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REVIEW PAPER OPEN ACCESS

Characterization of *Bacillus sphaericus* binary proteins for biological control of *Culex quinquefasciatus* mosquitoes: a review

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Abstract

The larvicidal action of the entomopathogen *Bacillus sphaericus* towards *Culex quinquefasciatus* is due to the binary (Bin) toxin protein present in crystals, which are produced during bacterial sporulation. However, the molecular basis for binary and receptor recognition is not well understood. In this review we attempted to discuss the general biology of this species and concentrate on the genetics and physiology of toxin production and it's processing for the production of biopesticides. In addition, larvicide of *B. sphaericus* is unique in that it consists of two proteins of 42 (BinA) and 51(BinB) kDa, both of which are required for toxicity to mosquito larvae midgut and these binary proteins are cleaved by proteases, yielding peptides of 39 kDa and 43 kDa, respectively that form the active toxin. These associate bind to the receptor, a α-glucosidase on the midgut microvilli, and cause lysis of midgut cells after internalization. Besides, Bin toxin can increase the toxicity of other mosquitocidal proteins and may be useful for both increasing the activity of commercial bacterial larvicides. Recently, 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. Finally, the availability of Bin toxins of *B. sphaericus* and newly discovered mosquitocidal protein offers the potential for constructing recombinant bacterial insecticides for more effective biopesticides for the biological control of mosquito vectors.

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Introduction

Binary toxin is produced by highly toxic strains of *Bacillus sphaericus* (Bs), and has been used as a bio-insecticide to control mosquito larvae (Baumann *et al.,* 1991). Toxicity is high against larvae of *Culex* and *Anopheles* mosquitoes, but low or nontoxic to *Aedes* larvae (Charles *et al.,* 1996). The binary toxin consists of two components, BinA (42 kDa) and BinB (51 kDa), and both are required at equimolar amounts to exert maximal biological activity (Baumann *et al.,* 1991; Porter *et al.,* 1993). Basically, the use of commercial bacterial larvicides to control nuisance and vector mosquitoes has grown rapidly over the past two decades, and these are now used instead of synthetic chemical insecticides in many countries (Becker, 2000; Fillinger and Lindsay, 2006). In this case, two bacteria are used as active ingredients in these larvicides, *Bacillus thuringiensis* ssp. *Israelensis* (Bti) and *B. sphaericus* (Bs). Interestingly, both have the advantage of being much more specific than chemical insecticides, having little effect on non-target organisms (Delécluse *et al.,* 2000). In particular, *Bacillus thuringiensis* ssp. *israelensis* typically kills only the larvae of mosquitoes, black flies, and to some extent, closely related nematoceran dipteran larvae such as those of chironomids (Glare and O'Callaghan, 2000). Besides, the target spectrum of Bs is more limited, restricted to mosquitoes, and even among these, it is ineffective against many. Most *Culex* species are highly sensitive to Bs, but within the genera *Aedes*, *Ochlerotatus* and *Anopheles*, some species are highly sensitive, whereas others show minimal sensitivity (Delécluse *et al.,* 2000). Bs formulations are more effective than Bti in polluted waters, where many different important *Culex* species breed. Moreover, Bs also has longer residual activity than Bti formulations in these habitats (Davidson *et al.,* 1984; Nicholas *et al.,* 1987; Kramer, 1990; Charles *et al.,* 1996). The principal protein responsible for Bs activity is the binary toxin, commonly referred to as Bin, which like those of Bti is produced during sporulation (Davidson, 1995). In particular, Bin is a very potent mosquitocidal protein consisting of two separate proteins that work together, BinA and BinB, which are, respectively, the toxic and binding moieties (Charles *et al.,* 1996). In more general terms, sensitivity to Bs is primarily dependent upon the presence of a-glucosidase, the 'receptor' or docking protein for BinB, on the midgut microvillar brush border membrane of sensitive species (Darboux *et al.,* 2001). Unfortunately, because Bin is in essence a single toxin, resistance to it can evolve quickly. In fact, where Bs has been used intensively for control of *Culex* species in China and Thailand, very high levels of mosquito resistance, as high as 50 000-folds, have evolved within a few years (Yuan *et al.,* 2000; Mulla *et al.,* 2003). In the present review we will summarize the recent literature on *B. sphaericus* with major emphasis on the larvicidal toxins of this species in *Culex spp* as biocontrol agents.

General information of *Culex quinquefasciatus*

Culex quinquefasciatus has an important role in the spread of diseases worldwide, and, in Bangladesh, this species is the major vector of lymphatic filariasis which remains an endemic disease in some urban areas (WHO, 1985). Generally, the status of *Culex sp.* as a disease vector has greatly increased in recent years the spread of the West Nile virus in the Americas. However, field trials have proved its effectiveness for reducing population density in areas where *Culex* is a source of nuisance or vector of diseases (Hougard *et al.,* 1993; Kumar *et al.,* 1996; Regis *et al.,* 2000). In the past many decades, vector control programmes to reduce transmission of the disease have been totally dependent on chemical insecticides. As a result, *Bacillus sphaericus* is the most successful biological larvicide commercially available to control *Culex*. Recently, the bacterial mosquito larvicides, *Bacillus thuringiensis var. israelensis* and *Bacillus sphaericus*, are identified as alternate tools and are being used for effective control of vector mosquitoes of filariasis (Gunasekaran *et al.,* 2000; Lee and Zairi, 2005; Medeiros *et al.,* 2005). It has been proved to be very effective against *Cx. quinquefasciatus,* the vector of

bancroftian filariasis, breeding in habitats prevalent in urban and peri-urban areas (Gunasekaran *et al.,* 2000; Medeiros *et al.,* 2005; Mwangangi *et al.,* 2011). After its application, the spores and crystals of the bacterium are ingested by mosquito larvae present in the breeding habitats and are eventually killed by the action of the crystal toxins. Apart from the larvicidal effect, reduced infection and infectivity of W. bancrofti filarial parasite is reported in *Cx. Quinquefasciatus* emerged from natural breeding habitats treated with B. sphaericus (Gunasekaran *et al.,* 2000). There are some previous reports that crystal proteins of *B. sphaericus* and *B. thuringiensis* are toxic to parasitic nematodes (Kotze *et al.,* 2005). Besides, anti-parasitic molecules have been reported to be upregulated in mosquitoes, especially in *Cx. quinquefasciatus,* after infection with filarial parasite (Paily *et al.,* 2007).

General information of *Bacillus sphaericus* (Bs)

Bacillus sphaericus (Bs) is a Gram-positive, sporeforming bacterium that can produce mosquitocidal toxins, particularly against *Culex* spp (Nicolas *et al.,* 1987; Yousten, 1984; Cheong & Yap, 1985). Unlike *Bacillus thuringiensis* var. *israelensis* (Bti), which has been used worldwide for mosquito control, Bs possesses the ability to survive in polluted water, and its toxicity appears to persist for a longer time (Mulligan *et al.,* 1980; Mulla *et al,.* 1982). In addition, Bs spores can recycle in *Culex* larvae. In the larval midgut, spores can germinate and multiply, leading to production of new spores which are released into the aquatic environment as the larval cadaver disintegrates (Mulligan *et al.,* 1980; Nicolas *et al.,* 1987). Particularly, the bacterial mosquito larvicide, *B. sphaericus*, is a biocontrol agent ideal for the control of both Anopheles sp. as well as *Culex sp*. of mosquitoes, because of its prolonged killing action (Singh and Prakash, 2009; Kovendan *et al.,* 2011; Raghavendra *et al.,* 2011). *B. sphaericus* has an additional, useful attribute in its ability to persist in polluted aquatic (Davidson *et al.,* 1984). In this study, we revealed that *B. sphaericus* is used for the biological control of mosquitos (Lacey and Undeen, 1986).

Taxonomy and general physiology of *B. sphaericus*

B. sphaericus are the presence of spherical spores, the inability to grow anaerobically, and a negative reaction on a variety of tests developed primarily for the classification of the family Enterobacteriaceae (Claus and Berkeley, 1986; Yousten, 1984). Moreover, the use of entomopathogenic microorganisms appears to be one of the promising alternatives, and microorganisms such as *Bacillus sphaericus* and *Bacillus thuringiensis ssp. israelensis* have been quite effective against different mosquito species (Federici *et al.,* 2007). Specifically, mosquitocidal strains of *B. sphaericus* can be divided into two groups on the basis of their toxicity to mosquito larvae (Baumann *et al.,* 1991). Strains which are highly toxic make a parasporal crystal, whereas strains with low toxicity lack a crystal. Furthermore, the high- and the low-toxicity strains are related by DNA homology values of over 79%, a finding consistent with their placement into a single species (Krych *et al.,* 1980).

Biochemistry of *B. sphaericus* crystal proteins

The crystal of *B. sphaericus* is a parallelepiped (de Barjac, 1988). Initially, the interior shows a crystalline lattice structure with striations about 6.3 nm apart (de Barjac, 1988; Yousten and Davidson, 1982). Then, the crystal is surrounded by an envelope (de Barjac, 1988; Yousten and Davidson, 1982) similar in appearance to that surrounding the crystals of *B. thuringiensis* (Fitz-James, 1984). Conversely, the envelope appears to be retained upon dissolution of the crystal matrix in the larval gut or after treatment with alkali (de Barjac, 1988; Yousten and Davidson, 1982). The relation between growth, sporulation, crystal formation, and toxicity for mosquito larvae has been studied for strains 1593 (Myers *et al.,* 1979), 2297 (Kalfon *et al.,* 1984; Yousten and Davidson, 1982), and 2362 (Broadwell and Baumann, 1986). However, the 51- and

42-kDa proteins were absent in the exponential phase of growth and appeared in approximately equal amounts during sporulation (Broadwell and Baumann, 1986; Charles *et al.,* 1988). Although both these toxins are required in equimolar concentrations for maximal toxicity (Baumann *et al.,* 1991), BinA alone has also been shown to be mildly toxic to the *Culex* larvae (Charles *et al.,* 1997; Hire *et al.,* 2009). As several strains of *B. sphaericus* have been found to exist in nature, which differ in the toxicity profile towards mosquito larvae. It is therefore important to have a systematic approach to isolate potent strains of this bacterium to exploit them as an effective biocontrol agent for mosquito control.

Isolation and purification of crystal proteins

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of partially purified crystal preparations from strain 2362 indicated that the major constituents were proteins of 122, 110, 51, and 42 kDa (Baumann *et al.,* 1985). The crystal preparation was solubilized by treatment with alkali (Davidson, 1983), and the 51- and 42-kDa proteins were purified to electrophoretic homogeneity by gel filtration through Sephadex G-200 followed by DEAE-agarose chromatography and gel filtration through Bio-Rad P-100 or P-60 (Baumann *et al.,* 1985). The N-terminal sequence of 40 amino acids of the 42-kDa protein was determined; the 51-kDa protein had a variety of N termini, thereby precluding sequence determination. Antisera prepared to the 51- and 42-kDa proteins were used in Ouchterlony immunodiffusion experiments and Western blots. Proteins immunologically related to the 51- and the 42-kDa proteins of *B. sphaericus* 2362 were detected in other highly toxigenic strains of this species, but not in strains which had low or no larvicidal activity (Baumann *et al.,* 1991).

Amino acid sequence of Bin proteins

The two major ORFs on the 3.5-kb HindIII fragment code for proteins of 448 and 370 amino acids with deduced molecular masses of 51 and 42 kDa, respectively. These genes from five strains of *B. sphaericus* have been sequenced (Baumann *et al.,* 1991). Importantly, three contain identical 51- and 42-kDa proteins; two have 51-kDa proteins that differ by 3 to 5 amino acids and 42-kDa proteins that differ by 1 to 5 amino acids (Berry *et al.,* 1989). Depending on the variety of this species, considerable sequence divergence has been found. However, a comparison of the amino acid sequences of the 51- and 42-kDa proteins with the sequences of representative *B. thuringiensis* crystal proteins active against members of the orders Diptera, Lepidoptera, or Coleoptera showed no significant sequence similarity (Baumann *et al.,* 1988), thereby indicating that the 51- and 42-kDa proteins of *B. sphaericus* constitute a separate family of insecticidal toxins.

Morphology of 51- and 42-kDa proteins

Thin sections of sporulating cells containing pUE382 (which produces both the 51- and the 42-kDa proteins) were examined by the electron microscope. *B. subtilis* DB104 contained large amorphous inclusions, whereas *B. sphaericus* 718 and SSII-1 contained crystals indistinguishable from those of *B. sphaericus* 2362 (Baumann *et al.,* 1991). This suggests the presence of a factor(s) absent in *B. subtilis* necessary for ordered aggregation of the 51- and 42-kDa proteins.

Larvicidal activity of 51- and 42-kDa proteins in *Culex*

Both the 51- and the 42-kDa proteins are necessary to kill 50% of the larvae of Culex pipiens (Baumann *et al.,* 1991). Strikingly, the spore-cell-amorphous inclusion complex of *B. subtilis* DB104 (pUE382) had an LC_{50} threefold lower than that of the *B. sphaericus* 2362 spore-cell-crystal complex.

Properties of Bs binary toxin

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). Morphologically, the principal toxin in these strains is the Bin toxin, which is composed of two proteins, a 51-kDa binding domain and a 42-kDa toxin domain, that co-crystallize into a single small parasporal body. However, 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-kDa and 42-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; Delécluse *et al.,* 2000). Although, the target spectrum of Bs is more limited than that of Bti, being restricted to mosquitoes, but its highest activity is against *Culex* and certain *Anopheles* species (Delécluse *et al.,* 2000). Moreover, 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). 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. In fact, resistance to Bs has already been reported in field populations of *Culex* mosquitoes in Brazil, China, France and India (Silva-Filha *et al.,* 2004; Mulla *et al.*, 2003; Yuan *et al.,* 2000), with resistance levels in some areas of China reported as> 20 000-fold. Finally, approximately equal amounts of each protein were required for maximal toxicity.

Mode of action and host specificity of toxin

Interaction between the subunits is essential to achieve full toxicity against larvae and the toxin seems to form oligomers (Regis *et al.,* 2001; Smith *et al.,* 2005). In addition, action of Bin toxin on *Culex quinquefasciatus* larvae depends on the recognition and binding of BinB subunit to specific receptors named Cqm1, which are 60-kDa a-glucosidases located on the apical membrane of midgut epithelium cells by a glycosylphosphatidylinositol (GPI) anchor (Silva-Filha *et al.,* 2004; Romão *et al.,* 2006). Toxin binding to Cqm1 receptors is followed by major cytopathological effects on the epithelium, and it is likely the toxin is able to form pores on cell membrane, although larval death occurs by mechanisms that are still unknown (Schwartz *et al.,* 2001; de Melo *et al.,* 2008). A number of studies revealed that the action of the crystal toxin on susceptible larvae involved the following series of reaction. The larvae of the target insect ingest crystal proteins from water. The crystal proteins are solubilized and activated under the combination of alkaline pH and proteinase of the larval midgut. Active toxins bind to apical microvilli of midgut cells via a glycosyl-phosphatidyl inositol anchor. After binding of toxin to the receptor site, a part of the toxin inserts into the membrane lipid bilayer forming ionic-selective channel or pore, which lead to entry of water into the cell and exit of ions and other larger components, leading to swelling and lysis of the cell by a colloid-osmotic lysis mechanism (Baumann *et al.,* 1985 and 1991; Knowles and Ellar, 1987; Charles, 1987; Singh and Gill, 1988; Davidson, 1988; Broadwell *et al.,* 1990; De Barjac, 1990; Baumann and Baumann, 1991; Oei *et al.,* 1992; Davidson, 1995; Charles *et al.,* 1996; Regis *et al.,* 2001; Manceva *et al.,* 2004; Smith *et al.,* 2005). The most drastic cytological changes caused by *Bs* toxin causes large vacuoles in the midgut cells and mitochondrial swelling. Meanwhile, late damage of neural tissues and skeletal muscles has also been reported.

Bioassays involving mosquito larvae

The ratio of the 51- to the 42-kDa protein necessary for maximal toxicity to mosquito larvae was determined by performing bioassays in which the relative amounts of amorphous inclusions containing each of the separate proteins were varied. Importantly, bioassay is a routine method to detect and compare toxicity of various Bs

strains. In this assay, the potency is expressed as LD_{50} (50% lethal dose) calculated from the amount of sample that kills 50% of mosquito larvae (Baumann *et al.,* 1991). Hence, a lower LD_{50} value equates with higher toxicity. Since the bioassay assesses the toxicity on mosquito larvae, it can provide a direct correlation with the bioactivity of a toxin preparation (Charles, 1987; Yousten and Davidson, 1982). However, the bioassay test is expensive, time-consuming, and subject to some variability.

Production of *Bacillus sphaericus* biopesticides

Bs is an aerobic rod shaped endospore forming bacterium with the endospore in a swollen terminal or subterminal position (Gordon *et al.,* 1973). It is widely distributed in soil and water habitats. Besides, some strains of Bs form crystal protein during sporulation and they are pathogenic to mosquito larvae. Bs produces Btx during the sporulation and comprises 42- (Bin A) and 51- KDa (Bin B) (Baumann et al., 1991, Charles *et al.,* 1996, Humphreys and Berry, 1998). However, Mtx toxins are produced during the vegetative growth and they are associated with the cell membrane of Bs (Liu *et al.,* 1996). Generally, Mtx toxins are three types, Mtx1, Mtx2 and Mtx3, with molecular masses of 100-, 31.8- and 35.8-kDa, respectively. Most of highly toxic strains synthesize Btx toxin and may contain one or more of Mtx toxins.

Lauge scale production of Bs

Bs grows in a culture medium containing sources of carbon and nitrogen as well as mineral salts. The growth of *Bs* can be described by three phases: Vegetative growth (exponential phase), transition phase and sporulation phase. During the sporulation phase, each cell liberates one spore and a protein toxin crystal. Because of the economic importance of Bs as powerful biological control agents against harmful insect pests, special attention was paid to elucidate and optimize growth conditions of Bs that leading to the highest yields of their toxins. Salama *et al.* (1983) and Sachdeva *et al.* (1999) reported that the commercial application of organism depends on the cost of raw materials, strain efficiency, fermentation cycle, maintenance of process parameters, bioprocessing of fermentation fluid, and formulation of the final product. Specially, the cost of raw materials is one of the principal costs involved in overall Bs production. Therefore, local production of this insecticide in developing countries should depend on the use of production media made of cheap, locally available sources including agro-industrial by-products (Ampofo, 1995). For large scale production of Bs, different approaches were investigated to construct media that could support good production of spores and toxins at reasonable costs. Various agricultural and industrial by-products used as raw material in Bs production were citrus peels, wheat bran, corn meal, seeds of dates, beef blood, silkworm pupal skin, ground nut cake, cane molasses, fish meal, cotton seed meal, soybean meal, residues from chicken slaughter house, fodder yeast, cheese whey and corn steep liquor (Salama *et al.,* 1983; Obeta and Okafor, 1983; Mummigatti and Raghunathan, 1990; Lee and Seleena, 1991; Sachdeva *et al.,* 1999; Foda *et al.,* 2002 and 2003). Recently, other wastes such as sludge and broiler poultry litter were utilized for biopesticides production (Adams *et al.,* 2002; Vidyarthi *et al.,* 2002). In general, two methods of fermentations are used for production of microbial products, submerged fermentation and solid state fermentation.

Recombinant bacteria for mosquito control

More recently, recombinant DNA techniques have been used to improve bacterial insecticide efficacy by increasing the synthesis of mosquitocidal proteins and by enabling toxin combinations from different bacteria produced within single strains (Federici *et al.,* 2007). 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. Significantly,

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 Bin protein (Park *et al.,* 2005). 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.

Safety of Bs insecticides

Entomopathogens like chemical insecticides must be evaluated for their safety to both animals and humans. However, microbial safety tests concentrate on acute toxicity and vertebrate infectivity, while chemical safety tests focus on acute toxicity, neurotoxicity and carcinogenicity. According to De Barjac (1990), Priest (1992), WHO (1999), Siegel (2001), Abdullah (2002) and Mittal (2003), Bs is completely safe to other nontarget organisms, human, animals, wildlife and environment and they are suitable for community use.

Future prospects and Conclusions

Bacterial insecticides have been tested with limited use for the control of vector mosquitoes for more than two decades (Lacey, 2007). Using entomopathogenic bacteria to control mosquitoes is a promising environmentally friendly alternative to chemical insecticides (Park and Federici, 2009). In this case, the most widely used alternative control agents for mosquitoes are the insecticidal spore-forming bacteria, *Bacillus thuringiensis* subsp. *israelensis* and *Bacillus sphaericus* (Federici *et al.,* 2006; Park *et al.,* 2010). The 51- and 42-kDa mosquitocidal crystal proteins of *B. sphaericus* are unique among bacterial insect toxins in that they (i) act as a binary toxin when tested against mosquito larvae, (ii) have a low sequence similarity, and (iii) are distinct from all of the cloned and sequenced insect toxins of *B. thuringiensis.* The present review indicates that the characterization of these toxins by the techniques of genetic engineering and molecular biology for constructing a range of recombinant bacterial insecticides which would be more effective biopesticides than chemical insecticides for control of mosquito vectors in nature.

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