Renewal Assessment Report

Bacillus thuringiensis subsp. *aizawai* *strain GC-91*

Volume 3 – B.2 Biological properties

July 2018

Rapporteur Member State: The Netherlands

Co-Rapporteur Member State: Germany

Version history

When	What
July 2018	Initial RAR

Table of contents

B Summary of the data and information

Introduction

Bacillus thuringiensis subsp. *aizawai* GC-91 (in the following abbreviated as Bta GC-91) was included in Annex I of Council Directive 91/414/EEC in 2008. Subsequently Regulation (EC) No 1107/2009 repealed and replaced Directive 91/414/EEC and the active substance Bta GC-91 was deemed to be approved under that Regulation and included in the Annex to Regulation (EC) No 540/2011. EFSA delivered its conclusions on *Bacillus thuringiensis* ssp. *aizawai* (strains ABTS-1857, GC-91) on the 19 December 2012 (published January 2013). Based on this new information available, no need to change the conditions of approval of Bta GC-91 was identified. The Commission filed on 13 December 2013 an updated review report for Bta GC-91 to the Standing Committee on the Food Chain and Animal Health for examination.

The approval of Bta GC-91 under the Regulation (EC) No 1107/2009 expires 30 April 2019. In accordance with the same Regulation the original notifier Mitsui AgriScience International SA/NV has filed to the Commission an application for the renewal of the approval of the active substance Bta GC-91 on 30 April 2016.

Bacillus thuringiensis subsp. *aizawai* GC-91 (in the following abbreviated as Bta GC-91) is a transconjugant strain originating from a Bta and a Bt subsp. *kurstaki* strain. Bta in general occurs ubiquitous in soils on plants as well as in infested insects. Bta acts highly specific against insect species of the order Lepidoptera and is not expected to have any harmful effects on beneficials and other non-target species of other insect orders. The insecticidal activity of Bta is mainly attributed to spore bound insecticidal pro-toxins (Cry toxins) which are ingested by the target pests and activated under alkaline conditions in the midgut of the larvae.

As the manufacturing process of Bta GC-91 has not been changed since original approval, all data submitted for the original approval of the strain are considered fully applicable for the current evaluation.

Besides new information, the submitted dossier includes all data, which have been presented in the DAR (Jan 2008) and DAR addendum (Nov 2012). This information is marked grey in this RAR with a clear indication where the information is originating from.

B.2 Biological properties of the micro-organism

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

B.2.1.1 Historical background

Information from DAR and DAR addendum (May 2007, February 2013) *B. thuringiensis* is a widespread bacterium (Martin & Travers, 1989) which has been isolated from soil (DeLucca et al., 1981; Martin & Travers, 1989; Ohba & Aizawa, 1986), insect habitats (Brownbridge & Margalit, 1986; Asimeng & Mutinga, 1992), insect larvae (Dulmage, 1970), stored products (DeLucca et al., 1982) and leaf surfaces (Smith and Couche, 1991). Most strains used in commercial production of microbial insecticides have been isolated from diseased insects (DeLucca et al., 1981).

The number of *B. thuringiensis* strains and insecticidal toxins has increased rapidly. It is estimated that 60,000 isolates are held in collections throughout the world (Glare & O'Callaghan, 2000).

Strain GC-91 is not a naturally occurring strain but a product of a crossing or conjugation between the parental strains HD-191-A2 and HD-135-4S that differ in their flagella serotype as well as in their δ-endotoxin genes, using the conjugation-like plasmid transfer system reported by González et al. (1982). The parental strains HD-191-A2 (flagella serotype kurstaki) and HD-135-4S (flagella serotype aizawai) were derived from the wild types HD-191 and HD-135. Plasmid conjugation is a natural process which allows one bacterium to donate genetic material to another.

To produce the new strain, the parental strains HD-191-A2 and HD-135-4S, differing in their flagella serotype and δ-endotoxin genes, were grown together in mixed culture to effect conjugation-like plasmid transfer. The mixed culture was then diluted and transferred to a solid medium (nutrient agar) to obtain single colonies. Selective grow-

ing conditions, DNA fingerprinting, and protein analyses were subsequently used to screen the resulting progeny for those bacteria that produced the desired combination of Cry toxins. Colonies of the strain GC-91 were microscopically selected by the increased size of the parasporal crystal. Reference is also made to the accompanying drawing in the US patent no 5,063,055 (Burges et al. 1991, US patent) which shows plasmid profiles of the wild-type, donor strain, recipient strain and recombinant strain GC-91. Eight plasmid bands can be determined indicating recombinant extrachromosomal material (Burges et al., 1991).

It has been found that strain GC-91 shows an improved insecticidal activity against certain lepidopterous pest species and has an effectively broadened spectrum of activity (Burges et al., 1991).

No unusual morphological, physiological, pesticidal or resistance characteristics of GC-91 which differ from classical description of the species *Bacillus thuringiensis* are known.

New data

No substantial new information is submitted for renewal of the strain according to Regulation (EC) 1107/2009. Products containing Bta GC-91 are currently registered all over Europe for control of lepidopteran pests in field and greenhouse crops. For more details on existing uses, please refer to 1.5.4 in Volume 1.

B.2.1.2 Origin and natural occurrence

Information from DAR and DAR addendum (May 2007, February 2013) In general, *Bacillus thuringiensis* is indigenous in many environments (Bernhard et al., 1997; Martin & Travers, 1989). Bt strains have been isolated mainly from environments associated with insect populations or plant material, but also from a range of habitats all over the world (e.g. Sweden: Landén et al., 1994; Spain: Iriarte et al., 1998; Bel et al. 1997; Korea: Kim et al., 1998; Brasil: Valicente & Barreto, 2003; Chile: Vásquez et al., 1995).

Bernhard et al. (1997) characterized the natural isolates of *Bacillus thuringiensis* in eight different countries from 5 different habitats. The majority of the isolates (45 %) originated from stored products whereas, only 25 % originated from soil. The majority of isolates with insecticidal activity were found in mushroom compost and stored products. No correlation between the activity against representative Lepidoptera, Diptera and Coleoptera and the origin or the type of material samples could be found, indicating a relatively ubiquitous distribution of the selected activities and of *B. thuringiensis* in general. A high number of isolates had no effects against all test insects as has been found in other surveys (e.g. Ohba & Aizawa, 1986). Martin & Travers (1989) analysed soil samples from all over the world and demonstrated that Bt could be collected from almost everywhere, from the beach as well as from desert or tundra habitats. Furthermore, they found that the presence of insects did not predict the presence of *B. thuringiensis* in soil samples.

Damgaard et al. (1997a) found Bt isolates active against insects of the orders Lepidoptera, Diptera and Coleoptera on cabbage foliage and Damgaard et al. (1998) showed that Bt isolates were naturally present in the phylloplane of grass foliage collected from pasture.

The results of numerous studies indicate that Bt is a ubiquitous soil microbe as well as a common inhabitant of the phylloplane (Smith & Couche, 1991).

New data

Bt as a species occurs naturally in a range of environmental compartments such as soils, plant surfaces and infected insects.

A literature review aiming to define background levels of Bt in the environment was done within the frame of the preparation of the EFSA Scientific opinion on the Risk for public health related to *B. cereus* and other *Bacillus* spp. including *B. thuringiensis* in food stuff published in 2016[1]. A summary on recorded background levels reported therein is provided below.

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

Confirming information already presented during the first evaluation of the strain, it was concluded that members of the *B. cereus* group occur ubiquitous in the environment in soil, plants, sediments, water, invertebrates and mammals. In soils, 0 - 50% of the *B. cereus* group isolates affiliate with Bt reaching levels of up to 5×10^5 CFU/g soil. On plants, the populations vary between 0 and 6×10^4 CFU/g with a mean density of 100 CFU/g in areas not previously treated with Bt. A summary of recorded background levels can be found in **Table 2.1.2.-1** below.

Table 2.1.2-1 Natural background levels of Bt in different environmental compartments

Environmental compartment*	**Density of *B. cereus* group members including Bt**	**Reference**
Soil		
Cultivated soils UK	2×10^4 CFU/g	Collier et al. (2005, cited in EFSA Scientific Opinion)
Cultivated soils Denmark	2×10^5 CFU/g	Hendriksen et al. (2006, cited in EFSA Scientific Opinion)
Cultivated soils UK	5×10^5 CFU/g	Raymond et al. (2010, cited in EFSA Scientific Opinion)
Agricultural soils France	$2.7 \times 10^4 – 1.7 \times 10^5$CFU/g	Brillard et al. (2015, cited in EFSA Scientific Opinion)
Danish soils	$4 \times 10^4 – 2 \times 10^5$ CFU/g, mean 10^5 CFU/g	Hendriksen et al. (2011, cited in EFSA Scientific Opinion)
Rice field	4.23 –6.52 10^5 CFU/g	Chatterjee et al. (2007, cited in EFSA Scientific Opinion
Water		
Rainwater	$1.4 \times 10^1 – 3.2 \times 10^2$ CFU/L	Brillard et al. (2015, cited in EFSA Scientific Opinion)
Groundwater	1.5 – 9.1 CFU/L	
Plants/crops		
Broad-leaf dock	2×10^4 CFU/g	Collier et al. (2005, cited in EFSA Scientific Opinion)
Curly kale	Max. 6×10^4 CFU/g, mean 3×10^2 CFU/g	Hendriksen (2011)**
Cauliflower leaves	80 – 1700 CFU/cm^2 leaf	Damgaard et al. (1994, cited in EFSA Scientific Opinion)
Rice	Max. 23 CFU/g	Ankolekar et al. (2009, cited in EFSA Scientific Opinion)
Maize and bean leaves	0.46 - 1.5 spores/cm²	Jara et al. (2006, cited in EFSA Scientific Opinion)
Rice	2 - 11.2 CFU/g	Kim et al. (2014, cited in EFSA Scientific Opinion)
Food		
Vegetables and fruits	10 – 11000 CFU/g	Frederiksen et al. (2006, cited in EFSA Scientific Opinion)
Ready to eat food (48,901 samples)	0 - 10^4 CFU/g, usually below 10^3 CFU/g	Rosenquist et al. (2005, cited in EFSA Scientific Opinion)

Environmental compartment*	Density of *B. cereus* group members including Bt	Reference
Spices, paprika, allspice, peppercorns, and mixed spices	3 to 240 MPN/g	Hariram and Labbé (2015, cited in EFSA Scientific Opinion)

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

*References were also provided for dairy products but were not included here, as this is not of relevance for use of the strain for pest control in agriculture

**Presentation of data shown at Second Biopesticides Steering Group Seminar on the Fate in the Environment of Microbial Control Agents and Their Effects on Non-target Organisms, summarized in Series of Pesticides No. 64. At page 125 of the cited paper for the presentation: Fate of microbials in the environment, A structural model for explanation of the fate By Niels Bohse Hendriksen.

B.2.2 Information on target organism(s)

Information from DAR and DAR addendum (May 2007, February 2013) Specifity of subspecies of *B. thuringiensis* is dependent on the δ-endotoxins which are produced. The spectra of toxins produced by isolates of *B. thuringiensis* varies greatly. The crystal proteins exhibit highly specific insecticidal activity (reviewed by Höfte & Whiteley, 1989). Höfte & Whiteley have classified the different Cry proteins denoting ICPs (insecticidal crystal proteins) toxic to various insects and invertebrate groups as follows: Cry I are toxic to lepidopterans, Cry II are toxic to lepidopterans and dipterans , Cry III are toxic to coleopterans, Cry IV are toxic to dipterans. Cry V and Cry VI are toxic to nematodes (Feitelson et al., 1992; Wei et al., 2003).

Strain GC-91 is highly specific to insect species of the lepidopteran order and shows an increased insecticidal activity compared to its parental strains or to the most frequently commercially used bacterial strain HD-1 (Table 2.1.2-2).

Table 2.1.2-2 Insecticidal activity of strain GC-91, parental and reference strains (Burges et al. (1991)

Insect species	LC50 µg bacteria**/g of insect food				
	HD-1*	HD-191	HD-135	HD-135-4S	GC-91
Galleria mellonella	2,600	3,500	20	64	18.4
Heliothis armigera	42	48	228	845	44
Heliothis virescens	8.6	5.8	205	2000	4.8
Spodoptora littoralis	5,780	>10,000	445	694	330
Pieris brassicae	0.64	0.98	1.2	100	0.72
Mamestra brassicae	1,510	>10,000	185	282	162

* HD-1 is the bacterial strain used in most commercial *Bacillus thuringiensis* products for control of lepidopterous larvae. It is available for example, from the U.S. Department of Agriculture and the Institute Pasteur

** Bacteria were cultured and harvested for bioassay according to the method of Dulmage et al. (1971)

Whereas Btk HD-1 is one of the most useful insecticidal strains, because it exhibits powerful toxicity to various lepidopteran larvae, the Bta strain GC-91 is more effective against larvae of *Spodoptera* species (Moar et al., 1995).

New data

The information provided previously is considered acceptable to cover current requirements. Therefore, no new data are submitted for renewal of Bta GC-91 under Regulation (EC) 1107/2009.

B.2.2.1 Description of target organism(s)

Information from DAR and DAR addendum (May 2007, February 2013)

Strain GC-91 is currently used as an insecticide against a wide range of lepidopteran pest species. With this Report, the control of the European Grape Vine Moth (*Lobesia botrana*) and the Grape Bud Moth (*Eupoecelia ambiguella*) in grapes is intended.

Infestation appears with superficial damage on remnants of flowers, partially eaten shrivelled fruits, and rotting fruits with contamination by larval frass and webbing.

English name: European Grape Vine Moth, Mediterranean Vine Moth

Latin name: *Lobesia botrana*

Adult moths are characterized by a 12- 13 mm wide span and 6-7 mm body length. The forewing is whitish brown, with intricate brown and purplish grey patterns, a dark brown median band, and a fringe border of dense olive green hairs.

The female moth lays her eggs singly on grape blossoms, on young grapes, or on the vine. In very warm and dry areas the female lays 300 or more eggs at a rate of more than 35 per day. In spring the eggs hatch in 7-11 days, and in summer in 3-5 days. Larvae of the first generation feed on buds and on flowers; later generations feed on developing fruit. The larva has a pale yellowish brown head; a slender body varying from greenish yellow to light brown. Mature larvae are 10-12 mm long, 2 mm wide. The pupa is dark brown. The pupal cocoon consists of white-grey silk threads, and is 8-10 mm long, 3 mm wide. Many young larvae die, but mortality among older larvae is very low. Pupae of the first generation occur in the fruit or in a fold of a leaf. Overwintering pupae of second or later generations occur beneath bark in fissures of the stock. Adults first emerge in April or May; final generations in August to September. There are two generations in central Europe with a third generation further south. Under favourable conditions up to four generations may occur. Development ceases at temperatures below 10.5°C. Several weeks may pass between the emergence of the first and last moth of the same generation.

English name: European Grape Berry Moth

Latin name: *Eupoecelia ambiguella*

Adult moths are characterised by a 14-18 mm wing span. The forewing is white, strongly infused with orange yellow, and exhibits a dark brown, broad median band. *Eupoecilia ambiguella* has one or two generations per year, and sometimes three further south in Europe, and in Asia. The larva usually overwinters in a portable case made of leaf fragments attached to the base of the trunk, or in fissures on the vine. Adults emerge in May; second generation adults occur at the beginning of July, and third generation adults in October. The female lays the eggs singly. The moth flies from dusk to dawn. The eggs are deposited, in the evening in humid sheltered sites, on the flower buds or, for the second generation, on the fruits. The embryonic development lasts 8 to 12 days. Larvae feed on flowers and later on developing fruit. The head of the larva is dark brown to black; its body varies from olive green to reddish brown, with a dark brown prothoracic plate, and a yellowish anal plate. Pupation occurs in April or May and in June or July, but a few larvae may pupate in late autumn. The colour of the pupa is light reddish brown. Pupal stage lasts 14 days.

New information

Bta GC-91 is used against *Cydia pomonella* in pome fruits, *Tuta absoluta* in solanaceous fruits as well as against *Spodoptera* spp. in turf and sports. In addition, use against *Lobesia botrana* and *Eupoecilia ambiguella* in grapes is intended but for this use, all relevant information have been already submitted before.

Cydia pomonella

English name: Codling Moth

The codling moth is one of the most important pests in apple orchards. The greyish moth with a wing spread of about 2 cm and a characteristic cross band of chocolate brown deposits lays 50-75 eggs on the leaves, twigs and fruits. The egg laying period extends from the end of spring to summer. After hatching (one to three weeks later),

the first larval instars walk on the fruit, test the fruit by shallow stings and look for a site to enter the fruit (through the side of the apple, the calyx or near the stalk). The lesions are therefore visible from the end of the spring until the beginning of autumn. A partial entry of the larvae causes stings which alter the fruit quality. After complete penetration in the fruit, the larva bores a tunnel to the core of the fruit, and after complete development, exits (3 to 5 weeks later). They leave the fruit and seek suitable places for hiding, such as underneath bits of loose bark and other protected places mainly on the tree and seldom in the debris on the ground. Here cocoons are spun and pupation follows. Depending on climate, one, two or even more generations each year (in warm regions) are possible (Little, 1957). Hibernation takes place in the form of diapausing larvae.

The larvae of the codling moth injure and contaminate the fruits by eating: the wormy fruit is familiar to every one. Fruits very often drop prematurely, the remaining ones are not marketable. Mainly apples are attacked but to a smaller extent also pears, walnuts and occasionally other fruits may be affected.

Tuta absoluta

English name: tomato leaf miner and South American tomato moth

Tuta absoluta is well-known as a serious pest of tomato crops in Europe and South America.

Tuta absoluta is a micro lepidopteron moth with high reproductive potential. There are about 10–12 generations per year. The total life cycle is completed within 30-35 days. Adults are nocturnal and hide between leaves during the day time. Adults are 5-7 mm long and with a wingspan of 8-10 mm Adult females lay eggs on host plants and mature female could lay up to 260 eggs before completing life cycle. Eggs are small cylindrical, creamy white to yellow, 0.35 mm long. Egg hatching takes place 4-6 days after egg lying. The larvae is cream in colour with characteristic dark head. Four larval instars develop. Larvae do not enter diapause when food is available. Pupation may take place in the soil, on the leaf surface or within mines. *Tuta absoluta* can overwinter as eggs, pupae or adults depending on environmental conditions. The most important identifying characters are the filiform antennae (bead like antennae), silverfish-grey scales and characteristic black spots present in anterior wing. The larvae become greenish to light pink in second to fourth instars. Larval period is most damaging period; itis completed within 12-15 days.

The larva feeds voraciously upon tomato plants, producing large galleries in leaves, burrowing in stalks, and consuming apical buds and green and ripe fruits. It is capable of causing a yield loss of 100%.

Spodoptera littoralis

English name: African Cotton Leafworm or Egyptian Cotton Leafworm, Mediterranean Brocade

S. littoralis is a noctuid moth found widely in Africa and Mediterranean Europe. It is often a pest on vegetables, fruits, flowers and other crops.

The forewings are brown with irregular markings, and they span up to 40 mm (1.57 inch). The hind wings, visible when spread, are whitish with darker margins.

The caterpillar is variable in colour, from dark green to blackish-grey or brown, with longitudinal stripes; it is hairless, and, when fully developed, up to 50 mm (1.97 inch) long.

The larvae of the cotton leafworm feed voraciously on almost all plant organs. Generally, young leaves are preferred, but when they have been consumed, other parts (e.g. stems, buds or pods) are attacked too. An infestation frequently leads to complete defoliation. Besides devouring the leaves, the caterpillars interfere with plant development by destroying growth points and flowers.

They bore into buds and fruits and feed inside them, soiling them with frass. In cotton, the bolls will be hollowed out, which often causes them to wilt and drop. In tomato, capsicum and similar crops, fruits that are attacked in this way are not only severely damaged, but also contain a lot of excrement and thus become unsuitable for human consumption. In corn, the larvae mine inside the stems and may feed on young kernels in the ear.

On light soil, they can continue feeding during the daytime, when they hide underground. In this case, subterranean plant parts (e.g. the pods and kernels of groundnut) will be attacked. Root vegetables may become unmarketable due to large holes.

Eggs are laid in batches of several hundred on the plant surface. Each egg mass is of about 3-7 mm (0.12-0.27 inch) diameter and appears hairy, because the female covers it with brownish-yellow scales produced from the tip of its abdomen. Fecundity is high: about 2,000-3,000 eggs are produced over a 6-8 day period.

Two to five days after oviposition, the larvae hatch and quickly disperse over their host plant. Normally, there are six larval instars. The older ones feed only at night and hide in the soil during the day. When they have exhausted

their food source, the caterpillars sometimes migrate in large numbers towards other, as yet undamaged plants. Pupation, too, takes place in the soil, about 2-5 cm (0.78-1.97 inch) deep, inside a loose cocoon, and lasts about 7-10 days. The adults are active at night and mate several times.

The development times can sometimes be much longer at lower temperatures; eggs may need up to 10 days, the larvae three months and pupation 4 weeks. If this is the case, there may be an additional instar. This occurs for example in areas where cotton leafworm is only found in glasshouses. It can diapause in the pupal stage, but cannot withstand repeated frost. In the tropics, more than 12 generations per year are possible and population densities may become huge. In Egyptian cotton fields, several 10,000 egg masses per acre have been counted.

B.2.2.2 Mode of action

Information from DAR and DAR addendum (May 2007, February 2013) 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 (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 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; 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 common to all ICPs (Insecticidal Crystal Proteins) (Schwartz et al., 1991; Schnepf et al., 1998). Spore germination and proliferation of the vegetative cells into the haemocoel may result in septicaemia, contributing to mortality of the insect larvae.

B. thuringiensis is a poor infectious agent and rarely recycles. While vegetative cells and spores will be produced in cadavers, *B. thuringiensis* has rarely been recorded causing natural epizootics (Aronson, 1993) and the transmission from diseased to healthy insects has been shown to be poor or non-existent (Burges, 1982). *B. thuringiensis* spores can remain viable for years in soil, but applied as a spray, the δ-endotoxins are rapidly degradable and endospores are rapidly inactivated when exposed to UV radiation (Griego & Spence, 1978; Pusztai et al., 1991).

The insecticidal activity of *B. thuringiensis* products is not necessarily reflected by the spore count, since the number and amount of the crystal proteins produced per bacterial cell can vary. The formulation of each *B. thuringiensis* product is bio-assayed against an accepted international standard using the test insect *Trichoplusia ni* (Dulmage et al., 1971). To be able to compare different formulations of *B. thuringiensis*, the potency is defined by the international units IU/mg product. The infective dose of a *B. thuringiensis* product depends on the susceptibility of the target insect and the specificity and composition of δ-endotoxins.

The mean biopotency from 5 batches of the strain GC-91 is 53,436 IU / mg. The different Cry proteins expressed by the strain GC-91 are Cry1Ac, Cry1C, Cry1D and Cry2A.

Each type of Cry1 toxin has a unique spectrum of activity and targets only a small range of lepidopteran species. Within the small target ranges there are dramatic differences in potency against species that are often closely related (Yamamoto & Iizuka, 1983; Höfte & Whiteley, 1989; Luo et al., 1999). The potency of a Cry1 toxin can significantly decrease as the larvae age (Raussell et al., 2000). Variations in the potencies of Cry1 toxins for different lepidopteran species and different larval stages may reflect the differences in any one of the prebinding, binding and pore-forming events required for full potency. Gilliland et al. (2002) demonstrated a positive correlation between the increase of resistance to Cry1Ac and Cry1Ba during larval development with fewer binding sites in third-instar BBMV (brush border membrane vesicles) than in neonate BBMV.

B. thuringiensis is relatively ubiquitous. Bernhard et al. (1997) provided several explanations for this:

- Distribution by humans on a large scale from storing and transportation of agricultural products between continents.

- Distribution by natural causes. By producing highly robust endospores, it is likely for *B. thuringiensis* to survive non-anthropogenic transport by water, wind and migrating animals.

- Molecular parasitism/ symbiosis.

In conclusion, *B. thuringiensis* strains produce Cry proteins with insecticidal properties. These proteins are contained as inert crystals in Bt products. Activation of the insecticidal form occurs through several steps: After ingestion by insect larvae, the crystals are solubilised in the alkaline conditions of the midgut and liberate the protoxin which is still inactive. Protoxins are cleaved at particular sites by specific proteases from the host. The active toxins then bind to specific receptors in the brush border membrane of the midgut epithelium columar cells which apparently determines the host specificity of Cry proteins. After binding, the toxins form oligomers which insert into the cell membrane, creating pores which facilitate the passage of ions and water into the epithelium cells. This subsequently results in cell death, and finally in swelling and lysis of the host. Several groups of binding proteins with different specificity for various Cry proteins are described in the literature. Pigott and Ellar (2007) summarized the different groups of binding proteins: The best characterised group of Cry binding proteins are aminopeptidase N (APN) receptors, which in most cases show specificity for a small group or even single Cry proteins. Binding of Cry proteins by APN results in pore formation and subsequent increased ion permeability. Expression of APN coding genes from *Manduca sexta* in *Drosophila* results in susceptibility to Cry proteins, showing that APN are involved in susceptibility to Cry proteins. Similarly, silencing of APN-encoding genes results in reduced susceptibility to Cry1Ca in *Spodoptera litura* larvae.

Cadherins represent the second group of binding proteins. Binding was described in different species and for several Cry proteins. Apparently, Cadherins are involved in both susceptibility to Cry proteins and in the specificity. Alkaline phosphatases from different lepidopteran larvae are able to bind to Cry proteins, but their role in toxicity is still not known. Two further glycoproteins are discussed as Cry binding proteins, but again, their role in the mode of action remains to be elucidated. Glycolipids were characterised as Cry-binding proteins in nematodes. These glycosyl transferases apparently synthesize components responsible for Cry protein sensitivity, but this mode of action seems to be different from the one observed in insects.

The mode of action of Cry proteins is still controversial, which is not surprising due to the variability between different Cry proteins, their (putative) receptors, and the host species that were studied. Currently, three models are discussed to explain the mode of action:

Bravo et al (2007) conclude that activated Cry protein monomers bind to a specific cadherin on the host cell surface. Subsequently, the Cry protein is cleaved by a membrane bound protease and then specifically binds to APN and inserts into the membrane which finally results in formation of membrane pores.

Zhang et al (2005) favour the hypothesis that apoptosis in involved in the mode of action of Cry proteins in their hosts. Binding of monomeric Cry1Ab to a cadherin triggers a Mg2+ dependent signalling pathway which is mediated by stimulation of a G protein, adenyl cyclase, increased cyclic AMP levels and activation of protein kinase A that ultimately leads to cytoskeleton degradation, ion channel activation and finally cell death.

The third model proposed by Jurat-Fuentes et al. (2006, cited by Pigott and Ellar 2007) consists of a combination of the two others. Binding of activated Cry1Ac to a cadherin-like protein results in triggering a phosphatase-mediated signal pathway. Finally, pore formation results in activation of cell death signalling pathways. However, it remains to be elucidated whether insertion of the APN bound Cry protein and pore formation is the cause of the apoptosis signalling pathway, or whether this signalling results in membrane breakdown. Disruption of the midgut epithelium and release of the cell contents finally leads to the death of the larvae. The mode of action of Cry5Ba to nematodes is apparently different, involving gycosyl transferases and specific Cry proteins that do not have an effect on insects even if they share some sequence homologies (Pigott and Ellar 2007).

Another aspect in the mode of action of Cry proteins is the involvement of midgut bacteria in the mode of action. As shown by Broderick et al. (2006, 2009), removal of gut bacteria from *Lymantria dispar* larvae resulted in a loss of susceptibility to Cry proteins. Susceptibility was restored by inoculation with cultivated gut bacteria which in the absence of Cry proteins do not have detrimental effects on their hosts. The authors conclude that septicemia which is often observed after treatment of lepidopteran larvae with Cry proteins which results in insect death is due to colonisation of the hemocoel by naturally occurring gut bacteria after permeabilization of the midgut epithelium by the action of the Cry proteins.

Taken together, the complexity of the mode of action clearly shows that many different components (APN, cadherins, membrane structure and endogenous microflora) have to be simultaneously present to observe an effect of Cry proteins on host cells. Various signalling pathways interact after binding of the activated Cry proteins, leading to a response that is specific both for the Cry protein and the hosts. Finally, damage at the cellular level is transferred to the whole larvae by the involvement of endogenous gut bacteria. This excludes the possibility of an effect of Cry proteins on other than insect cells from the order of Lepidoptera, Coleoptera, and Diptera.

New information

The insecticidal activity of Bt in general, including Bta, is mainly attributed to spore bound insecticidal pro-proteins (cry toxins) which are ingested by the target pests (lepidopteran larvae) and activated under alkaline conditions in the midgut of the larvae. The activated cry toxins interact with the midgut epithelium cells of susceptible insects forming channels or pores disturbing the trans-membrane potential and resulting in colloid-osmotic lysis of the cells. Spore germination and proliferation of the vegetative cells into the haemocoel may result in septicaemia, contributing to mortality of the insect larvae. However, according to studies of Broderick et al. (2006 and 2009) septicaemia is not only related to outgrowth of Bt but also due to extensive proliferation of insect midgut bacteria.

Apart from the cry toxins several other insecticidal proteins produced by Bt and contributing to their mode of action have been described during the last years. A summary of currently known Bt insecticidal toxins and their activity is provided in Palma et al. (2014). The most important information is summarized in **Table 2.2.2-1**. It has to be noted that mechanisms of action of the insecticidal toxins are in most cases not fully understood and usually different models are proposed and controversially discussed. In addition to known substances like the spore bound *cry* and *cyt* proteins, and the *vip* and *sip* toxins which are only produced during vegetative growth of Btk, putative insecticidal toxins are included in the paper of Palma et al. (2014) for which the mechanism and the host range is largely unknown. They are also summarized in the table below for reasons of completeness.

Note applicant: No references have been provided concerning the mean biopotency and expression of the different Cry proteins of strain GC-91. Applicant please address this point.

Table 2.2.2-1 Overview of Bt insecticidal proteins according to Palma et al. (2014)

Name	Mechanism of action	Target
Spore bound crystal proteins		
Cry toxins (three domain and non-three domain versions)	1) Lysis of midgut epithelian cells via unspecific pore formation accompanied by septemica 2) Pore formation by sequential binding to cadherin receptors 3) Activation of signalling pathway leading to necrotic cell death mediated by binding to cadherin receptors	Lepidoptera, Diptera, Coleoptera, Hemiptera, Rhabditida human cancer cells, bacteria/archaea, protozoans
Cyt toxins	General cytolytic/hemolytic activity by detergent action and/or pore formation	Diptera, Coleoptera
Secreted toxins		
Vip1/*Vip2* (binary) toxins	Proteolytic activation of the cell-binding precursor (*Vip1*) followed by translocation of the toxic component (*Vip2*) into the cytoplasm, destruction of filamentous actin, cell death by cytoskeletal disarrangement	Coleoptera, Hemiptera
Vip3 toxin	Precursor activation, binding to midgut epithelian cells causing lysis, gut paralysis and larval death	Lepidoptera
Vip4 toxin	Unknown, structure is very similar to *Vip1*	Unknown
Sip toxin	Pore formation, mechanisms largely unknown	Coleoptera
Further potential insecticidal proteins		
41.9 kDa Protein	Unknown	Unknown
Sphaericolysins and alveolysins	Unknown	Largely unknown, activity described for *Spodoptera litura* and *Blattela germanica*
Beta Exotoxin	Inhibition of DNA-dependent RNA polymerase	Unspecific toxicity against wide range of insects as well as

Name	**Mechanism of action**	**Target**
		mammals
Enhacin-like proteins	Enhancement of toxicity of *cry* toxins	Largely unknown, activity described for *Helicoverpa armigera*
P19/P20 helper proteins	Collaboration for stable production of parasporal crystals, enhancement of production and stabilizing of *Cyt* toxin	Largely unknown, activity described for *Aedes aegypti,* likely unspecific

Cited literature abstracts:

Report: KMA 2.2.2/01 - Broderick, N.A., Raffa, K.F., Handelsman,J. (2006), published report Proc Natl Acad Sci U S A., 103(41):15196-15199

Title: Midgut bacteria required for *Bacillus thuringiensis* insecticidal activity

Abstract

Bacillus thuringiensis is the most widely applied biological insecticide and is used to manage insects that affect forestry and agriculture and transmit human and animal pathogens. This ubiquitous spore-forming bacterium kills insect larvae largely through the action of insecticidal crystal proteins and is commonly deployed as a direct bacterial spray. Moreover, plants engineered with the cry genes encoding the *B. thuringiensis* crystal proteins are the most widely cultivated transgenic crops. For decades, the mechanism of insect killing has been assumed to be toxin-mediated lysis of the gut epithelial cells, which leads to starvation, or *B. thuringiensis* septicemia. Here, we report that *B. thuringiensis* does not kill larvae of the gypsy moth in the absence of indigenous midgut bacteria. Elimination of the gut microbial community by oral administration of antibiotics abolished *B. thuringiensis* insecticidal activity, and reestablishment of an *Enterobacter* sp. that normally resides in the midgut microbial community restored *B. thuringiensis*-mediated killing. *Escherichia coli* engineered to produce the *B. thuringiensis* insecticidal toxin killed gypsy moth larvae irrespective of the presence of other bacteria in the midgut. However, when the engineered *E. coli* was heat-killed and then fed to the larvae, the larvae did not die in the absence of the indigenous midgut bacteria. *E. coli* and the *Enterobacter* sp. achieved high populations in hemolymph, in contrast to *B. thuringiensis*, which appeared to die in hemolymph. Our results demonstrate that *B. thuringiensis*-induced mortality depends on enteric bacteria.

****

Report: KMA 2.2.2/02 - Broderick, N.A., Robinson, C.J., McMahon, M.D., Holt, J., Handelsman, J., Raffa, K.F. (2009), published report

BMC Biology, 7(11):1-9

Title: Contributions of gut bacteria to *Bacillus thuringiensis*-induced mortality vary across a range of Lepidoptera

Abstract

Background

Gut microbiota contribute to the health of their hosts, and alterations in the composition of this microbiota can lead to disease. Previously, we demonstrated that indigenous gut bacteria were required for the insecticidal toxin of *Bacillus thuringiensis* to kill the gypsy moth, *Lymantria dispar*. *B. thuringiensis* and its associated insecticidal toxins are commonly used for the control of lepidopteran pests. A variety of factors associated with the insect host, *B. thuringiensis* strain, and environment affect the wide range of susceptibilities among Lepidoptera, but the interaction of gut bacteria with these factors is not understood. To assess the contribution of gut bacteria to *B. thuringiensis* susceptibility across a range of Lepidoptera we examined larval mortality of six species in the presence and absence of their indigenous gut bacteria. We then assessed the effect of feeding an enteric bacterium isolated from *L. dispar* on larval mortality following ingestion of *B. thuringiensis* toxin.

Results

Oral administration of antibiotics reduced larval mortality due to *B. thuringiensis* in five of six species tested. These included *Vanessa cardui* (L.), *Manduca sexta* (L.), *Pieris rapae* (L.) and *Heliothis virescens* (F.) treated with a formulation composed of *B. thuringiensis* cells and toxins (DiPel), and *Lymantria dispar* (L.) treated with a cell-free formulation of *B. thuringiensis* toxin (MVPII). Antibiotics eliminated populations of gut bacteria below detectable levels in each of the insects, with the exception of *H. virescens*, which did not have detectable gut bacteria prior to treatment. Oral administration of the Gram-negative *Enterobacter* sp. NAB3, an indigenous gut resident of *L. dispar*, restored larval mortality in all four of the species in which antibiotics both reduced susceptibility to *B. thuringiensis* and eliminated gut bacteria, but not in *H. virescens*. In contrast, ingestion of *B. thuringiensis* toxin (MVPII) following antibiotic treatment significantly increased mortality of *Pectinophora gossypiella* (Saunders), which was also the only species with detectable gut bacteria that lacked a Gram-negative component. Further, mortality of *P. gossypiella* larvae reared on diet amended with *B. thuringiensis* toxin and *Enterobacter* sp. NAB3 was generally faster than with *B. thuringiensis* toxin alone.

Conclusion

This study demonstrates that in some larval species, indigenous gut bacteria contribute to *B. thuringiensis* susceptibility. Moreover, the contribution of enteric bacteria to host mortality suggests that perturbations caused by toxin feeding induce otherwise benign gut bacteria to exert pathogenic effects. The interaction between *B. thuringiensis* and the gut microbiota of Lepidoptera may provide a useful model with which to identify the factors involved in such transitions.

****

Report: KMA 2.2.2/03 - Palma, L., Munoz, D., Berry, C., Murillo, J., Caballero, P. (2014), published report

Toxins 2014, 6(12), 3296-3325

Title: *Bacillus thuringiensis* Toxins: An Overview of Their Biocidal Activity

Abstract *Bacillus thuringiensis* (Bt) is a Gram positive, spore-forming bacterium that synthesizes parasporal crystalline inclusions containing Cry and Cyt proteins, some of which are toxic

against a wide range of insect orders, nematodes and human-cancer cells. These toxins have been successfully used as bioinsecticides against caterpillars, beetles, and flies, including mosquitoes and blackflies. Bt also synthesizes insecticidal proteins during the vegetative growth phase, which are subsequently secreted into the growth medium. These proteins are commonly known as vegetative insecticidal proteins (Vips) and hold insecticidal activity against lepidopteran, coleopteran and some homopteran pests. A less well characterized secretory protein with no amino acid similarity to Vip proteins has shown insecticidal activity against coleopteran pests and is termed Sip (secreted insecticidal protein). Bin-like and ETX_MTX2-family proteins (Pfam PF03318), which share amino acid similarities with mosquitocidal binary (Bin) and Mtx2 toxins, respectively, from *Lysinibacillus sphaericus*, are also produced by some Bt strains. In addition, vast numbers of Bt isolates naturally present in the soil and the phylloplane also synthesize crystal proteins whose biological activity is still unknown. In this review, we provide an updated overview of the known active Bt toxins to date and discuss their activities.

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

Information from DAR and DAR addendum (May 2007, February 2013)

Host Specificity

Most *B. thuringiensis* strains are active against Lepidoptera. Some strains however are toxic against dipteran and coleopteran species. Not only different strains, but also different crystal proteins occurring in a single strain vary in insecticidal spectra (reviewed in Höfte & Whiteley, 1989). Numerous δ-endotoxins produced by *B. thuringiensis* have been identified and grouped on the basis of homology and insect specificity (Schnepf et al., 1998). The Cry I toxins are a group of δ-endotoxins that principally target lepidopteran species, including several important crop pests. Cry II toxins are effective against Lepidoptera and Diptera, Cry III toxins are effective against Coleoptera and Cry IV toxins kill Diptera. Cry toxin proteins fall into two size categories: 140-130 kDa (Cry I, Cry IVA, Cry IVB) and around 70 kDa (Cry II, Cry III, Cry IVD).

The insecticidal specificity is not only influenced by the type and total composition of crystal proteins (Aronson et al., 1991), but also by the composition of the midgut lumen. Jaquet et al. (1987) demonstrated that the activity of crystals from several *B. thuringiensis* strains against *Heliothis virescens* larvae was enhanced by prior dissolving in vitro, whereas such dissolving had no influence on the activity against *Pieris brassicae* larvae. This showed that solubility of the midgut juice is a factor codetermining crystal protein specificity and is determined by the interaction between the midgut environment and the crystal composition. A reduction in solubility is also speculated to be one potential mechanism for insect resistance (McGaughey & Whalon, 1992).

Like solubility, proteolysis can influence the activity of the proteins released from *B. thuringiensis* crystals. Haider et al. (1986) found that there was a differential processing of a single crystal protein by treatment with different insecticidal midgut juices. Proteins from *B. thuringiensis* serotype aizawai IC1, toxic to larvae of both lepidopteran and dipteran species, retain their activity after proteolytic treatment with lepidopteran gut juice. However, toxic fragments obtained by proteolysis with dipteran gut juices appeared only toxic to larvae of dipteran species.

The major determinant of the specificity of the crystal proteins are the postulated crystal protein receptors on the midgut epithelium cell membrane. Several immunological methods identified the luminal brush border of larval midgut as the primary target of the toxic fragments, and in vitro studies showed a correlation between toxicity and the occurrence of high affinity binding sites on the membrane vesicles (reviewed in Honée & Visser, 1993). The results suggested that high toxicity might result from high affinity or from the high number of receptors present.

The ability of a toxin to form a membrane pore presumably is also a determinant of its specificity. Wolfersberger (1991), demonstrated that the toxicity is correlated with the pore forming ability to inhibit K+ gradient driven amino acid uptake. Thus although receptor binding is an important step in the mechanism of toxic action of the crystal proteins, the efficiency of pore formation also influences the toxic potency of crystal proteins (reviewed in Honée & Visser, 1993).

Bta strain GC-91 contains genes for the different polypeptides: Cry1Ac, Cry1C, Cry1D and Cry2A, resulting in a main efficacy against insects of the lepidopteran order (refer to Point IIM 2.3.2). This order is one of the most de-

structive causes of economic loss to food crops in the world. Although *B. thuringiensis* δ-endotoxins are effective insecticidal proteins, there are several agronomically important insects that are less sensitive to their actions (MacIntosh et al., 1990). Delta-endotoxins were found to have insufficient effects against *Spodoptora* species such as the fall armyworm or beet armyworm (*S. frugiperda* and *S. exigua*) or the cutworm (*Agrotis ipsilon*).

Effects on Non-Target Organisms

B. thuringiensis strains are able to produce different δ-endotoxins, which are highly specific against certain target organisms. As mentioned above, special Cry protein groups determine the activity against different orders of insects. Bta strain GC-91 contains genes of the Cry1 and Cry2 group indicating an insecticidal activity only against lepidopteran and dipteran species (refer to Points IIM 2.3 and IIM 2.3.2).

Sears et al. (2001) demonstrated, that the effects of *B. thuringiensis* toxins are highly specific not only against an insect order but also against insect species within the same order. Their risk assessment, taking into account data from laboratory and field studies, indicate that the actual risk to monarch butterfly (*Danaus plexippus*) populations (larvae as well as adults) is negligible.

Owing to their specific mode of action (refer to Point IIM 2.3.2), Bt strains are unlikely to pose any hazard to humans or other vertebrates or to the great majority of non target invertebrates. Regular quality control testing during production ensures that products are free from impurities which could be hazardous to species other than the target pests (refer to Doc J-IIM 1.4.4). They are also safe for use in aquatic environments including drinking-water reservoirs for the control of mosquito, black fly and nuisance insect larvae (WHO, 1999).

Effects on Human and Animals

With the exception of case reports on ocular and dermal irritation, no adverse effects have been reported after occupational exposure to *B. thuringiensis* products (reviewed in WHO; reviewed in Siegel, 2001). General studies on humans and animals have shown that *B. thuringiensis* and the agricultural use of *B. thuringiensis* products have an excellent safety record. In the medical literature there is no case report associating commercially used *B. thuringiensis* directly with food poisoning (Siegel, 2001).

During the production process *B. thuringiensis* strain GC-91 is harvested at the end of its exponential growth phase and spores are spray dried to a technical powder by removing the culture filtrate subsequent to the fermentation process. Therefore *Bacillus cereus*-like toxins or other metabolites, released into the fermentation broth, are not likely to occur. Production batches are subsequently examined for microbial and non-microbial impurities in the Fermentation Monitoring Program, including quality control analysis. Neither microbial impurities nor toxic metabolites could be detected, indicating that there is no hazard for human and animals.

New information

Information submitted previously is still considered valid and sufficient to cover current data requirements.

Effects on human health

A literature search according to EFSA (2011)[2] aiming to retrieve references on possible toxic or pathogenic effects of Bta in humans was carried out but did not reveal any references which could potentially alter the risk assessment for Bta GC-91 as they are not changing the List of EU agreed endpoints for the strain. For more details of the search and the results please refer to Seehase 2016 (provided as KMA 5.1/01, see B6). References considered relevant and reliable are summarised under the respective data points in the human toxicity section B6. Available strain-specific studies confirm the absence of toxicity and pathogenicity of Bta GC-91 in test animals.

Already during first evaluation it was controversially discussed that *Bacillus thuringiensis*, as a member of the *B. cereus* group has the ability to produce *B. cereus*-like enterotoxins. This property could eventually lead to certain disease symptoms related to the diarrhoeal type of food borne poisoning usually caused by *B. cereus*. However, available information (including studies on different commercial Bt strains) indicate that commercial strains have a

[2] Guidance of EFSA: Submission of scientific peer-reviewed open literature for the approval of pesticide active substances under Regulation (EC) No 1107/2009. EFSA Journal 2011;9(2):2092

lower toxigenic potential compared to pathogenic *B. cereus* strains or would not be able to survive the human gastrointestinal passage. For more details please refer to B.2.8 below.

Effects on non-target species

A literature search according to EFSA (2011)[3] covering the last 10 years was conducted. The search strategy aimed to find all recent (from 2006 onwards) references that are of ecotoxicological relevance, regarding possible effects of Bta on non-target organisms. References considered relevant and reliable are summarised under the respective data points in the ecotoxicologcal section B9. For more details please refer to Schöbinger (2016, KMA 8.1/01, see B9). Available strain-specific studies confirm the absence of toxicity and pathogenicity of Bta GC-91 in the test animals.

Taken together it can be concluded that Bta GC-91 does not have any toxic or pathogenic effects in non-target organisms including birds, fish, daphnids, algae, aquatic plants, terrestrial plants, bees and other non-target arthropods, earthworms and soil microbial communities.

Effects on non-target lepidopteran species

By the literature search according to EFSA guidance no relevant reports referring to the risk of Bta to non-target lepidopteran species was obtained. See above for more details.

It can be assumed that the risk for non-target lepidopteran species following treatment with Bta is rather low as exposure only occurs in off-field areas, otherwise the species would be identified as pests and would be a potential target for Bta treatment. Exposure in off-field habitats is low and can only occur due to spray drift. A worst case exposure assessment is provided in the ecotoxicologcal section B9.

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

Information from DAR and DAR addendum (May 2007, February 2013)

The life cycle of Bta strain GC-91 is described on species level.

Bacillus thuringiensis is a ubiquitous micro-organism that colonizes a range of habitats and environments and can be found in two different stages. Under favourable conditions regarding moisture, temperature and nutrients, the basic metabolizing cell type is the vegetative cell that is actively growing and dividing. When a population of vegetative cells passes out of the exponential phase of growth, usually as a result of nutrient depletion, the differentiation of endospores begins. Endospores are formed intracellularly and are liberated after lysis of the parent cells. Typically one endospore is formed per vegetative cell. Mature spores have no detectable metabolism, a state that is described as cryptobiotic. They are highly resistant to environmental stresses such as high temperatures, strong acids, disinfectants, lack of nutrients and water, etc. Endospores have proven to be the most durable type of cell found in nature and in their cryptobiotic state of dormancy they can remain viable for extremely long periods of time. Applied as a spray, the δ-endotoxins are rapidly degradable and endospores are rapidly inactivated when exposed to UV radiation (Griego & Spence, 1978; Pusztai et al., 1991). An endospore will germinate and form a vegetative cell, when favourable conditions return for the growth of these cells and generations of vegetative cells will again thrive as long as the appropriate nutrients and environmental conditions exist. When nutrients begin to run out, endospores are again produced. Vegetative cells and endospores can each constitute colony-forming units (CFU). Any spore would have to germinate and become vegetative before a colony formation could take place. A spore is not able to replicate itself and form other spores. Before germination an endospore has to pass through an extended period of dormancy. This period can be shortened by exposure to a high temperature (WHO, 1999).

The transformation of dormant spores into vegetative cells can be described in three stages:

Activation: A reversible process that prepares the spore for germination and usually results from treatments like heating or exposure to certain chemical stimuli.

[3] Guidance of EFSA: Submission of scientific peer-reviewed open literature for the approval of pesticide active substances under Regulation (EC) No 1107/2009. EFSA Journal 2011;9(2):2092

Germination: The breaking of the spore stage involves the swelling, rupture of the spore coat, loss of resistance to deleterious environmental factors and increase of metabolic activity.

Outgrowth: Development into a vegetative cell by remerging new components from the spore coat.

New data

The information provided previously is considered acceptable to cover current requirements. Therefore, no new data are submitted for renewal of Bta GC-91 under Regulation (EC) 1107/2009.

B.2.5 Infectiveness, dispersal and colonisation ability

Information from DAR and DAR addendum (May 2007, February 2013)

The infectiveness, dispersal and colonization ability, are discussed at species level, as no indication emerges from literature that response to environmental stressors is a strain dependent trait for Bta.

During sporulation at the end of the exponential growth, *B. thuringiensis* produces inclusion bodies which are composed of proteins known as ICPs, Cry proteins or δ-endotoxins. These Cry toxins are highly toxic to a wide variety of important agricultural and health related insect pests (Bravo, 1997). Insecticidal crystal proteins (ICPs) from *Bacillus thuringiensis* have been used as biopesticides for the last 40 years. In recent years a number of insecticidal proteins expressed during the vegetative growth phase of *B. thuringiensis* have been identified (Estruch et al., 1996; Yu et al., 1997; Selvapandiyan et al., 2001). These secreted vegetative insecticidal proteins (VIPs) have also shown insecticidal activity against a wide spectrum of lepidopteran insects and also coleopteran pests (Estruch et al., 1996). The vip3A gene encodes a 88-kDa protein that is secreted into the supernatant fluid by *B. thuringiensis* and displays acute bioactivity towards the black cutworm, the fall and beet armyworm and a range of other lepidopteran insects which could mainly not be affected by the crystal *B. thuringiensis* δ-endotoxins (Estruch et al., 1996).

During the production process *B. thuringiensis* strain GC-91 is harvested at the end of its exponential growth phase and spores are spray dried to a technical powder by removing the culture filtrate subsequent to the fermentation process. Therefore vegetative insecticidal proteins, released into the fermentation broth, are not likely to occur and are therefore negligible.

New data

A literature review aiming to assess the fate of Bta in the environment was done within the frame of the preparation of the EFSA Scientific opinion on the Risk for public health related to *B. cereus* and other *Bacillus* spp. *including B. thuringiensis* in food stuff published in 2016[4]. From this document, the following can be summarised: In soils, half-life times between 100 and 200 days have been reported repeatedly. However, several authors recorded persistence of the spores, though at low levels for 2, 4, 7 or even 13 years. At leaves, half-life times are notably shorter ranging between 16 and 38 hours with more or less complete disappearance recorded between 15 and 60 days after application.

This is in agreement with the outcome of the subspecies specific literature search (Cornelese, 2016; KMA 7.1/01, see B8) carried out according to EFSA guidance[5] and presented in the environmental fate section B8.

Available information on Bt/Bta in general and strain specific data confirm that under normal circumstances, non-target organisms or humans are not infected by Bta GC-91.

More detailed information on the environmental requirements (temperature, pH, humidity, nutrition requirements) for survival and the possible effect of factors such as temperature, UV light, pH and the presence of certain substance on the stability of relevant toxins are described in the fate part (B8). It is generally agreed that *B. thuringiensis* and its secondary metabolites are not persistent in soil water and air. The mobility of *B. thuringiensis* and the spores can be considered limited. Factors restricting field persistence are UV-mediated degradation of spores, rain fall and plant growth (dilution effects), lack of nutrients and low humidity.

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

[5] Guidance of EFSA: Submission of scientific peer-reviewed open literature for the approval of pesticide active substances under Regulation (EC) No 1107/2009. EFSA Journal 2011;9(2):2092

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

Information from DAR and DAR addendum (May 2007, February 2013)

Information regarding closely related species

The *Bacillus cereus* group, also known as *B. cereus sensu lato*, is a subdivision of the Bacillus genus and consists of eight formally recognised species: *B. cereus sensu stricto, B. anthracis, B. thuringiensis, B. weihenstephanensis, B. mycoides, B. pseudomycoides, B. cytotoxicus* and *B. toyonensis*.

Note RMS: Applicant please provide a reference concerning the formally recognised members of the *Bacillus cereus* group.

B. thuringiensis is a member of the *Bacillus cereus* group which comprises closely related gram positive bacteria that exhibit highly divergent pathogenic properties. The most closely related species are *B. cereus* and *B. anthracis*.

B. cereus is a common spore forming, motile ubiquitous soil bacterium and an opportunistic human pathogen, causing contamination problems in the dairy industry and paper mills (Drobniewski, 1993).

B. anthracis, also a spore forming but not motile soil bacterium, is a virulent pathogen of mammals and is the causative agent of anthrax. Endospores enable the bacterium to survive indefinitely in soils and other environments. The spores are resistant to many disinfectants, but are found to be susceptible to 2% glutaraldehyde formaldehyde and 5% formalin (Anonymous, 2004).

B. cereus, *B. thuringiensis* and *B. anthracis* are distinguished principally by their plasmid content. These bacteria have highly similar 16S and 23S rRNA sequences indicating that they have diverged from a common evolutionary line relatively recently. Extensive genomic studies have shown that there is no taxonomic basis for separate species status (Carlson et al., 1996; Helgason et al., 2000). *B. thuringiensis* is characterized by its pathogenicity for lepidopteran insects (moths and caterpillars) and by production of an intracellular parasporal crystal in association with spore formation. The crystal proteins responsible for the insect pathogenicity are almost invariably plasmid encoded (Schnepf et al., 1998). Similarly, the pathogenicity of *B. anthracis* is also associated with the presence of two plasmids, pXOq and pXO2, the former coding for the anthrax toxin and the latter for capsule formation (Drobniewski, 1993). The virulence genes of *B. cereus*, on the other hand, are chromosomal (Prüß et al., 1999; Guttmann & Ellar, 2000; Ivanova et al., 2003). Unlike B. *thuringiensis* strains, *B. cereus* strains and *B. anthracis* strains lack parasporal inclusions. *B. anthracis* is additionally distinguishable from *B. thuringiensis* by its sensitivity to ampicillin, non-mobility and its requirement of thiamine for growth.

Among closely related species, provide information on pathogenicity to plants, animals or humans

Nothing is known about plant pathology of *B. cereus* and *B. anthracis*.

Bacillus anthracis

Anthrax is primarily a disease of herbivorous mammals. They become infected with *Bacillus anthracis* by ingesting the spores on forage plants. The spores can end up on the plants by being blown in dust from the soil or can be deposited on leaves by flies that have been feeding on anthrax-infected carcasses. The primary route of infection for herbivorous animals is therefore via the gut. *B. anthracis* spores are highly resistant to environmental extremes and can live in the soil, where they are mainly found, for many years. Humans can become infected with anthrax by handling animal products from infected animals or by inhaling anthrax spores from contaminated animal products.

In humans the disease takes one of three forms, depending on the route of infection.

- *Cutaneous anthrax*

accounts for more than 95 % of cases worldwide. It results from infections through skin lesions. Skin infection begins as a raised itchy bump that resembles an insect bite but within 1-2 days develops into a vesicle and then a painless ulcer, usually 1-3 cm in diameter, with a characteristic black necrotic (dying) area in the centre. Lymph glands in the adjacent area may swell. About 20% of untreated cases of cutaneous anthrax will result in death. Deaths are rare with appropriate antimicrobial therapy.

- Intestinal anthrax

results from ingestion of spores, usually in infected meat and is characterized by an acute inflammation of the intestinal tract. Initial signs of nausea, loss of appetite, vomiting, fever are followed by abdominal pain, vomiting of blood, and severe diarrhoea. Intestinal anthrax results in death in 25% to 60% of cases.

- Pulmonary anthrax

results from inhalation of spores. Initial symptoms may resemble a common cold. After several days, the symptoms may progress to severe breathing problems and shock. Pulmonary anthrax is usually fatal.

Anthrax can also be spread by eating undercooked meat from infected animals but is not known to spread from one person to another person. Communicability is not a concern in managing or visiting patients with pulmonary anthrax. Although anthrax can be found globally, it is more common in South and Central America, Southern and Eastern Europe, Asia, Africa, the Caribbean and the Middle East.

Bacillus cereus

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.

Note RMS: No reference has been provided concerning the information that the actual contribution of *B. cereus* and *B. thuringiensis* to gastrointestinal and non-gastrointestinal diseases is currently unknown. Applicant please address this point.

B. cereus is frequently isolated as a contaminant of various foods and can occasionally be an opportunistic human pathogen (Drobniewski, 1993; Helgason et al., 2000). The consumption of foods that contain more than 10^5 CFU *B. cereus* per gram may result in food poisoning (Kramer & Gilbert, 1989; Anonymous, 2005). However, in some outbreaks, lower numbers in the food (10^3 – 10^4 CFU/g) were reported (Anonymous, 2005; Rosenquist et al., 2005). Food borne poisoning caused by other *Bacillus* spp. has always been linked to high numbers of cells/spores in the food vehicle (equal to more than 10^6 CFU per g).

B. cereus causes two types of food borne intoxications.

The diarrhoeal type is manifested primarily by abdominal cramps and diarrhoea with an incubation period 8 to 16 hours after consumption of contaminated food. It is associated with a variety of foods, including meat, vegetable dishes, sauces, pastas, desserts and dairy products. It has been suggested that starch hydrolysis and the production of enterotoxins may be linked to the diarrhoeal symptoms (Shinagawa et al., 1984).

The "short-incubation" or emetic form of the poisoning is characterized by ingestion of rice- and pasta-based food. Fried rice is a leading cause of *B. cereus* emetic food type poisoning in the USA. *B. cereus* is frequently present in uncooked rice, related to ecological, economical and cultural factors: *B. cereus* is a common soil bacterium and contaminates rice plants in the paddy field. When the rice containing *B. cereus* spores is cooked, heat stable spores may survive. If cooked rice is subsequently held at room temperature, vegetative forms of *B. cereus* multiply and heat stable toxin is produced that can survive brief heating, such as stir frying. Toxin production is enhanced by the addition of protein in the form of egg or meat. The emetic syndrome has a short incubation period of 1 to 5 h, during which the emetic toxin induces nausea, vomiting, abdominal cramps, and also diarrhoea in about one-third of patients. Incubation periods as short as 15 min and as long as 12 h have been reported (Kramer & Gilbert, 1989). Supportive therapy is rarely needed and antimicrobial therapy is not required. The syndrome is self-limiting, and the patient recovers within 24 h. It resembles *Staphylococcus aureus* food poisoning in both its symptomatology and incubation period.

Among closely related species, provide information on formation of toxic metabolites: structure, stability, conditions under which they are formed, mode of action

Bacillus anthracis

Virulence of most *B. anthracis* strains is associated with two mega plasmids. The 110-MDa plasmid pXO1, encoding the structural genes pag, lef, and cya is required for synthesis of the anthrax toxin proteins, edema factor (cya),

lethal factor (lef), and protective antigen (pag). The smaller plasmid pXO2 (60 MDa) carries the genes capA, capB, and capC required for the synthesis of an antiphagocytic poly-D-glutamic acid capsule (Okinaka et al., 1999). These proteins act in binary combinations to produce the two anthrax toxins: edema toxin (a protective antigen and edema factor) and lethal toxin (a protective antigen and lethal factor).

The protective antigen (pag) first binds to receptors on host cells and is cleaved by a protease creating a binding site for either lethal factor (lef) or edema factor (cya).

The lethal factor is a protease that inhibits mitogen-activated kinase-kinase. At low levels, lef inhibits the release of proinflammatory cytokines such as interleukin-1 (IL-1), tumor necrosis factor-alpha, (TNF-alpha), and nitric oxide (NO). This may initially reduce immune responses against the organism and its toxins. But at high levels, lef is cytolytic for macrophages, causing release of high levels of interleukin-1 (IL-1), tumour necrosis factor-alpha (TNF-alpha) and nitric oxide (NO). Excessive release of these cytokines can lead to a massive inflammatory response and a shock cascade, similar to septic shock.

Edema factor is an adenylate cyclase that generates cyclic AMP in host cells, impairs phagocytosis, and inhibits production of TNF and interleukin-6 (IL-6) by monocytes. This most likely impairs host defences and is responsible for the fluid accumulation seen in anthrax.

The polypeptide capsule blocks phagocytosis.

Bacillus cereus

The formation of toxic metabolites is favoured by the storage of frequently heat-treated foods (recipe dishes, stews, purées...) and other food categories (salad, sprouts, orange juice, mayonnaise dressing) due to failure in refrigeration and/or too long delay before consumption of foodstuffs (Anonymous, 2005).

The diarrhoeal type of food poisoning is caused by enterotoxins formed by vegetative *B. cereus* in the small intestine (Granum & Lund, 1997). The fact that *B. cereus* spores can survive the conditions of the gastrointestinal tract and adhere to the gut epithelium may be another contributing factor (Drobniewski, 1993; Andersson et al., 1998). Enterotoxin activity is susceptible to proteolytic degradation and is thermo labile. The enterotoxins of *B. cereus* are synthesized during the late logarithmic phase at an optimum temperature of 32°C to 37°C and at a pH of 7.5.

At present, four endotoxins produced by *B. cereus* have been described: haemolysin (HBL), non-haemolysin (NHE), enterotoxin-T (bceT) and cytotoxin-K.

The most extensively studied enterotoxin is HBL, a three-component haemolysin (Beecher & Wong, 1994; 1997). It consists of two lytic components (L1 and L2) and a binding component (B). This toxin has hemolytic, dermonecrotic, and vascular permeability activities and is considered the primary virulence factor in B. cereus diarrhoea (Beecher et al., 1995a). The HBL operon includes the three known proteins, the lytic components L2 and L1, encoded by hblC and hblD, and the binding component B, encoded by hblA (Heinrichs et al., 1993; Ryan et al., 1997). HBL causes a potent dermonecrotic VP reaction in rabbit skin (Beecher & Wong 1994). It is also cytotoxic to Chinese hamster ovary cells (Beecher et al., 1995a) and retinal tissue (Beecher et al., 1995b). HBL produces a distinct and unusual discontinuous hemolysis pattern in blood agar gels (Beecher & Macmillan, 1990; 1991).

The combination of all three components of HBL is required for maximal activity against all of the cells and tissues so far tested. None of the individual components has exhibited any toxic activity.

Another enterotoxin gene, bceT, has been cloned and sequenced (Agata et al., 1995). It encodes only one component. This toxin exhibits Vero cell cytotoxicity and was positive in vascular permeability assays, but is not hemolytic. It causes fluid accumulation in the ligated rabbit ileal loop test and is lethal to mice after intravenous injection (Agata et al., 1995). To date, however, the enterotoxin has not been related to outbreaks of food borne disease.

Besides these two enterotoxins, *B. cereus* produces another three-component enterotoxin, called NHE (non-haemolytic enterotoxin) (Lund & Granum, 1996; 1997). The three components are different from the HBL. The three toxic proteins A, B and C are encoded by the three genes nheA, nheB and nheC organised in one operon. The toxin is non-hemolytic, but it has also been involved in food poisoning (Lund & Granum, 1997).

The most recently described enterotoxin from *B. cereus* is Cytotoxin K. It is a single component protein enterotoxin showing necrotic and haemolytic activity and is highly toxic to epithelial cells as shown with human Caco-2 cells, a cell line with mixed large and small bowel phenotypes (Hardy et al., 2001). *In vitro* studies have revealed that the toxin is able to form pores in lipid bilayers which might indicate that the mode of action of the toxin is the formation of pores in the epithelial cells, causing fluid release and destruction of the epithelial cells leading to necrosis (Hardy et al., 2001). In structure, as well as in mode of action, cytotoxin-K resembles that of *Staphylococcus aureus* α–toxin or *Clostidium perfringens* β–toxin (Lund et al., 2000).

The emetic toxin, or vomiting factor, is a highly stable peptide of less than 10 kDa which is thermo stable (surviving temperatures of 126°C for 90 min.), resistant to proteolytic degradation and stable at pH 2-11 (Granum, 2001). It is named cereulide and consists of a ring structure of three repeats of four amino and/or oxy acids (D-O-Leu-D-Ala-O-Val-L-Val)3. It is formed during the late exponential to stationary growth phase (and may be associated with sporulation) at optimal temperatures of 25 to 30°C but not above 40°C. It has been suggested that it may be a breakdown product from food stuffs supporting the growth of *B. cereus* (Turnbull 2005). Chemically, cereulide is closely related to the ionophore valinomycin (Agata et al., 1994). Mikkola et al. (1999) demonstrated that cereulides induce the formation of K+ channels, disturbing the osmotic balance and permeability of the membrane. Cereulide is at least as potent a K+ ionophore as valinomycin, suggesting that this property is responsible for the toxic effects. Regarding their study it is the first non proteinaceous ionophore shown to be involved in food poisoning.

Bacillus thuringiensis

Occasionally *B. thuringiensis* strains are responsible for human infections similar to those caused by strains of *B. cereus* (Jackson et al., 1995; Damgaard et al., 1997b). Prüß et al. (1999) found several *B. thuringiensis* strains encoding enterotoxic compounds, however the ability to produce enterotoxins and the toxic amount varies from strain to strain. In the medical literature there is no case report associating commercially used *B. thuringiensis* directly with food poisoning (Siegel, 2001).

What distinguishes the three members of the *B. cereus* group functionally are mostly genes carried on plasmids.The loss of the plasmid both of *B. anthracis* and *B. thuringiensis* make them indistinguishable to *B. cereus* by morphological and biochemical methods. The reverse process could also be possible; i.e. a *B. cereus* gaining a *B. thuringiensis* plasmid becomes also indistinguishable to *B. thuringiensis* by morphological and biochemical methods (González et al., 1982).

There are several methods available to verify micro-organisms at strain level. Molecular determination of specific *B. thuringiensis* strains can be performed by a combination of three techniques: For instance, comparison of results from hybridization experiments, cry PCR, and RAPD-analyses led to clear identification of *B. thuringiensis* ssp. *kurstaki* strain HD-1 (Hansen et al., 1998; Valadares de Amorim et al., 2001). Using these three methods together, also *B. thuringiensis* ssp. *aizawai* strains can be distinguished from other *B. thuringiensis* subspecies, as well as from *B. cereus* strains.

New data

As a member of the *B. cereus*-group, Bta is closely related to *B. anthracis* and *B. cereus*. In particular the close relationship to *B. cereus*, and a possible production of *B. cereus* enterotoxins related to the diarrheal type of *B. cereus* associated foodborne intoxication and difficulties to distinguish *B. thuringiensis* from *B. cereus* were identified as areas of concern by EFSA during first approval of Bta GC-91. For more details, please refer to Point MA 2.8 below.

The knowledge about phylogenetic relationships within the *B. cereus* group evolved considerably during the last 10 years. A literature review on the current taxonomy of the *B. cereus* group was done within the frame of the preparation of the EFSA Scientific opinion on the Risk for public health related to *B. cereus* and other *Bacillus* spp. including *B. thuringiensis* in food stuff published in 2016[6]. A summary of the information reported therein is provided below.

The phylogenetic relationship within the group is based on genome sequences while genetic determinants for specific traits, resulting in assignment to a certain species, like the Cry toxins for *B. thuringiensis* or the anthrax and emetic toxins for *B. anthracis* and *B. cereus*, respectively, are located at plasmids. Therefore, there is growing consensus in the scientific community that all strains of the *B. cereus* group should be considered a single species with the currently defined species being regarded as subspecies.

However, currently, the *B. cereus*-group consists of eight formally recognised species: *B. cereus sensu stricto* (or *B. cereus* as it is usually called), *B. anthracis, B. thuringiensis, B. weihenstephanensis, B. mycoides, B. pseudomycoides, B. cytotoxicus* and the new species *B. toyonensis*.

B. mycoides, B. pseudomycoides and *B. toyonensis* have not been described to have the potential to cause foodborne diseases. As *B. weihenstephanensis* do carry genes coding for endotoxins generally associated with *Bacillus cereus* (Stenfors et al., 2002) it may not be tenable to exclude the potential to cause foodborne diseases for *B. wei-*

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

henstephanensis B. cytotoxicus is known to produce the highly cytotoxic variant of CytK, the CytK-1, which is not produced by any of the other members of this group.

Note RMS: Could applicant confirm whether that *B. mycoides, B. pseudomycoides* and *B. toyonensis* have not yet been described to have the potential to cause foodborne diseases.

Various typing methods have been used to study the phylogenetic relationship of the members of the *B. cereus* group, including AFLP, MLEE and MLST. Guinebretiere et al., (2008) applying different genotypic and phenotypic criteria attributed 425 *B. cereus* group strains obtained from various ecological niches to seven major groups (I-VII). *B. thuringiensis* strains are present in five of these seven groups. Guinebretiere et al (2010), based on cytotoxicity tests and toxin gene distribution concluded that the ability of *B. cereus* group strains to cause food poisoning varies according to their phylogenetic affiliation with the groups defined in Guinebretiere et al. (2008), rather than with the species affiliation. The grouping has been confirmed by Tourasse et al. (2011) combining MLST and AFLP data of 2143 strains in a super tree. A procedure to assign *B. cereus* group strains to one of these seven genetic groups using the sequence of the panC gene is described in Guinebretiere et al. (2010) and an online tool has been developed which is available at https://www.tools.symprevius.org/Bcereus/english.php. The phylogenetic relationship of the 3193 analysed *B. cereus* group strains (including all fully sequenced and publicly available genomes) are now available in the HyperCat database (updated November 2015) at http://mlstoslo.uio.no/ (see **Figure 2.6-1,** obtained from EFSA Scientific Opinion, 2016[6]). Sequence data available for three of the Bt strains currently registered in Europe (Btk ABTS351, Btk HD1 and Btk Dipel) place all of them in group IV. This group is the largest one, with 498 *B. thuringiensis* and 316 *B. cereus sensu stricto* strains with a great part of them originating from soil, grassland and leaves from European countries, and from USA and Asia. Still, there are also *B. cereus* group strains isolated from dairies and food and from hospitals and patients in group IV. *B. anthracis* and most of the *B. cereus* isolates from patients, however, belong to group III which, together with group I, with which *B. cytotoxicus* affiliates, have the highest toxigenic potential. The risk for food poisoning is still considered high for group IV and decreases with group with V and VI, the latter with having the lowest or even no toxigenic potential Guinebretiere et al. (2010).

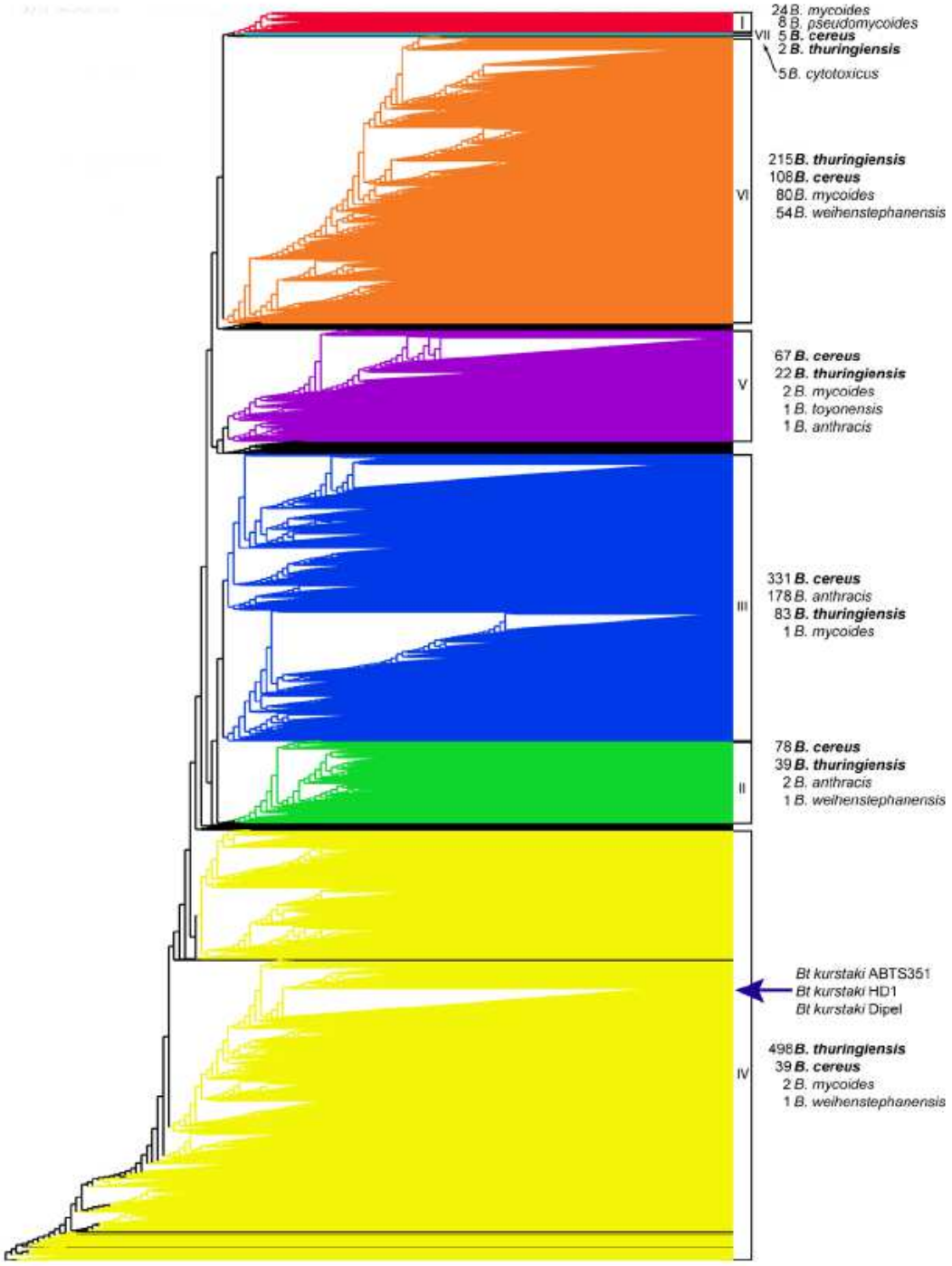

Figure 2.6-1 Phylogenetic relationship between 3193 *B. cereus* group isolates (http://mlstoslo.uio.no/) based on MLST and AFLP typing data. Strains from each group defined in Guinebretiere et al. (2008) are given different colours. Those outside any group are in black. Picture obtained from EFSA Scientific Opinion on the Risks for public health related to the presence of *Bacillus cereus* and other *Bacillus* spp. including *Bacillus thuringiensis* in foodstuffs (EFSA Journal, 2016[7]).

Cited literature abstracts:

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

Report:	KMA 2.6/01 - Guinebretiere, M.-H., Thompson, F.L., Sorokin, A., Normand, P., Dawyndt, P., Ehling-Schulz, M., Svensson, B., Sanchis, V., Nguyen-The, C., Heyndrickx, M., De Vos, P. (2008), published report Environ Microbiol., 10(4):851-865
Title:	Ecological diversification in the *Bacillus cereus* group
Abstract	The *Bacillus cereus* Group comprises organisms that are widely distributed in the environment and are of health and economic interest. We demonstrate an 'ecotypic' structure of populations in the *B. cereus* Group using (i) molecular data from Fluorescent Amplified Fragment Length Polymorphism patterns, ribosomal gene sequences, partial panC gene sequences, 'psychrotolerant' DNA sequence signatures and (ii) phenotypic and descriptive data from range of growth temperature, psychrotolerance and thermal niches. Seven major phylogenetic groups (I to VII) were thus identified, with ecological differences that provide evidence for a multiemergence of psychrotolerance in the *B. cereus* Group. A moderate thermotolerant group (VII) was basal to the mesophilic group I, from which in turn distinct thermal lineages have emerged, comprising two mesophilic groups (III, IV), an intermediate group (V) and two psychrotolerant groups (VI, II). This stepwise evolutionary transition toward psychrotolerance was particularly well illustrated by the relative abundance of the 'psychrotolerant' rrs signature (as defined by Pruss et al.) copies accumulated in strains that varied according to the phylogenetic group. The 'psychrotolerant' cspA signature (as defined by Francis et al.) was specific to group VI and provided a useful way to differentiate it from the psychrotolerant group II. This study illustrates how adaptation to novel environments by the modification of temperature tolerance limits has shaped historical patterns of global ecological diversification in the *B. cereus* Group. The implications for the taxonomy of this Group and for the human health risk are discussed.

****

Report:	KMA 2.6/02 - Guinebretiére, M.-H., Velge, P., Couvert, O., Carlin, F., Debuyser, M.-L., Nguyen-The, C. (2010), published report J Clin Microbiol., 48(9):3388-3391
Title:	Ability of *Bacillus cereus* Group Strains to cause food poisoning varies according to Phylogenetic affiliation (Groups I to VII) rather than species affiliation
Abstract	Cytotoxic activity levels of culture filtrates and toxin distributions varied according to the phylogenetic group (I to VII) within the *Bacillus cereus* group, suggesting that these groups are of different clinical significance and are more suitable than species affiliations for determining food poisoning risk. A first-line, simple online tool (https://www.tools.symprevius.org/Bcereus/english.php) to assign strains to the different phylogenetic groups is presented.

****

Report:	KMA 2.6/03 - Tourasse, N.J., Helgason, E., Klevan, A., Sylvestre, P., Moya, M., Haustant, M., Okstad, O.A., Fouet, A., Mock, M., Kolsto, A.-B. (2011), published report Food Microbiol., 28(2):236-244
Title:	Extended and global phylogenetic view of the *Bacillus cereus* group population by combination of MLST, AFLP, and MLEE genotyping data
Abstract	The *Bacillus cereus* group of bacteria includes species that can cause food-poisoning or spoilage, such as *B. cereus*, as well as *Bacillus anthracis*, the cause of anthrax. In the present report we have conducted a multi-datatype analysis using tools from the HyperCAT database (http://mlstoslo.uio.no/) that we recently developed, combining data from multilocus sequence typing (Tourasse et al., 2010), amplified fragment length polymorphism, and multilocus enzyme electrophoresis typing techniques. We provide a comprehensive snapshot of the

B. cereus group population, incorporating 2213 isolates including 450 from food and dairy products, in the form of both phylogenetic supertrees and superclusters of genetically closely related isolates. Our main findings include the detection of phylogenetically separated groups of isolates possibly representing novel evolutionary lineages within the *B. cereus* group, a putative new branch of *B. anthracis*, as well as new groups of related strains containing both environmental and clinical isolates. In addition, the multi-datatype analysis revealed to a larger extent than previously recognized that food-borne isolates can share identical genotyping profiles with strains from various other origins. Altogether, the global analysis confirms and extends the results underlining the opportunistic nature of *B. cereus* group organisms, and the fact that isolates responsible for disease outbreaks and contamination of foodstuffs can originate from various genetic backgrounds.

Report: Stenfors, LP; Mayr, R; Scherer, S; Granum, PE (2002). Pathogenic potential of fifty *Bacillus weihenstephanensis* strains. FEMS Microbiology Letters. Volume 215 (1), 1 September 2002, 47–51. https://dx.doi.org/10.1111/j.1574-6968.2002.tb11368.x

Abstract: The aim of this study was to evaluate the food poisoning potential of strains of the new species in the Bacillus cereus group, *B. weihenstephanensis*. Fifty strains were tested for cytotoxicity in a Vero cell assay, and 23 of the strains were also tested for production of enterotoxin components with commercial antibody kits, and for presence of enterotoxin gene components by polymerase chain reaction (PCR). The majority of the strains (72%) were not cytotoxic, although all of the strains that were tested with PCR and commercial kits had part of at least one of the *B. cereus* enterotoxins Hbl, Nhe or CytK.

B.2.7 Genetic stability and factors affecting it

Information from DAR and DAR addendum (May 2007, February 2013)

Genetic stability (mutation rate of traits related to the mode of action), factors affecting genetic stability; micro-organism's capacity to transfer genetic information to another

The extent and the frequency of the exchange of genetic material among *B. thuringiensis* strains and species is still under discussion and depends on several environmental factors and growth conditions.

Bacterial conjugation is a mechanism of genetic exchange that requires cell-to-cell contact in the mating medium. *B. thuringiensis* strains typically harbour multiple extra chromosomal plasmids, some of which contain insecticidal crystal protein (ICP) genes and some of which are capable of being transferred by a conjugation-like plasmid transfer process first described by González et al. (1982).

Plasmids are not essential for the survival of *B. thuringiensis* and certain plasmids in a given *B. thuringiensis* strain can be spontaneously cured, i.e. one or more plasmids may be lost. These characteristics facilitate the generation of strains that contain more potent ICP combinations or of strains having broadened insecticidal activity spectra (Carlton, 1993).

To improve maintenance of δ-endotoxin genes in the environment, the ability of *B. thuringiensis* to exchange plasmids is a very important factor. Plasmid exchange allows a greater amount of the desired Cry protein to accumulate in the strain's crystals. This occasionally results in increased effectivity range or potency. Though not well documented, patent applications claim that the combination of Cry gene types in a strain can have surprising synergistic activity or toxicity to a pest not killed by parental gene types. More expected is the result where a lepidopteran-active gene and coleopteran-active gene are harboured in the same bacterium, producing a strain with combined insect toxicity (Adang, 1991).

In the literature there are several studies describing the plasmid transfer of *B. thuringiensis* strains in a special mating broth. The frequency of plasmid transfer is considered to be dependent on the growth and the environmental conditions (Thomas et al., 2000). Thomas et al. (2000) showed that the mobilization temperature varied between *B. thuringiensis* strains. *B. thuringiensis* kurstaki HD1 mobilized pBC16 better at temperatures ≤ 30°C whereas pBC16 was mobilized better from *B. thuringiensis tenebrionis* at higher temperatures (between 30 and 37°C), prob-

ably due to differences in their plasmid composition. In contrast to the effect of temperature on plasmid transfer, no clear pH optimum was observed. Vilas-Bôas et al. (2000) showed that the acid pH of soil does not alter the conjugation process. A pH value ranging from alkaline to neutral condition did not hinder the conjugation process either (Furlaneto et al., 2000). However, both growth and plasmid transfer were inhibited at high salt levels. In experiments with mating broth using *B. thuringiensis* strains, Furlaneto et al. (2000) showed conjugation frequencies of 10^{-4} to 10^{-8}. These results are in agreement with those observed by other authors (Jarrett & Stephenson, 1990; Andrup et al., 1998; Vilas-Bôas et al., 1998).

All these studies implicate that the growth of donor and recipient cells together results in higher transfer ratio (Jarrett & Stephenson, 1990), and that optimal growth conditions result in the greatest transfer ratios and the highest numbers of transconjugants (Thomas et al., 2000).

When *B. thuringiensis* enters soil environments, most of the cells do not acquire enough nutrients to be able to sustain growth and they enter the sporulation phase (Thomas et al. 2000). Formation of spores is a major factor which prevents plasmid exchange with other *Bacillus* species. Thomas et al. (2000) could not detect any plasmid mobilization in soil at any time. Conjugation-mediated gene exchange between *B. thuringiensis* strains has been detected in soil, but generally only after some kind of manipulation, such as addition of bentonite clay (van Elsas et al., 1987) or soil sterilization (Vilas-Bôas et al., 1998). Although Haack et al. (1996) detected transfer in nonsterile soil, their data suggested that this happened when the sizes of the donor and recipient populations increased by as much as 2 log units. This is consistent for mobilization of pBC16 in broth cultures (Thomas et al., 2000) rather than in soil, in which *B. thuringiensis* and most bacilli occur predominantly as spores or decline in number.

There is no significant evidence for the horizontal plasmid transfer between *Bacillus* species and the extent of genetic exchange between strains under realistic natural conditions. Although gene exchange between *B. thuringiensis* and *B. cereus* could be demonstrated under laboratory conditions (González et al., 1982), Vilas-Bôas et al. (2002) could not find evidence that such transfer occurs randomly in sympatric natural isolates of *B. cereus* and *B. thuringiensis*. In a remarkable hierarchal population genetics analysis, Duncan et al. (1994) found that populations of *Bacillus subtilis* were genetically differentiated from sympatric populations of *B. licheniformis*, despite the known potential for genetic exchange between these species was only demonstrated in laboratory experiments. Helgason et al. (1998) demonstrated a very high diversity of *B. cereus* and *B. thuringiensis* strains in multilocus genotypes indicating that *B. cereus* and *B. thuringiensis* exhibit a low degree of clonality and that exchange of genetic material mainly occurs within each *Bacillus* taxa. The level of recombination between *Bacillus* species was found to be much lower than within the same species (Vilas-Bôas et al., 2002).

Battisti et al. (1985) reported mating between strains of *B. thuringiensis*, *B. cereus* and *B. anthracis* involving transference of small and large plasmids, but only under laboratory conditions in culture broth.

The risk of genetic exchange between *Bacillus* species in natural soils is considered to be very low due to several facts:

- Formation of spores is a major factor that prevents plasmid exchange. Genetic exchange occurs only in the vegetative phase of the bacterium. *B. thuringiensis* products mainly consist of endospores and sporulation only occurs under favourable conditions.

- Favourable conditions are not given due to surface application regarding the susceptibility of *B. thuringiensis* spores to UV radiation.

- The insertion of a *B. thuringiensis* plasmid into e.g. a *B. cereus* strain implicates no competitive advantage for *B. cereus* by producing insecticidal crystal proteins. Since *B. cereus* does not possess extrachromosomal plasmids, the exchange would be one-way. There is no risk for *B. thuringiensis* to achieve genetic parts of *B. cereus* encoding human toxic metabolites.

- Plasmids are not essential for the survival of *B. thuringiensis* and the loss of the plasmid only implicates the loss of insecticidal activity with no additional hazard for humans and the environment.

The potential for altering the Bta strain GC-91 via conjugation during the fermentation process is extremely low due to the shear force by aeration and agitation requirements of the Technical Powder fermentation. Conjugation requires a stable unity between mating bacteria which is broken by mechanical disruption. The identification of a cross-contaminated fermentation is done utilizing the Fermentation Monitoring Program (FMP). Should any of the analyses indicate the presence of contaminants or other impurities, the batch is isolated and investigated. Pending the results, the batch is either released (non-pathogenic, low titer), or inactivated and disposed of.

During the manufacture of the Technical Powder GC-91, human pathogens have never been associated with its manufacture, however indigenous bacteria have been found occasionally, albeit at a low titer, which is why the FMP is utilized to prevent or minimize their occurrence.

New data

As already stated during first evaluation of Bta GC-91, the possibility of exchange of genetic material before and during production of the technical material/end-use product is very unlikely. For manufacturing of Bta GC-91 technical material a culture maintenance program is applied to ensure that only genetically unchanged and pure subcultures of the mother culture are used for fermentation. This means that during the production fermentation steps cultures are used which are at most one to two passages away from the original lyophilised stock culture of the strain. Each passage from the original stock culture for preparing working culture lines is subjected to a battery of tests to ensure consistency of the starting material for fermentation. The genetic stability of the strain is ensured and verified by special quality control procedures which include a wide range of methods and procedures as described in volume 4. The potential for altering Bta GC-91 via conjugation during the fermentation process is extremely low due to the shear force by aeration and agitation requirements of the Technical Powder fermentation. Conjugation requires a stable unity between mating bacteria which is broken by mechanical disruption. In addition, only Bta GC-91 is present in the fermentation broth as contaminated batches identified during the fermentation monitoring will be discarded. A spontaneous loss of plasmids carrying the genetic information for Cry proteins would lead to a loss in insecticidal activity of the strain what would be directly detected by the bioassay applied to each production batch.

There is no reasonable experimental approach and no specific study guideline available to assess whether Bta GC-91 may transfer genetic material to other microorganisms after field application. Therefore, a literature search according to EFSA (2011)[8] guidance was carried out to obtain any new information relevant for this data point. The search was conducted using the DIMDI database provided by the German Institute of Medical Documentation and comprised searches in MEDLINE, BIOSIS, CAB and SCISEARCH databases. The search was done at subspecies level and included typical terms targeting potential mechanisms of genetic exchange occurring in bacteria. As Bta GC-91 is a transconjugant strain between a Bta and a Bt subsp. *kurstaki* strain, and both Bt subspecies considered very similar with their physiological requirements, the search was extended to Btk. Of the 64 obtained references five were subjected to full text assessment and four are summarized below as they were considered relevant and reliable. For more details, please refer to the literature review report by Süß (2016, KMA 2.7/01; see B.2.10).

Bizarri & Bishop (2008) studied whether Btk strains, either sprayed onto soil during sowing of plants, or directly applied to the phylloplane, are able to colonize leaves and to exchange genetic material in soil and in the phyllosphere. The experiments demonstrated that genetic exchange cannot be excluded but the authors raised three points which should be kept in mind when interpreting the results:

- The material sprayed onto the plants contained vegetative cells only, what is a rather artificial means as Btk is usually applied as a spore suspension. Hence, germination and growth would be required to allow a transfer of genetic material as observed in the study.
- The plasmid used in the study is very small compared to the ones harbouring the determinants for Cry proteins.
- The determined transfer ratio does not allow concluding whether it was a result of multiple transfer events or of extensive multiplication of a few transconjugants carrying the marker plasmid.

Yuan et al. (2007) studied the potential of gene exchange between a Btk donor strain and members of the *B. cereus* group as recipient strains (Btk, *B. cereus* and *B. mycoides*) in Lepidopteran larvae. The study indicates that dead insect larvae are an appropriate niche for germination and growth of Btk spores upon infection via the diet. Also exchange of a plasmid carrying genetic determinants for a Cry protein and erythromycin resistance was observed. However, compared to the high infectious dosage of 10^9 spores/g diet, the transfer ratio was rather low (9.3×10^{-7} to 1.2×10^{-6} CFU/donor) in particular when compared to possible exposure for Bta GC-91.

Maximum exposure = maximum density of spores in the spraying liquid:

- Maximum application rate/min water volume = 2.0 kg Agree 50 WG in 500 L water (use in tomato)
- Considering 3.3×10^{13} CFU/kg this would result in 1.3×10^{11} CFU/L = 1.3×10^{8} CFU/mL in the spraying liquid

[8] Guidance of EFSA: Submission of scientific peer-reviewed open literature for the approval of pesticide active substances under Regulation (EC) No 1107/2009. EFSA Journal 2011;9(2):2092

- Assuming a density of 1 g/mL this would correspond to 1.3×10^8 CFU/g for the potential donor strain what is still one order of magnitude below the rate used in the study.

In addition, the same high density of indigenous *Bacillus* spp. to which genetic material could be transferred would be needed, but will never occur at plants. Please refer to **Table 2.1.2-1** for natural background levels of Bt on plants.

Santos et al. (2010) also demonstrated that Btk is able to transfer a plasmid to closely related species under *in vitro* and *in vivo* conditions in *Bombyx mori* larvae. However, also here, the amount of Btk applied was extremely high. Under artificial conditions cultures with an OD of 1.0 were used and for experiments in *B. mori*, the larvae fed on leaf discs containing 10^9 CFU of each, the donor and the recipient strain. It is clear that such high levels are applied in laboratory studies to assess whether the possibility for gene transfer exists but it does not reflect the conditions occurring after field application of Bta GC-91.

Donnarumma et al. (2010) demonstrated that gene fragments of commercial Btk strains artificially introduced into soil can be transferred to indigenous soil bacteria. The conditions used in this study reflect realistic conditions upon use of a Btk product in forestry as samples were taken five years after spraying campaigns in cork oaks in Sardinia, Italy. However, also here, transfer frequencies were extremely low and the transfer of a certain gene fragment, in this case a fragment of the *cry1A* gene, did not have any consequences as the recipient strains were not able to produce the corresponding insecticidal proteins.

In 2013, EFSA published an External Scientific Report on Literature search and data collection for Risk Assessment on human health[9] which also covered the issue of genetic stability of microorganisms used a plant protecting agents. In this report two further publications on genetic stability of Btk are mentioned which are Zhan et al. (2007) and Yuan et al. (2010). Both references were also obtained during the search for Bta GC-91 but were excluded for relevance as they refer to conjugation under laboratory conditions in nutrient broth only. In addition, there are several publications on possible exchange of genetic material of Bt subsp. *israelensis*. The only report which could have any relevance might be a study of Van der Auwera et al. (2007) studying the conjugative behaviour of Bti in LB medium, milk and rice pudding. The authors used the pXO16 and paW63 conjugative system and the mobilisable plasmid pC194 in bi- and tri-parental mating experiments in the different matrices. They found that plasmids were mobilised and transferred via conjugation at significant levels in pudding and milk. Tenfold higher transfer frequencies were observed in milk compared to LB broth. However, the food matrices studied are considered not relevant for use of Bta GC-91 as plant protecting agent in vegetable, grape and orchard crops. It is therefore not further considered here. Another study tested whether DNA can be transferred between bacteria within a rat's intestinal tract, but no evidence was found for this (Wilcks and Jacobsen, 2010).

None of the literature references from the EFSA External Scientific Report is submitted with the dossier as they were all excluded for relevance. Therefore, no study summary and no abstract is provided. The full bibliography of the references is given here below.

Full bibliography of references on genetic stability from EFSA External Scientific Report (Hackl, et al., 2015; Table S7):

Van der Auwera, G.A., Timmery, S., Hoton, F., Mahillon, J., 2007. Plasmid exchanges among members of the *Bacillus cereus* group in foodstuffs. International Journal of Food Microbiology, 113, 164-172.

Wilcks, A., Jacobsen, B.B., 2010. Lack of detectable DNA uptake by transformation of selected recipients in monoassociated rats. BMC research notes, 3, 49.

Yuan Y, Zheng D, Hu X, Cai Q and Yuan Z, 2010. Conjugative transfer of insecticidal plasmid pHT73 from *Bacillus thuringiensis* to *B. anthracis* and compatibility of this plasmid with pXO1 and pXO2. Applied and Environmental Microbiology, 76, 468-473.

[9] Evelyn Hackl, Margit Pacher-Zavisin, Laura Sedman, Stefan Arthaber, Ulla Bernkopf, Günter Brader, Markus Gorfer, Birgit Mitter, Aspasia Mitropoulou, Monika Schmoll, Willem van Hoesel, Elisabeth Wischnitzky, and Angela Sessitsch, 2015. Literature search and data collection on RA for human health for microorganisms used as plant protection products Reference. EFSA supporting publication 2015:EN-801. 173 pp.

Zhang Q, Sun M, Xu Z and Yu Z, 2007. Cloning and characterization of pBMB9741, a native plasmid of *Bacillus thuringiensis* subsp. *kurstaki* strain YBT-1520. Current microbiology, 55, 302-307.

The summaries of the literature references obtained by the literature search done for Bta GC-91 are provided below.

Note RMS: Concerning the literature search in regard to whether or not Bta GC-91 may transfer genetic material to other microorganisms after field application the search strategy included transformation, transduction, and conjugation but not transposon.

Besides transformation (uptake of exogenous DNA), conjugation (exchange of DNA between bacterial cells) and transduction (mediated by viruses) another driver for DNA-exchange between two bacterial cells are so-called integrative and conjugate elements (ICEs). These mobile genetic elements which are also referred to as conjugative transposons are able to switch their location within the genome but can also be transferred into a recipient genome via conjugation. ICEs often contain antibiotic-resistance, symbiosis or virulence genes, thereby contributing to bacterial evolution by conferring new phenotypes to their recipients.

Applicant please addresss this point.

RMS Conclusion:

From the references obtained by the literature search for Bta GC-91 it can be concluded, that transfer of genetic material cannot be completely ruled out upon use of the strain as pest control agent in agricultural settings but the likelihood is rather low because the event requires germination and growth of the applied GC-91 spores at a high level and the presence of competent recipient vegetative cells at a high level. Even if the rare event of DNA transfer occurs the risk is acceptable as Bta GC-91 does not have the capacity to produce any other compounds than indigenous Bt already present in the environment. In addition, Bta GC-91 is not a multi-resistant strain. This means that a transfer of genetic material would not related to a spread of antibacterial resistance.

Summary/abstracts of cited literature references:

Report:	KMA 3.5/01 - Süß, J. (2016)
Title:	Literature review on *Bacillus thuringiensis* subsp. *aizawai* GC-91 Biological properties
Document No:	2281385_MA_02_01
Abstract	Not available

****

Report:	KMA 2.7/02–Bizzarri, M. F., Bishop, A. H. (2008), published report Toxins 2014, 6(12), 3296-3325 Microb Ecol., 56(1):133-139
Guideline:	Not specified
GLP:	No
Abstract	Seedlings of clover (*Triflorium hybridum*) were colonized by *Bacillus thuringiensis* when spores and seeds were co-inoculated into soil. Both a strain isolated in the vegetative form from the phylloplane of clover, 2810-S-4, and a laboratory strain, HD-1, were able to colonize clover to a density of about 1000 CFU/g leaf when seeds were sown in sterile soil and to a density of about 300 CFU/g leaf in nonsterile soil. A strain lacking the characteristic insecticidal crystal proteins produced a similar level of colonization over a 5-week period as the wild type strain,

indicating that crystal production was not a mitigating factor during colonization. A small plasmid, pBC16, was transferred between strains of *B. thuringiensis* when donor and recipient strains were sprayed in vegetative form onto leaves of clover and pak choi (*Brassica campestris* var. *chinensis*). The rate of transfer was about 0.1 transconjugants/recipient and was dependent on the plant species. The levels of *B. thuringiensis* that naturally colonized leaves of pak choi produced negligible levels of mortality in third instar larvae of *Pieris brassicae* feeding on the plants. Considerable multiplication occurred in the excreted frass but not in the guts of living insects. Spores in the frass could be a source of recolonization from the soil and be transferred to other plants. These findings illustrate a possible cycle, not dependent on insect pathology, by which *B. thuringiensis* diversifies and maintains itself in nature.

Materials & Methods

The experiment assessing plasmid transfer between two Btk strains was conducted under greenhouse conditions. Therefore, surface sterilized clover and pak choi seedling were planted onto sterilized and unsterilized soil samples and grown for 4 weeks at 25°C and a 12:12 hours photoperiod. To produce an appropriate donor strain for the experiment, Btk isolate 65-S-35, previously obtained from clover, was electroporated with the tetracycline encoding plasmid pBC16. pBC16 is a small plasmid that can be mobilized if the donor strain also contains one or more large conjugative plasmids. The recipient was a streptomycin-resistant mutant of strain 2810-S-4 which was also obtained from clover. The two strains were cultured separately, harvested, washed in phosphate buffered saline (PBS), mixed equally to obtain suspensions with approximately 106 CFU/mL and sprayed onto the plants till run-off. It is noteworthy that spraying with done with vegetative cells. One week after spaying, leaf samples were collected, weighed, stomached for 10 min in PBS, and pelleted. The pellet was re-suspended in PBS and submitted to plating on peptone dextrose agar (PDA) containing tetracycline, streptomycin or both substances to detect donor, recipient and transconjugant colonies. Other experiments of the study are not presented here.

Findings

Preliminary experiments introducing donor and recipient strain into soil at the time of sowing clover and pak choi resulted in transconjugants in the seedlings as well as in the soil (data not shown in report). When sprayed onto leafs, transconjugants were obtained from clover and pak choi grown on sterilised and clover grown in non-sterilised soil. The transfer ratio was approximately 0.1 in all cases. No transconjugants were obtained from pak choi grown in non-sterilised soil. Please see **Figure 2.7-1** below**.**

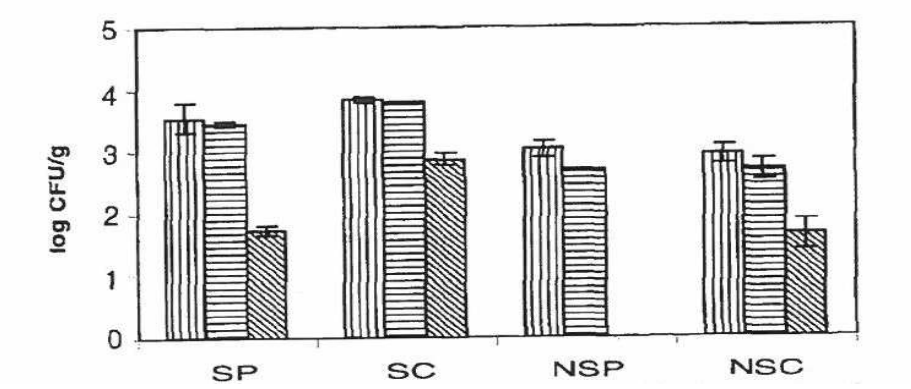

Figure 2.7-1 Transfer of plasmids between Btk strains in the phylloplane on pak choi (SP) and clover (SC) grown on sterile and non-sterile (NSP and NSC) soils. Recipient strain (vertical stripes), donor strain (horizontal stripes), transconjugant strain (diagonal stripes).

Discussion & conclusion

This study indicates that Btk strains, when sprayed onto plant leaves might be able to exchange genetic material to a certain extent. However, according to the authors, three points need to be kept in mind:

- The material sprayed onto the plants contained vegetative cells only, what is a rather artificial means as Btk is usually applied as a spore suspension. Hence, germination and growth would be required to allow a transfer of genetic material as observed in the study.

- The plasmid used in the study is very small compared to the ones harbouring the determinants

for cry proteins.

- The determined transfer ratio does not allow concluding whether it was a result of multiple transfer events or of extensive multiplication of a few transconjugants carrying the marker plasmid.

****

Report:	KMA 2.7/03–Yuan, Y.M., Hu, X.M., Liu, H.Z., Hansen, B.M., Yan, J.P., Yuan, Z.M., (2007), published report Arch Microbiol.,187(6):425-431
Title:	Kinetics of plasmid transfer among *Bacillus cereus* group strains within lepidopteran larvae
Guideline:	Not specified
GLP:	No
Abstract:	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.
Materials & Methods	Donor and recipient strains: The erythromycin-resistant strain KT0 (pHT73-EM^R) carrying a *cry1Ac* gene was used as donor strain in the experiments. All recipient strains were isolated before (see **Table 2.7-1** below) and were all rifampicin resistant. The strains were cultivated in Luria Bertani medium (LB) with appropriate antibiotics (erythromycin and rifampicin) at 100 µg/mL. For the experiments vegetative and sporulated cultures of the donor and the recipients were prepared. The cultures were pelleted and washed and adjusted to a final density of 10^{10} CFU/mL. The cultures were than mixed with artificial diet to get a final density of 10^9 CFU/g insect diet. The target insects used were stable, susceptible *S. exigua*, *H. armigera* and *P. xylostella* colonies. The experiments were carried out in 24-whole-plates. The third instar larvae of the different colonies were first infected with the donor and than with the respective recipient strain for 2 hours each via the diet. The infection procedure was repeated after 48 hours. Parallel treatments without donor or recipient strains were used as control. The infections were done in two replicates and repeated at three different days. At least 96 larvae were infected in each strain combination. The plates were incubated at 26 ± 1°C, humidity of 85% and a photoperiod of 12:12 hours. Five infected larvae were taken at time point 0, 24 and 48 hours during the infection and then every 4th hour post infection. Sampled larvae were weighted, surface sterilized (70% ethanol), washed and homogenized. Dilution suspensions (10^{-2} - 10^{-6}) were plated on nutrient agar containing erythromycin, rifampicin or a combination of both to enumerate donor, recipients and transconjugant strains, respectively. In parallel, dilution suspensions were subjected to heat treatment (70°C for 20 min) before plating to enumerate sporulated cultures of donor, recipient and transconjugant strains. In addition to CFU counts, DNA was extracted to carry out random amplified polymorphic DNA analyses (RAPD) and PCR analyses of erythromycin resistance and *cry 1Ac* genes. Spore crystal mixtures of sporulated cultures were collected by centrifugation and submitted to SDS page protein analysis. Statistical analyses were applied to assess significance of the plasmid transfer.

Table 2.7-1 Overview of recipient strains used in transconjugation experiments

Strains	Characteristics of recipients	Source/referenc
B. thuringiensis subsp. *kurstaki* AW05R	Containing pHT73, but cured of pAW63	Hu et al. (2004)
B. thuringiensis subsp. *kurstaki* DMU67R	Indistinguishable from *B. thuringiensis* subsp. *kurstaki* HD1 by RAPD fingerprinting	Pedersen et al. (
B. cereus MADM 1279R	Efficient in vitro conjugation recipient, isolated from sugar candy	Hu et al. (2004)
B. cereus CIP 5832R	Probiotic strain	Hu et al. (2004)
B. mycoides DSMZ 2048R	Type strain	Hu et al. (2004)
B. cereus AND2022R	*B. cereus* ATCC 33018R, efficient in vitro conjugation recipient	Hu et al. (2004)

Findings

H. armigera, *S. exigua* and *P. xylostella* larvae infected with sporulated cultures of the donor and recipient strains started dying at about 44, 44 and 50 h with 90% of the larvae found dead after 50, 52 and 56 h, respectively. Newly dead and alive larvae only contained spores. Highest spore germination levels were recorded in dead larvae at 52 - 56 hours after infection and biomass of vegetative cells increased. No insecticidal activity and no increase in biomass were observed in larvae infected with vegetative cells throughout the 5 days post infection period. In the contrary, CFU counts decreased by three orders of magnitude after the 2nd infection cycle from 4.9×10^5 CFU/mg larvae at 48 h to 2.6×10^2 CFU/mg larvae at 96 h.

Plasmid transfer was detected between the donor and fours of the six recipient strains (Btk AW05R, *B. cereus* MADM1278R, *B. cereus* AND2022R and Btk DMU67R). No transconjugants were detected after larvae have fed on vegetative cultures. At the treatment dosage of 10^9 spores/g diet spore numbers of donor strains in the different larvae reached values between $(4.5 \pm 2.1) \times 10^5$ to $(3.7 \pm 1.88) \times 10^6$ CFU/mg larvae at 48 h. CFU counts of recipient strains were in the same range ($(3.7 \pm 1.03) \times 10^5$ to $(2.2 \pm 0.61) \times 10^6$ CFU/mg larvae). Due to spore germination and proliferation the CFU numbers of donor and recipients increased by approximately 2 orders of magnitude within 48 h after infection (donor strain: $(4.3 \pm 1.28) \times 10^7$ to $(2.4 \pm 0.15) \times 10^8$ CFU/mg larvae, recipient strains: $(4.3 \pm 1.27) \times 10^6$ to $(8.4 \pm 3.95) \times 10^7$ CFU/mg larvae). Please also refer to **Figure 2.7-2.** Plasmid transfer was detected initially at 44-52 h in the dead larvae. The biomass of the transconjugants increased from 3.4 - 49.1 CFU/mg larvae after 72 h to 11.4 - 71.9 CFU/mg larvae after 120 h. Highest transfer ratios were in the range of 9.3×10^{-7} to 1.2×10^{-6} CFU/donor).

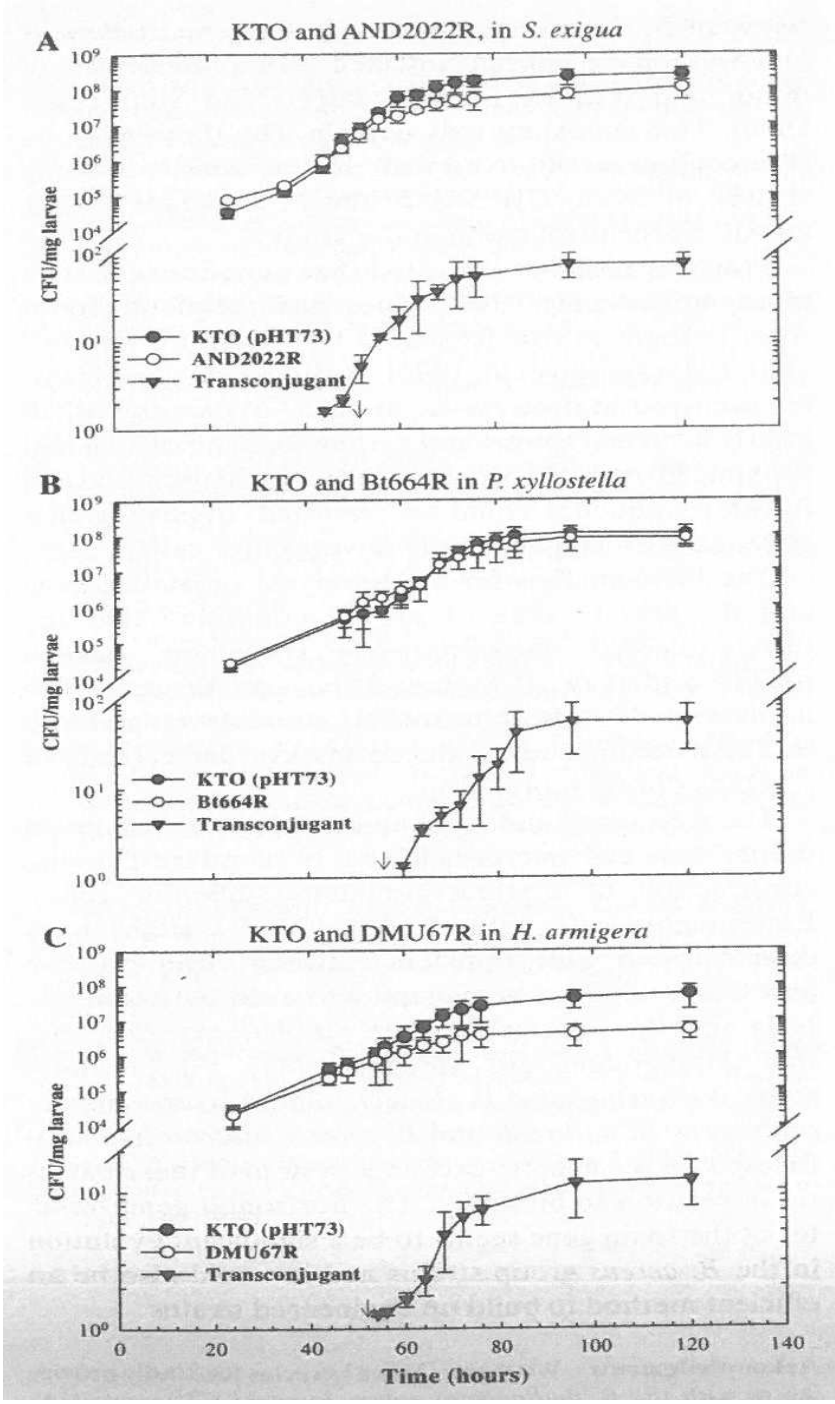

Figure 2.7-2 Population development of donor, recipients and transconjugant strains in three insect species. Infection took place via artificial diet containing 10^9 spores/g of the donor (KTO) and recipient strains. The arrow indicates the time point of spore germination.

Discussion & conclusion

The study proves that after infection, dead insect larvae are a suitable niche for germination and proliferation of Btk spores and that an exchange of plasmids among members of the *B. cereus* group may occur during vegetative growth of the populations at low levels.

****

Report: KMA 2.7/04– Santos, C.A., Vilas-Bôas, G.T., Lereclus, D., Suzuki, M.T., Angelo, E.A., Arantes, O.M.N., (2010), published report
J Invertebr Pathol, 105(2):171-175

Title: Conjugal transfer between *Bacillus thuringiensis* and *Bacillus cereus* strains is not directly correlated with growth of recipient strains

Guideline: Not specified

GLP: No

Abstract: *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-Em) 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.

Materials & Methods: *B. thuringiensis* subsp. *kurstaki* strain KT0 (pHT37-EMR) was used as donor strain in all experiments. The strain harbours the 75 kb resident plasmid pHT37-EMR carrying the *cry1A* gene tagged with an *ermC* gene conferring erythromycin resistance. One Bt strain lacking *cry1* and seven *B. cereus* strains served as recipient strains, all of them carrying either a streptomycin or rifampicin resistance. Details on the strains used are summarized in **Table 2.7-2** below. Vegetative growth was determined in Luria Berthani medium (LB) and incubation at 30°C with continuous shaking for 72 hours. Sporulated cultures were prepared in Bacto-Pepton medium (BP) using the same cultivation conditions. Isolation of streptomycin and rifampicin-resistant strains was done by adding the antibiotics to agar plates at 200 and 100 µg/mL, respectively. Btk KT0 was cultured in LB medium containing 100 µg/mL erythromycin. Plasmid stability in the exconjugants during vegetative growth was assessed in brain-heart infusion medium (BHI).

Table 2.7-2 Properties of Bt and *B. cereus* strains used in the study

Strains	Characteristics	Source
B. thuringiensis var. *kurstaki* KT0 (pHT73-Em^R)	Donor, Cry$^+$, Em^R	[a]
B. thuringiensis var. *thuringiensis* 407-1	Recipient, Pig$^+$, Cry$^-$, Sm^R	[b]
B. cereus D1 4430	Recipient, Sm^R	[c]
B. cereus 569	Recipient, Sm^R	[c]
B. cereus 388	Recipient, Rif^R	[c]
B. cereus 433	Recipient, Rif^R	[c]
B. cereus ATCC 14579	Recipient, Sm^R	[d]
B. cereus ATCC 10987	Recipient, Sm^R	[d]
B. cereus MADM 1279R	Recipient, Rif^R	[e]

Cry$^+$: produces an insecticidal crystal; Cry$^-$: does not produce an insecticidal crystal; Pig$^+$: produces a brown pigment; Em^R: erythromycin resistant; Sm^R: streptomycin resistant; Rif^R: rifampicin resistant.
[a] Vilas-Bôas et al. (1998).
[b] This study.
[c] INRA-Génétique Microbienne et Environnement, Guyancourt/France.
[d] American Type Culture Collection, Rockville, MD, USA.
[e] National Environmental Research Institute, Roskilde/Denmark.

Plasmid transfer in broth

Equal volumes (250 µL) of the donor and recipient strains were grown in LB medium to an OD600

of 1.0, added to 7 mL of fresh, pre-warmed LB medium and incubated for 2 h. Appropriate dilutions were plated on LB plates containing erythromycin, streptomycin or rifampicin or combinations of erythromycin and streptomycin/rifampicin to detected donor, recipient and transconjugant strains. Mating that did not yield exconjugants were repeated and the entire mating broth was concentrated and plated.

Exconjugants derived from recipient strains of Btt 407-1 were tested for resistance to erythromycin, streptomycin, the presence of crystals and production of production of brown pigment; those derived from *B. cereus* were tested for resistance to erythromycin and streptomycin or rifampicin (depending on the property of the recipient) and the presence of crystals. Only strains that successfully received the pHT73-Em^R on one-recipient matings were submitted to two-recipient mating experiments. These systems were composed of the donor and two recipient strains, Btt 407-1 and a rifampicin-resistant *B. cereus* strain. Mating conditions and assessments were the same as described above.

Plasmid transfer on nitrocellulose filters

Broth cultures of donor and recipient strains (0.5 mL), grown to an OD_{600} of 1.0 were mixed on membrane filters. The membranes were dried and transferred to LB agar without antibiotics. Incubation was done at 30°C for 24 hours. The mating mixture was resuspended in 1 mL of LB and spread on selective LB agar plates containing appropriate mixtures of antibiotics to detect exconjugants. In addition, dilutions were plated onto LB ager containing rifampicin and streptomycin to determine CFU numbers of recipient strains.

Bacterial growth and plasmid transfer in B. mori

3rd instar silkworm larvae, maintained in individual boxed, were fed on a mulberry leaf disk, containing 10^9 CFU of the recipient strain. After 12 h at 25°C, the larvae were transferred to leaves treated with 10^9 CFU of the donor strain, sporulated culture without heat treatment and incubation was continued until the larvae died. In the case of larvae feeding on non-toxic strains (all expect for the donor strain) were killed mechanically. The experiment was performed in triplicate. Larvae feeding on untreated leaf disks served as control and were also killed mechanically. Bacteria were recovered from seven dead larvae immediately after they have died/were killed and from seven additional dead larvae 24 h later. The larvae were crushed and diluted in sterile saline. Untreated and heat-treated dilutions were plated onto LB plates supplemented with erythromycin, rifampicin, streptomycin or appropriate combinations thereof to count donor, recipient and exconjugant strains. Two-recipient matings were carried out as described above with the same strain combinations as described for two-mating approaches in broth.

Plasmid stability

Exconjugant strains were tested for segregational stability of pHT73-Em^R during vegetative growth by exponential growth for 100 generations by repeated sub-culturing (every 10-15 generations) in selective BHI medium.

Findings

The various strains behaved differently in the *B. mori* larvae. All sampled larvae contained between 10^3 and 10^5 CFU/larvae at T_0. Vegetative cells of the Bt strains and some of the *B. cereus* strains grew significantly between T_0 and T_{24}. However, only the two Bt strains showed complete spore germination in the dead larvae. More details on the growth behaviour are summarized in **Table 2.7-3** below.

Table 2.7-3 Development of *B. cereus* and *B. thuringiensis* strains in carcasses of *B. mori*. Only Btk KT0 was toxic to the larvae. In all other cases the larvae were killed mechanically. Times 0 and 24 represent the time in hours after larval dead.

Strains	Bacterial growth in *B. mori* carcasses (CFU larva^{-1})[a]			
	Total CFU[b]		Number of spores[c]	
	t_0	t_{24}	t_0	t_{24}
B. thuringiensis var. *thuringiensis* 407-1	[a]1.1 ± 0.30 × 10^4	[b]6.8 ± 0.36 × 10^6	[a]1.9 ± 0.28 × 10^2	[b]0
B. cereus 433	[a]6.3 ± 0.69 × 10^4	[b]5.6 ± 0.73 × 10^5	[a]3.5 ± 0.70 × 10^3	[b]2.9
B. cereus 388	[a]9.5 ± 0.55 × 10^3	[a]1.5 ± 0.52 × 10^4	[a]1.9 ± 0.43 × 10^3	[a]4.7
B. cereus MADM 1279R	[a]7.2 ± 0.37 × 10^3	[a]1.1 ± 0.61 × 10^4	[a]7.5 ± 0.05 × 10^2	[a]1.3
B. cereus D1 4430	[a]1.5 ± 0.96 × 10^5	[b]2.8 ± 1.25 × 10^6	[a]1.3 ± 0.59 × 10^4	[b]1.0
B. cereus ATCC 10987	[a]7.4 ± 0.87 × 10^4	[a]3.1 ± 0.56 × 10^4	[a]1.1 ± 0.72 × 10^4	[b]8.9
B. cereus 569	[a]6.3 ± 0.66 × 10^4	[b]4.2 ± 0.26 × 10^5	[a]3.7 ± 0.71 × 10^4	[b]4.5
B. cereus ATCC 14579	[a]1.0 ± 0.41 × 10^4	[a]2.6 ± 0.40 × 10^4	[a]4.2 ± 1.0 × 10^2	[a]4.4
B. thuringiensis var. *kurstaki* KT0 (pHT73-Em^R)	[a]1.2 ± 0.38 × 10^5	[b]6.6 ± 0.36 × 10^8	[a]1.4 ± 0.55 × 10^4	[b]0

±Standard error (n = 3).
[a] Same letters indicate no significant difference as assessed by the Tukey test (P < 0.05).
[b] Samples without heat treatment.
[c] Samples with heat treatment (80 °C for 20 min).

In broth, only four of the recipient strains produced exconjugants in the one-recipient mating experiments, namely, Btt 407-1, and the *B. cereus* strains 433, 388 and MADM 1279R. Mating rates (= ratio of exconjugants to recipient cells = ER/R) ranged between 5×10^{-6} and 1.7×10^{-4}. When submitted to two-recipient experiments in broth with the Btt 407-1 strain used in all combinations, there were no differences detected in the mating rates for the three settings. When combined with *B. cereus* MADM 1279R, neither Btt 407-1 nor the *B. cereus* strain produced exconjugants. In experiments on nitrocellulose filters all strains produced transconjugants but the range of transfer frequencies was much wider compared to those observed in broth (10^{-2} to 10^{-9}). In the *in vivo* experiments in *B. mori* exconjugants were not detected for all recipients strains (two out of the eight failed) but the transfer frequencies were highest under these conditions (10^{-4} - 10^{-1} EC/R). The recipient strain with the best conjugation capacities (*B. cereus* 433) was not the one that grew best in *B. mori* larvae. The authors therefore concluded that although the presence of vegetative cells is essential for conjugation, there is no positive association between bacterial multiplication and of the recipient and conjugation capacity. The plasmid pHT73-EM^R was stable in all exconjugants (88.5 - 99.3) with Btt showing highest stability. All experimental data are summarized in **Table 2.7-4** below.

Table 2.7-4 Transfer frequencies in broth, nitrocelluslose filters and *B. mori* larvae in one-recipient and two-recipient matings and stability of plasmid pHT73-EmR in exconjugants after 100 generations

Recipient strains	Conjugation frequencies[a]				
	LB broth		Nitrocellulose filter	*B. mori* larvae	
	One-recipient *mating*	Two-recipient mating[b]		One-recipient mating	Two-recipient mating[b]
B. thuringiensis var. *thuringiensis* 407-1	1.7 ± 0.43 × 10^{-4}	1.8 ± 0.14–2.6 ± 0.45 × 10^{-4}	1.4 ± 0.49 × 10^{-2}	4.8 ± 0.53 × 10^{-2}	4.0 ± 0.33–7.5 ± 0.40 × 10^{-2}
B. cereus 433	7.9 ± 0.60 × 10^{-5}	4.0 ± 0.71 × 10^{-5}	3.1 ± 0.07 × 10^{-3}	4.1 ± 1.3 × 10^{-1}	1.8 ± 0.42 × 10^{-1}
B. cereus 388	5.0 ± 0.33 × 10^{-6}	2.3 ± 0.44 × 10^{-6}	4.3 ± 0.13 × 10^{-4}	2.5 ± 0.72 × 10^{-2}	8.8 ± 0.63 × 10^{-3}
B. cereus MADM 1279R	1.5 ± 0.20 × 10^{-5}	NC[c]	8.1 ± 0.86 × 10^{-4}	1.0 ± 0.9 × 10^{-2}	7.6 ± 0.80 × 10^{-3}
B. cereus D1 4430	NC	NT	3.7 ± 0.58 × 10^{-9}	2.6 ± 0.37 × 10^{-4}	NT
B. cereus ATCC 10987	NC	NT	9.7 ± 0.15 × 10^{-6}	NC	NT
B. cereus 569	NC	NT	1.7 ± 0.40 × 10^{-8}	1.8 ± 0.21 × 10^{-3}	NT
B. cereus ATCC 14579	NC	NT	1.1 ± 0.90 × 10^{-9}	NC	NT

±Standard error (n = 3).
NC: no conjugation detected.
NT: not tested.
[a] Conjugation frequencies were calculated as the ratio of exconjugants to recipient cells (EC/R).
[b] The second recipient strain was *B. thuringiensis* var. *thuringiensis* 407-1.
[c] No plasmid transfer detected, either in MADM 1279R or in 407-1 strains.

Discussion & conclusion

Conjugational transfer of the plasmid pHT73-EM^R among members of the *B. cereus* group (Btk, Btt and *B. cereus*) was demonstrated under *in vitro* and *in vivo* conditions. Depending on the conditions, not all recipient strains produced exconjugants and transfer frequencies varied considerable. The conjugation capacities were shown to be not related to the ability for in vivo grow on *B. mori* larvae.

****

Report:	KMA 2.7/05– Donnarumma, F., Paffetti, D., Stotzky, G., Giannini, R., Vettori, C. (2010), published report Soil Biology & Biochemistry, 42:1329-1337
Title:	Potential gene exchange between *Bacillus thuringiensis* subsp. *kurstaki* and *Bacillus* spp. in soil *in situ*
Guideline:	Not specified
GLP:	No
Abstract:	The possible transfer of genes from *Bacillus thuringiensis* subsp. *kurstaki* (Btk) to indigenous *Bacillus* spp. was investigated in soil samples from stands of cork oak in Orotelli (Sardinia, Italy) collected 5 years after spraying of the stands with a commercial insecticidal preparation (FORAY 48B) of Btk. Two colonies with a morphology different from that of Btk were isolated and identified as *Bacillus mycoides* by morphological and physiological characteristics and by 16S rDNA analysis. Amplification by the polymerase chain reaction (PCR) of the DNA of the two isolated *B. mycoides* colonies with primers used for the identification of the Btk *cry* genes showed the presence of a fragment of 238 bp of the cry1Ab9 gene that had a similarity of 100% with the sequence of the cry1Ab9 gene present in GenBank, indicating that the isolates of *B. mycoides* acquired part of the sequence of this gene from Btk. No cells of Btk or *B. mycoides* carrying the 238-bp fragment of the cry1Ab9 gene were isolated from samples of unsprayed control soil. However, the isolates of *B. mycoides* were not able to express the partial Cry1Ab protein. Hybridization with probes for IS231 and the cry1Ab9 gene suggested that the inverted repeated sequence, IS231, was probably involved in the transfer of the 238-bp fragment from Btk to *B. mycoides*. These results indicate that transfer of genes between introduced Btk and indigenous *Bacillus* spp. can occur in soil under field conditions.
Materials & Methods	Soil from cork oak forests in Orotelli, Sardinia, Italy, were treated with a commercial Btk product (FORAY 48B, cells, spores and insecticidal protoxins of Btk HD-1, 12400 Bill IU/mg) in May 1993. Btk and total indigenous bacteria were counted in the Orotelli soils and in soils not treated with FORAY 48B 5 years after the spraying. At each location, 36 soil samples were taken along a transect of 450 m, from the 2 - 20 cm depth with a steel cylinder. For isolation of Btk, 5 g of the samples were added to 20 mL Luria-Bertani medium (LB), shaken for 4 hours at 30°C at 200 rpm. After 5 min. of settling the soil suspensions were transferred into sterile flasks. For counting of endopsores, one mL of the supernatant was heated to 80°C for 10 min and 0.1 mL of appropriate dilutions were plated on a semi-selective Nutrient agar (NA) containing 5 mg/mL of polymyxin B sulfate and 4 µg/mL of penicillin G, to facilitate the selection of Btk and 200 µg/mL cycloheximid to inhibit the growth of fungi. Isolated were checked by PCR analysis to ensure that they represent Btk. Culturable indigenous bacteria were enumerated in unheated soil supernatants used for isolation of Btk by plating 0.1 mL of appropriate dilutions on NA containing 200 µg/mL cycloheximid. DNA was extracted from colonies of Btk and *B. mycoides* grown for 16-18 hours in LB medium and submitted to PCR amplification targeting different *cry* genes including specific ones encoding insecticidal proteins which are active against Lepidoptera. DNA probes targeting the IS231 region and a fragment from the *Cry1Ab9* gene were obtained by amplification with specific primers and purification using a commercial kit. Amplification products of IS231 and Cry1Ab9 were submitted to restriction enzyme analysis and Southern blotting. In addition, amplificates of the 16S rDNA genes and the *cry1Aab9* gene were purified and sequenced. The obtained sequences were compared to those available in the NCBI GenBank and the Ribosomal Database Project (RDP) using the BLAST search tool and RDP utilities, respectively. Multiple alignments of the *cry1Ab9* partial sequence with the ones obtained from NCBI, the ones present in the Btk control and variants of *cry* genes and their published allellic variations were carried out using the CLUSTAL-X program. Amino acid sequences were aligned with the CLUSTALW program. In addition, the Cry1Ab protein was isolated from sporulated cultures of Btk and *B. mycoides* grown in T3 medium for five days (3 g/L tryptone, 2 g/L tryptose, 1.5 g/L yeats extract, 7.1 g/L Na_2HPO_4 and 10^{-5} M $MnCL_2 \times 4\ H_2O$). Verification was done using a Lateral Flow Quickstick specific for Cry1Ab endotoxin, detection limit < 10 ppb.

Findings	Thirty-six soil samples from the Orotelli and from the control area were analysed 5 years after treatment with the Btk-based formulation FORAY 48B. A total of $1.2 \pm 0.58 \times 10^2$ cells of Btk/cm³ soil were isolated with the semi-selective medium from the treated area. No Btk was detected in the soil form the control area. Two colonies were obtained with this medium from the treated area which were totally different from Btk. According to morphological and physiological characteristics, the colonies were identified as B. *mycoides*. This was confirmed by 16S rDNA gene sequencing. Amplification with primers for the identification of cry genes revealed the presence of a 238-bp fragment of the *cry1Ab9* gene in the two *B. mycoides* strains. However, none of the other primer pairs targeting *cry1Aa1* and *cry2Ab2* gave a signal in the *B. mycoides* colonies. Restriction analyses of the *cry1Ab9* gene of the two B. mycoides colonies was identical with those for Btk and the obtained sequences showed 100% similarity to Btk *cry1Ab9* sequences available in GeneBank. Amino acid sequences of *cry* genes (from *cry 1* to *cry22*) where multiply aligned among them and with the predicted amino acid sequence of the *cry1Ab9* fragment present in the *B. mycoides* strain and the Btk control. It turned out that the sequence is a putative conserved structural domain of the Cry 1 protein. The authors concluded that a nucleotide sequence from sprayed Btk has been transferred to indigenous *B. mycoides* cells. Primers targeting genes encoding Lepidopteran specific Cry proteins which can be used to presumably predict the insecticidal activity of a Btk strain were applied to the Btk control and the two *B. mycoides* strains. It turned out, that only a part of the *cry 1Ab* gene was transferred. This assumption was confirmed by the immunological assay with Quicksticks. The *cry1A* genes reside on large plasmids in Bt and are flanked by two sets of inverted repeated sequences that presumably provide mobility to the *cry* genes (*IS231* and *IS232*). *IS231* was also detected in the two *B. mycoides* strains and the authors concluded that it could have been involved in the transfer and insertion of the *cry1Ab9* fragment from the FORAY 48B strain to the *B. mycoides* strains.
Discussion & conclusion	The observations of the study indicate that bacteria artificially introduced into soil may transfer genetic material to indigenous bacteria belonging to another species. However, the transfer frequency was extremely low as only 2 cells/cm³ from a total of 10^5 cells/cm³ of indigenous bacteria received the DNA fragment under evaluation. In addition, the transfer did not have any consequence as the *B. mycoides* strains were not able to produce insecticidal proteins.

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

Information from DAR and DAR addendum (May 2007, February 2013) As a general remark: Commercial *B. thuringiensis* products do not contain metabolites that are considered hazardous to humans and the environment. However, some *B. thuringiensis* subspecies and strains may produce an assortment of antibiotics, enzymes, metabolites and toxins during the vegetative growth and sporulation stages that are biologically active and may have effects on both target and non-target organisms.

During vegetative growth, some *B. thuringiensis* subspecies like Bt *thuringiensis*, Bt *galleriae* and Bt *darmstadiensis*, produce ß-exotoxin, an ATP analogue, water soluble and heat stable metabolite. Beta-exotoxin is an inhibitor of RNA polymerase and acts competitively with ATP in various biological processes. It is toxic to almost all forms of life including humans and has a broad spectrum of insecticidal activity. (WHO, 1999).

More specifically the Bta strain GC-91 does not produce metabolites toxic for human health. This is confirmed by the quality control tests during the fermentation and production process, respectively. The working vials will be tested for the amount of ß-exotoxins in the fermentation broth by HPLC analysis, fly test and mice injection. Furthermore, after the fermentation process the spores will be spray dried while the nutrient broth is discarded. If any metabolites would occur, they would be removed from the Technical Powder consequently (Chen & Hargrove, 2003).

In a study cunducted in 2004, by using the appropriate controls no ß-exotoxin could be determined in the production substrates of strain GC-91. The detection limit of ß-exotoxin by fly larvae assay was 0.028 µg/mL. The positive control, Btt produced 191µg/mL to 378 µg/mL of ß-exotoxins (Chen, 2004).

New data

Due to the long use of Bt/Bta for plant protection purposes there exists huge information about metabolites produced by the species/subspecies.

Available information will be provided following three categories:

- Metabolites involved in the mode of action (see B.2.8.1)

- Metabolites of possible concern for human health (see B.2.8.2)

- Other metabolites potentially produced by Bt (see B.2.8.3)

For all points available information on which metabolites are potentially produced and possible harmful effects on human health are presented. Information about which metabolites are produced are obtained from a free search for Bta metabolites, metabolites identified during the EFSA peer review of Bta GC-91 and metabolites identified for Bt in general in the two EFSA External Scientific Reports on Literature search and data collection for microorganisms used as plant protecting agents either for the Risk assessment related to human health (Hackl et al. 2015)[10] or the environment (Mudgal et al., 2013)[11]. In addition, information from the recently published EFSA Scientific Opinion on the Risk for public health related to *B. cereus* and other *Bacillus* spp. including Bt[12] is provided. Information about possible harmful effects were obtained through a literature search according to EFSA guidance[13] combining the metabolites with typical search terms related to effects on human health. The search was done using the DIMDI data base and covered the last ten years. More details can be found in the evaluation of the literature search in the human toxicology section B.6 (Seehase, 2016, KMA 5.1/01, see B.6).

B.2.8.1 Metabolites involved in the mode of action

Relevant information about insecticidal proteins produced by *Bacillus thuringiensis* including Bta are presented in a review paper of Palma et al. (2014) which is summarised in B.2.2.2 above. The insecticidal activity of Bt in general, including Bta, is mainly attributed to spore bound insecticidal crystal (cry) and cytolytic (Cyt) pro-proteins (also called δ-endotoxins) which are ingested by the target pests (lepidopteran larvae) and activated under alkaline conditions in the midgut of the larvae. To date, *B. thuringiensis* has been reported to produce many insectidical crystal proteins, classified into 74 Cry groups and 3 Cyt groups.

(http://www.lifesci.sussex.ac.uk/Home/Neil_Crickmore/Bt/; November, 2012).

Apart from the cry and cyt toxins there are several other insecticidal proteins produced by Bt and contributing to their mode of action: insecticidal toxins produced during vegetative growth (vegetative insecticidal protein: Vip, against lepidopteran, coleopteran and some homopteran pests) and secreted insecticidal proteins (Sip, against coleopteran pests).

Palma et al. (2014) included in their paper also some putative insecticidal toxins for which the mechanism and the host range is largely unknown. So far there are no clear indications that they are part of the Mode of Action of Bta GC91.

All metabolites involved in the mode of action were included in a literature search aiming to identify references providing information about toxic effects of insecticidal toxins produced by Bta (Seehase, 2016, KMA 5.1/01, see B.6). The obtained references confirm the absence of toxicity to humans and mammals.

EFSA published an External Scientific Report based on a literature search targeting human health related data for microorganisms used as active substances in plant protection products (Hackl et al., 2015)[10]. Based on available literature information the authors concluded that Cry-toxins are not of concern for human health.

[10] Evelyn Hackl, Margit Pacher-Zavisin, Laura Sedman, Stefan Arthaber, Ulla Bernkopf, Günter Brader, Markus Gorfer, Birgit Mitter, Aspasia Mitropoulou, Monika Schmoll, Willem van Hoesel, Elisabeth Wischnitzky, and Angela Sessitsch, 2015. Literature search and data collection on RA for human health for microorganisms used as plant protection products Reference. EFSA supporting publication 2015:EN-801. 173 pp.

[11] Mudgal S, De Toni A, Tostivint C, Hokkanen H, Chandler D; Scientific support, literature review and data collection and analysis for risk assessment on microbial organisms used as active substance in plant protection products –Lot 1 Environmental Risk characterization. EFSA supporting publications 2013:EN-518. [149 pp.]. Available online: www.efsa.europa.eu/publications

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

[13] Guidance of EFSA: Submission of scientific peer-reviewed open literature for the approval of pesticide active substances under Regulation (EC) No 1107/2009. EFSA Journal 2011;9(2):2092

Onose et al. (2008) studied the sub-chronic toxicity of the Cry1Ab protein in rats and did not observe any toxic effect. Shimada et al (2006) tested *in vitro* the effect of Cry1Ab protein on several mammalian intestinal epithelia; it binds less strongly to the bovine and porcine cellular brush border membrane vesicle (BBMV) than to the silkworm BBMV (human cells not tested) and does not adversely affect the membrane potential of bovine, porcine and human intestine cells. Berlitz et al. (2012) administered orally by gavage the supernatant or the bacterial suspension of a *Bt thuringiensis* and *Bta* isolate to rats. The supernatant appears not to contain detectable amounts of ß-exotoxins. They found that the substances did not exhibit toxicity in the test animals. Obeidat et al. (2012) investigated the hemolytic potential of ß-exotoxins and δ-endotoxins of different Bt strains to develop a non-hemolytic, ß-exotoxin-free strain with insecticidal activity. The study suggests that insecticidal δ-endotoxins of certain Bt strains may have hemolytic properties. However, the δ-endotoxins itself were tested by solubilising the pellet containing spores and parasporal inclusions under alkaline circumstances (pH of 10). This is mimicking the insect gut, were the protoxins (inclusion bodies) will be converted proteolytically into smaller toxic δ-endotoxins under the alkaline conditions in the insect midgut. Humans and other mammals will not be directly exposed to these δ-endotoxins as their stomach is acidic.

In conclusion, confirming information provided previously, there is no indication that metabolites involved in insecticidal activity of Bta GC-91 pose a risk for human health or the environment.

Summary/abstracts of literature references:

Report: KMA 2.8/01 - Palma, L., Munoz, D., Berry, C., Murillo, J., Caballero, P. (2014), published report
Toxins 2014, 6(12), 3296-3325

Title: *Bacillus thuringiensis* Toxins: An Overview of Their Biocidal Activity

Abstract *Bacillus thuringiensis* (Bt) is a Gram positive, spore-forming bacterium that synthesizes parasporal crystalline inclusions containing Cry and Cyt proteins, some of which are toxic against a wide range of insect orders, nematodes and human-cancer cells. These toxins have been successfully used as bioinsecticides against caterpillars, beetles, and flies, including mosquitoes and blackflies. Bt also synthesizes insecticidal proteins during the vegetative growth phase, which are subsequently secreted into the growth medium. These proteins are commonly known as vegetative insecticidal proteins (Vips) and hold insecticidal activity against lepidopteran, coleopteran and some homopteran pests. A less well characterized secretory protein with no amino acid similarity to Vip proteins has shown insecticidal activity against coleopteran pests and is termed Sip (secreted insecticidal protein). Bin-like and ETX_MTX2-family proteins (Pfam PF03318), which share amino acid similarities with mosquitocidal binary (Bin) and Mtx2 toxins, respectively, from *Lysinibacillus sphaericus*, are also produced by some Bt strains. In addition, vast numbers of Bt isolates naturally present in the soil and the phylloplane also synthesize crystal proteins whose biological activity is still unknown. In this review, we provide an updated overview of the known active Bt toxins to date and discuss their activities.

****

Report: KMA 2.8/02 – Onose, J-I., Imai, T., Hasumura, M., Ueda, M., Ozeki, Y., Hirose, M. (2008), published report
Food Chem Toxicol., 46(6), 2184-2189

Title: Evaluation of subchronic toxicity of dietary administered Cry1Ab protein from *Bacillus thuringiensis* var. *kurstaki* HD-1 in F344 male rats with chemically induced gastrointestinal impairment

Guideline: Not specified

GLP: No

Abstract *Bacillus thuringiensis* (Bt) proteins are developed for genetically modified crops and the Bt proteins demonstrate no evidence of toxicity by the oral route in traditional animal models. However, the possible toxicity of Bt proteins under conditions of reduced gastric acid secretion and/or small

intestinal damage has not been investigated. In the present study, we therefore evaluated following four F344 rat groups with a purified Bt protein Cry1Ab from *B. thuringiensis* var. *kurstaki* HD-1. Gastrointestinal impairment (GI) alone and GI + Bt protein fed (GI + Bt) groups were given i.p. injections of famotidine to reduce gastric acid secretion twice a day at 30 mg/kg body weight in weeks 2 and 4. GI and GI + Bt groups were additionally fed diets containing 80 ppm indomethacin for induction of intestinal damage during weeks 1 and 3. Bt alone and GI + Bt groups were also fed diet containing Bt protein Cry1Ab at a concentration of 10 ppm in weeks 2 and 4. A no treatment control group was also included. At the end of week 4, all animals were euthanized under ether anesthesia, blood samples were collected for hematology and serum biochemistry and a complete necropsy was performed. No significant changes indicative of toxicity of the Bt protein Cry1Ab used here were noted with any of the parameters investigated. In conclusion, no significant toxicological effects were detected in this subchronic gastrointestinal impairment rat model.

Material and Methods

Species 32 male F344 rats, aged 6 weeks, housed in groups of 4; water and basal diet ad libitum

Groups Untreated control

Cry1Ab from *Bacillus thuringiensis* var. *kurstaki* HD1 (= Bt)

Indomethacin and famotidine to induce Gastro-intestinal impairment (= GI)

Bt + GI

Study design:

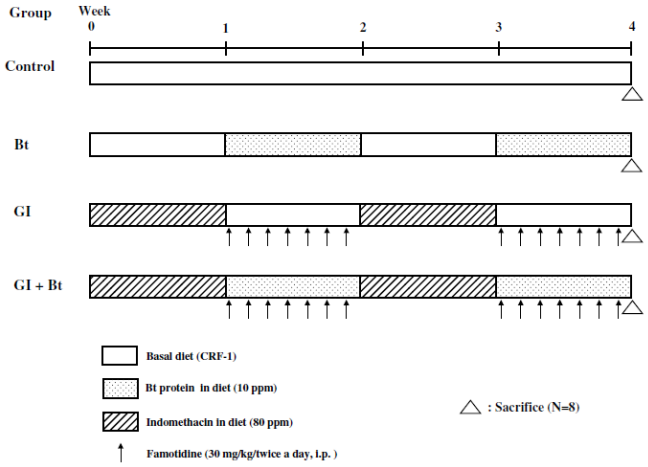

Figure 2.8-1 Experimental design

Group (C) and (D) received i.p. injection of famotidine twice a day at 30 mg/kg bw during week 2 and 4 as well as 80 ppm indomethacine via basal diet during week 1 and 3. Rats of group (B) and (D) received a basal diet containing 10 pm Cry1Ab on weeks 2 and 4. Control groups (A) and (C) received basal diet alone. Control and Bt group rats were given i.p. injection with saline as for famotidine administration. After week 4, all animals were fasted overnight prior to euthanization. Hematology and serum biochemistry was performed as well as a complete necropsy followed by histopathological examination.

Statistics The Student's or Welch's t-test after application of the F-test for homogeneity of variance were employed. Significance was inferred at the 5%, 1% and 0.1% levels.

Findings: Neither deaths nor deterioration in general conditions were observed in any animals throughout the experimental period. Significant reduction of body weight gain was noted in the GI ($p < 0.01$) and GI + Bt ($p < 0.05$) group. Food consumption was decreased or showed a tendency for decrease in the GI and GI + Bt groups during the indomethacin treatment in weeks 1 and 3. Under the physiological and pathological conditions in the present study, dietary administration of Bt protein Cry1Ab exerted no significant effect on any parameters except for the lower serum AST level in the Bt and GI + Bt groups as compared to the control. However, no changes in organ weights or histopathological changes were observed in related organs, such as the heart, liver and kidneys. In-

terpretation of relatively small changes in AST in toxicological studies should be made with caution, as the variability of this parameter can be quite wide in healthy animals.

Conclusion: No significant toxicological effect of refined Bt protein Cry1Ab was observed with and without the present gastrointestinal impairment.

****

Report: KMA 2.8/03 – Shimada, N., Miyamoto, K., Kanda, K., Murata, H. (2006), published report
In Vitro Cellular & Developmental Biology - Animal, 42(1):45-49

Title: *Bacillus thuringiensis* insecticidal Cry1Ab toxin does not affect the membrane integrity of the mammalian intestinal epithelial cells: An *in vitro* study

Guideline: Not specified

GLP: No

Abstract The mammalian intestinal epithelium has been found, based on in vivo experiments, to be resistant to insecticidal Cry toxins, which are derived from *Bacillus thuringiensis* and fatally damage insect midgut cells. Thus, the toxins are commonly used as a genetic resource in insect-resistant transgenic plants for feed. However, Cry toxins bind to the cellular brush border membrane vesicle (BBMV) of mammalian intestinal cells. In this study, we investigated the affinity of Cry1Ab toxin, a lepidopteran-specific Cry1-type toxin, to the cellular BBMV of two mammalian intestinal cells as well as the effect of the toxin on the membrane potential of three mammalian intestinal cells compared to its effects on the silkworm midgut cell. We found that Cry1Ab toxin did bind to the bovine and porcine BBMV, but far more weakly than it did to the silkworm midgut BBMV. Furthermore, although the silkworm midgut cells developed severe membrane potential changes within 1 h following the toxin treatment at a final concentration of 2 µg/mL, no such membraneous changes were observed on the bovine, porcine, and human intestinal cells. The present in vitro results suggest that, although Cry1Ab toxin may bind weakly or nonspecifically to certain BBMV components in the mammalian intestinal cell, it does not damage the cell's membrane integrity, thus exerting no subsequent adverse effects on the cell.

Material and Methods: Cry1Ab toxin was isolated from a recombinant *E. coli* strain harbouring the *cry1Ab* gene of Btk HD-1. The purified protein was activated by trypsin digestion and purified via ion-exchange liquid chromatography. The protein content was determined using the DC Protein Assay.

Silkworm BBMV were isolated from 5th instar larvae, bovine and porcine BBMV from small intestine of Holsteins, Japanese Black cows and crossbred pigs. Quality of the BBMV preparations was tested by measuring aminopeptidase activity.

Cry1Ab binding assays (to BBMV) were done following two approaches: the coprecipitation and the BIAcore assay. For the coprecipitation assay, BBMV were incubated with Cry1Ab toxin in PBS for 1 hour at room temperature. Separation of bound and free toxin was done via centrifugation. The bound toxin was washed, resuspended and submitted to Western Blot analysis. In the BIAcore assay, BBMV were immobilized at a sensor chip. Afterwards, Cry1Ab protein was injected over the immobilized membrane for 5 min. Determination of bound toxin was done via resonance measurements and finally, the amount of bound toxin was calculated.

Cry1Ab toxin effects on the membrane potential were assessed I, silkworm midgut (SM), bovine gut (BG), porcine gut (PG) and human intestinal epithelial (HIE) cell lines.

Findings: In the coprecipitation assay the Cry1Ab toxin bound less strongly to the bovine and porcine BBMV than to the silkworm BBMV. The results were confirmed by calculation of binding of the toxin to BBMV based on the BIAcore assay. A pronounced change in the membrane potential of SM cells was observed while there were no changes detected when BG, PG and IEH were treated either with phosphate buffered saline (as control treatment) or Cry1Ab toxin from Btk.

Conclusion: The results suggest that Cry1Ab toxin do not have the potential to damage intestine cells of cattle, pigs or human.

****

Report: KMA 2.8/04 – Obeidat, M., Khyami-Horani, H., Al-Momani, F. (2012), published report African Journal of Biotechnology, 11(46):10504-10512

Title: Toxicity of *Bacillus thuringiensis* beta-exotoxins and delta-endotoxins to *Drosophila melanogaster*, *Ephestia kuhniella* and human erythrocytes

Guideline: Not specified

GLP: No

Abstract A total of 73 *Bacillus thuringiensis* (Bt) strains were screened for the presence of non-hemolytic insecticidal ß-exotoxin-free δ-endotoxins. Out of them, 45 Bt strains produced δ -endotoxins with specific insecticidal activity against *Drosophila melanogaster* and/or *Ephestia kuhniella* larvae. The thermostable ß-exotoxin was observed only in 15 Bt strains and appeared to exhibit dual non-specific insecticidal activity against both *D. melanogaster* and *E. kuhniella* larvae and showed *in vitro* hemolysis for human erythrocytes. It was found that ß-exotoxin was produced by Bt strains belonging to five serovars (*israelensis, kenyae, kurstaki, pakistani, and tohokuensis*) and two non-serotypable strains. This result suggests that β-exotoxin production is a strain-specific property rather than a serovar-specific property. To our knowledge, this is the first study that demonstrates ß-exotoxins production association with Bt strains belonging to serovars *israelensis, pakistani*, and *tohokuensis*. The plasmid DNA profiles of some ß-exotoxin producing Bt strains shared large plasmid patterns which may have the common ß-exotoxin regulatory gene(s). It was found that 16 local Bt strains, 15 of which belonged to five serovars (*aizawai*, *israelensis, kurstaki, morrisoni*, and *pakistani*) and one was autoagglutinated strain, produced non-hemolytic insecticidal ß-exotoxin-free δ-endotoxins. Based on random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR), the genotypic relatedness among these 16 Bt strains was investigated. The strains were grouped into two clusters. Bt strains within serovars *israelensis* were grouped in two subclusters, Bt strains within serovars *aizawai* were genomically homogeneous and clustered together, while the other serovars were grouped together in one subcluster. The autoagglutinated strain was clustered within serovar *israelensis*. Thus, these δ-endotoxins can be developed for the use in Bt-based insecticidal preparations.

Material and Methods: A total of 78 previously isolated Bt strains from Jordanian habitats were used in the study. They belonged to the 14 serovars *aizawai entomocidus, higo, israelensis, jordanica kenyae, kumamotoensis, kurstaki, malaysiensis, morrisoni, pakistani, sooncheon, thuringiensis*, and *tohokuensis*, one autoagglutinated, and four nonserotypable (NSP) strains. In addition, three reference strains obtained from the International Entomopathogenic *Bacillus* Collection Center (IEBC), Institute Pasteur, Paris including T03A005 (serovar *kurstaki* HD1), T07001 (serovar *aizawai*) and T14001 (serovar *israelensis*) were used in this study.

The strains were grown in T_3 medium (0.3% tryptone, 0.2% tryptose, 0.15% yeast extract, 0.05 M sodium phosphate, and 0.005% $MnCl_2$) for 24 hours at 37°C. Spores and parasporal inclusion bodies were harvested by centrifugation. The supernatants were autoclaved and subjected to ß-exotoxin bioassays while the pellet containing δ-endotoxins and the spores were solubilized at a pH of 10, filtered and after determination of the protein content submitted to the bioassays. Both substances were subjected to insect bioassays and hemolytic activity assessments. The insect assays are not further described here. Hemolytic activity of δ-endotoxin and β-exotoxin of Bt strains that exhibited larvicidal activity was investigated in this study to be tested on normal human erythrocytes by spectrophotometric method. Blanks were prepared by including the vehicle components (all added materials were included but not the toxins). Positive controls using hemolysing buffer (170 mM Tris base, pH 7.2, 0.83% NH_4Cl) were run in parallel. The results were observed and recorded as +++: high hemolysis (A540 > 1.0 > 30% hemolysis); ++: moderate hemolysis (1.0 > A540 > 0.5; 30% > hemolysis > 15%); +: low hemolysis (0.5 > A540 > 0.2; 15% > hemolysis > 5%), and -: non-hemolytic (0.2 ≥ A540; 5% ≥ hemolysis). No further experimental details are provided here.

Findings: Out of the 78 strains, 45 exhibited insecticidal activity and where submitted to hemolytic assays. Out of these, 29 strains produced hemolytic δ-endotoxins, including strains belonging to the serovars *aizawai, israelensis, kurstaki and thuringiensis*. Please refer to **Table 2.8-1**.

Only 17 strains produced heat stable ß-exotoxins. These strains belonged to the serovars *israelensis, kenyae, kurstaki, pakistani*, and *tohokuensis*. In addition, ß-exotoxin production with hemolytic activity was observed in the two NSP strains. Please refer to **Table 2.8-2**.

Table 2.8-1 Larvicial and hemolytic activity of Bt δ-endotoxins

Bt Serovar[a]	Bt Strain	LC_{50}[b] (ng/ml)		Degree of erythrocytes hemolysis[c]
		D. melanogaster	*E. kuhniella*	
aizawai	J44	3.70 (3.06-4.14)	0	-
entomocidus	J115	0.29 (0.11-0.41)	0	++
higo	J77	5.19 (4.54-5.96)	0	+++
	J19	1.85 (1.21-2.49)	0	+++
	J21	1.11 (0.47-1.96)	0	+++
	J40	3.39 (2.03-4.93)	0	-
	J45	3.70 (3.06-4.81)	0	-
	J50	3.33 (1.93-4.36)	0	++
	J56	1.48 (0.52-2.02)	0	-
	J59	5.93 (4.29-7.57)	0	+
israelensis	J60	5.20 (4.55-7.13)	0	-
	J62	2.22 (1.58-3.07)	0	-
	J63	0.17 (0.14-0.29)	0	-
	J66	1.48 (0.84-2.27)	0	+++
	J67	4.44 (3.32-5.55)	0	+++
	J70	4.07 (2.96-5.68)	0	-
	J74	0.27 (0.13-0.39)	51.8 (40.6-65.2)	+
	J78	4.81 (4.16-5.63)	0	-
jordanica	J112	1.13 (0.46-1.78)	0	++
	J13	4.47 (3.32-5.56)	37.1 (24.7-51.3)	+++
	J15	1.11 (0.53-1.76)	0	++
kenyae	J30	5.96 (5.29-7.19)	25.9 (19.4-33.2)	+++
	J37	0.19 (0.14-0.33)	22.2 (15.8-31.6)	+++
	J81	0.26 (0.18-0.43)	0	++
kumamotoensis	J51	4.81 (4.16-5.65)	0	+++
	J06	1.85 (1.22-2.75)	0	+
	J17	4.83 (4.13-5.66)	43.4 (21.8-59.1)	+++
	J25	5.56 (4.92-6.68)	8.13 (1.7-14.5)	+++
	J26	0.18 (0.13-0.31)	0	-
kurstaki	J33	0.23 (0.13-0.38)	0	++
	J34	4.81 (4.16-5.45)	33.3 (17.8-49.6)	+++
	J35	0.27 (0.17-0.41)	44.4 (34.8-52.8)	-
	J36	4.81 (4.16-5.92)	0	-
	J41	5.19 (4.54-6.32)	0	++
	J49	5.90 (5.27-7.55)	0	+
malaysiensis	J20	3.33 (2.13-4.53)	0	+
	J80	4.81 (4.19-5.45)	0	++
morrisoni	J29	0	48.1 (35.7-62.5)	-
	J28	5.19 (4.52-6.79)	0	+++
pakistani	J52	0.19 (0.10-0.39)	0	-
	J139	0.30 (0.23-0.43)	59.3 (43.1-78.0)	-

Table 2.8-2 Larvicial and hemolytic activity of Bt δ-endotoxins-continued

sooncheon	J18	2.22 (1.57-3.33)	0	+++
thuringiensis	J23	5.89 (5.29-7.53)	25.7 (18.8-37.3)	+
	J69	0	24.6 (16.6-36.2)	+
Autoagglutinated	J71	0.74 (0.47-1.38)	0	-
Bti	T14001	5.48 (4.84-6.12)	0	++
Btk HD1	T03A005	3.62 (3.28-4.57)	42.3 (34.2-53.4)	-

[a] *Bti* is the reference strain *B. thuringiensis* serovar *israelensis* IEBC No. T14001. *Btk* HD1 is the reference strain *B. thuringiensis* serovar *kurstaki* HD1 IEBC No. T03A005. [b] Numbers between parentheses are 95% fiducial limits. [c]The degree of hemolysis was graded as +++ (high), ++ (moderate), + (low), and – (non-hemolytic).

Table 2.8-3 Larvicial and hemolytic activity of Bt ß-exotoxins

Bt Serovar[a]	Bt Strain	Mortality[b] % of β-exotoxins against		Degree of erythrocytes hemolysis[c]
		D. melanogaster	*E. kuhniella*	
israelensis	J21	$77.8\pm11.1d^{ef}$	22.2 ± 11.1^{bc}	+++
	J38	74.1 ± 12.8^{def}	63.0 ± 6.4^{e}	++
	J39	66.7 ± 0.0^{de}	25.9 ± 6.4^{bc}	+++
	J50	74.1 ± 12.8^{def}	88.9 ± 0.0^{f}	+++
	J53	88.9 ± 11.1^{ef}	100 ± 0.0^{g}	++
	J82	74.1 ± 12.9^{def}	63.0 ± 6.4^{e}	+
kenyae	J15	100 ± 0.0^{f}	88.9 ± 11.1^{f}	++
	JN23	55.6 ± 11.2^{cd}	18.5 ± 6.4^{b}	+
	J81	100 ± 0.0^{f}	100 ± 0.0^{g}	++
kurstaki	J31	88.9 ± 0.0^{ef}	66.7 ± 11.1^{e}	+++
	J32	85.2 ± 17.0^{ef}	100 ± 0.0^{g}	++
	J33	63.0 ± 6.4^{cde}	18.5 ± 6.4^{b}	+
	J49	51.9 ± 6.5^{bcd}	25.9 ± 6.4^{bc}	++
pakistani	J28	100 ± 0.0^{f}	88.9 ± 11.1^{f}	+
tohokuensis	J72	25.9 ± 6.4^{b}	40.7 ± 6.4^{d}	+++
Nonserotypable	J43	40.7 ± 6.4^{bc}	55.6 ± 11.2^{de}	+
	JN71	74.1 ± 12.8^{def}	25.9 ± 6.4^{bc}	++
Bta	T07001	77.8 ± 11.1^{def}	66.7 ± 0.0^{e}	++
Control	C	0.0^{a}	0.0^{a}	-

[a]*Bta* is the reference strain *B. thuringiensis* serovar *aizawai* IEBC No. T07001. [b] The mortality was corrected according to Abbott's formula and represented as means ± SD. Means ± SD within column followed by the same letter are not significantly different (Tukey's studentized range test: $\alpha = 0.05$). [c]The degree of hemolysis was graded as +++ (high), ++ (moderate), + (low), and – (non-hemolytic).

Conclusion: The results suggest that only part of the strains produced ß-exotoxins which in all cases had hemolytic effects to human erythrocytes. This was not the case for δ-endotoxins which were non-hemolytic in 16 strains, however 29 strains produced hemolytic δ-endotoxins. Based on additional phylogenetic analysis the authors concluded that production of insecticidal toxins with hemolytic activity is a strain specific property.

****

Report	KMA 2.8/05 Berlitz, D.S., Giovenardi, M., Fiuza, L.M., published report Neotropical Biology and Conservation, 2006, 1(1), pp. 35-38
Title:	Toxicology effects of delta-endotoxins and beta-exotoxins of *Bacillus thuringiensis* in Wistar rats
Guideline:	No
GLP:	No
Abstract:	*Bacillus thuringiensis* is an entomopathogen consisting of toxic proteins, such as the δ- endotoxins specific to insects. However, some subspecies can produce β-exotoxins that are non-specific and toxic to the vertebrates. So, in this research the toxicology effect of two isolates of *B. thuringiensis* in Wistar rats have been assessed. In the in vivo assays the animals have been given 3×10^{10} UFC/mL of *B. thuringiensis thuringiensis* and *B. thuringiensis aizawai* and supernatant bacterial suspensions orally. The excrements and the stomachic content of the animals have been collected and analysed in SDS-PAGE (10%). The analysed data show protein fragments between 151 kDa and 28 kDa, seeing that the faecal samples show only one track of peptides in the treatments when compared with the control group. The assessments of the stomach of the treated rats carried out under stereomicroscope have not shown alterations when compared with the control. These results indicate that the toxins which can be found in both *B. thuringiensis* species used in these assays can be degraded by the gastrointestinal conditions of the animals and have not presented oral toxic effect to this mammalian species under the conditions which the experiments had been carried out.
Species/ Strain	Male adult wistar mice aged 80 and 100 days ***Remark RMS: the entire article, except for the summary, indicates that (Wistar) mice are used. Since there is no mice strain called Wistar, it is assumed that the authors used Wistar rats for their experiment.***
Test item	*B. thuringiensis aizawai* from the commercial product Xentari® (Hokko do Brasil Indústria Química e Agropecuária Ltda.) *B. thuringiensis thuringiensis* isolate with H1 serovar (provided by the International Entomopathogenic Bacillus Centre, Pasteur Institute, Paris)
Test groups:	5 treatment groups comprising 30 animals: 400 µl of Bt *aizawai* suspension applied, at a concentration of 3×10^{10} UFC/mL 400 µl of Bt *thuringiensis* suspension applied, at a concentration of 3×10^{10} UFC/mL 1000 µl of Bt *aizawai* supernatant applied, at a concentration of 3×10^{10} UFC/mL 1000 µl of Bt *thuringiensis* supernatant applied, at a concentration of 3×10^{10} UFC/mL 1000 µl distilled water (=control)
Study design:	Treatments were performed by gavage at different times: 0, 12, and 24 h Each animal´s total faeces collection, according to the treatment, was made 12, 24, and 36 h after their application (HAT). The animals were killed 24 and 36 HAT, and their stomach contents removed for analysis in SDS-PAGE 10% to detect toxic proteic fragments of B. thuringiensis. Stereomicroscopic analysis of the murine stomach´s was performed.
Statistics:	Not reported
Findings:	Both tested isolates did not show peptides corresponding to the β-exotoxin. Compared to control, stereomicroscopic analysis of stomachs of the animals treated with Bt suspensions showed no anomalies. Protein fragments between 151 kDa and 28 kDa were found in the SDS-Page of stomach contents of animals treated with suspension and supernatant.
Conclusion:	The toxins which can be found in both *B. thuringiensis* species used in these assays can be degraded by the gastrointestinal conditions of the animals and have not presented oral toxic

effect to rats under the provisions described.

B.2.8.2 Metabolites of possible concern for human health

The presence of *B. cereus* enterotoxin genes in commercial Bt strains is a well known phenomenon which was already discussed during first evaluation of Bta GC-91 and is still under discussion by the European authorities.

Only recently EFSA published a Scientific Opinion on the Risk for public health related to *B. cereus* and other *Bacillus* spp. including *B. thuringiensis* in food stuffs[14]. There a complete overview is given about current knowledge on virulence factors potentially produced by members of the *B. cereus* group including Bt. A short summary and the most important information on the biological activity of the toxins as well as information if the substance is potentially produced by *B. thuringiensis* are provided below in **Table 2.8-4.** The authors came to the conclusion that neither cereulide, the emetic toxin of *B. cereus,* nor the highly cytotoxic form of CytK, namely CytK1, are produced by Bt. CytK2, however is not considered to be involved in enterotoxicity of *B. cereus* group strains. All other enterotoxins could be potentially produced by members of this species. Other virulence factors such as sphingomyelase or Haemolysin II have so far not been detected in Bt. It is noteworthy that in the EFSA Scientific Opinion it is noted that no definitive demonstration has been provided for the actual role of the enterotoxins (alone or in combination) in the diarrheal syndrome.

Table 2.8-4a Potential virulence factors produced by members of the *B. cereus* group

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

Toxin	Biological activity	Remarks	Produced by some *B. thuringiensis* strains	Potentially produced by Bta GC-91 (reference)
Cereulide	Emetic syndrome	-	No	No
Enterotoxins				
Non-hemolytic enterotoxin (NHE)	Cytolytic activity against erythrocytes and epithelian cells, Diarrheal type of *B. cereus* food poisoning	Three components, biological activity requires at least two or even all three components which bind sequential to the host cell	Yes	Genes present (Hansen et al., 2011)
Hemolysin BL (HBL)	Hemolytic and dermonecrotic activity	Involved in non-GI infections	Yes	Genes present (Hansen et al., 2011)
PlcR	Global regulator for transcription of enterotoxin genes	-	Yes	Genes present (Hansen et al., 2011)
Cyt K	Pore forming toxin, severe necrotic enteritis (CytK1 only)	Two variants, CytK1 /CytK2, CytK1 highly cytotoxic, CtyK2 not involved in enterotoxicity	CytK1: no CytK2: yes	CytK1: no CytK2: yes
Other virulence factors				
Sphingomyelinase	Synergistic interaction with NHE and HBL, described symptoms in patients: sepsis and endophtalmitis	Lethal to mice	Not yet observed	
Haemolysin II	Apoptosis in macrophages	-	Not yet observed	No (Hansen et al., 2011)
InhA1	Escaping of *B. cereus* from macrophages	-	Not yet observed	
NprA	Metalloprotease, immune evasion and tissue degradation by *B. anthracis*	-	Not yet observed	

The literature search on possible toxic effects of metabolites potentially produced by Bta also covered *B. cereus*-enterotoxins. After the rapid and full text assessment two references were identified being relevant for the data point and are summarised below (Kim et al., 2015; Wilcks et al., 2006a). More details can be found in the evaluation of the literature in the human toxicology section B.6 (Seehase, 2016; KMA 5.1/01).

Kim et al. (2015) screened commercial Bt strains as well as a set of other Bt and *B. cereus* reference strains for the presence of genetic determinants of enterotoxic, emetic and hemolytic activity and found that the corresponding genes are widespread in all strains. Wilcks et al. (2006a) performed experiments in which rats with associated human-intestinal-microflora, were fed with commercial Bt strains, namely *B. thuringiensis* ssp. *kurstaki* DMU67R or *B. thuringiensis* ssp. *israelensis* HD567, either in the form of spores (10^7 spores/animal/day, untreated or heat-treated) or as vegetative cells (10^7 – 10^8 cells/animal/day) for 5 days. Although spores germinated to a certain extent, no *in vivo* production of enterotoxins was detected by application of Vero cell assays to intestinal samples from animals fed with either of the strains. Full study summaries are provided below.

Discussion and conclusion

It has been confirmed that Bta GC-91 does not have the capability to produce cereulide and the highly cytotoxic type 1 of CytK (see Volume 4).

It is well known that commercial Bt strains, including Bta GC-91, harbour the genetic material of *B. cereus* enterotoxin genes. However, there are several factors which need to be considered when referring to a particular strain harbouring genetic determinants for a certain virulence factor. To act as a pathogen causing disease symptoms, the microorganism needs to have the ability to:

1) persist on treated crops until the moment of consumption of the harvested good at relevant levels

2) survive the gastrointestinal passage

3) germinate and grow in the host

4) adhere to and invade the intestinal wall

5) produce toxins in the host at relevant levels

So far, there is no evidence that commercial Bt strains fulfil all these preconditions required to cause disease effects in humans.

1) Persistence on treated crops until consumption

From published, partly strain specific data for commercial Bt strains including strain GC-91, it is obvious that field persistence of the spores and even more of living cells and insecticidal proteins is rather restricted. In particular on crops, where the microorganisms face exposure to drought and solar radiation as well as competition with indigenous microbial populations, persistence is low. Growth, if occurring, is restricted to infested insects, as this is the major niche for Bt. In accordance, on treated crops, half life times of spores are reported to range between some hours (16) and some days (maximum 10 days) and complete disappearance is recorded between 15 and 60 days.

It is generally agreed that persistence of Bt populations on plant surfaces is low. Half-life times recorded in the literature range from some hours to a maximum of 10 days (e.g. Griego & Spence, 1978; Pedersen et al., 1995; Dent et al., 1993; Martin, 1994). Factors restricting field persistence are UV-mediated degradation of spores, rain fall and plant growth (dilution effects), lack of nutrients and low humidity. Natural levels of Bt on plant surfaces range between 3 and nearly 1000 CFU/g or cm² (Smith & Couche, 1991; Ignoffo et al., 1974; Hostetter et al., 1975). These studies indicate also that Bt populations quickly decrease to background levels upon treatment.

Multiplication of B. thuringiensis ssp. kurstaki does not seem to play a role outside the host organisms. As seen from the data on persistence presented below, no multiplication occurs on leaves due to sensitivity to solar radiation, foliage exudates and microbial competition. Spore germination and growth was observed in sterile soils, when amended with nutrients, but never in natural bulk soils (Akiba et al., 1986; Saleh et al., 1970; Petras & Casida, 1985). Vegetative cells disappeared rapidly within 1-2 days after inoculation, but cells were able to form spores (Akiba 1986). Germination of spores occurs only if conditions are appropriate, which is only the case after ingestion by insects (Pedersen et al., 1995) or earthworms, or in the rhizosphere of several, but not all plants (Hendriksen & Hansen, 2002). Long term persistence in soil occurs at levels close to background levels (Hendriksen & Hansen, 2002).

In the Table B.2.8-5a a summary of data on environmental persistence of Bt spores

See for more details and references the environmental fate section B.8 and .

Table B.2.8-5b Half-live of Bt spores and protoxins in foliage/crops

Compound	Experimental approach	Germination/ growth	Half-live time	Factors affecting Bt loss	References
Foliage/crops					
Spores	Cabbage	No	16 h	Solar radiation, rain fall, plant growth, leaf temperature, vapour pressure	Pedersen et al., 1995[1]
	Soy bean	No	< 24 h		Ignoffo et al., 1974[1]
	California redbud, different commercial formulations	No	0.58-1.85 d		Pinnock et al., 1974[1]
	Field, different crops	No	24-48 h*		Leong et al., 1980[1]
	Depending on formulation	Not indicated#	4 -10 d		Dent et al., 1993
	Maize, beans (greenhouse)	No	3 days*		Sánchez-Yáñez & Peña-Cabriales, 2000[2]

Com-pound	Experimental approach	Germination/ growth	Half-live time	Factors affecting Bt loss	References
	Ornamental tree	No	1-3 days		Hostetter et al., 1975[2]
	Potato, tomato, green pepper, and eggplant leafs	No	1 day*		Martin, 1994[2]
	Grapes (spraying of commercial products)	Not indicated#	At time of harvest: 10^2 - 10^4 CFU/g		Bae et al., 2004[2]
	Cannot be retrieved	No	16-38 h		Hansen et al.,1996[3]
	Broccoli	No	1 month		Madsen et al., 2011[3]
	Celery		<<1 month		Hendriksen et al., 2011[3]
	Cotton, amaranth, rice	No	120 h		Wang et al.,2014[3]
Protoxin	Pecan tree	-	14.3-24.4 h		Sundaram et al., 1997[1]
	Tomato	-	< 48 h		Walgenbach et al., 1991[1]
	Cotton	-	< 48 h		Wilson et al., 1983 (cited in Walgenbach et al., 1991)[1]
	Field, different crops	-	48-96 h*		Leong et al., 1980[1]

* no half-live times provided, therefore data for complete disappearance are given

† Adsorption of free toxins produced by transgenic plants avoids microbial degradation, insecticidal activity remaining after 234 d

not explicitly mentioned but data suggest absence of germination and growth

[1] OECD dossier M-IIM, Section 5 form DAR

[2] OECD dossier M-IIM Section 4 from DAR

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

[4] Current dossier M-MA section 7 below

According to Bae et al., 2004, Bt counts on fresh fruits and vegetables range between 10^2 and 10^4 CFU/g, because at the time of harvest, *B. thuringiensis* populations on grapes varied between 10^2 and 10^4 CFU/g based on experiments using Bt-based biopesticides (Dipel and Delfin) and 4 times of application (latest 5 days before harvest), depending on vineyard.

However, it was not confirmed that those levels have been solely due to the use of Bt-based biopesticides.

It has to be kept in mind also that this is still in the range of the normal level of *B. cereus*-group bacteria in food (please refer to B. 2.1.2 above).

The main reason for the restricted field persistence of insecticidal Bt strains might be their high degree of adaptation to host insects where they preferably complete their life cycle. Du & Nickerson (1996) investigated differences in activation/germination abilities of the Cry-producing Btk strains HD-1 and HD-73 and Cry^- mutants of the two strains. Spores from Cry-producing strains germinated slower compared to their Cry^- counterparts. In the presence of specific insect receptors, in the contrary, they were three times faster and also completed germination. Besides slow germination, it was also demonstrated that Cry^+ spores were less resistant to environmental stressors, such as heat, UV light and osmotic stress, when compared to Cry^- spores. The authors concluded that in terms of a distinction between *B. cereus* and *B. thuringiensis*, Bt has acquired a plasmid, a crystal and also a modified spore and that the trade-off for higher insect pathogenicity is a decreased spore resistance. Extremely low germination rates in the

absence of any activator (heat, alkaline conditions, germinant) in particular when compared to its Cry-deficient counterpart, has been repeatedly shown for Btk strain HD-1 (Abdoarrahem et al., 2009; King et al., 2012), a strain which is closely related to all commercial Bt strains. Taken together it can be concluded, that insecticidal Bt strains, such as the commercial strain Bta GC-91, are less competitive compared to non-Cry producing *B. cereus* group members. This might be the main reason for their restricted field persistence and leads to the assumption that germination and growth in either environment apart from host insect guts is rather unlikely.

Table 2.8-5: Physiological differences between spores formed by Cry-producing and Cry-deficient strains of the *B. cereus* group (after Du and Nickerson, 1996)

Spore characteristics	Presence and/or value in	
	Cry$^+$ spores (Bt)	**Cry $^-$ spores***
Protoxin on surface	Yes	No
Heat resistance	Lower	High
UV resistance	Lower	High
Osmotic resistance	Lower	High
Germination in 0.25 M acetate	No	Yes
Heat activation	Yes	Yes
Alkaline activation	Yes	No
Binding of insect BBMVs[#]	Yes	No
Spore coat dimensions	Thin	Thick

*Data on Cry$^-$ spores include data form Cry-deficient Bt and *B. cereus*
[#] BBMV: Brush Border Membrane Vesicles obtained from the gut of *M sexta*

Remark RMS:

In general, high densities of MBCA's are not likely to cause any adverse effect in natural systems. Based on ecological knowledge and experience derived from past and current MBC applications its seems reasonable to assume that population densities shifts and effects will not be different from those in the overall microbial communities due to natural environmental variation. In the common case where the control agent is applied at high dose to be effective against the target, population levels decline to "normal range levels" after application and proliferation or spread beyond the target situation is not apparent. According to the RMS the evaluation should not be based on a fear that released organisms will spread and become dominant in the environment and may have negative effects on other in the natural environment or even at humans as the fear is also connected to the fear that people get exposed to the organisms themselves or the metabolites they might produce in the food chain. Strains of micro-organisms used as MBCA's originate from natural microbial communities present in crops and nature, where populations fluctuate spread and adapt to changing environments as all organisms do in natural systems. The application of MBCA's only enhance the local abundance in order to do their antagonistic work against plant diseases or pests.

2) Survival during gastrointestinal passage

Available literature information indicates that survival of Bt spores may be affected by gastrointestinal passage. Hansen et al. (2011) for example, made an attempt to develop new approaches which could be useful to study the actual toxigenic potential of commercial Bt strains, one of which was Bta GC-91. They based their approaches on the fact that virulence of *B. cereus* group strains and as a result the development of gastrointestinal disease in humans depends on various factors including survival through stomach passage, germination and growth in the intestine, adhesion to and invasion to epithelian cells, expression of virulence factors etc. To assess the ability to survive the gastrointestinal passage they carried out two experiments. One mimicked gastric conditions (simulated gastric fluid at pH 2.0, 3.4 and 5.0, respectively) and the other conditions in the human intestine (simulated gastric fluid, pH

5, bile acids, micro aerobic conditions). In both experiments GC-91 had an initial 2-3 log values drop in growth, which stabilised thereafter. For more details, please refer to the full study summary of Hansen et al. (2011) below.

There are some studies assessing the fate of *B. cereus* group bacteria in rats or under conditions, mimicking gastro-intestinal passage but most of the studies were carried out with *B. cereus* strains and not with Bt. As there are differences between *B. cereus* and Bt strains indicated by Hansen et al. (2011) the information might not be fully applicable. One reference however, also focusses on commercial Bt strains (Wilcks et al. 2006a; obtained by the literature search according to EFSA guidance). In the study, rats with associated human-intestinal-microflora, were fed with commercial Bt strains (Btk DMU67R identical to HD1, and Bti HD567), either in the form of spores (10^6 - 10^7 spores/animal/day, untreated or heat-treated) or as vegetative cells (10^7 – 10^8 cells/animal/day) for 4 days. The study however, rather focussed on germination and growth than on persistence and will be discussed in more detail in the next point. What was obvious, is that when administered as vegetative cell, Btk poorly survived the gastric passage, whereas Bti vegetative cells did not survive gastric passage.

A similar experiment was carried out by Wilcks et al. (2006b) with a pathogenic *B. cereus* strain but also here the focus was on germination and growth. Ceuppens et al. (2012a) studied the population dynamics of *B. cereus* in a gastrointestinal simulation experiment. In the experiment the gastrointestinal passage was mimicked in 5 phases: (i) the mouth, (ii) the stomach, with gradual pH decrease and fractional emptying, (iii) the duodenum, with high concentrations of bile and digestive enzymes, (iv) dialysis to ensure bile reabsorption, and (v) the ileum, with competing human intestinal bacteria. Two clinical isolates from faeces and two food isolates of *B. cereus* were subjected to the gastro passage at a load of 10^7 spores/mL. As shown by other authors, spore population levels were not affected by the gastrointestinal passage.

3) Germination and growth in the human intestine

In general, it is expected that insecticidal Bt strains, being highly adapted to their insect hosts and requiring specific conditions for spore outgrowth, are unlikely to germinate and multiply in the human intestine.

However, germination, but not growth was observed in at least two Bt strains. In the study by Wilcks et al (2006a), the density of the two commercial Bt strains, one of which was a Btk strain (DMU67R) and the other one a Bti strain (HD567), was assessed in intestine samples and faeces upon oral gavage of human-flora-associated rats for 4 days. In animals fed spores, *B. thuringiensis* cells were detected in faecal and intestinal samples of all animals, 1 day after the last administration. Vegetative cells of Btk poorly survived the gastric passage, whereas Bti vegetative cells did not survive gastric passage. In 5/6 animals, Btk but not Bti, was detectable two weeks post administration in faeces samples, when feeding the animals the spores. One specific animal differed from the rest by a two log higher density of Btk DMU67R cells in the faecal samples (10^4 CFU/g) being observed at the end of the experiment. A similar tendency was observed in intestine samples. Btk but not Bti was still detectable in the samples two weeks after the last dosage and the animal having the high counts in faeces also had significant higher CFU numbers in intestine samples. Heat treatment of intestinal samples of this animal, thereby killing the vegetative cells but not the spores, revealed the presence of a high percentage of living vegetative cells. The same was observed in samples from 3 animals fed Bti untreated spores at sacrifice on day 5 post treatment. It can be concluded that vegetative cells and spores can survive the gastric passage, but the rate and extend depends on the Bt strain. Moreover, spores that survive the gastric passage do germinate in the gut.

For pathogenic *B. cereus* strains it is already known that they are able to germinate and grow in the gut.

The same authors as above also studied the fate and effect of a potentially pathogenic *B. cereus* strain (F4433/73R), isolated from a diarrhoeal food poisoning case, in the intestine of human-flora associated rats (Wilcks, et al., 2006b). *B. cereus* spores survived the gastric barrier well, and were in some cases detected in faeces up to two weeks after ingestion. Only in a single case, spore counts in excreted faeces increased towards the end of the experiment indicating that germination and multiplication occurred.

Also in experiments carried out by Ceuppens et al. (2012a, already mentioned above), germination and growth of a pathogenic *B. cereus* strain under simulated gastrointestinal conditions was observed. Germination started at the end of the duodenum phase and dialysis. When no competing intestinal bacteria were added during the ileum phase significant outgrow of the viable *B. cereus* cells was observed. However, in the presence of intestinal microflora no proliferation was detectable due to competition and/or inhibition by the indigenous microbes. Based on their observations, the authors concluded that *B. cereus*-induced diarrhea is not caused by massive *B. cereus* proliferation and

toxin production but by localized growth at the host´s mucus layer or epithelial surface. Based on the data obtained for the behaviour of the *B. cereus* strains in the presence/absence of the intestinal microflora the authors concluded that indigenous microbes act as a natural defense barrier and may play an important role in human susceptibility to diarrheal food poisoning. Actually, in another study the same authors demonstrate that the number, the nutrition status and the composition of the intestinal microflora may affect competition with *B. cereus* in the human intestine (Ceuppens et al., 2012b).

4) Adherence to and invasion of epithelian cells

Adhesion and invasion experiments were performed by Hansen et al. (2011) using vegetative bacterial cells of commercial Bt (including Bta GC-91) and pathogenic *B. cereus* strains in two different mammalian cell lines, namely HT29-MTX (grown for 28 - 29 days) and Caco-2 (grown for 18 - 19 days). While adhesion was demonstrated for all strains, for some strains even at high levels, invasion rates were rather low. There were no pronounced differences noted between the strains under investigation. However, in which trait the strains differed significantly was their action against Caco-cell monolayers. Generally, all strains where able to detach the monolayers but the time and also the dose required to see the effect was strongly strain specific. The pathogenic *B. cereus* strains were most aggressive while commercial Bta (GC-91) and Bti strains, independent from the dosage tested, always acted rather slowly. One of the commercial Bt strains (Btk) switched from an aggressive phenotype at high dosages tested to a less aggressive phenotype at low dosages. The grouping of the strains was confirmed by assessing their potential to compromise the epithelian cell barrier. It is assumed that during infection with a pathogenic microorganism the integrity of the epithelian monolayer may be compromised leading to diarrhea. Measuring the trans-epithelial electrical resistance (= TEER) it turned out that the strains acting less aggressive against Caco-cells (Bta GC-91 and commercial Bti strain) also showed a low potential to cause damage of epithelium cells. Pathogenic *B. cereus* strains in the contrary had a high toxigenic potential. The same was observed for the other two commercial strains, Btk and Btt. However, it is noteworthy that these latter two strains did not well survive conditions in the human stomach and intestine. Although all these experiments were carried out *in vitro* they clearly demonstrated differences between Bt strains commercially used for pest control and pathogenic *B. cereus* strains (Hansen et al., (2011). It has to be noted that based on the results of the study, the authors concluded that the toxigenic potential of Bta GC-91 is comparable to that of non-pathogenic reference strains.

5) Production of enterotoxins

The presence of a certain enterotoxin gene does not necessarily mean that it is expressed, and even if it is expressed under optimal conditions in the laboratory it might not be expressed in the human gastrointestinal tract.

Phelps and McKillip (2002) for example studied the presence of enterotoxin genes in various *Bacillus* spp., also outside the *B. cereus* group, as well as their expression using commercial immunoassay kits (Oxoid RPLA and Tecra BDE). In the study, a Btk strain proved to be the most unusual by exhibiting negative results on the commercial RPLA kit, which detects the L2 subunit of the hemolytic enterotoxin, a gene (hblC) for which this isolate demonstrated a positive PCR amplicon.

Damgaard (1995) studied the expression of enterotoxins in commercial Bt strains compared to a pathogenic *B. cereus* strain using the commercial Tecra VIA kit and found that for example strain GC-91 (designated Agree (=Turex) strain in the publication) had a titre of 21 (18-27) while the pathogenic strain *B. cereus* F4433/73 had a titre of 1629 (1350-2051). The titre is giving the reciprocal of the highest dilution in which the substances were still detectable. This indicates that Bta GC-91 is capable of producing enterotoxins under the right conditions, but at a much lower capacity that a pathogenic Bc strain. Damgaard also indicated that several Bt commercial products including Turex (=Agree) contained enterotoxin (no kwanitative measurements), but the toxin is heat-labile, as boiling the product for 12 minutes resulted in a negative enterotoxin test. Moreover, the enterotoxin is deactivated by the low pH in the stomach and duodenum.

Hansen et al. (2011) in their assay also investigated the expression of virulence genes during attachment of the commercial Bt strains and the pathogenic *B. cereus* strains to Caco-cells but the experiments revealed several shortcomings. Amongst others, was the density of attached cells too low to extract sufficient amounts of rRNA for the analysis and was expression of housekeeping genes instable, making an interpretation of the obtained results extremely difficult.

In most of the studies discussed in Points 2) and 3), namely, Wilcks et al. (2006a and b) studying commercial Bt and pathogenic *B. cereus* strains and Ceuppens et al. (2012) studying different *B. cereus* strains, attempts were made to measure production of enterotoxins under *in vivo* or simulated *in vivo* conditions (rat intestine samples or laboratory

culture mimicking the conditions in the intestine). The authors used either commercial kits (BCET-RPLA, Duopath and VIA-BDE) targeting the hemolytic and/or the non-hemolytic type of *B. cereus* enterotoxins or cytotoxicity assays with Vero cells, or both. Independent of the experimental approach and the method used for detection of the substances and despite germination and proliferation of the tested strains partly to high densities (e.g. in Ceuppens, et al., 2012a), production of toxins could not be demonstrated in any of the experiments.

The results of the 5 steps described above, needed for a micro-organism to act as a pathogen causing disease symptoms, are summarised for Bta GC-91 below:

1) persist on treated crops until the moment of consumption of the harvested good at relevant levels:

Bt spores do not perist on harvested goods, with complete disappearance between 15-60 days. However, for crops with short harvest to market time, levels of Bt between 10^2 and 10^4 CFU/g were measured. Levels of Bta GC-91 as high as 10^2 and 10^4 CFU/g can therefore not be excluded.

2) survive the gastrointestinal passage

Survival of the gastic passage seems to be dependant on the Bt strain. For GC-91, simulated human intestine circumstances reduced but not completely stopped growth, hence it cannot be excluded that CG-91 spores will survice the human gastric passage.

3) germinate and grow in the host

For some Bt strains is was demonstrated that spores could germinate in human-flora-associated rats. No growth was observed. For CG-91 it cannot be excluded that spores will germinate.

4) adhere to and invade the intestinal wall

In *in vitro* experiments Bta GC-91 was able to adhere to intestinal walls cells, and cause some damage. However, Bta GC-91 was acting the least aggressive against Caco-cells and also showed the lowest potential to cause damage of epithelium cells, when compared to the other Bt and Bc strains tested. The authors concluded that the toxigenic potential of Bta GC-91 is comparable to that of non-pathogenic reference strains.

5) produce toxins in the host at relevant levels

Independent of the experimental approach and the method used for detection of the substances and despite germination and proliferation of the tested strains partly to high densities, production of toxins could not be demonstrated in any of the experiments.

For GC-91 the occurrence of steps 1-3 cannot be excluded. However, each step will reduce the amount of GC-91 going to the next step. In *in vitro* experiments adhesion and damage to intestinal cells was seen for GC-91, but it was not significantly different from the negative controls. Moreover, steps 1-4 are still only the precursors for a possible production of toxins. Each step is more or less limiting the eventual possibility of enterotoxin production. Moreover, toxin production could not be demonstrated in any of the experiments, indicating that food-poisening is more likely to be produced by toxins already present in the food, rather then being formed after ingestin of the Bc group micro-organism.

From the available information it can therefore be concluded, that the likelihood for a commercial Bt strain, actually acting as a human pathogen and inducing diarrheal disease symptoms is rather unlikely.

Taking into account the available information, the EFSA BIOHAZ panel concluded in its opinion (EFSA, 2016[15]), that most cases of food-borne outbreaks caused by the *B. cereus* group have been associated with bacterial concentrations above 10^5 CFU/g foodstuff. The Panel concluded that the levels of *B. cereus* that can be considered as a risk for consumers are also likely to be valid for *B. thuringiensis*. It is currently discussed whether a safety limit for Bt on harvested goods/foodstuff should be established based on these information for *B. cereus*. The main argument for using the same value for Bt and *B. cereus* is that the two species cannot be distinguished by clinical diagnostics used in the described cases and that it is therefore unclear what is the actual contribution of Bt in foodborne disease outbreaks. However, due to the following reasons the approach is not justified:

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

a) Pathogenicity assessment leading to a certain safety value for a microbial active substance used for plant protection purposes can only be done at strain level as pathogenicity is a strain specific trait (SANCO/10754/2005 rev.5, 2005; Hackl et al., 2015[16]; EFSA, 2016[18])

b) Commercial Bt strains, including Bta GC-91, underwent extensive pathogenicity testing, confirming that the strains are not toxic, pathogenic or infective upon either route of exposure (please refer to info provided in MA Section 5 and MP Section 8)

c) For all commercial Bt strains, including Bta GC-91, methods are currently available for unequivocal identification of the strain distinguishing them from other *Bacillus* sp. including *B. cereus* and even from other Btk strains (please refer to MA, Section 1). Already during Annex I inclusion of Bta GC-91, AFLP patterns demonstrated that the strain is well separated from pathogenic strains in the *B. cereus* group (please refer to OECD dossier, Doc IIM Section 1, Point IIM 1.3.3).

d) Bt in general and this particularly applies to commercial Bt strains, which were selected for insecticidal activity, are first and foremost insect pathogens. As a result, insecticidal Bt strains are better adapted to complete their life cycle in infested host insects but not in other environmental compartment including the human intestine. Insecticidal proteins of Bt comprise up to 30% of the total cellular protein content, creating a large energy demand on the microorganism, which in turn affect strain characteristics such as the ability for spores to germinate and resist environmental stresses (Du & Nickerson, 1996; please also refer to the points already discussed above)

e) Commercial Bt strains differ from pathogenic *B. cereus* strains with regard to their physiology:

- they have lower germination rates and germination in general, is strongly restricted to conditions in the host insect gut (Du & Nickerson, 1996; Abdoharrahem et al., 2009, King et al., 2012)

- they have lower growth rates than pathogenic *B. cereus* strains (Hansen et al., 2011)

- they grow less well at high temperatures (Hansen et al., 2011)

- they grow less well at microaerobic conditions (Hansen et al., 2011)

- Hansen et al. (2011) even concluded that pathogenic *B. cereus* strains might be better adapted to survival in the human body than commercial Bt strains.

f) Commercial Bt strains have a much lower toxigenic potential than pathogenic *B. cereus* strains, which is particularly true for Bta GC-91.

- Pathogenic *B. cereus* strains have a higher potential to adhere to living surfaces (Auger et al., 2009)

- Pathogenic *B. cereus* strains act more aggressive against mammalian cells and have a higher potential to damage human epithelium cells during gastrointestinal passage than commercial Bt strains, in particular at low densities (Hansen et al., 2011). Based on their observations, Hansen et al. (2011) concluded that Bta GC-91 has a low toxigenic potential which is comparable to that of non-pathogenic reference strains.

- Pathogenic *B. cereus* strains produce much higher amounts of enterotoxins under optimal laboratory conditions than commercial Bt strains do. This was demonstrated for Bta GC-91 for example by Damgaard (1995, summarised below)

- Commercial Bt strains are not able to produce the emetic toxin cereulide (Kim et al., 2015; EFSA Scientific Opinion, 2016[17]). Thus, direct intoxication with cereulide will not be an issue for Bt strains but might have been the reason for foodborne disease in many reported outbreaks related to *B. cereus*.

g) 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" written by Ben Raymond (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 con-

[16] Evelyn Hackl, Margit Pacher-Zavisin, Laura Sedman, Stefan Arthaber, Ulla Bernkopf, Günter Brader, Markus Gorfer, Birgit Mitter, Aspasia Mitropoulou, Monika Schmoll, Willem van Hoesel, Elisabeth Wischnitzky, and Angela Sessitsch, 2015. Literature search and data collection on RA for human health for microorganisms used as plant protection products Reference. EFSA supporting publication 2015:EN-801. 173 pp.

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

sumed 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. 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 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 (Hernandez, Ramisse and Ducoureau 1998). That isolate did indeed pose a greater risk to mice than biopesticidal strains of Bt (Hernandez et al. 2000). Crucially, the Bt konkukian can be firmly placed in the anthracis clade and is distantly related to all the biopesticidal strains (Han et al. 2006; Raymond et al. 2010b; Raymond and Bonsall 2013); it is also

not demonstrably pathogenic to insects. Based on the ecological differentiation across the Bc group, we would not recommend licensing any Bt products that show a similar biological affinity to *B. anthracis*.

Here below, the abstract 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 GC-91 together with the proven absence of pathogenicity of the strain indicates that the risk for consumers following use of Bta GC-91 for pest control in agricultural settings is acceptable.

Summary and abstracts of cited literature:

Here below, full summaries of articles referring to Btk are provided as well as abstracts of articles referring to other species and sub-species. Apart from Kim et al. (2015) and Wilcks et al. (2006a) all references were obtained by a free literature search in PUBMED (http://www.ncbi.nlm.nih.gov/pubmed).

Report: KMA 2.8/06 – Kim, M.J., Han, J.K., Park, J.S., Lee, J.S., Lee, S.H., Cho, J.I., Kim, K.S. (2015), published report

Various enterotoxin and other virulence factor genes widespread among *Bacillus cereus* and *Bacillus thuringiensis* strains

J. Microbiol. Biotechnol, 25(6), 872–879

Guideline: Not specified

GLP: No

Abstract Many strains of *Bacillus cereus* cause gastrointestinal diseases, and the closely related insect pathogen *Bacillus thuringiensis* has also been involved in outbreaks of diarrhea. The diarrheal diseases are attributed to enterotoxins. Sixteen reference strains of *B. cereus* and nine commercial and 12 reference strains of *B. thuringiensis* were screened by PCR for the presence of 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*), and a pleiotropic transcriptional activator gene (*plcR*). These genes encode various enterotoxins and other virulence factors thought to play a role in infections of mammals. Amplicons were successfully generated from the strains of *B. cereus* and *B. thuringiensis* for each of these sequences, except the *ces* gene. Intriguingly, the majority of these *B. cereus* enterotoxin genes and other virulence factor genes appeared to be widespread among *B. thuringiensis* strains as well as *B. cereus* strains.

Material and Methods: Five commercial *B. thuringiensis* strains isolated from five different biopesticide products obtained from local retail establishments in rural areas of the Republic of Korea were screened in this study. A strain of each of the following commercial *B. thuringiensis* subspecies was also screened: *kurstaki*, *aizawai*, *israelensis*, and *tenebrionis*. Twelve and 16 reference strains of Bt and *B. cereus* (Bc), respectively, were screened in this study. Bt ATCC 33679, Bt ATCC 35646, Bt ATCC 19266, Bt ATCC 19268, Bt ATCC 13367, Bt ATCC 13366, Bt ATCC 11429, Bc ATCC 21366, Bc ATCC 21768, Bc ATCC 10876, Bc ATCC 21772, Bc ATCC 11778, Bc ATCC 10702, Bc ATCC 13061, Bc ATCC 14579, Bc ATCC 21769, and Bc ATCC 21771 were obtained from the American Type Culture Collection (Manassas, VA, USA). Bt KCTC 1508, Bt KCTC 1510, Bt KCTC 1511, Bt KCTC 1512, Bt KCTC 1513, and Bc KCTC 1094 were obtained from the Korean Collection for Type Cultures (Daejeon, Korea). Bc KFRI 181 was obtained from the Korea Food Research Institute (Sungnam, Korea). Bc IFO 3514, Bc IFO 3563, Bc IFO 3001, and Bc IFO 3003 were obtained from the Institute for Fermentation (Osaka, Japan). All bacterial strains were grown at 30°C on nutrient agar or in nutrient broth with shaking for preparation of template DNA for PCR screening.

Template DNA for PCR screening was prepared by processing 5 mL of culture grown for 18 h at 30°C, using a QIAamp DNA Mini Kit from Qiagen.

PCR analyses were carried out to detect 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*), and a pleiotropic transcriptional activator gene (*plcR*) among *B. cereus* and *B. thuringiensis* strains.

PCR reaction mixtures for amplification of sequences encoding toxins and putative virulence factors contained 5 µL of template DNA (25 ng), 10 mM Tris-HCl (pH 8.3), 10 mM KCl, 0.2 mM each deoxynucleoside triphosphate, 2.5 mM $MgCl_2$, 1 µM each primer, and 0.5 U of Taq DNA polymerase (Solgent Co., Daejeon, Korea). The optimized PCR conditions were as follows: a single denaturation step of 3 min at 95°C; 35 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 45 sec, and extension at 72°C for 1.5 min; and a final extension at 72°C for 5 min. To validate the results, all PCR amplifications were performed a minimum of three times. After DNA amplification, PCR fragments were analyzed by submarine gel electrophoresis, stained, and visualized under UV illumination. Suitable molecular size markers were included in each gel. To identify cases in which poor quality of template DNA caused amplification failure, the quality of any DNA extract that failed to amplify in a specific reaction was examined by attempting amplification with a pair of universal primers designed to amplify a region of the 16S rRNA gene. Negative controls were included with all amplifications. Suitable controls such as buffer, media, PCR mixtures, and template DNA were used to detect any false-positive or false-negative reactions.

Findings: The occurrence of genes encoding the enterotoxic HBL complex (*hblA, hblC*, and *hblD*), the nonhemolytic enterotoxin (*nheA, nheB*, and *nheC*) in the reference strains of Bt and Bc is summarised in **Table 2.8-6.**

Five and three of the commercial strains, respectively, harboured either one or two of the *hbl* genes while other Bt reference strains harboured at least 2 or even 3 of the genes. Only two of the Bt strains did not contain the genetic determinants for the *hbl* gene complex. Interestingly, most of the Bc reference strains only contained single genes and the number of strains containing three of the genes was considerably lower than within the Bt reference strains. All commercial Bt strains were shown to contain two or three genes of the *nhe* complex. The findings for the remaining Bt and Bc strains very similar.

Table 2.8-6: Occurrence of enterotoxigenic hbl and nhe genes in *B. cereus* and *B. thuringiensis*

Strain	Frequencies of *hbl* genes (%)							
	hblA	*hblC*	*hblD*	*hblA + hblC*	*hblA + hblD*	*hblC + hblD*	*hblA + hblC + hblD*	None
B. thuringiensis								
Commercial (n = 9)	5 (56)	0 (0)	0 (0)	0 (0)	3 (33)	0 (0)	0 (0)	1 (11)
Reference (n = 12)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	3 (25)	8 (67)	1 (8)
Total (n = 21)	5 (24)	0 (0)	0 (0)	0 (0)	3 (14)	3 (14)	8 (38)	2 (10)
B. cereus								
Reference (n = 16)	1 (6)	1 (6)	4 (25)	0 (0)	3 (19)	1 (6)	2 (13)	4 (25)
	Frequencies of *nhe* genes (%)							
	nheA	*nheB*	*nheC*	*nheA + nheB*	*nheA + nheC*	*nheB + nheC*	*nheA + nheB + nheC*	None
B. thuringiensis								
Commercial (n = 9)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (22)	7 (78)	0 (0)
Reference (n = 12)	0 (0)	0 (0)	0 (0)	3 (25)	1 (8)	3 (25)	5 (42)	0 (0)
Total (n = 21)	0 (0)	0 (0)	0 (0)	3 (14)	1 (5)	5 (24)	12 (57)	0 (0)
B. cereus								
Reference (n = 16)	1 (6)	0 (0)	1 (6)	1 (6)	0 (0)	2 (13)	9 (56)	2 (13)

The occurrence of other enterotoxigenic and emetogenic genes in the Bt and Bc strains is summarized in **Table 2.8-7**. Most of the strains harboured the genes encoding Cytotoxin K and enterotoxin T. All commercial strains lacked the *entFM* genes while *entS* genes were widespread in all Bt strains. The distribution of the genes in Bc reference strains was very similar. For none of the strains under investigation the presence of the *ces* gene was demonstrated. Genes for hemolytic activity and the pleiotropic transcriptional activator gene were found to be widespread in all strains (**Table 2.8-8**).

Table 2.8.-7 Occurrence of other enterotoxigenic and emetogenic genes in *B. cereus* and *B. thuringiensis*

Strain	Cytotoxin K (*cytK*)	Enterotoxin T (*bceT*)	Enterotoxin FM (*entFM*)	Enterotoxin S (*entS*)				Cereulide (*ces*)
				TY123/TY124	TY123/TY125	TY123/TY126	TY123/TY127	
B. thuringiensis								
Commercial (n = 9)	7 (78)	8 (89)	0 (0)	9 (100)	4 (44)	0 (0)	0 (0)	0 (0)
Reference (n = 12)	12 (100)	12 (100)	10 (83)	10 (83)	9 (75)	1 (8)	12 (100)	0 (0)
Total (n = 21)	19 (91)	20 (95)	10 (48)	19 (91)	13 (62)	1 (5)	12 (57)	0 (0)
B. cereus								
Reference (n = 16)	13 (81)	14 (88)	12 (75)	13 (81)	9 (56)	0 (0)	15 (94)	0 (0)

Table 2.8-8 Occurrence of hemolytic genes and pleiotropic transcriptional activator gene in *B. cereus* and *B. thuringiensis*.

	Hemolysin A (*hlyA*)	Hemolysin II (*hlyII*)	Hemolysin III (*hlyIII*)		Phosphatidylinositol -specific phospholipase C (*plcA*)	Cereolysin AB		Cereolysin O (*cerO*)	Pleiotropic transcriptional activator (*plcR*)
			bchem1/4	bchem2/3		*cerA*	*cerB*		
B. thuringiensis									
Commercial (n = 9)	9 (100)	5 (56)	4 (44)	5 (56)	9 (100)	8 (89)	7 (78)	7 (78)	9 (100)
Reference (n = 12)	10 (83)	1 (8)	12 (100)	11 (92)	11 (92)	6 (50)	11 (92)	9 (75)	12 (100)
Total (n = 21)	19 (90)	6 (29)	16 (76)	16 (76)	20 (95)	14 (67)	18 (86)	16 (76)	21 (100)
B. cereus									
Reference (n = 16)	16 (100)	4 (25)	14 (88)	15 (94)	12 (75)	6 (38)	9 (56)	8 (50)	16 (100)

Conclusion: Genes encoding for enterotoxic and hemolytic activity are widespread in Bt and Bc including commercial Bt strains. None of the commercial strains and also none of the other reference strains appear to be able to produce cereulide.

****

Report: KMA 2.8/07 Wilcks, A., Hansen, B.M., Hendriksen, N.B., Licht, T.R. (2006a), published report
Persistence of *Bacillus thuringiensis* bioinsecticides in the gut of human-flora-associated rats
FEMS Immunol Med Microbiol, 48(3):410-418

Guideline: Not applicable

GLP: No

Abstract The capability of two bioinsecticide strains of *Bacillus thuringiensis* (ssp. *israelensis* and ssp. *kurstaki*) to germinate and persist in vivo in the gastrointestinal tract of human-flora-associated rats was studied. Rats were dosed either with vegetative cells or spores of the bacteria for 4 consecutive days. In animals fed spores, *B. thuringiensis* cells were detected in faecal and intestinal samples of all animals, whereas vegetative cells only poorly survived the gastric passage. Heat-treatment of intestinal samples, which kills vegetative cells, revealed that B. thuringiensis spores were capable of germination in the gastrointestinal tract. In one animal fed spores of B. thuringiensis ssp. kurstaki, these bacteria were detected at high density (10^3 - 10^4 CFU/ g faecal and intestinal samples) even 2 weeks after the last dosage. In the same animal, passage of *B. thuringiensis* ssp. *kurstaki* to the spleen was observed; however, no other adverse effects were observed. Denaturing gradient gel electrophoresis of PCR-amplified bacterial 16S rRNA genes in faecal samples revealed no major effect of *B. thuringiensis* on the composition of the indigenous gut bacteria. Additionally, no cytotoxic effect was detectable in gut samples by Vero cell assay.

Material and Methods: The rifampicin-resistant *B. thuringiensis* ssp. *kurstaki* (Btk) DMU67R identical to HD1 and a spontaneous streptomycin-resistant *B. thuringiensis* ssp. *israelensis* (Bti) HD567 were used for inoculation of the animals. When not stated otherwise, the strains were grown in Luria–Bertani medium (LB) supplemented with 50 µg/mL rifampicin or 100 µg/mL streptomycin, respectively. For production of spores the bacteria were grown in sporulation media containing 20 µg/mL rifampicin or 40 µg/mL streptomycin, respectively, for about 1 week at 30°C.

Six germfree human-flora-associated Sprague-Dawley rats (7 - 9 weeks old) per group each were dosed for 4-consecutive days with B. thuringiensis strains either (1) irradiated spores (control), (2) untreated spores, (3) heat-treated spores (mimicking heating of food), or (4) vegetative cells.

Rats fed Btk received either 10^7 spores (untreated or heat-treated) or 10^7 – 10^8 vegetative cells per day. Animals dosed with Bti HD567 received 10^8 untreated spores, 10^6 heat-treated spores, or 10^8 vegetative cells. Half of the animals were sacrificed at day 5, and the remaining half at day 18.

Faecal and intestinal samples (duodenum, ileum, caecum, and colon) as well as samples of spleens and livers were diluted in saline supplemented with 0.1% peptone. For enumeration of *B. cereus*-like bacteria, dilutions were plated on Bacillus cereus Selective Agar. The same media supplemented with either 50 µg/mL rifampicin or 100 µg/mL streptomycin were used for enumeration of Btk DMU67R and Bti HD567, respectively. For counting of spores, dilutions of intestinal samples taken at sacrifice were treated at 80°C for 15 min to kill the vegetative cells and enumerated in the same way. Lactobacilli, coliforms and enterococci as well as total aerobic and anaerobic

germs were enumerated after appropriate incubation on selective media.

For DGGE analysis of fecal samples, DNA was extracted from samples of two animals from each group at 3 independent days before dosage, at 3 days during the week of dosage and at 3 independent days after dosage. DNA extracts were submitted to PCR with universal primer sets and subsequent DGGE analysis to obtain intestine microbial community profiles. Prominent DGGE bands were extracted from the DGGE gels and sequenced to identify the dominant species in the communities.

Intestinal samples were studied using the Vero Cell assay and the BCET-RPLA toxin detection kit from Oxoid to investigate in vivo toxin production.

Findings: *B. thuringiensis* cells were detected in faecal and intestinal samples of all animals treated with spores, whereas vegetative cells only poorly survived the gastric passage. No difference between Btk recovered from faecal samples of rats dosed with untreated and those dosed with heat-treated spores was detectable. In 5/6 animals fed spores, Btk but not Bti, was detectable 2 weeks post administration Additionally, in one animal fed spores of Btk were detected at high density (10^3 – 10^4 CFU/g faecal and intestinal samples) even 2 weeks after the last dosage (**Table 2.8-9**). Bti HD567 untreated spores were found at a higher density in the small intestine than observed for Btk DMU67R while at day 18 the density was below the detection limit in all samples independently of whether spores or vegetative cells were administered (**Table 2.8-9**).

Table 2.8-9 Presence of Btk DMU67R in intestinal samples from HFA rats, dosed either with untreated spores, heat-treated spores, or vegetative cells.

	Density of DMU67R (log CFU g^{-1} intestinal content)					
	Day 5			Day 18		
Intestinal sample	Untreated spores	Heat-treated spores	Vegetative cells	Untreated spores	Heat-treated spores	Vegetative cells
Duodenum	2.49 ± 0.47	2.64 (1/3)*	†	†	2.53 ± 1.31 (2/3)	†
Ileum	3.32 ± 0.25	2.60 ± 0.96	†	†	3.39 (1/3)	†
Caecum	5.70 ± 0.12	5.31 ± 0.14	1.30 (1/3)	1.30 ± 0.00 (2/3)	2.11 ± 1.15	†
Colon	5.82 ± 0.13	5.73 ± 0.17	1.43 ± 0.38	1.30 (1/3)	2.64 ± 0.91 (2/3)	†

*Number in brackets indicates how many animals were positive out of total number of animals tested if this differs from three.
†Number of cells was below detection limit (10 CFU g^{-1} intestinal sample).

Table 2.8-10 Presence of Bti HD567 in intestinal samples from HFA rats, dosed either with untreated spores, heat-treated spores, or vegetative cells.

	Density of HD567 (log CFU g^{-1} intestinal content)					
	Day 5			Day 18		
Intestinal sample	Untreated spores	Heat-treated spores	Vegetative cells	Untreated spores	Heat-treated spores	Vegetative cells
Duodenum	4.36 ± 0.34	1.60 (1/3)*	†	†	†	†
Ileum	5.85 ± 0.27	2.96 ± 0.59	†	†	†	†
Caecum	5.79 ± 0.06	3.47 ± 0.51	†	†	†	†
Colon	5.84 ± 0.07	3.44 ± 0.60	†	†	†	†

*Number in brackets indicates how many animals were positive out of total number of animals tested if this differs from three.
†Number of cells was below detection limit (10 CFU g^{-1} intestinal sample).

Intestinal samples from animal no. 15 fed Btk DMU67R were heat treated to kill bacteria in the vegetative state. This revealed that 90% of the cells found in the small gastrointestinal tract (duodenum and ileum) were present as vegetative cells. In the large intestinal samples (caecum and colon) the percentage of vegetative cells was lower (Figure 2.8-2). Similar results were obtained for animals fed spores of Bti HD567.

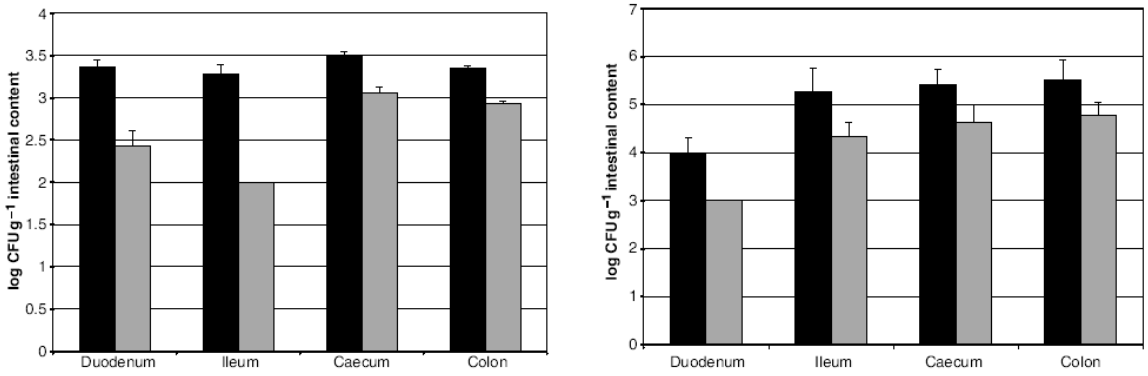

Figure 2.8-2 Heat-treatment of intestinal samples from animal 15 fed Btk DMU67R untreated spores at sacrifice on day 18 of the experiment (left) and of samples from 3 animals fed Bti HD567 untreated spores at sacrifice on day 5 of the experiment (right). Black columns represent the untreated samples, whereas grey columns represent the heat-treated (80°C, 15 min) samples. The experiment was performed twice.

Denaturing gradient gel electrophoresis of PCR-amplified bacterial 16S rRNA genes in faecal samples revealed no major effect of Btk and Bti on the composition of the indigenous gut bacteria.

In none of the animals fed either of the two investigated *B. thuringiensis* strains were enterotoxins observed in samples from the intestine.

Conclusion: Although germination of spores was detected and it is known that both of the investigated B. thuringiensis strains produce enterotoxins in vitro, no in vivo production of enterotoxins was detected by application of Vero cell assays to intestinal samples from animals fed with either of the strains.

****

Report:	MA 2.8/08 - Du, C., Nickerson, K.W. (1996), published report Applied and Environmental Microbiology, 62(10):3722-3726
Title:	*Bacillus thuringiensis* HD-73 Spores Have Surface-Localized Cry1Ac Toxin: Physiological and Pathogenic Consequences
Abstract	Spores from Cry^+ strains of *Bacillus thuringiensis* bound fluorescein isothiocyanate-labeled antibodies specific for the 65-kDa activated Cry 1Ac toxin, whereas spores from *Bacillus cereus* and Cry^- strains of *B. thuringiensis* did not. The Cry^+ spores could be activated for germination by alkaline conditions (pH 10.3), whereas Cry^- spores could not. Once the surrounding exosporia had been removed or permeabilized, Cry^+ spores were able to bind the toxin receptor(s) from insect gut brush border membrane vesicle preparations, and their germination rates were increased ca. threefold in the presence of brush border membrane vesicles. A model is presented whereby in the soil the Cry toxins on the spore surface are protected by the exosporium while in the gut they are exposed and available for binding to the insect receptors. This model explains why the disulfide-rich C terminus of the cry genes is so highly conserved even though it is removed during the processing of the protoxin to the activated toxin. It also highlights the trade-off resulting from having Cry toxins located on the spore surface, i.e., decreased spore resistance versus enhanced insect pathogenesis.

****

Report:	MA 2.8/09 - Abdoarrahem, M.M., Gammon, K., Dancer, B.N., Berry, C. (2009), published report Applied and Environmental Microbiology, 75(19):6410-6413
Title:	Genetic Basis for Alkaline Activation of Germination in *Bacillus thuringiensis* subsp. *israelensis*
Abstract	Differences in activation between spores from strains of *Bacillus thuringiensis* subsp. *israelensis* with and without the toxin-encoding plasmid pBtoxis are demonstrated. Following alkaline activation, the strain bearing pBtoxis shows a significantly greater germination rate. Expression of just three genes constituting a previously identified, putative ger operon from this plasmid is sufficient to produce the same phenotype and characterizes this operon as a genetic determinant of alkaline activation.

****

Report:	MA 2.8/10 - King, P.J.H., Ong, K.H., Sipen, P., Mahadi, N.M. (2012), published report African Journal of Biotechnology, 11(56):11925-11930
Title:	Toxicity of local Malaysian *Bacillus thuringiensis* subspecies *kurstaki* against *Plutella xylostella*
Abstract	The toxicity effect of *Bacillus thuringiensis* against *Plutella xylostella* is well established. However, effective B. *thuringiensis* strain especially local isolate is not well tested. In this study local strain *B. thuringiensis* subspecies *kurstaki*, SN5 was assessed for its effectiveness against *P. xylostella* 3rd instar larvae. Other factors such as spore germination, spore coat, L-alanine-adenosine (LAA) and streptomycin were evaluated with their possible effects on the toxicity of *B. thuringiensis* cry protein. The result of the study showed that SN5 spore exhibit higher toxicity than the commercial strain, HD-1. L-Alanine-adenosine not only improves rate of spore germination but also synergy effect of spore-crystal mix by increasing toxicity of the mixture. These results demonstrating the potential of local isolate in managing P. xylostella and its potential effect can be increase by adding LAA.
Justification	The reference is included as it contains information about germination rates of HD-1 and a Cry-deficient variant of HD-1, namely 4D8.

****

Report:	KMA 2.8/11 Hansen, B.M., Thorsen, L., Nielsen-LeRoux, C., Wilcks, A., Hendriksen, N.B. (2011), published report Pesticides Research 136 2011 Danish Ministry of the Environment, Environmental Protection Agency
Title:	New experimental approaches for human risk assessment of microbial pest control agents – exemplified by the bacterium *Bacillus thuringiensis*
Abstract	The aim of the project has been to investigate three different types of pathogenicity models for their ability to assess virulence in microorganisms used as pest control agents. The three models were the nematode *Caenorhabditis elegans*, *Galleria mellonella* larvae and functional mammalian cell-models. Four different strains were chosen as primary objects for the investigations; these strains originate from the products Agree (Bta strain GC-91, in the study indicated as Bt50), Vectobac, Dipel and Novodor. To be able to compare these four product strains in relation to virulence, four related strains, which are supposed to be pathogenic, were selected (positive control strains). A further two strains were

selected that were supposed to be avirulent.

The nine selected strains were tested under different *in vitro* conditions to study their potential to survive in the gastrointestinal tract of humans – the first prerequisite of a *Bacillus thuringiensis* strain to cause disease. Besides the survival under gastrointestinal conditions, i.e. survival in gastric and small intestinal environments, the strains were also studied at low and high temperatures. Previous studies have shown that pathogenic strains grow better at low and high temperatures compared to environmental strains. This means that studying the temperature profile of a strain can give an indication on the predisposition of the strain to cause disease in humans.

There is a tendency in the nine strains investigated, that strains isolated from either food involved in outbreaks or from patients have better growth capacity at high temperatures and better survival at gastrointestinal conditions than strains used commercially in plant protection products.

Bt *aizawai* GC-91 has limited growth at 43°C, survives well at the stomach conditions, affects the Caco-cells, and to a lesser extent the HT-29 MTX cells, and has a limited effect on the PoM2 cells after 22 hours (if gentamicin is added 1 h post infection). It has some, but low, effects on *Galleria* and the nematode *C. elegans*. From an overall point of view this strain is more comparable with the negative control strains than with the positive controls. GC-91 seems therefore to have a pathogenic potential which is lower, than the strains from pathogenic cases, especially the two strains isolated from blood and a gastrointestinal case.

The results obtained with the developed methodology show, that the bacteria with regard to virulence toward the nematode, can be divided into four categories, and that they all got a higher virulence than an *E. coli* strain, which are considered to be avirulent. The strain with the lowest virulence is Bt50 (Bta GC-91) from Agree.

In order to get an idea about the speed of the strains to develop in the host, the spore germination capacity was first analysed. The results showed a variation between strains at 3 hours post ingestion, while at 24 hours all strains had largely germinated. Virulence following oral infection was measured for all ten strains with one or several doses. Generally we did not find any strong difference among strains. Meanwhile, we found a more pronounced strain difference when the strains were used for infection by injection into the hemocoel.

Functional mammalian cell models

The intestine is composed of epithelial cells which are bound closely together, forming a barrier. If you have diarrhea or suffer from an intestinal illness, this barrier has been compromised. By using a cell model of the gut it is possible to measure the electrical resistance across a cell layer (transepithelial electrical resistance, TEER), which provides a measure of how close the epithelial cells are bound. It has previously been shown that probiotic bacteria may increase the TEER, while pathogenic bacteria can reduce the TEER in the model system (Klingberg et al. 2005). The current work has used two types of epithelial cell lines, Caco-2 and HT29-MTX that are both of human origin. The Caco-2 is a cancer cell line that differentiates itself during growth, so that it physically and biochemically resembles the adsorptive epithelial cells of the small intestine. In comparison HT29-MTX is a "goblet" cell type that produces a protective layer of mucus during prolonged culturing. A macrophage cell line PoM2 of porcine origin, has also been used to measure the bacteria's ability to circumvent the immune system (machrophage engulfment).

Results of this study show that all the investigated strains (*B. thuringiensis* product strains, a probiotic and pathogenic strains) reduces the transepithelial electrical resistance (TEER) of Caco-2 and HT29-MTX cell monolayers. All the strains caused morphological changes / damage to the epithelial as observed by microscopy. The results indicate that all investigated bacteria including the probiotic control have pathogenic potential. By examining the effect of the dose of bacteria, however, it was clear that bacteria could be differentiated based on the speed with which they damaged the mammalian cells, particularly at low infectious dose. By use of the functional mammalian cell models, two product strains (including Bta GC-91) could be placed primarily in the group of bacteria with low potential for virulence (were "slower" than the probiotic bacterium).

Material and

RT-PCR detection of virulence expression

Primers used for RT-PCR reactions were designed using the primer Express software (Applied Bio-

Methods

systems) or the CLC main workbench software version 5 (CLC Bio, Aarhus, Denmark). RNA was isolated according to Tri Reagent (Ambion) protocol. RNA samples were treated with the Turbo DNAse kit (Ambion) according to the manufacturer's instructions. The reverse transcription was performed on 1 µg of RNA using The High Capacity cDNA RTPCR kit according to the guidelines given by the supplier (Applied Biosystems) or the Stratagen Affinity Script QPCR cDNA synthesis Kit (La Jolla, CA, USA). RT-PCR was set up in MicroAmp Fast Optical 96-Well Reaction Plates (Applied Biosystems) using the PowerSYBRgreen Master Mix (Applied Biosystems). The reactions were prepared in triplicates in a total volume of 20 µL; the final concentrations of primers were 500 nM. Plates were covered with MicroAmp Optical AdhesiveFilm (Applied Biosystems). Amplification of PCR products and recording of fluorescence were done with the 7500 Fast Real-Time PCR system (Applied Biosystems) using the following program: 1 cycle at 95°C for 15 min, 40 cycles at 95°C for 15 sec followed by 75°C for 1 min, and finally a dissociation stage consisting of 1 cycle at 95°C for 15 s, 57°C for 1 min, 95°C for 15 s. Alternatively Q-RT-PCR analysis were also realized by SYBR green system Kit BrilliantII SYBR Green QPCR master Mix using the equipment from Applied Biosystems 7900HT, on the experimental Platform at INRA, Jouy en Josas. Quantitative expression was performed by relative expression as based on Ct delta Ct. The results are analysed by the software SDS2.3 (Applied Biosystems).

Temperature growth characteristics and survival under gut/stomach conditions

Growth at low temperatures

One colony from a fresh LB (Luria-Bertani, Oxoid) plate was streaked onto a new LB plate, and incubated aerobic and micro aerobic at 10ºC. Growth was measured once a week for up to three weeks. The experiment was performed twice.

Growth at high temperatures

One colony from a fresh LB plate was streaked onto a new LB plate, which was incubated aerobic and micro aerobic at 37, 43, 45 and 50ºC for a period up to 5 days. The experiment was performed up to three times.

Survival under simulated gastric conditions

One mL of an o/n culture was added to 100 mL SGF (Simulated gastric fluid; 2 g NaCl and 3.2 g pepsin (Sigma, P7000) in 7 mL 12 M HCl. Sterile MilliQ was added up to 1 L. pH was adjusted with HCl and NaOH to the desired values) in 250 mL sterile bottles. The bottles were incubated up to 4 hours at 37ºC. Samples (200 µL) were taken regularly and diluted in MRD (Maximum Recovery Diluent, Difco) and spotted (20 µL) on LB agar plates. The plates were incubated overnight at 30ºC. SGF with three different pH values: 2.0, 3.4 and 5.0 was tested twice.

Survival under simulated small intestinal conditions

One mL of an overnight culture (grown at 37ºC) was added to 40 mL pre-warmed (37ºC) SGF, pH 5.0 and incubated for 30 min at 37ºC. To the culture 50 mL double strength LB (37ºC) and 10 mL sterile filtrated bile acid (B8631, Sigma) solutions (1.5 or 3.0 g/L) was added. The culture was incubated under micro-aerophilic conditions at 37ºC for up to 4 hours. Samples (1 ml) were drawn at 0, 1, 2 and 4 hours, diluted in MRD and spotted on LB agar plates.

Detection of enterotoxin HBL using a commercial kit

O/n cultures were established in BHI broth supplemented with 1% glucose (BHIG) at 270 rpm and 32°C. For production of enterotoxic substances, 20 µL of bacterial culture was added per mL BHIG, and the cultures were cultivated at 270 rpm and 32°C for six hours. The culture was centrifuged at 15000 g for 3 min at 4ºC, the supernatant was sterile filtrated and for detection of the L2 component of HBL, the BCET-RPLA toxin detection kit from Oxoid (TD0950) was used as recommended by the manufacturer.

Caenorhabditis elegans *model*

Synchronized *C. elegans* populations were washed from the growth medium with sterile water and

10 µL nematode suspensions were transferred to a number of wells in a 96 well microtiter plate, to reach 15 – 20 nematodes per 10 µL per well. The plates were incubated at 15°C 30 in a sealed box at 100% relative humidity to avoid evaporation. The bacteria to be tested were inoculated in 2 mL liquid BHI substrate in 14 mL Falcon tubes, and incubated o/n at room temperature and 275 rpm. 20 µL (BHI, NGM, T3 or water) +/-50 µg/mL ampicillin +/-2, 5% Laked Horse Blood (Oxoid SR0048C) were added to the wells. The ampicillin was added to inhibit growth of *E. coli* OP50, and only allowing the *B. cereus* group bacteria to grow, as an absolute majority of *B. cereus* group bacteria are resistant to penicillins. The horse blood was added to imitate a situation where bacteria had got access to blood in the human body. The bacteria were diluted in water (125, 250 or 500 times) and 10 µL diluted suspensions were added to the wells. In control wells with the *E. coli* OP50 no ampicillin was added. The following days, the numbers of living nematodes in the wells were counted. In liquid, living nematodes had at least two bends, while dead nematodes had only one or no bend. The survival data were analyzed by ANOVA on ranked data.

Galleria mellonella *model*

Infections assays were run both with spores and vegetative bacteria. 25 larvae per dose (about 200 mg each) were infected orally with 10 µL of either a suspension with spore or vegetative bacteria alone or mixed with Cry1C toxin as a synergy factor, at 3 microgram per larva. Mortality was scored at 6, 24 ad 48 hours post infection following incubation at 37°C. All tests are repeated at least 3 times. Control larvae were fed with PBS buffer alone or Cry1C in buffer alone. Although the project are mainly concerned with infection related to interaction with the intestinal barriers, the relative virulence among strains was also investigated following infection by injection of spores and vegetative bacteria into the hemocoel of the insect larvae. 10 microliter of spore suspension containing various doses of spores (3000, 10000, 30000) in PBS buffer using a cutting headed needle. Larvae are incubated at 37°C and mortality is scored over 3 days.

Determination of spore germination

Spore germination assays were performed with 2 × 2 larvae per strain (repeated three times) by homogenisation of whole larvae in 10 mL of PBS buffer 3 and 24 hours post infection. The suspensions are divided into two, and one is submitted to heat chock (78°C for 12 min). These analyses are run with and without addition of Cry1C toxin to the spores. Determination of level of spore germination is done by plating of various dilutions of the two suspensions. The CFU (colony forming units) were counted and the percentage of spores germinated (not heat resistant) in larvae was determined. The infections are done with high doses of vegetative bacteria from LB culture. About 15 mL at OD = 650 nm between 1.2 to 3 are centrifuged and suspended in Cry1C toxin (0.3 mg/mL). The obtained dose is (5×10^7 to 2×10^8). 20 larvae are infected and incubated at 37°C. 6 hours post infection 5 larvae are dissected and the midgut is immediately and gently homogenized in 100 microliter buffer. 10 microliter of this is sampled for estimation of bacterial content, and the remaining is transferred to liquid nitrogen and stored at -70°C. The five midguts are pooled together before total RNA extraction and bacterial estimation is done on the 5 pooled 10 microliter samples. The remaining 5 × 90 microliter are frozen in liquid nitrogen and stored at -70°C until extraction of total RNA following the protocol of the TriReagent (Ambion) RNA extraction followed by DNAase treatment. Extraction was done if the amount of bacteria in the pool was at least 1×10^8.

Expression of virulence genes

The reverse transcription (RT), was performed using the Stratagen Kit and random hexamers. Thus, the obtained cDNA was a mixture of fragments amplified from mRNA from both bacteria and *G. mellonella.* The quality of RNA and cDNA, was verified by Nano and Pico Agilent chips. Extractions and analysis were done twice from 5 larvae infected with the same bacteria preparation and at least one time from a second infection for each strain. Before running SYBR-green Q-PCR, endpoint PCR was performed in order to get a first indication for gene expression. Q-PCR was only performed if a positive result was found by endpoint PCR.

Mammalian cell model

Morphology, adhesion and invasion

The human colon adenocarcinoma cell line Caco-2 and mucus producing HT29-MTX were used. Caco-2 cells were seeded in 24 well plates (Nunc, Denmark) at a density of 1×10^5 cells per well and incubated for 7-22 days. Growth media without antibiotics was changed every 2 - 3 days. Effect of bacterial cell density on morphology of Caco-2 cells was observed every hour from 0 h of infection using bright field microscopy. Adhesion and invasion experiments using Caco-2 (17 - 18 days) or HT29- MTX (28 - 29 days) were performed using 24 well culture plates (Nunc). Infection was done with approximately 104 bacteria per well and for 3 hours at 37°C, 5% CO_2, 95% air. The number of adhering bacterial cells per well was determined by removing the growth media, washing the wells 3 times with PBS, pH 7.0, lysing with 1 mL sterile MQ-water and by using the drop plate method. The number of invading cells per well was determined by removing the growth media and incubating at room temperature for 40 - 50 minutes together with 1 mL 100 or 200 µg gentamicin/mL PBS. Wells were washed with PBS, cells were lysed with MQ-water and CFU/well was determined as above. Experiments were repeated at least 2 times in duplicate.

TEER

Measurements of the transepithelial electrical resistance (TEER) of Caco-2 or HT29-MTX monolayers seeded on Transwell filter inserts (0.4 µm pore size, 12 mm) in 12-well plates (diameter: 22.1 mm; Corning Incorporated, Sigma Denmark and infected with approximately 103 CFU of *Bacillus*/mL on the apical side was conducted as previously described. Each assay was conducted at least twice (more than two different passages) with triplicate determinations. Average TEER values at time 0 h was 940 Ωcm^2 for Caco-2 and 270 Ωcm^2 for HT29-MTX. The statistical significance of the effect of bacterial infection on TEER (HT29-MTX) was analyzed by two way ANOVA combined with a Bonferroni post-test using the GraphPad Prism Software version 5 (GraphPad Software, Inc. La Jolla, California, USA). The level of significance was $p = 0.05$.

Mitochondrial activity

Mitochondrial function may serve as an index of living metabolically active cells. The effect of various *Bacillus* spp. on the mitochondrial function of PoM2 macrophage/Monocytes was examined using the Methylthiazolyldiphenyl-tetrazolium bromide (MTT) –assay (3-(4,5- dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (Sigma) as described by Bergamini et al. (1992). Initially PoM2 cells were seeded in 96 well plates, 0.33 cm^2 well areas (Nunclon surface, Nunc, Denmark) at a density of 2×10^5 cells per well and incubated for 3 days at 37°C, 5% CO_2, 95% air. The monolayer was washed 3 times with PBS pH 7.0, and fresh media was added before infection with bacteria. Bacteria were added at 102 or 104 cells per well (moiety of infection of 1:600 or 1:6) and incubation was for 22 hours at 37°C in a humidified atmosphere of 5% CO_2, 95% air. After infection for 22 h, the wells were carefully washed with PBS, and media with antibiotics was added (gentamicin 100 µg/mL, penicillin 100 U/m), and streptomycin 1 mg/mL). Alternatively gentamicin was added after 1 h of infection at a concentration of 100 µg per mL in order to kill the bacteria. Then twenty µL MTT at 15 mg/mL was added to wells containing 180 µl growth media, and incubation was for 75 min at 37°C, 5% CO2. Then 100 µL was removed and replaced by 100 µl of 10% Triton X-100, 0.4% concentrated HCl in isopropanol to dissolve the formazan crystals. The plate was read at 590 nm using a multiscan plate reader. The experiments were performed in triplicates on 2 - 5 separate occasions. Percent survival was calculated: $100 \times OD_{590\ nm}$ (exposed cells)/$OD_{590\ nm}$ (control cells).

Expression of virulence genes

Sampling for RNA extraction from bacterial cells was performed for adhesion/invasion experiments with Caco-2 cells. Sampling was performed at 3 and 4 hours of adhesion/invasion by removing the supernatant from the wells (without washing). Thereafter 1 mL RNAlater (Ambion) was added, and pipeting was performed until the Caco-2 layer was disrupted. The samples including supernatant (spinned down at 4000 g, 2 min, 37°C) and resuspended in 1 mL RNAlater were then stored overnight at 4°C and then at -20°C or -80°C until further use. Sampling was performed on 3 - 4 separate occasions. Samples were also collected from a TEER experiment, where RNAlater (Ambion) was added to the apical side of the monolayer 2 hours after infection with bacteria. The suspension was

pipetted up and down, until the monolayer was disrupted.

Findings

Upon initial screening of a hugh culture collection the following strains were used for further investigation based on their history, temperature characteristics and MLST analysis. The study included four pathogenic strains derived from foodborne illness outbreaks, two negative control strains and four commercial Bt strains. Please refer to **Tables 2.8-11, 2.8-12 and 2.8-13.**

Table 2.8-11 Pathogenic strains

Consecutive strain number	Internal DMU strain number	Original Strain name	Origin	Reference or source
bc 11	Bt 676	MADM 1291	Birthday cake	Marilena de Muro, Brazil
bc14	Bt 698	MADM 1561	Cooked chicken	Marilena de Muro, Brazil
bc25	Bt 1202	B-05	Blood, Patient 8	Gaur et al., 2001
bc38	Bt 642	B4-ac	Gastro-intestinal	Agata et al., 1995

Table 2.8-12 Negative control strains

Consecutive strain number	Internal DMU strain number	Original Strain name	Origin	Reference or source
bt48	Bt 959	Bt 407 PlcR	Inactivated PlcR regulator	Salamitou et al., 2000
bc49	Bt 1254	*B. cereus* var. *toyoi* (CNCM I-1012/NCIMB 40112)	Probiotic	A free sample from Rubinum, Spain

Table 2.8-13 Product strains (Bt50 = Bta GC-91 included in the study)

Consecutive strain numbers	Internal DMU strain number	Product name	Serotype	Original Strain name	Reference or source
bt50	600	Agree 50WP	*aizawai*	GC-91	Certis
bt52	1253	Vectobac-12AS	*israelensis*	AM 65-52	A free sample from Borregaard, Bioplant, DK
bt53	1255	Dipel	*kurstaki*	ABTS-351	Valent BioSciences
bt55	1256	Novodor	*tenebrionis*	NB-176	Free sample from "Andermatt-Biocontrol AG", CH

RT-PCR detection of virulence expression

With only some exceptions all of the *Bacillus* strains transcribed all of the genes investigated. Q-PCR quantification revealed increasing amounts of transcripts by time.

Growth at low and high temperatures

All strains grew well at 10°C under both aerobic and micro aerobic conditions. In high temperature experiments, all strains involved in human illness grow fine under all temperature (37 - 50°C) and oxygen conditions (aerobic/microaerobic). Growth of commercial strains was already impaired at 45°C and more or less completely inhibited at 50°C (**Table 2.8-14**).

Table 2.8-14 Results of growth experiments at high temperature

Strain	Growth observed at day							
	37°C Aerobic	37°C micro	43°C aerobic	43°C micro	45°C aerobic	45°C micro	50°C aerobic	50°C micro
Bt48	1/1	1/1	1/1	1/1	1/1	1/1/	1/6/6	1/6/-
Bt50	1/1	1/1	1/1	1/1	1/1	1/1	5/-/-	1/-/-
Bt52	1/1	1/1	1/1	1/2	1/2	2/6/6	5/3/-	5/6/2
Bt53	1/1	1/1	1/1	1/2	1/1	1/2	1/6/-	1/-/6
Bt55	1/1	1/1	1/1	1/1	1/2	1/6/6	2/-/6	5/6/-
Bc11	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
Bc14	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
Bc25	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
Bc38	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1

*Micro: micro aerobic conditions. Disease causing isolates: red; Commercial strains: green; Probiotic strains: blue. No growth: -. The experiment was performed up to three times, and the result of each experiment is separated by /

Survival under simulated gastric conditions

Only results from pH 2.0 are shown (**Figure 2.8-3**), since at pH3.5 and 5.0 no pronounced difference could be observed between the three categories of strains (virulence attenuated (bt48), product and disease isolates). At pH 5.0 all strains were growing, and at pH 3.5 an initial drop of 0.5 – 3 log was observed for all strains, and afterwards the strains where stably maintained. At pH 2.0, all strains have an initial drop of 2 - 3 log values. Most of the strains stabilize after this drop; this includes all the disease isolates (except bc14), the positive strains and the strains from bt50 (Agree) and bt52 (Vectobac). The strain from Dipel (53) was most affected by the low pH, and is undetectable after four hours.

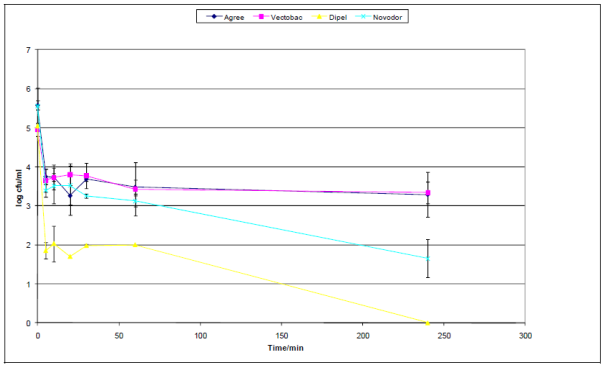

Figure 2.8-3 Persistence of the selected commercial strains in simulated gastric fluid at pH 2.0.

After adding bile salt at 0.3 g/L the growth of the strains is affected (**Figure 2.8-4**), however there are no pronounced differences between the strains.

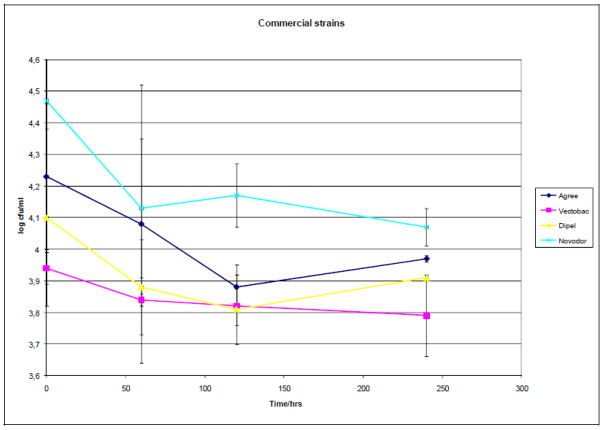

Figure 2.8-4 Persistence of the selected commercial strains in 0.3 g/L bile acids

Detection of enterotoxin HBL using a commercial kit

B. thuringiensis strain GC-91 was capable of producing enterotoxins, or at least the L2 component of enterotoxin HBL under optimal laboratory conditions. L2 was detected up to 1:64 diluted culture, which suggests its high concentration (**Table 2.8-15**).

Table 2.8-15 Detection of the L2 component of HBL in the ten selected strains under optimal growth conditions using the Oxoid BCET-RPLA kit

Strain	Dilution factor*
Bt48	4/2
Bt50	64/64
Bt52	8/8
Bt53	128/128
Bt55	128/128
Bc11	16/32
Bc14	ND
Bc25	128/128
Bc38	64/128

*** Reciprocal value of the highest dilution factor where the assay was still positive. ND: not detected. The strains were tested twice, and the results of the two experiments are separated by /.**

Caenorhabditis elegans model

After inoculating the nematodes with bacteria, the surviving nematodes were counted at days 1 and 3. The analysed bacteria are grouped according to their virulence/pathogenicity to the nematodes in four categories (1, 2, 3 and 4), so that 4 has the highest virulence. The least pathogenic bacterium (category 1) was *B. thuringiensis* subsp *aizawai* GC-91 (**Figure 2.8-5**). Category 2 contained most of the bacteria: bc 11 (MADM 1291), bc14 (MADM 1561), bt48 (Bt407-PlcR), strain bt52 (*B. thuringiensis* subsp. *israelensis*) and strain bt53 (*B. thuringiensis* subsp. *kurstaki*, **Figure 2.8-6**). Category 3 bacteria were: Strain bc25 (B-05) and strain bc38 (B4-ac). The bacterium being the most virulent (category 4) was strain bt55 (*B. thuringiensis* subsp. *tenebrionis*) likely due to nematicidal activity of crystal proteins. Strain bt48 (Bt407-PlcR), the strain with an interrupted PlcR regulator which activates many virulence functions, and as such was expected to be a negative control strain. This presumed negative control strain also grouped together with two of the potential positive control strains and two product strains. If, however the assumption is correct, that PlcR controls the

majority of human virulence functions, it could be concluded from these data that that all five strains in category 2 are nonpathogenic to humans.

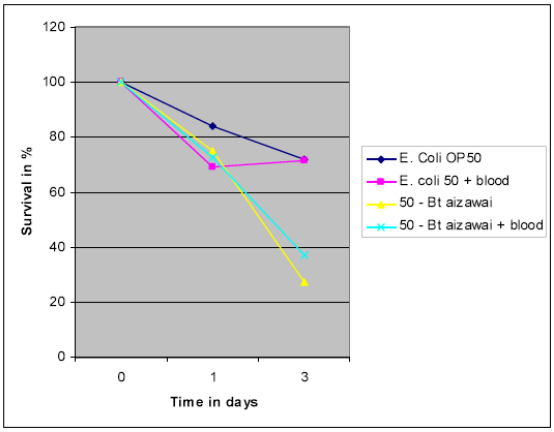

Figure 2.8-5 Nematode survival after exposure to *E. coli* and *B. thuringiensis* GC-91

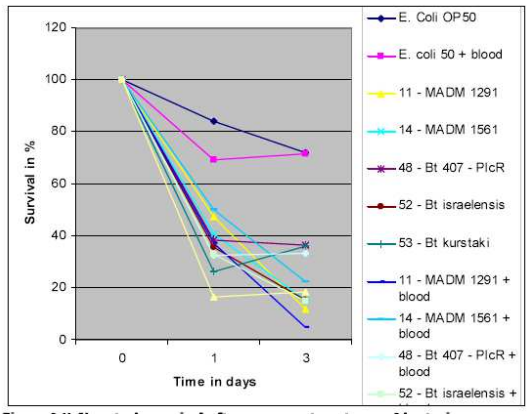

Figure 2.8-6 Nematode survival after exposure to category 2 bacteria

Galleria mellonella model

Since the infectious stage of Bt and Bc group bacteria can be as both spores and vegetative bacteria and because Bt in biopesticides are mainly found as spores in the commercial formulations and because the Probiotic strains are also supposed to be used as spore preparations, we wanted to analyse the spore germination capacity in the intestine of *G. mellonella* larvae. After 3 h germination of most strains was scarce or even not detectable apart from the pathogenic and the commercial Btk strain Bt53 (**Table 2.8-17**). The results after 24 hours demonstrate that all strains are able to germinate in the insect.

Table 2.8-16 Percentage of spore germination at 3 and 24 hours post oral infection of *G. mellonella*

	3H post infection in living larvae		24H post infection with Cry1C	
Strains	Without Cry1C	With Cry1C	Living larvae	Dead larvae
Bc11 (MADM 1291)	4 ±1	79±10	84±5	96±4
Bc 14 (MADM 1561)	51±5	83±6	ND	99±1
Bc 25 (B-05)	89±5	90±6	ND	99±1
Bc 38 (B-04)	ND	ND	ND	ND
Bc 49	ND	10±5	ND	55±5
Bt 50 (Bta, Agree)	ND	2±1	ND	65±5
Bt 51 (Bta, Xentari)	ND	25±5	ND	66±4
Bt 52 (Bti, Vectobac)	1±1	76±5	31±3	94±5
Bt 53 (Btk, Dipel)	73±10	90±5	68±5	99±1
Bt 55 (Btt, Novodor)	ND	1±1	ND	70±3
Bt407 cry- WT	ND	40±6	ND	80±10
Bt48 (Bt407δ *Plcf*)	93±5	96±3	ND	99±1

The mortality was assessed after oral feeding of *G. mellonella* with bacterial spores or vegetative cells. All Bc strains even in association with Cry1C, result in larval mortality, in maximum, between 27% and 37%, while Bt stains are resulting in higher values running from 50 ± 10% for Bt407, 63 ± 10% for Bt50 and up to 84 ± 15% for Bt55 and 50 ± 25% for Bt53. As expected the Bt407 PlcR mutant (strain bt48) showed a low mortality. There were not significant differences in mortality of the larvae when vegetative cells of the commercial strains were administered at a level of 10^6 (mortality between 39 and 68%). Further tests were run with higher levels of vegetative cells for few strains (Bc11, Bt48, Bt53) all resulting in high larval mortality of 80%, except for the *plcR* mutant strain Bt48.

In order to compare the infection and virulence level between oral infection and direct injection into the hemocoel, injection was done with several doses of spores and vegetative bacteria (1000, 10000 and 30000, respectively). The test were run at least 2 - 3 times and the mortality is is shown as percentage plus SD of the mean in **Figure 2.8-7 and 8** for vegetative cells and spores, respectively. The results show that all strains confer a certain level of virulence to *Galleria* larvae and that there is variation between mortality obtained with vegetative cells and spores. All strains confer high mortality at the highest dose used (**Figure 2.8-7**). For infections with spores (**Figure 2.8-8**) there are stronger differences among strains and larval mortality is lower than with vegetative bacteria. Bt50 and Bt52 only confer low mortality.

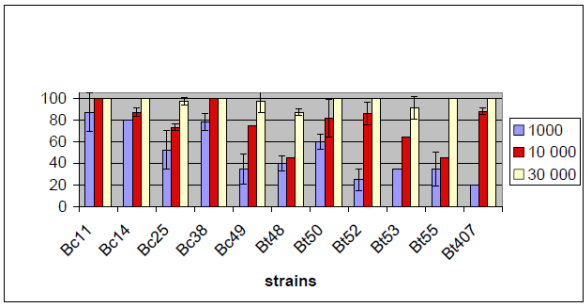

Figure 2.8-7 Mortality in % at 48 h post injection of *Bacillus* vegetative cells (10^3, 10^4 and 3×10^4) into the *Galleria mellonella* hemocoel.

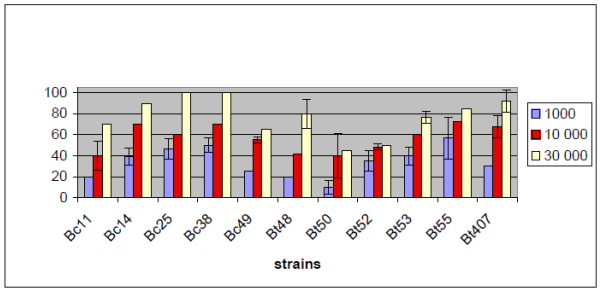

Figure 2.8-8 Mortality in % at 48 h post injection of *Bacillus* spores (10^3, 10^4 and 3×10^4) into the *Galleria mellonella* hemocoel.

RNA was extracted from the larvae 3 and 6 h after infection to investigate the expression of virulence genes *in vivo*. After 3 h no expression of virulence genes was detectable apart from *plcR* for strain Bt53. The results for the 6 h samples show that most of the studied genes are expressed in the midgut of *Galleria* and that the expression profile is quite similar to that found *in vitro*. Strain Bt407 appeared to have the highest *in vivo* expression for all genes (**Table 2.8-17**). Higher expression was observed for Bt53 and Bc11 than for Bc49 (Probiotic strain) for NheB and CytK toxins genes although the *nheB* is well expressed in the Bc49 strain too. For the metalloprotease InhA2 (also part of the PlcR regulon) the results do not permit to make a comparison since the primers were not 100 efficient on all strains. Nevertheless, the gene is well expressed in both Bt407 and Bt53, while much lower expression is observed in Bc11 and as expected in Bt 48.

Table 2.8-17 Relative in vivo expression (%) of virulence genes of Bt/Bc in the *Galleria* midgut. Data are based on normalised Ct values for a number of genes using Bt407 gene expression as base (100%) and *rboB* and *tpi* for normalisation.

Gene / Strain		hblC	cytK	nheB
Bt 407	*in vivo*	100	100	100
	in vitro	100	100	100
Bt48 (Bt 407 *plcR*)	*in vivo*	7,9	1	1,6
	in vitro	0,9	0,1	0,5
Bt53	*in vivo*	43,1	68,6 [a]	101[a]
Bc 49	*in vivo*	1,2	0	31,1
Bc11	*in vivo*	0	13,4	71,5 [a]

a = not significantly different from Bt407. All the other values are significantly different (p-value 0.01, from Bt407 (Quiagen REST Q-RT –PCR software). Yellow background is from *in vitro* expression.

Functional mammalian cell model

Effect of the bacteria on Caco-2 cell morphology

The experiments in this section were performed to investigate the effect of the selected bacteria on Caco-2 cell physiology as detected by visual inspection by brightfield microscopy. It is seen from the **Figure 2.8-9** that the Caco-2 cells are changed/detached when infected with strain bc11 and bc25 for 4 h, while the monolayer was unchanged as compared to control cells when infected with strain Bt50 for 4 h.

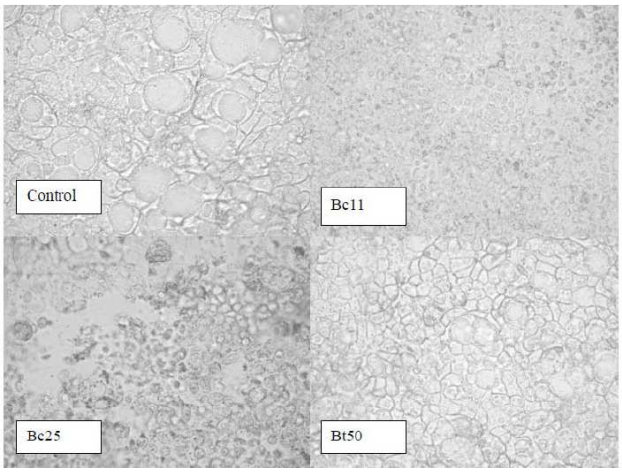

Figure 2.8-9 Morphological changes to Caco-2 cells infected with various *Bacillus* spp. (vegetative cells) For 4 hours at 37°C, 5% CO2.

From **Figure 2.8-10** it can be observed that all the bacteria examined, including the Bt commercial strains bt50, bt52 and bt53, destroyed (detached) the Caco-2 monolayer within 3 - 9 h depending on the strain and concentration of bacterial cells used. Infection with different doses of bacteria revealed that strains bt50 and bt52 (encircled in red in **Figure 2.8-10**) generally were the least aggressive by being slowest in detaching the Caco-2 monolayer.

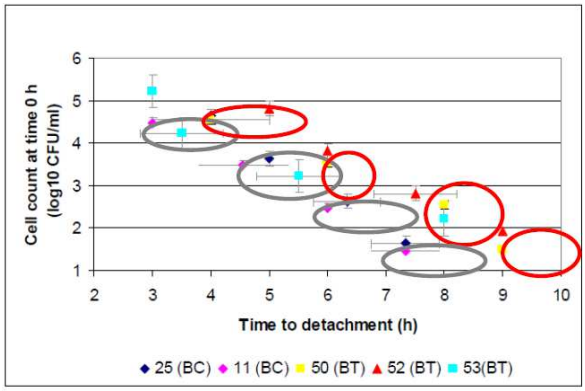

Figure 2.8-10 Effect of five different bacteria on the physiology of Caco-2 monolayers as observed by bright field microscopy. The bacteria were added at different concentrations, and the monolayer was observed for a maximum of nine hours. Results are shown as averages of 2 - 3 independent experiments with standard deviations.

Adhesion and invasion

As described in the introduction, good adhesion ability can be an indicator of probiotic as well as pathogenic potential, depending on the type of bacteria examined. Invasion ability is a pathogenic trait. Adhesion and invasion experiments were performed using vegetative bacterial cells and two different mammalian cell lines, HT29-MTX (grown for 28 - 29 days) and Caco-2 (grown for 18 - 19 days).

As shown in **Figure 2.8-11**, 3 hours of co-incubation with mucus covered HT29-MTX cells resulted in an increase in bacterial cell counts to between 5.8×10^5 and 1.2×10^7 CFU/well. The Bacteria adhered with between 6.4×10^3 and 2.2×10^6 CFU/well. Invasion was generally very low (< 400 cells/well). Three hours of co-incubation with Caco-2 cells resulted in an increase in bacterial cell counts to between 1.6×10^6 and 1.8×10^7 CFU/well. The Bacteria adhered with approximately 104 - 106 cells/well. Similar to HT29-MTX, invasion by the bacteria was generally very low. The weaker detachment effect (rounding of cells) was observed when cells were infected with strains bt48 and

bt50, even though these strains adhered with relatively high numbers as compared to the other bacteria.

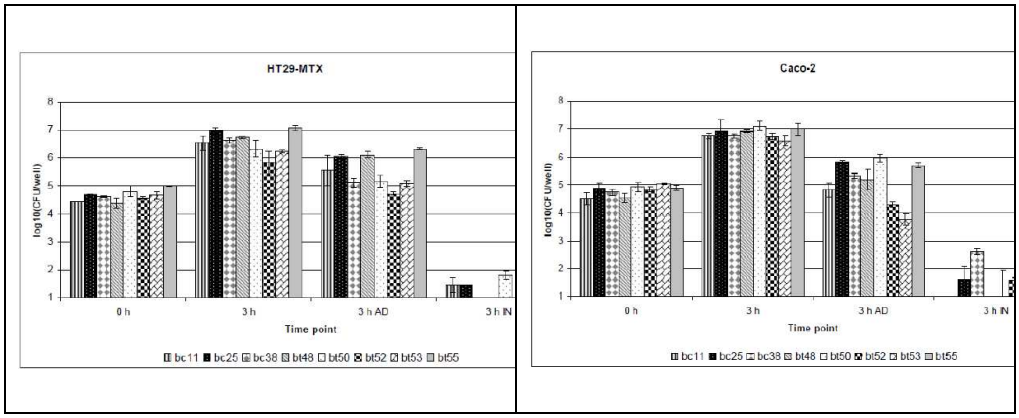

Figure 2.8-11 HT29-MTX cells (28 - 29 Days) and Caco-2 cells (17 - 18 days) infected with eight different bacteria (vegetative cells) for 3 hours at 37°C, 5% CO_2. Data is the average of 2 independent experiments performed in duplicate. Data are shown with standard deviations. AD = Adhesion, IN = Invasion.

TEER

The human gut is lined with a monolayer of polarized intestinal epithelial cells. During infection with a pathogenic microorganism the integrity of the monolayer may be compromised leading to e.g. diarrhea. In our model system, mimicking the polarized intestinal epithelial barrier, transepithelial electrical resistance (TEER) measurements were used to evaluate the integrity of the intestinal polarized epithelial monolayer of Caco-2 or HT29-MTX cells. A decrease in TEER will indicate that the integrity of the epithelial barrier is compromised (weakened) by the bacteria used for infection, whereas an increase will indicate a strengthening of the barrier.

As shown in **Figure 2.8-12**, all of the tested *Bacillus* strains were able to decrease the TEER to 60% of the initial value within 2½ - 3 to approximately 5 - 6 hours depending on the strain. The ability to reduce the TEER decreased in the following order bt53, bc11, bt48, bc25, bc38 = bt55 ($p > 0.05$), bt50 ($p > 0.05$) and bt52 when HT29-MTX were infected, while the order was bt53, bc25, bc38, bt50 = bt55 = bc49, bc11, bt48 and bt52 when Caco-2 cells were infected.

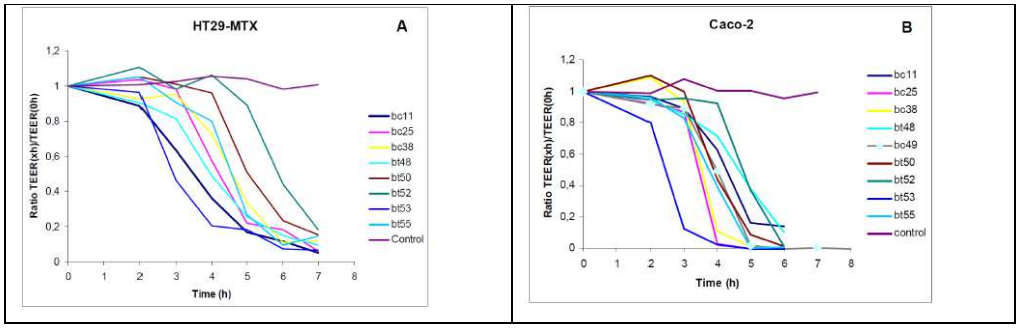

Figure 2.8-12 TEER of polarized HT29-MTX and Caco-2 monolayers exposed to various *Bacillus* spp. at a oncentration of 1×10^4 CFU/well (bc25, bc38, bt50, bt52, bt53, bt55) and 3×10^3 CFU/well (bc11, bt48). TEER (Ω/cm^2) is expressed as the ratio of TEER at time t in relation to the initial value (at time zero [t0]) for each series.

Mitochondrial activity

Macrophages are recruited to sites of infection. One role of macrophages is to engulf and kill pathogenic bacteria. If the bacteria kill the macrophages it is a sign of virulence. Mitochondrial function can be used as an indicator of viability. Results for the mitochondrial function of PoM2 mono-

cyte/machrophages determined 22 h post infection with the strains. by use of the MTT- assay indicate that in most instances, even though the bacteria were added at very low numbers (moiety of infection of 1:600) the macrophages were not able to engulf and kill them. If gentamicin was added 1 h post infection to kill the bacteria, the macrophage survival was much higher for most of the bacterial strains tested as compared to when gentamicin was added 22 h post infection (data not shown).

Expression of virulence genes

Virulence gene expression of the strains exposed to Caco2 monolayers was measured by use of Real time PCR (RT-PCR). As shown in **Table 2.8-19**, it was not possible to detect gene expressions from the adhering Bc11 cells at 3 h and 4 of infection, however the non adherent Bc11 cells (floating above the Caco-2 cells) expressed the virulence gene *cytK* at 3 and 4 h of infection (combined from different experiments). Bt50 cells expressed all the virulence genes, except for *hlyll*. Strain Bt52 (non adherent cells) expressed the virulence genes *cytK* and *nheB* even though no visible change could be observed to the Caco-2 cells. Strain Bt53 (non adherent cells) expressed all the virulence associated genes except for *hlyII* and *inhA2* at 3 h of infection (Caco-2 cells strongly detached).

Table 2.8-18 Gene transcriptions of various Bc and Bt strains sampled at 0, 3 and 4 h of infection of differentiated Caco-2 cells (17 - 18 days) at 37°C. Gene transcriptions (CT-values, indicated as either present (+) or absent (-)) were detected by use of SYBRG technology

				Virulence genes							House keeping genes	
Date	Strain/sample	Cell count	Caco-2	*plcR*	*nheB*	*hblC*	*cytK*	*ilsA*	*inhA2*	*hlyII*	*tpi*	*pta*
	Bc11/0h		none	-	(+)	-	+	(+)	-		+	+
	Bc11/3h		detach	-	-	-	+	-	+		+	-
	Bc11/3h Ad			-/-	-/-	-/-	-/-	-/-	-/-		-/-	-/-
9/3-10	Bc11/3h	$2{,}3*10^6$	detach				-				-	-
	Bc11/3h Ad	$1{,}7*10^2$					-				-	-
	Bc 11/4h	$9{,}5*10^6$	strong				+				+	+
	Bc 11/4 Ad	$9{,}7*10^4$	detach				-				-	-
9/3-10**	Bt48/3h	$2{,}8*10^5$	none				?				?	+
	Bt48/3h Ad	$6{,}5*10^3$					-				?	-
	Bt48/4h	$1{,}3*10^7$					-				-	-
	Bt48/4h Ad	$6{,}5*10^1$					-				+	+
8/10-09	Bt50/0h	$8.5*10^4$	none	+	+	+	+	-	(+?)	-	+	(+)
	Bt50/3h	$1.4*10^7$		+	+	-	+	+	(+?)	-	+	
	Bt50/3h AD	$9.6*10^1$		(+?)	(+?)	-	+	-	?	-	+	
22/10-09	Bt52/0h	$6.9*10^4$	none		-		-				(+)	-
	Bt52/3h	$5.7*10^6$			+		+				+	+
	Bt52/3h AD	$2.1*10^4$		-	+	-	(+)	-			+	
7 and 8/10-09	Bt53/0h	$1.1*10^1$	none	+	+	+	+	+	-	-	+	-
	Bt53/3h	$4.1*10^6$	Strong detach	+/nd	+/+	+/(+)	+/+	+/nd	(+?)/nd	-/nd	+/+	(+)/+
	Bt53/3h AD	$6.4*10^2$		-	-	-	-	-	-	-	(+)	-
8/10-09	Bt55/0h	$7.9*10^4$	none	+/+	+/+	+/+	+/+	+/-		+/+	+/+	(+)/-
	Bt55/3h	$1.1*10^7$	detach	-/-	-/+	-/-	-/-	-/-		-/-	-/-	-/-
	Bt55/3h AD	$5.2*10^1$		-/-	-/-	-/nd	-/nd	-/-		-/-	-/-	-/-

Conclusions

There is a tendency in the nine strains investigated, that strains isolated from either food involved in outbreaks or from patients have better growth capacity at high temperatures and better survival at gastrointestinal conditions than strains used commercially in plant protection products.

Summing up all the obtained results by the cluster analysis, if the four strains from the microbial pest control products (Bt50, Bt52, Bt 53, Bt 55) are compared mutually and with the four strains from contaminated food, blood and a gastrointestinal case (Bc11, Bc14, Bc25, Bc38) and the negative control (Bt48) and the strain from a probiotic (Bc49), it can be concluded that:

- Bt50 and Bt52 the Bt *aizawai* and *israelensis* strains from Agree and Vectobac behave in many ways similar in the different models. They have limited growth at 43°C, survive well at the stomach conditions, affect to different extent the Caco-cells, and to a lesser extent the HT-29 MTX cells, and have a limited effect on the PoM2 cells after 22 hours (if gentamicin is added 1 h post infection), they have some, but low, effects on *Galleria* and the nematode *C. elegans*. From an overall point of view are these two strains more comparable with the negative control strains than with the positive controls. They seem therefore to have a pathogenic potential which are lower, than the strains from pathogenic cases, especially the two strains isolated from blood and a gastrointestinal case (Bc25 and Bc38).

- Bt53 and Bt55 the Bt *kurstaki* and *tenebrionis* strains from Dipel and Novodor behave in many ways also similarly in the models. They grow at 43°C, are affected by the stomach conditions, they

negatively affect all of the mammalian cell lines, and Galleria, while different effects on the nematode C. elegans are observed, as Bt55 has a considerable negative effect on the nematodes, while Bt53 has a much lesser pronounced effect. From an overall point of view are these two strains more comparable with the strains from the pathogenic cases (food poisoning and somatic), than with the negative controls. They seem therefore to have a pathogenic potential which does not differ from the potential of the strains from the pathogenic cases. Their main difference from the "non pathogenic" strains (bt48, bc49) is that they are affected by the acidic stomach conditions.

Table 2.8-19 Characterization of studied Bc and Bt strains: *in vitro* growth at different temperatures and in stimulated stomach conditions and presence/expression of enterotoxin genes.

Strains	N° in the project	Bc11	Bc14	Bc25	Bc38	Bt48	Bc49	Bt50	Bt52	Bt53	Bt55	Bt407 Cry-
	Original name	MADM1291	MADM1561	B-05	B4-ac	(Bt407 Cry- ΔplcR)	*Toyoi*	*Aisawai*	*israelensis*	*kurstaki*	*tenebrionis*	
	Cluster (Guinebretières,)	No fit	III	IV	IV	III (?)	III	IV	(IV)	IV	(IV)	III(?)
	Origins/function	Contaminated food		Blood	Gastro intestin	Neg control	Pro-biotic	Product strains				Positive control
								Agree	Vectbac	Dipel	Novodor	
GROWTH												
Temperature tolerance (growth at)	43°C micro aerob	1	5	5	5	1		1	1	4	3	nd
	30°C micro aerob	2	4	5	5	3		3	3	5	4	nd
	10°C micro aerob	5	5	5	5	5		5	3	5	5	nd
Stomach (survival)	Acid pH 2	5	5	5	5	5		5	5	2	3	nd
	Bile salt (0.3g/L)	4	3	4	4	5		4	4	4	4	nd
Growth rates in LB-broth at 37C	µmax (doublings/h)	3.7	5.3	3.5	3.1	2.5		2.2	2.7	1.4	2.8	nd
ENTEROTOXINS												
Strains		Bc11	Bc14	Bc25	Bc38	Bt48	Bc49	Bt50	Bt52	Bt53	Bt55	Bt407
Presence and expression of « enterotoxins »	Enterotoxins	NheB = N				HblC=H		CytK = C		HlyII=h		
		N H C h	N H C h	N H C h	N H C h	N H C h	N H C h	N H C h	N H C h	N H C h	N H C h	N H C h
	Presence	+ - + +	+ - + +	+ + + -	+ + + -	+ + + +	+ + + -	+ + + -	+ + + +	+ + + +	+ + + +	+ + + +
	Expression *in vitro*	+ - + +	nd	nd	nd	+ + + +	+ + +	nd	+ + + +	+ - - -	nd	+ + + +
	Expression *in vivo Gm*	+ - + +	nd	nd	nd	low +	+ + nd	nd	nd	+ + + +	nd	+ + + +
	Expression *in vivo Caco2*	+ - + +	+ - + +	nd	nd	low +	+ + nd	nd	+ + + +	nd	nd	+ + + +

ues : 5 is highest growth , 1 is lowest 0 = no effect; + = presence, - =absence of genes or expression, nd= not determined

Table 2.8-20 Summarized results from the studied virulence models exposed to Bc and Bt strains

Strains	N° in the project	Bc11	Bc14	Bc25	Bc38	Bt48	Bc49	Bt50	Bt52	Bt53	Bt55	Bt407 Cry-
	Original name	MADM1291	MADM1561	B-05	B4-ac	(Bt407 Cry- ΔplcR)	*Toyoi*	*Aisawai*	*israelensis*	*kurstaki*	*tenebrionis*	
	Cluster following (Guinebretières et al. 2008, 2010))	No fit	III	IV	IV	III (?)	III	IV	(IV)	IV	(IV)	III (?)
	Origins/function	Contaminated food		Blood	Gastro intestin	Neg control	Pro-biotic	Product strains				Pos control
								Agree	Vectbac	Dipel	Novodor	
CELL LINES												
Caco2 Intestinal (Human)	TEER	3	nd	4	4	3	4	4	3	5	4	3
	Adhesion	4	nd	5	4	4	nd	5	3	4	5	nd
	Detachment/cytotox	4	nd	4	4	2	nd	2	0	5	4	nd
HT-29 MTX Intestinal Mucus (human)	TEER	4	nd	3	3	4	nd	3	2	5	3	nd
	Adhesion	5	nd	5	4	5	nd	4	3	4	5	nd
	Invasion	1	nd	1	0	0	nd	1	0	2	0	nd
	Detachment	4	nd	0	4	0	nd	0	0	5	4	nd
PoM2 Macrophages (porcine)	Cytotox/mitochondial activity Gentamycin after 22 h	2-5	nd	5	5	3	0	4-5	4-5	5	5	5
PoM2 Macrophages (porcine)	Cytotox/mitochondial activity Gentamycin after 1 h	3	nd	0-2	0-2	3	1	0-1	0-1	4-5	3-4	3
INVERTEBRATE MODEL												
Galleria (Gm) insects larvae mortality at 37°C	*In vivo* Spore germination (3H)	4	4	5	4	5	nd	1	3	5	2	3
	Oral Cry1C+ spores	3	3	3	4	1	3	nd	3	5	4	4
	Oral Cry1C+ vegetative	5	5	5	4	2	nd	nd	4	5	5	5
	Hemocoel spores $1x10^4$	3	5	4	5	3	4	2	3	4	3	4
	Hemocoel vegetative $1x10^3$	5	5	3	4	2	2	2	2	2	2	2
	Hemocoel vegetative $1x10^4$	5	5	4	5	2	4	4	3	2	3	5
C.elegans nematode	Virulence /mortality	2	2	3	3	2	nd	1	2	2	5	nd

s : 5 is highest viruelnce ; 1 is lowest; 0= no effect; nd= not determined

****

Report:	KMA 2.8/12 - Wilcks, A., Hansen, B.M., Hendriksen, N.B., Licht, T.R. (2006b), published report FEMS Immunol Med Microbiol.; 46(1):70-77
Title:	Fate and effect of ingested *Bacillus cereus* spores and vegetative cells in the intestinal tract of human-flora-associated rats
Abstract	The fate and effect of *Bacillus cereus* F4433/73R in the intestine of human-flora-associated rats was studied using bacteriological culturing techniques and PCR-denaturing gradient gel electrophoresis in combination with cell assays and immunoassays for detection of enterotoxins. In faecal samples from animals receiving vegetative cells, only few *B. cereus* cells were detected. Spores survived the gastric barrier well, and were in some cases detected up to 2 weeks after ingestion. Selective growing revealed no major changes in the intestinal flora during passage of *B. cereus*. However, denaturing gradient gel electrophoresis analysis with universal 16S rRNA gene primers revealed significant changes in the intestinal microbiota of animals dosed with spores. Vero cell assays and a commercial kit (BCET-RPLA) did not reveal any enterotoxin production from *B. cereus* F4433/73R in the intestinal tract.

****

Report:	KMA 2.8/13 - Ceuppens, S., Uyttendaele, M., Drieskens, K., Heyndrickx, M., Rajkovic, A., Boon, N., Van de Wiele, T. (2012a), published report Appl Environ Microbiol., 78(21):7698-7705
Title:	Survival and Germination of *Bacillus cereus* Spores without Outgrowth or Enterotoxin Production during In Vitro Simulation of Gastrointestinal Transit
Abstract	To study the gastrointestinal survival and enterotoxin production of the food-borne pathogen *Bacillus cereus*, an *in vitro* simulation experiment was developed to mimic gastrointestinal passage in 5 phases: (i) the mouth, (ii) the stomach, with gradual pH decrease and fractional emptying, (iii) the duodenum, with high concentrations of bile and digestive enzymes, (iv) dialysis to ensure bile reabsorption, and (v) the ileum, with competing human intestinal bacteria. Four different *B. cereus* strains were cultivated and sporulated in mashed potato medium to obtain an inoculum of 7.0 log spores/mL. The spores showed survival and germination during the in vitro simulation of gastrointestinal passage, but vegetative outgrowth of the spores was suppressed by the intestinal bacteria during the final ileum phase. No bacterial proliferation or enterotoxin production was observed, despite the high inoculum levels. Little strain variability was observed: except for the psychrotrophic food isolate, the spores of all strains survived well throughout the gastrointestinal passage. The in vitro simulation experiments investigated the survival and enterotoxin production of *B. cereus* in the gastrointestinal lumen. The results obtained support the hypothesis that localized interaction of *B. cereus* with the host's epithelium is required for diarrheal food poisoning.

****

Report:	KMA 2.8/14 - Ceuppens, S., Van de Wiele, T., Rajkovic, A., Ferrer-Cabaceran, T., Heyndrickx, M., Boon, N., Uyttendaele, M. (2012b), published report Int J Food Microbiol.; 155(3):241-246
Title:	Impact of intestinal microbiota and gastrointestinal conditions on the in vitro survival and growth of *Bacillus cereus*
Abstract	Ingestion of *B. cereus* can result in diarrhea, if these bacteria survive gastrointestinal passage and achieve growth and enterotoxin production in the small intestine. The gastrointestinal survival of vegetative cells and spores of the diarrheal food poisoning strain *B. cereus* NVH 1230-88 was investigated during in vitro batch experiments simulating the stomach, duodenum and ileum using simulation media and competing intestinal microbiota. All spores and

approx. 30% of the vegetative *B. cereus* cells survived the 2 h incubation in gastric medium with pH 4.0. Sterile intestinal medium induced germination of spores and enabled outgrowth of vegetative cells to approx. 7 log CFU/mL. The behaviour of *B. cereus* in the intestinal environment with competing intestinal bacteria was determined by their relative concentrations. Besides the numbers of intestinal bacteria, the nutrition and composition of the intestinal community were also very important for the growth inhibition of *B. cereus*.

****

Report: MA 2.8/15 - Auger, S., Ramarao, N., Faille, C., Fouet, A., Aymerich S., Gohar, M. (2009) Applied and Environmental Microbiology, 75(20):6616-6618

Title: Biofilm Formation and Cell Surface Properties among Pathogenic and Non-pathogenic Strains of the *Bacillus cereus* Group

Abstract Biofilm formation by 102 Bacillus cereus and B. thuringiensis strains was determined. Strains isolated from soil or involved in digestive tract infections were efficient biofilm formers, whereas strains isolated from other diseases were poor biofilm formers. Cell surface hydrophobicity, the presence of an S layer, and adhesion to epithelial cells were also examined.

****

Report: MA 2.8/16 - Phelps, R.J., McKillip, J.L. (2002) Applied and Environmental Microbiology, 68(6):3147-3151

Title: Enterotoxin production in natural isolates of Bacillaceae outside the *Bacillus cereus* group

Abstract Thirty-nine *Bacillus* strains obtained from a variety of environmental and food sources were screened by PCR for the presence of five gene targets (hblC, hblD, hblA, nheA, and nheB) in two enterotoxin operons (HBL and NHE) traditionally harbored by *Bacillus cereus*. Seven isolates exhibited a positive signal for at least three of the five possible targets, including *Bacillus amyloliquefaciens*, *B. cereus*, *Bacillus circulans*, *Bacillus lentimorbis*, *Bacillus pasteurii*, and *Bacillus thuringiensis* subsp. *kurstaki*. PCR amplicons were confirmed by restriction enzyme digest patterns compared to a positive control strain. Enterotoxin gene expression of each strain grown in a model food system (skim milk) was monitored by gene-specific reverse transcription-PCR and confirmed with the Oxoid RPLA and Tecra BDE commercial kits. Lecithinase production was noted on egg yolk-polymyxin B agar for all strains except *B. lentimorbis*, whereas discontinuous beta hemolysis was exhibited by all seven isolates grown on 5% sheep blood agar plates. The results of this study confirm the presence of enterotoxin genes in natural isolates of *Bacillus* spp. outside the *B. cereus* group and the ability of these strains to produce toxins in a model food system under aerated conditions at 32 degrees C.

****

Report: KMA 2.8/17 Damgaard, P.H. (1995), published report
FEMS Immunology and Medical Microbiology 12(3-4):245-250

Title: Diarrhoeal enterotoxin production by strains of *Bacillus thuringiensis* isolated from commercial *Bacillus thuringiensis*-based insecticides

Abstract

Strains of *Bacillus cereus* and *B. thuringiensis* were tested by the Tecra VIA kit for the ability to produce a diarrhoeal enterotoxin. The strains of *B. thuringiensis* were isolated from commercial *B. thuringiensis*-based insecticides (Bactimos™, DiPel™, Florbac™ FC, Foray™ 48B, Novodor™ FC, Turex™, VecTobac™, XenTari™). The production of diarrhoeal enterotoxin varied by a factor of more than 100 among the different strains tested. *B. cereus* (F4433/73) produced the highest amount of enterotoxin and the *B. thuringiensis* strain isolated from DiPel™ the lowest. The products were tested for their content of diarrhoeal enterotoxin and all products, except MVP™ which does not contain viable *B. thuringiensis* spores, contained diarrhoeal enterotoxins. The results indicate a potential risk for gastroenteritis outbreak caused by *B. thuringiensis*.

Material and Methods**:**

The strains used included a diarrhoeal enterotoxin-producing *B. cereus* strain (F4433/73), a *B. thuringiensis* strain (NRRL B-4066) isolated from a fatal case of bovine mastitis and *B. thuringiensis* strain (HD-1). From different commercial *B. thuringiensis*-based insecticides (Bactimos, Forbac FC, Foray 48B and Novodor FC (Novo Nordisk, Bagsvxrd, Denmark); Turex (Ciba Geigy. Greensboro. USA); DiPel, VecTobac and XenTari (Abbott, Chicago, USA)), *B. thuringiensis* was isolated by transferring with a sterile loop one drop of product to a nutrient agar plate (Difco), followed by incubation at 30°C until sporulation

Assay for diarrhoeal enterotoxigenic activity of pure cultures using Tecra VIA

Brain Heart Infusion Broth (BHI; Difco) 20 ml, supplemented with 0.1% (w/v) glucose, were inoculated with 0.2 mL of a 24 h culture and incubated at approximately 100 rev for 18 h at 30°C. After incubation, the cultures were centrifuged (12 min. 4300 × g, 5°C) and the supernatant fluid was tested for the content of the diarrhoeal toxin. The *Bacillus* diarrhoeal enterotoxin visual immunoassay kit (Tecra diagnostics, Roseville, Australia) was used for detection of the diarrhoeal toxin. The toxin assay is a sandwich enzyme-linked immunosorbent (ELISA) analysis kit. Serial dilutions of the supernatant fluid were made in order to determine toxin titre of the fluid. Uninoculated BHI was used as a negative control, and enterotoxin-positive controls were provided with the kit. Response was measured by absorbance at 405 nm using a T1M-10 plate-reader (Life Technologies, Roskilde. Denmark). The individual samples were considered to be positive when the assays in the positive and negative control had proven valid, and the samples had an absorbance greater than or equal to 0.2.

Assay for diarrhoeal enterotoxigenic content in B. thuringiensis-based insecticides using Tecra VIA

B. thuringiensis products 0.5 g, including the product MVPTM (Mycogen, San Diego. USA) were suspended in 10 mL of sterile water and vigorously shaken for 10 min at room temperature. Suspensions were centrifuged (12 min. 4300 × g, 5°C) followed by passage through 0.22 µm syringe filter (Nalgene, USA). Samples of denaturated, heat-labile enterotoxin were prepared by placing tubes of product filtrates in a boiling-water-bath for 12 min. The filtrated was then tested for the content of the diarrhoeal toxin by using the *Bacillus* diarrhoeal enterotoxin visual immunoassay kit (Tecra diagnostics. Roseville, Australia). The toxin assay is a sandwich enzyme-linked immunosorbent (ELISA) analysis kit. Uninoculated BHT was used as a negative control and enterotoxin-positive controls were provided with the kit. Response was measured by absorbance at 405 nm using a TIM-10 plate-reader. The individual samples were considered to be positive when the assays in the positive and negative control had proven valid, and the samples had an absorbance greater than or equal to 0.2.

Findings: The reference *B. cereus* strain F4433/73, isolated from a typical diarrhoeal, food poisoning outbreak and known to cause diarrhoea in monkey feeding assays, showed the highest diarrhoeal enterotoxin titre of the strains tested in these experiments (**Table 2.8-21**).

Of the *B. thuringiensis* strains tested for the production of the diarrhoeal enterotoxin all reacted positively. Three strains exceeded a titre of 100 (HD-1, Bactimos and VecTobac), whereas the rest showed low to moderate production. There is however no doubt about the ability of these strains to produce the diarrhoeal enterotoxin (**Table 2.8-21**). Turex is the same as Agree, containing Bta GC-91.

Table 2.8-21 Titre of *Bacillus* diarrhoeal enterotoxin of 18 h cultures, determined using Tecra VIA.

Strain/Product	Titre
B. cereus F4433/73	1629 (1350-2051)
B. thuringiensis HD- I	182 (120-367)
NRRL B-4066	86 (60-148)
Bactimos	242 (194-321)
DiPel	14 (13-15)
Florbac FC	15 (14-17)
Foray 48B	56 (46-71)
Novodor FC	80 (57-136)
Turex (= Bta GC-91)	21 (18-27)
VecTobac	120 (100-151)
XenTari	23 (18-33)

Again using the Tecra VIA kit, all commercial *B. thuringiensis*-based insecticides, except for MVP, were shown to contain diarrhoeal enterotoxin (**Table 2.8-22**). MVP contains only the δ-endotoxin encapsulated in dead *Pseudomonas fluorescens* cells and contains no *B. thuringiensis* spores. In line with the heat sensitivity of the enterotoxin the degeneration of the toxin was seen by boiling for 12 min of the *B. thuringiensis* products.

Table 2.8-22 Response of commercial products tested by Tecra VIA for contents of enterotoxin.

Product	Untreated	Boiled
Bactimos	+	-
DiPel	+	-
Foray 48B	+	-
Turex	+	-
Xentari	+	-
MVP	-	-

Conclusions *B. thuringiensis* has been shown to produce a diarrhoeal enterotoxin, capable of causing food poisoning, although toxin production was low in some of the tested strains. It would therefore be reasonable to suggest that strains used in commercial *B. thuringiensis*-based insecticides be tested in specific assays to determine the amount of enterotoxin produced, as a natural part of the product registration procedure. The current guidelines for testing of active substances and products of microbiological pesticides for EU registration do not contain experiments that would reveal the production of diarrhoeal enterotoxin. It could also be suggested that only diarrhoeal enterotoxin-negative strains are to be used in insecticides; this is the case with 0-exotoxin producing strains, where toxin-positive strains have been banned in USA since 1971 due to their human toxicity.

B.2.8.3 Other metabolites produced by *Bacillus thuringiensis* subsp. *aizawai*

In the External Scientific Report on Literature review and data collection on microbial active substances which can be used for the environmental risk characterisation, the following metabolites have been identified for *B. thuringiensis* without any consideration of the subspecies.

- iturin A, bacillomycin D, mycosubtilisin, surfactin, fengycin, entomocin 110, zwittermycin for which the target are bacteria and fungi

- ß-1,3-glucanase and chitinase ChiS, ChiL which are enzymes and target other microorganisms such as fungi.

No single reference was obtained when the above mentioned metabolites were combined with Btk and typical search terms aiming to identify information about possible toxic effects. All relevant details can be found in the evaluation of the Literature Review Report in B6 (Seehase, 2016, KMA 5.1/01, see B6).

B.2.9 Antibiotics and other anti-microbial agents

Information from DAR and DAR addendum (May 2007, February 2013) Resistance/sensitivity to antibiotics / anti-microbial agents used inhuman or veterinary medicine

The susceptibility of the strain GC-91 to six clinically used antimicrobials was determined using the National Committee for Clinical Laboratory Standards (NCCLS) disk susceptibility test (Barbera, 1993). In both studies, the *Staphylococcus aureus* strain ATTC 25923 was used as positive control organism.

Table 2.9-1 shows the sensitivity to antibiotics of GC-91. It shows high sensitivity (large inhibition zones >19-20 mm) to Chloramphenicol, Erythromycin, Streptomycin and Tetracycline and resistance to Penicillin.

Table 2.9-1 Antibiotic Sensitivity Tests of the Bta strain GC-91

	Zone Diameter (mm) (1)		
Antibiotic	*S. aureus*(2)	GC-91	Resistance Pattern of GC-91
Chloramphenicol	NR	23	S
Clindamycin	NR	18	I
Erythromycin	25	24	S
Penicillin G	29	0	R
Streptomycin	NR	19	S
Tetracycline	NR	21	S

(1) Average of two plates
(2) Control Strain , *Staphylococcus aureus* (ATCC 25923)

NR Not reported
S Susceptible
R Resistant
I Intermediate

New data:

The above presented data are considered valid to provide information about resistance patterns of Bta GC-91 to commonly used antibiotics. However, for the assessment of antibiotic resistance in the EU it would be preferable to use broth dilution minimum inhibitory concentration (MIC) methodology against relevant antibiotics since acceptable MICs specifically for *Bacillus* ssp. have been established for regulatory use in the EU whereas no acceptance criteria exist for the NCCLS method.

To be in compliance with the EFSA guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance[18] additional antibiotics were tested following standard procedures for antibiotic testing by deep agar diffusion tests using BD BBL Sensi-Disc Antimicrobial Susceptibility Test Discs from VWR (Cheng & Chen, 2016). The results are presented in Table 2.9-2 below and demonstrate that Bta GC-91 is susceptible to gentamycin and vancomycin, shows intermediate sensitivity to kanamycin and is resistant to ampicillin. From the obtained resistance patterns the following can be concluded:

- Bta GC-91 is sensitive or at least intermediate susceptible to all antibiotics recorded in the EFSA guidance document for *Bacillus* spp. used in feed additives (chloramphenicol, tetracycline, streptomycin, clindamycin, erythromycin, streptomycin, kanamycin, gentamycin and vancomycin)

- is not multi-resistant and

- in the unlikely case of human infection appropriate treatment methods are available.

Table 2.9-2 New Antibiotic Sensitivity Tests of the Bta GC-91 (Cheng & Chen, 2016)

Antibiotic Disc	**Code**	**Disc Potency [µg]**	**Measured zone of inhibition diameter in mm (zone diameter interpretive)**	
			S. aureus	**GC-91**
Ampicillin	AM10	10	20 (S)	9 (R)
Gentamycin	GM10	10	27 (S)	21 (S)
Kanamycin	K30	30	28 (S)	16 (I)
Vancomycin	VA30	30	23 (S)	18 (S)

Zone diameter interpretative
S Susceptible
R Resistant
I Intermediate

[18] EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP); Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. EFSA Journal 2012;10(6):2740. [10 pp.] doi:10.2903/j.efsa.2012.2740. Available online: www.efsa.europa.eu/efsajournal

B.2.10 References relied on

Literature search
A literature search with regard to biological properties was performed

This report summarizes the search and selection process of open peer-reviewed literature for *Bacillus thuringiensis* subsp. *aizawai* GC-91 (Bta GC-91).

Bta GC-91 is a transconjugant strain of a *Bacillus thuringiensis* subsp. *aizawai* (Bta) and a *Bacillus thuringiensis* subsp. *kurstaki* (Btk) strain. Therefore, the literature search was extended to the subspecies *kurstaki*. It is also considered that Bta and Btk are closely related and are very similar with regard to their biological properties and physiological requirements. References on Btk are therefore considered fully applicable for the evaluation of Bta strain GC-91.

The search strategy was based on a multi-concept approach. For details regarding the search strategy and the results obtained, please refer to Point 4 and Point 5 of this Literature Review Report.

The selection process resulted in the classification of the available reports in the four categories recommended by the EFSA (2011)[19] guideline:

1) Studies that are relevant to the data requirement and that provide data for establishing or refining risk assessment parameters. These studies should be considered for reliability.
2) Studies that are relevant to the data requirement, but in the opinion of the applicant provide only supplementary information that does not alter existing risk assessment parameters.
3) Studies for which relevance cannot be clearly determined.
4) Studies of no relevance

The relevance criteria applied are reported in Point 3.2 of this Literature Review Report.
The reliability assessment for relevant studies was done according to the recommendations of the EFSA (2011)[1].

The overall results are shown below on **Table 2-1**.

Table 2-1 Results of the study selection process for Section 2 and 3

Data requirement capture in the search:	**n**
Total number of summary records retrieved after all searches of peer-reviewed literature	**330**
Number of summary records excluded from the search after rapid assessment for relevance	**307**
Total number of full-text documents assessed in detail	**23**
Number of studies excluded from further consideration after detailed assessment of relevance	**7**
Number of studies not excluded for relevance after detailed assessment	**16**

[19] Guidance of EFSA: Submission of scientific peer-reviewed open literature for the approval of pesticide active substances under Regulation (EC) No 1107/2009. EFSA Journal 2011;9(2):2092

3 Protocol: objective of the review and criteria used

3.1 Objective

The review was made in order to identify scientific peer-reviewed open literature on the active substance *Bacillus thuringiensis* subsp. *aizawai* GC-91 with respect to the genetic stability and the possible development of resistances in the target pests.

3.2 Criteria for relevance and reliability

The criteria for relevance and reliability used are summarised below in **Table 3.2-1.** Only studies that were considered relevant were assessed for reliability.

Table 3.2-1 Criteria for relevance and reliability used in the review

Relevance criteria
• Property investigated was relevant for data requirements of Regulation (EC) No 1107/2009 • Identification of the species and subspecies referred to as *Bacillus thuringiensis* subsp. *aizawai* and *kurstaki* • Relevant information on the development of resistance in the target pests • Relevant information on the genetic stability of *Bacillus thuringiensis* subsp. *aizawai* and *kurstaki*
Reliability criteria
Minimum information reported e.g.: Test item or related compound Test species relevant Clear and comprehensive description of material and methods, incl. duration, replicates, test conditions Definition of endpoints Presentation of result Guideline compliance Further criteria: statistical power verification of measurement methods and data control of experimental variables that could affect measurements universality of the effects in validated test systems using relevant animal strains and appropriate routes of exposure biological plausibility of results uniformity among substances with similar attributes and effects

4 Search methods and results

The literature research was conducted on the DIMDI database provided by the German Institute of Medical Documentation and comprised searches in MEDLINE; BIOSIS, CAB and SCISEARCH databases. Search strategy aimed to find all recent (from 2006 onwards) references relevant for each of the topics: genetic stability and development of resistance in then target pests.

Keywords used:

Genetic stability (also refer to **Table 4-1**): *Bacillus thuringiensis* AND (*aizawai* OR *kurstaki*) AND (((gene? OR plasmid OR DNA) AND ((stability OR transfer OR uptake OR exchange)) OR mutation OR conjugation OR transduction OR transformation OR recombination OR mating))) NOT: cotton OR maize OR engineer? OR clone? OR regulat? OR transgen?

Development of resistance (also refer to **Table 4-1**): *Bacillus thuringiensis* AND (*aizawai* OR *kurstaki*) AND resistan?

Obtained references were first subjected to a *rapid assessment* based on title and the abstract. Summary records that appeared to be relevant passed to a second step in which a detailed assessment of full text documents was conducted.

In both types of assessment articles referring to transgenic Bt-toxin producing plants or an assessment of Cry toxins only were excluded. In addition, in the search for resistance development, articles reporting on laboratory experiments selecting for resistance in insect populations were also excluded.

Details on the search strategies are presented in **Table 4-1**. Results are listed in **Table 4-2**.

Table 4-1 Search process for peer-reviewed open literature in bibliographic databases

Database	BA00	CV72	SCISEARCH	MEDLINE
Justification for choosing the source	BIOSIS Previews covers worldwide literature in the field of biology (zoology, botany, microbiology), human and veterinary medicine, biochemistry, pharmacology, toxicology, and environmental sciences, especially from Northern America and Europe. It corresponds to the printed Biological Abstracts and Biological Abstracts/RRM (Reports, Reviews, Meetings). BA00: 2000 to date	CAB Abstracts covers worldwide literature of agriculture and related sciences including biotechnology, veterinary medicine, nutrition, medicine and forestry sciences. Sources include approx. 9000 international journals, books, conference proceedings, and patents. CV72: 1972 to present	SciSearch covers worldwide literature in the fields of science, technology, and medicine. The database contains all citations published in "Science Citation Index Expanded". Sources include approx. 6650 international journals of 150 disciplines including Clinical Medicine and Life Sciences. IS00: 2000 to date	MEDLINE (Medical Literature Analysis and Retrieval System Online) covers worldwide literature on every area of medicine, including dental medicine, veterinary medicine, psychology, and public health. The database corresponds to the printed "Index Medicus" and to some other printed material. Sources include approx. 4800 international journals. ME00: 2000 to date
Date of the search	11.07.2016			
Date span of the search	2006 - 2016			
Language limit	English – Spanish – French - German			
Search type	Expert Search			
Search strategy	Search: Genetic stability			
	Search 1	(FT=*Bacillus thuringiensis* AND FT=*aizawai* AND (((FT=gene? OR FT=plasmid OR FT=DNA) AND (FT=stability OR FT=transfer OR FT=uptake OR FT=exchange)) OR FT=mutation OR FT=conjugation OR FT=transduction OR FT=transformation OR FT=recombination OR FT=mating)))		
	Search 2	(FT=*Bacillus thuringiensis* AND FT=*kurstaki* AND (((FT=gene? OR FT=plasmid OR FT=DNA) AND (FT=stability OR FT=transfer OR FT=uptake OR FT=exchange)) OR FT=mutation OR FT=conjugation OR FT=transduction OR FT=transformation OR FT=recombination OR FT=mating)))		
	Search 3	(FT=cotton OR FT=maize OR FT=engineer? OR FT=clone? OR FT=regulat? OR FT=transgen?)		
	Search 4	2 NOT 3		
	Search: Development of resistance			
	Search 5:	((FT=*Bacillus thuringiensis* AND FT=*aizawai*) AND FT=resistan?))		
	Search 6:	((FT=*Bacillus thuringiensis* AND FT=*kurstaki*) AND FT=resistan?))		

* Use of „?“ at the end of keyword will lead to an expansion of the search criteria at DIMDI database

Table 4-2 Search results

Search strategy	Search terms	Number of hits	After removal of duplicates*
1	(FT=*Bacillus thuringiensis* AND FT=*aizawai* AND (((FT=gene? OR FT=plasmid OR FT=DNA) AND (FT=stability OR FT=transfer OR FT=uptake OR FT=exchange)) OR FT=mutation OR FT=conjugation OR FT=transduction OR FT=transformation OR FT=recombination OR FT=mating)))	**66**	**44***
2	(FT=*Bacillus thuringiensis* AND FT=*kurstaki* AND (((FT=gene? OR FT=plasmid OR FT=DNA) AND (FT=stability OR FT=transfer OR FT=uptake OR FT=exchange)) OR FT=mutation OR FT=conjugation OR FT=transduction OR FT=transformation OR FT=recombination OR FT=mating)))	223	
3	(FT=cotton OR FT=maize OR FT=engineer? OR FT=clone? OR FT=regulat? OR FT=transgen?)	4047660	
4	**1 NOT 2**	**92**	**64***
5	(FT=*Bacillus thuringiensis* AND FT=*aizawai*) AND FT=resistan?	**24**	**17***
6	(FT=*Bacillus thuringiensis* AND FT=*kurstaki*) AND FT=resistan?	**366**	**241***
Total number of hits			**330****

* Duplicate references identified by the title in several databases were deleted from the list.
** Duplicate references due to searches for the two different subspecies of Bt were deleted from the list
* Use of „?“ at the end of keyword will lead to an expansion of the search criteria at DIMDI database

5 Results of the study selection process

The relevance criteria were applied in the order presented in **Table 3.2-1** to sort out references that could be relevant (*rapid assessment* based on titles and abstracts).

Results were selected by evaluating and sorting all entries.

After the *rapid assessment*, 21 references were identified as being potentially relevant to subjected to a *detailed assessment* of the full-text documents.

The overall results of the literature research are presented in **Table 5-1.**

Table 5-1 Results of the study selection process

Data requirement capture in the search:	**n**
Total number of summary records retrieved after all searches of peer-reviewed literature	**330**
Number of summary records excluded from the search after rapid assessment for relevance	**307**
Total number of full-text documents assessed in detail	**23**
Number of studies excluded from further consideration after detailed assessment of relevance	**7**
Number of studies not excluded for relevance after detailed assessment	**16**

Table 5-2 and Table 5-3 below present all relevant studies subjected to a detailed assessment of full-text documents and considered relevant an d reliable (n = 16) by data requirement and by author, respectively.

Table 5-4 presents all the studies not included in the dossier after detailed assessment of full-text documents (n = 7).

Table 5-2 **List of bibliographical references identified as potentially relevant and studies of unclear relevance included in the dossier after detailed assessment of full-text documents for relevance: ordered by data requirements**

EU point	Author	Year	Title	Source	Relevant to the data requirement? Yes/No	Does the new information alter the risk assessments or List of Endpoints? Yes/No	Reliability Yes/No
MA 2.7	Bizzarri M. F., Bishop A. H.	2008	The ecology of *Bacillus thuringiensis* on the phylloplane: colonization from soil, plasmid transfer, and interaction with larvae of *Pieris brassicae*	Microb Ecol., 56(1):133-139	Yes	No	Yes
MA 2.7	Yuan Y.M., Hu X.M., Liu H.Z., Hansen B.M., Yan J.P., Yuan Z.M.	2007	Kinetics of plasmid transfer among *Bacillus cereus* group strains within lepidopteran larvae	Arch Microbiol.,187(6):425-431	Yes	No	Yes
MA 2.7	Santos C.A., Vilas-Bôas G.T., Lereclus D., Suzuki M.T., Angelo E.A., Arantes O.M.	2010	Conjugal transfer between *Bacillus thuringiensis* and *Bacillus cereus* strains is not directly correlated with growth of recipient strains	J Invertebr Pathol, 105(2):171-175.	Yes	No	Yes
MA 2.7	Donnarumma, F., Paffetti, D., Stotzky, G., Giannini, R., Vettori, C.	2010	Potential gene exchange between *Bacillus thuringiensis* subsp. *kurstaki* and *Bacillus* spp. in soil *in situ*	Soil Biology & Biochemistry, 42(8):1329-1337	Yes	No	Yes
MA 3.5	Jiang, T., Wu, S., Yang, T., Zhu, C., Gao, C.	2015	Monitoring field populations of *Plutella xylostella* (Lepidoptera: Plutellidae) for resistance to eight insecticides in China	Florida Entomologist, 98(1):65-73	Yes	No	Yes
MA 3.5	Xia, Y., Lu, Y., Shen, J., Gao, X., Qiu, H., Li, J.	2014	Resistance Monitoring for eight insecticides in *Plutella xylostella* in central China	Crop Protection 63:131-137	Yes	No	Yes
MA 3.5	Wang, L., Li, X.-F. , Zhang, J., Zhao, J.-Z., Wu, Q.-J., Xu, B., Zhang, Y.-J.	2007	Monitoring of resistance for the diamondback moth to *Bacillus thuringiensis* Cry1Ac and Cry1Ba toxins and a	Journal of Applied Entomology 131(7):441–446	Yes	No	Yes

EU point	Author	Year	Title	Source	Relevant to the data requirement? Yes/No	Does the new information alter the risk assessments or List of End-points? Yes/No	Reliability Yes/No
			Bt commercial formulation.				

EU point	Author	Year	Title	Source	Relevant to the data requirement? Yes/No	Does the new information alter the risk assessments or List of End-points? Yes/No	Reliability Yes/No
MA 3.5	Gong, Y., Wang, C., Yang, Y., Wu, S., Wu, Y.	2010	Characterization of resistance to *Bacillus thuringiensis* toxin Cry1Ac in *Plutella xylostel*la from China	J Invertebr Pathol., 104(2):90-96.	Yes	No	Yes
MA 3.5	Mittal, A., Kumari, A., Kalia, V., Kumar Singh, D., Gujar, G.T.	2007	Spatial and temporal baseline susceptibility of diamondback moth, *Plutella xylostella* (Linnaeus) to *Bacillus thuringiensis* spore Crystal mixture, purified Crystal Toxins and mixtures of cry Toxins in India	Biopesticides International, 3(1):58-70	Yes	No	Yes
MA 3.5	Sarmiento, G.M., Ocampo, V.R.	2010	Variability in response to insecticides of field populations of diamondback moth, *Plutella xylostella* (Linnaeus), in the Philippines	Phillip Ent, 24(1):39-76	Yes	No	Yes
MA 3.5	Pereira, S. G.; Sannaveerappanavar, V. T.; Murthy, M. S.	2006	Geographical variation in the susceptibility of diamondback moth, *Plutella xylostella* L. (Lepidoptera: Yponomeutidae) to *Bacillus thuringiensis* products and acylurea compounds	Resistant Pest Management Newsletter, 15(2):26-28	Yes	No	Yes
MA 3.5	Sannaveerappanavar, V.T., Virktamath, C.A.	2006	Resistance to insecticides in an Indian strain of diamondback moth, *Plutella xylostella* (L.) (Lepidoptera: Yponomeutidae)	Resistant Pest Management Newsletter, 15(2):32-35	Yes	No	Yes
MA 3.5	Zago, H.B., Siqueira, H.A., Pereira, E.J., Picanco, M.C., Barros, R.	2014	Resistance and behavioural Response of *Plutella xylostella* (Lepidoptera: Plutellidae) populations to *Bacillus thuringiensis* formulations	Pest Management Science, 70(3):488-495	Yes	No	Yes
MA 3.5	Franklin, MT., Ritland, C.E., Myers, J.H.	2010	Spatial and temporal changes in genetic structure of green-	Molecular Ecology, 19(6):1122-1133	Yes	No	Yes

EU point	Author	Year	Title	Source	Relevant to the data requirement? Yes/No	Does the new information alter the risk assessments or List of End-points? Yes/No	Reliability Yes/No
			house and filed populations of cabbage looper, *Trichoplusia ni*				
MA 3.5	Janmaat, A., Franklin, M., Myers, J.H.	2015	Resistance of cabbage loopers to Btk in a greenhouse setting: occurrence, spread and management	CAB International 2015. Bt resistance (eds. M. Soberón, Y. Gao and A. Bravo), Chapter 5:49-55	Yes	No	Yes
MA 3.5	Kalia, V., Kumari, A., Mittal, A., Singh, B.P., Nair, R., Gujar, G.T.	2006	Temporal Variation in susceptibility of American Bollworm, *Helicoverpa armigera* to *Bacillus thuringiensis* (BT) var. *kurstaki* HD-73, its Cry1Ac Toxin and Bt cotton	Pesticide Research Journal, 18(1):47-50	Yes	No	Yes

Table 5-3 **List of bibliographical references identified as potentially relevant and studies of unclear relevance included in the dossier after detailed assessment of full-text documents for relevance: ordered by authors**

Author	EU point	Year	Title	Source	Relevant to the data requirement? Yes/No	Does the new information alter the risk assessments or List of Endpoints? Yes/No	Reliability Yes/No
Bizzarri M. F., Bishop A. H.	MA 2.7	2008	The ecology of *Bacillus thuringiensis* on the phylloplane: colonization from soil, plasmid transfer, and interaction with larvae of *Pieris brassicae*	Microb Ecol., 56(1):133-139	Yes	No	Yes
Donnarumma, F., Paffetti, D., Stotzky, G., Giannini, R., Vettori, C.	MA 2.7	2010	Potential gene exchange between *Bacillus thuringiensis* subsp. *kurstaki* and *Bacillus* spp. in soil *in situ*	Soil Biology & Biochemistry, 42(8):1329-1337	Yes	No	Yes
Franklin, MT., Ritland, C.E., Myers, J.H.	MA 3.5	2010	Spatial and temporal changes in genetic structure of greenhouse and filed populations of cabbage looper, *Trichoplusia ni*	Molecular Ecology, 19(6):1122-1133	Yes	No	Yes
Gong, Y., Wang, C., Yang, Y., Wu, S., Wu, Y.	MA 3.5	2010	Characterization of resistance to *Bacillus thuringiensis* toxin Cry1Ac in *Plutella xylostel*la from China	J Invertebr Pathol., 104(2):90-96.	Yes	No	Yes
Janmaat, A., Franklin, M., Myers, J.H.	MA 3.5	2015	Resistance of cabbage loopers to Btk in a	CAB International 2015. Bt resistance (eds. M. Soberón,	Yes	No	Yes

Author	EU point	Year	Title	Source	Relevant to the data requirement? Yes/No	Does the new information alter the risk assessments or List of End-points? Yes/No	Reliability Yes/No
			greenhouse setting: occurrence, spread and management	Y. Gao and A. Bravo), Chapter 5:49-55			
Jiang, T., Wu, S., Yang, T., Zhu, C., Gao, C.	MA 3.5	2015	Monitoring field populations of *Plutella xylostella* (Lepidoptera: Plutellidae) for resistance to eight insecticides in China	Florida Entomologist, 98(1):65-73	Yes	No	Yes
Kalia, V., Kumari, A., Mittal, A., Singh, B.P., Nair, R., Gujar, G.T.	MA 3.5	2006	Temporal Variation in susceptibility of American Bollworm, *Helicoverpa armigera* to *Bacillus thuringiensis* (BT) var. *kurstaki* HD-73, its Cry1Ac Toxin and Bt cotton	Pesticide Research Journal, 18(1):47-50	Yes	No	Yes
Mittal, A., Kumari, A., Kalia, V., Kumar Singh, D., Gujar, G.T.	MA 3.5	2007	Spatial and temporal baseline susceptibility of diamondback moth, *Plutella xylostella* (Linnaeus) to *Bacillus thuringiensis* spore Crystal mixture, purified Crystal Toxins and mixtures of cry Toxins in India	Biopesticides International, 3(1):58-70	Yes	No	Yes
Pereira, S. G.; Sannaveerappa-	MA 3.5	2006	Geographical variation in	Resistant Pest Management Newsletter,	Yes	No	Yes

Author	EU point	Year	Title	Source	Relevant to the data requirement? Yes/No	Does the new information alter the risk assessments or List of End-points? Yes/No	Reliability Yes/No
navar, V. T.; Murthy, M. S.			the susceptibility of diamondback moth, *Plutella xylostella* L. (Lepidoptera: Yponomeutidae) to *Bacillus thuringiensis* products and acylurea compounds	15(2):26-28			
Sannaveerappanavar, V.T., Virktamath, C.A.	MA 3.5	2006	Resistance to insecticides in an Indian strain of diamondback moth, *Plutella xylostella* (L.) (Lepidoptera: Yponomeutidae)	Resistant Pest Management Newsletter, 15(2):32-35	Yes	No	Yes
Santos C.A., Vilas-Bôas G.T., Lereclus D., Suzuki M.T., Angelo E.A., Arantes O.M.	MA 2.7	2010	Conjugal transfer between *Bacillus thuringiensis* and *Bacillus cereus* strains is not directly correlated with growth of recipient strains	J Invertebr Pathol, 105(2):171-175.	Yes	No	Yes
Sarmiento, G.M., Ocampo, V.R.	MA 3.5	2010	Variability in response to insecticides of field populations of diamondback moth, *Plutella xylostella* (Linnaeus), in the Philippines	Phillip Ent, 24(1):39-76	Yes	No	Yes
Wang, L., Li, X.-F. , Zhang, J., Zhao, J.-Z., Wu,	MA 3.5	2007	Monitoring of resistance for the diamond-	Journal of Applied Entomology 131(7):441–446	Yes	No	Yes

Author	**EU point**	**Year**	**Title**	**Source**	**Relevant to the data requirement?** **Yes/No**	**Does the new information alter the risk assessments or List of End-points?** **Yes/No**	**Reliability** **Yes/No**
Q.-J., Xu, B., Zhang, Y.-J.			back moth to *Bacillus thuringiensis* Cry1Ac and Cry1Ba toxins and a Bt commercial formulation.				
Xia, Y., Lu, Y., Shen, J., Gao, X., Qiu, H., Li, J.	MA 3.5	2014	Resistance Monitoring for eight insecticides in *Plutella xylostella* in central China	Crop Protection 63:131-137	Yes	No	Yes
Yuan Y.M., Hu X.M., Liu H.Z., Hansen B.M., Yan J.P., Yuan Z.M.	MA 2.7	2007	Kinetics of plasmid transfer among *Bacillus cereus* group strains within lepidopteran larvae	Arch Microbiol.,187(6):425-431	Yes	No	Yes
Zago, H.B., Siqueira, H.A., Pereira, E.J., Picanco, M.C., Barros, R.	MA 3.5	2014	Resistance and behavioural Response of *Plutella xylostella* (Lepidoptera: Plutellidae) populations to *Bacillus thuringiensis* formulations	Pest Management Science, 70(3):488-495	Yes	No	Yes

Table 5-4 **List of bibliographical references excluded from the dossier after detailed assessment of full-text documents for relevance: ordered by authors**

Author	Year	Title	Source	Relevant to the data requirements? Yes/No	Does the new information alter the risk assessments or List of Endpoints? Yes/No	Reliability Yes/No/n.a	Justification
Beuls, E., Van Houdt, R., Leys, N., Dijkstra, C., Larkin, O., Mahillon, J.	2009	*Bacillus thuringiensis* conjugation in simulated Microgravity	Astrobiology, 9(8):797-805	No	No	n.a.	The article reports about mating experiments between Bt strains assessing exchange of the conjugative plasmid pAW63 and its ability to mobilise the non-conjugative plasmid pUB110. There are two reasons why the article was excluded from the dossier after the full text assessment: 1) the experiments were only carried out in culture broth, 2) the experiments were carried out under conditions simulating microgravity. These conditions are not considered relevant to assess a possible exchange of genetic material of Btk upon use as ppp.
Franklin, M.T., Nieman, C.L., Janmaat, A.F., Soberon, M., Bravo, A., Tabashnik, B. E., Myers, J.H.	2009	Modified *Bacillus thuringiensis* Toxins and a hybrid *B. thuringiensis* strain Counter greenhouse-selected resistance in *Trichoplusia ni*	Applied and Environmental Microbiology, 75(17):5739-5741	No	No	n.a.	The article describes experiments in which the response of resistant and susceptible strains of *Trichoplusia ni* to different commercial Bt-based products, native and modified Cry toxins is assessed in the laboratory. The article does not include records of resistant field or greenhouse population but the resistant strains used in the experiments where obtained via directed selection of resistance in the laboratory. It is therefore considered not relevant for the data point.
Janmaat, A. F	2007	Development of resistance to the biopesticide *Bacillus thuringiensis*	Book: Biological control: a global perspective, Chapter 19:179-184	No	No	n.a.	The article describes results of a monitoring of Bt resistance in *T. ni* populations in greenhouses and is considered relevant. However, the

Author	**Year**	**Title**	**Source**	**Relevant to the data require quirements?** **Yes/No**	**Does the new information alter the risk assessments or List of Endpoints?** **Yes/No**	**Reliability** **Yes/No/n.a**	**Justification**
		kurstaki					same data set is presented in Janmaat et al. (2015) in which the development of the resistance over the years is presented. The older article is therefore excluded from the dossier.
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, 20(1):62-69	No	No	n.a.	The study describes the tri-trophic interaction between the target pest *T. ni*, the host plant and Btk and resistance patterns related to the host plant. In the experiment resistant (and susceptible) strains were used which were obtained trough directed selection. The article does not provide any information about resistance of the original field population and is therefore considered not relevant for the data point.
Riley, D.G.	2013	Insecticide Rotations for the Management of Lepidopteran Pests in Cabbage and Collards	Journal of Entomology, 49(2):130-143	No	No	n.a.	The article describes field trials in collard and cabbage. Assessed was control of different lepidopteran pests by rotational application of different insecticides. The results only refer to determination of the efficacy of the different treatment programs but not to recording of resistant insect populations.
Sukonthabhirom, S., Siripontangmun, S.	2012	Toxicity of insecticides on diamondback moth from three areas in Thailand.	Proceedings Seaveg 2012, p:97-103	No	No	n.a.	The study reports on tests for toxicity of different insecticides to *P. xylostella* populations from different locations in Thailand over a period of 4 years. Based on LC_{50} values obtained from leaf dip bioassays, toxicity ratios and resistance factors were calculated. The study is considered not reliable because the assessment of resistance was not

Author	**Year**	**Title**	**Source**	**Relevant to the data require quirements?** **Yes/No**	**Does the new information alter the risk assessments or List of Endpoints?** **Yes/No**	**Reliability** **Yes/No/n.a**	**Justification**
							based on comparison of LC_{50} values between susceptible and field populations. Instead, data from one year were compared to those of the previous year.
Tamez-Guerra, P., Damas, G., Iracheta, M.M., Oppert, B., Gomez-Flores, R., Rodriguez-Padilla, C.	2006	Differences in susceptibility and physiological fitness of Mexican field *Trichoplusia ni* strains exposed to *Bacillus thuringiensis*	Journal of Economic Entomology, 99(3):937-945	No	No	n.a.	The article reports on laboratory experiments with different *P. xylostella* strains which do not present original field populations as all of them underwent a kind of selection procedure. No information is provided about resistance patterns in field populations. The article is therefore considered not relevant for the data point.

Note RMS:

1) References relied on, 4. Search methods and results, p. 85-87: There are some inconsistencies regarding the literature search. In general there are several synthax errors in regard to the brackets used in the search strategy. This may indicate that the literature search was not adequately performed but may perhaps also not have influenced the search results.

While the search strategy concerning genetic stability contains in total six opening brackets and seven closing ones on page 86 it contains 5 opening brackets and 6 six closing ones on page 87 (Table 4-1). Similarly, the search strategy for the development of resistances contains in total only two opening brackets but three closing ones.

2) References relied on, 4. Search methods and results, Table 4-1, p. 87: Please indicate what FT stands for! Full title? Full text? Any other?

3) References relied on, 4. Search methods and results, Table 4-1, p. 87: Concerning Search 4, shouldn't it be 1 AND 2 NOT 3 (instead of 2 NOT 3)? According to the search method outlined before the search strategy was Bacillus thuringiensis AND (aizawai OR kurstaki) AND […]. If the search strategy here would have been 2 NOT 3 as indicated all the keywords to be excluded from the search would still have been part of the search results under Search 1.

4) References relied on, 4. Search methods and results, Table 4-2, p. 88: Search strategy 4 here clearly differs from Table 4-1 (1 NOT 2 instead of 2 NOT 3). Again, shouldn't it be 1 AND 2 NOT 3? If the search strategy here would have been 1 NOT 2 as indicated all the results from search 2 (concerning the subspecies kurstaki) would have been excluded.

5) References relied on, 4. Search methods and results, Table 4-2, p. 88: Something is strange about the total number of results. If search strategy 4 is indeed 1 AND 2 NOT 3 then the total number of results amounts to 64 + 17 + 241 = 322 already after removing of duplicates. How then can the total number be even higher than that (i.e. 330) if in addition duplicate references due to searches for the two different subspecies of Bt were deleted from the list?

As the search strategy as outlined is somewhat confusing applicant please clarify and address these 5 points.

Data point CADDY (ongoing numbering)	Author(s)	Year	Title Owner, Report No. Source (where different from owner) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously submitted Y/N* If Y => old data point
KMA 2.1/01	Martin, P.A.W., Travers, R.S.	1989	WORLDWIDE ABUNDANCE AND DISTRIBUTION OF BACILLUS THURINGIENSIS ISOLATES not available, not applicable Applied and Environmental Microbiology, 55, 2437-2442 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.1
KMA 2.1/02	DeLucca, A.J., Simonson, J.G., Larson, A.D.	1981	BACILLUS THURINGIENSIS DISTRIBUTION IN SOILS OF THE UNITED STATES not available, not applicable Canadian Journal of Microbiology, 27, 865-870 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.1
KMA 2.1/03	Ohba, M., Aizawa, K.	1986	DISTRIBUTION OF BACILLUS THURINGIENSIS IN SOILS OF JAPAN not available, not applicable Journal of invertebrate Pathology, 47, 277-282 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.1
KMA 2.1/04	Brownbridge, M., Margalit, J.	1986	NEW BACILLUS THURINGIENSIS STRAINS ISOLATED IN ISRAEL HIGHLY TOXIC TO MOSQUITO LARVAE not available, not applicable Journal of invertebrate Pathology, 48, 216-222 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.1
KMA 2.1/05	Asimeng, E.J., Mutinga, M.J.	1992	ISOLATION OF MOSQUITOTOXIC BACTERIA FROM MOSQUITO-BREEDING SITES IN KENYA not available, not applicable Journal of the American Mosquito Control Assocation, 8, 86-88 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.1
KMA 2.1/06	Dulmage, H.T.	1970	INSECTICIDAL ACTIVITY OF HD-1, A NEW ISOLATE OF BACILLUS THURINGIENSIS VAR. ALESTI not available, not applicable Journal of invertebrate Pathology, 15, 232-239 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.1

Data point CADDY (ongoing numbering)	Author(s)	Year	Title Owner, Report No. Source (where different from owner) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously submitted Y/N* If Y => old data point
KMA 2.1/07	DeLucca, A.J., Palmgren, M.S., Ciegler, A.	1982	BACILLUS THURINGIENSIS IN GRAIN ELEVATOR DUSTS not available, not applicable Canadian Journal of Microbiology, 28, 452-456 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.1
KMA 2.1/08	Glare, T.R., O'Callaghan, M.	2000	BACILLUS THURINGIENSIS: BIOLOGY, ECOLOGY AND SAFETY not available, not applicable John Wiley and Sons Inc New York GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.1
KMA 2.1/09	Smith, R.A., Couche, G.A.	1991	THE PHYLLOPLANE AS A SOURCE OF BACILLUS THURINGIENSIS VARIANTS not available, not applicable Applied and Environmental Microbiology, 57, 311-315 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.1
KMA 2.1/10	González, J.M., Brown, B.J., Carlton, B.C.	1982	TRANSFER OF BACILLUS THURINGIENSIS PLASMIDS CODING FOR DELTA-ENDOTOXIN AMONG STRAINS OF B. THURINGIENSIS AND B. CEREUS not available, not applicable Proc Natl Acad Sci USA, 79, 6951-6955 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.1
KMA 2.1/11	Burges et al.	1991	UNITED STATES PATENT FOR GC-91 not available, 5063055 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.1
KMA 2.1.2/01	Bernhard, K., Jarrett, P., Meadows, M., Butt, J., Ellis, D.J., Roberts, G.M., Pauli, S., Rodgers, P., Burges, H.D.	1997	NATURAL ISOLATES OF BACILLUS THURINGIENSIS: WORLDWIDE DISTRIBUTION, CHARACTERIZATION, AND ACTIVITY AGAINST INSECT PESTS not available, not applicable Journal of invertebrate Pathology, 70, 59-68 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.2

Data point CADDY (ongoing numbering)	Author(s)	Year	Title Owner, Report No. Source (where different from owner) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously submitted Y/N* If Y => old data point
KMA 2.1.2/02	Martin, P.A.W., Travers, R.S.	1989	WORLDWIDE ABUNDANCE AND DISTRIBUTION OF BACILLUS THURINGIENSIS ISOLATES not available, not applicable Applied and Environmental Microbiology, 55, 2437-2442 GLP/GEP: no Published: yes **Submitted in: KMA 2.1/01**	no	no	not protected	-	Y KIIM 2.2
KMA 2.1.2/03	Landén, R., Bryne, M., Abdel-Hameed, A.	1994	DISTRIBUTION OF BACILLUS THURINGIENSIS STRAINS IN SOUTHERN SWEDEN not available, not applicable World J Microbiol Biotechnol, 10, 45-50 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.2
KMA 2.1.2/04	Iriarte, J., Bel, Y., Ferrandis, M.D., Andrew, R., Murillo, J., Ferré, J., Caballero, P.	1998	ENVIRONMENTAL DISTRIBUTION AND DIVERSITY OF BACILLUS THURINGIENSIS IN SPAIN not available, not applicable System Appl Microbiol, 21, 97-106 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.2
KMA 2.1.2/05	Bel, Y., Granero, F., Alberola, T.M., Martínez-Sebastián, M., Ferré, J.	1997	DISTRIBUTION, FREQUENCY AND DIVERSITY OF BACILLUS THURINGIENSIS IN OLIVE TREE ENVIRONMENTS IN SPAIN not available, not applicable System Appl Microbiol, 20, 652-658 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.2
KMA 2.1.2/06	Kim, H.S., Lee, D.W., Woo, S.O., Yu, Y.M., Kang, S.K.	1998	SEASONAL DISTRIBUTION AND CHARACTERIZATION OF BACILLUS THURINGIENSIS ISOLATED FROM SERICULTURAL ENVIRONMENTS IN KOREA not available, not applicable Journal of General and Applied Microbiology, 44, 133-138 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.2

Data point CADDY (ongoing numbering)	Author(s)	Year	Title Owner, Report No. Source (where different from owner) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously submitted Y/N* If Y => old data point
KMA 2.1.2/07	Valicente, F.H., Barreto, M.R.	2003	BACILLUS THURINGIENSIS SURVEY IN BRAZIL: GEOGRAPHICAL DISTIBUTION AND INSECTICIDAL ACTIVITY AGAINST SPODOPTERA FRUGIPERDA (J.E. SMITH) (LEPIDOPTERA: NOCTUIDAE) not available, not applicable Neotropical Entomology, 32 (4), 639-644 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.2
KMA 2.1.2/08	Vásquez, M., Parra, C., Hubert, E., Espinoza, P., Theoduloz, C., Meza-Basso, L.	1995	SPECIFICITY AND INSECTICIDAL ACTIVITY OF CHILEAN STRAINS OF BACILLUS THURINGIENSIS not available, not applicable Journal of invertebrate Pathology, 66, 143-148 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.2
KMA 2.1.2/09	Ohba, M., Aizawa, K.	1986	DISTRIBUTION OF BACILLUS THURINGIENSIS IN SOILS OF JAPAN not available, not applicable Journal of invertebrate Pathology, 47, 277-282 GLP/GEP: no Published: yes **Submitted in: KMA 2.1/03**	no	no	not protected	-	Y KIIM 2.2
KMA 2.1.2/10	Damgaard, P.H., Hansen, B.M., Pedersen, J.C., Eilenberg, J.	1997	NATURAL OCCURRENCE OF BACILLUS THURINGIENSIS ON CABBAGE FOLIAGE AND IN INSECTS ASSOCIATED WITH CABBAGE CROPS not available, not applicable Journal of Applied Microbiology, 82, 253-258 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.2
KMA 2.1.2/11	Damgaard, P.H., Abdel-Hameed, A., Eilenberg, J., Smits, P.H.	1998	NATURAL OCCURRENCE OF BACILLUS THURINGIENSIS ON GRASS FOLIAGE not available, not applicable World J Microbiol Biotechnol, 14, 239-242 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.2

Data point CADDY (ongoing numbering)	Author(s)	Year	Title Owner, Report No. Source (where different from owner) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously submitted Y/N* If Y => old data point
KMA 2.1.2/12	Smith, R.A., Couche, G.A.	1991	THE PHYLLOPLANE AS A SOURCE OF BACILLUS THURINGIENSIS VARIANTS not available, not applicable Applied and Environmental Microbiology, 57, 311-315 GLP/GEP: no Published: yes **Submitted in: KMA 2.1/09**	no	no	not protected	-	Y KIIM 2.2
KMA 2.2/01	Höfte, H., Whiteley, H.R.	1989	INSECTICIDAL CRYSTAL PROTEINS OF BACILLUS THURINGIENSIS not available, not applicable Microbiol Rev, Jun, 242-255 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.3
KMA 2.2/02	Feitelson, J.F., Payne, J., Kim, L.	1992	BACILLUS THURINGIENSIS: INSECTS AND BEYOND not available, not applicable Biotechnology, 10 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.3
KMA 2.2/03	Wei, J.Z., Hale, K., Carta, L., Platzer, E., Wong, C., Fang, S.-C., Aroian, R.V.	2003	BACILLUS THURINGIENSIS CRYSTAL PROTEINS THAT TARGET NEMATODES not available, not applicable Proc Natl Acad Sci USA, 100, 2760-2765 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.3
KMA 2.2/04	Burges et al.	1991	UNITED STATES PATENT FOR GC-91 not available, 5063055 GLP/GEP: no Published: yes **Submitted in: KMA 2.1/11**	no	no	not protected	-	Y KIIM 2.3
KMA 2.2/05	Dulmage, H.T.	1970	INSECTICIDAL ACTIVITY OF HD-1, A NEW ISOLATE OF BACILLUS THURINGIENSIS VAR. ALESTI not available, not applicable Journal of invertebrate Pathology, 15, 232-239 GLP/GEP: no Published: yes **Submitted in: KMA 2.1/06**	no	no	not protected	-	Y KIIM 2.3

Data point CADDY (ongoing numbering)	**Author(s)**	**Year**	**Title Owner, Report No. Source (where different from owner) GLP or GEP status Published or not**	**Vertebrate study Y/N**	**Data protection claimed Y/N**	**Justification if data protection is claimed**	**Owner**	**Previously submitted Y/N* If Y => old data point**
KMA 2.2/06	Moar, W.J., Pusztai-Carey, M., van Faassen, H., Bosch, D., Frutos, R., Rang, C., Luo, K., Adang, M.J.	1995	DEVELOPMENT OF BACILLUS THURINGIENSIS CRYIC RESISTANCE BY SPODOPTERA EXIGUA (HÜBNER) (LEPIDOPTERA: NOCTUIDAE) not available, not applicable Applied and Environmental Microbiology, Jun, 2086-2092 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.3
KMA 2.2.2/01	Schnepf, E., Crickmore, N., van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D.R., Dean, D.H.	1998	BACILLUS THURINGIENSIS AND ITS PESTICIDAL CRYSTAL PROTEINS not available, not applicable Microbiol Mol Biol Rev, 62, 775-806 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.3.2
KMA 2.2.2/02	Höfte, H., Whiteley, H.R.	1989	INSECTICIDAL CRYSTAL PROTEINS OF BACILLUS THURINGIENSIS not available, not applicable Microbiol Rev, Jun, 242-255 GLP/GEP: no Published: yes **Submitted in: KMA 2.2/01**	no	no	not protected	-	Y KIIM 2.3.2
KMA 2.2.2/03	Hofmann, C., Vanderbruggen, H., Höfte, H., van Rie, J., Jansens, S., van Mellaert, H.	1988	SPECIFICITY OF BACILLUS THURINGIENSIS DELTA-ENDOTOXINS IS CORRELATED WITH THE PRESENCE OF HIGH-AFFINITY BINDING SITES IN THE BRUSH BORDER MEMBRANE OF TARGET INSECT MIDGUTS not available, not applicable Proc Natl Acad Sci USA, 85, 7844-7848 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.3.2
KMA 2.2.2/04	Hofmann, C., Lüthy, P., Hütter, R., Pliska, V.	1988	BINDING OF THE DELTA ENDOTOXIN FROM BACILLUS THURINGIENSIS TO BRUSH-BORDER MEMBRANE VESICLES OF THE CABBAGE BUTTERFLY (PIERIS BRASSICAE) not available, not applicable Eur J Biochem, 173, 85-91 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.3.2

Data point CADDY (ongoing numbering)	**Author(s)**	**Year**	**Title Owner, Report No. Source (where different from owner) GLP or GEP status Published or not**	**Vertebrate study Y/N**	**Data protection claimed Y/N**	**Justification if data protection is claimed**	**Owner**	**Previously submitted Y/N* If Y => old data point**
KMA 2.2.2/05	Schwartz, J.L., Garneau, L., Masson, L., Brousseau, R.	1991	EARLY RESPONSE OF CULTURED LEPIDOPTERAN CELLS TO EXPOSURE TO DELTA-ENDOTOXIN FROM BACILLUS THURINGIENSIS: INVOLVEMENT OF CALCIUM AND ANIONIC CHANNELS not available, not applicable Biochimica et Biophysica Acta, 1065, 250-260 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.3.2
KMA 2.2.2/06	Aronson, A.I.	1993	THE TWO FACES OF BACILLUS THURINGIENSIS: INSECTICIDAL PROTEINS AND POST-EXPONENTIAL SURVIVAL not available, not applicable Mol Microbiol, 7, 489-496 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.3.2
KMA 2.2.2/07	Burges, H.D.	1982	CONTROL OF INSECTS BY BACTERIA not available, not applicable Parasitology, 84, 79-117 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.3.2
KMA 2.2.2/08	Griego, V.M., Spence, K.D.	1978	INACTIVATION OF BACILLUS THURINGIENSIS SPORES BY ULTRAVIOLET AND VISIBLE LIGHT not available, not applicable Applied and Environmental Microbiology, 35, 906-910 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.3.2
KMA 2.2.2/09	Pusztai, M., Fast, P., Gringorten, L., Kaplan, H., Lessard, T., Carey, P.R.	1991	THE MECHANISM OF SUNLIGHT-MEDIATED INACTIVATION OF BACILLUS THURINGIENSIS CRYSTALS not available, not applicable Biochemical Journal, 273, 43-47 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.3.2
KMA 2.2.2/10	Dulmage, H.T., Boening, O.P., Rehnborg, C.S., Hansen, G.D.	1971	A PROPOSED STANDARDIZED BIOASSAY FOR FORMULATIONS OF BACILLUS THURINGIENSIS BASED ON THE INTERNATIONAL UNIT not available, not applicable Journal of invertebrate Pathology, 18, 240-245 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.3.2

Data point CADDY (ongoing numbering)	**Author(s)**	**Year**	**Title** **Owner, Report No.** **Source (where different from owner)** **GLP or GEP status** **Published or not**	**Vertebrate study Y/N**	**Data protection claimed Y/N**	**Justification if data protection is claimed**	**Owner**	**Previously submitted Y/N*** **If Y => old data point**
KMA 2.2.2/11	Yamamoto, T., Iizuka, T.	1983	TWO TYPES OF ENTOMOCIDAL TOXINS IN THE PARASPORAL CRYSTALS OF BACILLUS THURINGIENSIS KURSTAKI not available, not applicable Archives of Biochemistry and Biophysics, 227, 233-241 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.3.2
KMA 2.2.2/12	Luo, K., Banks, D., Adang, M.J.	1999	TOXICITY, BINDING, AND PERMEABILITY ANALYSES OF FOUR BACILLUS THURINGIENSIS CRY1 DELTA-ENDOTOXINS USING BRUSH BORDER MEMBRANE VESICLES OF SPODOPTERA EXIGUA AND SPODOPTORA FRUGIPERDA not available, not applicable Applied and Environmental Microbiology, Feb, 457-464 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.3.2
KMA 2.2.2/13	Rausell, C., Martinez-Ramirez, C.M., García-Robles, I., Real, M.D.	2000	A BINDING SITE FOR BACILLUS THURINGIENSIS CRY1AB TOXIN IS LOST DURING LARVAL DEVELOPMENT IN TWO FOREST PESTS not available, not applicable Applied and Environmental Microbiology, Apr, 1553-1558 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.3.2
KMA 2.2.2/14	Gilliland, A., Chambers, C.E., Bone E.J., Ellar D.J.	2002	ROLE OF BACILLUS THURINGIENSIS CRY1 DELTA ENDOTOXIN BINDING IN DETERMINING POTENCY DURING LEPIDOPTERAN LARVAL DEVELOPMENT not available, not applicable Applied and Environmental Microbiology, Apr, 1509-1515 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.3.2

Data point CADDY (ongoing numbering)	**Author(s)**	**Year**	**Title Owner, Report No. Source (where different from owner) GLP or GEP status Published or not**	**Vertebrate study Y/N**	**Data protection claimed Y/N**	**Justification if data protection is claimed**	**Owner**	**Previously submitted Y/N* If Y => old data point**
KMA 2.2.2/15	Bernhard, K., Jarrett, P., Meadows, M., Butt, J., Ellis, D.J., Roberts, G.M., Pauli, S., Rodgers, P., Burges, H.D.	1997	NATURAL ISOLATES OF BACILLUS THURINGIENSIS: WORLDWIDE DISTRIBUTION, CHARACTERIZATION, AND ACTIVITY AGAINST INSECT PESTS not available, not applicable Journal of invertebrate Pathology, 70, 59-68 GLP/GEP: no Published: yes **Submitted in: KMA 2.1.2/01**	no	no	not protected	-	Y KIIM 2.3.2
KMA 2.2.2/16	Pigott, C.R., Ellar, D.J.	2007	ROLE OF RECEPTORS IN BACILLUS THURINGIENSIS CRYSTAL TOXIN ACTIVITY not available, not applicable Microbiol Mol Biol Rev, 71, 255-281 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.3.2
KMA 2.2.2/17	Bravo, A., Gill, S.S., Soberon, M.	2007	MODE OF ACTION OF BACILLUS THURINGIENSIS CRY AND CYT TOXINS AND THEIR POTENTIAL FOR INSECT CONTROL not available, not applicable Toxicon, 49, 423-435 (online version 1-18) GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.3.2
KMA 2.2.2/18	Zhang, X., Candas, M., Griko, N.B., Taussig, R., Bulla, L.A. Jr.	2006	A MECHANISM OF CELL DEATH INVOLVING AN ADENYLYL CYCLASE/PKA SIGNALING PATHWAY IS INDUCED BY THE CRY1AB TOXIN OF BACILLUS THURINGIENSIS not available, not applicable Proc Natl Acad Sci USA, 103, 9897-9902 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.3.2
KMA 2.2.2/19	Broderick, N.A., Raffa, K.F., Handelsman,J.	2006b	MIDGUT BACTERIA REQUIRED FOR BACILLUS THURINGIENSIS INSECTICIDAL ACTIVITY not available, not applicable Proc Natl Acad Sci USA, 103, 15196-15199 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.3.2

Data point CADDY (ongoing numbering)	**Author(s)**	**Year**	**Title Owner, Report No. Source (where different from owner) GLP or GEP status Published or not**	**Vertebrate study Y/N**	**Data protection claimed Y/N**	**Justification if data protection is claimed**	**Owner**	**Previously submitted Y/N* If Y => old data point**
KMA 2.2.2/20	Jurat-Fuentes, J.L., Adang, M.J.	2006	CRY TOXIN MODE OF ACTION IN SUSCEPTIBLE AND RESISTANT HELIOTHIS VIRESCENS LARVAE not available, not applicable Journal of invertebrate Pathology, 92, 166-171 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.3.2
KMA 2.2.2/21	Broderick, N.A., Robinson, C.J., McMahon, M.D., Holt, J., Handelsman, J., Raffa, K.F.	2009b	CONTRIBUTIONS OF GUT BACTERIA TO BACILLUS THURINGIENSIS-INDUCED MORTALITY VARY ACROSS A RANGE OF LEPIDOPTERA not available, not applicable BMC Biology, 7, 1-9 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.3.2
KMA 2.2.2/01 (KMA 2.2.2/22)	Palma, L., Munoz, D., Berry, C., Murillo, J., Caballero, P.	2014	BACILLUS THURINGIENSIS TOXINS: AN OVERVIEW OF THEIR BIOCIDAL ACTIVITY not available, not applicable Toxins, 6, 3296-3325 GLP/GEP: no Published: yes	no	no	not protected	-	N
KMA 2.2.2/02 (KMA 2.2.2/23)	Broderick, N.A., Raffa, K.F., Handelsman,J.	2006a	MIDGUT BACTERIA REQUIRED FOR BACILLUS THURINGIENSIS INSECTICIDAL ACTIVITY not available, not applicable Proc Natl Acad Sci USA, 103, 15196-15199 GLP/GEP: no Published: yes	no	no	not protected	-	N
KMA 2.2.2/03 (KMA 2.2.2/24)	Broderick, N.A., Robinson, C.J., McMahon, M.D., Holt, J., Handelsman, J., Raffa, K.F.	2009a	CONTRIBUTIONS OF GUT BACTERIA TO BACILLUS THURINGIENSIS-INDUCED MORTALITY VARY ACROSS A RANGE OF LEPIDOPTERA not available, not applicable BMC Biology, 7, 1-9 GLP/GEP: no Published: yes	no	no	not protected	-	N
KMA 2.3/01	Höfte, H., Whiteley, H.R.	1989	INSECTICIDAL CRYSTAL PROTEINS OF BACILLUS THURINGIENSIS not available, not applicable Microbiol Rev, Jun, 242-255 GLP/GEP: no Published: yes **Submitted in: KMA 2.2/01**	no	no	not protected	-	Y KIIM 2.4

Data point CADDY (ongoing numbering)	Author(s)	Year	Title Owner, Report No. Source (where different from owner) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously submitted Y/N* If Y => old data point
KMA 2.3/02	Schnepf, E., Crickmore, N., van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D.R., Dean, D.H.	1998	BACILLUS THURINGIENSIS AND ITS PESTICIDAL CRYSTAL PROTEINS not available, not applicable Microbiol Mol Biol Rev, 62, 775-806 GLP/GEP: no Published: yes **Submitted in: KMA 2.2.2/01**	no	no	not protected	-	Y KIIM 2.4
KMA 2.3/03	Aronson, A.I., Han, E.-S., McGaughey, W., Johnson, D.	1991	THE SOLUBILITY OF INCLUSION PROTEINS FROM BACILLUS THURINGIENSIS IS DEPENDENT UPON PROTOXIN COMPOSITION AND IS FACTOR IN TOXICITY TO INSECTS not available, not applicable Applied and Environmental Microbiology, 57, 981-986 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.4
KMA 2.3/04	Jaquet, F., Hütter, R., Lüthy, P.	1987	SPECIFICITY OF BACILLUS THURINGIENSIS DELTA-ENDOTOXIN not available, not applicable Applied and Environmental Microbiology, Mar, 500-504 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.4
KMA 2.3/05	McGaughey, W.H., Whalon, M.E.	1992	MANAGING INSECT RESISTANCE TO BACILLUS THURINGIENSIS TOXINS not available, not applicable Science, 258, 1451-1455 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.4
KMA 2.3/06	Haider, M.Z., Knowles. B.H., Ellar, D.J.	1986	SPECIFICITY OF BACILLUS THURINGIENSIS VAR. COLMERI INSECTICIDAL DELTA-ENDOTOXIN IS DETERMINED BY DIFFERENTIAL PROTEOLYTIC PROCESSING OF THE PROTOXIN BY LARVAL GUT PROTEASES not available, not applicable Eur J Biochem, 156, 531-540 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.4
KMA 2.3/07	Honée, G., Visser, B.	1993	THE MODE OF ACTION OF BACILLUS THURINGIENSIS CRYSTAL PROTEINS not available, not applicable Entomol Exp Appl, 69, 145-155 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.4

Data point CADDY (ongoing numbering)	Author(s)	Year	Title Owner, Report No. Source (where different from owner) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously submitted Y/N* If Y => old data point
KMA 2.3/08	Wolfersberger, M.G.	1991	INHIBITION OF POTASSIUM-GRADIENT DRIVEN PHENYLALANINE UPTAKE IN LARVAL LYMANTRIA DISPAR MIDGUT BY TWO BACILLUS THURINGIENSIS DELTA-ENDOTOXINS CORRELATES WITH THE ACTIVITY OF THE TOXINS AS GYPSY MOTH LARVICIDES not available, not applicable Journal of Experimental Biology, 161, 519-525 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.4
KMA 2.3/09	MacIntosh, S.C., Stone, T.B., Sims, S.R., Hunst, P.L., Greenplate, J.T., Marrone, P.G., Perlak, F.J., Fischhoff, D.A., Fuchs R.L.	1990	SPECIFICITY AND EFFICACY OF PURIFIED BACILLUS THURINGIENSIS PROTEINS AGAINST AGRONOMICALLY IMPORTANT INSECTS not available, not applicable Journal of invertebrate Pathology, 56, 258-266 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.4
KMA 2.3/10	Sears, M.K., Hellmich, R.L., Stanley-Horn, D.E., Oberhauser, K.S., Pleasants, J.M., Mattila, H.R., Siegfried, B.D., Dively, G.P.	2001	IMPACT OF BT CORN POLLEN ON MONARCH BUTTERFLY POPULATIONS: A RISK ASSESSMENT not available, not applicable Proc Natl Acad Sci USA, 98 (21), 11937-11942 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.4
KMA 2.3/11	World Health Organization	1999	ENVIRONMENTAL HEALTH CRITERIA 217 - MICROBIAL PEST CONTROL AGENT BACILLUS THURINGIENSIS not available, not applicable WHO World Health Organization GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.4
KMA 2.3/12	Siegel, J.P.	2001	THE MAMMALIAN SAFETY OF BACILLUS THURINGIENSIS-BASED INSECTICIDES not available, not applicable Journal of invertebrate Pathology, 77, 13-21 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.4

Data point CADDY (ongoing numbering)	**Author(s)**	**Year**	**Title** **Owner, Report No.** **Source (where different from owner)** **GLP or GEP status** **Published or not**	**Vertebrate study Y/N**	**Data protection claimed Y/N**	**Justification if data protection is claimed**	**Owner**	**Previously submitted Y/N*** **If Y => old data point**
KMA 2.3/13	Wraight, C.L., Zangerl, A.R., Carroll, M.J., Berenbaum, M.R.	2000	ABSENCE OF TOXICITY OF BACILLUS THURINGIENSIS POLLEN TO BLACK SWALLOWTAILS UNDER FIELD CONDITIONS not available, not applicable Proc Natl Acad Sci USA, 97, 7700-7703 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.4
KMA 2.4/01	Griego, V.M., Spence, K.D.	1978	INACTIVATION OF BACILLUS THURINGIENSIS SPORES BY ULTRAVIOLET AND VISIBLE LIGHT not available, not applicable Applied and Environmental Microbiology, 35, 906-910 GLP/GEP: no Published: yes **Submitted in: KMA 2.2.2/08**	no	no	not protected	-	Y KIIM 2.3.2
KMA 2.4/02	Pusztai, M., Fast, P., Gringorten, L., Kaplan, H., Lessard, T., Carey, P.R.	1991	THE MECHANISM OF SUNLIGHT-MEDIATED INACTIVATION OF BACILLUS THURINGIENSIS CRYSTALS not available, not applicable Biochemical Journal, 273, 43-47 GLP/GEP: no Published: yes **Submitted in: KMA 2.2.2/09**	no	no	not protected	-	Y KIIM 2.3.2
KMA 2.4/03	World Health Organization	1999	ENVIRONMENTAL HEALTH CRITERIA 217 - MICROBIAL PEST CONTROL AGENT BACILLUS THURINGIENSIS not available, not applicable WHO World Health Organization GLP/GEP: no Published: yes **Submitted in: KMA 2.3/11**	no	no	not protected	-	Y KIIM 2.5
KMA 2.5/01	Bravo, A.	1997	 not available, not applicable Journal of Bacteriology, 179, 2793-2801 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.5

Data point CADDY (ongoing numbering)	**Author(s)**	**Year**	**Title Owner, Report No. Source (where different from owner) GLP or GEP status Published or not**	**Vertebrate study Y/N**	**Data protection claimed Y/N**	**Justification if data protection is claimed**	**Owner**	**Previously submitted Y/N* If Y => old data point**
KMA 2.5/02	Estruch, J.J., Warren, G.W., Mullins, M.A., Nye, G.J., Craig, J.A., Koziel, M.G.	1996	VIP3A, A NOVEL BAC. THUR.VEGETATIVE INSECTICIDAL PROTEIN WITH A WIDE SPECTRUM OF ACTIVITES AGAINST LEPIDOPTERAN INSECTS not available, not applicable Proc Natl Acad Sci USA, 93, 5389-5394 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.5
KMA 2.5/03	Yu, C.-G., Mullins. M.A., Warren, G.W., Koziel, M.G., Estruch, J.J.	1997	THE BACILLUS THURINGIENSIS VEGETATIVE INSECTICIDAL PROTEIN VIP3A LYSES MIDGUT EPITHELIUM CELLS OF SUSCEPTIBLE INSECTS not available, not applicable Applied and Environmental Microbiology, 63, 532-536 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.5
KMA 2.5/04	Selvapandiyan, A., Arora, N., Rajagopal, R., Jalali, S.K., Venkatesan, T., Singh, S.P., Bhatnagar, R.K.	2001	TOXICITY ANALYSIS OF N- AND C-TERMINUS-DELETED VEGETATIVE INSECTICIDAL PROTEIN FROM BACILLUS THURINGIENSIS not available, not applicable Applied and Environmental Microbiology, 67, 5855-5858 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.5
KMA 2.6/01	Drobniewski, F.A.	1993	BACILLUS CEREUS AND RELATED SPECIES not available, not applicable Clinical Microbiology Reviews, published by the American Society for Microbiology, 6, 324-338 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.7
KMA 2.6/02	Anonymous	2004	ANTHRAX FACT SHEET not available, not applicable CFSPH Center for Food Security and Public Health, 1-4 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.7

Data point CADDY (ongoing numbering)	Author(s)	Year	Title Owner, Report No. Source (where different from owner) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously submitted Y/N* If Y => old data point
KMA 2.6/03	Carlson, C.R., Johansen, T., Kolsto, A.B.	1996	THE CHROMOSOME MAP OF BAC. THUR. SUBSP. CANADENSIS HD224 IS HIGHLY SIMILAR TO THAT OF THE BAC. CEREUS TYPE STRAIN ATCC 14579 not available, not applicable FEMS Microbiology Letters, 141, 163-167 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.7
KMA 2.6/04	Helgason, E., Okstad, O.A., Caugant, D.A., Johansen, H.A., Fouet, A., Mock, M., Hegna, I., Kolsto, A.B.	2000	BACILLUS ANTHRACIS, BACILLUS CEREUS, AND BACILLUS THURINGIENSIS-ONE SPECIES ON THE BASIS OF GENETIC EVIDENCE not available, not applicable Applied and Environmental Microbiology, 66, 2627-2630 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.7
KMA 2.6/05	Schnepf, E., Crickmore, N., van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D.R., Dean, D.H.	1998	BACILLUS THURINGIENSIS AND ITS PESTICIDAL CRYSTAL PROTEINS not available, not applicable Microbiol Mol Biol Rev, 62, 775-806 GLP/GEP: no Published: yes **Submitted in: KMA 2.2.2/01**	no	no	not protected	-	Y KIIM 2.7
KMA 2.6/06	Prüß, B.M., Dietrich, R., Nibler, B., Märtlbauer, E., Scherer, S.	1999	THE HEMOLYTIC ENTEROTOXIN HBL IS BROADLY DISTRIBUTED AMONG SPECIES OF THE BACILLUS CEREUS GROUP not available, not applicable Applied and Environmental Microbiology, 65, 5436-5442 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.7
KMA 2.6/07	Guttmann, D.M., Ellar, D.J.	2000	PHENOTYPIC AND GENOTYPIC COMPARISONS OF 23 STRAINS FROM THE BACILLUS CEREUS COMPLEX FOR A SELECTION OF KNOWN AND PUTATIVE B. THURINGIENSIS VIRULENCE FACTORS not available, not applicable Ferdinand Enke Verlag Stuttgart, 188, 7-13 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.7

Data point CADDY (ongoing numbering)	Author(s)	Year	Title Owner, Report No. Source (where different from owner) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously submitted Y/N* If Y => old data point
KMA 2.6/08		2003	GENOME SEQUENCE OF BACILLUS CEREUS AND COMPARATIVE ANALYSIS WITH BACILLUS ANTHRACIS not available, not applicable Nature, 423, 87-91 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.7
KMA 2.6/09	Kramer, J.M., Gilbert, R.J.	1989	BACILLUS CEREUS AND OTHER BACILLUS SPECIES not available, not applicable Foodborne Bacterial Pathogens, 21-70 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.7.1
KMA 2.6/10	Anonymous	2005	OPINION OF THE SCIENTIFIC PANEL OF BIOLOGICAL HAZARDS ON BACILLUS CEREUS AND OTHER BACILLUS SPP IN FOODSTUFFS not available, not applicable The EFSA Journal, 175, 1-48 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.7.1
KMA 2.6/11	Rosenquist, H., Smidt, L., Andersen, S.R., Jensen, G.B., Wilcks, A.	2005	OCCURRENCE AND SIGNIFICANCE OF BACILLUS CEREUS AND BACILLUS THURINGIENSIS IN READY-TO-EAT FOOD not available, not applicable FEMS Microbiology Letters, 250, 129-136 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.7.1
KMA 2.6/12	Shinagawa, K., Konuma, H., Kurata, H., Tanabayshi K., Matsusaka, N.	1984	SURVEILLANCE OF RAW MEAT PRODUCTS AND MEAT-PRODUCT ADDITIVES FOR CONTAMINATION WITH BACILLUS CEREUS AND ENTEROTOXIGENICITY OF THE ISOLATED STRAINS not available, not applicable Journal of the Faculty of Agriculture, Iwate University, 17, 175-182 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.7.1

Data point CADDY (ongoing numbering)	Author(s)	Year	Title Owner, Report No. Source (where different from owner) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously submitted Y/N* If Y => old data point
KMA 2.6/13	Okinaka, R.T., Cloud, K., Hampton, O., Hoffmaster, A.R., Hill, K.K., Keim, P., Koehler, T.M., Lamke, G., Kumano, S.K., Mahillon, J., Manter, D., Martinez, Y., Ricke, D., Svensson, R., Jackson, P.J.	1999	SEQUENCE AND ORGANIZATION OF PX01, THE LARGE BACILLUS ANTHRACIS PLASMID HARBORING THE ANTHRAX TOXIN GENES not available, not applicable Journal of Bacteriology, 181, 6509-6515 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.7.2
KMA 2.6/14	Granum, P.E., Lund, T.	1997	BACILLUS CEREUS AND ITS FOOD POISONING TOXINS not available, not applicable FEMS Microbiology Letters, 157, 223-228 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.7.2
KMA 2.6/15	Andersson, A., Granum, P.E., Rönner, U.	1998	THE ADHESION OF BACILLUS CEREUS SPORES TO EPITHELIAL CELLS MIGHT BE AN ADDITIONAL VIRULENCE MECHANISM not available, not applicable Int J Food Microbiology, 39, 93-99 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.7.2
KMA 2.6/16	Beecher, D. J., Wong, A. C. L.	1994	IMPROVED PURIFICATION AND CHARACTERIZATION OF HEMOLYSIN BL, A HEMOLYTIC DERMONECROTIC VASCULAR PERMEABILITY FACTOR FROM BACILLUS CEREUS not available, not applicable Infect Immun, 62, 980-986 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.7.2
KMA 2.6/17	Beecher, D. J., Wong, A. C. L.	1997	TRIPARTITE HEMOLYSIN BL FROM BACILLUS CEREUS. HEMOLYTIC ANALYSIS OF COMPONENT INTERACTION AND MODEL FOR ITS CHARACTERISTIC PARADOXICAL ZONE PHENOMENON not available, not applicable Journal of Biological Chemistry, 272, 233-239 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.7.2

Data point CADDY (ongoing numbering)	Author(s)	Year	Title Owner, Report No. Source (where different from owner) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously submitted Y/N* If Y => old data point
KMA 2.6/18	Beecher, D.J., Schoeni, J.L., Wong, A.C.L.	1995	ENTEROTOXIC ACTIVITY OF HEMOLYSIN BL FROM BACILLUS CEREUS not available, not applicable Infect Immun, 63, 4423-4428 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.7.2
KMA 2.6/19	Heinrichs, J.H., Beecher, D.J., MacMillan, J.D., Zilinskas, B.A.	1993	MOLECULAR CLONING AND CHARACTERIZATION OF THE HBLA GENE ENCODING THE B COMPONENT OF HEMOLYSIN BL FROM BACILLUS CEREUS not available, not applicable Journal of Bacteriology, 175, 6760-6766 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.7.2
KMA 2.6/20	Ryan, P.A., Macmillan, J.D., Zilinskas, B.A.	1997	MOLECULAR CLONING AND CHARACTERIZATION OF THE GENES ENCODING THE L1 AND L2 COMPONENTS OF HEMOLYSIN BL FROM BACILLUS CEREUS not available, not applicable Journal of Bacteriology, 179, 2551-2556 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.7.2
KMA 2.6/21	Beecher, D.J., Pulido J.S., Barney, N.P., Wong, A.C.L.	1995	EXTRACELLULAR VIRULENCE FACTORS IN BACILLUS CEREUS ENDOPHTHALMITIS: METHODS AND IMPLICATION OF INVOLVEMENT OF HEMOLYSIN BL not available, not applicable Infect Immun, 63, 632-639 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.7.2
KMA 2.6/22	Beecher, D. J., Macmillan, J. D.	1990	A NOVEL BICOMPONENT HEMOLYSIN FROM BACILLUS CEREUS not available, not applicable Infect Immun, 58, 2220-2227 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.7.2
KMA 2.6/23	Beecher, D. J., Macmillan, J. D.	1991	CHARACTERIZATION OF THE COMPONENTS OF HEMOLYSIN BL FROM BACILLUS CEREUS not available, not applicable Infect Immun, 59, 1778-1784 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.7.2

Data point CADDY (ongoing numbering)	Author(s)	Year	Title Owner, Report No. Source (where different from owner) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously submitted Y/N* If Y => old data point
KMA 2.6/24	Agata, N., A., Ohta, M, Mori, M., Isobe, M.	1995	A NOVEL DODECADEPSIPEPTIDE, CEREULIDE, IS AN EMETIC TOXIN OF BACILLUS CEREUS not available, not applicable FEMS Microbiology Letters, 129, 17-20 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.7.2
KMA 2.6/25	Lund, T., Granum, P.E.	1997	COMPARISON OF BIOLOGICAL EFFECT OF THE TWO DIFFERENT ENTEROTOXIN COMPLEXES ISOLATED FROM THREE DIFFERENT STRAINS OF BACILLUS CEREUS not available, not applicable Microbiology, 143, 3329-3336 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.7.2
KMA 2.6/26	Hardy, S.P., Lund, T., Granum, P.E.	2001	CYTK TOXIN OF BACILLUS CEREUS FORMS PORES IN PLANAR LIPID BILAYERS AND IS CYTOTOXIC TO INTESTINAL EPITHELIA not available, not applicable FEMS Microbiology Letters, 197, 47-51 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.7.2
KMA 2.6/27	Lund, T., Granum, P.E.	1996	CHARACTERISATION OF A NON-HAEMOLYTIC ENTEROTOXIN COMPLEX FROM BACILLUS CEREUS ISOLATED AFTER A FOODBORNE OUTBREAK not available, not applicable Ferdinand Enke Verlag Stuttgart, 141, 151-156 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.7.2
KMA 2.6/28	Lund, T., De Buyser, M.L., Granum, P.E.	2000	A NEW CYTOTOXIN FROM BACILLUS CEREUS THAT MAY CAUSE NECROTIC ENTERITIS not available, not applicable Mol Microbiol, 38, 254-261 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.7.2
KMA 2.6/29	Granum, P.E.	2001	BACILLUS CEREUS not available, not applicable Food Microbiology, 373-381 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.7.2

Data point CADDY (ongoing numbering)	Author(s)	Year	Title Owner, Report No. Source (where different from owner) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously submitted Y/N* If Y => old data point
KMA 2.6/30	Turnbull, P.C.B.	2005	BACILLUS not available, not applicable Internet, http://gsbs.utmb.edu/microbook/ch015.htm GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.7.2
KMA 2.6/31	Agata, N., Mori, M., Ohta, M., Suwan, S., Ohtani, I., Isobe, M.	1994	A NOVEL DODECADEPSIPEPTIDE, CEREULIDE, ISOLATED FROM BACILLUS CEREUS CAUSES VACUOLE FORMATION IN HEP-2 CELLS not available, not applicable FEMS Microbiology Letters, 121, 31-34 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.7.2
KMA 2.6/32	Mikkola, R., Saris, N.E.L., Grigoriev, P.A., Andersson, M.A., Salkinoja-Salonen, M.S.	1999	IONOPHORETIC PROPERTIES AND MITOCHONDRIAL EFFECTS OF CEREULIDE- THE EMETIC TOXIN OF B. CEREUS not available, not applicable Eur J Biochem, 263, 112-117 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.7.2
KMA 2.6/33	Jackson, S.G., Goodbrand, R.B., Ahmed, R., Kasatiya, S.	1995	BACILLUS CEREUS AND BACILLUS THURINGIENSIS ISOLATED IN A GASTROENTERITIS OUTBREAK INVESTIGATION not available, not applicable Lett Appl Microbiol, 21, 103-105 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.7.2
KMA 2.6/34	Damgaard, P.H., Granum, P.E., Bresciani, J., Torregrossa, M.V., Eilenberg, J., Valentino, L.	1997	CHARACTERIZATION OF BACILLUS THURINGIENSIS ISOLATED FROM INFECTIONS IN BURN WOUNDS not available, not applicable FEMS Immunol Med Microbiol, 18, 47-53 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.7.2
KMA 2.6/35	Siegel, J.P.	2001	THE MAMMALIAN SAFETY OF BACILLUS THURINGIENSIS-BASED INSECTICIDES not available, not applicable Journal of invertebrate Pathology, 77, 13-21 GLP/GEP: no Published: yes **Submitted in: KMA 2.3/12**	no	no	not protected	-	Y KIIM 2.7.2

Data point CADDY (ongoing numbering)	**Author(s)**	**Year**	**Title Owner, Report No. Source (where different from owner) GLP or GEP status Published or not**	**Vertebrate study Y/N**	**Data protection claimed Y/N**	**Justification if data protection is claimed**	**Owner**	**Previously submitted Y/N* If Y => old data point**
KMA 2.6/36	González, J.M., Brown, B.J., Carlton, B.C.	1982	TRANSFER OF BACILLUS THURINGIENSIS PLASMIDS CODING FOR DELTA-ENDOTOXIN AMONG STRAINS OF B. THURINGIENSIS AND B. CEREUS not available, not applicable Proc Natl Acad Sci USA, 79, 6951-6955 GLP/GEP: no Published: yes **Submitted in: KMA 2.1/10**	no	no	not protected	-	Y KIIM 2.7.2
KMA 2.6/37	Hansen, B.M., Damgaard, P.H., Eilenberg, J., Pedersen, J.C.	1998	MOLECULAR AND PHENOTYPIC CHARACTERIZATION OF BACILLUS THURINGIENSIS ISOLATED FROM LEAVES AND INSECTS not available, not applicable Journal of invertebrate Pathology, 71, 106-114 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.7.2
KMA 2.6/38	Valadares de Amorim, G., Whittome, B., Shore, B., Levin, D.B.	2001	IDENTIFICATION OF BACILLUS THURINGIENSIS SUBSP. KURSTAKI STRAIN HD1-LIKE BACTERIA FROM ENVIRONMENTAL AND HUMAN SAMPLES AFTER AERIAL SPRAYING OF VICTORIA, BRITISH COLUMBIA, CANADA, WITH FORAY 48B not available, not applicable Applied and Environmental Microbiology, 67, 1035-1043 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.7.2
KMA 2.6/01 (KMA 2.6/39)	Guinebretiere, M.-H., Thompson, F.L., Sorokin, A., Normand, P., Dawyndt, P., Ehling-Schulz, M., Svensson, B., Sanchis, V., Nguyen-The, C., Heyndrickx, M., De Vos, P.	2008	ECOLOGICAL DIVERSIFICATION IN THE BACILLUS CEREUS GROUP not available, not applicable Environmental Microbiology, 10, 851-865 GLP/GEP: no Published: yes	no	no	not protected	-	N

Data point CADDY (ongoing numbering)	**Author(s)**	**Year**	**Title Owner, Report No. Source (where different from owner) GLP or GEP status Published or not**	**Vertebrate study Y/N**	**Data protection claimed Y/N**	**Justification if data protection is claimed**	**Owner**	**Previously submitted Y/N* If Y => old data point**
KMA 2.6/02 (KMA 2.6/40)	Guinebretiére, M.-H., Velge, P., Couvert, O., Carlin, F., Debuyser, M.-L., Nguyen-The, C.	2010	ABILITY OF BACILLUS CEREUS GROUP STRAINS TO CAUSE FOOD POISONING VARIES ACCORDING TO PHYLOGENETIC AFFILIATION (GROUPS I TO VII) RATHER THAN SPECIES AFFILIATION not available, not applicable Journal of Clinical Microbiology, 48, 3388-3391 GLP/GEP: no Published: yes	no	no	not protected	-	N
KMA 2.6/03 (KMA 2.6/41)	Tourasse, N.J., Helgason, E., Klevan, A., Sylvestre, P., Moya, M., Haustant, M., Okstad, O.A., Fouet, A., Mock, M., Kolsto, A.-B.	2011	EXTENDED AND GLOBAL PHYLOGENETIC VIEW OF THE BACILLUS CEREUS GROUP POPULATION BY COMBINATION OF MLST, AFLP, AND MLEE GENOTYPING DATA not available, not applicable Food Microbiology, 28, 236-244 GLP/GEP: no Published: yes	no	no	not protected	-	N
	STENFORS, LP; MAYR, R; SCHERER, S; GRANUM, PE	2002	(2002). PATHOGENIC POTENTIAL OF FIFTY BACILLUS WEIHENSTEPHANENSIS STRAINS. FEMS MICROBIOLOGY LETTERS. VOLUME 215 (1), 1 SEPTEMBER 2002, 47–51.					
KMA 2.7/01	González, J.M., Brown, B.J., Carlton, B.C.	1982	TRANSFER OF BACILLUS THURINGIENSIS PLASMIDS CODING FOR DELTA-ENDOTOXIN AMONG STRAINS OF B. THURINGIENSIS AND B. CEREUS not available, not applicable Proc Natl Acad Sci USA, 79, 6951-6955 GLP/GEP: no Published: yes **Submitted in: KMA 2.1/10**	no	no	not protected	-	Y KIIM 2.10
KMA 2.7/02	Carlton, B.C.	1993	GENETICS OF BT INSECTICIDAL CRYSTAL PROTEINS AND STRATEGIES FOR THE CONSTRUCTION OF IMPROVED STRAINS not available, not applicable American Chemical Society, 326-334 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.10

Data point CADDY (ongoing numbering)	Author(s)	Year	Title Owner, Report No. Source (where different from owner) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously submitted Y/N* If Y => old data point
KMA 2.7/03	Adang, M.J.	1991	BACILLUS THURINGIENSIS INSECTICIDAL CRYSTAL PROTEINS: GENE STRUCTURE, ACTION AND UTILIZATION not available, not applicable Biotechnology for Biological Control of Pests and Vectors, Publisher: CRC Press, 3-24 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.10
KMA 2.7/04	Thomas, D.J.I., Morgan, J.A.W., Whipps, J.M., Saunders, J.R.	2000	PLASMID TRANSFER BETWEEN THE BACILLUS THURINGIENSIS SUBSPECIES KURSTAKI AND TENEBRIONIS IN LABORATORY CULTURE AND SOIL AND IN LEPIDOPTERAN AND COLEOPTERAN LARVAE not available, not applicable Applied and Environmental Microbiology, 66, 118-124 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.10
KMA 2.7/05	Vilas-Bôas, L.A., Vilas-Bôas, G.F.L.T., Saridakis, H.O., Lemos, M.V.F., Lereclus, D., Arantes O.M.N.	2000	SURVIVAL AND CONJUGATION OF BACILLUS THURINGIENSIS IN A SOIL MICROCOSM not available, not applicable FEMS Microbiol Ecol, 31, 255-259 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.10
KMA 2.7/06	Furlaneto, L., Saridakis, H.O., Arantes, O.M.N.	2000	SURVIVAL AND CONJUGAL TRANSFER BETWEEN BACILLUS THURINGIENSIS STRAINS IN AQUATIC ENVIRONMENT not available, not applicable Brazilian Journal of Microbiology, 31, 233-238 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.10
KMA 2.7/07	Jarrett, P., Stephenson, M.	1990	PLASMID TRANSFER BETWEEN STRAINS OF BACILLUS THURINGIENSIS INFECTING GALLERIA MELLONELLA AND SPODOPTERA LITTORALIS not available, not applicable Applied and Environmental Microbiology, 56, 1608-1614 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.10

Data point CADDY (ongoing numbering)	Author(s)	Year	Title Owner, Report No. Source (where different from owner) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously submitted Y/N* If Y => old data point
KMA 2.7/08	Andrup, L., Smidt, L., Andersen, K., Boe, A.	1998	KINETICS OF CONJUGATIVE TRANSFER: A STUDY OF THE PLASMID PXO16 FROM BACILLUS THURINGIENSIS SUBSP. ISRAELENSIS not available, not applicable Plasmid, 40, 30-43 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.10
KMA 2.7/09	Vilas-Bôas, G.F.L.T., Vilas-Bôas, L.A., Lereclus D., Arantes, O.M.N.	1998	BACILLUS THURINGIENSIS CONJUGATION UNDER ENVIRONMENTAL CONDITIONS not available, not applicable FEMS Microbiol Ecol, 25, 369-374 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.10
KMA 2.7/10	van Elsas, J. D., Govaert, J. M., van Veen, J. A.	1987	TRANSFER OF PLASMID PFT30 BETWEEN BACILLI IN SOIL AS INFLUENCED BY BACTERIAL POPULATION DYNAMICS AND SOIL CONDITIONS not available, not applicable Soil Biol Biochem, 19 (5), 639-647 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.10
KMA 2.7/11	Haack, B.J., Andrews, R.E., Loynachan, T.E.	1996	TN916-MEDIATED GENETIC EXCHANGE IN SOIL not available, not applicable Soil Biol Biochem, 28 (6), 765-771 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.10
KMA 2.7/12	Vilas-Bôas, G., Sanchis, V., Lereclus, D., Lemos, M.V.F., Bourguet, D.	2002	GENETIC DIFFERENTIATION BETWEEN SYMPATRIC POPULATIONS OF BACILLUS CEREUS AND BACILLUS THURINGIENSIS not available, not applicable Applied and Environmental Microbiology, 68, 1414-1424 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.10
KMA 2.7/13	Duncan, K.E., Ferguson, N., Kimura, K., Zhou, X., Istock, C.A.	1994	FINE-SCALE GENETIC AND PHENOTYPIC STRUCTURE IN NATURAL POPULATIONS OF BACILLUS SUBTILIS AND BACILLUS LICHENIFORMIS: IMPLICATIONS FOR BACTERIAL EVOLUTION AND SPECIATION not available, not applicable Evolution, 48, 2002-2025 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.10

Data point CADDY (ongoing numbering)	**Author(s)**	**Year**	**Title Owner, Report No. Source (where different from owner) GLP or GEP status Published or not**	**Vertebrate study Y/N**	**Data protection claimed Y/N**	**Justification if data protection is claimed**	**Owner**	**Previously submitted Y/N* If Y => old data point**
KMA 2.7/14	Helgason, E., Caugant, D.A., Lecadet, M.M., Chen, Y., Mahillon, J., Lövgren, A., Hegna, I., Kvaloy, K., Kolsto, A.B.	1998	GENETIC DIVERSITY OF BACILLUS CEREUS/BACILLUS THURINGIENSIS ISOLATES FROM NATURAL SOURCES not available, not applicable Current Microbiology, 37, 80-87 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.10
KMA 2.7/15	Battisti, L., Green, B.D., Thorne, C.B.	1985	MATING SYSTEM FOR TRANSFER OF PLASMIDS AMONG BACILLUS ANTHRACIS, BACILLUS CEREUS AND BACILLUS THURINGIENSIS not available, not applicable Journal of Bacteriology, 162, 543-550 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.10
KMA 2.7/01 (KMA 2.7/16)	Süß, J.	2016	LITERATURE REVIEW ON BACILLUS THURINGIENSIS SUBSP. AIZAWAI STRAIN GC-91 BIOLOGICAL PROPERTIES Certis USA LLC, 2281385_MA_02_01 GAB Consulting GmbH, Heidelberg, Germany GLP/GEP: no Published: no	no	yes	New data for active ingredient, not previously submitted nor evaluated	CEU	N
KMA 2.7/02 (KMA 2.7/17)	Bizzari, M.F., Bishop, A.H.	2007	THE ECOLOGY OF BACILLUS THURINGIENSIS ON THE PHYLLOPLANE: COLONIZATION FROM SOIL, PLASMID TRANSFER, AND INTERACTION WITH LARVAE OF PIERIS BRASSICAE not available, not applicable Microb Ecol, 56, 133-139 GLP/GEP: no Published: yes	no	no	not protected	-	N
KMA 2.7/03 (KMA 2.7/18)	Yuan, Y.M., Hu, X.M., Liu, H.Z., Hansen, B.M., Yan, J.P., Yuan, Z.M.	2007	KINETICS OF PLASMID TRANSFER AMONG BACILLUS CEREUS GROUP STRAINS WITHIN LEPIDOPTERAN LARVAE not available, not applicable Archives of Microbiology, 187, 425-431 GLP/GEP: no Published: yes	no	no	not protected	-	N

Data point CADDY (ongoing numbering)	**Author(s)**	**Year**	**Title** **Owner, Report No.** **Source (where different from owner)** **GLP or GEP status** **Published or not**	**Vertebrate study Y/N**	**Data protection claimed Y/N**	**Justification if data protection is claimed**	**Owner**	**Previously submitted Y/N*** **If Y => old data point**
KMA 2.7/04 (KMA2.7/19)	Santos, C.A., Vilas-Boas, G.T., Lereclus, D., Suzuki, M.T., Angelo, E.A., Arantes, O.M.N.	2010	CONJUGAL TRANSFER BETWEEN BACILLUS THURINGIENSIS AND BACILLUS CEREUS STRAINS IS NOT DIRECTLY CORRELATED WITH GROWTH OF RECIPIENT STRAINS not available, not applicable Journal of Invertebrate Pathology, 105, 171-175 GLP/GEP: no Published: yes	no	no	not protected	-	N
KMA 2.7/05 (KMA 2.7/20)	Donnarumma, F., Paffetti, D., Stotzky, G., Giannini, R., Vettori, C.	2010	POTENTIAL GENE EXCHANGE BETWEEN BACILLUS THURINGIENSIS SUBSP. KURSTAKI AND BACILLUS SPP. IN SOIL IN SITU not available, not applicable Soil Biology and Biochemistry, 42, 1329-1337 GLP/GEP: no Published: yes	no	no	not protected	-	N
KMA 2.8/01	World Health Organization	1999	ENVIRONMENTAL HEALTH CRITERIA 217 - MICROBIAL PEST CONTROL AGENT BACILLUS THURINGIENSIS not available, not applicable WHO World Health Organization GLP/GEP: no Published: yes **Submitted in: KMA 2.3/11**	no	no	not protected	-	Y KIIM 2.6
KMA 2.8/01 (KMA 2.8/03)	Palma, L., Munoz, D., Berry, C., Murillo, J., Caballero, P.	2014	BACILLUS THURINGIENSIS TOXINS: AN OVERVIEW OF THEIR BIOCIDAL ACTIVITY not available, not applicable Toxins, 6, 3296-3325 GLP/GEP: no Published: yes **Submitted in: KMA 2.2.2/01**	no	no	not protected	-	N
KMA 2.8/02 (KMA 2.8/04)	Onose, J.-I., Imai, T., Hsumura, M., Ueda, M., Ozeki, Y., Hirose, M.	2008	EVALUATION OF SUBCHRONIC TOXICITY OF DIETARY ADMINISTERED CRY1AB PROTEIN FROM BACILLUS THURINGIENSIS VAR KURSTAKI HD-1 IN F344 MALE RATS WITH CHEMICALLY INDUCED GASTROINTESTINAL IMPAIRMENT not available, not applicable Food Chem Toxicol, 46, 2184-2189 GLP/GEP: no Published: yes	no	no	not protected	-	N

Data point CADDY (ongoing numbering)	**Author(s)**	**Year**	**Title Owner, Report No. Source (where different from owner) GLP or GEP status Published or not**	**Vertebrate study Y/N**	**Data protection claimed Y/N**	**Justification if data protection is claimed**	**Owner**	**Previously submitted Y/N* If Y => old data point**
KMA 2.8/03 (KM; 2.8/05)	Shimada, N., Miyamoto, K., Kanda, K., Murata, H.	2006	BACILLUS THURINGIENSIS INSECTICIDAL CRY1AB TOXIN DOES NOT AFFECT THE MEMBRANE INTEGRITY OF THE MAMMALIAN INTESTINAL EPITHELIAL CELLS: AN IN VITRO STUDY not available, not applicable In Vitro Cellular & Developmental Biology - Animal, 42, 45-49 GLP/GEP: no Published: yes	no	no	not protected	-	N
KMA 2.8/04 (KMA 2.8/06)	Obeidat, M., Khyami-Horani, H., Al-Momani, F.	2012	TOXICITY OF BACILLUS THURINGIENSIS BETA-EXOTOXINS AND DELTA-ENDOTOXINS TO DROSOPHILA MELANOGASTER, EPHESTIA KUHNIELLA AND HUMAN ERYTHROCYTES. not available, not applicable African Journal of Biotechnology, 11(46), 10504-10512 GLP/GEP: no Published: yes	no	no	not protected	-	N
KMA 2.8/05 (KMA 2.8/07)	Berlitz, D.S., Giovenardi, M., Fiuza, L.M.	2006	TOXICOLOGY EFFECTS OF DELTA-ENDOTOXINS AND BETA-EXOTOXINS OF BACILLUS THURINGIENSIS IN WISTAR RATS not available, not applicable Neotropical Biology and Conservation, 1(1), pp. 35-38 GLP/GEP: no Published: yes	no	no	not protected	-	N
	Raymond, B. and Federici, B.A.	2017	In defence of *Bacillus thuringiensis*, the safest and most successful microbial insecticide available to humanity—a response to EFSA *FEMS Microbiology Ecology*, 93, 2017, fix084	no	no	not protected	-	RMS
KMA 2.8/06 (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 not available, not applicable Microbial Biotechnology, 25(6), pp. 872-879 GLP/GEP: no Published: yes	no	no	not protected	-	N

Data point CADDY (ongoing numbering)	**Author(s)**	**Year**	**Title** **Owner, Report No.** **Source (where different from owner)** **GLP or GEP status** **Published or not**	**Vertebrate study Y/N**	**Data protection claimed Y/N**	**Justification if data protection is claimed**	**Owner**	**Previously submitted Y/N*** **If Y => old data point**
KMA 2.8/07 (KMA 2.8/09)	Wilcks, A., Hansen, B.M., Hendriksen, N.B., Licht, T.R.	2006a	PERSISTENCE OF BACILLUS THURINGIENSIS BIOINSECTICIDES IN THE GUT OF HUMAN-FLORA-ASSOCIATED RATS not available, not applicable FEMS Immunol Med Microbiol, 48, pp. 410-418 GLP/GEP: no Published: yes	yes	no	not protected	-	N
KMA 2.8/08 (KMA 2.8/10)	Du, C., Nickerson, K.W.	1996	BACILLUS THURINGIENSIS HD-73 SPORES HAVE SURFACE-LOCALIZED CRY1AC TOXIN: PHYSIOLOGICAL AND PATHOGENIC CONSEQUENCES not available, not applicable Applied and Environmental Microbiology, 62, 3722-3726 GLP/GEP: no Published: yes	no	no	not protected	-	N
KMA 2.8/09 (KMA 2.8/11)	Abdoarrahem, M.M., Gammon, K., Dancer, B.N., Berry, C.	2009	GENETIC BASIS FOR ALKALINE ACTIVATION OF GERMINATION IN BACILLUS THURINGIENSIS SUBSP. ISRAELENSIS not available, not applicable Applied and Environmental Microbiology, 75, 6410-6413 GLP/GEP: no Published: yes	no	no	not protected	-	N
KMA 2.8/10 (KMA 2.8/12)	King, P.J.H., Ong, K.H., Sipen, P., Mahadi, N.M.	2012	TOXICITY OF LOCAL MALAYSIAN BACILLUS THURINGIENSIS SUBSPECIES KURSTAKI AGAINST PLUTELLA XYLOSTELLA not available, not applicable African Journal of Biotechnology, 11, 11925 - 11930 GLP/GEP: no Published: yes	no	no	not protected	-	N
KMA 2.8/11 (KMA 2.8/13)	Hansen, B.M., Thorsen, L., Nielsen-LeRoux, C., Wilcks, A., Hendriksen N.B.,	2011	NEW EXPERIMENTAL APPROACHES FOR HUMAN RISK ASSESSMENT OF MICROBIAL PEST CONTROL AGENTS - EXEMPLIFIED BY THE BACTERIUM BACILLUS THURINGIENSIS not available, not applicable Danish EPA Pestcides Research, 136 2011, 1-128 GLP/GEP: no Published: yes	no	no	not protected	-	N

Data point CADDY (ongoing numbering)	**Author(s)**	**Year**	**Title Owner, Report No. Source (where different from owner) GLP or GEP status Published or not**	**Vertebrate study Y/N**	**Data protection claimed Y/N**	**Justification if data protection is claimed**	**Owner**	**Previously submitted Y/N* If Y => old data point**
KMA 2.8/12 (KMA 2.8/14)	Wilcks, A., Hansen, B.M., Hendriksen, N.B., Licht, T.R.	2006b	FATE AND EFFECT OF INGESTED BACILLUS CEREUS SPORES AND VEGETATIVE CELLS IN THE INTESTINAL TRACT OF HUMAN-FLORA-ASSOCIATED RATS not available, not applicable FEMS Immunol Med Microbiol, 46, 70-77 GLP/GEP: no Published: yes	no	no	not protected	-	N
KMA 2.8/13 (KMA 2.8/15)	Ceuppens, S., Uyttendaele, M., Drieskens, K., Heyndrickx, M., Rajkovic, A., Boon, N., Van de Wiele, T.	2012	SURVIVAL AND GERMINATION OF BACILLUS CEREUS SPORES WITHOUT OUTGROWTH OR ENTEROTOXIN PRODUCTION DURING IN VITRO SIMULATION OF GASTROINTESTINAL TRANSIT not available, not applicable Applied and Environmental Microbiology, 78, 7698-7705 GLP/GEP: no Published: yes	no	no	not protected	-	N
KMA 2.8/14 (KMA 2.8/16)	Ceuppens, S., Van de Wiele, T., Rajkovic, A., Ferrer,Cabaceran, T., Heyndrickx, M., Boon, N., Uyttendaele, M.	2012	IMPACT OF INTESTINAL MICROBIOTA AND GASTROINTESTINAL CONDITIONS ON THE IN VITRO SURVIVAL AND GROWTH OF BACILLUS CEREUS not available, not applicable Int J Food Microbiology, 155, 241-246 GLP/GEP: no Published: yes	no	no	not protected	-	N
KMA 2.8/15 (KMA 2.8/17)	Auger, S., Ramarao, N., Faille, C., Fouet, A., Aymerich S., Gohar, M.	2009	BIOFILM FORMATION AND CELL SURFACE PROPERTIES AMONG PATHOGENIC AND NONPATHOGENIC STRAINS OF THE BACILLUS CEREUS GROUP not available, not applicable Applied and Environmental Microbiology, 75, 6616-6618 GLP/GEP: no Published: yes	no	no	not protected	-	N
KMA 2.8/16 (KMA 2.8/18)	Phelps, R.J., McKillip, J.L.	2002	ENTEROTOXIN PRODUCTION IN NATURAL ISOLATES OF BACILLACEAE OUTSIDE THE BACILLUS CEREUS GROUP not available, not applicable Applied and Environmental Microbiology, 68, 3147-3151 GLP/GEP: no Published: yes	no	no	not protected	-	N

Data point CADDY (ongoing numbering)	**Author(s)**	**Year**	**Title Owner, Report No. Source (where different from owner) GLP or GEP status Published or not**	**Vertebrate study Y/N**	**Data protection claimed Y/N**	**Justification if data protection is claimed**	**Owner**	**Previously submitted Y/N* If Y => old data point**
KMA 2.8/17 (KMA 2.8/19)	Damgaard, P.H.	1995	DIARRHOEAL ENTEROTOXIN PRODUCTION BY STRAINS OF BACILLUS THURINGIENSIS ISOLATED FROM COMMERCIAL BACILLUS THURINGIENSIS-BASED INSECTICIDES not available, not applicable FEMS Immunol Med Microbiol, 12, 245-250 GLP/GEP: no Published: yes	no	no	not protected	-	N
KMA 2.9/01	Barbera, P.W.	1993	NCCLS STANDARDIZED DISK SUSCEPTIBILITY TEST OF CGA-237218 TECHNICAL (BACILLUS THURINGIENSIS VAR. STRAIN GC-91) Certis USA LLC, CG-5 IIT Res. Inst. Life Scie.Operation, Chicago 60616-3799, USA GLP: yes Published: no	no	no	not protected	CEU	Y KIIM 2.12
KMA 2.9/01 (KMA 2.9/02)	Cheng, M., Chen, C.-Y.	2016	ANTIBIOTICS SENSITIVITY OF CONDOR STRAIN EG 2348 AND TUREX STRAIN GC-91 Certis USA LLC, not stated Certis U.S.A., L.L.C., Columbia, Maryland GLP/GEP: no Published: no	no	yes	New data for active ingredient, not previously submitted nor evaluated	CEU	N