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Nitrogen-fixing cyanobacteria as gene delivery system for expressing mosquitocidal toxins of *Bacillus thuringiensis* ssp. *israelensis*

S. Boussiba[1*], X-Q. Wu[1], E. Ben-Dov[2], A. Zarka[1] & A. Zaritsky[2]
[1]*Microalgal Biotechnology Laboratory, Albert Katz Department of Dryland Biotechnologies, Blaustein Institute for Desert Research, Ben-Gurion University at Sede-Boker, 84990, Israel*
[2]*Department of Life Sciences, Ben-Gurion University of the Negev, P.O. Box 653, Be'er-Sheva 83105, Israel*

(*Author for correspondence; phone +972-7-6596795; fax +972-7-6596802;
e-mail sammy@bgumail.bgu.ac.il)

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Abstract

Classical biological control is the most successful and promising way to replace chemical pesticides. The subspecies *israelensis* of *Bacillus thuringiensis* (Bti) is a safe and efficient agent to control mosquito larvae and hence mosquito-borne diseases. One approach to overcome the low efficacy and short half-life in nature of current formulations of Bti is by expressing the toxin genes in recombinant cyanobacteria as a delivery system. Attempts to express Bti toxin in cyanoabcteria have been carried out during the last ten years. Toxicities of the transgenic strains were however very low, even under regulation of strong promoters, too low to be effective in vivo. Two Bti Cry proteins have recently been co-expressed in the filamentous nitrogen-fixing cyanobacterium *Anabaena* PCC 7120, resulting in clones with the highest toxicities and stabilities ever reached so far. However, to obtain a long-lasting preparation, it would be useful to express Bti toxin genes in cyanobacterial strains isolated from nature. This approach requires development of a system for effective transformation into such strains. Releasing such recombinant strains to open environments is still a major obstacle in exploiting this biotechnology.

Abbreviation Bti – *Bacillus thuringiensis* ssp. *israelensis*

Introduction

Research in applied phycology has achieved much success (Richmond, 1986; Vonshak, 1997), but still faces challenges. While mass culture relies on the advantage of microalgae over other conventional agricultural products, it is limited to a number of natural species (Apt & Behrens, 1999). Their commercial potential largely depends on the reduction of production costs, which is equivalent to improvement of the quality of microalgal strains. Extensive screening of novel strains with high biotechnological value have been conducted worldwide over the decades with very limited economic gains. Attempts to improve desired characteristics of existing strains by chemical mutagenesis were hindered due to the poor plasticity of genetic traits and reduced stability of selected mutations in microalgae. Various aspects of strain improvement for commercial purposes have been achieved in cyanobacteria with recombinant DNA techniques (Tandeau de Marsac & Houmard, 1993; Elhai, 1994; Vermaas, 1996). For example, herbicide resistance was conferred to prevent annihilation of indigenous species by heavy application of herbicides in rice fields (Golden & Haselkorn, 1985; Haselkorn, 1992; Windhovel et al., 1997). Use of genetically modified cyanobacteria for degradation of residual pesticides in water bodies, a potential serious hazard to pub-

lic health, is another such example (Kuritz & Wolk, 1995).

Another attractive approach is to engineer cyanobacteria for mosquito control (Boussiba & Wu, 1995). Mosquitoes and blackflies are vectors of infectious diseases such as malaria, filariasis, encephalitis, dengue fever, yellow fever, hence they constitute a threat to public health (Porter et al., 1993; Margalith & Ben-Dov, 2000). Effective control and prevention of such epidemic diseases, a persistent major public concern, is achieved by reducing population sizes of the transmitting vectors. Their impact on civil life has been partially alleviated by the heavy use of pesticides.

Chemical insecticides used to control mosquitoes cause ecological problems and enhance resistance development of the pests. Biological control is thus considered a permanent and environment-friendly alternative. The subspecies *israelensis* of the bacterium *Bacillus thuringiensis* (Bti) is a safe and efficient agent to control mosquito larvae. One approach to overcome the low efficacy and short half-life in nature of current formulations of Bti is use of genetically engineered microorganisms (Porter et al., 1993). Cyanobacteria are likely candidates to be exploited as gene delivery systems for heterogeneously expressing Bti toxins to control mosquito borne diseases.

This review aims to summarize work carried out in this topic. It describes the bacterium Bti and its toxins, lists the advantages of cyanoabcteria as gene delivery systems, and recites the investigations expressing Bti toxins in cyanobacteria. In addition, it points out some of the problems hampering this biotechnology such as transformation of indigenous isolates and impact to the environment of releasing genetically modified microorganisms.

Bacillus thuringiensis ssp. *israelensis* (Bti) as a biopesticide against mosquitoes

Bti, the most valuable biological agent for controlling mosquito and blackfly larvae (Lacey & Undeen, 1986), was discovered in the Negev region of Israel over two decades ago (Margalit, 1990). It is highly specific against water-dwelling dipteran species while causing no harm to other organisms including predators of the vectors. Bti's capacity to synthesize insecticidal crystal proteins (ICPs) has spurred intensified research which led to a comprehensive understanding of their structure and function, the encoding genes and their expression (Margalith & Ben-Dov, 2000). This knowledge, consequently, improved the efficiency of biocontrol formulations, the construction of effective toxins and delivery systems. The ICPs reside in a crystal (δ-endotoxin) synthesized during sporulation, which is composed of 4 major polypeptides (Cry4Aa, Cry4Ba, Cry11Aa, Cyt1Aa) (Porter et al., 1993; Margalith & Ben-Dov, 2000). Synergism exists between combinations of the polypeptides (Angsuthanasombat et al., 1992; Crickmore et al., 1995; Poncet et al., 1995). All the genetic information responsible for toxicity is located on a 137 kb plasmid (pBtoxis) (Ben-Dov et al., 1996, 1999).

The use of Bti is limited by its low persistence under field conditions, where it does not reproduce. The major reasons for low efficacies of current Bti preparation are sinking to the bottom of the water body, absorption to silt particles and organic matters, inactivation of its toxins by sunlight and consumption by other organisms (Margalith & Ben-Dov, 2000).

Advantage of cyanobacteria as Bti toxin delivery systems

Much effort has been expended around the world to solve these problems. Improving the formulations of Bti to enhance its persistence in natural habitats of dipteran larvae and isolating new mosquitocidal strains or species are such examples. One high-tech approach is cloning the genes responsible for toxicity for expression in other organisms that are ingested by the targets (Porter et al., 1993; Yap et al., 1994; Thanabalu et al., 1992; Liu et al., 1996; Khampang et al., 1999). Among these are cyanobacteria, which are abundant in nature (Thiery et al., 1991), resist a range of environmental conditions and some species of which.can float in the upper layer of water bodies (Boussiba & Wu, 1995). Shuttle vectors harboring cyanobacterial replicons have been constructed and used in a number of strains (Wolk et al., 1984; Porter, 1986).

Previous attempts to control mosquitoes by recombinant cyanobacteria

The last decade saw some progress in expressing mosquitocidal toxin genes of *Bacillus* sp. in cyanobacteria. In a pioneering work, the binary toxin genes from *B. sphaericus* 1593M were expressed in *E. coli*, *B. subtilis* and the unicelluar cyanobacterium *Synechococcus* PCC 7942 (Tandeau de Marsac et al., 1987).

All three transgenic organisms displayed reasonable levels of larvicidal activity against *Culex pipiens* larvae despite expression from their original promoter. Soon afterwards, *cry4Ba* from Bti was expressed in *Synechococcus* PCC 7002 through the shuttle vector pAQE19LPCΔSal under regulation of the cyanophycocyanin (CPC) promoter (Angsuthanasombat & Panyim, 1989). The larvicidal activity against *Aedes aegypti* of the transgenic cells was very low due to degradation of the product, Cry4Ba. The same gene was later placed under control of P_{psbA}, which originated from the chloroplast *psbA* of *Nicotiana tabacum* (Chungjatupornchai, 1990). The construct was directed and integrated into a specific chromosomal site of *Synechocystis* PCC 6803. The transformed cells were not toxic to mosquito larvae, but concentrated extracted proteins were, with comparable toxicity to that expressed in *E. coli* (mean LC_{50} of 1.3 μg mL^{-1}). Western blot as well as ELISA analyses revealed that the amount of 126 kDa protein was about 0.2% of the total soluble protein. Similarly, cells of the same *Synechocystis* (PCC 6803) transformed with the same vector carrying *cyt1Aa* showed no toxicity at all; in this case, however, the protein extract was also not toxic. Intact mRNA of *cyt1Aa* was detected by Northern hybridization, but its overall level was very low, at least 5 times less than of *cry4Ba* (Chungjatupornchai, 1990). The third cyanobacterial species to express Cry4Ba was *Synechococcus* PCC 7942 (Soltes-Rak et al., 1993; 1995), with a similarly disappointing result even after replacement of the original regulatory sequences of Bti with indigenous corresponding sequences of *petF1* (Ferredoxin apoprotein). Detailed analyses suggested that the low expression in this case could have resulted from a significant bias in codon usage, absence of a full-length transcript, inefficient translation or internal ribosomal binding site. The inability of cyanobacterial lysates to degrade the intact *E. coli*-expressed Cry4Ba has precluded the possibility of *in situ* proteolysis; the apparent degradation has likely resulted from premature termination of transcription or translation (Soltes-Rak et al., 1993, 1995).

Cyanobacteria, which proliferate in water bodies such as rice fields (Boussiba, 1991), may thus provide an alternative for prolonged delivery of Bti δ-endotoxin to mosquito larvae that breed in the same habitat. Toxicities of the transgenic unicellular cyanobacteria were however very low, even under the regulation of strong promoters, too low to be effective in vivo. Certain improvements have been reported by Murphy & Stevens (1992), and Stevens et al. (1994). The gene coding for Cry11Aa was fused in frame with the first 6 codons of *cpcB* and cloned into shuttle vector pAQE19ΔSal for expression in *A. quadruplicatum* PR6 under P_{cpcB} promoter. The fused protein was stable in *A. quadruplicatum* PR6 cells, which was readily ingested by the freshly hatched *C. pipiens* larvae, but toxicity was also low. It was the first genetically engineered cyanobacterium that killed mosquito larvae by feeding living cells.

In parallel to the use of unicellular cyanobacteria, the filamentous nitrogen fixing cyanobacterium *Anabaena* PCC 7120 was used for expressing the *B. sphaericus* binary toxin genes (Xudong et al., 1993). Transformed cells carrying the larvicidal toxin genes (*p51* and *p42*) from *B. sphaericus* 2297 were bioactive upon feeding mosquito larvae, with higher toxicity against *C. pipiens* than against *Anopheles sinensis*. The much higher toxicity in the latter study than in Tandeau de Marsac's (1987) can be attributed to the multicellular character of *Anabaena* compared to the unicellular *A. nidulans* originally used. It is conceivable that a filament of many expressing cells inflicts damage to ingesting larva that is more harmful; this proposition is supported by the higher toxicity of Bti when bioencapsulated in the ciliate protozoan *Tetrahymena pyriformis* (e.g. Manasherob et al., 1994).

Expression of Bti *cry* genes in *Anabaena* PCC 7120

The broader spectrum of toxicity against mosquito larvae of Bti than *B. sphaericus* (Skovmand & Sanogo, 1999) makes the former more useful. Three of the Bti toxin genes (*cry4Aa*, *cry11Aa* and *p20*) had been isolated and cloned in all seven possible combinations for expression in two vectors of *E. coli* (Ben-Dov et al., 1995). The two most toxic clones (pHE4-ADR, with all three genes, and pHE4-4AD, without *p20*) were subcloned into *E. coli-Anabaena* shuttle vector for expression under control of the strong constitutive promoter *PpsbA* (Elhai, 1993) or tandem promoters *PpsbA*-P_{A1} (Wu et al., 1997). The final constructs, under control of the tandem promoters, were designated pSBJ2 and pSBW2 (Wu et al., 1997). They were introduced into *Anabaena* PCC 7120 by tri-parental conjugal mating. Two clones with pSBJ2 and 4 with pSBW2 were found toxic to larvae of *A. aegypti*, and then studied as described (Wu et al., 1997). This study was the first to co-express several Bti Cry proteins in this cyanobacterial species, and resulted in clones

with the highest toxicities ever reached so far (LC_{50} of about 0.3 μg total soluble protein per mL^{-1}). The high toxicities remained surprisingly stable during 3 years of constant cultivation, whether under drug selection or not (to be published elsewhere). The possibility to avoid the demand for antibiotic resistant genes is crucial for field applicability of these transgenic strains. Furthermore, a sensitive method for tracing the transgenic cyanobacteria after release by gene amplification with several pairs of primers targeted uniquely to each of the Bti toxin genes was developed (Ben-Dov et al., 1997).

The expressed proteins in the transgenic organisms were analyzed by SDS-PAGE and Western hybridization with antisera directed against each of them or against Bti whole crystals. Cry11Aa was detected in both strains, but in different quantities, as in *E. coli*. In *Anabaena*, large amounts of Cry11Aa were found in both the pellet and supernatant of cell crude extracts, but only trace Cry4Aa was detected in the pellet. The abundance of Cry11Aa and synergism with trace amount of Cry4Aa seem to account for the high toxicity. P20, that was readily detected in the 3 *E. coli* strains carrying *p20*, pHE4-ADR, pHE4-AR and pHE4-DR, in mass ratios of 1:2:10, respectively, was not found in the transgenic *Anabaena*.

Large differences in toxicities exist among the clones expressing mosquitocidal toxin genes in cyanobacteria (Table 1, and Soltes-Rak et al., 1995; Sangthongpitang et al., 1997), from non-toxic to very toxic, by feeding with intact cells with LC_{50} as high as 10^5 cells mL^{-1}. The bioassay conditions were varied over a wide range from feeding newly hatched larvae during 6 days, to 3rd instar larvae for 24 h (as in standard assays). These marked differences in bioactivity and stability among the reported clones can be explained by various reasons as follows. Previous work expressed a single or binary toxin, mostly in unicellular cyanobacteria. The very high bioactivity achieved by Wu et al. (1997) can thus be due to co-expressing in a filamentous strain two toxin genes that synergize each other (Porter et al., 1993; Poncet et al., 1995). Moreover, the codon usage of Bti's *cry* genes fortuitously resembles that of *Anabaena* PCC7 120, rather than all those unicellular strains (Wada et al., 1992; Soltes-Rak et al., 1995). The poor expression of Bt *cry* genes in higher plants has been greatly improved by modifying them to match the codon usage of the transgenic plants (Perlak et al., 1991, 1993; van der Salm et al., 1994; van Aarssen et al., 1995). Such codon usage bias may well account for the poor expression of Bti *cry* gene in unicellular cyanobacteria.

Transformation of indigenous filamentous cyanobacteria

Laboratory strains such as *Anabaena* sp. PCC 7120, not adapted to natural environments, may be inferior in nature because they are selected against (by competition with indigenous species) too quickly to yield the desired control efficacy. Reintroducing recombinant indigenous species that express cloned *cry* genes into their natural habitat would provide a solution to this issue. Two major problems are often encountered in establishing a transformation system for a newly isolated strain, restriction endonuclease activities and host range of shuttle plasmids (Elhai, 1994; Moser et al., 1993; Soper & Reddy, 1994). Strong restriction activities in the host often chop foreign incoming DNA before it is established. To overcome this barrier, two general approaches have been suggested, i.e. methylating DNA prior to transfer (Elhai et al., 1997) or using restriction-minus mutants, such as the commonly used *E. coli* strains showing very high transformation efficiencies. Endogenous plasmids, found in many strains of cyanobacteria (Houmard & Tandeau de Marsac, 1988), are often not suitable for replication in several strains of high interest, such as *Spirulina* sp. (Vachhani & Vonshak, 1997). New vectors should therefore be constructed for transforming indigenous strains. The cargo or incoming plasmids include two types of widely used plasmids for genetic transformation, replicative (shuttle) and integrative (suicide). Shuttle vectors are either derivatives of an indigenous plasmid of a recipient or a broad host range plasmid RSF1010, which could propagate in a wide range of microorganisms, including a limited number of unicellular and filamentous cyanobacteria (Houmard & Tandeau de Marsac, 1988; Mermet-Bouvier et al., 1993). An example of an indigenous plasmid is pDU1 from *Nostoc* sp. that has been used for constructing a number of shuttle vectors for transforming *Anabaena* sp. (Thiel, 1994). Both types of plasmid are compatible for cyanobacterial transformation.

Integrative vectors, lacking a cyanobacterial replicon, such as transposon-based plasmids (Mermet-Bouvier et al., 1993) have also been used in transformation of cyanobacteria. These are designed for recombination between a cloned gene of interest or a fragment of genomic DNA and a homologous region

Table 1. List of larvicidal cyanobacteria carrying toxin genes

Cyanobacterial strain	Toxin gene(s)	Promoter(s)	Mode of feeding	Larval species instar	Mortality % in 24 h	Mortality % in 48 h (72 h)[a]	Ref.
A. quadruplicatum PR-6	*cry4Ba*	P_{cpcB}	Sonicated cells (10 mg protein mL^{-1})	*Ae. aegypti* 2nd	ND	45	Angsuthanasombat Panyim (1989)
A. quadruplicatum PR-6	*cry11Aa*	P_{cpcB}	$2{\cdot}10^7$ cells mL^{-1} in 12h intervals	*C. pipiens* 1st	0	0 (10)	Murphy & Stevens (1992)
A. quadruplicatum PR-6	*cry11Aa*	P_{cpcB}	$2{\cdot}10^7$ cells mL^{-1} in 12-h intervals	*Ae. aegypti* 1st	45	75 (95)	Stevens et al. (1994)
Synechococcus PCC 7942	*cry4Ba*	P_{lac}-P_{Bti} P_{Bti} or P_{petF1}	$7.5{\cdot}10^7$ cells mL^{-1}, daily	*C. restuans* 1st	0	20–90 (50–95)	Soltes-Rak et al. (1993)
Synechococcus PCC 7942	*B. sphaericus p42+p51*	original promoter	One dose, sonicated cells (1 μg mL^{-1})	*C. pipiens* 2nd	45–50	100	Tandeau de Marsac et al. (1987)
Synechococcus PCC 6301	*B. sphaericus P42+p51*	*Prbc*L	One dose, $1.3–2.1{\cdot}10^5$ cells mL^{-1}	*C. quinque-fascitus* 2-day old, 6-day old	–	50	Sangthongpitang et al. (1997)
Synechocystis PCC 6803	*cry4Ba* or *cyt1Aa*	P_{psbA}	Soluble proteins (1 mg mL^{-1})	*Ae. aegypti* 3rd	ND	30	Chungjatupornchai (1990)
Anabaena PCC 7120	*B. sphaericus p42 + p51*	original promoter	One dose $4.1{\cdot}10^5$ cells mL^{-1}	*C. pipiens 2nd*	92	100	Xudong et al. (1993)
Anabaena PCC 7120	*cry4Aa + cry11Aa + p20*	P_{psbA}-P_{A1}	One dose, $5{\cdot}10^5$ cells mL^{-1}	*Ae. aegypti* 3rd	100	100	Wu et al. (1997)

a: percent of mortality after 72 h exposure
ND: not determined.

on the chromosome. They are typically used either for gene inactivation by targeted mutagenesis or for integration of a second copy of a gene into the chromosome forming a merodiploid. In unicellular species, such as *Synechococcus*, the replacement frequency of a chromosomal gene by double crossover between plasmid and chromosome is about 100–1000 fold higher than single crossover (Thiel, 1994), by which plasmid is added to the genome. In contrast, recombination following conjugation yields primarily merodiploids in *Anabaena* sp., the result of single crossover (Thiel, 1994).

Concerns related to release of genetically-modified microorganisms

Risks associated with release of recombinant microorganisms into the environment are well discussed in the literature (e.g. Porter et al., 1993; Gustaffson & Jansson, 1993; Stephenson & Warnes, 1996; Giddings, 1998). The major risks identified include unintentional spread of transgenes into other organisms (i.e. through horizontal gene transfer) and adverse ecological impacts of the introduced strains. In the case of insecticidal organisms, the development of resistance among target species is to be seriously considered.

For a number of reasons, these risks are minimal concerning the application in nature of recombinant cyanobacterial strains carrying the Bti's Cry toxin genes. Firstly, a dried formulation with cells killed during the process can be used. It can be argued that the transgenic organisms carry unaltered Bti toxins, already shown to be highly specific against larvae of mosquito and black flies, and harmless to all non-target organisms tested so far, including other insects, fish, frogs, crustaceans, and mammals, not to mention humans (Margalith & Ben-Dov, 2000). Moreover, Bti, hence its Cry toxins and their encoding genes occur naturally in the environment. These facts, coupled with the observations that no resistance was developed in field populations of mosquitoes and

no adverse effects on human or animal health have been detected after more than 15 years of large-scale, world-wide, and extensive field use of Bti (Margalith & Ben-Dov, 2000), further argue for the safety of using recombinant cyanoabcterial strains as biocontrol agents.

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