Cytolytic Toxin Cyt1Aa of *Bacillus thuringiensis* Synergizes the Mosquitocidal Toxin Mtx1 of *Bacillus sphaericus*

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Using the shuttle vector pBU4, the mosquitocidal toxin gene *mtx1* from *Bacillus sphaericus* strain SSII-1 was introduced into an acrystalliferous strain of *B. thuringiensis* both individually and in combination with the accessory protein gene *p20* and the cytolytic protein gene *cyt1Aa* from *B. thuringiensis* subsp. *israelensis*. Bioassay results indicated that the recombinants B-pMT4(Mtx1) and B-pMT9(Mtx1), both individually containing *mtx1*, had moderate toxicities to binary toxin susceptible and binary toxin resistant *Culex quinquefasciatus* larvae during the vegetative growth stage, but that their toxicities declined rapidly during the sporulation phase. The LC$_{50}$ values were 2.5 and 4.8 mg/ml respectively, against 3–4 instar susceptible and resistant larvae for the final sporulated cultures of recombinants B-pMT9(Mtx1), and little toxicity was detected for B-pMT4(Mtx1). Meanwhile, the recombinant B-pMPX2(Mtx1+Cyt1Aa) expressing Mtx1, P20 alone, and Cyt1Aa in combination had stable toxicities during both the vegetative phase and the sporulation phase, with a LC$_{50}$ ranging from 0.45–0.58 mg/ml. Furthermore, expression of Cyt1Aa appeared to enhance the activity of Mtx1 to target mosquito larvae, suggesting a synergism between Cyt1Aa and Mtx1 toxins.

**Key words:** 100-kilodalton mosquitocidal toxin; synergism; *Bacillus sphaericus*; Cyt1Aa; resistance

*Bacillus sphaericus* has been successfully applied worldwide in urban *Culex quinquefasciatus* control for more than 10 years. The main mosquitocidal activity is due to the presence of a binary crystal toxin (Bin), which is produced and assembled as parasporal bodies during sporulation. But, this toxin has a relatively narrow spectrum of action, having high toxicity against *Culex* species, moderate toxicity against *Anopheles* species, and a range of toxicities to mosquitoes of the genus *Aedes* from high (*Aedes atropalpus, Aedes nigromaculis*) to little or no toxicity (*Aedes aegypti*). Previous studies indicate that there is only one specific Bin receptor in larval midgut brush border membrane (BBMF), and that the target mosquitoes can evolve strong resistance to this toxin after long-term continuous pressure both in the laboratory and under field conditions. The appearance of high level resistance to Bin in target mosquitoes has hindered the further application of *B. sphaericus* as a microbial mosquitocidal agent, and thus it is necessary to discover and develop new toxins to supplement Bin for future mosquito control.

In addition to the binary crystal toxin, some toxic *B. sphaericus* strains can produce another mosquitocidal toxin (Mtx1, 100-kilodalton) in their vegetative growth stage. It has been found that the N-terminal region of this toxin has significant homology with the catalytic subunits of ADP-ribosyltransferase toxins, such as the pertussis toxin S1 subunit, and hence it is assumed that this toxin has a different mode of action from Bin. Although purified Mtx1 has high activity against a variety of mosquitoes, comparable with Bin, the native *B. sphaericus* strain produces only low quantities of Mtx1 in vegetative cells under the regulation of a weak vegetative promoter of the *mtx1* gene, and no Mtx1 is deposited in parasporal crystals. Furthermore, Mtx1 can easily be cleaved into non-active peptides because of its liability to a proteinase(s) secreted during bacterial growth; hence little Mtx1 toxin is detected in the final whole culture of toxic strains. Only in a proteinase-deficient strain of *B. sphaericus*, can this toxin be expressed and accumulated efficiently with a final whole culture of toxic strains. Therefore, this toxin might be a potential candidate for further mosquito control, especially for Bin resistant mosquito control if its expression and stability can be improved.

Recently, the cytolytic toxin Cyt1Aa toxin from *B. thuringiensis* subsp. *israelensis* (*B.t.i*) was effectively co-expressed with other toxins to delay and overcome the resistance of mosquitoes to Cry4A and Bin as well as the resistance of the cottonwood leaf beetle, *Chrysomela scripta*, to Cry3Aa. With co-expression...
of accessory protein P20 and Cyt1Aa, Bin has been found not only to have activity against Bin resistant mosquitoes, but also to have a higher toxicity to susceptible mosquito larvae than Bin alone. It has been suggested that a mixture of different toxins might be more effective than a single toxin and might delay rapid onset of resistance, but no data on the interaction or synergism of Cyt1Aa and Mtx1 has been recorded. In this study, the Mtx1 toxin was effectively co-expressed or synergism of Cyt1Aa and Mtx1 has been recorded. In this study, the Mtx1 toxin was effectively co-expressed

### Materials and Methods

**Bacterial strains and plasmids.** B. sphaericus strain SSII-1 and an acrystalliferous strain 4Q7 of B.t.i. were obtained from the Institut Pasteur (Paris, France), and the latter was used as the recipient strain in the transformation experiments. Escherichia coli TG-1 was used as the recipient strain for subcloning experiments. A 4Q7 recombinant B-CW-1 (Bin), which contains a Bin gene from B. sphaericus strain C3-41, was used as a control in mosquito bioassays. The shuttle vector pBU4 was used for gene cloning and expression, kindly provided by Dr. A. Delecluse of the Institut Pasteur. P20 and Cyt1Aa genes from B.t.i., was constructed by Li et al. The bacterial strains and plasmids used in this study are listed in Table 1.

**Mosquito colonies.** A susceptible laboratory C. quinquefasciatus colony (SLCq) was established from a laboratory-reared colony maintained at Hubei Academy of Medical Sciences (Wuhan, China) for more than 10 years. A Bin resistant C. quinquefasciatus colony (RLCq) was selected with B. sphaericus C3-41 for 13 generations in the laboratory, with a resistance ratio of 142,000-fold Bin. Larvae of all colonies were reared in enamel pans filled with dechlorinated tap water and fed with a mixture of yeast powder and wheat mill. The pupae were removed every day from the pans and placed in screen cages for emergence. The adults were allowed to feed on a 10% sucrose solution and the females were fed with blood from mice. All larvae and adults were held at 26–28 °C under a photoperiod of 12:12 (L:D) h.

**Culture conditions.** All recombinant E. coli strains were grown in Luria Bertani (LB) broth supplemented with tetracycline (12.5 μg/ml) when appropriate. B.t.i recombinants were grown in G-Tris broth liquid medium until 20% sporangial lysis for the sporulated culture preparation and in LB broth for 24 h for the vegetative culture preparation; all media were supplemented with tetracycline (12.5 μg/ml). B. sphaericus SSII-1 strain was grown in MBS broth for 48 h for the sporulated culture preparation and in LB broth for 24 h for vegetative culture preparation.

**Construction of recombinant plasmids and their transformation.** Plasmids were isolated from E. coli by the standard alkaline lysis procedure. Cloning experiments and restriction enzyme analyses were carried out as described by Sambrook et al. An approximately 3.8-kb PstI fragment from pXP33, which contains the mtx1 gene with its native promoter and transcriptional

### Table 1. Plasmids and Strains Used in This Study

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<thead>
<tr>
<th>Plasmids</th>
<th>Description</th>
<th>References</th>
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<tbody>
<tr>
<td>pXP33</td>
<td>Plasmid containing the mtx1 from B. sphaericus SSII-1</td>
<td>[4]</td>
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<tr>
<td>pBU4</td>
<td>Shuttle vector containing tet&lt;sup&gt;B&lt;/sup&gt; and amp&lt;sup&gt;B&lt;/sup&gt; determinants</td>
<td>[12]</td>
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<tr>
<td>pMT9</td>
<td>Derivative of pBU4 containing mtx1 in forward orientation</td>
<td>This work</td>
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<tr>
<td>pMT4</td>
<td>Derivative of pBU4 containing mtx1 in reverse orientation</td>
<td>This work</td>
</tr>
<tr>
<td>pBA30</td>
<td>Derivative of pBU4 containing cyt1Aa and p20</td>
<td>[7]</td>
</tr>
<tr>
<td>pMPX2</td>
<td>Derivative of pMT9 containing the cyt1Aa, p20 and mtx1</td>
<td>This work</td>
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<tr>
<th>Strains</th>
<th>Description</th>
<th>References</th>
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<tr>
<td>B.s SSII-1</td>
<td>B. sphaericus wild-type strain containing mtx1 gene</td>
<td>Institut Pasteur</td>
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<tr>
<td>B.t.i 4Q-7</td>
<td>B. thuringiensis subs. israelensis acrystalliferous strain</td>
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<tr>
<td>E. coli TG-1</td>
<td>F' (traD36, proAB&lt;sup&gt;B&lt;/sup&gt;, LacP, lacZ, ΔM15)</td>
<td></td>
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<tr>
<td>B-pMT9(Mtx1)</td>
<td>B.t.i 4Q-7 containing the plasmid pMT9</td>
<td>This work</td>
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<td>B.t.i 4Q-7 containing the plasmid pMT4</td>
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<tr>
<td>B-pMPX2(Mtx1+C)</td>
<td>B.t.i 4Q-7 containing the plasmid pMPX2</td>
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<tr>
<td>B-pBA30(Cyt1Aa)</td>
<td>B.t.i 4Q-7 containing the plasmid pBA30</td>
<td>[7]</td>
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<tr>
<td>B-CW-1(Bin)</td>
<td>B.t.i 4Q-7 expressing binary toxin from B. sphaericus</td>
<td>[11]</td>
</tr>
<tr>
<td>B-pBU4</td>
<td>B.t.i 4Q-7 containing the plasmid pBU4</td>
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B. thuringiensis and the Health Organization. The bioassays were undertaken by a standard method recommended by the World Sporulated culture preparations of B. sphaericus were collected by centrifugation for 5 min at 15,000 g. Spore-crystal mixtures were harvested by centrifugation for 5 min at 12,000 g, washed twice with cold 0.1 M sodium phosphate buffer, and resuspended in ice-cold deionized water. Proteins were separated by SDS–PAGE in a 12% acrylamide gel, and resuspended in ice-cold deionized water. Proteins containing 12.5 mg protein were loaded per lane. Electrophoresis was carried out in a constant current of 30 mA for B. thuringiensis 4Q7 by electroporation as described by Shi et al., giving recombinant B. t.i. strains designated B-pBU4, B-pBA30(Cyt1Aa), B-pMT4(Mtx1), B-pMT9(Mtx1) and B-pMPX2(Mtx1+Cyt1Aa) which contain plasmids pBA30(Cyt1Aa) (encoding Cyt1Aa and P20) and pMT4 in which the transcription terminator, was inserted into shuttle vector pBU4, resulting in plasmid pMT9 in which the transcription direction is the same as that of the lacZ gene in vector pBU4, and plasmid pMT4 in which the transcription direction is in the opposite orientation. The 4.2-kb Sal1-SacI fragment, which contains the cyt1Aa and p20 genes from plasmid pBA30, was ligated with the plasmid pMT9, giving rise to plasmid pMPX2 containing the mtx1, cyt1Aa and p20 genes (Fig. 1). All recombinant plasmids and control plasmid pBU4 were introduced into acrystaliferous strain B. thuringiensis 4Q7 by electroporation as described by Shi et al., giving recombinant B. t.i. strains designated B-pBU4, B-pBA30(Cyt1Aa), B-pMT4(Mtx1), B-pMT9(Mtx1) and B-pMPX2(Mtx1+Cyt1Aa) which contain plasmids pBU4, pBA30, pMT4, pMT9 and pMPX2 respectively.

Protein analysis. For protein analysis, all the B. thuringiensis transformants were grown at 30°C in LB broth containing 12.5 μg/ml tetracycline until 20% sporangium lysis. Spore-crystal mixtures were collected by centrifugation for 5 min at 6,000 × g, then washed twice and resuspended in ice-cold deionized water. Proteins were separated by SDS–PAGE in a 12% acrylamide gel, which was fixed and stained with Coomassie brilliant blue R250 after electrophoresis. For electron microscopy, B. thuringiensis recombinant strains were grown in LB broth at 30°C until sporulation, then the spore-crystal mixtures were harvested by centrifugation for 5 min at 6,000 × g. Cells were fixed in 2% glutaraldehyde followed by 1% OsO4. The dehydrated samples were set in Epon for slicing and subsequently collected on Formvar-coated nickel grids, and examined with a Hitachi H7000FA electron microscope.

Toxicity bioassays. The toxicities of vegetative and sporulated culture preparations of B. sphaericus SSII-1 and the B. thuringiensis recombinants were determined by a standard method recommended by the World Health Organization. The bioassays were undertaken by placing groups of 25 larvae from 3rd to early 4th instar in 100 ml of distilled water in 125 ml plastic cups with the desired concentration of samples. At least 5 concentrations giving mortality between 2 and 98% were tested, and mortality was recorded after 48 h. One drop of larval food was added to each cup. Tests were replicated on at least 3 different days. Fifth percent and 90% lethal concentration (LC50 and LC90 respectively) was determined using probit analysis with 95% confidence limits. LC50 and LC90 were expressed in mg final whole culture/ml.

For comparison of the toxicity among the B.t.i. recombinants and B. sphaericus SSII-1, 2.0 and 0.7 mg sporulated culture preparations of B-pBA30(Cyt1Aa), B-pMT9(Mtx1), B-MTX2(Mtx1+Cyt1Aa), and a vegetative culture preparation of SSII-1 respectively were added to 100 ml of distilled water in 125 ml plastic cups containing 25 larvae from 3rd to early 4th instar. Larval mortality was recorded every 2–3 h until 48 h. Each dose had four repeats and the tests were replicated on at least 3 different days.

Results

Coexpression of Mtx1 and Cyt1Aa in B. thuringiensis SDS–PAGE showed that both B.t.i recombinant B-pBA30(Cyt1Aa) (encoding Cyt1Aa and P20) and B-pMPX2(Mtx1+Cyt1Aa) (encoding Cyt1Aa, P20 and Mtx1) produced a peptide of 28 kDa during sporulation, consistent with production of Cyt1Aa. Besides this peptide, there was little difference between strains B-pMPX2(Mtx1+Cyt1Aa) and B-pBA30(Cyt1Aa). No obvious 100-kDa or 97-kDa band representing Mtx1 was observed in the vegetative or sporulated cultures of B-pMPX2(Mtx1+Cyt1Aa) and B-pMT9(Mtx1) (Fig. 2).

Electron microscopy revealed no inclusion of crystal in the sporulated recombinants B-pMT9(Mtx1) or B-pMT4(Mtx1) (data not shown), while in the recombinants B-pMPX2(Mtx1+Cyt1Aa) and B-pBA30-(Cyt1Aa), there was apparently a rhomboidal crystalline inclusion body (Fig. 3) as is to be expected in a bacterium producing Cyt1Aa. Twenty μl of spore-crystal suspensions were subjected to sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis, followed by staining with Coomassie blue. Lane M, protein molecular weight marker 1, B-pBU4; 2, B-pMT9(Mtx1); 3, B-pBA30(Cyt1Aa); 4, B-pMPX2(Mtx1+Cyt1Aa); 5, crystal of Cyt1Aa.
Toxicity of recombinants

Bioassay results revealed that in the vegetative growth stage, the *B.t.i* recombinants containing the *mtx1* gene alone and the naturally occurring *B. sphaericus* SSII-1 strain have moderate toxicities to 3–4 instar *C. quinquefasciatus* mosquito larvae, with LC$_{50}$ values varying from 0.20 mg/ml to 0.52 mg/ml for the Bin susceptible colony and from 0.30 mg/ml to 1.5 mg/ml for the Bin resistant colony. The toxicity of these native and recombinant Mtx1 producing bacteria against both susceptible and resistant colonies clearly declined when they developed into sporulation, with an LC$_{50}$ value of 2.5 mg/ml and 7.4 mg/ml for strain B-pMT9(*Mtx1*), and 1.5 mg/ml and 1.4 mg/ml for SSII-1. Little toxicity was detected in sporulated B-pMT4(*Mtx1*), in which the transcription direction of *mtx1* was opposite to that of *lacZ*. Although the vegetative and sporulation cultures of B-pBA30(*Cyt1Aa*) have no significant high toxicity against susceptible and resistant mosquito larvae, the sporulated B-pBA30(*Cyt1Aa*) culture might enhance the mosquitocidal activity of vegetative and sporulated B-pMT9(*Mtx1*) against both colonies. The mixtures of vegetative B-pMT9(*Mtx1*) and sporulated B-pBA30-(*Cyt1Aa*) (in a ratio of 1:1 in volume) have similar toxicities to both mosquito colonies, with LC$_{50}$ values of 0.45 mg/ml and 0.61 mg/ml respectively. Importantly, the mixture of sporulated B-pMT9(*Mtx1*) and sporulated B-pBA30(*Cyt1Aa*) gave LC$_{50}$ values of 1.6 mg/ml and 1.9 mg/ml to both mosquito colonies, toxicities somewhat higher than that of B-pMT9(*Mtx1*) alone (2.5 mg/ml). For strain B-pMPX2(*Mtx1+ Cyt1Aa*), toxicity remained stable both in vegetative and sporulation cultures, particularly in the sporulation stage, where its toxicities were clearly higher than strains B-pMT9(*Mtx1*) and B-pMT4(*Mtx1*), as well as for the mixture of sporulated B-pBA30(*Cyt1Aa*) and B-pMT9(*Mtx1*) (Table 2). In addition, it was noted that all recombinants producing Cyt1Aa (B-pBA30 or B-pMPX2) induced the larvae to congregate as early as 10 min after exposure at high treatment dosages.

**Time course mortality with Cyt1Aa and Mtx1**

In treatment dosages of 2.0 and 0.7 mg/ml of culture preparation respectively, different recombinants induced different time-course mortalities against the susceptible mosquito colony, as indicated in Fig. 4. The vegetative culture preparation of *B. sphaericus* SSII-1 gave rise to nearly 100% and 90% larval mortality respectively within 48 h, but no significant larval mortality was observed until 6 h of exposure at the two concentrations. However, death of the infected mosquitoes began within 1 h following the addition of preparations of B-pMPX2(*Mtx1+ Cyt1Aa*) and B-pBA30(*Cyt1Aa*), both producing Cyt1Aa protein. At the same two dosages, B-pMT9 produced low larval death over the period of the experiment and mortality was not observed until late in the bioassay at the higher concentration (Fig. 4). Furthermore, B-pMPX2(*Mtx1+ Cyt1Aa*) caused mortality as high as that of SSII-1 in 48 h, whilst B-pBA30(*Cyt1Aa*) caused only low mortality. In these experiments, the combination of Mtx1 and Cyt1Aa administered together from the same recombinant B-pMPX2(*Mtx1+ Cyt1Aa*) clearly showed a level of toxicity that is more than an additive effect of the two toxins individually (B-pMT9 and B-pBA30) and this was particularly evident at dosage of a 0.7 sporulated culture mg/ml (Fig. 4B).

**Discussion**

The toxicity profile of *B.t.i* recombinants carrying the *mtx1* gene alone, B-pMT4(*Mtx1*) and B-pMT9(*Mtx1*) has some similarity to that seen in *B. sphaericus* SSII-1, appearing predominantly during vegetative growth and then declining greatly during sporulation. Nevertheless B-pMT9 retained partial mosquito larvicidal activity during the sporulation phase. The difference between the two constructs might be caused by the influence on the expression of Mtx1 of the vector-derived *lacZ* promoter, especially during the sporulation phase. In contrast to the situation with the recombinants carrying the *mtx1* gene alone, the toxicity of B-pMPX2(*Mtx1+ Cyt1Aa*) remained stable whether in the vegetative phase or during sporulation. This observation is consistent with the results from expression of Mtx1 in a proteinase-deficient strain of *B. sphaericus*. Furthermore, B-pMPX2(*Mtx1+ Cyt1Aa*) can induce higher mosquito larval mortality than either B-pMT9(*Mtx1*) or B-
Evidence of the presence of a crystal in B-pBA30(Cyt1Aa) and B-pMPX2(Mtx1+Cyt1Aa) and the results of SDS–PAGE indicated that the presence or absence of Mtx1 (Fig. 2, Fig. 3), did not significantly affect expression or crystal formation of Cyt1Aa. In addition, the presence of the cyt1Aa gene had no apparent influence on the expression of Mtx1 in the vegetative phase, based on the bioassay results.

Although SSII-1 has moderate toxicity to the targets both in vegetative and sporulation stages, it requires several hours to kill mosquito larvae, even in high concentrations. The same pattern has been observed for recombinant B-pMT9(Mtx1), which expressed Mtx1 alone. In contrast, at the same dilution of B-pMPX2(Mtx1+Cyt1Aa) and pBA30(Cyt1Aa), both producing Cyt1Aa protein, mosquito larvae began to congregate within 10 min of exposure, then became insensitive to external stimulation until death.

The presence of Mtx1 and Cyt1Aa simultaneously, either as a mix of B-pMT9(Mtx1) and B-pBA30(Cyt1Aa) or in strain B-pMPX2(Mtx1+Cyt1Aa) appeared to produce greater toxicity than strains producing either toxin alone. This was particularly clear when sporulating cultures were used and in the time course experiments. The effect was more pronounced for B-pMPX2(Mtx1+Cyt1Aa), which co-expresses Mtx1, Cyt1Aa and P20 in the same cell, than for the combination of strains B-pMT9(Mtx1) and B-pBA30(Cyt1Aa). These results imply either that there is a synergism of action of the two toxins against the susceptible C. quinquefasciatus
colony or that there is stabilisation of the Mtx1 toxin when co-expressed with Cyt1Aa and P20. In the latter case, the effect might be contributed by P20, which might function as a chaperone for Mtx1, protecting it from proteolytic attack during sporulation, with or without subsequent synergism between the two toxins. The details of these possible interactions among Cyt1-Aa, Mtx1 and P20 require further characterization.

**Acknowledgments**

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