A REVIEW

The safety of *Bacillus* species as insect vector control agents

F. A. Drobniewski

Public Health Laboratory Service, Dulwich Hospital, East Dulwich Grove, London, UK

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1. Introduction, 101
2. A taxonomic dilemma, 102
3. *Bacillus thuringiensis* toxins, 103
4. *In vivo* testing, 104
5. *Bacillus thuringiensis* and *B. sphaericus* as pathogens, 104
6. Conclusions, 106
7. References, 106

1. INTRODUCTION

One of the success stories of international co-operation in the control of infectious diseases has been the World Health Organization's (WHO) Onchocerciasis Control Programme (OCP) in West Africa; the use of *Bacillus thuringiensis* (BT) toxins has been an important component of the programme (Burges 1981; Anon. 1987; Guillet 1990; Webb 1992; Drobniewski 1993a).

Onchocerciasis, or 'river blindness', is a chronic filarial disease caused by the parasitic nematode worm *Onchocerca volvulus*, and is transmitted by blackflies of the *Simulium* genus. The WHO estimates that over 90 million people are at risk from acquiring the disease, that there is an overall prevalence of 18 million people and that 1000000 cases of blindness have been caused by onchocerciasis (Anon. 1987; Guillet 1990; Webb 1992; Drobniewski 1993a).

The OCP has demonstrated the effectiveness of BTI under field conditions, against all the larval stages of *Simulium* blackflies and shown it to be environmentally friendly, affecting few non-target species, and exhibiting no cross-resistance with the chemical insecticides used by the OCP. It has been a remarkably successful policy with vector transmission interrupted in an area of 600 000 km² and no blindness reported among the 9 million children born within the boundaries. Approximately 30 million people have been protected from disease and 3.25 million people initially infected by *O. volvulus* are now parasite-free (Burges 1981; Anon. 1987; Guillet et al. 1990; Webb 1992; Drobniewski 1993a). In a world with increasing famine, 15 million hectares of additional cultivatable land have been created.

The use of another species producing mosquitocidal
toxins, *B. sphaericus*, may also increase over the next decade (Hofte and Whiteley 1989; Singer 1990; Davidson and Yousten 1990) but other entomopathogenic species such as *B. lentimorbus*, *B. popillae* and *B. larvae* have not found widespread use.

Within the OCP, between 1981 and 1988, over 2 million litres of BTI were used (Guillet et al. 1990) and obviously on this scale an agent must be both effective and completely safe for human use. This paper reviews the experimental evidence for the safety of *B. thuringiensis* and to a lesser extent, *B. sphaericus*, the evidence of pathogenicity to man, and established clinical cases. It will also explore the problems of identifying *Bacillus* isolates in a clinical context particularly in the developing world where laboratory facilities may be limited and consider how valid species designations are within the genus *Bacillus*.

2. A TAXONOMIC DILEMMA

*Bacillus* spp. are ubiquitous organisms that successfully occupy a wide variety of ecological niches. Members of the genus are strict aerobes and are genetically heterogenous with the DNA G + C mol % concentration of strains varying from 32 to 69% (Kramer and Gilbert 1989; Turnbull et al. 1990; Drobniewski 1993b). Most strains are catalase-positive, motile, with peritrichous flagella, and possess the ability to sporulate in air which differentiates members of the genus from clostridia.

The genus is divided into three groups depending on the morphology of the spore and sporangium. *Bacillus thuringiensis*, *B. cereus*, *B. megaterium*, *B. anthracis*, and *B. cereus* var. *mycoides* occupy Group 1, i.e Gram-positive organisms which produce central or terminal-sited ellipsoid or cylindrical spores that do not distend the sporangia. These species are all found in the 'large-cell' subgroup in which the width of the vegetative cells is ≥ 1 μm (Kramer and Gilbert 1989; Turnbull et al. 1990). *Bacillus thuringiensis* is therefore morphologically similar to all the medically-important isolates of *Bacillus*. Group 2 species are Gram-variable and have swollen sporangia with central or terminal ellipsoid spores. The principal members of this group are *B. circulans*, *B. macerans*, *B. polymyxa*, *B. popillae*, *B. larvae*, *B. lentimorbus*, *B. alvei*, *B. stearothermophilus* and *B. brevis*. *Bacillus sphaericus* (Group 3) is morphologically easier to distinguish from other *Bacillus* species in that spherical, terminally or sub-terminally sited spores are located within a swollen sporangia.

The taxonomic relationship between and within species in Group 1 is not clear. Serological studies have shown significant cross-agglutination between spore antigens of *B. cereus*, *B. anthracis* and *B. thuringiensis* (Lamanna and Eisler 1960; Lamanna and Jones 1961) and the flagellar antigens of *B. cereus* and *B. thuringiensis* (Turnbull et al. 1990). Enzyme electrophoretic patterns and numerical phenetic analysis have also emphasized the close relationship between these species (Baptist et al. 1978; Priest 1988).

Studies of DNA–DNA hybridization, despite some inconsistency in the overall relatedness of strains and technical difficulties, suggest considerable chromosomal similarity between *B. anthracis* and some non-anthrax bacilli (Somerville and Jones 1972; Kaneko et al. 1978; Seki et al. 1978). The development of nucleotide probes has been of value in, for example, veterinary services demonstrating that isolates which were loosely designated 'anthrax-like' were in fact *B. anthracis* which had lost plasmids (Turnbull 1992).

Recent research based on a comparative study of 16S rRNA sequences from *B. anthracis* Sterne and *B. cereus* emetic strain NCTC 1143 showed that 1446 bases, or 94 % of the total sequence, were identical (Ash and Collins 1992). *Bacillus thuringiensis* and *B. cereus* var. *mycoides* differed from each other and from *B. anthracis* and *cereus* by less than nine nucleotides (Ash and Collins 1992). Sequencing of the 23S rRNA genes derived from PCR-amplification of chromosomal DNA from *B. anthracis* and an emetic strain of *B. cereus* showed that they are almost identical (Ash et al. 1991). This similarity has prompted the view that *B. anthracis*, *B. thuringiensis* and *B. cereus* are all varieties of a single species. Conversely DNA–DNA hybridization experiments have demonstrated that *B. sphaericus* is an extremely heterogenous species, and it may in fact be a group of species (Krych et al. 1980).

In the diagnostic laboratory differentiating between *Bacillus* species can be difficult although discrimination based on a combination of morphological characters, and the API 20E and API 50 CHB test strips (bio Merieux) can be quite successful (Logan and Berkeley 1984; Bryant et al. 1985; Logan et al. 1985). Novel techniques such as pyrolysis mass spectrometry which are available in reference laboratories have been able to discriminate some closely related species; for example, 53 strains of *B. subtilis*, *B. pumilis*, *B. licheniformis* and *B. amyloliquefaciens* could be distinguished although the spectra were influenced by media composition, culture maturity and whether the cultures had sporulated (Shute et al. 1984, 1988).

Within the developing world (and often, also, in the developed world), the few existing microbiology laboratories cannot justify the expense of investigating an isolate that might be a contaminant. Although a large number of phenotypic tests are used to distinguish between species, in practice sometimes only a single feature is used to separate them, such as the crystalline parasporal inclusion of *B. thuringiensis*. Not all strains produce crystals, however, and crystalliferous mutants occur readily, as a result of the loss of toxin-encoding plasmids (Gonzalez et al. 1981), producing organisms that are practically indistinguishable from
3. **BACILLUS THURINGIENSI S TOXINS**

The crystalline toxins exist as protoxins which become solubilized in the alkaline gut contents of dipteran, lepidopteran and coleopteran insect larvae and undergo proteolytic cleavage to form active toxins. The parasporal inclusions of *B. sphaericus* similarly require solubilization and proteolytic activation. *Bacillus thuringiensis* toxins interact with mid-gut membranes causing physiological changes which lead to paralysis, cessation of feeding and eventually death (Thomas and Ellar 1983; Ellar *et al.* 1986; Knowles and Ellar 1986; Anon. 1987; Gill *et al.* 1987; Wolfsberger *et al.* 1987; Hofmann *et al.* 1988; Hofte and Whiteley 1989; Drobniowski and Ellar 1988a, 1989; Chilcott *et al.* 1990; Van Rie *et al.* 1990; Wolfsberger 1990). The disruption of the mid-gut epithelium allows mixing of alkaline gut contents with haemolymph, reducing the gut pH and permitting bacterial spore germination in the nutrient-rich mixture.

The majority of these toxins have a molecular weight of 65 to 135 kDa and over 40 toxins have been cloned, expressed in other bacteria and plants, and sequenced (recently reviewed by Aronson (1993) and Hofte and Whiteley (1989)). From sequence and toxicity data a classification scheme was formulated (Hofte and Whiteley 1989) in which there were 13 'CRY' or crystal groups which were homologous but which exhibited toxicity against different target larvae. In *vitro* these activated toxins were cytolytic to cells from the target and related larval species. A fourteenth 'CYT' group was created for a 27 kDa toxin from the var. *israelensis* crystal, which by contrast was mosquito-cidal, haemolytic and cytolytic to many mammalian and insect cell lines, but which did not share sequence homology with CRY toxins (Ellar *et al.* 1985; Hofte and Whiteley 1989). It has become apparent that the CYT designation can be applied to a broader group that includes at least five hydrophobic proteins which share the above properties: the 27 kDa protoxins (processed to an active toxin of 25 kDa) of BTI and var. *morrisoni* PG14 (Ward *et al.* 1986; Earp and Ellar 1987; Chilcott and Ellar 1988); the 28 kDa protoxin of var. *darmstadiensis* 73-E10-2 which is processed to a 23 kDa toxin (Drobniowski and Ellar 1989); the 25 kDa protoxin of var. *kyushuensis* which forms an active toxin of 23 kDa (Knowles *et al.* 1992); and probably, the 27 kDa toxin of var. *fukuokaensis* (Yu *et al.* 1991). The var. *israelensis* and var. *morrisoni* 27 kDa toxins have been cloned and sequenced and differ by only one nucleotide (Earp and Ellar 1987; Galjart *et al.* 1987). However, a change in only a few key amino acids has a significant effect on the insect specificity of the toxin (Haider and Ellar 1987). The CYT toxins of var. *darmstadiensis* and var. *kyushuensis* are immunologically related, and in common with the var. *israelensis* 27 kDa toxin can be neutralized by, and release entrapped markers from, phospholipid containing liposomes (Gill *et al.* 1987; Drobniowski and Ellar 1988a, 1989; Yu *et al.* 1991; Knowles *et al.* 1992). The mode of action at the molecular level has been facilitated by the purification, or cloning, of individual cytolytic toxins, in place of solubilized crystal mixtures. Activated crystal toxin extracts (Ellar *et al.* 1986; Knowles and Ellar 1987; Drobniowski and Ellar 1988a), and more significantly purified toxins (Chilcott and Ellar 1988; Drobniowski and Ellar 1988a, 1989) bind to insect cell membranes forming transmembrane oligomeric pores of 1 nm radius initially, leading to an influx of electrolytes and water, and colloid-osmotic lysis. Planar lipid bilayer experiments indicate that the toxins form cation-selective channels (Knowles 1992). The CRY toxins are believed to act in the same way (although this has been fiercely debated) displaying saturable binding to specific membrane receptors, which have been identified on culture cells in *vitro* and on mid-gut epithelium in *vivo* (Knowles and Ellar 1986; Wolfsberger *et al.* 1987; Hofmann *et al.* 1988; Wolfsberger 1990; Van Rie *et al.* 1990; Garczynski *et al.* 1991). The crystal structure of a coleopteran specific toxin at 2.5 Å resolution has been described (Li *et al.* 1991) reinforcing the view that the toxins have distinct functional domains, and that conserved hydrophobic regions, and conformational changes in amphipathic helices, are important for toxin insertion and pore formation in membranes (Ellar *et al.* 1985, 1986; Aronson 1993).

Vegetative cells of *B. thuringiensis* produce several exotoxins and enzymes which may be important in the septicaemia produced in target larvae after the bacterial spores have germinated. They are usually absent from commercial preparations and include: thuringolysin, which like the
homologous toxin cereolysin, produced by *B. cereus*, is a thiol-activated cytolsin; a 'louse factor' which is toxic to lice, an α-toxin which is toxic to mice as well as lepidopteran larvae; and a heat-stable adenine nucleotide, the β-exotoxin which can substitute for ATP in many cellular reactions and has a broad activity spectrum including invertebrates and vertebrates (Holmes and Monro 1965; Krieg 1971; Forsberg 1976).

*Bacillus cereus* is a cause of significant wound and ocular infections and produces haemolysins and phospholipases that are probably important virulence determinants. The thiol-activated cytolsins of both *B. thuringiensis var. kurstaki* and *B. cereus* have been purified and shown to be biologically, physiochemically and immunologically identical (Honda et al. 1991). If environmental contamination of wounds by BT increased, thuringolysin and other toxins might be of significance in the establishment of infection. In man, listeriolysin O, the thiol-activated cytolsin of *Listeria monocytogenes*, has a role in resisting phagocytic destruction of the organism (Bielecki et al. 1990). It is interesting to speculate whether thuringolysin has a similar role in the establishment of BT septicaemia within insect larvae following disruption of the mid-gut and spore germination. It is conceivable that cereolysin and thuringolysin might play a similar role in any human infections.

4. IN VIVO TESTING

Several varieties of *B. thuringiensis* and *B. sphaericus* have been extensively tested in mammals, and also in human volunteers, and shown to be safe (Burges 1981). In the 1950s rats fed $2 \times 10^{12}$ BT spores per kg and human volunteers fed $3 \times 10^8$ spores per d for 5 d showed no ill effects (Fisher and Rosner 1959). This makes biological sense as toxins given per os, or formed by vegetative cells derived from ingested spores, would be degraded in the stomach as they are not acid stable. Only those with a specific absence or reduction of gastric acidity following gastric surgery, antacid or ulcer-healing medication could face any risk.

Rats did succumb to *B. thuringiensis var. kurstaki* and var. *israelensis* but only after $10^7$ to $10^8$ viable organisms were injected intracerebrally (Warren et al. 1984; Siegel and Shadduck 1990a,b). There was no direct evidence for infection as no replication occurred (Siegel and Shadduck 1990a,b) and the mortality could be explained by the elaboration of bacterial waste metabolites in sensitive areas of the brain. Local abscesses have been noted on subcutaneous injection of BTI but these may have been related simply to persistence of foreign antigen as autoclaved material produced the same effect. These experiments may be relevant in that *B. cereus*, which is a human pathogen, can produce cerebral and wound abscesses (Turnbull et al. 1990; Drobniewski 1993b). Morbidity and mortality were virtually absent from aerosol, oral or intraperitoneal administration of different BTI formulations. BTI spores remained viable in mammalian tissue for lengthy periods of time, however, and their isolation is likely to increase wherever these agents are used (Allen and Wilkinson 1969; Siegel and Shadduck 1990a).

When the BTI δ-endotoxin crystal is solubilized in alkaline buffer and administered intravenously or subcutaneously to mice, however, it is lethal at a dose of 5–30 μg g⁻¹ body weight (Thomas and Ellar 1983). The extract is also haemolytic, as are similar extracts of other crystals such as var. *darmstadiensis* 73-E10-2 (Thomas and Ellar 1983; Chillcott and Ellar 1988; Drobniewski and Ellar 1988a,b, 1989; Padua et al. 1990). It must be stressed that these experimental conditions mimic the alkaline milieu of insect guts rather than those of the human intestine.

Experimental evaluation of *B. sphaericus* has also shown that the organism is generally a safe one although some formulations can persist after ocular instillation and may act as irritants (Siegel and Shadduck 1990a). No acute or chronic toxicity was noted when different preparations containing up to $10^8$ viable organisms were administered to mice, rats or guinea pigs via intravenous, intraperitoneal, intracerebral, oral, percutaneous or inhaled routes (De Barjac et al. 1979, 1987; Siegel and Shadduck 1990a). Two *B. sphaericus* isolates did produce lesions when injected intracerebrally or intracutaneously, but no bacterial multiplication was observed and these lesions were probably due to the accumulation of metabolites and/or toxins at the inoculum site. Nevertheless, *B. sphaericus* is a proven human pathogen and some caution must be exercised before its wide-scale use.

5. BACILLUS THURINGIENSI S AND *B. SPHAERICUS* AS PATHOGENS

Whilst the pathogenic potential of *B. anthracis* is well known, increasingly non-anthrax members of the genus have been recognized as pathogens with isolated reports of severe infections documented to the beginning of this century and probably earlier (Lacorte 1932; Farrar 1963; Pearson 1970; Isaacson et al. 1976; Gordon 1977; Tuazon et al. 1979; Samples and Buettner 1983; Banerjee et al. 1988; Green et al. 1990; Drobniewski 1993b).

*Bacillus* species are common laboratory contaminants of blood cultures with estimates varying from 0.1 to 0.9% of submitted cultures (Pearson 1970), and are frequently present in mixed culture, as when isolated from wound specimens for example, making interpretation of their clinical significance difficult. The retrieval of *B. thuringiensis* var. *israelensis* and *Acinetobacter calcoaceticus* var. *anitratus* from an infection after an accidental inoculation injury
Table 1 Infection caused by *Bacillus thuringiensis* and *Bacillus sphaericus* 

| Infection                          | Organism       | Result       | Reference               | Notes and risk factors |
|------------------------------------|----------------|--------------|-------------------------|------------------------|
| Mastitis                           | BT             | Death        | Gordon 1977             | Bovine infection       |
| Corneal ulcer                      | BT             | Visual loss  | Samples and Buettner 1983 | See text               |
| Web-space of hand (needle-stick injury) | BTI           | Recovered    | Warren *et al.* 1984    | Co-infection with *Acinetobacter* sp.; Benzylpenicillin, erythromycin, gentamicin therapy; see text|
| Pericarditis, pleuritis, peritonitis | BS            | Death        | Lacorte 1932             | Nephrotic syndrome; no antimicrobial therapy |
| Bacteraemia, meningitis, endocarditis | BS            | Recovered    | Farrar 1963             | Rheumatic heart disease, chronic alcoholism; penicillin therapy. |
| Bacteraemia meningitis             | BS             | Dead         | Allen and Wilkinson 1969 | Cephalothin, kanamycin, antimicrobial therapy |
| Pseudotumour of lung               | BS             | Dead         | Isaacson *et al.* 1976  | Asthmatic; several prior chest infections; ampicillin, co-trimoxazole, gentamicin and steroids |
| Bacteraemia                        | BS             | Recovered    | Banerjee *et al.* 1988  | Immunosuppressed; imipenem, gentamicin and vancomycin therapy |

* All infections occurred in man apart from the case of bovine mastitis.

BT, *Bacillus thuringiensis*; BTI, *B. thuringiensis* var. *israelensis*; BS, *Bacillus sphaericus*.

Illustrates this point; proteases from the latter organism could have produced toxin activation under what might be very rare conditions (Warren *et al.* 1984). Severe systemic illness caused by the closely-related *B. cereus* is primarily associated with intravenous drug abusers, the immunosuppressed, those with underlying malignancies and neonates. Combining any of these factors potentially increases this risk. Immunosuppression is not uncommon in West Africa because of the high prevalence of chronic parasitological diseases such as malaria, and the prevalence of immunosuppressive diseases, particularly measles, in early infancy. One can speculate that this might predispose individuals to opportunistic BTI infections that would not occur in the immunocompetent.

There have been few population-based studies to evaluate post-exposure contamination and infection. One recent prospective study examined routine clinical isolates from individuals living in an area of Oregon aerially sprayed for two successive growing seasons with *B. thuringiensis* var. *kurstaki*. The population within the area was 80 000 and 55 var. *kurstaki* isolates were found from a variety of body sites. Of these, 52 were judged to be contaminants but it was argued that in three cases the organism could have been the pathogen responsible for infection (Green *et al.* 1990).

Clinical infections caused by non-anthrax species, such as *B. cereus*, *B. circulans*, *B. coagulans*, *B. subtilis* and *B. licheniformis* include local infections of burns, wounds and the eye, bacteraemia and septicaemia, meningitis, cerebral abscesses and ventricular shunt-associated infections, pulmonary infection, endocarditis and pericarditis, and toxigenic food-poisoning (Lacorte 1932; Farrar 1963; Pearson 1970; Isaacson *et al.* 1976; Gordon 1977; Tuazon *et al.* 1979; Samples and Buettner 1983; Banerjee *et al.* 1988; Green *et al.* 1990; Drobniewski 1993b). *Bacillus cereus*, for example, is a significant cause of severe ophthalmic lesions including corneal ring abscesses, keratitis, endophthalmitis, and panophthalmitis. Infections are very aggressive and
even with prompt antimicrobial therapy, enucleation of the eye and blindness are the usual sequelae (Drobniewski 1993b). One of the two clinical cases attributed to BT infection involved an isolation from a corneal ulcer but vegetative bacilli were not seen in the material initially taken from the ulcer and subsequent growth on culture medium might have been due to laboratory contamination (Samples and Buettner 1983). In experiments involving topical ocular instillation in rabbit conjunctivae of BTI and B. sphaericus, organisms continued to be recovered 1 week and 8 weeks later respectively (Siegel and Shadduck 1990a) although there was no evidence for infection. However, some commercial powders can cause minor local irritation and conjunctival discharge which should not be confused with infection (Siegel and Shadduck 1990a). Bacillus cereus is an acknowledged ocular pathogen and its similarity to B. thuringiensis should necessitate vigilance in monitoring ocular material for the presence of BTI.

Bacillus cereus also causes diarrhoeal and emetic food poisoning syndromes; BT has not produced any human gastrointestinal illness although fluid production has been demonstrated in ligated rabbit ileal loops as occurs with the enterotoxin of B. cereus (Kramer and Gilbert 1989).

Proven cases of entomopathogens causing clinical disease in mammals including man are extremely rare. Table 1 lists the principal cases of severe illness associated with B. thuringiensis and B. sphaericus infection.

6. CONCLUSIONS

Both B. thuringiensis and B. sphaericus are entomopathogens which can cause disease in man. The number of reported cases is tiny, although this is probably an underestimate due to inadequate diagnostic laboratory facilities, failure to speciate Bacillus isolates, the mixed microbiological nature of some clinical specimens, and the rejection of clinically significant isolates as contaminants. The widespread use of B. thuringiensis var. israelensis and the ubiquitous nature of Bacillus species suggest that the risk to public health remains extremely small, particularly in comparison to the benefit accrued to a community. It would nevertheless be prudent for clinical isolates, particularly from ocular lesions in which Bacillus species have a distinct pathogenic role, and which might be missed against the background of for example, onchocerciasis-generated blindness, to be carefully evaluated for the presence of var. israelensis wherever this agent has been widely used. At present there is no good evidence to discontinue the use of B. thuringiensis in the developed and developing world on grounds of risk to human health. The introduction of new varieties and toxin mixtures, such as those derived from recombinant techniques, should not be assumed safe on the basis of previous work and should be carefully evaluated. The pathogenic role of B. sphaericus may be a greater hinderance to its more widespread use at the present time. Prospective or retrospective studies would be needed to confirm the safety of any new agent, or combination of agents, so that an adequate assessment of the risk-to-benefit ratio can be obtained. New formulations also contain emulsifiers and dispersal agents which should be assessed for human toxicity as well.

The use of any novel entomopathogenic Bacillus toxin needs to weigh the likely environmental and commercial benefit against any potential health risk from the toxin, and where appropriate, to consider the considerable indirect medical advantage accrued through elimination of disease carrying vector organisms.

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