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 proteinaceous inclusions that are active larvicidal factors for mosquitoes. These are viable alternatives to toxic chemicals for mosquito control measures[1,2]. 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[3] and in *B. sphaericus* in particular[4] 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*[4-9], *Bacillus subtilis*[10], *Anacystis nidulans*[11], sub-toxic strains of *B. sphaericus*[9] and *Bacillus thuringiensis* var. *israeliensis*[12] and expressed with varying degrees of success. We have previously reported the cloning of two mosquito larvicidal genes of *B. sphaericus* 1593 M in *E. coli*[4]. 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[4-12]. 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 gene(s) 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 gene(s), which may play a role in achieving high levels of biological activity of the expressed gene product.

Methods and materials

The malE 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*$^+$/*proC*::Tn5 (*lacIZYA*) *U169 hsdS20 ara14 galK2 rpsl20 xyl5 mtl1 supE44 leu*(*6?*)) were generous gifts from Riggs, New England Biolabs, Beverly, Mass, USA[13,14]. The recombinant plasmid pAS233HT and *B. sphaericus* 1593M were from our previous study[4].

Purification of the mini-crystal from *B. sphaericus* 1593M on sodium bromide gradient and alkali solubilization with 50 mM NaOH were as described earlier[15]. 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 coomassie blue staining were cut out and used as antigens[16].

Mosquito larvicidal assays against *Culex quinquefasciatus* larvae (II instar) were carried out as described earlier[4,16]. The lethal concentration for 50% mortality (LC_{50}) in 24 hours was determined using probit analysis[17,18]. 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*[19]. These were solubilized with 8 M urea. After solubilization, the urea concentration in the sample was reduced by diluting 10

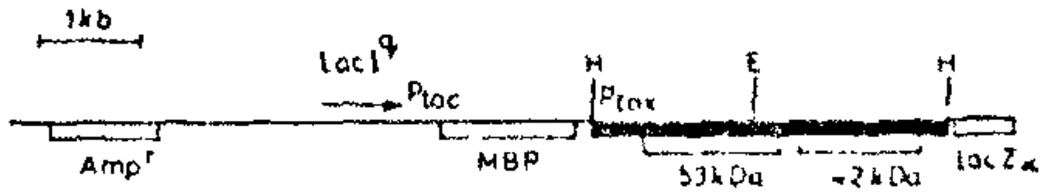

Figure 1. Linear map of the recombinant plasmid pARS. Thick lines represent *B. sphaericus* insert DNA, open boxes indicate open reading frames (ORFs). E-*EcoRI*, H-*HindIII*, P-*PstI*

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 al*[20]. SDS-PAGE was based on the method of Laemmli[21], 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 phosphatase[22].

Results and discussion

A DNA fragment of *B. sphaericus* 1593M coding for the larvicidal factors from pAS233HT was recloned into the *Hind*III 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 *lac*I 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 (LC_{50} value; 19 ng ml^{-1}) was comparable to that of the parental strain, *B. sphaericus* 1593M (LC_{50} 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 ***b***), 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 larvae[15,23], whereas the purified 53-kDa peptide is non-toxic. Further, it has also been

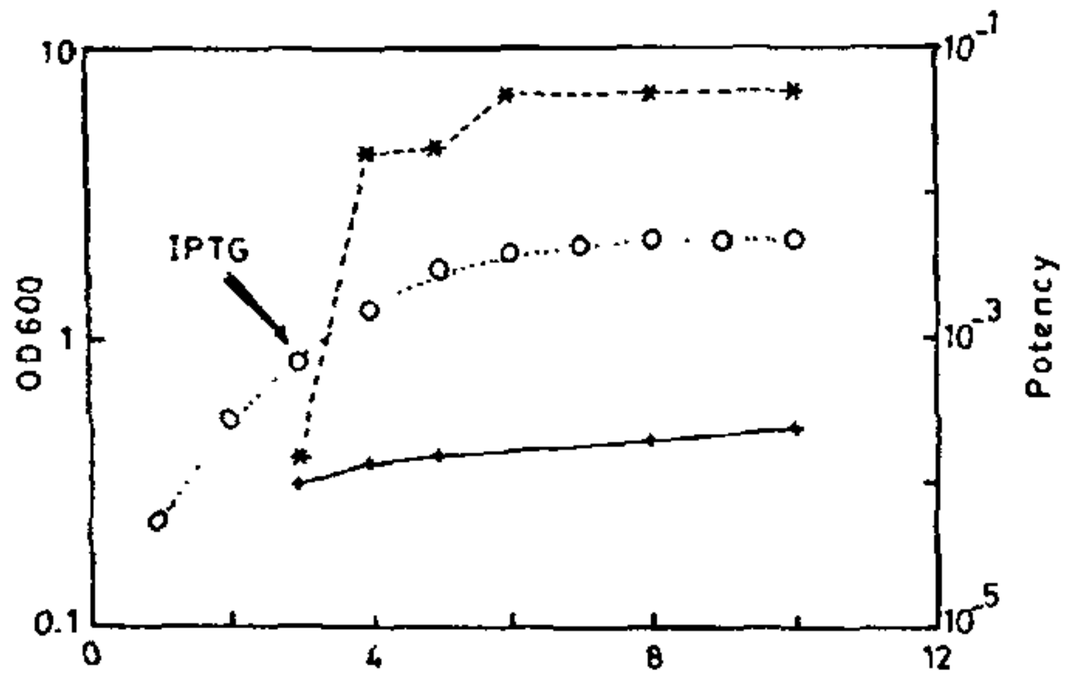

Figure 2. Growth and expression of larvicidal activity of *E. coli* containing the recombinant plasmid pAR5, with and without induction by IPTG. The strain was grown in Luria Bertani broth (tryptone 10 $g\,l^{-1}$, yeast extract 5 $g\,l^{-1}$, and NaCl 5 $g\,l^{-1}$) supplemented with 100 $\mu g\,ml^{-1}$ ampicillin. The cells were induced with 1 mM IPTG at an optical density of 0.8–1.0 (600 nm).

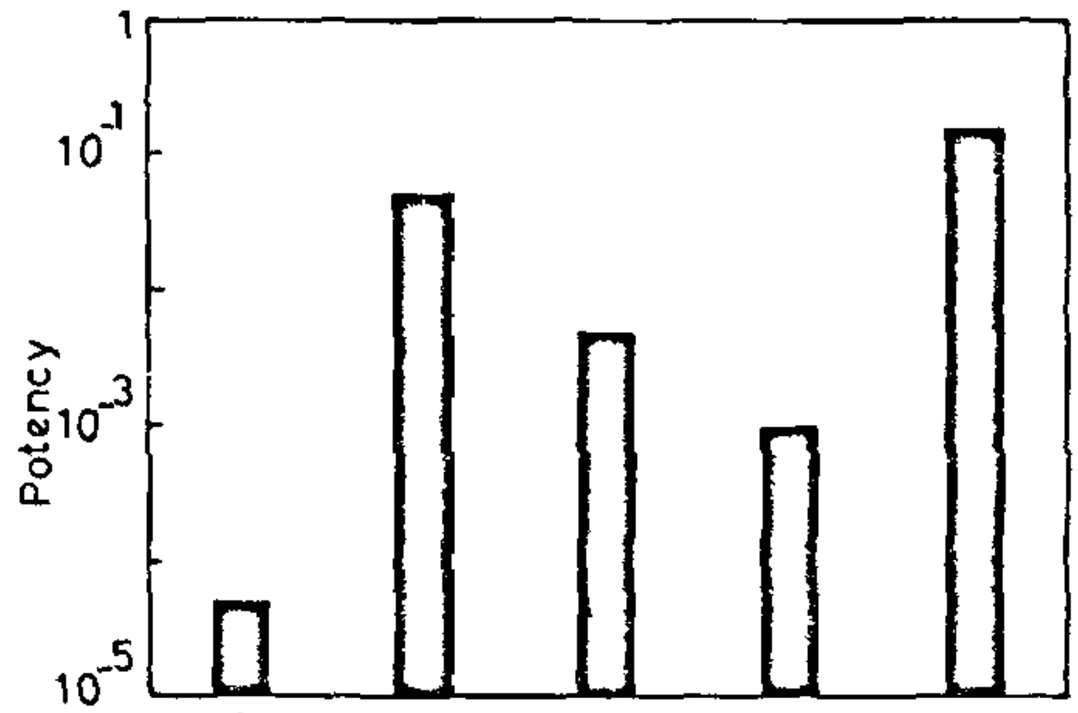

Figure 3. Comparison of the potency of the mosquito larvicidal activities of *B. sphaericus* and the recombinant *E. coli* (pAR5) under different conditions of growth and induction. Cells were grown in LB 1. pAR5, uninduced, 2. pAR5 induced with IPTG, 3 pAR5 induced with 2.5% w/v lactose present from the beginning; 4 pAR5 induced with 2.5% w/v lactose at an optical density of 0.8–1.0 (600 nm). BS. *B. sphaericus* 1593M.

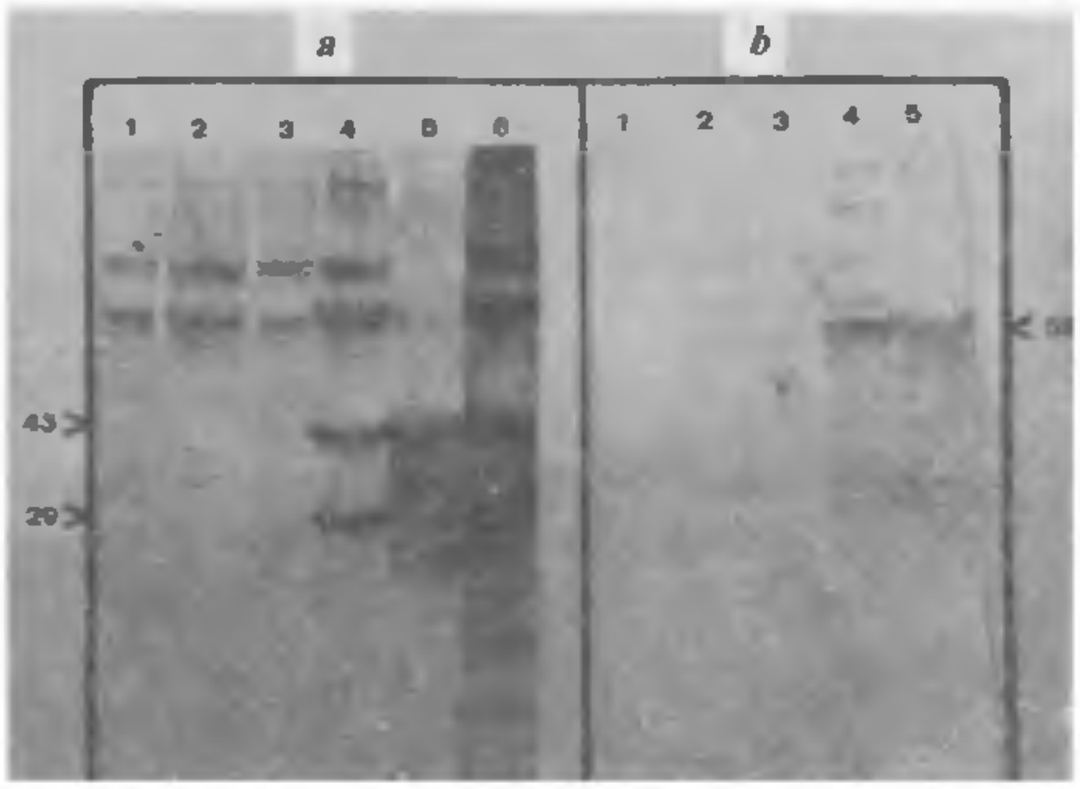

Figure 4. Western blots of proteins from cells harbouring the plasmids pPR683 and the recombinant pAR5: Proteins from urea solubilized cell extracts (150 μg per slot) and purified toxin (alkali solubilized 50 μg), were separated on a 10% SDS-PAGE and probed with: Panel *a*: anti 43-kDa serum; Panel *b*: anti 53-kDa serum, relevant molecular weights (in kDa) based on standards are indicated. Lane 1. pPR683 uninduced; Lane 2. pPR683 induced with IPTG; Lane 3. pAR5 uninduced; Lane 4. pAR5 induced with IPTG; Lane 5. Purified crystal toxin alkali solubilized. Lane 6. pRK43 induced with IPTG.

reported that 43-kDa peptide expressed in *B. subtilis* can be toxic to mosquito larvae[24]. In order to assign the larvicidal activity to the 43-kDa peptide or both the peptides, the 1.8 kb *Eco*RI–*Hind*III fragment of the 3.6-kb *Hind*III 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[8], 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* 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.*[9] as well as by us (unpublished data) that when the biocidal genes are placed directly downstream of *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 *Hind*III 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 antisera, identifying them as cleavage products of 43-kDa peptide[10]. 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[25,9], 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[15].

It has been established for the first time from our laboratory that at least two toxin genes exist in *B. sphaericus*[4], one corresponding to that reported by Baumann *et al.*[8] and the other confirmed by Thanabalu *et al.*[26]. 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[27]. Further work is in progress in this direction for the optimization of the parameters for economic production of bioinsecticidal factors.

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