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Review

Recombinant bacteria for mosquito control

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Summary

Bacterial insecticides have been used for the control of nuisance and vector mosquitoes for more than two decades. Nevertheless, due primarily to their high cost and often only moderate efficacy, these insecticides remain of limited use in tropical countries where mosquito-borne diseases are prevalent. Recently, however, recombinant DNA techniques have been used to improve bacterial insecticide efficacy by markedly increasing the synthesis of mosquitocidal proteins and by enabling new endotoxin combinations from different bacteria to be produced within single strains. These new strains combine mosquitocidal Cry and Cyt proteins of *Bacillus thuringiensis* with the binary toxin of *Bacillus sphaericus*, improving efficacy against *Culex* species by 10-fold and greatly reducing the potential for resistance through the presence of Cyt1A. Moreover, although intensive use of *B. sphaericus* against *Culex* populations in the field can result in high levels of resistance, most of this can be suppressed by combining this bacterial species with Cyt1A; the latter enables the binary toxin of this species to enter midgut epithelial cells *via* the microvillar membrane in the absence of a midgut receptor. The availability of these novel strains and newly discovered mosquitocidal proteins, such as the Mtx toxins of *B. sphaericus*, offers the potential for constructing a range of recombinant bacterial insecticides for more effective control of the mosquito vectors of filariasis, Dengue fever and malaria.

Key words: *Bacillus thuringiensis* subsp. *israelensis*, *Bacillus sphaericus*, Cyt protein, Cry protein, *B. sphaericus* binary toxin, transcript stabilization, chaperone, recombinant bacterial larvicide.

Introduction

Despite advances in medical science and new drugs, mosquito-borne diseases, including malaria, filariasis, dengue and the viral encephalitides, remain the most important diseases of humans, with an estimated two billion people worldwide living in areas where these are endemic (World Health Organization, 1999a). Thus, there is an urgent need for new agents and strategies to control these diseases. Potential strategies include vaccines and transgenic mosquitoes refractive to the causative disease agents, but, in the near future, control efforts will rely on insecticides.

Since World War II, disease control methods have relied heavily on broad-spectrum synthetic chemical insecticides to reduce vector populations. However, synthetic chemical insecticides are being phased out in many countries due to insecticide resistance in mosquito populations. Furthermore, many governments restrict chemical insecticide use owing to concerns over their environmental effects on non-target beneficial insects and especially on vertebrates through contamination of food and water supplies. As a result, the World Health Organization (1999b) is facilitating the replacement of these chemicals with bacterial insecticides through the development of standards for their registration and use.

Vector control products based on bacteria are designed to control larvae. The most widely used are VectoBac® and Teknar®, which are based on *Bacillus thuringiensis* subsp. *israelensis* (Bti). In addition, VectoLex®, a product based on *Bacillus sphaericus* (Bs), has come to market recently for the control of mosquito vectors of filariasis and viral diseases. These products have achieved moderate commercial success in developed countries, but their high cost deters use in many developing countries. Moreover, concerns have been raised about their long-term utility due to resistance, which has already been reported to *B. sphaericus* in field populations of *Culex* mosquitoes in several different countries.

The insecticidal properties of these bacteria are due primarily to insecticidal proteins produced during sporulation. In Bti, the key proteins are Cyt1A, Cry11A, Cry4A and Cry4B, whereas Bs produces a single binary toxin commonly referred to as Bin. Of particular interest among these proteins is Cyt1A, which synergizes and delays resistance to mosquitocidal Cry proteins and can be used to overcome resistance to Bs, as well as extend its spectrum of activity to, for example, the yellow fever mosquito *Aedes aegypti* (Fig. 1). In addition to Bti and Bs, mosquitocidal proteins have been identified in other

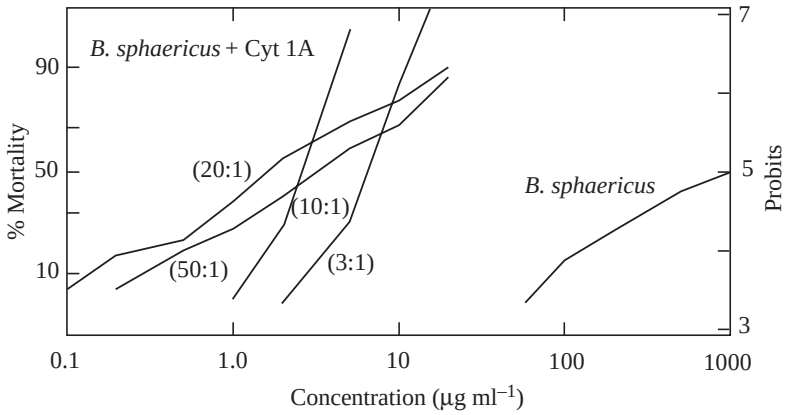

Fig. 1. Toxicity of *Bacillus sphaericus* (strain 2362) alone and in combination with a recombinant strain of *Bacillus thuringiensis* that only produces Cyt1A against fourth instars of *Aedes aegypti*. The figure illustrates dose–response regression lines of technical powder combinations (*B. sphaericus*: *B. thuringiensis*) of different ratios of the two bacterial strains.

species, such as *B. thuringiensis* subsp. *jegathesan*; these also offer promise for use in new types of larvicide.

Aside from this variety of mosquitocidal proteins, several genetic elements have been identified that, when used in combination with strong Bt promoters, can be used to improve efficacy by increasing endotoxin synthesis significantly. The most important of these are the STAB/SD sequence, a nine-nucleotide polypurine sequence that improves transcript stability and thus endotoxin synthesis, and a 20-kDa protein that occurs naturally in the Cry11A operon. This protein enhances net synthesis of Cry11A and other proteins and apparently acts as a molecular chaperone.

The biochemical and toxicological differences between mosquitocidal Bt and Bs toxins prompted several attempts during the late 1980s and 1990s to construct recombinant bacteria that combined the best properties of these species. However, none of the resultant recombinants had efficacy sufficiently improved over wild-type strains to warrant commercial development. The prospects for developing recombinant bacteria with high efficacy suitable for commercial development have improved recently due to the availability of genetic elements for improving endotoxin synthesis, a greater range of mosquitocidal proteins and the development of a better understanding of the toxicological properties of Cyt1A. In this overview, we first describe the properties of Bti and Bs and summarize previous research on improving bacteria for mosquito control. We then go on to show how new knowledge and technologies have been used to create recombinant bacteria that have much better potential for use in operational mosquito control programs owing to their very high efficacy and built-in resistant management properties based on Cyt1A. The literature on Bt, Bti and Bs is extensive and thus, in this overview, we cite review papers to guide interested readers to the original literature.

Properties of Bti

In 1976, a new subspecies, *B. thuringiensis* subsp. *israelensis*, that proved highly insecticidal to larvae of mosquitoes and blackflies was isolated from a mosquito breeding site in Israel (Goldberg and Margalit, 1977). Bti has an LC_{50} in the range of 10–13 ng ml^{-1} against the fourth instar of many mosquito species. Bti's parasporal body differs substantially from the classic Bt bipyramidal crystal that is toxic to lepidopteran larvae. It contains four major proteins – Cyt1A (27.3 kDa), Cry4A (128 kDa), Cry4B (134 kDa) and Cry11A (72 kDa) – in three different inclusion types assembled into a spherical parasporal body held together by lamellar envelope (Ibarra and Federici, 1986; Fig. 2). Studies of the amino acid sequences of Bti's Cry proteins have shown that they are related to other Cry endotoxins (Crickmore et al., 1998; Schnepf et al., 1998). However, Cyt1A differs markedly from the Cry proteins in its amino acid sequence and toxicology. It is highly cytolytic to a range of vertebrate and invertebrate cells *in vitro*, having an affinity for unsaturated fatty acids in the lipid portion of cell membranes (Thomas and Ellar, 1983). While it may act by

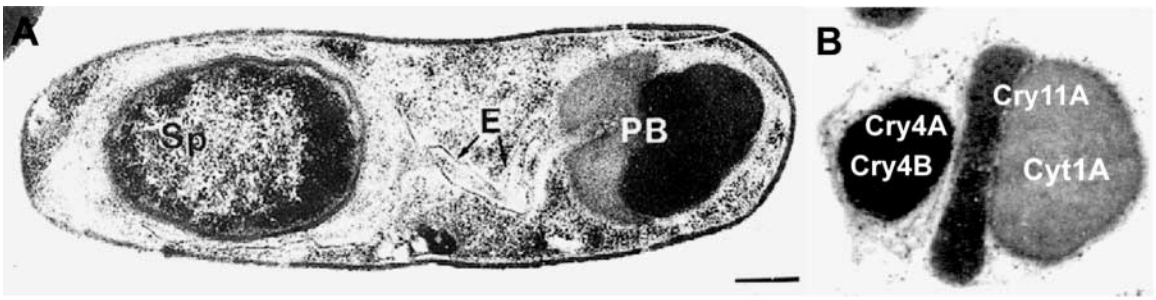

Fig. 2. (A) Sporulating cell of *Bacillus thuringiensis* subsp. *israelensis* and (B) a typical parasporal body of this species showing individual toxin inclusions and their toxin composition. Sp, spore; E, exosporium; PB, parasporal body.

forming transmembrane pores, other evidence suggests it has a detergent-like mode of action, perturbing the membrane by binding to specific fatty acids (Butko et al., 1997).

Despite our uncertainty about Cyt1A's mode of action, it is an extremely important protein with respect to mosquito control. Numerous studies have revealed that Bti's high toxicity is due to synergistic interactions among its Cry proteins and especially between Cyt1A and the Cry proteins (Wu and Chang, 1985; Ibarra and Federici, 1986; Crickmore et al., 1995; Wirth et al., 2000a,b). Even more importantly, recent studies suggest that Cyt1A can delay resistance to Cry proteins in mosquitoes (Georghiou and Wirth, 1997) and overcome resistance to these if it develops. For example, resistance levels to Cry11A of >900-fold in laboratory populations of *Culex quinquefasciatus* were suppressed completely when Cry11A was combined with Cyt1A in a 3:1 ratio (Wirth et al., 1997). In addition, Cyt1A can overcome very high levels of resistance to the Bin toxin of *B. sphaericus* 2362 when combined with this species (Wirth et al., 2000a) and can extend its target spectrum to *A. aegypti* (Wirth et al., 2000b). Recent studies using fluorescent dyes have shown that the lack of sensitivity in *B. sphaericus*-resistant *C. quinquefasciatus* is due to the absence of the Bin toxin receptor in the midgut microvillar membrane (Darboux et al., 2002). Studies in our laboratory have shown that Cyt1A forms lesions in this membrane that enable the Bin toxin to enter these cells and exert toxicity (Fig. 3).

The high efficacy that Bti showed in laboratory and field trials during the early 1980s led rapidly to its development as a commercial bacterial larvicide for control of mosquito and blackfly larvae (Mulla, 1990; Becker and Margalit, 1993). Four commercial products, VectoBac® (Valent Biosciences, Libertyville, IL, USA), Teknar® (Valent Biosciences, Libertyville, IL, USA), Bactimos® (Bayer Research, Triangle Park, NC, USA) and Acrobe® (Becker Microbial Products, Plantation, FL, USA) are used in many countries for the control of vector and nuisance mosquitoes and blackflies. Teknar® and VectoBac® proved to be particularly important for the World Health Organization's Onchocerciasis Control Program in West Africa, where they have been used for almost two decades to control the blackfly vectors of *Onchocerca volvulus*, which causes river blindness in humans (Becker, 2000).

Despite its intensive use in numerous mosquito and blackfly ecosystems and the development of resistance under intensive selection in the laboratory, resistance to Bti has not been reported in the field (Becker and Ludwig, 1993). Laboratory studies suggest that this lack of resistance is due primarily to the presence of Cyt1A in the parasporal body (Georghiou and Wirth, 1997; Wirth et al., 1997). Cyt1A's capacity to synergize endotoxin proteins, including the *B. sphaericus* Bin toxin against resistant and non-sensitive mosquitoes (Wirth et al., 2000a), and to delay resistance are important properties for the improvement of mosquito larvicides.

Properties of Bs

Many mosquitocidal strains of *B. sphaericus* have been isolated over the past 30 years, and the most toxic of these, including strains 1593 and especially 2362, belong to flagellar serotype 5a5b (Charles et al., 1996; Delécluse et al., 2000). The principal toxin in these strains is the Bin toxin, which is composed of two proteins, a 51.4-kDa binding domain and a 41.9-kDa toxin domain, that co-crystallize into a single small parasporal body. Strain 2362 has an LC_{50} of 18 ng ml^{-1} against the fourth instar of *Culex* mosquitoes (Baumann et al., 1991). After ingestion by a mosquito larva, the 51.4-kDa and 41.9-kDa proteins are cleaved by proteases, yielding peptides of 43 kDa and 39 kDa, respectively, that form the active toxin (Baumann et al., 1991; Charles et al., 1996). These associate, bind to the receptor, an α-glucosidase on the midgut microvilli (Darboux et al., 2001), and cause lysis of midgut cells after internalization (Davidson, 1988).

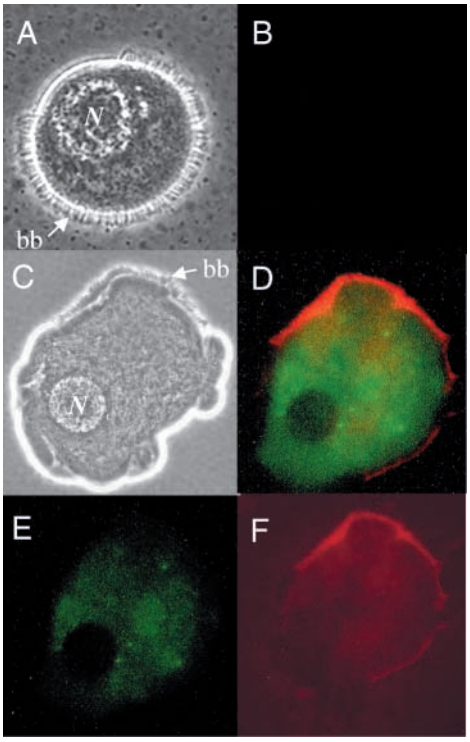

Fig. 3. Binding of Bin and Cyt1A to midgut epithelial cells of Bin-resistant fourth instars of *Culex quinquefasciatus* five hours after treatment. (A) Phase-contrast micrograph of a posterior midgut epithelial cell of a Bin-resistant larva fed only Bin labeled with Oregon Green. (B) The same cell as in A viewed with an Oregon Green filter, showing that little or no Bin bound to the microvilli or entered the cell. (C–F) Micrographs of a single posterior midgut epithelial cell from a Bin-resistant larva fed a 1:1 mixture of Bin and Cyt1A. Bin was labeled with Oregon Green, and Cyt1A with Rhodamine Red-X. (C) Phase contrast without filter; (D) superimposed micrographs taken with Oregon Green and Rhodamine Red-X filters; (E) micrograph of the cell using Oregon Green filter showing Bin throughout the cytoplasm, with no Bin detectable bound to the microvilli. (F) Micrograph of the cell taken using Rhodamine Red-X filter showing Bin bound primarily to the microvilli. N, nucleus; bb, microvilli of the brush border.

In addition to the binary toxin, many strains of Bs produce other mosquitocidal toxins during vegetative growth that are referred to as Mtx toxins. Two of these have been well studied – Mtx (100 kDa) and Mtx2 (30.8 kDa) – but are not as toxic as the Bin toxin (Delécluse et al., 2000).

The target spectrum of Bs is more limited than that of Bti, being restricted to mosquitoes, and its highest activity is against *Culex* and certain *Anopheles* species (Delécluse et al., 2000). Some important species of *Aedes*, such as *A. aegypti*, are not very sensitive to Bs, whereas others, for example, *Aedes atropalpus* and *Aedes nigromaculis*, appear to be quite sensitive (Delécluse et al., 2000). Moreover, although strain 2362 was isolated from a blackfly (*Simulium damnosum*) adult, Bs strains have little or no activity against nematoceran flies other than mosquitoes. Nevertheless, Bs does appear to have better initial and residual activity than Bti against mosquitoes in polluted waters. As a result, a commercial formulation, VectoLex® (Abbott Laboratories), based on strain 2362 is marketed in many countries, especially to control *Culex* larvae in polluted waters. A disadvantage of Bs strains is that the Bin toxin is, in essence, a single toxin. Laboratory studies have shown that it is much more likely to result in resistance than Bti. In fact, resistance to Bs has already been reported in field populations of *Culex* mosquitoes in Brazil, China, France and India (Sinègre et al., 1994; Rao et al., 1995; Silva-Filha et al., 1995; Yuan et al., 2000), with resistance levels in some areas of China reported as >20 000-fold.

Other mosquitocidal bacteria

The discovery and successful use of Bti and Bs strains in mosquito and blackfly control programs stimulated a worldwide search for more potent isolates of these and other bacteria. Many isolates of Bt and Bs, and even other bacterial species such as *Clostridium bifermentans*, have been discovered with mosquitocidal properties. Most of these produce toxins related to those already known (Delécluse et al., 2000). One of the more interesting Bt isolates is the PG-14 isolate of *B. thuringiensis* subsp. *morrisoni* discovered in the Philippines. This isolate is as toxic as Bti and produces the same complement of endotoxin proteins (Cyt1A, Cry4A, Cry4B and Cry11A) plus an additional 144-kDa Cry1 protein toxic to lepidopterans. Another interesting isolate is *B. thuringiensis* subsp. *jegathesan* from Malaysia (Delécluse et al., 2000). This isolate produces a complex of seven Cry and Cyt proteins, several of which are related to those of Bti but have different toxicological properties. One of these is Cry11B, a protein of 80 kDa that is approximately 10-fold more toxic to mosquitoes than the related Cry11A protein that occurs in Bti (Delécluse et al., 1995). The discovery of proteins such as Cry11B demonstrates the value of searching for new insecticidal isolates, even if the new strains prove not to be as effective as Bti and Bs.

Initial recombinant mosquitocidal bacteria

The existence of highly mosquitocidal strains of Bti and Bs, each with a unique set of toxins, as well as other mosquitocidal bacteria suggested that it might be possible to construct improved recombinant bacteria that combined the best properties of these. For example, a basic principle of resistance management is that it is more difficult to develop resistance to a multiplicity of toxins than to a single toxin. In addition, multiple toxins with different modes of action have the potential to be less prone to resistance than toxins with the same mode of action. As noted above, studies of Bti have validated the multiplicity and different modes of action principles for mosquitocidal bacteria, as has the development of resistance in the field to Bs for the single *versus* multiple toxin principle. Thus, there were two obvious strategies for making improved recombinant mosquitocidal bacteria: (1) introduce Bti or related mosquitocidal endotoxin genes into the best Bs strains and (2) introduce Bs toxin genes into Bti. Both of these approaches have been to construct a variety of Bt and Bs recombinants that produce different combinations of Bt and Bs proteins. The next two sections review the properties of these recombinants.

Bti endotoxins in Bs

Most recombinants made to date have introduced the Cry or Cyt proteins of Bti and related mosquitocidal subspecies into Bs, with Bs 2297 being the typical host. In general, production of Bti or other Bt toxins in recombinant Bs strains made these considerably more toxic to mosquito species insensitive to Bs, such as *A. aegypti*, or to species normally sensitive to Bs but that had developed resistance to the Bs Bin toxin, such as *C. quinquefasciatus*. Yet most Bs recombinants producing Cry or Cyt toxins were either equal in toxicity to parental strains (i.e. Bti or Bs) or only slightly more toxic or were unstable.

In one of the first sets of Bs/Bti recombinants, a Bti DNA fragment encoding the Cry11A- and Cyt1A-encoding genes was cloned into pPL603E and introduced into Bs 2362 by protoplast transformation (Bar et al., 1991). One recombinant produced Cyt1A, Cry11A and the Bs Bin toxin and was 10-fold more toxic to *A. aegypti* than parental Bs 2362 but was not nearly as toxic to this species as Bti. Initially, this recombinant appeared to be stable, but it was eventually found to be unstable (Bar et al., 1998).

In two other early recombinants, a plasmid containing *cry4B* was transformed into Bs strains 1593 and 2297 by protoplast transformation. Parental Bs 1593 and Bs 2297 strains had low toxicity to *A. aegypti*. However, production of Cry4B in the transformants increased toxicity to this species 100-fold (Trisrisook et al., 1990), making Bs transformants as toxic to *A. aegypti* as Bti. Against *Anopheles dirus* and *C. quinquefasciatus*, the Cry4B Bs transformants were similar in toxicity to the parental strains, being slightly more or less toxic depending on the recombinant strain and mosquito species tested.

In a related study, the *cry4B* or *cry11A* genes of Bti were transferred into Bs 2297 by electroporation using the shuttle vector pMK3 (Poncet et al., 1994). The parental Bs 2297 strain

was non-toxic to *A. aegypti*, whereas the Bs Cry4B and Bs Cry11A 2297 transformants were both moderately toxic to this species but not as toxic as Bti. In this study, it was found that the Cry4B transformant was approximately 10-fold more toxic to *A. aegypti* than the Cry11A transformant, and the authors suggested that the higher toxicity of the former was due to synergism between the Cry4B and the Bs binary toxin.

A more recent attempt to improve Bs used the transposon Tn917 to insert the major Bti toxin-encoding genes or fragments thereof into the chromosome of Bs 2362 (Bar et al., 1998). A series of recombinants was obtained that produced one or more of the Bti proteins in Bs 2362 along with the Bs binary toxin. As in previous studies, although not as toxic as Bti, many of the Bs 2362 recombinants obtained in this study were as much as 10-fold more toxic to *A. aegypti* than the parental Bs 2362 strain. However, against *C. quinquefasciatus* and *A. gambiae*, the recombinant toxicity was only in the range of parental Bs 2362 or Bti. In another study, integrative plasmids were used to introduce the *cry11A* gene into Bs 2362, resulting in recombinants that produced both the Bs binary toxin and Cry11A (Poncet et al., 1997). These recombinants were much more toxic to *A. aegypti* than parental Bs 2297 and were similar in toxicity to the parental strain against *C. quinquefasciatus*. However, one of the Cry11A Bs 2297 recombinants [2297(::pHT5601)] had toxicity to *Anopheles stephensi* that was comparable with that of Bti. In addition, the recombinant 2297(::*cry11A*) partially suppressed resistance to Bs 2297 in a strain of *C. quinquefasciatus* from India resistant to Bs 1593. Similar results were obtained when either the *cry11A* or *cry11B* (from *B. thuringiensis* subsp. *jegathesan*) or both of these genes were inserted into the chromosome of Bs 2297 using integrative plasmids (Servant et al., 1999). The production of Cry11A and/or Cry11B along with the Bs 2297 binary toxin increased the toxicity of this strain against *A. aegypti*, depending on the specific recombinant, from 5-fold to 11-fold. Against *Culex pipiens*, most recombinants were similar in toxicity to parental Bs 2297, although one (2297*pro::cry11Ba*) was about twice as toxic. Recombinants producing Cry11A and/or Cry11B were

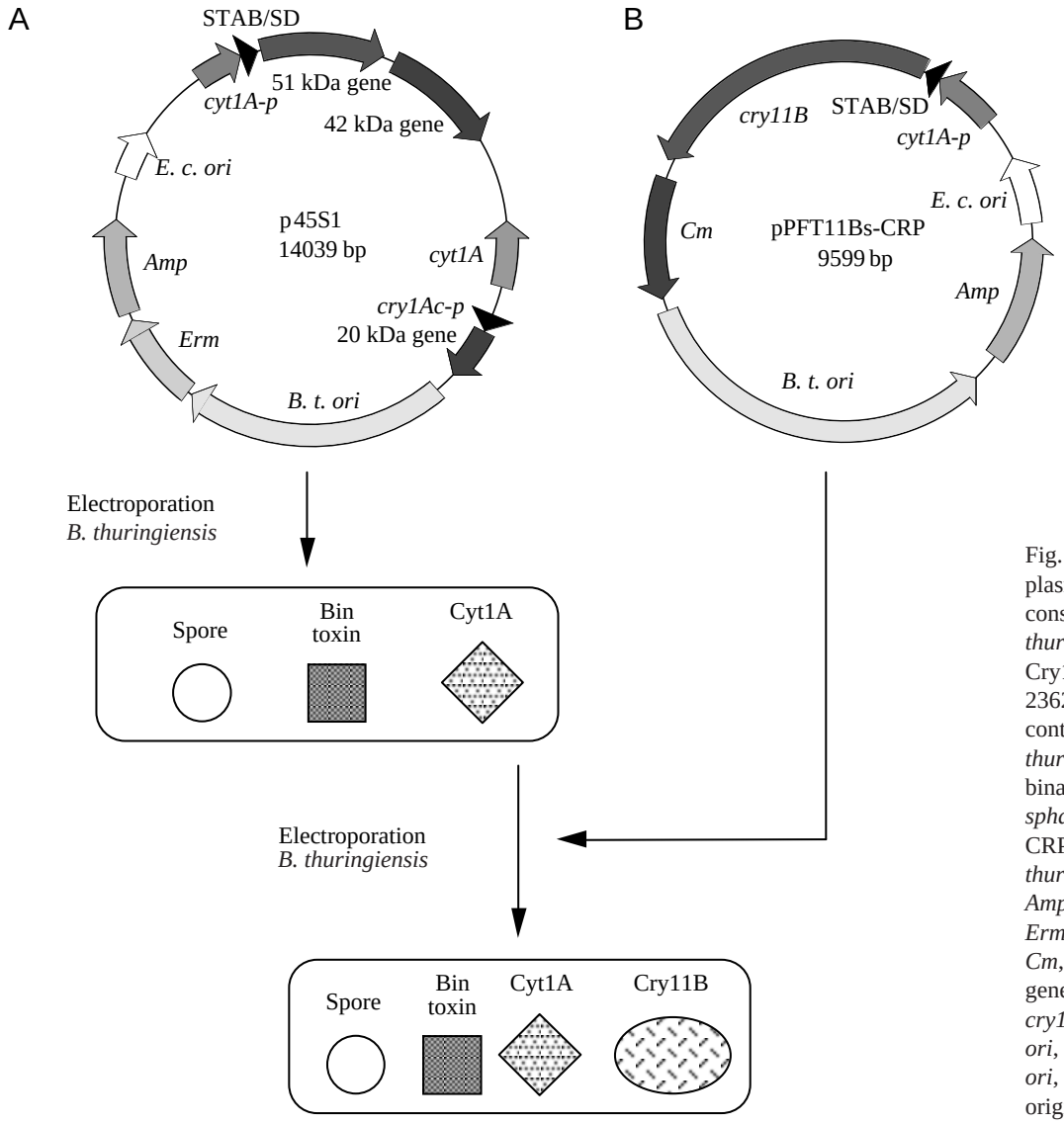

Fig. 4. Maps of recombinant plasmids and strategy for constructing a strain of *Bacillus thuringiensis* that produces Cyt1A, Cry11B and the *Bacillus sphaericus* 2362 binary toxin. (A) p45S1 containing *cyt1A* from *B. thuringiensis* subsp. *israelensis* and binary toxin gene from *B. sphaericus* 2362. (B) pPFT11Bs-CRP containing *cry11B* from *B. thuringiensis* subsp. *jegathesan*. *Amp*, ampicillin-resistant gene; *Erm*, erythromycin-resistant gene; *Cm*, chloramphenicol-resistant gene; *cyt1A-p*, *cyt1A* promoters; *cry1Ac-p*, *cry1Ac* promoters; *E. c. ori*, *E. coli* replication origin; *B. t. ori*, *B. thuringiensis* replication origin.

able to partially suppress resistance to Bs 2297 in different populations of *C. pipiens*.

A different type of Bs/Bt recombinant was constructed by using the shuttle vector pMK3 to insert the *cyt1Ab1* gene from *B. thuringiensis* subsp. *medellin* into Bs 2297 (Thiéry et al., 1998). The production of the Bs 2297 binary toxin together with Cyt1Ab did not improve toxicity to *A. aegypti* or *C. pipiens*. However, the recombinant strain was able to restore the sensitivity of Bs-resistant populations of *C. pipiens* and *C. quinquefasciatus* by 10–20-fold.

Because they contained broad-spectrum mosquitocidal Cry proteins, the Bs recombinants described above were typically considerably more toxic to *A. aegypti* than were parental Bs strains. However, none of these recombinants was better than Bti against this species, and only a few were more toxic to *Culex* and *Anopheles* species than the parental Bs strains. Nevertheless, these studies were very valuable because they resulted in techniques for constructing recombinants and showed that the various proteins of Bti and other Bt subspecies could be produced in substantial quantities in different strains of Bs. Moreover, they showed that producing Bt Cry and Cyt proteins in Bs extended its target spectrum to *A. aegypti* and partially suppressed Bs resistance in *Culex* species. Although not tested under field conditions, based on laboratory studies with Bti, it is probable that Bs strains containing Cry and/or Cyt toxins, even if not as effective as Bti, would be less prone to resistance and therefore useful for *Culex* control in polluted waters.

Bs binary toxin in Bti

The strategy of improving mosquitocidal bacteria by producing the Bs binary toxin in Bti has been used much less frequently, primarily because Bti already has a broad host spectrum and is more effective than Bs against most mosquito species. In addition, Bs is more effective and persists better than Bti in polluted waters, so improving the host spectrum and toxicity of Bs with Bti proteins had the potential for producing an excellent recombinant strain. It is not clear at this point which host is the best for optimizing toxin production and achieving broad-spectrum mosquito control. It may be that Bti is the best for some endotoxin combinations, targets and ecological situations and Bs for others. But producing the Bs binary toxin in Bti along with its normal toxin complement, especially using some of the enhancing elements, shows that very effective recombinants that use Bt as a host cell can be constructed.

In the first study where Bs toxins were produced in Bti, the binary toxin of Bs 1593 was cloned into the shuttle vector pBU4, yielding pGSP10 (Bourgouin et al., 1990). This plasmid was then transformed into the 4QS-72 strain of Bti, a strain that only contains the large plasmid encoding the Cyt and Cry endotoxins typically found in this subspecies. Analysis of the recombinant Bti strain showed that it produced the standard Bti toxins in normal amounts along with the 51.4-kDa and 41.9-kDa peptides of the Bs binary toxin. When tested against *A. aegypti*, *C. pipiens* and *A. stephensi*, the toxicity of the recombinant was no better than that of either the parental Bti or Bs strains.

In the above study, Bs promoters were used to express the Bs binary toxin in Bti, and none of the enhancing elements identified after this study was published were present in the plasmid used to produce the Bs binary toxin in Bti. Electron microscopy indicated that only small crystal of the binary toxin was produced in the Bti transformants. This could account for the lack of improved toxicity.

More recently, we have taken a different strategy in which we use Bt promoters and genetic elements that enhance toxin synthesis in Bt to produce both Bs and Bt proteins in Bti. Using this strategy, we have achieved significant improvement in the levels of Bt and Bs endotoxins synthesized in Bti and improvements in toxicity that were correspondingly higher against *C. quinquefasciatus*. Examples of two improved Bti strains will be used to illustrate this strategy. In the first, we constructed a strain of Bti that produced a combination of Cyt1A, Cry11B and the Bs Bin toxin, all in large quantities (Park et al., 2003). To engineer this strain, we constructed two plasmids, each of which contained a different selectable

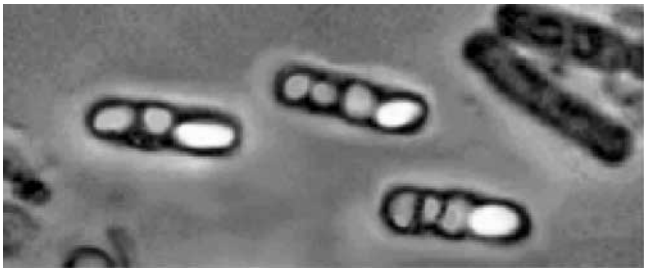

Fig. 5. Phase-contrast micrograph of *Bacillus thuringiensis* subsp. *israelensis* strain 4Q7/p45S1-11B, which produces crystals of Cry11B, Cyt1A and the *Bacillus sphaericus* binary toxin.

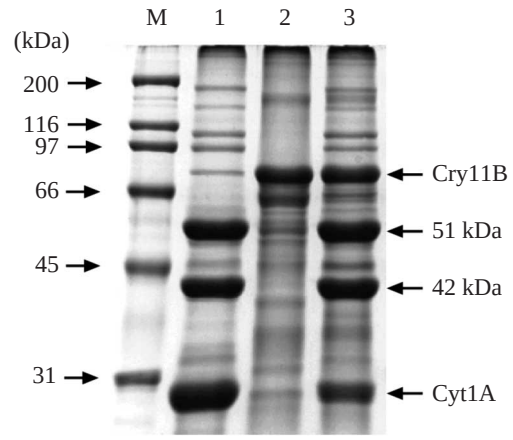

Fig. 6. Analysis of endotoxin content in recombinant strains of *Bacillus thuringiensis*. M, molecular mass marker; lane 1, *B. thuringiensis* subsp. *israelensis* 4Q7 producing Bs binary toxin and Cyt1A (4Q7/p45S1); lane 2, *B. thuringiensis* subsp. *israelensis* 4Q7 producing Cry11B (4Q7/pPFT11Bs-CRP); lane 3, *B. thuringiensis* subsp. *israelensis* 4Q7 producing Cry11B, Cyt1A and Bs binary toxin (4Q7/p45S1-11B).

Table 1. *Toxicity of recombinant and* Bacillus thuringiensis *subsp.* israelensis *(Bti) 4Q7 strains producing the* B. sphaericus *(Bs) binary toxin,* B. thuringiensis *Cry11B and/or Cyt1A against fourth instar* Culex quinquefasciatus *and* Aedes aegypti *in comparsion with wild-type strains*

Strain	Toxin combination	LC_{50} (fiducial limits) (ng ml^{-1})	Slope (± S.E.M.)
Culex quinquefasciatus			
4Q7/pPFT11Bs-CRP	Cry11B	9.2 (6.8–12.1)	2.0±0.2
4Q7/p45S1	Bin + Cyt1A	3.7 (2.5–5.2)	1.5±0.2
4Q7/p45S1-11B	Cry11B + Bin + Cyt1A	1.7 (1.2–2.5)	1.5±0.2
Bti IPS-82	Cry4A + Cry4B + Cry11A + Cyt1A	7.9 (6.2–9.7)	3.4±0.6
Bs 2362	Bin	12.6 (8.7–17.5)	1.4±0.2
Aedes aegypti			
4Q7/pPFT11Bs-CRP	Cry11B	28.5 (17.7–45.2)	2.6±0.5
4Q7/p45S1	Bin + Cyt1A	265.5 (214.9–321.6)	3.0±0.4
4Q7/p45S1-11B	Cry11B + Bin + Cyt1A	37.2 (30.5–44.8)	3.0±0.4
Bti IPS-82	Cry4A + Cry4B + Cry11A + Cyt1A	10.2 (8.0–21.9)	5.6±1.6
Bs 2362	Bin	Not tested	–

marker for expression in Bti (Fig. 4). The first plasmid (P45S1) contained as its key elements the Bs Bin toxin operon, under the control of cyt1A promoters and the STAB/SD sequence, along with the *cyt1A* gene and the 20-kDa chaperone-like protein, under the control of the *cry1Ac* promoters (Wu and Federici, 1993, 1995; Park et al., 1998). This plasmid had, as its selectable marker, erythromycin resistance. The second plasmid (pPFT11Bs-CRP) contained the *cry11B* gene under the control of *cyt1A* promoters and the STAB/SD sequence. The first plasmid was transferred into a Bti crystal minus strain (4Q7) by electroporation, and the transformants were selected on brain heart infusion agar containing erythromycin. Subsequently, the second plasmid (pPFT11BS-CRP) was transferred into this transformant by electroporation and was selected for on plates containing chloramphenicol. Analysis of this double transformant by microscopy and SDS-PAGE showed that it produced large quantities of all three endotoxins (Figs 5, 6). Bioassays of this double transformant against fourth instars of *C. quinquefasciatus* showed that it had an LC_{50} of 1.7 ng ml^{-1}, making it approximately fourfold as toxic as Bti (LC_{50}=7.9 ng ml^{-1}) and approximately sixfold as toxic as Bs 2362 (LC_{50}=12.6 ng ml^{-1}). While the activity of this recombinant was much better against this species than Bti or Bs, against *A. aegypti* the toxicity was much lower than Bti (Table 1).

As the second example, we transformed the p45S1 plasmid described above, which produces large amounts of the Bs Bin toxin, into the acrystalliferous strain of Bti (4Q7) as well as into IPS-82, a strain of Bti that produces the normal complement of Bti toxins. Both of these strains produced very large quantities of the Bs Bin toxin, as assessed by SDS-PAGE analysis (Fig. 7). Both of these transformants had markedly improved toxicity in comparison with wild-type strains of Bti or Bs 2362, each being at least 10-fold more toxic against

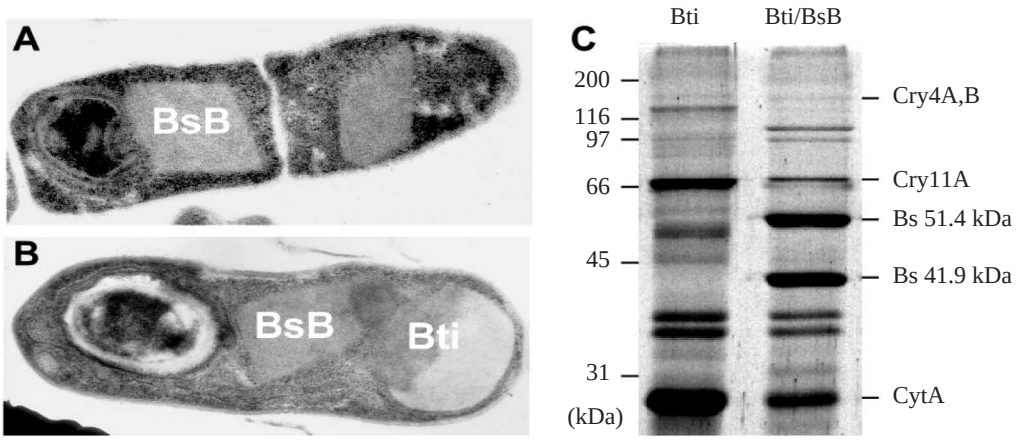

Fig. 7. Recombinant strains of *Bacillus thuringiensis* subsp. *israelensis* (Bti) that produce the *Bacillus sphaericus* (Bs) 2362 binary toxin. (A) Acrystalliferous strain transformed with a plasmid that produces the Bs 2362 binary toxin using *cyt1A* promoters and the STAB/SD mRNA stabilizing sequence. (B) IPS-82 transformed with the same plasmid. A large crystal of the Bs binary toxin and a typical Bti crystal are obvious in the sporulated cell. (C) SDS-PAGE analysis of IPS-82 (lane Bti) and IPS-82 producing the Bti proteins and the Bs binary toxin (lane Bti/BsB).

Table 2. *Toxicity to fourth instar* Culex quinquefasciatus *of* Bacillus thuringiensis *subsp.* israelensis *(Bti) strains that produce high levels of the* B. sphaericus *(Bs) binary toxin in comparison with wild-type Bti and Bs*

Bacterial strain	Toxin composition	LC_{50} (ng ml^{-1})	Improvement[a]
Bti IPS-82 + Bs Bin	Cry4A + Cry4B + Cry11A + Cyt1A + Bs Bin	1.5±0.4	13×
Bti 4Q7 + Bs Bin	Bs Bin	1.4±0.4	10×
Bti IPS-82	Cry4A + Cry4B + Cry11A + Cyt1A	19.5±3.5	
Bs 2362	Bs Bin	15.0±3.4	

[a]Improvement represents the factor by which the toxicity per unit mass of lyophilized fermentation product is improved compared with that of the wild-type strains. The Bti IPS-82 + Bs Bin strain is compared with Bti IPS-82, a wild-type strain, and the Bti 4Q7 + Bs Bin strain is compared with Bs 2362, a wild-type strain.

fourth instars of *C. quinquefasciatus* as assessed by comparison of their LC_{50}s per unit fermentation medium (Table 2). However, there was no substantial improvement in toxicity against fourth instars of *A. aegypti*.

Assessment and future prospects

The advent of recombinant DNA technology is now having an enormous impact on agriculture and medicine and it is appropriate that the ability to manipulate and recombine genes with this technology be applied to improving larvicides for vector control. The first steps in this direction made in the 1980s and 1990s are now bearing fruit owing to new discoveries regarding the properties of Cyt proteins, the availability of a wider range of mosquitocidal proteins and the identification of a variety of genetic regulatory elements that can be used to enhance synthesis of these proteins. In many ways, these new recombinant bacteria are almost ideal insecticides. They are as potent as many synthetic chemical insecticides yet are much less prone to resistance, as they typically contain a mixture of endotoxins with different modes of action. For example, the recombinant Bti species discussed above produce Cyt1A, Cry proteins and the Bs Bin toxin, each type with a different mode of action. Significantly, Cyt1A adds the important trait of making it difficult for the mosquitoes to develop resistance to these strains, something not achieved with chemical insecticides. The existing recombinants also have what can be considered disadvantages in that they do not show significantly improved activity against aedine and anopheline mosquitoes in comparison to Bti. But it may be possible to overcome this limitation using some of the newly discovered mosquitocidal proteins such as the Mtx proteins (Delécluse et al., 2000) and peptides such as the trypsin-modulating oostatic factor (Borovsky et al., 1993), which could be easily engineered for high expression in recombinant bacteria. While other microbial technologies such as recombinant algae and other bacteria are being evaluated, it has yet to be shown that these are as efficacious and environmentally friendly as Bti and Bs. One of the more remarkable traits of the latter bacteria, especially in comparison to chemical insecticides, is that they are highly specific for mosquitoes and their relatives. Lastly, we must realize that the initial recombinants described above are really the first steps in a process that should ultimately lead to much better insecticidal bacteria. By combining the genes from a variety of organisms, it should ultimately be possible to design 'smart' bacteria that will seek out and kill larvae of specific vector mosquitoes. While this seems far-fetched at this point, the rate at which advances are made with recombinant DNA technology is routinely underestimated. Thus, recombinant bacteria show excellent promise for development and use in operational vector control programs, hopefully within the next few years.

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