Efficient Expression of a 100-Kilodalton Mosquitocidal Toxin in Protease-Deficient Recombinant *Bacillus sphaericus*

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The expression of the 100-kDa mosquitocidal toxin (Mtx) during vegetative growth and sporulation in nine different mosquito-larvicidal strains of *Bacillus sphaericus* has been analyzed. In five out of the nine strains the 100-kDa toxin was found to be expressed predominantly in the vegetative phase of growth, and in all nine strains the level of the toxin in sporulated cells was very low or undetectable. Strains in four out of the six DNA homology groups of *B. sphaericus* produced intracellular and extracellular proteases, which degraded the 100-kDa toxin, during sporulation. The 100-kDa toxin gene was expressed by using its native promoter on a multicopy number plasmid in *B. sphaericus* 1693 (protease negative) and *B. sphaericus* 13052 (protease positive). High levels of the 100-kDa toxin were produced in vegetative cells of both strains as well as in sporulated cells of protease-negative strain 1693, which is in contrast to the low levels of the 100-kDa toxin produced in sporulated cells of protease-positive strain 13052. Thus, the small amount of the 100-kDa toxin in sporulated cells of the nine mosquito-larvicidal strains is probably due to degradation of the 100-kDa toxin synthesized during vegetative growth by a protease(s) produced during sporulation. *B. sphaericus* 1693 transformed with the 100-kDa toxin gene was as toxic to mosquito larvae during both vegetative growth and sporulation as the natural high-toxicity strains of sporulated *B. sphaericus*. Therefore, it is conceivable that protease-negative strains of *B. sphaericus* expressing Mtx and other toxins may form the basis of an alternative to the natural high-toxicity strains for mosquito control.

Bacillus sphaericus is an aerobic spore-forming bacillus, several strains of which are pathogenic to mosquito larvae (3, 6, 17). *B. sphaericus* strains have been classified into five groups based on DNA homology, with group II further subdivided into IIA and IIB, and all the toxic strains are in group IIA (10). The toxic strains have been further subdivided into low-toxicity strains (50% lethal concentration [LC_{50}], $\sim 10^5$ cells per ml) and high-toxicity strains (LC_{50}, 10^2 to 10^3 cells per ml) (3). *B. sphaericus* SSII-1 is a low-toxicity strain (19) in which toxicity is produced initially in the vegetative phase of growth before the onset of sporulation (15, 16), but the toxicity was found to be markedly unstable (15). In contrast to strain SSII-1, the high-toxicity strains of *B. sphaericus*, for example, strains 1593 (20), 2362 (25), and 2297 (26), develop relatively stable, high toxicity at the onset of sporulation (5).

The high-toxicity strains of *B. sphaericus* have been shown to produce binary toxins with protein components of 41.9 and 51.4 kDa, and both these proteins are required for toxicity to mosquito larvae (4). A gene encoding a 100-kDa toxin designated Mtx from the low-toxicity strain *B. sphaericus* SSII-1 has been cloned and sequenced (24). Genes encoding the binary toxin are distributed among the high-toxicity strains, while the gene encoding the 100-kDa toxin is widely distributed among both the low- and high-toxicity strains (24). The purified, recombinant 97-kDa toxin (the 100-kDa toxin minus a putative signal peptide) was found to have an LC_{50} of 15 ng/ml against *Culex quinquefasciatus* mosquito larvae (23), which is an LC_{50} of the same order as that of the binary toxins from *B. sphaericus*. Thus, the low toxicity of *B. sphaericus* SSII-1 is not due to a low specific activity of the 100-kDa toxin but could be due to

poor expression and/or a low level of stability of the toxin during sporulation in this strain.

As the *mtx* promoter resembles vegetative promoters, the present study was undertaken to test the idea that Mtx synthesized during vegetative growth of *B. sphaericus* is degraded by proteases during sporulation. Several lines of evidence confirmed this notion, and unexpectedly, strains in two groups of *B. sphaericus* which do not produce protease activity against Mtx have been identified. Efficient expression of the cloned *mtx* gene was obtained in both vegetative cells and sporulated cultures of *B. sphaericus* 1693 (protease negative), suggesting that this strain is worth investigating as a vehicle for delivering Mtx (and other toxins which are susceptible to proteases) to mosquito larvae.

MATERIALS AND METHODS

Bacterial strains. *B. sphaericus* SSII-1 was obtained from E. W. Davidson, Arizona State University, Tempe; strains Kellen Q and 31 were obtained from A. A. Yousten, Virginia Polytechnic Institute and State University, Blacksburg; strain 1593M was obtained from J. Szulmajster, Centre National de la Recherche Scientifique, Gif sur Yvette, France; strain 13052, a restriction-negative mutant of strain 1593 (14), was obtained from W. F. Burke, Arizona State University; group I, III, IV, and V strains were obtained from F. G. Priest, Heriot Watt University; and all other *B. sphaericus* strains were obtained from H. de Barjac, Pasteur Institute, Paris, France.

Cell extract preparation. *B. sphaericus* strains were grown in Luria-Bertani broth (LB) at 30°C for 24 h to isolate proteins expressed in the vegetative phase and in NYSM (15) at 30°C for 48 h to isolate proteins expressed during the sporulation phase of growth. The cells were harvested, and the culture supernatant (the supernatant fraction) was transferred to a fresh tube. The cell pellet was washed with phosphate-buffered saline (PBS), resuspended in PBS, and then sonicated for 1 min. After harvesting, the cells were always maintained on ice to prevent proteolysis, and 1 mM EDTA was included in the PBS when proteins were being isolated from sporulated cells for Western blot (immunoblot) analysis. After sonication, the cell extract was centrifuged at $12,000 \times g$. The clarified cell extract (the soluble fraction) was transferred to a fresh tube, while the pellet was resuspended in PBS (the pellet fraction). The protein concentration was measured with the Bio-Rad protein assay kit.

Polyclonal antibodies. The 97-kDa derivative of Mtx was purified with the

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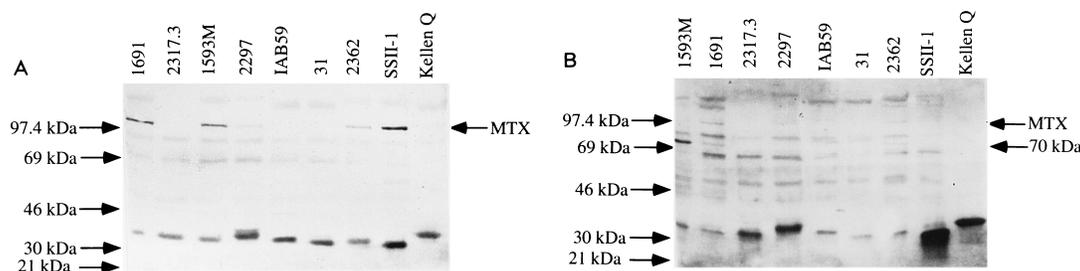
FIG. 1. Western blot analysis of vegetative (A) and sporulated (B) cells of indicated *B. sphaericus* strains. Cell soluble extract (50 μg) was fractionated in a 0.1% SDS–10% polyacrylamide gel, transferred onto a nylon membrane, and probed with purified polyclonal antibodies against Mtx. Molecular mass markers are shown at the left. MTX indicates the position of the 100-kDa toxin, and 70 kDa indicates the position of a putative partial breakdown product of the 100-kDa toxin.

glutathione *S*-transferase expression system as described earlier (23). Polyclonal antibodies against the purified 97-kDa toxin were raised by subcutaneous and intramuscular injection of the purified 97-kDa protein in a rabbit. After a total of four injections with an interval of 3 weeks between each injection, the rabbit was bled by cardiac puncture and the blood was processed as described previously (8). The polyclonal antibodies were purified with an affinity column with the purified 97-kDa protein as the antigen (8).

Western blot analysis. The cell soluble fraction or the cell pellet fraction prepared as described above was boiled with protein sample loading buffer (11) and resolved in a 0.1% sodium dodecyl sulfate (SDS)–10% polyacrylamide gel. The resolved proteins were transferred onto a nylon membrane (Immobilon-p; Millipore) with a Sartorius semidry blot apparatus at a constant current of 60 mA. The membrane was washed four times with PBS, and nonspecific binding sites were blocked with 5% skim milk in PBS. Subsequently, the membrane was incubated with purified polyclonal antibodies in 5% skim milk in PBS at 4°C overnight. The membrane was then washed four times with PBS and incubated with anti-rabbit antibodies coupled with peroxidase in 1% bovine serum albumin and PBS at room temperature. The membrane was developed with an ECL kit used as described by the manufacturer (Amersham International, plc).

Protease assays. *B. sphaericus* strains from each of the six different DNA homology groups (10) were grown at 30°C in 6 ml of NYSM. After 28 h of incubation, 1 ml was removed from each culture, the cells were harvested, and the protein concentration of the culture supernatant was determined. After 48 h of incubation, the remaining culture was centrifuged, the culture supernatant was discarded, and the cell soluble fraction was prepared as described above but without including 1 mM EDTA during the preparation.

Five micrograms of purified 97-kDa protein was incubated overnight (16 h) at 30°C with samples of culture supernatant or cell soluble fraction (1 μg of protein of each) from various *B. sphaericus* strains. The mixture was analyzed by electrophoresis in a 0.1% SDS–10% polyacrylamide gel. After electrophoresis, the gel was fixed and stained with Coomassie blue.

Cloning and expression of the 100-kDa toxin in *B. sphaericus*. In order to express Mtx in *B. sphaericus*, the 100-kDa toxin gene was cloned from *B. sphaericus* SSII-1 (24) into plasmid vector pHV1431 (9). pHV1431 is a 10.9-kb shuttle vector with both pBR322 and pAMB1 origins of replication as well as a chloramphenicol resistance gene for selection in *Bacillus subtilis*.

The 3.3-kb *Cla*I fragment containing the 100-kDa toxin gene with its natural promoter and transcription terminator was excised from pXP33 (24), the cohesive ends were made blunt ended, and the fragment was ligated into pHV1431 at the *Eco*RV site. After transformation of *Escherichia coli* DH5α, one recombinant plasmid (designated pC35) was selected after restriction analysis for electroporation into *B. sphaericus* strains. Group III and IV strains of *B. sphaericus* as well as *B. sphaericus* 13052 were tested for their natural resistances to chloramphenicol by inoculating 1 drop of overnight cultures of the various strains in LB containing various concentrations of chloramphenicol. *B. sphaericus* cells were prepared for electroporation as described previously (21). Transformants were selected and subsequently grown in chloramphenicol at 4 μg/ml (strain 1693) or 10 μg/ml (strain 13052). All recombinant DNA techniques were performed as described previously (12).

Toxicity assays. The mosquito-larvicidal activities of *B. sphaericus* strains were assayed with first- or second-instar larvae of laboratory-reared *C. quinquefasciatus* mosquitoes. To test the toxicity of vegetative cells, the cells were grown in LB for 24 h at 30°C, while to test the toxicity of sporulated cells, the cells were grown in NYSM at 30°C for 48 h. Cells from 6 ml of culture were harvested by centrifugation, washed once with 0.85% NaCl, and resuspended in 1 ml of 0.85% NaCl. The A_{550} was measured, and the cell suspension was diluted to an A_{550} of 0.5. Aliquots of this suspension were added to cups with 10 larvae in 10 ml of water. Toxicity assays were carried out in triplicate for each concentration of cells, and the total concentration of cells in each assay was kept at 10^8 cells per ml by adding nontoxic *B. sphaericus* cells. The number of cells was determined by plating dilutions on L agar plates and incubating at 30°C for 16 h. Mortality was recorded after 48 h, and the LC_{50} was calculated (13).

RESULTS

Expression patterns of Mtx in naturally occurring *B. sphaericus* strains. We have previously cloned and characterized a 100-kDa mosquitocidal toxin gene from *B. sphaericus* SSII-1 designated *mtx*. This gene or a related gene is widely distributed among the low- and high-toxicity strains of *B. sphaericus* (24). In order to elucidate the contribution of Mtx to the toxicity of *B. sphaericus*, we checked for the expression of this toxin by Western blot analysis of vegetative and sporulated cell extracts of various *B. sphaericus* strains previously shown to have the *mtx* gene (24). With purified polyclonal antibodies against the recombinant 97-kDa toxin (the 100-kDa toxin lacking the N-terminal signal sequence), preliminary Western blot analysis showed that the Mtx protein was associated predominantly with the soluble fraction in vegetative cells of strain SSII-1 but was undetectable in both pellet and soluble fractions of sporulated cells of this strain (data not shown). In strain Kellen Q, Mtx was undetectable in both the soluble and pellet fractions of vegetative and sporulated cells (data not shown).

Thus, the cell soluble fractions (50 μg of protein) of vegetative and sporulated cells from nine different toxic strains of *B. sphaericus* were prepared and probed with polyclonal antibodies against the 97-kDa toxin to compare the Mtx protein levels. Extracellular proteases of *B. sphaericus* are completely inhibited in the presence of 1 mM EDTA (5) and in our assay system (data not shown). Thus, in order to prevent proteolysis, 1 mM EDTA was included in PBS when proteins from sporulated cells were being isolated. Mtx protein was absent in vegetative cells and spores of four of the nine strains tested (2317.3, IAB59, 31, and Kellen Q) (Fig. 1). However, five *B. sphaericus* strains (1691, 1593M, 2297, 2362, and SSII-1) were found to have clearly detectable levels of Mtx in their vegetative cells (Fig. 1A). In contrast, the sporulated cells of strains 2362, 2297, 1593M, and 1691 had very low levels of Mtx, which could only be detected by overexposing the membrane (Fig. 1B). In sporulated cultures of strain SSII-1, Mtx could not be detected (Fig. 1B) even though vegetative cells of this strain produced the highest levels of Mtx in all the strains tested (Fig. 1A). Besides the 100-kDa toxin, variable amounts of a 70-kDa protein, which might represent an intermediate in the processing or degradation of Mtx, were observed in sporulated cultures of strains 2362, 1593M, and 1691 (Fig. 1B; see also below). The distinct band at 32 kDa was due to nonspecific binding of antibodies, as it was also found in the nontoxic strain 7054 (see Fig. 5A).

Evidence for proteases in *B. sphaericus* which degrade Mtx. We next investigated the possibility that the very small amounts of Mtx in sporulated cells of strains 2362, 2297, 1691, and 1593M and the lack of this toxin in sporulated SSII-1 could
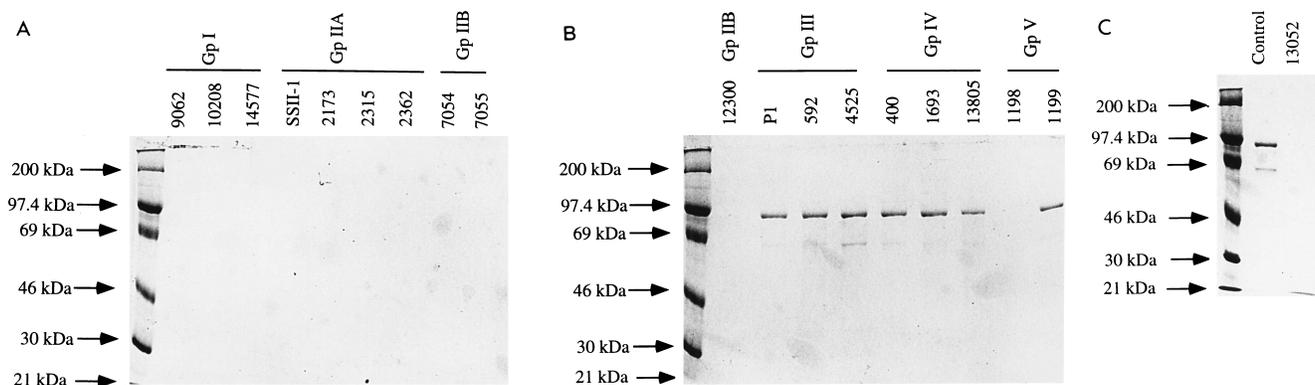
FIG. 2. Analysis of extracellular proteases produced by 28-h sporulated cultures of indicated *B. sphaericus* strains. Extracellular protein (1 μg) was incubated with 5 μg of purified 97-kDa toxin at 30°C for 16 h. The mixture was analyzed in a 0.1% SDS–10% polyacrylamide gel. In panel C, Control refers to untreated 97-kDa toxin; 13052 is a group IIA strain (cf. panel A). Molecular mass markers are shown at the left.

be due to degradation of Mtx (synthesized during the vegetative phase of growth) by a protease(s) produced at the onset of sporulation. *B. sphaericus* has been previously shown to produce both extracellular proteases (5, 7) and intracellular proteases (1). The extracellular protease levels have been shown to be maximal at 28 h in sporulated cultures of strain 2362 (5). Thus, both vegetative cell extracts and 28-h sporulated culture supernatants from the nine strains were incubated with the purified recombinant 97-kDa toxin. All nine strains were found to produce extracellular protease activity during sporulation, which completely degraded the 97-kDa protein in 16 h, whereas vegetative cell extracts did not degrade the 97-kDa toxin (data not shown).

Some *B. sphaericus* strains do not produce proteases which degrade Mtx. In order to assess the significance of proteases in the expression of Mtx during sporulation as well as to enhance the expression and stability of Mtx, it was important to discover a protease-negative *B. sphaericus* strain. The presence of extracellular and intracellular proteases which degrade the 97-kDa mosquitocidal toxin was determined in at least two strains of *B. sphaericus* from each of the six different DNA homology groups (10). Almost all the 28-h sporulated cultures of strains analyzed from DNA homology groups I, IIA, IIB, and V but none from groups III and IV produced an extracellular protease(s) which completely degraded the 97-kDa protein (Fig. 2).

Cell soluble fractions derived from 48-h sporulated cultures

of many of the strains from groups I, IIA, IIB, and V caused partial breakdown of the 97-kDa toxin (Fig. 3). In most of these strains, the 97-kDa protein was apparently processed to 70- and 27-kDa peptides by the soluble extract, but the amount of these peptides was often smaller than the starting amount of the 97-kDa protein, indicating that there was complete toxin degradation as well as partial processing (Fig. 3A and B). In contrast, there was no detectable degradation when the 97-kDa protein was incubated with soluble cell extracts from group III or IV *B. sphaericus* strains (Fig. 3B). Together, these results indicate that *B. sphaericus* strains in DNA homology groups III and IV are devoid of proteases capable of degrading Mtx (Fig. 2 and 3).

Expression of cloned *mtx* gene in protease-positive and protease-negative strains of *B. sphaericus*. The fate of Mtx synthesized during vegetative growth could next be compared in sporulated cultures of protease-positive and protease-negative *B. sphaericus*. However, none of the protease-negative group III and IV strains are toxic to mosquito larvae and they all lack the *mtx* gene. Therefore, in order to express Mtx in protease-negative strains, the complete *mtx* gene, including its native promoter and transcription terminator, was subcloned from plasmid pXP33 (24) into the multicopy number shuttle vector pHV1431 (9) to give plasmid pC35 (Fig. 4). The abilities of the vector pHV1431 to transform *B. sphaericus* 1693 (group IV); 4525, PI, and 592 (group III); 9062, 10208, and 14577 (group I); 7054, 7055, and 12300 (group IIB); 1198 (group V); and
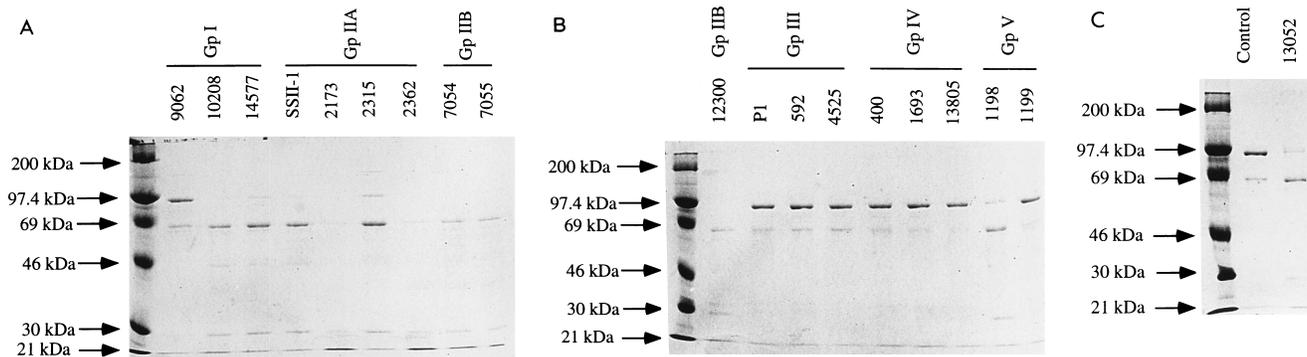

FIG. 3. Analysis of intracellular proteases produced by 48-h sporulated cultures of various *B. sphaericus* strains. Cell soluble protein (1 μg) was incubated with 5 μg of purified 97-kDa toxin at 30°C for 16 h. The mixture was analyzed in a 0.1% SDS–10% polyacrylamide gel. In panel C, Control refers to untreated 97-kDa toxin; 13052 is a group IIA strain. Molecular mass markers are shown at the left.
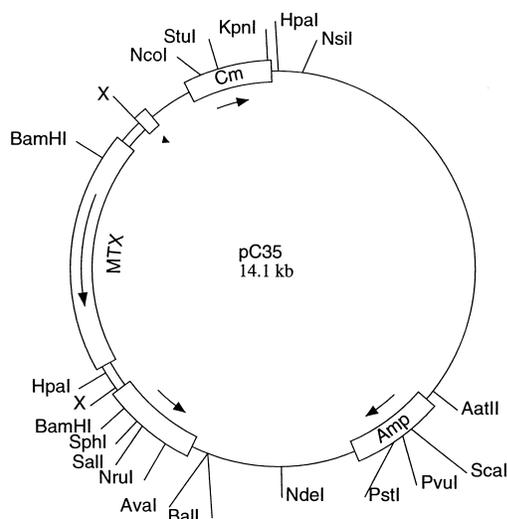
FIG. 4. Restriction map of plasmid pC35. The 3.25-kb *Cla*I fragment from plasmid pXP33 (24) was ligated into the *Eco*RV site of plasmid pHV1431 (9) after the cohesive ends of the *Cla*I site had been made blunt ended. X denotes the *Eco*RV and *Cla*I sites.

TABLE 1. LC$_{50}$s of vegetative and sporulated cells of *B. sphaericus* against larvae of *C. quinquefasciatus*

B. sphaericus strain	LC$_{50}$ (cells/ml)[a]	
	Vegetative cells	Sporulated cells
2362	6.3×10^4	4.1×10^3
SSII-1	3.6×10^5	3.2×10^{5b}
13052.pHV1431	5.1×10^5	3.6×10^3
13052.pC35	8.8×10^3	3.7×10^3
1693.pHV1431	—[c]	—
1693.pC35	5.6×10^3	4.0×10^3

[a] LC$_{50}$s were determined with *C. quinquefasciatus* larvae at 48 h. Toxicity assays were carried out in triplicate, and the results given are averages of two independent assays.
[b] Mosquito-larvicidal activity of sporulated SSII-1 may be due to other unidentified toxins, since Mtx was only detected in vegetative cells (see Results).
[c] —, nontoxic.

13052 (group IIA) were tested, but only 1693 and 13052 could be transformed with this plasmid. These two strains were then transformed with the recombinant plasmid pC35 (Fig. 4), and the transformants (1693.pC35 and 13052.pC35) were grown in LB as well as in NYSM for the analysis of Mtx expression. Both transformants were found to produce high levels of Mtx in vegetative cells, as shown by Western blotting, with strain 13052.pC35 producing more of the toxin than 1693.pC35 (Fig. 5A). Conversely, when the sporulated cultures of these recombinant strains were analyzed by Western blotting, the level of Mtx in protease-negative 1693.pC35 was much higher than that in protease-positive 13052.pC35 because of the sharp decline in the amount of Mtx in 13052.pC35 (Fig. 5B).

In order to determine the fate of Mtx produced during vegetative growth in strain 13052.pC35, a time-point analysis of its expression in 13052.pC35 was performed. This strain was grown in NYSM, and samples were analyzed at 4-h intervals for the expression of Mtx. Detectable amounts of Mtx were first observed during vegetative growth at 20 h, peaked at 24 h, and then declined thereafter during sporulation (Fig. 5C). This clearly shows that Mtx synthesized during the vegetative phase

of growth of 13052.pC35 was being degraded during sporulation.

Mosquito-larvicidal activity of recombinant strains of *B. sphaericus*. The toxicity of recombinant *B. sphaericus* strains with and without the *mtx* gene was determined with *C. quinquefasciatus* mosquito larvae and compared with that of the naturally occurring strains of *B. sphaericus*. Vegetative cells of nontoxic strain 1693 harboring plasmid pC35 were toxic to mosquito larvae, and vegetative cells of recombinant strain 13052.pC35 were ~58-fold more toxic than vegetative cells of the vector control 13052.pHV1431, suggesting that Mtx was being produced during vegetative growth of these recombinant strains (Table 1). The protease-negative strain 1693.pC35 was found to be equally toxic to larvae in both vegetative and sporulation phases of growth (Table 1), consistent with the Western blot results of Mtx protein yield (Fig. 5A and B). It is interesting that the observed toxicity of sporulated 1693.pC35 was similar to that of spores of *B. sphaericus* 2362, a natural high-toxicity strain producing large amounts of the binary toxin during sporulation (3). It is also noteworthy that the toxicity of vegetative cells of recombinant strain 1693.pC35 was an order of magnitude greater than the toxicity of vegetative cells of strain 2362 (Table 1).

DISCUSSION

Our results show that Mtx is expressed predominantly in the vegetative phase of growth in five strains of *B. sphaericus* tested
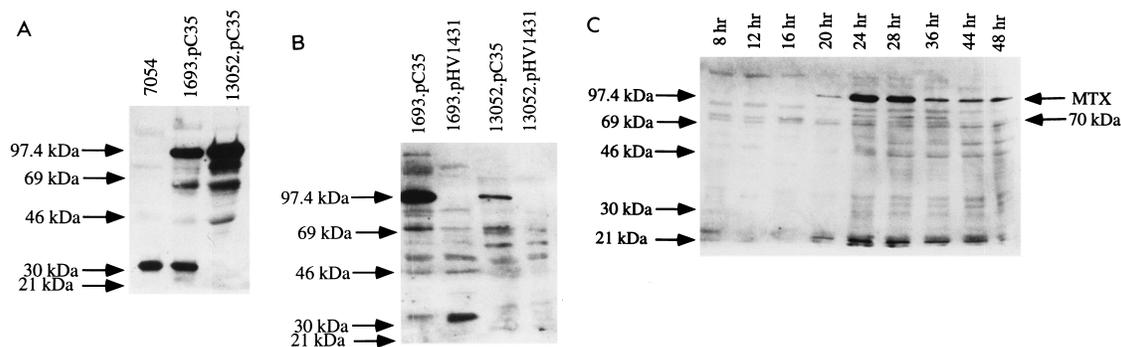

FIG. 5. Western blot analysis of vegetative and sporulated cells of *B. sphaericus* 1693.pC35 and 13052.pC35. Proteins were resolved in 0.1% SDS–10% polyacrylamide gels. (A) Vegetative cells. Strain 7054 is a nontoxic strain shown as a control. (B) Sporulated cells. pHV1431 is the plasmid vector without the *mtx* gene. (C) Time-point study of synthesis and degradation of Mtx during vegetative growth and sporulation of *B. sphaericus* recombinant strain 13052.pC35. MTX marks the position of the 100-kDa toxin, and 70 kDa marks the position of a putative partial breakdown product of the 100-kDa toxin.

(SSII-1, 2362, 2297, 1593M, and 1691), which is consistent with the identification of a vegetative promoter upstream of the *mtx* gene in strain SSII-1 (24). The level of Mtx in vegetative cells varied among these five strains but could not be detected in four other strains (IAB59, 31, Kellen Q, and 2317.3). The variation in the yield of Mtx among the nine strains could be due to differences in *mtx* promoter strength or to other factors such as the presence of proteases in vegetative cells of some of the strains. Although protein extracts from vegetative cells of the nine strains did not degrade the 97-kDa toxin from SSII-1 during long incubations, it is still possible that the native Mtx protein in strains IAB59, 31, Kellen Q, and 2317.3 is susceptible to proteases in these strains because of variations in the Mtx amino acid sequence.

In contrast to the situation in vegetative cells, the levels of Mtx in sporulated cultures of 2362, 2297, 1593M, and 1691 were extremely low and could not be detected in sporulated cells of SSII-1, which had the highest level of Mtx in vegetative cells. In sporulated cultures of 2362, 1593M, and 1691, small amounts of an additional 70-kDa protein which was relatively abundant in strain 1593M were observed. Since the purified 97-kDa toxin of strain SSII-1 is processed to 70- and 27-kDa peptides by mosquito-larval gut proteases (23), it is possible that the 70-kDa protein is derived from Mtx in these three strains by in vivo proteolytic processing.

Strains belonging to DNA homology groups I, IIA, IIB, and V produced intracellular and extracellular proteases during sporulation which were capable of degrading the purified, recombinant 97-kDa toxin from SSII-1, whereas strains in groups III and IV were completely devoid of such proteases. The degradation of the 97-kDa toxin by extracellular proteases of group I, II, and V strains was complete, while the degradation by intracellular proteases was only partial; in some strains a smaller amount of the 97-kDa toxin was still present together with additional 70- and 27-kDa peptides. It will be recalled that a 70-kDa peptide was also synthesized in sporulated cells of 2362, 1593M, and 1691, as shown by Western blotting. Many insecticidal toxins are activated by proteolytic cleavage of protoxins (2, 17), and Mtx has been shown to be cleaved into 70- and 27-kDa peptides by mosquito-larval gut proteases (23). It remains to be determined whether the generation of these peptides by mosquito gut or *B. sphaericus* proteases represents cleavage activation of this toxin.

If proteases are responsible for the processing and/or degradation of Mtx during sporulation of mosquito-larvicidal *B. sphaericus*, then Mtx synthesized during vegetative growth should be degraded during sporulation in protease-positive but not in protease-negative strains transformed with the *mtx* gene. This is exactly what was observed, since during vegetative growth recombinant strains 1693.pC35 (protease negative) and 13052.pC35 (protease positive) both produced large amounts of Mtx, which declined dramatically during sporulation of 13052.pC35 but remained essentially unchanged in 1693.pC35. A time-point study of the fate of Mtx in the protease-positive strain 13052.pC35 confirmed that Mtx synthesized during vegetative growth was proteolytically degraded at the onset of sporulation, and again some processing of Mtx to a 70-kDa peptide was observed. This difference in 100-kDa toxin expression was not due to a significant difference between strains 1693 and 13052 in the sporulation rate (data not shown). An intriguing idea is that the synthesis of Mtx during vegetative growth and, conversely, the synthesis of the binary toxin during sporulation could be devices used by natural mosquito-larvicidal strains of *B. sphaericus* to delay or prevent the development of larval resistance to killing by these toxins. This speculation relies on the reasonable assumption that Mtx and the unrelated binary toxin bind to different mosquito-larval gut receptors and have different modes of action (17, 22).

The level of expression of the *mtx* gene in the vegetative cells of 13052.pC35 was higher than that in vegetative cells of 1693.pC35. This could be due to the presence of the endogenous *mtx* gene in strain 13052, a difference in the SSII-1 *mtx* promoter strength, or a difference in the copy number of plasmid pC35. These differences in expression during vegetative growth do not, however, affect the main conclusion that in many mosquito-larvicidal strains of *B. sphaericus*, the 100-kDa toxin synthesized during vegetative growth is proteolyzed during sporulation.

Finally, the mosquito-larvicidal activities of natural and recombinant *B. sphaericus* strains were compared with the yields of Mtx protein observed during vegetative growth and sporulation (Table 1). The toxicities of recombinant strain 1693.pC35 expressing Mtx were the same in both growth phases, which is consistent with the similar yields of Mtx observed in the Western blot analysis. Because the native promoter of the *mtx* gene resembles a vegetative promoter (24), the similar high levels of toxicity due to Mtx found in both vegetative and sporulated cells of 1693.pC35 strongly suggest that Mtx synthesized during vegetative growth remains stable in sporulated cells owing to the lack of toxin-degrading proteases. The levels of mosquitocidal activity were also high and not significantly different in vegetative and sporulated cells of 13052.pC35, but unlike strain 1693 this strain harbors an endogenous binary toxin gene highly expressed during sporulation, which makes it difficult to judge the contribution of the cloned *mtx* gene to toxicity during the sporulation phase.

The toxicity of vegetative and sporulated cells of the protease-negative strain 1693.pC35 against *C. quinquefasciatus* mosquito larvae was as high as the toxicity of sporulated cells of the natural high-toxicity *B. sphaericus* strains such as 2362 (Table 1) (5). These natural strains, which develop high toxicities during sporulation and much lower toxicities in the vegetative phase, have potential for the control of *Culex* mosquito larvae (17, 18). As 1693.pC35 produces the same high toxicity to mosquito larvae during vegetative growth and sporulation and the recombinant 100-kDa toxin is stable during sporulation, it is conceivable that protease-negative strains of *B. sphaericus* expressing Mtx and other toxins may form the basis of an alternative to the natural high-toxicity strains for mosquito control.

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