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TRANSFER OF TOXIN - CODING MEGAPLASMID OF *BACILLUS THURINGIENSIS* SUBSP. *THOMPSONI* INTO *BACILLUS SPHAERICUS*

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ABSTRACT: Mosquito borne diseases form a major component of vector borne diseases from all over the world. Several control strategies have been adopted to control diseases transmitted by mosquitoes. 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. Both *Bacillus sphaericus* and *Bacillus thuringiensis* subsp.*thompsoni* produce insecticidal toxins during sporulation and are extensively used in the field for control of mosquito populations. All the known toxins of the latter organism are known to be encoded on its megaplasmids. In an attempt to combine the best properties of the two bacteria, a cry proteins (namely 34- and 40-kDa) encoding megaplasmid (~ 100 MDa) of *Bt* subsp.*thompsoni* was transferred to *Bacillus sphaericus* by conjugation. Many of the transconjugants reacted with antibody to the 34- and 40-kDa *Bt* subsp.*thompsoni* crystal toxins in western blotting and were more toxic to *Aedes* mosquitoes than the wild type *B. sphaericus.* The toxicity of the transconjugants was maintained through many transfers in the absence of selective pressure.
Keywords: *Bacillus.sphaericus*, *Bt* subsp.*thompsoni,* transconjugation, insecticidal toxins, megaplamid.

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 (WHO 1999). 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 (1999) is facilitating the replacement of these chemicals with bacterial insecticides through the development of standards for their registration and use.

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 Cry genes (Suzuki, et. al., 2004).

In this study, we obtained proof of the mating transfer strategy concept and the results indicated that plasmid transfer into *Bacillus sphaericus* is possible and enhances the toxicity of the resulting transconjugants.

MATERIALS AND METHODS

Strains, Culture Conditions and Isolation of mutants

Bacillus sphaericus soil isolate (sbs4) obtained from the previous study (NCBI Gene bank accession No: 234124) used to produce a restriction less mutant strain. The donor strain was *Bacillus thuringiensis* subsp.*thompsoni* HD 542 (BGSC, USA), with the conjugative megaplasmid (~ 100 MDa), which encodes cry genes for 34- and 40-kDa toxins and which is Streptomycin (str) and Chloramphenicol (cam) sensitive (Brown and Whitelay, 1992). *Bacillus thuringiensis* subsp.*israelensis* HD2 (BGSC, USA) was used as reference in the megaplasmid electrophoresis (Gonzalez *et al,* 1982). str^r, cam^r and restriction less (Res^-) mutant of *B.sphaericus* (sbs4) as recipient strain was isolated on nutrient agar plates containing streptomycin (100 µg ml^{-1}), chloramphenicol (10 µg ml^{-1}), after Ethyl Methane Sulfonate (EMS) mutagenesis (carried out as described by Jiaviriyaboonya 1999).

Production of Transconjugant

The donor strain *B. thuringiensis israelensis* (wild type: fig.1a) and the transconjugant strain *B. sphaericus* sbs4A1 mutant (a restriction less mutant: fig.1b) were allowed to grow up to their early log phase in a rotatory shaker for 3 hrs in their respective medium separately. The recipient cells were washed by centrifuging them at 3000 rpm for obtaining the cells free of streptomycin (str) and chloramphenicol (cam), which were added to their medium. Then the donor and recipient cells were mixed in a ratio of 4:1 respectively on a sterile nutrient broth adding without antibiotics. Then these mixed strains were allowed to grow for two days. These mixed cultures were taken and serially diluted after resuspending them in sterile distilled water and they were spread plated on the petri plates having nutrient agar with both streptomycin and chloramphenicol for isolation of the colonies having transconjugants. The presence of transconjugants was further estimated by the number of colony forming units and by performing bioassay from the isolated colonies. The confirmation of the transconjugants (Fig.1c) was done by observing single cells under the phase contrast microscope.

Colony forming unit enumeration, Isolation of endotoxin and bioassay of Transconjugant

The donor, the recipient and the transconjugants were inoculated in their 100 ml medium respectively and they were allowed for sporulation by keeping them in the shaker for 3 days at 37^0c. Then these sporulated cells were obtained separately by centrifuging them at 6000 rpm for 10 min. These cells were washed once with the sterile distilled water. These washed cells were suspended in 100ml sterile distilled water separately and from this about 10ml of each sporulated strain were added to 20 litre plastic container and kept in the natural environment for a month (Sarrafzadeh, et. al., 2005). Their sustainability in the natural water environment was observed by performing CFU count for the 10^{-5} dilutions from the experimental setup every week.

The endotoxin concentration of *Bt. israelensis*, *B.sphaericus* sbsA1 mutant and the transconjugants were estimated by performing Lowry's method of protein estimation. About 10 larvae of *Aedes aegypti* were taken in 100ml water with 100µl of suspension in it. This was used to study the larval mortality with respect to the endotoxin concentration. Ten larvae were introduced to the 100ml water where the sporulated culture was added for the CFU study and the mortality rate was recorded after 24 hrs for every week. Two sets were maintained separately for the bioassay and to measure the residual effect. (Su and Mulla 2004).

SDS-PAGE and Immunoblotting

The transconjugants obtained were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting as described by (Bora, et, al., 1994). Protein concentrations in the transconjugants were determined by the method of (Lowry, et, al.,1951) after the protein crystals had been solubilized at 37°C for 3 h in 50 mM NaOH (pH 11.7) containing 10 mM EDTA. SDS-PAGE analysis was carried out with a mighty Small vertical-slab unit, according to the method of Laemmli (1970). Proteins fractionated on SDS-PAGE gels were electrophoretically transferred on to a nitrocellulose membrane with a TE70 Semiphor semidry transfer unit. Immunoblotting was performed according to the method of Towbin et al. (31). Polyclonal rabbit antiserum directed against purified crystal toxins (34- and 40-kDa) of HD542 was used as the primary antibody. Goat anti-rabbit immunoglobulin conjugated to alkaline phosphatase served as the secondary antibody (Murty, 1994).

Microscopy

Phase-contrast light microscopy

Bacteria and crystal samples were pipetted or suspended in dH_2O onto a microscope slide, covered with an over glass and examined using a Nikon Optiphot phase-contrast microscope under an oil-immersion objective (100x). Photos were taken with a Nikon Coolpix 950 digital camera attached to the eyepiece.

Scanning Electron Microscopy

Crystal suspensions were pipetted onto glass cover slips, mounted using colloidal silver paint onto 1 cm diameter SEM stubs, and allowed to dry in a fume hood. Samples were then sputter-coated with 10 nm Au/Pd using a Polaron E5100 Sputter Coater, and viewed using a Philips XL30 FEG scanning electron microscope at 5 kV beam current. Images were saved in Tiff digital image format.

Plasmid analysis

Detection and isolation of large plasmids were performed using the method of (Jensen, et. al., 1996). The bacteria were grown in 7 ml LB broth overnight (12-16 h) at 30^0C. 2 ml of cells were pelleted and resuspended in 100 µl E buffer (15% (wt/v) sucrose, 40 mM Tris-hydroxide, 2 mM ethylenediamine tetra acetic acid (EDTA), pH 7.9). Cells were lysed by the addition of 200 µl lysing solution (3% (wt/v) sodium dodecyl sulfate (SDS), 50 mM Tris, 15% (wt/v) sucrose, pH 12.5). The lysate was heated at 60^0C for 30 min followed by the addition of 5 units proteinase K. The solution was inverted 20 times and incubated at 37^0C for 90 min. After phenol extraction and centrifugation, the upper aqueous layer was subjected to horizontal 0.5% agarose gel electrophoresis (6-10 V cm^-1) with 1XTBE buffer for about 6-8 h.

RESULTS AND DISCUSSION

Bacillus sphaericus has the advantages of its ability to persist and recycle in the field condition. It is more toxic to *Culex* sp. and least toxic to *Aedes* sp. In order to improve their biological spectrum the meaplasmid encoding genes for the crystal protein of *B.t.thompsoni* were transferred by a bacterial conjugation method. In the present work, a transconjugant *Bacillus sphaericus* sbs4A1 with the crystal toxins from *Bt. thompsoni* was produced from the above experiment.

Transconjugant production

Following the on-plate mating described above, a number of colonies were selected on plates containing streptomycin and chloramphenicol (to select for *B. sphaericus* since the parental *B. sphaericus* sbs4 and mutant of *B. sphaericus* sbs4 strains were streptomycin and chloramphenicol resistant). The str^r, cam^r colonies were allowed to sporulate and were examined by phase – contrast microscopy for the presence of crystals (Fig.1) and followed by preliminary bioassay for toxicity (data not shown). Based on the results, one transconjugant was selected for further study and it was designated as transconjugant sbs4A1.The presence of megaplasmid (~ 100 MDa) was confirmed by agarose gel electrophoresis (see below) and the presence of toxins (40 – and 34 - KDa) by SDS – PAGE (see below) in this transconjugant by comparing with the donor *Bt* subsp.*thompsoni* HD542 and recipient *B.sphaericus* mutant.

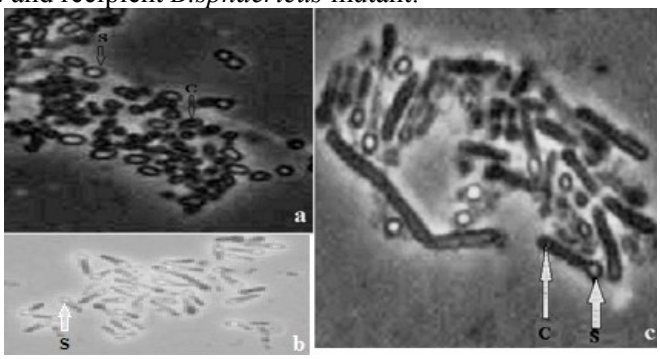

Fig.1: Phase contrast microscope image of a) *Bt. thompsoni* spores (s) and crystals (c) b) *B. sphaericus* sbs4 mutant spores (s) c) Transconjugant of *B.sphaericus* sbs4A1 mutant spore(s) with *Bt. thompsoni* crystal (c)

Toxicity

Transconjugant strain sbs4A1 and its parent *B. sphaericus* sbs4 and *B. thuringiensis* subsp. *thompsoni* were bioassayed with laboratory colonies of *Aedes aegypti* (Table.1). The estimation of the protein content of transconjugant also confirmed that the transconjugant of *Bacillus sphaericus* sbsA1 mutant has high toxicity to mosquito larvae of *Aedes aegypti* even at low level crystal protein concentration i.e.100 µg but the *Bacillus sphaericus* sbs4 mutant i.e. 120 µg shows comparatively low toxicity to mosquito larvae of *Aedes aegypti.* The mosquito larval control potential of the transconjugant *Bacillus sphaericus* sbs4A1 mutant was studied by a field bioassay along with the parental type strains such as wild type *B. t. thompsoni* and *Bacillus sphaericus* sbs4 mutant. It was found that the residual effect of the strains of *Bacillus sphaericus* sbs4 mutant and the transconjugant *B.sphaericus* sbs4A1 were high in field, indicating its persistence. However, the *Bacillus thuringiensis thompsoni* wild type was not persistence more than a week after incubation. The newly developed transconjugant *B.sphaericus* sbs4A1 was compared with the parental strains and it was found that the transconjugant strain maintained both toxicity and stability and in addition the ability to be sporulated with the crystal proteins for a longer period of time (Table 2).

Table 1: Comparison of larvicidal activity of *Bt. thompsoni*, *B.sphaericus* sbs4 mutant and transconjugant *B. sphaericus* sbs4A1 against *Aedes aegypti*

Days of exposure	Mortality rate (%)/ day		
	Bt. thompsoni HD542	*B. sphaericus mutant* sbs4	Transconjugant sbs4A1
1st day	98	36	90
7th day	54	32	87
14th day	0	30	83
21st day	0	34	78
28th day	0	31	80

Table 2: Persistance of *Bt. thompsoni*, *B.sphaericus* sbs4 mutant and transconjugant *B. sphaericus* sbs4A1 in breeding medium of *Aedes aegypti*

Days of exposure	Colony Forming Unit (cfu) per ml (x 10^{-5})		
	Bt. thompsoni HD542	*B. sphaericus mutant* sbs4	Transconjugant sbs4A1
1st day	272	532	590
7th day	1	657	481
14th day	0	912	228
21st day	0	107	412
28th day	0	126	263

Bacillus thuringiensis has been used widely to control mosquitoes which are vectors of human infectious diseases. The larvicidal activity of this organism is contained in a parasporal crystalline inclusion (δ-endotoxin) synthesized during sporulation .The growth and sporulation synthesis of δ- endotoxin and the toxicity against the larvae of *Aedes aegypti* and *Culex pipiens* were studied during fermentation of *B.thuringiensis* H14 in a 20 litre container by Sarrafzadeh *et al,* 2005. The synthesis of 65, 25 and 130KDa proteins started at 16, 18 and 24 hrs respectively. These proteins were enriched in different ways until the end of culture that is 48hrs. Toxicity in the course of the sporulation was different in the larvae of the both species. Maximal activity against *Aedes aegypti* was obtained at the end of the culture, whereas for *C. pipiens* the sample at 38hrs was active (Broadwell, 1986). Similarly in the present study also, it was shown the larvicidal effect was maximum by exhibiting 100% larval mortality in the treated natural breeding places. This effect was persisted for one month period of observation with 85% of the larvae reduction, indicating its capability of persistence in the environment.

In the present study, comparative mosquito larval bioassay data showed that the transconjugant *Bacillus sphaericus* sbs4A1 (containing *Bacillus thuringiensis thompsoni* crystals possessed high resistance and recycling ability in the breeding medium during bioassay.

This indicated that the transconjugant *B. sphaericus* sbs4A1did not lose their own characteristics and recycling ability but they obtain more toxic towards the *Aedes aegypti* larvae by their incorporation of *Bacillus thuringiensis thompsoni* crystal proteins. The level of toxicity of strain depends not only on the strains intrinsic properties but also on the test insect on the bacterial growth conditions and on the bioassay method (de Barjac and Charles, 1983).

Toxin production

Production of the *B. sphaericus* binary toxin and production of the *B. thuringiensis* subsp. *thompsoni* Cry toxins were analyzed by SDS-PAGE. Figure 2a shows the protein profiles of lysed sporulated cultures of *B. thuringiensis* subsp. *thompsoni* HD542 , the parental *B. sphaericus*, and transconjugant sbs4A1. There are two protein bands in the profiles of the transconjugant strains that are absent from the profiles of the parental *B. sphaericus* and appear to match bands in the profile of the *B. thuringiensis* subsp. *thompsoni* HD542 donor. Immunoblotting using antisera raised against these proteins confirmed the presence of *B. thuringiensis* subsp. *thompsoni* HD542 crystal toxins (40 and 34 - KDa) in the transconjugant sbs4A1 (Fig.2b). Electron micrograph of transconjugant sbs4A1 showed that if had acquired an additional small adjacent to the typical *B. sphaericus* crystal (fig.3).

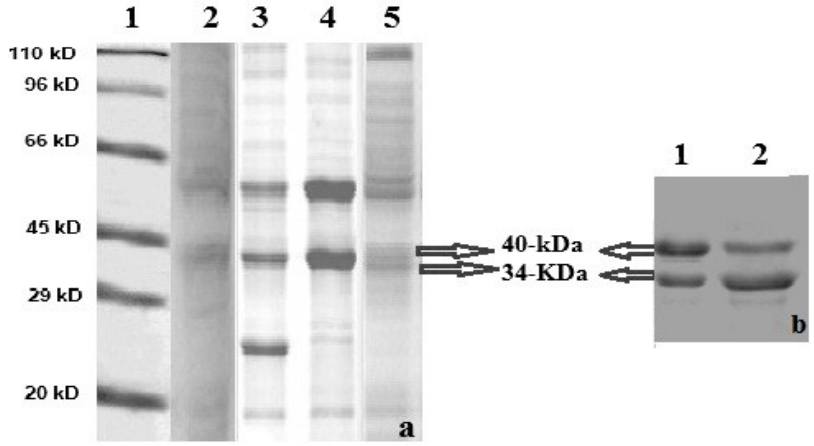

Fig.2: SDS-PAGE and Immunoblot analysis of the transconjugant *B.sphaericus* sbs4A1 a) Coomassie blue- stained 10 % SDS- Polyacrylamide gel. Lanes: 1. Marker, 2. *Bt. thompsoni*, 3.*B.sphaercius* sbs4 wild type, 4.*B.sphaercius* sbs4 mutant, 5. Transconjugant *B.sphaericus* sbs4A1 b) Immunoblotting with antibodies against solubilized crystals of *Bt.thompsoni* Lanes: 1. Crystal proteins of *Bt. thompsoni* and 2. Proteins from the transconjugant *B.sphaericus* sbs4A1. Molecular markers are indicated on the left.

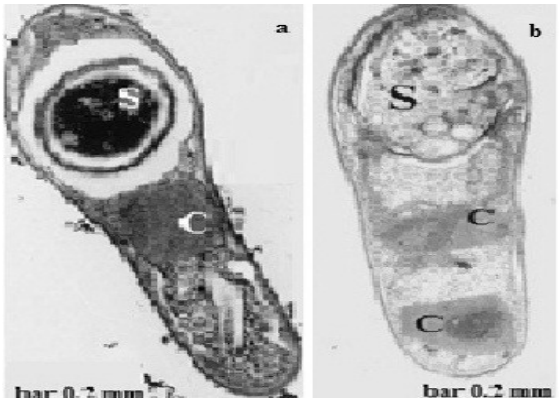

Fig.3: Electron microscope image of a) *B. sphaericus* sbs4 mutant spore (s) and crystal (c) b) Transconjugant of *B.sphaericus* sbs4A1 mutant spore(s) with *Bt. thompsoni* crystal (c)

Reports on the protein profile of *B.t. thompsoni* are scanty (Rang, et. al.,2000) showed that the simultaneous production of a 34 kDa and a 40 kDa protein was found to be required for the formation of inclusion bodies in *B.t. thompsoni*. The 34-kDa protein is active when produced alone and retains a large part, but not all, of the toxicity to codling moth . This same individual crystal protein was originally shown to be active against *M. sexta* and was included in the nomenclature of *B. thuringiensis* toxins as Cry15Aa1 (Crickmore, et. al,. 1998). The 40-kDa protein, which did not demonstrate any toxicity to *M. sexta* (Brown and Whiteley 1992), is still not considered in this classification restricted to active toxins. In agreement with these data, the transconjugant *B. sphaericus* sbs4A1, which simultaneously produces both crystal proteins of *B. thuringiensis* subsp. *thompsoni* along with bin toxins of *B.sphaericus*. Furthermore, the toxicity of the *Bt. thompsoni* strain producing both crystal proteins is about tenfold higher than that of the strain producing only the 34-kDa protein. BT-3440 was obtained by transforming BT-34 with a plasmid, i.e., pBU4, containing the 40-kDa protein gene and antibiotic resistance different from that of the plasmid already present in BT-34, i.e., pHT-3101. Therefore, the difference in toxicity between BT-34 and BT-3440 is necessarily a consequence of the addition of the 40-kDa gene. In addition, considering the total lack of toxicity of the 40-kDa protein to codling moth, the tenfold increase in toxicity cannot be related to a cumulative effect. This increase in toxicity after the addition of the non-active 40-kDa protein to the 34-kDa protein indicates a synergistic effect, the former potentiating the activity of the latter. Furthermore, the crystal proteins from *B. thuringiensis* subsp. *thompsoni* have been associated with unusual features for *B. thuringiensis* insecticidal proteins. The genes encoding these toxins share no homology with any other *B. thuringiensis* toxin genes and are present in an operon (Crickmore, et. al., 1998, Rang, et. al., 1997). However, unlike other polycistronic genes from *B. thuringiensis*, they are not associated with any other open reading frame, such as the molecular chaperon genes described in the *cry2* or *cry11* operons. The 34-kDa and 40-kDa proteins must be produced simultaneously from a single transcript to allow the formation of the characteristic pyramidal inclusion bodies, and interruption of either of the two genes will result in the loss of crystal formation. The simultaneous production of both proteins, but from different messenger RNAs, will also result in the loss of crystal formation (Dervyn, et. al., 1995). The binary toxins described in *B. sphaericus* (Charles *et al.* 1996) share many traits with the 34-kDa and 40-kDa proteins from *B. thuringiensis* subsp. *thompsoni*. The 41-kDa and 52-kDa crystal proteins from *B. sphaericus* work synergistically to make a typical crystal inclusion and make small-size amorphous inclusion bodies when produced separately. Like the 34-kDa protein from *B. thuringiensis* subsp. *thompsoni*, the 42-kDa protein from *B. sphaericus* is insecticidal, whereas the 51-kDa protein, like the 40-kDa protein described here, is not (Nicolas, et. al*.,* 1993). The 42-kDa protein is, however, about 160-fold less toxic than *B. sphaericus* inclusion bodies, whereas the 40-kDa protein described here is tenfold less toxic than whole crystals. However, this variation in the level of remaining toxicity must be considered with caution owing to large differences in protein preparation and bioassay procedures, as well as to difficulty of purifying individual components (Bauman and Bauman 1990).

The presence of both genes in an operon, the strict cooperative effect of these two proteins for formation of inclusion bodies, and the obligate simultaneous translation from a single transcript indicate the presence of a strong selection pressure for the simultaneous presence of both proteins. This is relevant with the conclusion from the bioassays experiments presented here that the 34-kDa and 40-kDa crystal proteins from *B. thuringiensis* subsp. *thompsoni* are likely to be the components of a binary toxin (Rang, et. al.,1997).

The binary toxin from *B. sphaericus* recognizes specific binding sites in mosquito midguts and was recently shown to form pores in planar lipid bilayers (Nielson and Charles 1993). The mechanism of potentiation and the mode of action of the 34-kDa and 40-kDa crystal proteins is not known yet and further research will be needed.

Plasmid profile

To examine the plasmid profile of transconjugant *B.sphaericus* sbs4A1, purified plasmids were separated in an agarose gel. The sizes of the donor, recipient and transconjugant plasmids were determined by comparison with sized plasmids isolated from B. thuringiensis HD-2 (Fig.4). Plasmid profile indicated the presence of toxin coding megaplasmid (100 MDa) of *Bt thompsoni* in the transconjugant *B.sphaericus* sbs4A1 This confirmed the transfer of the toxin coding plasmid at the time of conjugation. This offers the potential to transfer the toxin-coding plasmids to other species that may have useful environmental properties for enhanced insect control.

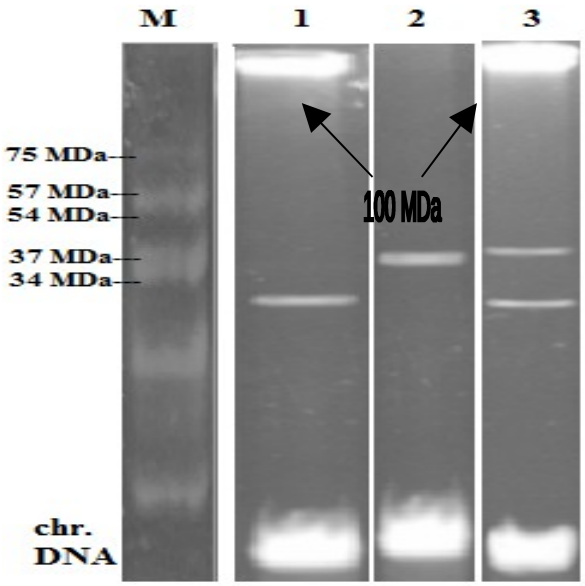

Fig.4: Plasmid DNA profile of a) *Bt. thompsoni* HD542 b) *B. sphaericus* sbs4 mutant c) Transconjugant of *B.sphaericus* sbs4A1 with *Bt. thompsoni* megaplasmid (~ 100 MDa). Molecular marker is indicated on the left. *Bt. thuringiensis* HD2 is used as a marker.

Five plasmids in *B. thuringiensis* subsp. *thompsoni*, ranging from 40 MDa to greater than 150 MDa, were observed, with four of the plasmids clustered around 100 to 150 MDa (Brown and Whiteley, 1992). But Carlton and Gonzalez (1985), who reported four plasmids in B. thuringiensis subsp. thompsoni ranging from 4 to 100 MDa. These authors may have used a different strain, or the larger plasmids may have been broken during isolation; in our study, no plasmids smaller than 30 MDa were ever observed. To determine which plasmid encoded the crystal protein genes, B. thuringiensis subsp. thompsoni plasmid DNA was transferred to nitrocellulose and hybridized with a radioactive probe specific to the genes encoding the 40- and 34-kDa proteins. The crystal protein genes were located on the predominant plasmid species of ~100 MDa. This observation agrees with the results of Carlton and Gonzalez (1984), who showed that an acrystalliferous strain of B. thuringiensis subsp. thompsoni resulted from the loss of a 100-MDa plasmid. In the present study, transconjugant which contains 100 MDa plasmid only expressed high toxicity towards the Aedes *aegypti* larvae (data not shown).

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. These results demonstrate that searching for novel strains is still a productive approach and that different kinds of insecticidal proteins can still be identified. *B. thuringiensis* remains, thus, a reliable source of novel insecticidal proteins to meet the growing need for novel specificities and novel modes of action to enlarge the host range and to manage insect resistance. Lastly, we must realize that the transconjugant obtained from the present study is really the first step 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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