Renewal
Assessment Report

Bacillus thuringiensis ssp. aizawai strain ABTS-1857

Volume 3 – B.2 Biological properties

Rapporteur Member State: The Netherlands

Co-Rapporteur Member State: Germany

Version history

When	What
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B Summary of the data and information

B.2 Biological properties of the micro-organism

Besides new information, this RAR includes the relevant data, which have been presented in the DAR (May 2007) and DAR addendum (April 2012). This information is marked grey.

B.2.1 History of the micro-organism and its uses. Natural occurrence and geographical distribution

B.2.1.1 Historical background

Bacillus thuringiensis is naturally occurring in the environment and has been isolated from a range of habitats including soil, phylloplane, dust, plant material and insects throughout the world.

Information from the DAR:
Bacillus thuringiensis is an extensively studied commercialised bacterium, forming the basis of over 90% of commercially available biopesticides. There are a number of commercial strains available, including the Valent Biosciences *Bacillus thuringiensis* subsp. *aizawai* strain ABTS-1857.
Thirty colonies of the ABTS-1857 strain were selected, pooled as a suspension, and lyophilised for long-term preservation in May 1990. All inoculum used in the production of ABG-6305 has been obtained from a pooled suspension of pure single colony isolates. Any subcultures from the original stock culture will be derived by mass transfer.

B.2.1.2 Origin and natural occurrence

Bacillus thuringiensis subsp. *aizawai* (Bta) is naturally occurring in soil. *Bacillus thuringiensis* subsp. *aizawai*, strain ABTS-1857 originates from a natural, indigenous wild type, isolated from soil taken from a lawn in Ephraim, Wisconsin (USA) in 1987 (Anon., 1995; Smith, 1990)

Information from the DAR:
Isolation of *Bacillus thuringiensis* strains from soil was accomplished using procedures designed to favour the germination of *Bacillus thuringiensis* -type bacilli over other micro-organisms which include six to ten species of spore-forming bacilli as well as non-sporulating bacteria, yeast and fungi. A short heat treatment (80^{o}C for 10 minutes) was utilized to activate spores, after which the samples were rapidly subcultured on NSYM agar medium.
Colonies of *Bacillus thuringiensis* -type bacilli were selected on the basis of colony morphology and growth characteristics typical of that species. Also, microscopic examination was used to identify the endotoxin protein crystal which differentiates *Bacillus thuringiensis* from *Bacillus cereus*, a common soil bacterium which is otherwise morphologically and biochemically indistinguishable.
In general, the XenTari strain was one of many strains, isolated according to the above method and placed into a collection. Screening of the collection was done using gene probes, which allowed isolates to be classified into groups having certain genes. At that time very few Bt gene sequences had been published. Two new, so far unpublished genes were identified in a Bta stain (a different isolate than XenTari). These were the 1C and 1D genes, which sequences were also used in screening. These genes were published a very short time later. Gene probes were used to select all the isolates from the collection that had 1C and 1D (i.e, Bta types). All these isolates were grown and bioassayed against *S. exigua*. Ultimately ABTS 5686 was chosen, based on many levels of evaluation. ABTS 5686 was

subsequently verified to be a clean/stable strain by checking several individual isolates for gene content and morphology. This "pooled isolate" culture was designated as ABTS 1857.

B.2.2 Information on target organism(s)

B.2.2.1 Description of target organism(s)

B. thuringiensis subsp. *aizawai* ABTS-1857 is applied to control Lepidopteran pests including foliar feeding caterpillar larva, *Chrysodeixis chalcites* PLUSCH (Northern EU), *Heliothis plusia* HELISP or *Spodoptera* 1SPODG (Southern EU), 1-4 instars (reference).

The target organism is Lepidopteran larvae including but not limited to: 1NOCTF, GNORAB, HELIAR, LAPHEG, PLUSCH, PYRUNU, SPODLI, SPODSP, HELISP(Reference?).

B.2.2.2 Mode of action

During the stationary phase of its growth cycle, *B. thuringiensis* forms parasporal crystalline inclusions. The crystal proteins of *B. thuringiensis* must be ingested to be effective against the target insect (in Schnepf et al., 1998). Upon ingestion of *B. thuringiensis* by the larvae, the crystalline inclusions dissolve in the larval midgut, releasing insecticidal crystal proteins (so called δ-endotoxins) of 27 to 140 kDa. Most of the crystal proteins are protoxins, converted proteolytically into smaller toxic polypeptides (Cry IAa, Cry IAb, Cry IC and Cry ID) under the alkaline conditions in the insect midgut. The activated Cry toxins interact with the midgut epithelium cells of susceptible insects (reviewed by Höfte & Whiteley, 1989, submitted in Point IIM 2.3; Schnepf et al, 1998). For several *B. thuringiensis* toxins, specific high-affinity binding sites on the apical brush border of the midgut of susceptible insects have been demonstrated to exist (Hofmann et al., 1988a; Hofmann et al., 1988b). After binding to the midgut receptors, they insert into the apical membrane to create ion channels, or pores, disturbing the osmotic balance and permeability. The regulation of the trans-membrane electric potential is disturbed. This can result in colloid-osmotic lysis of the cells, which is the main cytolytic mechanism that is common to all insecticidal crystal proteins (ICPs) (Schwartz et al., 1991; Schnepf et al., 1998). Spore germination and proliferation of the vegetative cell into the haemocoel may result in septicaemia, contributing to mortality of the insect larvae.

Additional information from the DAR:
Affected insects stop feeding and die from the combined effects of starvation and tissue damage (Copping, 1998). Different toxins bind to different receptors in different insect species with varying intensities. This explains species specificities as observed with two strains (XenTari and Florbac) showing bioactivity towards different target organisms (Nair *et al*, 1996).
Host range and gene contents of *Bacillus thuringiensis* strains are further explored by Porcar *et al* (2000) and Martinez & Caballero (2002).

B.2.3 Host specificity range and effects on species other than the target harmful organism

The strain ABTS-351 is highly specific against insect species of the order Lepidoptera.

Information from the DAR:
Larval Lesser Mealworm (*Alphitobius diaperinus* Coleoptera:Tenebriondae) were exposed to 0.0, 0.2, 0.5 and 5.0% w/w ABG-6305 in corn meal incubated at 25-28°C for 7 days. Results showed that none of the doses had any effect on the beetle larvae. (Jaronski, 1990a)
4-5 week old nymphal house crickets (*Acheta domestica*) were exposed to 0.0, 0.005, 0.05 and 0.25% w/w ABG-6305 in wheat bran incubated at 25°C for 7 days. Results showed that none of the doses had any effect on the young crickets (Jaronski, 1990b)

A literature search and review for the active substance was conducted and the report is submitted in the dossier. The following paper(s) relate to relevant information from this search and review in the current section:

Report KMA 2.3/01 Mohan M. *et al*. (2014). Relative toxicity of subspecies of *Bacillus thuringiensis* against lepidopterous insect pests of agricultural importance. Journal of Biological Control, Vol. 28, No. 4, pp. 197-203.
The relative toxicity of various subspecies of *Bacillus thuringiensis (Bt)* against important lepidopterous insect pests was determined. *Bt kurstaki* HD-I was toxic against all the five insect pests tested; however, with low toxicity against *Spodoptera litura.* Larvae of *Helicoverpa armigera* and *Pieris brassicae* were fivefold more susceptible to *Bt kurstaki* HD-73 that produces only Cry1Ac toxin. However, *Bt kurstaki* HD-73 was non-toxic against the larvae of S. *litura* and *Spilarctia obliqua. Bt aizawai* HD-137 was equally active against *S. litura* and *Sesamia inferens* but non-toxic against *R. brassicae* and *S. obliqua.* On the other hand *Bt tolworthi* strain was highly toxic against *R. brassicae* and *S. obliqua. Bt japonensis* T23 00 l was non-toxic to all the insect pests tested. Larval growth of *H. armigera* was severely inhibited at extremely low concentration of *Bt kurstaki* HD-73 (EC_{50} 0.07 ng/ml) and *Bt kurstaki* HD-I (EC_{50} 0.16 ng/ml). The toxicity of indigenous *Bt* strains belonged to subspecies *tolworthi* and *galleriae* were also promising against *H. armigera.*

RMS remark:
It is unclear whether this paper is relevant; the only Bta tested is referenced as HD-137. From the paper it can be concluded that host specificity is highly strain (hence endotoxin) specific.

B.2.4 Development stages/life cycle of the micro-organism

Bacillus cultures are found in nature in one of two states. They are found either as vegetative cells that are actively growing and dividing or as spores.

The spores are a resistant metabolically inactive resting form with a completely different fine structure, chemical composition and enzymatic constitution. The transformation of dormant spores into active vegetative cells occurs in three stages: (1.) Activation, (2.) Germination, and (3.) Outgrowth.

Additional information in DAR:
Bacillus cultures follow a characteristic pattern in the process of spore formation or sporulation. Spore formation normally commences when growth ceases due to a lack of nutrients or a shift in the environment. It is a complex process that may be divided into seven stages.
Stage:

1. An axial filament of nuclear material forms.
2. An inward folding of the cell membrane occurs to enclose part of the DNA to produce the forespore septum in an unequal cell division.
3. The membrane continues to grow and engulfs the immature spore in a second membrane.
4. Cortex is laid down in the space between the two membranes and both calcium and dipicolinic acid are accumulated.
5. A protein coat is then formed around the cortex
6. Maturation of the spore occurs with the completion of the coat synthesis and an increase in refractility and heat resistance.

7. Lytic enzymes destroy the sporangium releasing the spore.

Bacillus spores are resistant to desiccation, heat, ultraviolet irradiation and other environmental factors such as chemical disinfectants.

The transformation of dormant spores into active vegetative cells occurs in three stages: (1.) Activation, (2.) Germination, and (3.) Outgrowth.
Stage:

1. Activation is a reversible process that prepares the spore for germination and usually results from treatments like heating or exposure to certain chemical stimuli.
2. Germination involves the breaking of the spore state and involves spore swelling, the rupture or absorption of the spore coat, loss of resistance to heat and other stresses, loss of refractility, release of spore components and an increase in metabolic activity.
3. Outgrowth involves the protoplast making new components remerging form the spore coat and developing again into an active vegetative bacterium.

Generation time in model solution (i.e. microbiological media and controlled conditions) and generation time in nature (soil) are not known, and specific questions have been addressed to the Applicant.

B.2.5 Infectiveness, dispersal and colonisation ability

Bacillus thuringiensis is a ubiquitous micro-organism that colonizes a range of habitats and environments. Vegetative cells and crystal proteins of *Bacillus thuringiensis* are rapidly degraded by the actions of indigenous micro-organisms and also by the photodegradation effects of sunlight.

Bacillus thuringiensis is a poor infectious agent and rarely recycles.

Please refer to the human toxicology part (B.6), where *Bacillus thuringiensis* and food poisoning is discussed in detail. *B. cereus* and *B. thuringiensis* strains are very similar and, consequently have not been distinguished in cases of food poisoning using routine methodology. The EFSA Panel on Biological Hazards (BIOHAZ)[1] has recently concluded that the levels of *B. cereus* considered to be a consumer risk for are $>10^5$ organisms/g food (although this will be strain-specific). EFSA further recommend the use of genome sequencing to discriminate *B. cereus* from *B. thuringiensis* in food poisoning cases (EFSA, 2016). The document notes that there is no definitive evidence for the role of these toxins (alone or in combination) in the diarrhoeal syndrome.

Please also refer to the residue part (B.7). In the EFSA BIOHAZ document (EFSA, 2016) an extensive literature search was conducted by EFSA in order to obtain information on the presence and levels of *B. thuringiensis* in food. In general, the information concerning the detection of *B. thuringiensis* in foods was scarce, with a few studies covering this. In most studies, *B. cereus* group organisms were isolated from fruits and vegetables, and differentiation between *B. cereus* and *B. thuringiensis* strains was performed using conventional culture methods and molecular techniques.

BTa ABTS-1857 is an insect pathogen and does not have the propensity for growth under environmental conditions that would apply to strains of the *B. cereus* group (see B.7.1.1, B.2.5 and B.6.1). The EFSA BIOHAZ document indicates the occurrence of *Bacillus* species in raw materials used for food processing or in prepared foods such as soups, sauces, puddings, milk, meat and vegetables, is generally below 10^5 cells/g or mL food. It is stated in B.6.1 that there is strong evidence to show that *B. thuringiensis* does not grow and multiply in or on food. Residues trials (see B.7.2.2) demonstrate spore counts of BTa ABTS-1857 do not exceed this level when crops are treated according to the proposed GAP.

[1]EFSA Panel on Biological Hazards (BIOHAZ). Risks for public health related to the presence of *Bacillus cereus* and other *Bacillus* spp. including *Bacillus thuringiensis* in foodstuffs. EFSA Journal 2016;14(7):4524-4516.

A literature search and review for the active substance was conducted which is submitted in the dossier. The following paper(s) relate to relevant information from this search and review in the current section;

Report KMA 2.5/01 Ruan, L., Crickmore, N., & Sun, M. (2015). Is there sufficient evidence to consider *Bacillus thuringiensis* a multihost pathogen? Trends in Microbiology, 23(10): 587.
GLP No
Summary
Article is a short letter stating "Our main concern is the belief that Bt can 'proliferate' and 'thrive' outside of an insect or nematode host. We are just not convinced that there is currently sufficient evidence that this bacterium can do much more than simply survive outside of its primary host."

Information in DAR:
A detailed review of papers and reviews reported in the scientific literature, which address the environmental fate and behaviour of Bacillus thuringiensis, including Bacillus thuringiensis subsp. aizawai, Strain ABTS-1857 is provided in the DAR as summarised below.

Bacillus thuringiensis occurs naturally and ubiquitously in the environment. It is a common component of the soil micro-flora and has been isolated from most terrestrial habitats. In the natural environment, under favourable conditions, *Bacillus thuringiensis* cells exist in an active vegetative state where growth and colony formation can occur. When conditions for continued growth and survival become unsuitable, sporulation occurs, during which endospores and crystalline inclusions, or proteins are formed and the vegetative cells lyse. The endospores exist in a cryptobiotic state and can be quite durable. The crystalline proteins are the source of delta-endotoxins, which are damaging to highly specific insect species. When insects ingest crystal proteins, alkaline conditions in the gut initiate breakdown of the proteins, releasing the delta-endotoxins. These immediately begin to interfere with internal cell gut structure, leading to a cessation of feeding and eventual starvation. Unlike most insect pathogenic microbes, *Bacillus thuringiensis* is a poor infectious agent and rarely recycles. While vegetative cells and spores can be produced in insect cadavers, *Bacillus thuringiensis* has rarely been recorded causing natural epizootics in insects, leading to speculation that it is essentially a soil micro-organism that possesses incidental insecticidal activity.
Following commercial field applications of formulations containing *Bacillus thuringiensis* endospores and parasporal crystals, some viable spores are expected to survive. These can remain inactive and immobile in soil for several months or even years, during which time a natural breakdown occurs, resulting in gradual spore mortality. However, the vegetative cells and crystal proteins of *Bacillus thuringiensis* are far more rapidly degraded in soil. The actions of indigenous micro-organisms, which compete for nutrients and produce proteolytic enzymes that degrade the protoxin, lead to a rapid loss of potency and insecticidal activity in soil. The photodegradation effects of sunlight also affect the survival and growth of endospores and vegetative cells in the environment. *Bacillus thuringiensis* endospores rarely germinate in soil unless favourable conditions exist in combination such as neutral to alkaline pH, sufficient nutrient availability, favourable soil temperature and moisture content and lack of competition / predation from other soil micro-organisms. *Bacillus thuringiensis* is not therefore adapted to survive as an active member of the soil microbial community and the low potential for spore germination, growth and re-sporulation restricts population growth.
In many of the studies to investigate the behaviour of *Bacillus thuringiensis* in soil, which are reported in the scientific literature, spores and parasporal crystals were initially added to soil at concentrations far greater than is recommended for insect control in the field, in order to measure degradation over time. It is also expected that field applications of *Bacillus thuringiensis* subsp. *aizawai* to foliage will reduce potential soil exposure, resulting in lower soil concentrations, which are well below insecticidal levels. Degradation in soil is also likely to exceed the rate of acquisition from repeated foliar sprays, such that populations of applied *Bacillus thuringiensis* bacteria reaching the soil should decline over time to the fluctuating natural level.

Bacillus thuringiensis may survive to a limited extent in water. However, its survival and viability in the natural aquatic environment are influenced by the complex interaction of a number of biological, chemical and physical factors. Predation by protozoans and lower animal forms undoubtedly plays a significant role in controlling the population of *Bacillus thuringiensis* in the aquatic environment. The effects of solar radiation may destroy *Bacillus thuringiensis* endospores, crystal proteins and vegetative cells in the upper layers of an aquatic system and extremes of water temperature may have a detrimental effect on survival and insecticidal activity. The adsorption of bacterial cells to the sediment layer in the natural aquatic environment is also expected to occur. Spores are unlikely to be capable of germinating and multiplying in sediment and the crystalline proteins become inaccessible to insect larvae.
The *Bacillus thuringiensis* subsp. *aizawai*, XenTari WG formulation is not intended for direct application to water. Although there may be some potential for surface water exposure resulting from spray drift from field applications, spray drift from application to developed foliage is unlikely to be significant. Concentrations of *Bacillus thuringiensis*, which are deposited in surface water bodies, are therefore expected to be extremely diluted and well below insecticidal effect levels.

Potential atmospheric exposure of *Bacillus thuringiensis* may occur following commercial field applications. However, rapid atmospheric degradation is expected since inactivation by solar radiation is a key factor causing the degradation and loss of activity of *Bacillus thuringiensis* bacteria spores and crystal proteins. Atmospheric concentrations of *Bacillus thuringiensis* may be transported by spray drift and air currents before the spores and crystals on finer spray droplets settle out. However, as the spatial and temporal distribution of *Bacillus thuringiensis* in the environment is limited by its poor survival and limited ability to sustain infections in populations, atmospheric dispersal is likely to result in reduced inoculum density and subsequent toxicity levels.

B.2.6 Relationships to known plant or animal or human pathogens

The Bacillus cereus group, also known as *B. cereus sensu lato*, is a subdivision of the Bacillus genus and consists of several species, including *B. cereus sensu stricto* and *B. thuringiensis*. The current taxonomy of the B. cereus group and the status of separate species mainly rely on phenotypic characteristics. *Bacillus cereus* and *B. thuringiensis* strains are usually not discriminated in clinical diagnostics or food microbiology. Thus, the actual contribution of the two species to gastrointestinal and non-gastrointestinal diseases is currently unknown.

Some strains of *Bacillus thuringiensis* have been found to produce β-exotoxins and *Bacillus cereus* type enterotoxins. Mammalian safety tests against a range of small mammals have demonstrated a very low safety risk from direct exposure to Bt spores and δ endotoxins (see B.6. and B.9).

Please refer to the human toxicology part (B.6), where *Bacillus thuringiensis* and food poisoning is discussed in detail. *B. cereus* and *B. thuringiensis* strains are very similar and, consequently have not been distinguished in cases of food poisoning using routine methodology. The EFSA Panel on Biological Hazards (BIOHAZ) has recently concluded that the levels of *B. cereus* considered to be a consumer risk for are $>10^5$ organisms/g food (although this will be strain-specific). EFSA further recommend the use of genome sequencing to discriminate *B. cereus* from *B. thuringiensis* in food poisoning cases (EFSA, 2016). The document remarks that there is no definitive evidence for the role of these toxins (alone or in combination) in the diarrhoeal syndrome.
In the public literature RMS found a recent paper titled "In defence of *Bacillus thuringiensis*, the safest and most successful microbial insecticide available to humanity—a response to EFSA (2016)" written by Ben Raymond and Brian Federici (FEMS Microbiology Ecology, 93, 2017, fix084). RMS agrees with the conclusion of the paper that the recent controversial case of food poisoning in Germany presents no convincing evidence that Bt was the causative agent, since individuals with food poisoning had also consumed a dose of Bc sufficient to cause the observed level of infection. Overall, the MLST databases, the epidemiological studies and safety testing literature present a well-informed and coherent view of the biology and ecology of the Bc group. The arguments in the EFSA report, that we

do not understand the risks of consuming Bt spores, are therefore unfounded and overly cautious according to Raymond B and Federici B.A. 2017.An analysis of studies cited in EFSA's opinion used to question Bt safety (Rosenquist et al. 2005, Frederiksen et al. 2006) show not only do humans routinely eat high levels of this species, but that most of the strains (>80%) consumed are naturally occurring, not from biopesticides. Yet even at rates (>10^4 CFU/g), not considered acceptable under Danish guidelines, there is no evidence that consumption has ever resulted in food poisoning. Furthermore, strains of entomocidal Bt are not capable of infecting vertebrates at extremely high doses in controlled laboratory tests and there are no robust data to suggest that humans might be an exception. Phylogenetic analyses of ecological differentiation across the Bc group suggest that there are very few strains of Bt with elevated risks for vertebrates (Guinebreti `ere et al. 2010; Raymond et al. 2010b; Raymond and Bonsall 2013). This would include the subsp. konkukian, which was originally isolated from a soldier severely injured by a land mine (Hernan-dez, Ramisse and Ducoureau 1998). That isolate did indeed pose a greater risk to mice than biopesticidal strains of Bt (Hernandez et al. 2000
Here below, the abstract of the study written by Ben Raymond and Brian Federici (FEMS Microbiology Ecology, 93, 2017, fix084) is presented:
The Bacillus cereus group contains vertebrate pathogens such as B. anthracis and B. cereus and the invertebrate pathogen *B.thuringiensis* (Bt). Microbial biopesticides based on Bt are widely recognised as being among the safest and least environmentally damaging insecticidal products available. Nevertheless, a recent food-poisoning incident prompted a European Food Safety Authority review which argued that Bt poses a health risk equivalent to B. cereus, a causative agent of diarrhoea. However, a critical examination of available data, and this latest incident, provides no solid evidence that Bt causes diarrhoea. Although relatively high levels of B. cereus-like spores can occur in foods, genotyping demonstrates that these are predominantly naturally occurring strains rather than biopesticides. Moreover, MLST genotyping of >2000 isolates show that biopesticide genotypes have never been isolated from any clinical infection. MLST data demonstrate that B. cereus group is heterogeneous and formed of distinct clades with substantial differences in biology, ecology and host association. The group posing the greatest risk (the anthracis clade) is distantly related to the clade containing all biopesticides. These recent data support the long-held view that Bt and especially the strains used in Bt biopesticides are very safe for humans.

According to SANCO/10754/2005 rev.5, 2005, an assessment for a certain strain can be used for another strain only when there is sufficient evidence that the strains do not differ with regard to properties of potential relevance for human health. This is obviously not the case for commercial Bt and pathogenic *B. cereus* strains, as they do significantly differ in their toxigenic potential, but also in their physiology and their environmental behaviour. The low toxigenic potential of Bta ABTS 1857 together with the proven absence of pathogenicity of the strain indicates that the risk for consumers following use of Bta ABTS 1857 for pest control in agricultural settings is acceptable.

Information in DAR:
A detailed review of papers and reviews reported in the scientific literature, which address the relationship of the micro-organism *Bacillus thuringiensis*, including *Bacillus thuringiensis* subsp. *aizawai* to closely related species and potential for pathogenicity is provided (Anon., 2005; Confidential Information).

B.2.7 Genetic stability and factors affecting it

Genetic Stability of Bta strain ABTS-1857 in manufacturing

Strains of *Bacillus thuringiensis* are capable of plasmid and gene transfer. However, during manufacture, due to the rigorous sterilization of fermentation equipment between every production run, the control of source inoculum, and the constant monitoring of runs to ensure a pure culture during fermentation, any genetic transfers would likely be to other Bt cells of the same strain and therefore not expected to affect the final product.

During the production process the Bta ABTS-1857 strain is proved to be stable by regular quality control checks. The spontaneous loss of plasmids, carrying the information for the insecticidal active crystal proteins, would be detected in bioassays. The transfer of genetic material in the fermentation broth is very unlikely due to the absence of microbial impurities and the continuous stirring movements of the fermentation broth. The quality control of the production batches confirms the stability of the *B. thuringiensis aizawai* strain ABTS-1857.

The manufacturers of *Bacillus thuringiensis* (Bt) biopesticides have been using, in some case for decades, the same *B. thuringiensis* strains for the production of microbial plant protection products. During the production fermentation steps companies usually use cultures, which are at most one to two passages away from the original lyophilised stock culture. Each passage from the original stock culture for preparing working culture line is subjected to a battery of tests to ensure consistency of the starting material for fermentation.

The genetic stability of the relevant commercial *B. thuringiensis* starter cultures is ensured and verified by special quality control procedures which include a wide range of methods and procedures such as:

- Morphological , biochemical and activity characteristics
- Serotyping
- Characterisation of crystal proteins (insecticidal toxins)
- *Cry* toxin gene(s) identification by PCR
- Molecular typing methods (e.g. Amplified Fragment Length Polymorphism, AFLP)
- Plasmid profiling
- Gene sequencing of toxin related genes and genome sequencing
- Other molecular methods (e.g. qPCR)
- Assessment of Beta exotoxin
- Assessment of lack of mouse toxicity

Valent BioSciences Corp. maintains a cell bank of *Bacillus thuringiensis* subspecies *aizawai*, strain ABTS-1857. Cell Bank (CB) is defined as a permanent collection of cells derived from a single colony. For *Bt*a strain ABTS-1857, the CB is a lyophilized culture. *Bt*a strain ABTS-1857 cells can be stored indefinitely in this state.

For use in large-scale fermentation, a "master" stock culture batch is prepared from the CB. Under aseptic conditions, the CB is re-suspended in liquid medium. The flask is incubated to obtain actively growing cells. The cell suspension is then transferred into sterile vials containing a cryoprotectant and stored at approximately -80°C. Purity tests are performed on both the residual re-suspension of the CB and the cell suspension of the master stock culture

It is unlikely that unintentional ingredients will occur in fermentation of *Bt* as a result of spontaneous mutation or alternate forms of the MPCA. Spontaneous mutations in most cultures occur at very low rates and should be at too low of a concentration to pose any significant risk in the final product. Similarly, stability of the genes and plasmids can be associated with active crystal production in our *Bt* strains, as measured on preparation of new master stock culture batches, and measured by the consistent batch to batch quality of the active crystal protein by HPLC and/or insect bioassay monitoring. These data suggest insignificant risk associated with alternate forms of the MPCA.

As manufacturing always goes back to the lyophilized culture, any genetic changes do not carry forward. The strain is as identified and characterized through regulatory studies.

Genetic Stability of Bta strain ABTS-1857 in the field

Possibility of genetic transfer out in the field will be dependent on spore germination. Transfer of genetic material cannot occur unless there is germination, which is highly unlikely for Bta strain ABTS-1857 in soils.

Bta strain ABTS-1857 is most likely to germinate in lepidopteran larva. The kinetics of plasmid transfer was studied in the laboratory by Yuan *et al*. (2007; KMA 2.7/04), who did indicate that spore germination was only observed in killed larvae, at 44-56 hours post infection, and that conjugation transfer only occurred among vegetatively growing bacteria. The conjugational transfer ratios varied among different strain combinations used by and different lepidopteran larvae used Yuan et al. The highest transfer ratio reached 5.83×10^{-6} CFU/donor, given a diet of 10^9 spores/g of diet, much higher dose than would be experienced by insects in the field.

Thomas *et al*. (2000; KMA 2.7/03) monitored the plasmid transfer between *B. thuringiensis* subsp. *kurstaki* HD1 and *B. thuringiensis* subsp. *tenebrionis* under laboratory conditions *in vitro* in laboratory broth , in soil, and in insects. Plasmid transfer was observed in laboratory broth and in insects, but not in soil. For *B. thuringiensis* subsp. *kurstaki* HD1, which was used as donor strain, plasmid transfer was detected in dead susceptible lepidopteran insect (*Lacanobia oleracea*) larvae but not in the nonsusceptible coleopteran insect (*Phaedon chocleriae).*

Santos *et al*. (2010 ; KMA 2.7/02) looked at Btk and Bc conjugation in insect larvae. They found that *B. thuringiensis* strains germinated and multiplied more efficiently than *B. cereus* strains in insect larvae and only *B. thuringiensis* maintained complete spore germination for at least 24 h in B. mori larvae. Mating was least efficient in nutrient broth, indicative of little spore germination and transconjugants were detected for only four of the recipient strains, with frequencies between 10^-4 and 10^-6 transconjugants per recipient.

Hu *et al*.(2004; KMA 2.7/01) showed that cry1Ac of a Btk strain can be transferred to some *B. cereus* strains by natural conjugation and express insecticidal toxin. The highest stability of this *kurstaki* plasmid was found in the two *kurstaki* isolates. In those strains where conjugation could occur transfer frequencies to different recipients occurred in the range of 10^{-7} to 10^{-4} transconjugants per recipient.

Millennia of natural selection have led to establishing the environment in which *B. thuringiensis* cells find the best conditions for multiplication being in dead insect larvae, which might be considered as the ecological niche of these bacteria. Because *B. thuringiensis* is more adapted to develop the complete life cycle in a susceptible insect host than in other environments, we would not expect genetic transformation of *Bts* to occur on human food stuffs.

Regarding the concern posed by EFSA peer-review (EFSA Journal 2013;11(1):3063) about “potential transfer of genetic material from *Bacillus thuringiensis aizawai* strains to other organisms", it is necessary to clarify that:
A successful horizontal transfer would require stable insertion of chromosomal genes sequences into another bacterial genome and a selective advantage to be conferred on the transformed recipient cell. The only mechanism known to facilitate horizontal transfer of non-mobile, chromosomal DNA fragments among bacterial genomes is homologous recombination. This requires the presence of stretches of DNA sequences that are similar in the donor's DNA molecules that, in addition to substitutive gene replacement, would facilitate the insertion of non-homologous DNA sequences if their flanking regions share sequence similarity with the bacterial sequences in the recipient. Since the process requires actively growing, i.e. dividing cells, and cannot occur among dormant spores (which are the most frequent physiological status of *Bt* in soil), the likelihood of this kind recombination can be expected to be negligible. In addition to homology-based recombination processes, non-homologous (illegitimate) recombination that does not require similarity between the recombining DNA molecules is theoretically possible. Non-homologous recombination has rarely been described in bacteria. However, transformation rates for illegitimate recombination are considered to be 10^{10}-fold lower than for homologous recombination. Since actively dividing cells are required, this process, compared with

homologous recombination, is considered not to contribute significantly to horizontal gene transfer events for Bt spores. In comparison with the above-described homology-facilitated recombination processes, the contribution of illegitimate recombination would be extremely low.

Report KMA 2.7/01 Hu X, Hansen BM, Eilenberg J, Hendriksen NB, Smidt L, Yuan Z, Jensen GB. Conjugative transfer, stability and expression of a plasmid encoding a *cry*1Ac gene in *Bacillus cereus* group strains. FEMS Microbiology Letters 231 (2004) 45-52.

GLP

No

Summary

The plasmid pHT73 containing cry1Ac and tagged with an erythromycin resistance gene was transferred from *Bacillus thuringiensis* subspecies *kurstaki* KT0 to several *Bacillus cereus* group strains by conjugation. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and phase contrast microscopy showed that the transconjugants containing plasmid pHT73 could express Cry1Ac toxin and produce bipyramidal crystalline inclusion bodies during sporulation. The study demonstrated that pHT73 could be transferred to *B. thuringiensis* subsp. *kurstaki*, several *B. cereus* strains and *Bacillus mycoides*. Under non-selective conditions, the stability of the pHT73 plasmid in the transconjugants was found to be 58.2-100% after 100 generations and 4-96% after 200 generations. The variations are mainly caused by the choice of receptor strain.

Report KMA 2.7/02 Santos CA, Vilas-Bôas GT, Lereclus D, Suzuki MT, Angelo EA, Arantes O.M. (2010). Conjugal transfer between *Bacillus thuringiensis* and *Bacillus cereus* strains is not directly correlated with growth of recipient strains. Journal of Invertebrate Pathology, Vol. 105, No. 2, pp. 171-5.

GLP No

Summary

Bacillus thuringiensis and *Bacillus cereus* belong to the *B. cereus* species group. The two species share substantial chromosomal similarity and differ mostly in their plasmid content. The phylogenetic relationship between these species remains a matter of debate. There is genetic exchange both within and between these species, and current evidence indicates that insects are a particularly suitable environment for the growth of and genetic exchange between these species. We investigated the conjugation efficiency of *B. thuringiensis* var. *kurstaki* KT0 (pHT73-EmR) as a donor and a *B. thuringiensis* and several *B. cereus* strains as recipients; we used one-recipient and two-recipient conjugal transfer systems in vitro (broth and filter) and in *Bombyx mori* larvae, and assessed multiplication following conjugation between *Bacillus* strains. The *B. thuringiensis* KT0 strain did not show preference for genetic exchange with the *B. thuringiensis* recipient strain over that with the *B. cereus* recipient strains. However, *B. thuringiensis* strains germinated and multiplied more efficiently than *B. cereus* strains in insect larvae and only *B. thuringiensis* maintained complete spore germination for at least 24 h in *B. mori* larvae. These findings show that there is no positive association between bacterial multiplication efficiency and conjugation ability in infected insects for the used strains.

Report KMA 2.7/03 Thomas DJI, Morgan JAW, Whipps JM, Saunders JR (2000) Plasmid transfer between the *Bacillus thuringiensis* subspecies *kurstaki* and *tenebrionis* in laboratory culture and soil and in *Lepidopteran* and *Coleopteran larvae*. Appl Environ Microbiol 66:118–124

GLP No

Summary

Plasmid transfer between *Bacillus thuringiensis* subsp. *kurstaki* HD1 and *B. thuringiensis* subsp. *tenebrionis* donor strains and a streptomycin-resistant *B. thuringiensis* subsp. *kurstaki* recipient was studied under environmentally relevant laboratory conditions in vitro in laboratory broth, in soil, and in insects. Plasmid transfer was detected *in vitro* in laboratory broth at temperatures of 5 to 37°C, at pH 5.9 to 9.0, and at water activities of 0.965 to 0.995, and the highest transfer ratios (up to 10^{-1} transconjugant/donor) were detected within 4 h. In contrast, no plasmid transfer was detected in nonsterile

soil, and rapid formation of spores by the introduced strains probably contributed most to the lack of plasmid transfer observed. When a *B. thuringiensis* subsp. *kurstaki* strain was used as the donor strain, plasmid transfer was detected in killed susceptible lepidopteran insect (*Lacanobia oleracea*) larvae but not in the non-susceptible coleopteran insect *Phaedon chocleriae*. When a *B. thuringiensis* subsp. *tenerbrionis* strain was used as the donor strain, no plasmid transfer was detected in either of these insects even when they were killed. These results show that in larger susceptible lepidopteran insects there is a greater opportunity for growth of *B. thuringiensis* strains, and this finding, combined with decreased competition due to a low initial background bacterial population, can provide suitable conditions for efficient plasmid transfer in the environment.

Report KMA 2.7/04 Yuan YM, Hu XM, Liu HZ, Hansen BM, Yan JP and Yuan ZM (2007). Kinetics of plasmid transfer among *Bacillus cereus* group strains within lepidopteran larvae. Archives of Microbiology, Vol. 187, No. 6, pp. 425-431.
GLP No
Summary
The cry toxin encoding plasmid pHT73 was transferred from *Bacillus thuringiensis* subspecies *kurstaki* KT0 to six *B. cereus* group strains in three lepidopteran (*Spodoptera exigua*, *Plutella xyllostella* and *Helicoverpa armigera*) larvae by conjugation. The conjugation kinetics of the plasmid was precisely studied during the larval infection using a new protocol. The infections were performed with both vegetative and sporulated strains. However, larval death only occurred when infections were made with spore and toxin preparations. Likewise, spore germinations of both donor and recipient strains were only observed in killed larvae, 44–56 h post-infection. Accordingly, kinetics showed that gene transfer between *B. thuringiensis* strain KT0 and other *B. cereus* strains only took place in dead larvae among vegetatively growing bacteria. The conjugational transfer ratios varied among different strain combinations and different larvae. The highest transfer ratio reached 5.83×10^{-6} CFU/donor between the KT0 and the AW05R recipient in *Helicoverpa armigera*, and all transconjugants gained the ability to produce the insecticidal crystal. These results indicated that horizontal gene transfer among *B. cereus* group strains might play a key role for the acquisition of extra plasmids and evolution of these strains in toxin susceptible insect larvae.

Report KMA 2.7/05 Ferreira, L., Leme HP; Suzuki MT; Itano EN; Ono MO; Arantes OMN (2003). Ecological aspects of *Bacillus thuringiensis* in an oxisol. Scientia Agricola, 60(1): 19-22.
GLP No
Summary
Bacillus thuringiensis is a Gram positive, sporangial bacterium, known for its insecticidal capabilities. Survival and conjugation ability of *B. thuringiensis* strains were investigated; vegetative cells were evaluated in non-sterile soil. Vegetative cells decreased rapidly in number, and after 48 hours the population was predominantly spores. No plasmid transfer was observed in non-sterile soil, probably because the cells died and the remaining cells sporulated quickly. Soil is not a favourable environment for *B. thuringiensis* multiplication and conjugation. The fate of purified *B. thuringiensis* toxin was analysed by extractable toxin quantification using ELISA. The extractable toxin probably declined due to binding on surface-active particles in the soil.

Report KMA 2.7/06 Van der Auwera, G.A., Timmery, S., Hoton, F. and Mahillon, J (2007). Plasmid exchanges among members of the *Bacillus cereus* group in foodstuffs. International Journal of Food Microbiology, Vol. 113, No. 2, pp. 164-172.
GLP No
Summary
The *Bacillus cereus sensu lato* group is genetically very close and possesses a remarkable plasmid gene pool that encodes a variety of functions such as virulence and self-transfer capabilities. The potential for horizontal transfer among the various subspecies of this group, which includes the human opportunistic pathogens *B. cereus sensu stricto* and *B. anthracis* as well as the biopesticide *B. thuringiensis*, has led to growing concerns regarding food safety and public health. In this study, the conju-

gative behaviour of *B. thuringiensis* strains was compared in LB medium, milk and rice pudding using the pXO16 and pAW63 conjugative systems, as well as the mobilisable plasmid pC194, in bi- and triparental matings. Conjugation and mobilisation of these plasmids were shown to occur at significant levels in both food products, attaining the highest transfer frequencies in milk, with an approximately ten-fold increase in conjugative transfer in this growth medium as compared to liquid LB. Furthermore, the ability of an emetic strain of *B. cereus* to function as either plasmid donor or recipient partner in heterologous biparental matings with *B. thuringiensis* was demonstrated in these food matrices.

Report KMA 2.7/07 Short, F.L., Monson, R. E., & Salmond, G. (2015). A Type III protein-RNA toxin-antitoxin system from *Bacillus thuringiensis* promotes plasmid retention during spore development. RNA Biology, Vol. 12, No. 9, pp. 933-937.
GLP No
Summary
Members of the *Bacillus cereus sensu lato* group of bacteria often contain multiple large plasmids, including those encoding virulence factors in *B. anthracis*. *Bacillus* species can develop into spores in response to stress. During sporulation the genomic content of the cell is heavily compressed, which could result in counterselection of extrachromosomal genomic elements, unless they have robust stabilization and segregation systems. Toxin-antitoxin (TA) systems are near-ubiquitous in prokaryotes and have multiple biological roles, including plasmid stabilization during vegetative growth. It has been shown that a Type III TA system, based on an RNA antitoxin and endoribonuclease toxin, from plasmid pAW63 in *Bacillus thuringiensis* serovar *kurstaki* HD-73 can dramatically promote plasmid retention in populations undergoing sporulation and germination, and we provide evidence that this occurs through the post-segregational killing of plasmid-free forespores. The findings demonstrate that an extremely common genetic module can be used to ensure plasmid maintenance during stress-induced developmental transitions, with implications for plasmid dynamics in *B. cereus* s.l. bacteria.

B.2.8 Information on the production of metabolites (especially toxins)

Commercial products containing *Bacillus thuringiensis* subsp. *aizawai*, strain ABTS-1857 have been shown not to contain β-exotoxins or an high amount of enterotoxins.

One of the most significant features of *B. thuringiensis* is the formation of parasporal crystals during sporulation, which are typically comprised of several kinds of insecticidal crystal proteins (ICPs). Moreover, different strains can produce various ICPs having specific insecticidal activity. To date, *B. thuringiensis* has been reported to produce over 760 kinds of ICPs that are classified into 72 Cry groups (723 kinds) and 3 Cyt groups (37 kinds).
(http://www.lifesci.sussex.ac.uk/Home/Neil_Crickmore/Bt/; November, 2012).
Bacillus thuringiensis Cry and Cyt protein families are a diverse group of proteins with activity against insects of different orders - Lepidoptera, Coleoptera, Diptera and also against other invertebrates such as nematodes. Their primary action is to lyse midgut epithelial cells by inserting into the target membrane and forming pores.

The crystal proteins that account for the insecticidal activity of the particular submitted *Bta* strain ABTS-1857 have been identified and measured. This information is covered within the identity portion of the dossier and in the confidential section.

The crystal proteins have been extensively studied and their specificity due to insect gut binding sites determined. The biological activity of these toxins, their structure and composition was recently reviewed by Pardo-Lopez et al (2012; KMA 2.8/03) and Palma et al. (2014; KMA 2.8/04).

The Cyt endotoxins have been shown to have some activity against pathogens, for example Cyt1Aa is a delta-endotoxin protein that is produced by *Bacillus thuringiensis* subsp. *israelensis*. Cyt1Aa was

found to be bactericidal for *E. coli*, whereas it was bacteriostatic for *S. aureus* (Cahan et al. 2008; KMA 2.8/05)

Thuringiensin (Thu), also known as β-exotoxin, is a thermostable secondary metabolite secreted by some strains of *Bacillus thuringiensis* (Liu,et al. 2014; KMA 2.8/06). In contrast to the insecticidal crystal proteins, β-exotoxin is not a protein but a small molecule oligosaccharide. This metabolite is considered toxic as it is known to inhibit the biosynthesis of RNA polymerase, an enzyme essential to the transfer of genetic information in almost all organisms (Wiest et al. 2015; KMA 2.8/07). As such is tested for its absence in manufacturing of *Bta* strain ABTS-1857 (Benzon 2016; see volume 4)). Analysis for this metabolite constitutes part of the submission, showing the absence of this metabolite.

The enterotoxin question for Bta strain ABTS-1857 is being dealt with in the human toxicity section (B.6) and the confidential section based on genetics.

B. thuringiensis strains also contain genetics for enterotoxins which are suspected of being involved in food poisoning incidences. Among these toxins are 10 enterotoxigenic genes (hblA, hblC, hblD, nheA, nheB, nheC, cytK, bceT, entFM, and entS), one emetogenic gene (ces), seven hemolytic genes (hlyA, hlyII, hlyIII, plcA, cerA, cerB, and cerO) (Kim et al., 2015; KMA 2.8/08).

As stated in B.6 genetic analysis of *Bacillus thuringiensis* ssp. *aizawai* (strain ABTS 1857) presented in volume 4 confirms the absence of the emetic toxin gene from the genome of this particular strain.This is also supported by the study performed by Harriam (2015) who characterized Enterotoxigenic *Bacillus cereus* and *Bacillus thuringiensis* Spores in U.S. retail Spices by using a novel chromogenic agar, B. cereus spores were isolated from 77 samples (31%) and *B. thuringiensis* spores were isolated from 11 (4%) samples. Levels of B. cereus ranged from <3 MPN/g to 1600 MPN/g. B. thuringiensis ranged from 3.6 to 240 MPN/g with only one sample in the 100-1600 range - the rest were evenly distributed between ranges 1 to 10 and 10 to 100. Eighty four of the 88 spices tested possessed either one of the enterotoxin genes. None of the isolates tested positive for the emetic toxin (ces) gene (see B.6).

B. thuringiensis also secretes secondary metabolites with biological activities. One of these metabolites is Zwittermicin A. This natural antibiotic is a highly polar, water-soluble aminopolyol that was firstly isolated from *B. cereus*. The group of Handelsman has been the pioneer that isolated this metabolite for the first time. They detected that *B.cereus* had a biological effect against the fungal pathogens of plants. It protects alfalfa seedlings from damping-off caused by *Phytophthora medicaginis*, tobacco seedlings from *Phytophthora nicotianae*, cucumber fruits from rot caused by *Pythium aphanidermatum* and peanuts from *Sclerotinia minor*. When they analyzed the containing of this strain to view which was the active component they reported that zwittermicin A 1 was responsible for the biological activity of the strain (Silo-Suh et al 1998; KMA 2.8/09).

Bacillus thuringiensis has been studied extensively. The genetics for many of its metabolites is well characterized. How and when these metabolites are produced, still remains to be elucidated.
A literature search and review for the active substance was conducted which is submitted in this dossier. The following paper(s) relate to relevant information from this search and review in the current section;

Report KMA 2.8/01 de la Vega, L. M. et al. (2006). Purification and characterization of an exochitinase from *Bacillus thuringiensis* subsp *aizawai* and its action against phytopathogenic fungi. Canadian Journal of Microbiology, Vol. 52, No. 7, pp. 651-657.
GLP No
Summary
A chitinolytic enzyme from *Bacillus thuringiensis* subsp. *aizawai* has been purified and its molecular mass was estimated ca. 66 kDa by sodium dodecyl sulfate – polyacryamide gel electrophoresis (SDS–PAGE). The enzyme was able to hydrolyze chitin to chitobiosides but not carboxymethylcellulose, cellulose, pullulan, and laminarin. Optimal pH and temperature were detected at 6 and 50 °C, respectively. Stability, in the absence of substrate, was observed at temperatures less than 60 °C and pH be-

tween 5 and 8. Enzyme activity was significantly inhibited by K+ and EDTA and completely inhibited by Hg2+. Purified chitinase showed lytic activity against cell walls from six phytopathogenic fungi and inhibited the mycelial growth of both *Fusarium* sp. and *Sclerotium rolfsii.* The biocontrol efficacy of the enzyme was tested in the protection of bean seeds infested with six phytopathogenic fungi.

Report KMA 2.8/02 Hsieh, F-C. et al. (2008). Comparing methods for identifying *Bacillus* strains capable of producing the antifungal lipopeptide iturin A. Current Microbiology, Vol. 56, No. 1, pp. 1-5.
GLP No
Summary
Lipopeptides represent a unique class of bioactive microbial secondary metabolites, and iturin A shows attractive antibiotic properties among them. This study compares three methods, such as yeast/fungal growth inhibition assay, quantitative high-performance liquid chromatography (HPLC) and polymerase chain reaction (PCR) for identifying a number of *Bacillus* species that produce iturin A. The authors examined the feasibility of screening iturin A-producing *Bacillus* strains by PCR using specific primers for ituD and lpa-14 amplification. Twenty standard strains and 120 field-collected *Bacillus* spp. isolates were tested in this study. Four *B. subtilis* and one *B. circulan* strains from ATCC, and *B. amyloliquefaciens* B128, a known iturin A producer, exhibited positive results. Of the 120 field-collected isolates, 42 strains were positive. The potential of producing iturin A by these PCR-positive strains were then confirmed by conventional methods such as fungal growth inhibition assay and HPLC analysis. The consistency between results of PCR, HPLC, and fungal growth inhibition assay suggests that the PCR method could be used as an alternative tool for fast screening of iturin A-producing *Bacillus* strains from the environment. This is the first report of detecting iturin A production from *B. circulans.*

B.2.9 Antibiotics and other anti-microbial agents

The sensitivity to antibiotics was described in the confidential section in the DAR, however, without a detailed study evaluation. This is now provided below.

Strain ABTS-1857 was sensitive to gentamicin, kanamycin, erthyromycin, clindamycin, vancomycin, chloramphenicol and trimethoprim/sulfamethoxazole but not sensitive to penicillin, ampicillin or cephalothin. The susceptibility was determined using standard methodology (SOP 047T-12-043A; National Committee for Clinical Laboratory Standard, 1984). *Staphylococcus aureus* was also included in the test to verify the test procedure (Smith, 1990).

Table 2.9-1 Antibiotic Sensitivity Tests of the Bta strain ABTS-1857

	Zone Diameter (mm)				
Antibiotic	**Bta ABTS-1857**	**Btk HD-1**	**Bta ABTS-26**	**Bta ABTS-1883**	**Response[(1)]**
Gentamicin	25	24	24	23	S
Kanamycin	16	19	17	16	S
Erythromycin	31	28	31	27	S
Clindamycin	21	24	22	21	S
Penicillin	8	9	9	8	R
Ampocillin	8	10	10	8	R
Cephalothrin	9	9	10	8	R
Vancomycin	19	20	19	18	S
Chloramphenicol	25	27	25	24	S
Trimethoprim sulfamethoxa-zole	23	23	22	20	S

(1) Antibiotic response common for all four strains; R=resistent and S=sensitive.

B.2.10 References relied on

See B.6 MA for summary literature search.

Note RMS: RMS did not find a literature search for metabolites only or in combination with Bta. Therefore, applicant please perform a correct literature search on metabolites only or in combination with Bta based on the EFSA guidance

Data point	**Author(s)**	**Year**	**Title** **Company Report No.** **Source (where different from company)** **GLP or GEP status** **Published or not**	**Vertebrate study Y/N**	**Data protection claimed Y/N**	**Justification if data protection is claimed**	**Owner**
KMA 1.3/03	Böhm, M-E. et al	2016	Comparative Bioinformatics and Experimental Analysis of the Intergenic Regulatory Regions of Bacillus cereus hbl and nhe Enterotoxin Operons and the Impact of CodY on Virulence Heterogeneity. Frontiers in Microbiology, Vol. 7, Art. 768. GLP: No Published	N	N	-	-
KMA2.1 /01	Anon., 1995;						
KMA2.1/ 02	Smith, 1990						
KMA 2.3/01	Mohan et al.	2014	Relative toxicity of subspecies of Bacillus thuringiensis against lepidopterous insect pests of agricultural im-portance. Journal of Biological Control, Vol. 28, No. 4, pp. 197-203. GLP: No	N	N	-	-

			Published				
KMA 2.5/01	Ruan, L., Crickmore, N., & Sun, M.	2015	Is there sufficient evidence to consider Bacillus thuringiensis a multihost pathogen? Trends in Microbiology, 23(10): 587 GLP: No Published	N	N	-	-
KMA 2.7/01	Hu X, Hansen BM, Eilenberg J, Hendriksen NB, Smidt L, Yuan Z, Jensen GB.	2004	Conjugative transfer, stability and expression of a plasmid encoding a cry1Ac gene in Bacillus cereus group strains. FEMS Microbiology Letters 231 (2004) 45-52 GLP: No Published	N	N	-	-
KMA 2.7/02	Santos CA, Vilas-Bôas GT, Lereclus D, Suzuki MT, Angelo EA, Arantes O.M.	2010	Conjugal transfer between Bacillus thuringiensis and Bacillus cereus strains is not directly correlated with growth of recipient strains. Journal of Invertebrate Pathology, Vol. 105, No. 2, pp. 171-5. GLP: No Published	N	N	-	-
KMA 2.7/03	Thomas DJI, Morgan JAW, Whipps JM, Saunders JR	2000	Plasmid transfer between the Bacillus thuringiensis subspecies kurstaki and tenebrionis in laboratory culture and soil and in Lepidopteran and Coleopteran larvae. Appl Environ Microbiol 66:118–124 GLP: No Published	N	N	-	-

KMA 2.7/04	Yuan YM, Hu XM, Liu HZ, Hansen BM, Yan JP and Yuan ZM	2007	Kinetics of plasmid transfer among Bacillus cereus group strains within lepidopteran larvae. Archives of Microbiology, Vol. 187, No. 6, pp. 425-431. GLP: No Published	N	N	-	-
KMA 2.7/05	Ferreira, L., Leme HP; Suzuki MT; Itano EN; Ono MO; Arantes OMN	2003	Ecological aspects of Bacillus thuringiensis in an oxisol. Scientia Agricola, 60(1): 19-22 GLP: No Published	N	N	-	-
KMA 2.7/06	Van der Auwera, G.A., Timmery, S., Hoton, F. and Mahillon, J	2007	Plasmid exchanges among members of the Bacillus cereus group in foodstuffs. International Journal of Food Microbiology, Vol. 113, No. 2, pp. 164-172. GLP: No Published	N	N	-	-
KMA 2.7/07	Short, F.L., Monson, R. E., & Salmond, G.	2015	A Type III protein-RNA toxin-antitoxin system from Bacillus thuringiensis promotes plasmid retention during spore development. RNA Biology, Vol. 12, No. 9, pp. 933-937. GLP: No Published	N	N	-	-
KMA 2.8/01	de la Vega, L. M. et al.	2006	Purification and characterization of an exochitinase from Bacillus thuringiensis subsp aizawai and its action against phytopathogenic fungi. Canadian Journal of Microbiology, Vol. 52, No. 7, pp. 651-657. GLP: No Published	N	N	-	-

KMA 2.8/02	Hsieh, F-C. et al..	2008	Comparing methods for identifying Bacillus strains capable of producing the antifungal lipopeptide iturin A. Current Microbiology, Vol. 56, No. 1, pp. 1-5. GLP: No Published	N	N	-	-
KMA 2.8/03	Pardo-López, L., Soberón, M. & Bravo, A.	2013	*Bacillus thuringiensis* insecticidal three-domain Cry toxins: mode of action, insect resistance and consequences for crop protection FEMS Microbiol Rev 37 (2013) 3–22 GLP: No Published	N	N	-	-
KMA 2.8/04	Palma, L., Muñoz, D., Berry, C., Murillo, J. & Caballero, P.	2014	*Bacillus thuringiensis* Toxins: An Overview of Their Biocidal Activity Toxins (2014), *6*, 3296-3325 GLP: No Published	N	N	-	-
KMA 2.8/05	Cahan, R., Friman, H. & Nitzan, Y.	2008	Antibacterial activity of Cyt1Aa from *Bacillus thuringiensis* subsp. *israelensis* Microbiology (2008), 154, 3529–3536 GLP: No Published	N	N	-	-
KMA 2.8/06	Liu, X., Ruan, L., Peng, D., Li, L., Sun, M. & Yu, Z.	2014	Thuringiensin: A Thermostable Secondary Metabolite from *Bacillus thuringiensis* with Insecticidal Activity against a Wide Range of Insects Toxins (2014), 6, 2229-2238 GLP: No Published	N	N	-	-

KMA 2.8/07	Wiest, S.L.F., Pilz Júnior, H.L. & Fiuza, L.M.	2015	Thuringiensin: a toxin from *Bacillus thuringiensis* Bt Research (2015), Vol.6, No.4, 1-12 GLP: No Published	N	N	-	-
KMA 2.8/08	Kim, M-J., Han, J-K., Park, J-S., Lee, J-S., Lee, S-H., Cho, J-I. & Kim, K-S.	2015	Various Enterotoxin and Other Virulence Factor Genes Widespread Among *Bacillus cereus* and *Bacillus thuringiensis* Strains J. Microbiol. Biotechnol. (2015), 25(6), 872–879 GLP: No Published	N	N	-	-
KMA 2.8/09	Silo-Suh, L.A., Stabb, E.V., Raffel, S.J. & Handelsman, J.	1998	Target Range of Zwittermicin A, an Aminopolyol Antibiotic from *Bacillus cereus* Current Microbiology (1998), Vol. 37, pp. 6-11 GLP: No Published	N	N	-	-
KMA 3.5/01	Forrester, N.W	1994	Resistance management options for conventional Bacillus thuringiensis and transgenic plants in Australian summer field crops. Biocontrol Science and Technology (1994) 4, 549-553 GLP: No Published	N	N	-	-
KMA 3.5/02	Tabashnik, B.E.	1994	Evolution of resistance to Bacillus thuringiensis. Annual Review of Entomology, Vol. 39, pp. 47-79. GLP: No Published	N	N	-	-
KMA	Tang, J.D. et	1997	Inheritance, stability, and lack-of-fitness costs of field-selected resistance to Bacillus thuringiensis in Diamond-	N	N	-	-

3.5/03	al.		back moth (Lepidoptera: Plutellidae) from Florida. Journal of Economic Entomology, Vol. 90 (3), pp. 732-741. GLP: No Published				
KMA 3.5/04	Wirth, M.C. et al	2005	Cyt1A of Bacillus thuringiensis delays evolution of resistance to Cry11A in the mosquito Culex quinquefasciatus. Applied and Environmental Microbiology (2005) January 185-189 GLP: No Published	N	N	-	-
KMA 3.5/05	Janmaat, A. F. & Myers, J.H..	2007	Host-plant effects the expression of resistance to Bacillus thuringiensis kurstaki in Trichoplusia ni (Hubner): an important factor in resistance evolution. Journal of Evolutionary Biology, Vol. 20, No. 1, pp. 62-69. GLP: No Published	N	N	-	-
KMA 3.5/06	Wojda, I	2013	Galleria mellonella infected with Bacillus thuringiensis involves Hsp90. Central European Journal of Biology, Vol. 8, No. 6, pp. 561-569. GLP: No Published	N	N	-	-

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner
IIM 2.3.2/01	Schnepf, E., Crickmore, N., van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D.R. and Dean, D.H.	1998	BACILLUS THURINGIENSIS AND ITS PESTICIDAL CRYSTAL PROTEINS - Report-no. GLP: no Published: no		No		-
IIM 2.3.2/02	Höfte, H. and Whiteley, H.R.	1989	INSECTICIDAL CRYSTAL PROTEINS OF BACILLUS THURINGIENSIS - Microbiological Reviews, June 1989, p. 242-255 Report-no. GLP: no Published: yes **Submitted in: K IIM 2.3/01**				
IIM 2.3.2/03	Hofmann, C., Vanderbruggen, H., Höfte, H.; van Rie, J., Jansens, S. and van Mellaert, H.	1988a	SPECIFICITY OF BACILLUS THURINGIENSIS ENDOTOXINS IS CORRELATED WITH THE PRESENCE OF HIGH-AFFINITY BINDING SITES IN THE BRUSH BORDER MEMBRANE OF TARGET INSECT MIDGUTS - Proc. Natl. Acad. USA Vol. 85, pp. 7844-7848 Report-no. GLP: no Published: yes		No		-

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protec-tion claimed Y/N	Justification if data protection is claimed	Owner
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