Expression of the Mosquitocidal Toxins of Bacillus sphaericus and Bacillus thuringiensis subsp. israelensis by Recombinant Caulobacter crescentus, a Vehicle for Biological Control of Aquatic Insect Larvae

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In the quest for effective control of mosquitoes, attention has turned increasingly to strains of the bacteria Bacillus sphaericus and Bacillus thuringiensis subsp. israelensis, which produce potent toxins with specific mosquitocidal activities. However, sedimentation of the bacterial spores limits the duration of effective control after field application of these bacilli. We describe here the cloning of genes encoding the 51.4- and 41.9-kDa toxins from B. sphaericus 2297, the 100-kDa toxin from B. sphaericus SSII-1, and the 130-kDa toxin from B. thuringiensis subsp. israelensis into the broad-host-range plasmid pRK248 and the transfer of these genes for expression in Caulobacter crescentus CB15. The recombinant C. crescentus cells were shown to be toxic to mosquito larvae. Caulobacter species are ubiquitous microorganisms residing in the upper regions of aquatic environments and therefore provide the potential for prolonged control by maintaining mosquitocidal toxins in larval feeding zones.

The mosquito is the vector of a variety of widespread human diseases, such as malaria, filariasis, and dengue fever. The control of mosquito populations to limit the spread of disease, especially in the third world, has been targeted as an area of special importance by the World Health Organization (25). Early attempts to control mosquitoes used chemical pesticides with relatively low target specificity, but these compounds carry environmental hazards, and their repeated use has led to the development of resistance in target populations. More recently, biological pesticides which utilize highly specific insect pathogens have been developed (13). Among the new generation of biological control agents which are currently being deployed for control of mosquitoes are strains of Bacillus sphaericus and Bacillus thuringiensis. The highly toxic strains of B. sphaericus used in the field produce two proteins with masses of 51.4 and 41.9 kDa which are jointly responsible for toxicity (4, 8). These toxins are produced at the onset of sporulation and accumulate in parasporal crystalline inclusions (3, 7). Binding of the two proteins occurs in the gastric caecum and posterior midgut of Culex quinquefasciatus larvae (6, 8), causing disruption of the gut cells and larval death. In addition to the 51.4- and 41.9-kDa toxins, toxic B. sphaericus strains may produce a 100-kDa toxin during vegetative growth (16, 21). B. sphaericus is generally most toxic for mosquitoes of the genus Culex, with less activity against Anopheles species and least activity against Aedes species (5, 22). This mosquitocidal target spectrum is complemented by that of the 130-kDa toxin of B. thuringiensis subsp. israelensis. This protein is also produced during sporulation, is deposited as an endosporal crystal, and is most active against Aedes mosquitoes, with less toxicity to Anopheles species and least to Culex species (2). The site of the action of the 130-kDa toxin, like that of the B. sphaericus toxins, is in the larval gut, where the 130-kDa toxin causes cell lysis by a process of colloid osmotic lysis (12). The 130-kDa protein of B. thuringiensis subsp. israelensis is also toxic to the blackfly, a vector of onchocerciasis. One of the major factors limiting the duration of mosquito control following the application of B. sphaericus or B. thuringiensis strains is the rapid sedimentation of bacterial spores (9, 15). Since most mosquito larvae feed at or near the water surface, the effective larvicidal activity of the toxins is limited to the short period prior to spore sedimentation. Other workers have recognized this problem and have transferred insecticidal toxin genes from B. sphaericus (10) or B. thuringiensis subsp. israelensis (1) into cyanobacteria. In these studies, lysates produced by the sonication of recombinant cyanobacteria were shown to be toxic to mosquito larvae, but larvicidal activity of intact recombinant cyanobacteria was not demonstrated.

In this paper, we report the production of recombinant Caulobacter crescentus by transformation with broad-host-range plasmids carrying genes encoding larvicidal toxins from either B. sphaericus or B. thuringiensis subsp. israelensis. We further demonstrate the mosquito larvicidal activity of intact recombinant C. crescentus. Bacteria belonging to the genus Caulobacter were chosen for the following reasons. Caulobacter species are found in almost every aquatic habitat, and within these habitats they are found predominantly in the regions at or close to the water surface (18). In the flagellate swarmer stage, Caulobacter species are motile, thus allowing distribution through the habitat. In both the swarmer and the stalked stages, they are capable of attachment to solid particles at or near the water surface. Caulobacter species are able to persist and grow in environments with low nutrient concentrations (18). These bacteria

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are therefore ideal carriers for biological toxins aimed at the surface-feeding larvae of mosquitoes.

MATERIALS AND METHODS

Construction of clones. In order to prevent possible unrestricted transmission of toxin genes among microorganisms in the field, pRK248, a derivative of the broad-host-range plasmid RK2 lacking the genes necessary for self-transmission (23), was chosen as the cloning vector for transformation into C. crescentus. Fragments containing genes encoding mosquitocidal toxins were prepared for ligation into pRK248 (23) as follows.

(i) Clones encoding the 51.4- and 41.9-kDa toxins of B. sphaericus 2297. The fragment bearing the genes encoding the 51.4- and 41.9-kDa toxins was removed from the plasmid pCC2297-M (8) with SmaI and HindIII. This fragment was gel purified following electrophoresis in a 1% agarose gel and was made blunt ended by treatment with Klenow polymerase in the presence of all four deoxynucleotides. This fragment was ligated into EcoRI-cut, blunt-ended pRK248 to produce the plasmid pJC3 (Fig. 1A). The fragment bearing the toxin genes under the control of the tac promoter was excised from the plasmid pCK2297-M (17) with BamHI. This fragment was gel purified, made blunt ended, and ligated into EcoRI-cut, blunt-ended pRK248 to produce the plasmid pJK5 (Fig. 1B). To allow regulation of the tac promoter in C. crescentus, the fragment containing the lacF gene was removed from plasmid pMR1560 (19) by digestion with KpnI and PstI. This fragment was gel purified, made blunt ended by treatment with T4 DNA polymerase, and ligated into SmaI-cut, phosphatase-treated pCK2297-M to produce plasmids pCKI6 and pCKI7 with the lacF gene in opposite orientations. Fragments bearing the lacF gene and the genes encoding the 51.4- and 41.9-kDa toxins were removed from pCKI6 and pCKI7 by digestion with BamHI. These fragments were gel purified, made blunt ended, and ligated into EcoRI-cut, blunt-ended pRK248 to produce the plasmids pJK69 and pJK73, respectively (Fig. 1C).

(ii) Clones encoding the 130-kDa toxin of B. thuringiensis subsp. israelensis. The gene encoding the 130-kDa toxin from B. thuringiensis subsp. israelensis T14001 (WHO Collaborating Center for Entomopathogenic Bacteria, Institut Pasteur, Paris, France) was amplified from total DNA isolated from this strain by the polymerase chain reaction (PCR). The PCR using 40 cycles was carried out at the following temperatures and times: 95°C, 1 min; 40°C, 2 min; and 72°C, 3 min. PCR primers were designed from the sequence of Tungpradubkul et al. (24).

5′ primer: 5'-GGCGAATTCTAGAATTCTAGCTATTGGTTTA-3'
3′ primer: 5'-GGATCTGAGATTCATATCATATATTTTTATTTTTTTT-3'

The 5′ PCR primer includes a novel EcoRI site (nucleotides [nt] 4 to 9) upstream of the translational start codon (nt 10 to 12) and removes the naturally occurring EcoRI site within the coding sequence by a silent T-C mutation (nt 15). The PCR product was digested with EcoRI and HindIII, gel purified, and ligated into EcoRI-HindIII-cut pKK223-3 to place the gene for the 130-kDa toxin under the control of the
tac promoter. The resulting plasmid was shown to confer toxicity to *Escherichia coli* against *Aedes aegypti* and was designated pMJ22. A fragment carrying the toxin gene and the tac promoter was removed from this plasmid by digestion with SphI and HindIII, and after gel purification this fragment was ligated into Sph1-HindIII-cut pMJR1560 (which contains the lacI*"* gene) (19) to produce the plasmid pMJ11. The lacI*"* gene and the toxin gene were excised from pMJ11 by digesting the plasmid with KpnI and HindIII. The fragment containing these genes was gel purified, made blunt ended, and ligated into EcoRI-cut, blunt-ended pRK248 to produce the plasmid pKH49 (Fig. 2).

(iii) Clones encoding the 100-kDa toxin of *B. sphaericus* SSII-1. The *mtx* gene encoding the 100-kDa toxin of *B. sphaericus* SSII-1 was removed from the plasmid pXP33 (21) by digestion with StuI and HpaI. The fragment carrying the toxin gene and the *B. sphaericus* promoter was gel purified and ligated into EcoRI-cut, blunt-ended pRK248 to produce the plasmid pSS6 (Fig. 3).

**Transformation of C. crescentus.** The above constructs were transformed into *C. crescentus* CB15 cells (American Type Culture Collection) by electroporation. *C. crescentus* cells were prepared for this procedure by the method described by Dower et al. (11) for *E. coli*. *C. crescentus* CB15 was grown in PYE medium (0.2% peptone, 0.1% yeast extract) at 30°C to an optical density at 550 nm of 0.7. The cells were collected after chilling on ice for 15 min by centrifugation at 5,000 × g for 15 min. The cells were resuspended in 1 liter of cold water and pelleted as described before. Cells were resuspended in 20 ml of 10% glycerol and pelleted again as described above. Finally, the cells were resuspended in 20 ml of 10% glycerol and were stored at −70°C. Cells were thawed, and 1 μg of DNA (1 to 2 μl) was added to 40 μl of cells, mixed, and left on ice for 0.5 to 1 min. The mixture was transferred to a cold 0.2-cm electroporation cuvette, and electroporation was carried out at 2 to 4°C with a Bio-Rad gene pulser set at 2.5 kV, 25 μF, 200 ohms. After a single pulse at the above settings, 1 ml of PYE medium was added and the cells were allowed to regenerate at 30°C for 1 h. The cells were plated onto PYE agar containing 2 μg of tetracycline per ml and 10 μg of nalidixic acid per ml and incubated at 30°C for 48 to 72 h. Recombinant *C. crescentus* cells were screened for toxicity to mosquito larvae.

**Toxicity assays.** Mosquitocidal assays were performed as follows. Recombinant *E. coli* cells were grown overnight at 37°C in Luria broth containing 60 μg of ampicillin per ml or 2 μg of tetracycline per ml, as appropriate. Recombinant *C. crescentus* cells were grown at 30°C for 2 to 3 days in PYE medium containing 2 μg of tetracycline per ml and 10 μg of nalidixic acid per ml. When constructs containing the tac promoter were assayed, induction was carried out by harvesting cells and resuspending them in fresh medium containing 5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and continuing the incubation for 1 to 5 h. Cells from 5 ml of culture were harvested by centrifugation at 5,000 × g for 10 min, washed once with 0.85% saline, and finally resuspended in 1 ml of 0.85% saline. Aliquots of this cell suspension were added to 10 ml of water and 10 second-instar mosquito larvae. The final cell concentration in all assays was maintained at 5 × 10⁸ cells per ml by adding control *C. crescentus* or *E. coli* cells transformed with pRK248 to various amounts of the test recombinant. When the toxicity of *B. sphaericus* toxins was assayed, larvae of the mosquito species *C. quinquefasciatus* were used, while *A. aegypti* were used in the assay of recombinants carrying the *B. thuringiensis* subsp. *israelensis* toxin.

**RESULTS AND DISCUSSION**

Recombinant *C. crescentus* cells transformed with pJC3 (Fig. 1A) were produced which express the *B. sphaericus* 51.4- and 41.9-kDa toxins under the control of the *B. sphaericus* promoter. In order to enhance toxin production in recombinant bacteria, the genes encoding the 51.4- and 41.9-kDa toxins were placed under the control of the tac promoter (pJK5) (Fig. 1B). However, no recombinant *C.
were the 51.4-crescentus. ml. per while B. lacIq colonies indicate that (Fig. 1C), proteins from to construct to plating after of toxins with promoter pRK248. Blunt-ended restriction sites used in cloning procedures are denoted by an X. The toxin gene is under the control of the tac promoter in the presence of the lactose repressor gene (pKH49).

FIG. 2. Construction of a plasmid encoding the 130-kDa toxin of B. thuringiensis subsp. israelensis in the broad-host-range plasmid pRK248. Blunt-ended restriction sites used in cloning procedures are denoted by an X. The toxin gene is under the control of the tac promoter in the presence of the lactose repressor gene (pKH49).

crescentus colonies were produced after electroporation with this construct. High levels of expression of the toxin genes by these clones may have been inhibitory to the growth of recombinant cells. We therefore introduced the lacP0 gene encoding the lactose repressor protein into our constructs to reduce basal expression of the toxin genes while maintaining the ability to induce production of the toxins with IPTG once recombinants had been obtained. Plasmids pJK69 and pJK73, which contain the lacP0 gene, in addition to the toxins under the control of the tac promoter (Fig. 1C), were able to yield C. crescentus recombinants after plating onto PYE agar containing 2 μg of tetracycline per ml. However, no colonies formed when transformants were replated onto PYE agar containing 5 mM IPTG. This indicates that unrestricted expression of the 51.4- and 41.9-kDa proteins from the tac promoter prevents growth of C. crescentus. Recombinant C. crescentus cells expressing both the 51.4- and 41.9-kDa proteins of B. sphaericus under the regulation of the natural B. sphaericus promoter from plasmid pJC3 are toxic to mosquito larvae (Table 1). The level of toxicity was increased with the inducible tac promoter in plasmid pJK69. IPTG induction of 51.4- and 41.9-kDa toxin production from plasmid pJK69 resulted in a further increase in toxicity (approximately twofold) (Table 1). Recombinant C. crescentus cells containing plasmid pJK73 in which the lacP0 gene is in an orientation opposite to that of pJK69 were also toxic, although slightly less so than those containing pJK69. The reduced toxicity may be due to the absence of a strong transcriptional terminator following the lacP0 gene, leading to the production of antisense mRNA to the toxin genes and resulting in reduced translation of toxin mRNAs.

Although recombinant C. crescentus cells in which toxin expression is driven by the natural B. sphaericus promoter are toxic, the presence of the lacP0-regulated tac promoter improves effective control of mosquito larvae. C. crescentus cells transformed with plasmid pJK69 produce 50% lethal concentrations (LC50) of 2.2 × 105 and 1.9 × 105 cells per ml at 24 and 48 h, respectively, approximately 103-fold more toxic than E. coli JM109 transformed with the same plasmid. Although the toxicity of transformed C. crescentus is lower than that of B. sphaericus 2297 (LC50 3.5 × 102 spores per ml at 24 h) (3a), it may be possible to enhance the toxicity of the recombinant C. crescentus by the use of other promoters.

Strains of B. sphaericus have been used as control agents of mosquito larvae in the field (14), but their toxicity is greatest against Culex mosquitoes (5, 22). In order to provide effective control of all mosquito vectors, it is necessary to complement the activity of the 51.4- and 41.9-kDa toxins...

FIG. 3. Construction of a plasmid encoding the 100-kDa toxin of B. sphaericus SSII-1 in the broad-host-range plasmid pRK248. Blunt-ended restriction sites used in cloning procedures are denoted by an X. The toxin gene is under the control of the B. sphaericus SSII-1 promoter (pSS6).
TABLE 1. Assay of transformed C. crescentus cells expressing the B. sphaericus toxins against C. quinquefasciatus larvae

<table>
<thead>
<tr>
<th>Vector</th>
<th>Final conc of test cells (cells/ml)</th>
<th>% Survivors* after:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>24 h</td>
</tr>
<tr>
<td>pJC3</td>
<td>3.7 x 10^7</td>
<td>0*</td>
</tr>
<tr>
<td></td>
<td>1.9 x 10^7</td>
<td>21*</td>
</tr>
<tr>
<td></td>
<td>3.7 x 10^5</td>
<td>100*</td>
</tr>
<tr>
<td>pJK69 (uninduced)</td>
<td>1.3 x 10^7</td>
<td>0*</td>
</tr>
<tr>
<td></td>
<td>2.5 x 10^6</td>
<td>5*</td>
</tr>
<tr>
<td></td>
<td>2.5 x 10^5</td>
<td>63*</td>
</tr>
<tr>
<td>pJK69 (induced)</td>
<td>1.3 x 10^7</td>
<td>0*</td>
</tr>
<tr>
<td></td>
<td>2.6 x 10^6</td>
<td>0*</td>
</tr>
<tr>
<td></td>
<td>2.6 x 10^5</td>
<td>32*</td>
</tr>
<tr>
<td>pJK73 (uninduced)</td>
<td>1.5 x 10^7</td>
<td>0*</td>
</tr>
<tr>
<td></td>
<td>3 x 10^6</td>
<td>10*</td>
</tr>
<tr>
<td></td>
<td>3 x 10^5</td>
<td>97*</td>
</tr>
<tr>
<td>pJK73 (induced)</td>
<td>1.5 x 10^7</td>
<td>0*</td>
</tr>
<tr>
<td></td>
<td>2.9 x 10^6</td>
<td>0*</td>
</tr>
<tr>
<td></td>
<td>2.9 x 10^5</td>
<td>71*</td>
</tr>
<tr>
<td>pSS6</td>
<td>4.6 x 10^8</td>
<td>90\†</td>
</tr>
<tr>
<td>pRK248</td>
<td>5 x 10^8</td>
<td>100*</td>
</tr>
</tbody>
</table>

\* Data represent frequency of survival in five (*) and nine (†) assays, each performed in duplicate.

with other toxins which exhibit greater toxicity toward mosquitoes of the genus *Aedes*. We therefore chose to clone a toxin from *B. thuringiensis* subsp. *israelensis* which is toxic to these mosquitoes and has been used as a field control agent in many parts of the world (14). The gene encoding the *B. thuringiensis* subsp. *israelensis* 130-kDa toxin under the control of the tac promoter was transformed into *C. crescentus* on plasmid pKH49 (Fig. 2), and the resulting recombinants were toxic to *Aedes* larvae (Table 2).

We have recently cloned and sequenced a gene encoding a novel toxin present in *B. sphaericus* SS11-1 (21). The gene encoding this 100-kDa toxin is present in a wide range of *B. sphaericus* strains, including the highly toxic strains used in the field (21). The 100-kDa toxin expressed in *E. coli* shows activity against *C. quinquefasciatus* larvae of the same order as *E. coli* expressing the 51.4- and 41.9-kDa *B. sphaericus* toxins. In addition, *E. coli* expressing the 100-kDa toxin also show activity against *A. aegypti*, equivalent to approximately 1/10 their toxicity to *C. quinquefasciatus* (21). Recombinant *C. crescentus* cells containing the gene encoding the 100-kDa toxin under the control of its *B. sphaericus*

TABLE 2. Assay of transformed C. crescentus cells expressing the 130-kDa toxin of B. thuringiensis subsp. israelensis against A. aegypti larvae

<table>
<thead>
<tr>
<th>Vector</th>
<th>Final conc of test cells (cells/ml)</th>
<th>% Survivors* after:</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>24 h</td>
</tr>
<tr>
<td>pKH49</td>
<td>3.2 x 10^8</td>
<td>84</td>
</tr>
<tr>
<td>pRK248</td>
<td>5 x 10^8</td>
<td>100*</td>
</tr>
</tbody>
</table>

\* Data represent frequency of survival in four assays, each performed in duplicate.

The use of the insecticidal toxins of *B. sphaericus* and *B. thuringiensis* subsp. *israelensis* provides a specific and safe means for the control of mosquitoes. We have transformed genes encoding such toxins into *C. crescentus* CB15 and demonstrated the toxicity of the whole recombinant bacterium to mosquito larvae. The expression of a range of mosquitocidal toxin genes in *Caulobacter* species may provide an effective tool for broad-range control of mosquito pests in the field. This range of control of mosquitoes is important, as the different mosquito genera are vectors for specific diseases. Resistance among target insect populations to microbial toxins, including those of *B. thuringiensis* subsp. *israelensis*, has been reported (20). In order to prevent resistance from developing, it is important to develop a variety of toxins with overlapping target spectra for use in combination in the field. Toward this end, we have demonstrated that three mosquitocidal toxins can be expressed in *Caulobacter* species, a novel vehicle for the toxins intended to prolong the duration of effective mosquito control in the aquatic habitat.

ACKNOWLEDGMENTS

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REFERENCES


