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Cloning and expression of mosquitocidal endotoxin gene *cryIVB* from *Bacillus thuringiensis* var *israelensis* in the obligate methylotroph *Methylobacillus flagellatum*

ND Marchenko[1,2], GN Marchenko[1,3], LO Ganushkina[1,4] and RR Azizbekyan[1]

[1]*Institute of Genetics and Selection of Industrial Microorganisms, 1st Dorozhny proezd 1, Moscow, 113545 Russia*

The *cryIVB* gene from a new isolate of *Bacillus thuringiensis* var *israelensis* was cloned and sequenced. Two nucleotide replacements resulted in changing Asp[385]-Thr[386] to Glu[385]-Ser[386] were found in comparison with the previously sequenced *cryIVB* gene. Two genetic constructions were designed for expression of *cryIVB* in the obligate methylotroph *Methylobacillus flagellatum*. In the first construction, *cryIVB* was cloned under the strong inducible *lac* promoter and contained original ribosome binding site and 150 bp of 5′ transcribed but untranslated region. In the second construct, the first five codons of the *lacZ* gene were fused to the second codon of the *cryIVB* gene. Both *E. coli* and *M. flagellatum* harboring both constructs were toxic to insect larvae of *Anopheles stephensi* and *Aedes aegypti*. However, the toxicity of the methylotroph was about 450 times less. This study is the first attempt to use methylotrophs as an insecticidal endotoxin producer. *Journal of Industrial Microbiology & Biotechnology* (2000) **24,** 14–18.

Keywords: *B. thuringiensis*; delta-endotoxin; CryIVB; mosquitocidal toxin; methylotroph

Introduction

Bacillus thuringiensis var *israelensis* is an aerobic spore-forming bacterium, which produces crystalline inclusions at the outset of sporulation. These inclusions are toxic for larvae of numerous mosquito species and blackflies, which are vectors for several dangerous tropical diseases. *CryIVA*, *cryIVB*, *cryIVC*, *cryIVD* and *cytA* endotoxin genes are located in a 125-kb plasmid, and encode 134, 128, 78, 72 and 27 kDa proteins respectively [6]. They differ in structure and insecticidal activity spectrum, and a synergism between their activities was observed [10,19]. All endotoxin genes of the *B. thuringiensis* var *israelensis* have been cloned and the DNA sequences determined. The *cryIVB* gene was cloned by several investigators [9,22,28,32] and recombinant *Escherichia coli* cells harbored by the *cryIVB* gene have mosquitocidal activity [2,7].

Biological control of mosquito populations is highly effective without evidence of any harmful impact on the environment [5]. Despite the high toxicity of *B. thuringiensis* var *israelensis* against different mosquito species, its industrial use is limited by considerable disadvantages for extensive mosquito control programs [29]. The primary disadvantages are rapid settling, UV sensitivity, proteolysis of toxins, and the high cost of fermentation. The need for biological control of mosquitos has stimulated research into development of alternative hosts for expression of mosquitocidal genes [15,21,26,27,33]. The expression of the *B. thuringiensis* var *israelensis* larvicidal genes in the obligate methylotroph *Methylobacillus flagellatum* has been suggested as an effective means for controlling mosquito populations. *M. flagellatum* is able to grow on methanol or methylamine as sources of carbon and energy [12]. This bacterium has a high maximum growth rate with a coefficient of conversion of methanol into biomass of about 50% [3]. Moreover, this methylotroph is resistant to UV light, lacks toxin-degrading proteases, is phage-resistant, and has very low production costs. These properties make *M. flagellatum* an ideal producer for biotechnology industry. This bacterium has a potential to be used for overexpression of heterologous proteins [8], production of amino acids [25] and vitamins [23]. Here we describe cloning and sequencing of the *cryIVB* gene from a new isolate of *Bacillus thuringiensis* var *israelensis* 1–5 and its expression in the obligate methylotroph *M. flagellatum*.

Materials and methods

Strains and plasmids

A strain of *B. thuringiensis* var *israelensis* IPS82 was obtained from the collection of H Barjac (Pasteur Institute, Paris, France). *B. thuringiensis* var *israelensis* strain 1–5 was isolated from mosquito larvae collected in Middle Asia (Uzbekistan). Procedures for the isolation and identification of *B. thuringiensis* var *israelensis* 1–5 have been described [17]. *M. flagellatus* strains were grown in minimal medium with methanol as described previously [12]. For cloning experiments, the strains *M. flagellatum* and *E. coli* TG1 were used as recipients. *M. flagellatum* strains harboring recombinant plasmids were obtained by standard conjugative transfer from recombinant strains of *E. coli* TG1 also

Correspondence: G Marchenko, MSRC, SUNY at Stony Brook, Stony Brook, NY 11794, USA. E-mail: gmarchenko@usa.net
[2]Present address: Pathology Department, SUNY at Stony Brook, Stony Brook, NY 11794, USA
[3]Present address: Marine Science Research Center, SUNY at Stony Brook, Stony Brook, NY 11794, USA
[4]Present address: Institute of Medical Parasitology and Tropical Medicine, Moscow 119435, Russia
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containing the helper plasmid R751 [24]. For cloning experiments we used the plasmid pUC19 and broad host range vectors pNGM130K and pNGM131K (Figure 1). The construction and detailed description of the vectors pNGM130K and pNGM131K were reported previously [16].

General procedures

Plasmids from *M. flagellatum* and *E. coli* were isolated by the alkaline lysis method [20]. A slightly modified procedure was used for purification of plasmids from *B. thuringiensis* var *israelensis* [13]. To construct the *B. thuringiensis* var *israelensis* 1–5 125-kb plasmid library, the total plasmid DNAs were separated by electrophoresis in 0.3% low melting agarose. The excised band containing the 125-kb plasmid was melted, added to four volumes of *Xba*I buffer, and digested by *Xba*I endonuclease with conditions providing partial digestion. The partial *Xba*I digest was ligated into the *Xba*I site of the pUC19 vector after being processed by alkaline phosphatase. The ligation mix was transformed into *E. coli* strain TG1. White ampicillin-resistant transformants were then screened by colony hybridization with a radiolabeled probe for the presence of a homologous DNA sequence. The sequence of the 15-mer oligonucleotide 5′- AATTATAAAGATTGG-3′ was based upon the conservative sequences of the known *cryIVB* genes. The operations for cloning, construction of plasmid libraries, and gene-specific hybridization were used according to standard methods [20].

Western-blot analysis

Cultures being tested for endotoxin production were harvested after 24 h. Cells were centrifuged and then suspended in ice-cold 10 mM TE buffer (pH 7.5). Samples with equal concentrations of total protein (10 μg for *E. coli* and 100 μg for *M. flagellatum* cells) were separated by 8% SDS-PAGE. After electrophoresis, the proteins were transferred onto a nitrocellulose membrane. The CryIVB protein was detected with polyclonal anti-CryIVB antiserum (raised in white rabbits), that was supplied generously by A Shevelev (GNIIGenetika, Moscow, Russia).

DNA sequencing

Fragments for sequencing were recovered from low melting agarose gels and subcloned into M13 vectors BM20 or BM21 (Boehringer Mannheim, Mannheim, Germany). Single-stranded DNA templates were sequenced by the dideoxy chain termination method with fluorescent primers using the Applied Biosystem Model 370A DNA Sequencing System. The sequencing procedure was performed with a Techne PHC-2 CyclerA (Techne Ltd, Duxford, Cambridge, UK).

Biological assay

Bioassays were performed with five mosquito species: *Aedes aegypti*, *Culex pipiens*, *Anopheles stephensi*, *Anopheles sacharovi*, and *Anopheles atroparvus*. The strains of *B. thuringiensis* var *israelensis* 1–5, *M. flagellatum* and *E. coli* (with induction by 0.1 mM IPTG for 4 h) were har-

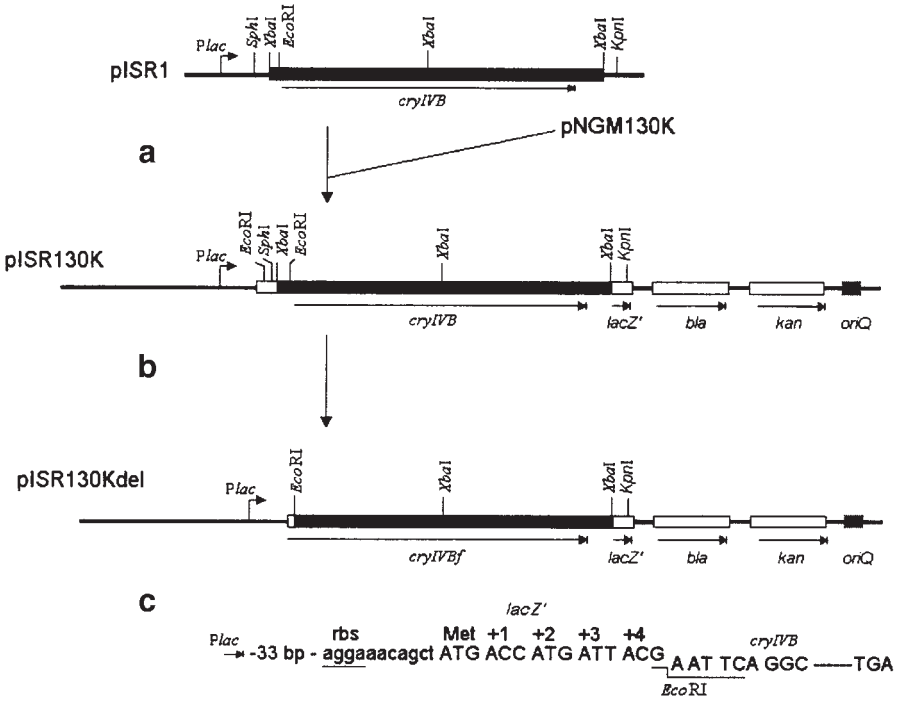

Figure 1 Schematic representation of the construction of the *lacZ*::*cryIVB* fusion. The plasmids are represented in a linear form with relevant restriction endonuclease sites being indicated above the lines for the insert (solid bar) or the vector DNAs (thick line or open bar). (a) The *cryIVB* gene was transferred from pISR1 to pNGM130K by *Sph*I–*Kpn*I double digestion to form pISR130K. (b) *Eco*RI deletion of the 5′-region *cryIVB* gene led to translational fusion with the *lacZ*′ gene. (c) Nucleotide sequence of the 5′-region of the chimerical gene *cryIVB*f.

vested after 24 h growth. Cells were centrifuged, washed twice with sterile 0.05 NaCl, and diluted in sterile water. Samples with equal total protein concentrations were serially diluted 10–10 000 times and assayed with third-instar mosquito-larvae. Mortality was recorded after 24, 48 and 72 h. Each dilution was tested in triplicate with 20 larvae in plastic dishes containing a final volume of 20 ml. The concentration of total cell protein in samples, which killed 50% of the exposed population in a standard bioassay (LC_{50}), was determined.

Results

Cloning of mosquitocidal endotoxin gene of B. thuringiensis var israelensis 1–5 into E. coli

Fifteen positive colonies were detected as result of the colony hybridization with radiolabeled probe. Plasmids from ten positive colonies contained the same 1.8-kb *Xba*I insertion, while five others carried a 3.7-kb insert with one internal *Xba*I site. All clones were tested for toxicity against *A. aegypti*. Two clones were found to be toxic to mosquito larvae. Restriction nuclease site analysis of plasmids from the selected clones showed that these recombinant plasmids contain the identical 3.7-kb *Xba*I fragment in the same orientation under control of the *lac* promoter of the vector. Restriction nuclease site maps of these fragments corresponded to the previously described *cryIVB* gene of mosquitocidal endotoxin [2]. A plasmid containing the complete gene was designed as pISR1 (Figure 1).

The nucleotide sequence of the cloned gene was compared to previously reported nucleotide sequences for mosquitocidal endotoxin genes. Analysis of the nucleotide sequence has shown that the (structural part) gene consists of 3408 bp and codes for a 127.8-kDa protein. The DNA sequence of the entire 3.7-kb *Xba*I fragment matched the published sequence of the *cryIVB* gene (GenBank accession number X07423) [9] with two exceptions. $T^{1311}A^{1312} \rightarrow A^{1311}T^{1312}$ replacements in the structural part of the gene were determined. Thus, the cloned gene from the new isolate of *B. thuringiensis* var *israelensis* 1–5 belongs to the CryIVB class of delta-endotoxins.

Cloning and expression of the cryIVB mosquitocidal endotoxin gene in the obligate methylotroph M. flagellatum

To study an expression of the *cryIVB* gene in the obligate methylotroph *M. flagellatum*, a DNA fragment containing the intact gene was subcloned into the broad host range vector pNGM130K (Figure 1). This vector contains the replicon of RSF1010 [4] and is stably maintained in a variety of Gram-negative hosts [4,16]. The *Sph*I-*Kpn*I digest of plasmid pISRI was ligated into the *Sph*I-*Kpn*I cut vector pNGM130K (Figure 1). The ligation mix was transformed into *E. coli* TGI (R751) cells and plated onto LB agar containing kanamycin, isopropyl-thio-β-galactoside (IPTG) and 5-bromo-4-chloro 3-indolyl-β-D-galactoside (X-gal). White clones harboring the hybrid plasmid pISR130K were selected. The plasmid pISR130K contains the *cryIVB* gene under the control of both the *lac* promoter and its own promoter. Plasmid pISR130K from *E. coli* TGI (R751, pISR130K) cells was conjugated into the obligate methylotroph *M. flagellatum*. The expression levels of the CryIVB protein in the recombinant *M. flagellatum* and *E. coli* cells were analyzed by Western blot analysis (Figure 2) and their insecticidal activity was assayed (Table 1).

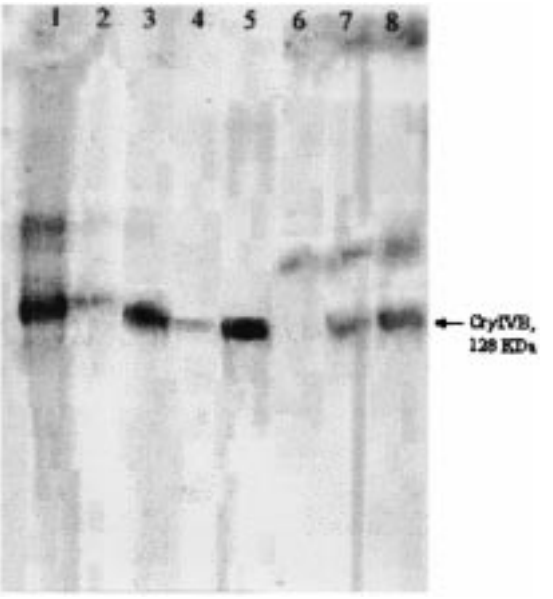

Figure 2 Immunoblot analysis of CryIVB expression in the *E. coli* and *M. flagellatum* cells. Samples with equal concentrations of total protein (10 μg for *B. thuringiensis* var *israelensis* 1–5 or *E. coli* and 100 μg for *M. flagellatum* cells) were separated by 8% SDS-PAGE and the immunoassay was carried out as described in the text. The arrow shows the position of CryIVB endotoxin. (1) *B. thuringiensis* var *israelensis* 1–5; (2) *E. coli* TG1 (pISR130K); (3) *E. coli* TG1 (pISR130K) + 4 h IPTG induction; (4) *E. coli* TG1 (pISR130Kdel); (5) *E. coli* TG1 (pISR130Kdel) + 4 h IPTG induction; (6) *M. flagellatum* (wild); (7) *M. flagellatum* (pISR130K); (8) *M. flagellatum* (pISR130K*del*).

To obtain the *lacZ′-cryIVB* fusion, the 5′-region of the *cryIVB* gene located on pISRI was deleted. Deletion of the small *Eco*RI fragment (150 bp) upstream of the initiation codon of the *cryIVB* gene resulted in a fusion of the first five codons of the *lacZ′* gene and the structural part of the *cryIVB* gene downstream of the second codon. The plasmid carrying the *lacZ′-cryIVB* fusion was designated pISR130K*del*. The nucleotide sequence of the promoter region of the hybrid *cryIVB* translational fusion gene is shown in Figure 1. The plasmid pISR130K*del* was conjugated into the *M. flagellatum* cells. Both recombinant plas-

Table 1 Toxicities of *E. coli* and *M. flagellatum* against third-instar *A. stephensi* and *A. aegypti*

Strain (plasmid)	Induction by IPTG	LC_{50} (μg total protein ml^{-1})[a]	
		A. stephensi	*A. aegypti*
E. coli TG1 (pISR 130K)	–	4.4	19.1
E. coli TG1 (pISR 130K)	+	0.095	0.42
E. coli TG1 (pISR 130Kdel)	+	0.075	0.37
M. flagellatum (pISR130K)	–	62.7	>200
M. flagellatum (pISR130Kdel)	–	35.2	>200

[a]Values are averages from three bioassays performed as described in the text.

mids, pISR130K and pISR130K*del*, were stable in the methylotroph. About 95% of colonies had stably maintained the plasmids after 20 passages of culture in the absence of an antibiotic selection.

To determine whether the amounts of the mosquitocidal endotoxin were comparable in the *E. coli* and *M. flagellatum* cells, proteins in the cell extracts were separated by SDS-PAGE and were probed with the CryIVB antiserum. Immunoelectrophoresis showed that cell lysates of the recombinant strains of *M. flagellatum* and *E. coli* produced immunoreactive bands with a molecular weight of approximately 130 kDa. This confirms that the recombinant proteins are identical to the original CryIVB protein from *B. thuringiensis* var *israelensis* 1–5. Quantitative analysis of the immunoreactive bands indicated that endotoxin production in *E. coli* depended on induction by IPTG. In both microorganisms minor differences in endotoxin production between cells harboring pISR130K or pISR130*del* were observed. Production of CryIVB fusion protein in *E. coli* (pISR130K*del*) was at a level more than 100 times greater than that found in *M. flagellatum* (pISR130K*del*) cells.

Insecticidal activity of the recombinant strains

The insecticidal activities of the parental and recombinant *M. flagellatum* and *E. coli* strains were determined by measuring the 50% lethal concentration. All recombinant strains were toxic to *A. stephensi* larvae, less toxic to *A. aegypti* larvae (Table 1), and exerted little or no toxic effect on *C. pipiens*, *A. sacharovi*, and *A. atroparvus* (data not shown). This indicates that recombinant *E. coli* cells grown under IPTG induction caused the highest level of toxicity. The insecticidal activity of recombinant *E. coli* strains was remarkably higher than that of the recombinant *M. flagellatum* strains. The toxicity of the recombinant *E. coli* and *M. flagellatum* strains harboring the plasmid pISR130K*del* was slightly higher than the toxicity of the strains harboring plasmid pISR130K. These results are consistent with the results of Western blot analysis.

Discussion

Recently, numerous attempts have been made to use transgenic organisms to express *B. thuringiensis* mosquitocidal endotoxins. By varying combinations of mosquitocidal genes, expression vectors, different promoter types, ribosome-binding sites (RBS), and terminator sequences, expression of mosquitocidal endotoxin genes was obtained in cyanobacterium [1,21,26,30,31], the gas-vacuolated strain *Ancylobacter aquaticus* [33], *Bacillus sphaericus* [11], *Caulobacter crescentus* [27] and the Gram-negative bacterium *Asticcacaulis excentricus* [15]. Genetically engineered bacteria are not currently widely being used to control mosquito populations. This study is the first attempt to use methylotrophs as an insecticidal endotoxin producer. The industrial importance of methylotrophs has been confirmed on many occasions for their capacity to overexpress foreign proteins, amino acids and vitamins [14].

The strain *B. thuringiensis* var *israelensis* 1–5 used in this work was shown previously to have biochemical, physiological and insecticidal properties slightly different from the widely distributed strain *B. thuringiensis* var *israelensis* IPS 82 [17]. The *cryIVB* gene from the new isolate has been cloned and sequenced. The nucleotide sequence of the cloned gene with two exceptions showed complete homology to the previously cloned [9] *cryIVB* gene (GenBank accession number X07423). T^{1311}-$A^{1312} \rightarrow A^{1311}$-$T^{1312}$ replacements in the structural part of the gene resulted in a change of Asp^{385}-Thr^{386} to Glu^{385}-Ser^{386}. Both amino acids were changed with chemically similar analogs. Thus, we do not consider that the changes exert a serious influence on the insecticidal properties of CryIVB. However, the insecticidal power of isolate *B. thuringiensis* var *israelensis* 1–5 is higher than was observed in *B. thuringiensis* var *israelensis* IPS 82 [17]. The cloning of the *cryIVB* gene into the broad host range vector pNGM130K allowed expression of the mosquitocidal endotoxin in different Gram-negative microorganisms including obligate methylotrophs.

A DNA fragment containing the *cryIVB* gene was cloned into pNGM130K downstream of the strong *lac* promoter resulting in the pISR130K plasmid. *E. coli* cells harboring pISR130K were highly toxic to *A. stephensi* and *A. aegypti* mosquito larvae only after induction by IPTG (Table 1). Hence, the cloned gene was expressed in *E. coli* under the *lac* promoter rather than its original promoter. Since vector pNGM130K does not have the *lac*I repressor gene, induction of the *lac*Z promoter in *M. flagellatum* had not been carried out. The bioassay data were consistent with Western blot analysis; the lysates of the *E. coli* cells harboring pISR130K produced an immunoreactive band with the same molecular mass as in the original strain *B. thuringiensis* var *israelensis* 1–5. In contrast, the toxicity of *M. flagellatum* cells harboring pISR130K was lower and the amount of CryIVB protein detected by immunoblot analysis was also lower. To enhance expression of the *cryIVB* gene in *M. flagellatum* cells, we created a chimerical *lacZcryIVB* construction in which the *B. thuringiensis* var *israelensis* promoter and ribosome binding site were replaced with the *lacZ* regulative elements. In this construction the first five codons of the *lacZ′* gene were fused to the second codon coding part of the *cryIVB* gene. A similar strategy was used to enhance the expression of the *cryIVB* gene in *Synechococcus* PCC 7942 by cloning the *cryIVB* gene as a translation fusion product under control of the ferredoxin gene (petF1) promoter and ribosome binding site [26]. Murphy and Stevens [18] achieved relatively high level expression of the *cryIVD* gene in cyanobacterium *Agmenellum quadruplicatum* by using translation fusion of the 5′ region of the β-phycocyanin gene (*cpcB*) and the coding sequence of the *cryIVD* gene.

The present results indicate the *E. coli* and *M. flagellatum* cells carrying the *cryIVB* or *lacZryIVB* fusion were toxic to mosquitoes. Among observed mosquito species (*A. aegypti*, *C. pipiens*, *A. stephensi*, *A. sacharovi* and *A. atroparvus*), native and recombinant CryIVB protein was most toxic to *A. stephensi* mosquito larvae (data not shown). However, deletion of the 5′ untranslated region of *cryIVB* gene did not lead to substantial increase of endotoxin production in either *E. coli* or *M. flagellatum*. A possible explanation for the lower expression of the *cryIVB* gene in *M. flagellatum* could be the weak promoter and/or the RBS site of the β-galactosidase gene for strong

expression in the methylotroph. The mosquitocidal activity produced by the constructed *M. flagellatum* strain demonstrates the potential of methylotrophs for the biological control of mosquitoes. Future work is needed to improve the toxicity of the *M. flagellatum* cells.

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