Enhanced expression of a mosquito larvicidal gene(s) of *Bacillus sphaericus* 1593M in *Escherichia coli*

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*Bacillus sphaericus* is known for its potential as a biocontrol agent to *Culex* and *Anopheles* species of mosquitoes. A 3.6-kb-DNA fragment of *B. sphaericus* 1593M encoding mosquito larvicidal activity has earlier been cloned by us in *E. coli*, using the vector pBR322 and the recombinant had a low larvicidal activity. This fragment has now been recloned into an expression vector of *E. coli*, pPR683. The resultant recombinant clone expressed high levels of larvicidal factors under the control of tac promoter at levels comparable to that of *B. sphaericus* 1593M. Since lactose can be an effective inducer of the larvicidal factors in this recombinant, an economic production of bioinsecticidal factors is forecasted.

Several strains of *Bacillus sphaericus* are known to possess proteaceous inclusions that are active larvicidal factors for mosquitoes. These are viable alternatives to toxic chemicals for mosquito control measures. However, the precise nature of the active peptides that contribute to the larvicidal activity is not fully understood. Added to this the presence of multiple larvicidal genes in Bacilli in general and in *B. sphaericus* in particular has necessitated the recombinant DNA approach for the identification and characterization of these active principles.

DNA sequences coding for larvicidal factors from *B. sphaericus* 1593M, 1593 and 2362 have been cloned in *Escherichia coli* and *B. sphaericus* respectively. Anacystis nidulans, sub-toxic strains of *B. sphaericus* and *Bacillus thuringiensis* var. *israelensis* and expressed with varying degrees of success. We have previously reported the cloning of two mosquito larvicidal genes of *B. sphaericus* 1593M in *E. coli*. The recombinant *E. coli* cells carrying the larvicidal genes were active against *Culex* and *Anopheles* species of mosquito larvae at a concentration of 1–10 μg protein per ml. One of these corresponded to the DNA sequence reported by others. In this recombinant the larvicidal activity was expressed when the insert was in either orientation, indicating that the larvicidal genes are transcribed from their native promoter(s).

However, in all these clones the expression of the mosquito larvicidal genes was poor. There are a number of possible reasons for the low expression of *B. sphaericus* genes in *E. coli*. These include; action of *E. coli* proteases on the foreign gene product, poor utilization of heterologous promoters, lowered ribosomal binding activity, and different codon usage preferences between the two organisms. In the present study we have demonstrated that recloning of the larvicidal gene (into a vector with strong inducible promoter) has resulted in enhanced expression of the larvicidal factors at levels comparable to the parental organism, *B. sphaericus*. In addition to the presence of a strong promoter, the vector also has flanking sequence coding for maltose binding protein, between the promoter and the larvicidal genes, which may play a role in achieving high levels of biological activity of the expressed gene product.

**Methods and materials**

The maie vector pPR683, which over-expresses maltose-binding protein (MBP) as β-galactosidase α-peptide fusion protein, has intervening multiple cloning sites between these two genes and their expression is under the control of a strong tac promoter and lacI locus (Figure 1). This vector has the capability to form MBP-foreign protein fusion peptide when the insert is fused 'in-frame'. This plasmid and the *E. coli* host PR722 (*F* · Δ (lacZ) E65 pro+ /prO C·Tn5 (lac1ZYA) U169 hsdS20 ara14 galK2 rpsl20 xyl5 mtl supE44 leu(6+)) were generous gifts from Riggs, New England Biolabs, Beverly, Mass, USA. The recombinant plasmid pAS233HHT and *B. sphaericus* 1593M were from our previous study.

Purification of the mini-crystal from *B. sphaericus* 1593M on sodium bromide gradient and alkali solubilization with 50 mM NaOH were as described earlier. Antisera to the 53-kDa and 43-kDa peptides of purified toxin were raised in rabbits. The crystal proteins were separated on SDS-polyacrylamide gels, bands identified after coomasie blue staining were cut out and used as antigens.

Mosquito larvicidal assays against *Culex quinquefasciatus* larvae (II instar) were carried out as described earlier. The lethal concentration for 50% mortality (LC₅₀) in 24 hours was determined using probit analysis. Controls included larvae fed with *E. coli* cells harbouring the vector plasmid alone and larvae starved for the same period.

Insoluble aggregates from *E. coli* clones were purified according to protocol of Babbit et al. These were solubilized with 8 M urea. After solubilization, the urea concentration in the sample was reduced by diluting 10

**Figure 1.** Linear map of the recombinant plasmid pAS5. Thick lines represent *B. sphaericus* insert DNA, open boxes indicate open reading frames (ORFś). *E. EcoRI*, *H HindIII*, P-PstI.
times with 50 mM Tris pH 7.5, dialysed extensively to remove urea and then the sample was concentrated by lyophilization.

Recombinant DNA techniques were essentially as described in Sambrook et al20. SDS-PAGE was based on the method of Laemmli21.10% acrylamide in separating and 4% in stacking gels were used. The proteins were electroblotted onto nitrocellulose paper and Western analysis was made using appropriate antibody, conjugated to alkaline phosphatase22.

Results and discussion

A DNA fragment of B. sphaericus 1593M coding for the larvicidal factors from pAS233HT was recloned into the HindIII site of the polylinker of the plasmid pPR683. One colony containing the recombinant plasmid pAR5 (Figure 1), 9.8 kb in size, was selected on the basis of its high mosquito larvicidal activity for further studies. The larvicidal activity was expressed under the control of tac promoter and lacI gene as seen by the large increase (500 fold) in the level of expression of the larvicidal genes after induction by IPTG (Figure 2). Under optimal condition of induction the biocide potency of the recombinant clone (LC50 value; 19 ng ml⁻¹) was comparable to that of the parental strain, B. sphaericus 1593M (LC50 value; 10 ng ml⁻¹).

When cells carrying pAR5 were grown in the presence of lactose (2.5% w/v) in LB broth from the beginning, the level of larvicidal activity was significant and only about 10 fold lower than the IPTG induced cells. However, if pAR5 was grown in LB in the absence of lactose, and later induced with lactose, the final larvicidal potency was much lower (Figure 3).

As the larvicidal activity of pAR5 was high, identification of the active peptides was undertaken by the use of anti-MBP serum and the sera raised against the major component peptides of the crystals of B. sphaericus (53 kDa and 43 kDa). In Western blots probed with anti-MBP serum large amounts of MBP and MBP-β-gal fusion peptide made by the vector pPR683 upon induction with IPTG were seen. The recombinant pAR5 also expressed MBP after induction and no higher-molecular-weight MBP-fusion peptides were seen.

Although the toxicity of the cells containing the recombinant plasmid pAR5 was high, the soluble fraction did not show significant presence of any peptide immunoreactive with the antiserum raised against 53- or 43-kDa peptides of crystals from B. sphaericus. However the insoluble aggregates, when solubilized in 8 M urea and renatured, revealed the presence of both these peptides in significant quantities. While the 53-kDa peptide was present as a distinct entity (Figure 4, panel a), the 43-kDa peptide can be seen to be degraded to a 29-kDa peptide (Figure 4, panel a).

Earlier a few reports suggested that 43-kDa peptide alone purified from B. sphaericus is enough to confer toxicity to mosquito larvae15,23, whereas the purified 53-kDa peptide is non-toxic. Further, it has also been
reported that 43-kDa peptide expressed in *B. subtilis* can be toxic to mosquito larvae. In order to assign the larvicidal activity to the 43-kDa peptide or both the peptides, the 1.8 kb *EcoRI*-HindIII fragment of the 3.6-kb HindIII fragment which has been shown to have the sequences coding for a few C-terminal amino acids of the 53-kDa peptide and the complete 43-kDa peptide, was subcloned into pPR683 in the same orientation as the tac promoter (Figure 1). Western blot analysis of the urea solubilized inclusion proteins of the clone containing the recombinant plasmid pRK43, probed with antiserum to the 43-kDa peptide, revealed the presence of both the 43-kDa peptide and its derivative 29-kDa peptide (Figure 4, panel a, lane 6). Nevertheless, this recombinant did not show toxicity to mosquito larvae even at very high concentrations (100 μg ml⁻¹).

The successful expression of the cloned larvicidal genes from *B. sphaericus* strain 1593M, at high levels by the use of the malE vector, has indicated that the reasons for the decreased expression of the heterologous genes in *E. coli* can be overcome by a combination of several features. It is obvious that the higher expression of larvicidal genes in pAR5 stems from an increased level of transcription from the tac promoter (in spite of the presence of the native promoter from *B. sphaericus*) as seen by the 500-fold increase of toxicity upon induction by IPTG. However it has been observed by Broadwell et al. as well as by us (unpublished data) that when the biocidal genes are placed directly downstream of the tac promoter the expression of these genes was much lower. Consequently additional factors like increased stability of the transcripts or translational products conferred by the flanking regions on both the sides of the inserted genes in this system has to be explored. MBP expressed as a part of the synthetic operon might be aiding the formation of inclusion bodies, thus protecting the peptides from host proteolysis and conferring increased stability. Further, the fact that mosquito larvae are filter feeders and crystalline inclusions may be more effective than soluble proteins might also be a reason for the increased potency seen in this recombinant.

Earlier studies have shown that the cloned *HindIII* fragment from *B. sphaericus* 2362 expressed several low-molecular-weight peptides ranging from 24 to 51 kDa in *B. subtilis*. The 27- and 24-kDa peptides were cross reactive only to the 43-kDa antiserum, identifying them as cleavage products of 43-kDa peptide. In our study also the 53-kDa peptide was expressed intact and most of the 43-kDa peptide seems to be degraded to a 29-kDa peptide in pAR5. Nevertheless these extracts still retained high larvicidal activity for *Culex quinquefasciatus*. As observed by earlier workers, the expression of both 53-kDa and 43-kDa (29 kDa) peptides appears to be essential for conferring mosquito larvicidal activity, as with pRK43 even overexpression of 43-kDa peptide was insufficient to confer toxicity. It has earlier been proved conclusively that the 53-kDa peptide in isolation could not confer toxicity to mosquito larvae.

It has been established for the first time from our laboratory that at least two toxin genes exist in *B. sphaericus*, one corresponding to that reported by Baumann et al. and the other confirmed by Thanabal et al. We have recently increased the expression levels of the product coded by the second larvicidal gene using the same vector host system (manuscript communicated). It is expected that when both the larvicidal genes are overexpressed together in one host, it should be possible to achieve even higher level of larvicidal activity in *E. coli*. These studies are currently in progress.

Since *B. sphaericus* does not utilize simple sugars, the introduction of the larvicidal gene(s) into sugar-utilizing bacterium is advantageous in terms of ease of production and also for continuous cultivation. As the formation of the larvicidal factor can be induced by lactose, which is far less expensive than IPTG, the cost effectiveness of the process is ensured. Recently such an approach to successfully optimize the expression of a foot and mouth virus coat protein by the manipulation of induction time with lactose has been reported. Further work is in progress in this direction for the optimization of the parameters for economic production of bioinsecticidal factors.

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RESEARCH COMMUNICATIONS


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ENHANCED EXPRESSION OF A SECOND MOSQUITO LARVICIDAL
GENE FROM B. sphaericus 1593M IN E.coli

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SUMMARY
Enhanced expression of a second mosquito larvicidal gene from
B. sphaericus 1593M in E.coli has been achieved by the recloning of
the DNA fragment encoding for larvicidal activity previously reported
by us, in a pHal vector system. The potency of this recombinant strain
was only 10 fold lower than the parental B. sphaericus 1593M strain. The
protein encoded was different from the previously reported larvicidal
gene products of B. sphaericus. Nevertheless, this protein is
recognized by the antiserum raised against crystal proteins. This
result has indicated the presence of multiple mosquito larvicidal genes
in B. sphaericus, a situation similar to that encountered with
B. thuringiensis toxins.

INTRODUCTION
In the course of sporulation some strains of Bacillus sphaericus
synthesize parasporal crystals, which contain several proteins that are
toxic to certain species of mosquito larvae on ingestion. The major
components of the crystal are peptides of molecular weight 122, 110, 51
and 42 kDa. The larvicidal genes coding for the 51 kDa and 42 kDa
peptides have been cloned, sequenced and expressed in a variety of
hosts (Baumann et al. 1985, 1987 and 1989; Hindley and Berry, 1987;
Souza et al. 1988; Tandeau de Marsac et al., 1987). Both peptides are
required for toxicity (Broadwell et al. 1990). The 122 and 110 peptides
were shown to originate from surface layer proteins of B. sphaericus and
do not contribute to the toxicity (Bowditch et al., 1989). The role of
other minor peptides present in the crystal is not clear.

Earlier we have reported the cloning of two dissimilar fragments
encoding for toxicity from B. sphaericus 1593M using pBR322 vector in
E.coli (Souza et al., 1988). One of these clones (pAS233HT) contained
the genes that encoded the above mentioned toxin peptides (51 & 42
kDa). Subsequently we have over expressed these toxic peptides in
E. coli achieving toxicity levels comparable to that of *B. sphaericus* (Rajamohan, 1990; Rajamohan et al., 1992).

Recently a DNA fragment from a sub toxic strain *B. sphaericus* (SSII-I) has been cloned into *E. coli* and sequenced (Thanabalu et al., 1991). The restriction enzyme map of this fragment was similar to the second mosquito larvicidal gene earlier reported by us (Souza et al., 1988). In this communication we report the enhanced expression of this second larvicidal gene and preliminary characterization of the peptide encoded by this fragment.

**MATERIALS AND METHODS**

The lacI complementation *E. coli* host PR722 and the pMAl vector pPR683 were kind gifts from Dr. Rigg (New England Biolabs). The vector pPR683 has a strong promoter (lac) and has the capability to form N-terminal and C-terminal fusion with maltose binding protein (MBP) and β-galactosidase with the insert encoded product when appropriately placed in the multiple cloning site (Chu di Guan et al., 1988; Maina et al., 1988). *B. sphaericus* 1593M and the plasmid pAS377CA, with 3.3 kb insert encoding for the second larvicidal factor, are from our previous study (Souza et al., 1988). Standard molecular cloning techniques were used. SDS-PAGE was according to Laemmli (1970) and Western blotting was according to Towbin et al. (1979). The antiserum for the Western blot was raised in rabbit against the total crystal proteins of *B. sphaericus* purified on sodium bromide gradient (Baumann et al., 1985). *B. sphaericus* was grown at 30°C in Luria broth with 100 µg/ml streptomycin and *E. coli* strains containing the plasmids were grown in Luria broth with 100 µg/ml ampicillin at 37°C. When necessary, the *E. coli* strains were induced with 1 mM IPTG at optical density of 1.0 (600nm). Determination of the concentration required to kill 50% of *Culex quinquifasciatus* larvae (LC50) was as described earlier (Souza et al., 1988).

**RESULTS**

1. An approximately 3.6 kb StuI - PstI fragment from pAS377CA was recloned directionally into pPR683 (BamHI(filled with Klenow fragment of *E. coli* DNA polymerase) - PstI]. The resulting recombinant plasmid was designated pASR23, and the strain carrying this plasmid, CBT-23 (Fig.1). Inspite of the presence of insert coded promoter (Thanabalu et al., 1991), the expression of the larvicidal activity in CBT-23 was under the control of the tac promoter as evidenced by a 100-fold increase in activity upon induction with IPTG (LC50 = 120 ng/ml at 6h post induction compared to 10 ng/ml in uninduced cells) Fig.2. This represents a 100 fold increased larvicidal activity compared to the
However, the LC50 value of sporulated culture of *B. sphaericus* 1593M was 10 ng/ml.

Fig. 1. Cloning strategy used to introduce the second toxin gene of *B. sphaericus* 1593M from pAS377CA into the aalE vector pPR683. B-BamHI, P-PstI, St-StuI, H-HindIII, Hp-HpaI, C-ClaI, E-EcoRI. Bold line indicate *B. sphaericus* DNA, open box indicate the coding region, and thin lines represent the vector DNA.

Fig. 2. Time course of toxin and biomass synthesis in uninduced and induced cultures of pASR23. The cultures were induced at an optical density of 1.0 with 1mM IPTG.

Potency = 1/LC50 (ng protein/ml)
Western blot analysis of whole cell extracts from sporulated culture of *B. sphaericus* 1593M (Lane 1) from induced cultures of *E. coli* harboring pPR683 (Lane 2), and pASR23 (Lane 3); (200 μg protein per well) probed with polyclonal antisera to crystal proteins of *B. sphaericus* 1593M. Molecular weights based on standard are indicated.

2. The kinetics of formation of the larvicidal principle, upon induction with IPTG, is outlined in Fig. 2. Maximal larvicidal activity was expressed after 6 h of induction. That the expressed peptide was unstable (probably due to host proteolysis) was indicated by a drastic decline in LC50 value from 120 ng/ml 10 h post induction to 20 μg/ml 16 h post induction.

3. Identification of the protein product(s) encoded by this gene was carried out by western blot analysis of the antigens, expressed by induced cultures of *E. coli* containing pASR23, using the antisera raised against the purified crystals of *B. sphaericus* 1593M. A 60 kDa peptide, cross reactive to the serum was detected only in the induced cultures of CBT-23 (Fig. 3. Lane 3) and not in the strain containing the vector plasmid only (Fig. 3. Lane 2).

When the extracts from CBT-23 were prepared with additional protease inhibitors TPCK (100 μg/ml), TLCK (50 μg/ml) and EGTA (2 mM) and processed immediately, a peptide of Ca. 85 kDa was detected in addition to the Ca. 60 kDa peptide (data not shown).
DISCUSSION

Previous studies indicated that most of the toxic activity of \textit{B. sphaericus} is associated with the crystal. Two major peptides of the crystal 41kDa and 52kDa are shown responsible for the toxic activity. These two peptides are needed synergistically to confer mosquito larvicidal activity (Broadwell et al., 1990). In our present studies, enhanced expression of a second toxin gene from \textit{B. sphaericus} has been achieved using the \textit{malE} vector of \textit{E.coli}. Though a 100 kDa peptide product of this gene was expected, based on the deduced aminoacid sequence of this insert (Thanabalu et al., 1991), only a peptide of Ca.60 kDa was detectable in Western blots (and a Ca.85 kDa peptide in carefully processed samples). Nevertheless the cells still exhibited larvicidal activity. This might be due to proteolytic cleavage of a larger precursor. The interesting aspect is the detection of these peptides by the crystal antiserum, though this peptide is not a major component of the crystal. This indicates that this peptide or its breakdown product(s) contribute to the overall larvicidal activity of the crystals of \textit{B. sphaericus}.

In conclusion, enhanced expression of the two sets of genes of \textit{B. sphaericus} in \textit{E.coli}, opens up possibility of combining these two toxin genes in one host and express them perhaps at levels far higher than \textit{B. sphaericus}. Further, the existence of multiple toxin genes in \textit{B. sphaericus} similar to that observed in \textit{B.thuringiensis} (Hofte and Whiteley, 1989) is established beyond doubt.

If the mode of action of these two toxins are different, then development of resistance by the mosquito larvae may also be delayed, making \textit{B. sphaericus} a biopesticide of considerable importance.
REFERENCES


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