformed with the mutant p51Y-L.314/L-F.317 were assayed in combination with cells containing mutations in gene 42 (p42S-A.104, p42K-R.267, p42S-A.104/K-R.267, and p42F-V.99). Activity against *C. quinquefasciatus* (not shown) and *A. aegypti* (Fig. 3C, D, and E, open triangles; Fig. 4C, D, and E, hatched bars) was enhanced over that produced by cells containing pCC2297-R.

DISCUSSION

The results of our assays using *B. sphaericus* spore preparations confirm the findings of Thiery and deBarjac (22), in that spores of strain 2362 (serotype H5a5b) are toxic to both *C. quinquefasciatus* (= *C. pipiens*) and *A. aegypti*, with a difference in susceptibility of these two mosquito species of ca. 1,000-fold. Thiery and deBarjac (22) found strains in serotypes H6 (IAB59) and H25 (2297) to be approximately equal in toxicity to strain 2362 against *C. quinquefasciatus* but nontoxic against *A. aegypti*, defined in their assays as less than 100% mortality at 48 h at a dilution of the culture of 1/100. In our assays spores from *B. sphaericus* 2297 or IAB59 exhibited low toxicity not exceeding 65% even at high concentrations. *E. coli* cells expressing both the 51.4- and 41.9-kDa toxins from strains 2362, 2297, and IAB59 were less toxic cell for cell than *B. sphaericus* spores. However, the temporal pattern of mortality over the 72-h bioassay period, and in particular the relative sensitivity of *A. aegypti* larvae to toxins of the three *B. sphaericus* strains, was similar whether the toxins were produced in *E.*

TABLE 6. Results of bioassays with *E. coli* expressing 51.4-kDa protein from strain 2297 in equal concentrations with cells expressing mutations in the 41.9-kDa protein against first-instar *C. quinquefasciatus* larvae[a]

Mutant plasmid	LC_{50} (cells of each type/ml) at:		% Change[b]
	24 h	48 h	
p42F-V.99	7.0×10^5	3.5×10^5	100
p42S-A.104	1.5×10^6	3.0×10^5	400
p42S-G.104	1.1×10^7	1.8×10^6	511
p42K-R.267	1.5×10^5	6.0×10^4	150
p42S-A.104/K-R.267	2.5×10^6	1.8×10^6	39

[a] p42S-A.104 and p42K-R.267 data are average of results of four assays; other data are averages of results of three assays.

[b] The increase in mortality between 24 and 48 h of bioassay is indicated as the percent change in LC_{50}: (LC_{50} at 24 h − LC_{50} at 48 h)/(LC_{50} at 48 h) × 100.

coli or in the native organism. These results suggest that the temporal pattern of mortality and the host range of the highly toxic *B. sphaericus* strains are due to the binary toxin (51.4- plus 41.9-kDa proteins) rather than to other toxins known to be produced by this organism (21). Three strains within the serotype H5a,5b (2362, 1593, and 2317.3) are identical in amino acid sequences in both the 51.4- and 41.9-kDa toxin proteins but differ from strains in serotypes H25 (2297) and H6 (IAB59) in several amino acids (Table 1) (4).

The 2297 51.4-kDa protein was mutated to be identical to the 2362 51.4-kDa protein in the two positions in which both 2297 and IAB59 differ from 2362 within the active, enzymatically processed toxin (amino acids 314 [Y→L] and 317 [L→F]; Table 1) (4, 6, 7, 18, 20). Enhanced overall activity to both *C. quinquefasciatus* and *A. aegypti* was produced, but the temporal pattern of toxicity to *C. quinquefasciatus* was unchanged. The 51.4-kDa protein has been found to be responsible for regional localization of the binary toxin in the larval gut and may be essential to internalization of the binary toxin in *C. quinquefasciatus* (19). In excess, the 51.4-kDa protein can inhibit toxin activity (13). The 51.4-kDa protein may be necessary for binding to the insect midgut and to overall toxicity but is not responsible for the temporal pattern of activity or the host range of the toxin. The increase in toxicity of the mutant 51.4-kDa protein may be due to an enhancement of toxin binding to target cells or to enhanced interaction with the 41.9-kDa protein.

The temporal pattern of mortality, the host range, and the phenomenon of growth retardation in *A. aegypti* appear to be related to the 41.9-kDa protein. Recently, Baumann and Baumann (1) have shown that the 41.9-kDa protein alone can lyse cultured *C. quinquefasciatus* cells, further suggesting that this protein is the most important in the overall mode of action of this binary toxin.

Position 267 of the 41.9-kDa protein is the second amino acid downstream of a region of complete homology between the 51.4- and 41.9-kDa toxin proteins (Ile-Gly-Ala-Phe-Gly) (2) and corresponds in relationship to this homology region to amino acid 314 of the 51.4-kDa protein. A change in amino acid 267 (K→R) of the 41.9-kDa protein, or in 314 (Y→L) along with 317 (L→F) of the 51.4-kDa protein, did not result in a change in the temporal pattern of activity against *C. quinquefasciatus* or marked enhancement of activity toward *A. aegypti*, but the 314.Y-L/317.L-F mutation in the 51.4-kDa protein did increase overall toxicity against both species. These results suggest that this homologous region may be important in the mode of action of the 51.4-kDa toxin protein but not necessarily in the host range.

The 41.9-kDa protein of strain 2297 differs from strain 2362 in five amino acids, whereas strain IAB59 differs from 2362 in only one of these, amino acid 104 (Table 1) (4). Changes in amino acid 104 produced changes in activity of this protein to both *C. quinquefasciatus* and *A. aegypti* larvae. A change from serine to alanine, rendering the protein identical to that of 2362 in this position, produced increased mortality and growth retardation in *A. aegypti* larvae. A change from serine to glutamic acid, producing a protein identical to that of IAB59 in this position, or from serine to glycine (which like alanine is a nonpolar residue but has not been identified in any known *B. sphaericus* 41.9-kDa toxin sequence) reduced the toxicity to *C. quinquefasciatus* and obliterated the activity to *A. aegypti*. A change at amino acid 99 from the 2297 sequence (phenylalanine) to that of 2362 (valine) resulted in slightly increased overall toxicity and growth retardation of *A. aegypti* larvae. The double mutation at positions 104 (S→A) and 267 (K→R) led to slightly increased overall toxicity to *A. aegypti* and markedly enhanced growth retardation of surviving *A. aegypti* larvae.

These results lead to the suggestion that the host range of *B. sphaericus* toxins may be due to amino acids centered around position 100 in the 41.9-kDa toxin protein. This suggestion is based upon the gain in two functions, overall *A. aegypti* mortality and growth retardation, caused by the change from serine to alanine (2297 to 2362) in position 104 or from phenylalanine to valine (2297 to 2362) in position 99. The failure of two other mutations in position 104 to produce increased activity against *A. aegypti* while resulting in a decrease in activity towards *C. quinquefasciatus* larvae also indicates the importance of this amino acid. Interestingly, the mutation S→E in position 104, a change from the 2297 to the IAB59 sequence at this position, caused an at least 250-fold decrease in toxicity to *C. quinquefasciatus*, whereas the wild-type IAB59 sequence shows toxicity of the same order as that of 2297 and 2362. The 41.9-kDa protein of IAB59 differs from that of 2362 only in position 104. This implies that some of the other amino acid changes between strains IAB59 and 2297 can in some way compensate for the reduction in toxicity caused by the S→E mutation at position 104.

Retardation of growth of *A. aegypti* larvae may be the result of feeding inhibition, as at least 50% of larvae exhibiting this symptom grew normally when removed from the *E. coli* preparation. Feeding inhibition has also been reported in lepidopteran larvae fed *Bacillus thuringiensis* toxins (14).

It has earlier been generalized that members of the genus *Aedes* are much less sensitive than *C. quinquefasciatus* or insensitive to *B. sphaericus* toxins (e.g., see references 9 and 10). *A. atropalpus* demonstrated a response to both *B. sphaericus* spores and cloned toxins similar to that of *C. quinquefasciatus* and did not respond to these toxins with growth retardation. *Aedes nigromaculis* was also found to be sensitive to *B. sphaericus* 1593 (17). Response to *B. sphaericus* toxin is therefore not uniform within the genus *Aedes*.

The marked differences in toxic activity of the 41.9-kDa protein from the three *B. sphaericus* strains to *A. aegypti* but not to *C. quinquefasciatus* or *A. atropalpus* suggest that the mode of action may be somewhat different in these mosquito species. Charles (8) observed differences in the ultrastructural changes induced by feeding *B. sphaericus* 2297 spores to highly susceptible *C. pipiens*, moderately sensitive *Anopheles stephensi*, and relatively insensitive *A. aegypti* larvae. Binding of fluorescence-labelled toxin containing both 51.4- and 41.9-kDa proteins was also found to differ among *C. quinquefasciatus*, *A. aegypti*, and *Anopheles* spp. larvae (11, 12). These observations also suggest that the mode of action may be different among these mosquito species.

B. sphaericus has excellent activity against susceptible mosquito larvae in the field and often exhibits long-term persistence. However, the major factor leading to reduced interest in this organism as a microbial insecticide is its limited mosquito host range, in particular its low activity against *A. aegypti* and related species. Localization of host range determinants to one of the binary toxin proteins and the apparent relationship of host range to specific amino acids within this protein suggest that further manipulation of this protein may eventually lead to an organism with satisfactory activity towards *A. aegypti* and other vector mosquitoes.

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