European Commission

Draft Renewal Assessment Report prepared according to the Commission Regulation (EU) N° 1107/2009

Microbial Pest Control Agent (MPCA) *Bacillus thuringiensis* subsp. *kurstaki* SA-12

Volume 3 B.2 (MPCA)

Biological properties

Rapporteur Member State: Denmark

Co- Rapporteur Member State: The Netherlands

Version history

When	What
2008	DAR
2011	Addendum to the DAR
2019	Initial RAR

Table of contents

B Summary of the data and information

B.2 Biological properties of the micro-organism 4

B.2.1 History of the micro-organism and its uses, natural occurrence and geographical distribution 4
B.2.1.1 Historical background 5
B.2.1.2 Origin and natural occurrence 6
B.2.2 Information on target organism(s) 8
B.2.2.1 Description of target organism(s) 8
B.2.2.2 Mode of action 10
B.2.3 Host specificity range and effects on species other than the target harmful organism 14
B.2.4 Development stages/life cycle of the micro-organism 18
B.2.5 Infectiveness, dispersal and colonisation ability 19
B.2.6 Relationships to known plant or animal or human pathogens 22
B.2.7 Genetic stability and factors affecting it 28
B.2.8 Information on the production of metabolites (especially toxins) 41
B.2.9 Antibiotics and other anti-microbial agents 85
B.2.10 References relied on 92

B.2 Biological properties of the micro-organism

INTRODUCTION

Bacillus thuringiensis subsp. *kurstaki* SA-12 (in the following abbreviated as Btk SA-12) was one of the existing active substances covered by the Regulation (EC) No 2229/2004 on the implementation of the fourth stage of the program of work referred to in Article 8(2) of Council Directive 91/414/EEC. In Annex I to Regulation (EC) No 2229/2004 the Commission designated Denmark as rapporteur Member State to carry out the assessment of Btk SA-12 on the basis of a joint dossier submitted for the Btk strains SA-11, SA-12 and EG 2348. The notifier for Btk SA-11 and SA-12 was Mitsui AgriScience International SA/NV while EG 2348 was notified by Mitsui AgriScience International SA/NV and Intrachem Bio Italia S.p.A. (now CBC (Europe) S.r.l.). In accordance with the provisions of Article 22(1) of Regulation (EC) No 2229/2004, Denmark submitted in January and February 2008 to the EFSA the draft assessment report, including, as required, a recommendation concerning the possible inclusion of Btk SA-12 in Annex I to the Directive. The Commission examined the draft assessment report, the recommendations by the rapporteur Member State and the comments received from other Member States in consultation with experts from a certain number of Member States. The Commission referred on 12 July 2008 a draft review report to the Standing Committee on the Food Chain and Animal Health, for final examination. The draft review report was finalized in the meeting of the Standing Committee on 12 July 2008. Subsequently Regulation (EC) No 1107/2009 repealed and replaced Directive 91/414/EEC and the active substance Btk SA-12, 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. *kurstaki* (strains ABTS-351, PB-54, SA-11, SA-12, EG2348) on the 16 December 2011 (published 23 February 2012). Based on this new information available, no need to change the conditions of approval of Btk SA-12 was identified. The Commission filed on 13 December 2013 an updated review report for Btk strains SA-11, SA-12 and EG 2348 to the Standing Committee on the Food Chain and Animal Health for examination.

The approval of Btk SA-12 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 Btk SA-12 on 30 April 2016. In accordance with Regulation (EU) 2016/183 the notifier submitted to the designated RMS Denmark, the co-RMS The Netherlands as well as to EFSA and Commission a dossier for renewal of Btk SA-12 considering the deadline stated in SANTE-2016-10616–rev. 3.

Btk SA-12 is a wild type strain originating from infested insects. Btk 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 Btk is mainly attributed to spore bound insecticidal pro-proteins (*Cry* toxins) which are ingested by the target pests and activated under alkaline conditions in the midgut of the larvae. The first assessment of the strain proved that it does not have any harmful effects on human or animal health or on groundwater or any unacceptable influence on the environment. The overall conclusion from EFSA (2012) confirms that no critical areas of concern are identified within the framework of the use which was supported.

As the manufacturing process of Btk SA-12 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.

For the renewal of the Btk strains SA-11, SA-12 and EG 2348 under Regulation (EC) 1107/2009, a separate dossier was submitted for each strain only including data, which have previously not been submitted or evaluated. Nevertheless, there is some information which is applicable to all three Btk strains, e.g. published information for Btk in general obtained during searches for peer reviewed literature according to EFSA Guidance (2011)[1] carried out for relevant sections.

In the following for ease of information, full study summaries/sections taken from the DAR (2008) or its Final Addendum (2011) are included if they are considered relevant for renewal of Btk SA-12. In order to facilitate discrimination between new data and data already evaluated during the first approval process, the headline "New Data" begins the section with data, which have previously not been submitted or evaluated. Data and their evaluations from the original DAR and addenda to the DAR are highlighted by grey background.

1 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.2.1 History of the micro-organism and its uses, natural occurrence and geographical distribution

B.2.1.1 Historical background

Probably the first description of *B. thuringiensis* was by Ishiwata, who, in 1901, described the etiological agent of "sotto disease" infecting cultues of silk producing larvae (Ishiwata S, 1901). However, the bacterium was not named until Berliner isolated a spore forming bacterium from *Anagasta* (*Ephestia*) *kuhniella* and named this organism *B. thuringiensis* in 1915.

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) (also refer to point B.2.1.2 below). Most strains used in commercial production of microbial insecticides have been isolated from diseased insects (deLucca et al., 1981). *Bacillus thuringiensis* produces one or more parasporal crystals (δ-endotoxins) which have been found to be primarily toxic for insect species in the orders *Coleoptera*, *Diptera* and *Lepidoptera*, but other targets have been described (Feitelson et al., 1992; de Castilhos-Fortes et al., 2002))

In 1962 Kurstak isolated a variety that was effective primarily against *Lepitoptera* and named it *B. thuringiensis kurstaki*. Another more potent strain of this variety was isolated from diseased mass-reared pink bollworm, *Pectinophora gossypiella* larvae by Dulmage (1970), who named it the HD-1 strain. Research and development of *B. thuringiensis* was strengthened considerably by Dulmage and Beegle of the USDA, who accumulated the first significant collection of strains (in Glare & O'Callaghan, 2000). The HD-1 strain was used as a standard for subsequent Bt studies and is still used in several products today.

The isolation of *B. thuringiensis* from samples is by enrichment, using heat treatment followed by plating a sample either onto a non-selective medium such as nutrient agar (Ohba & Aizawa, 1986) or onto a selective medium such as sodium acetate (Travers et al. 1987). Bacteria identified as *Bacillus cereus* are subsequently investigated by phase-contrast microscopy (Ohba & Aizawa, 1986) or microscopic examination of stained smears (Chilcott & Wigley, 1988). Crystal-forming *B. cereus* are named *B. thuringiensis* per definition.

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

RMS evaluation of section from the DAR 2008	We have no remarks to the information and references referred to in the original DAR of Btk strains SA-11, SA-12 and EG2348. We find the information relevant and still valid for renewal of Btk SA-12.

New information

Bacillus thuringiensis including *Bacillus thuringiensis* subsp. *kurstaki* have been used for decades for control of Lepidopteran pests in agricultural settings. Bt is considered the most successful insect pathogen and presently comprises ~ 2% of the worldwide insecticidal market (Bravo et al. 2011). Besides use of sprayable insecticides, Bt toxins have been successfully introduced in transgenic plants. However, the mode of action of Bt does not only rely on production of insecticidal proteins such as Cry proteins and others (please refer to Point B.2.2.2 below) but also on septicemia in the infected insect larvae. The multiple mode of action of Bt bacteria, compared to transgenic crops which often only produce single insecticidal proteins, renders the risk for resistance development in target insects lower. In the following, all information only refers to the bacterium *B. thuringiensis* subsp. *kurstaki* and not to transgenic plants or information about single insecticidal proteins.

Btk strain SA-12, as all other currently registered Btk strains, was approved in the EU in 2009. Before inclusion into Annex I of Regulation (EC) No 91/414, Btk products have been on the European market for decades. Products based on Btk strain SA-12 are currently registered all over Europe. For more details please refer to Vol 3 MP, B.3.3.

RMS comment	In relation to risk assessment of Btk strain SA-12 it is important to take into account that Btk strains have been used for decades for control of Lepidopteran pests in agricultural settings. CoStar WG has been approved in the EU since the mid 1990s. Products containing *Bacillus thuringiensis* subsp. *kurstaki* SA-12 are widely authorised in European countries. For details please refer to Vol 3 MP, B.3.3.

Cited literature references:

Report:	KMA 2.1.1/15 - Bravo, A., Likitvivatanavong, S., Gill, S.S., Soberon, M. (2011), published report
	Insect Biochemistry and Molecular Biology 41(7):423-431
Title:	*Bacillus thuringiensis*: A story of a successful bioinsecticide
Abstract	*Bacillus thuringiensis* (Bt) bacteria are insect pathogens that rely on insecticidal pore forming proteins, known as Cry and Cyt toxins to kill their insect larval hosts. At least four different non-structurally related families of proteins form the Cry toxin group of toxins. The expression of certain Cry toxins in transgenic crops has contributed to an efficient control of insect pests resulting in a significant reduction in chemical insecticide use. The mode of action of the three domain Cry toxin family involves sequential interaction of these toxins with several insect midgut proteins facilitating the formation of a pre-pore oligomer structure and subsequent membrane insertion that leads to the killing of midgut insect cells by osmotic shock. In this manuscript we review recent progress in understanding the mode of action of this family of proteins in lepidopteran, dipteran and coleopteran insects. Interestingly, similar Cry-binding proteins have been identified in the three insect orders, as cadherin, aminopeptidase-N and alkaline phosphatase suggesting a conserved mode of action. Also, recent data on insect responses to Cry toxin attack is discussed. Finally, we review the different Bt based products, including transgenic crops, that are currently used in agriculture.

Evaluation RMS	The reference is applicable and acceptable.

B.2.1.2 Origin and natural occurrence

Origin of *B. thuringiensis kurstaki* SA-12

The strain SA-12 (HD-119) was originally derived from the insect *Ephestia cantella* and was deposited in the ARS Culture Collection (also known as Northern Regional Research Laboratory (NRRL), at the Microbial Properties Research Unit, National Centre for Agricultural Utilization Research, Agricultural Research Service, U.S. Department of Agriculture Peoria, Illinois 61604 USA. under the reference number NRRL B-30791 by H.D. Burges.

Natural occurrence

Bacillus thuringiensis is indigenous in many environments (Bernhard et al., 1997; Chaufaux et al., 1997; Martin & Travers, 1989). Whereas *Bt* strains have been isolated mainly from environments associated with insect populations or plant material in the past, a large number of surveys have been conducted more recently, where *Bt* bacteria have been isolated from a range of habitats in many different countries (Martin & Travers, 1989; 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).

The subspecies *B. thuringiensis kurstaki* has been found in several regions Worldwide (e.g. Damgaard et al., 1997a; Ohba and Aizawa, 1978; DeLucca et al., 1981; 1982; Dulmage 1970; Martin and Travers 1989).

RMS evaluation of section from the DAR 2008	We have no remarks to the information and references referred to in the original DAR of Btk strains SA-11, SA-12 and EG2348. We find the information relevant and still valid for renewal of Btk SA-12.

New information

Btk as a species occurs naturally in a range of environmental compartments such as soils, plant surfaces and infected insects. Strain SA-12, for example, was isolated from infested insects.

A literature review aiming to define background levels of Btk 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[2]. A summary about recorded background levels reported therein is provided below.

Confirming information already presented during 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 Denmark	1×10^5 CFU/g	Raynond et al. (2010, cited in EFSA Scientific Opinion)
Forest soils (Jordania)	Up to 1.4×10^8 CFU/g	Al-monami and Obeidat, 2011
Agricultural soils France	4.43 - 5.23 log CFU/g	Brillard et al. (2015, cited in EFSA Scientific Opinion)
Danish soils	4×10^4 - 2×10^5 CFU/g, mean 10^5 CFU/g	Hendriksen et al. (2011, cited in EFSA Scientific Opinion)
Rice field	4.23 - 6.52 $\times 10^5$ CFU/g	Chatterjee et al. (2007, cited in EFSA Scientific Opinion
Water		
Rainwater	1.16 - 2.5 log CFU/L	Brillard et al. (2015, cited in EFSA Scientific Opinion)
Groundwater	0.17 - 0.96 log 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 et al. (2011, cited in EFSA Scientific Opinion)

[2] 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

Environmental compartment*	**Density of *B. cereus* group members including Bt**	**Reference**
Cauliflower leaves	80 – 1700 CFU/cm² 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)
Spices, paprika, allspice, peppercorns, and mixed spices	3 to 240 MPN/g	Hariram and Labbé (2015, cited in EFSA Scientific Opinion)

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

RMS evaluation and conclusion	Natural background levels from EFSA Scientific Opinion is provided for members of the *B. cereus* group but is applicable to all Btk strains including strain SA-12. 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.
Endpoint: Origin and natural occurrence, Background level	Btk as a species occurs naturally in a range of environmental compartments such as soils, plant surfaces and infected insects. Strain SA-12 was isolated from infested insects. Background populations of Bt in the environment were usually found in the range from 10^4 to 10^5 CFU/g in soil and 0 – 6 x 10^4 CFU/g on plants in areas not previously treated with Bt.

B.2.2 Information on target organism(s)

B.2.2.1 Description of target organism(s)

The *B. thuringiensis* subsp. *kurstaki* strain SA-11 is currently used as an insecticide against a wide range of lepidopteran pest species. With this dossier the control of the European Grape Vine Moth (*Lobesia botrana*) and the Grape Bud Moth (*Eupoecelis ambiguella*) (both belonging to the insect order Lepidoptera) in grapes is intended. For further information on the European Grape Vine Moth and the Grape Bud Moth please refer to the DAR.

RMS comment of section from the DAR 2008	In the original DAR the representative product of the active substance SA-11 and the target organisms the European Grape Vine Moth (*Lobesia botrana*) and the Grape Bud Moth (*Eupoecelis ambiguella*) were described in details. We don't find the information particular relevant for renewal of Btk SA-12.

New information

Btk strain SA-12 is used against *Tuta absoluta* in tomato, *Spodoptera littoralis* in turf and against *Cydia pomonella* in pome fruits.

Tuta absoluta

English name: tomato leafminer or South American tomato moth

Tuta absoluta is a micro lepidopteron moth of the family gelechiidae with high reproductive potential. There are about 10–12 generations per year. The total live cycle is completed with in 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 laying. 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 is the Filiform antennae (bead like antennae), silverfish-grey scales and characteristic black spots present in anterior wing. The larvae becoming greenish to light pink in second to fourth instars. Larval period is most damaging period which completed with in 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%.

Tuta absoluta is a devastating pest of Tomato. This pest is crossing borders and devastating tomato production in both protected and open fields. The infestation of *Tuta absoluta* is also reported on potato, eggplant and common beans.

Spodoptera littoralis

English name: African Cotton Leafworm or Egyptian Cotton Leafworm — also known as the Mediterranean Brocade in the UK

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 2000 - 3000 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.

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. 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.

RMS comment	*B. thuringiensis* subsp. *kurstaki* strain SA-12 is currently used as an insecticide against a wide range of lepidopteran pest species. With this dossier Btk strain SA-12 is used against *Tuta absoluta* in tomato, *Spodoptera littoralis* in turf and against *Cydia pomonella* in pome fruits.
Endpoint: Target organism(s)	Lepidopteran pests (GAP: *Tuta absoluta, Cydia pomonella*, *Spodoptera littoralis*)

B.2.2.2 Mode of action

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* vary 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) according to the old Cry toxin nomenclature. This information given by the notifier is according to the old Cry toxin nomenclature. The new Cry nomenclature is avaiable at http://www.lifesci.susx.ac.uk/home/Neil_Crickmore/Bt/, where also a table with old and new names is available.

During the stationary phase of its growth cycle, *B. thuringiensis* forms parasporal crystalline inclusions. The crystal proteins of *B. thuringiensis* must be ingested to be effective against the target insect (in Schnepf et al., 1998). Upon ingestion of *B. thuringiensis* by the larvae, the crystalline inclusions dissolve in the larval midgut, releasing insecticidal crystal proteins (so called δ-endotoxins) of 27 to 140 kDa. Most of the crystal proteins are protoxins, converted proteolytically into smaller toxic polypeptides under the alkaline conditions in the lepidopteran 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 that is common to all insecticidal crystal proteins (ICPs) (Schwartz et al., 1991; Schnepf et al., 1998). Spore germination and proliferation of the vegetative cell into the haemocoel may result in septicaemia, contributing to mortality of the insect larvae. The septicaemia might be the main mortality factor and other bacteria, including enteric bacteria, might be involved (Broderick et al., 2006).

In **Table 2.2.2-1** the different Cry proteins expressed by the strains SA-11, SA-12 and EG2348 are shown. The strains SA-11 and SA-12 contain the genes for the expression of the same crystal proteins but the amount of proteins expressed by each strain is different. That is the reason why SA-11 is more effective against *Spodoptora exigua* and SA-12 is more effective against *Heliothis virescens*. The strain EG2348 shows a different Cry protein pattern and is also more effective against *H. virescens*. For details on the quantitative composition of Cry proteins in the different strains refer to confidential information in Annex C.

Table 2.2.2-1 Crystal proteins of the *Bacillus thuringiensis* ssp. *kurstaki* strains*

Strain	SA-11	SA-12	EG2348
Cry Proteins	Cry1Aa	Cry1Aa	Cry1Aa
	Cry1Ab	Cry1Ab	-
	Cry1Ac	Cry1Ac	Cry1Ac
	Cry2Aa	Cry2Aa	Cry2Aa

* Yamamoto & Chen, 2006

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 in 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 (Rausell 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.

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 a specific test insect (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.

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 nonexistent (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).

RMS evaluation of section from the DAR 2008	The mode of action of Btk has been described in detail for the first approval of strain SA-12. Description of mode of action is primarily presented at a general species level and has not been described for the specific isolates. However, as mode of action is expected to be similar for cry proteins with specific activity, this is regarded as acceptable. We have no other remarks to the information and references referred to in the original DAR of Btk strains SA-11, SA-12 and EG2348. We consider the information relevant and still valid for renewal of Btk SA-12.

New information

The insecticidal activity of Btk 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 2.2.2-2**. 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.

Table 2.2.2-2 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 sepicaemia 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 *Blattella germanica*
Beta Exotoxin	Inhibition of DNA-dependent RNA polymerase	Unspecific toxicity against wide range of insects as well as mammals
Enhancin-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

RMS comment	The insecticidal activity of Btk is mainly attributed to spore bound insecticidal protoxins (Cry toxins) which are ingested by the target pests (Lepidopteran larvae) and activated under alkaline conditions in the midgut of the larvae. Apart from the Cry toxins, several other insecticidal proteins produced by Bt contribute to their mode of action
Endpoint: Mode of action	The insecticidal activity of Btk is mainly attributed to spore bound insecticidal protoxins (Cry toxins) which are ingested by the target pests (Lepidopteran larvae) and activated under alkaline conditions in the midgut of the larvae.

Cited literature abstracts:

Report: KMA 2.2.2/08 - 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.

Evaluation RMS	Evaluated in the DAR 2008, No new remarks

Report:	KMA 2.2.2/19 - 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.

Evaluation RMS	The reference is applicable and acceptable.

Report:	KMA 2.2.2/20 - 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.

Evaluation RMS	The reference is applicable and acceptable.

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

Host Specificity

Most *B. thuringiensis* strains, especially the subspecies *Bt kurstaki*, are active against *Lepidoptera.* Some strains however are toxic against dipteran and coleopteran species. Not only different strains, but also different crystal proteins occurring within 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 and 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 appear 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).

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 order of the target organism. *B. thuringiensis* ssp. *kurstaki*, for instance produce Cry 1 and Cry 2 proteins which are effective specifically against lepidopteran and dipteran species.

The three strains contain genes for the different polypeptides: Cry1Aa, Cry1Ab, Cry1Ac and Cry2A, resulting in a main efficacy against insects of the *Lepidopteran* order. A study with four different orders of insects, which includes targets and susceptible non-targets, carried out with the strains SA-11 and SA-12 also confirms the high specificity against the lepidopteran order, although up to 10% mortality to another insect order was observed (Cerf, 1990). See table B.2.1.3-1 below (from Cerf, 1990).

Owing to their specific mode of action, *B. thuringiensis* products are unlikely to pose any hazard to the great majority of non-target invertebrates provided that they are free from microbial impurities and biologically active products other than the insecticidal crystal proteins (ICPs). Regular quality control testing during production ensures products are free from such impurities.

The susceptibility of different orders of insects to the ICPs of *B. thuringiensis* ssp. *kurstaki* strain SA-11 and SA-12 is described in the report below. For further information about effects on non-target organisms, please refer to B.9.

Report : Cerf (1990): Susceptibility of four Orders of insects (Lepidoptera, Diptera, Coleoptera and Othroptera) to Technical Grade Active Ingredients (TGAI's), manufacturing products (MP's) and end-use products (EP's) produced from fermentation of *Bacillus thuringiensis* subspecies *kurstaki* (strains Int-15-313, SA110001C98-1-1 and SA-12), *israelensis* (strains SA-3 and SA3A), *aizawai* (strain SA-2) and *tenebrionis* (strain SA-10), Unpublished Report No. 90/03/12, 1990.

Guideline: EPA 151A-10, similar to 40 CFR, Sect. 160, Fed. Reg., August 17, 1989

GLP: Yes

Materials and Methods:

The study was conducted during the period 19.01.1990 – 12.03.1990, by Biological Development, Sandoz Crop Protection Corporation, Palo Alto, CA USA. The test organisms used were different strains and products of *B. thuringiensis kurstaki*, *aizawai, israelensis and tenebrionis*. The TGAI, MP and EP materials were bio assayed against four orders of Insects. The assays were conducted on *Manduca sexta* (order Lepidoptera) at 10 ppm in artificial diet; on *Leptinotarsa texana* (order Coleoptera) at 10,000 ppm on eggplant var. Black Beauty leaves; at 1ppm in tap water on *Aedes aegypti* (order Diptera) and at 10,000 ppm in cockroach diet on *Blattella germanica* (order Orthoptera).

Findings:

The strains SA-11 and SA-12 demonstrated negligible activity on insect representatives of the orders *Coleoptera*, *Diptera* and *Orthoptera* and significant activity on an insect representative of the order *Lepidoptera.* See table 2.1.3-1.

Conclusion: The insecticidal activity of the *B. thuringiensis kurstaki* strains SA-11 and SA-12 is highly specific to insect species of the lepidopteran order.

Table 2.1.3-1 Activity of the Technical Grade Active Ingredient, Manufacturing Product and End Product of the *B. thuringiensis* strains SA-11 and SA-12

Strain / Material	% Mortality[a]			
	Lepidoptera (*Manduca sexta*)	**Coleoptera (*Leptinotarsa texana*)**	**Diptera (*Aedes aegypti*)**	**Orthoptera (*Blattella germanica*)**
SA-11 / TGAI* (SA11001C98-1-1)	100	10	0	3.5
SA- 12 / TGAI*	97.4	10	10	0
SA-11 / MP** (SA11001C98-1-1	100	5	7.5	0
SA- 12 / EP***	100	17.5	0	0

TGAI*- technical grade active ingredient

MP**- manufacturing product

EP***- end product

[a] % Mortality of sample corrected for control mortality by Abbott's formula

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, EHC 217, 1999; reviewed in Siegel, 2001). *Bacillus thuringiensis* subsp. *kurstaki* Strain SA-11, SA-12 and EG2348 are not expected to be hazardous to mammals, as any adverse effects reported only occurred in exceptional cases.

General studies on humans and animals have shown that *B. thuringiensis* and the agricultural use of *B. thuringiensis* products have long safety record without known significant adverse effects. In the medical literature there is no case report associating commercially used *B. thuringiensis* directly with food poisoning (Siegel, 2001). However, analysis for the presence of crystalline δ-endotoxins is not normal practise in the medical diagnosis of *B. cereus* gastro-intestinal and somatic diseases. It can not be ruled out that in some cases *B. thuringiensis* may be the causative agent in gastrointestinal and somatic diseases, however, this is unknown and would be very difficult to investigate as cases of *B. cereus* caused diarhoea normally only have a duration of 1-2 days.

For detailed information about the toxicology of the Btk strain, see B.6

During the production process *B. thuringiensis* strains are harvested at the end of their 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 in the product. 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, animals and environment. For detailed information about the production process please refer to Annex C.

RMS evaluation of section from the DAR 2008	We have no remarks to the information and references referred to in the original DAR of Btk strains SA-11, SA-12 and EG2348. We find the information relevant and still valid for renewal of Btk SA-12.
RMS comment	It is generally agreed that Btk acts highly specific against members of the insect order of Lepidoptera. Some are also active against Diptera or Coleoptera. The activity spectrum of a certain strain is defined by the production of *cry* toxins, which are

	Cry1Aa, Cry1Ab, Cry1Ac and Cry2Aa for SA-12. Submitted data demonstrated high specificity against Lepidopteran species while no or very limited activity is exhibited against members of the Colepotera, Diptera and Orthoptera. During first evaluation, based on available studies and information on Btk in general, it was concluded that strain SA-12: - is not toxic, pathogenic or infective for humans or mammals and only few human health problems related to Btk have been recorded - does not cause any adverse effects on the environment - does not exhibit toxicity or pathogenicity in any other non-target organism including birds, fish, daphnids, algae, aquatic plants, terrestrial plants, bees and other non-target arthropods, earthworms and soil microbial communities.

New information

Effects on human health

A literature search according to EFSA (2011)[3] aiming to retrieve references on possible toxic or pathogenic effects of Btk in humans was carried out but did not reveal any references which could potentially alter the risk assessment for Btk SA-12 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. References considered relevant and reliable are summarised under the respective data points in Vol 3 MA B.6. Available strain-specific studies confirm the absence of toxicity and pathogenicity of SA-12 in test animals.

Already during the 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 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] was conducted in May 2016 covering the last 10 years. The search strategy aimed to find all recent (from 2006 onwards) references that are of ecotoxicological relevance, regarding possible effects of Btk on non-target organisms. In total, 10 references were considered relevant and reliable and are summarised under the respective data points in Vol 3 MA, B.9. There were reports testing effects of Btk on daphnids (1), bees or bumblebees (2) and non-target arthropods (6), none of them indicating any unacceptable risk. For more details please refer to Schöbinger (2016, Vol 3 MA, B.9.8). Available strain-specific studies confirm the absence of toxicity and pathogenicity of Btk SA-12 in the test animals.

Taken together it can be concluded that SA-12 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 Btk 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 Btk 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 Btk treatment. Exposure in off-field habitats is low and can only occur due to spray drift.

RMS comment	During first evaluation, based on available studies and information on Btk in general, it was concluded that strain SA-12: - is not toxic, pathogenic or infective for humans or mammals and only few and not

[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

	confirmed human health problems related to Btk have been recorded - does not cause any adverse effects on the environment - does not exhibit toxicity or pathogenicity in any other non-target organism including birds, fish, daphnids, algae, aquatic plants, terrestrial plants, bees and other non-target arthropods, earthworms and soil microbial communities.
Endpoint: Host specificity	It is generally agreed that Btk acts highly specific against members of the insect family of Lepidoptera. Some are also active against Diptera or Coleoptera. The activity spectrum of a certain strain is defined by the production of *cry* toxins. Btk SA-12 was shown to be active against Lepidopteran species only.

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

The data below is presented at species level and is not specific for the three *Bt. kurstaki* strains SA-11, SA-12 and EG2348. RMS regards this as acceptable, since we have no data indicating significant variations among individual strains.

Bacillus thuringiensis is a ubiquitous micro-organism that colonizes a range of habitats and environments and can be found in two different stages: as vegetative cells or spores.

Under favourable conditions regarding moisture, temperature and nutrients, the basic metabolizing cell type is the vegetative cell that is actively growing and proliferating. 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.
- 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.

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, which are highly toxic to a wide variety of important agricultural and health related insect pests as well as other invertebrates (Bravo, 1997).

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). In contrast to the widely investigated crystal proteins (Schnepf et al., 1998), 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 an 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. This could not be explained by the crystalline *B. thuringiensis* δ-endotoxins (Estruch et al., 1996).

New data

The information provided previously is considered acceptable to cover current requirements. Therefore, no new data are submitted for renewal of Btk SA-12 under Regulation (EC) 1107/2009.

RMS evaluation of section from the DAR 2008	We have no remarks to the information and references referred to in the original DAR of Btk strains SA-11, SA-12 and EG2348. We find the information relevant and still valid for renewal of Btk SA-12.
Endpoint: Life cycle	*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 proliferating. 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. The transformation of dormant spores into vegetative cells can be described in three stages: (i) Activation: a reversible process that prepares the spore for germination and usually results from treatments like heating or exposure to certain chemical stimuli; (ii) 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; (iii) Outgrowth: development into a vegetative cell by remerging new components from the spore coat.

B.2.5 Infectiveness, dispersal and colonisation ability

Unless specified the data in this section are presented at species level and not for the three specific strains SA-11, SA-12 and EG2348. RMS regards this as acceptable, since we have no data indicating significant variations among individual strains.

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 epiziootics (Aronson, 1993) and the transmission from diseased to healthy insects has been shown to be poor or nonexistent (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). However, long persistence in the soil environment of a *B. thuringiensis kurstaki* strain has been reported (Hendriksen & Hansen, 2002). These authors also reported the presence of vegetative *B. thuringiensis* subsp. *kurstaki* cells in the gut of earthworms and in the rhizosphere of certain plant species.

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

Distribution by humans on a large scale (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 nonanthropogenic transport by water, wind and migrating animals.

Molecular parasitism/ Symbiosis.

Several environmental factors can influence the viability and activity of the endospores and the crystal proteins of *B. thuringiensis* species.

There are some other environmental biotic and abiotic effects that can influence the viability and activity of the endospores and the crystal proteins of *B. thuringiensis* species.

Soil

Most of all the growth and the survival of *B. thuringiensis* in natural soils is affected by the nutrient availability and the presence of indigenous micro organisms (West et al. 1985). Additionally, soil pH and moisture play another important role in the population dynamics.

West et al. (1985) investigated the relative importance of pH, moisture, nutrient availability and indigenous micro organisms on survival of *B. thuringiensis* spores in soil. Populations in autoclaved soil increased rapidly in all treatments within 5 days, indicating that the cells can grow vegetatively, when easy accesible nutrients are available and no competing microorganisms are present. The addition of nutrients shows increasing populations of *B. thuringiensis* in natural as well as in autoclaved soil within the first 10 days and 18 hours, respectively, whereas the populations in the autoclaved soil increased much higher from log 4 to log 6-7.

Indicated by the data of the nutrient-supplemented soils, the growth of *B. thuringiensis* in natural soils is limited by the poor availability of nutrients. Though they are able to compete successfully for added nutrients with present indigenous bacteria (Saleh et al., 1970), the absence of suitable nutrients upon inoculation cause a lack of or reduced growth of *B. thuringiensis*. Spore germination has never been demonstrated in non-amended soil (Vilas-Bôas et al., 2000). West & Burges (1985) showed that amending soil with different organic fractions influenced growth and survival. Dried grass-supplemented soil resulted in 22 x increase in *B. thuringiensis* cells in soil over 64 days, while chicken manure reduced viability to 0.22 x the original level.

The fate of *Bt* in soil is likely dependent on microbial competition. The abundance of *Bt* rapidly diminishes in unsterilized soils but may increase in sterilized soils (West et al., 1985). When soil was treated at 10^5 cells per gram, *Bt* persisted at 10^3 cells per gram for 12-16 months. However, the proportion of *Bt* compared to other soil bacilli was reduced from 20-40% to about 10% indicating that Bt is not well-adapted to soil environments.

Pruett et al. (1980) have shown that, although 38% of the endospores remained viable in soil after 63 days, only 3% of the insecticidal activity remained. After 135 days, there were 23% of the original spores and no insecticidal activity. Other authors also showed a rapid decline in spore viability during the first few weeks after application (Petras & Casida, 1985; Pedersen et al., 1995).

These studies demonstrated that growth and survival of *B. thuringiensis* in natural soils is possible, although slow and often only under conditions favouring growth (reviewed by Addison, 1993). On the other hand, there are studies determining that *B. thuringiensis* cannot survive or grow well under most soil conditions (West et al., 1984a; 1984b; 1985; Akiba, 1986). Petras & Casida (1985) concluded that the high survival of *B. thuringiensis* spores after the first two weeks in soil seemed to be a result of their inability to germinate in soil. There is no evidence for the hypothesis that rapid germination ability of spores in soil conferred a survival advantage.

In consequence, the results of all these studies lead to the conclusion that the survival of *B. thuringiensis* in the soil is a dynamic process involving germination, cell division and sporulation in specific habitats and under changing conditions regarding soil type, crop type, micro flora, nutrients and fertilization (Hendriksen & Hansen, 2002).

Furhter more factors as temperature, pH, moisture and soiltype affects the survival of spores in soil.

Foliage

Insecticidal *B. thuringiensis* is considered to be highly sensitive to UV radiation, whereas *Bacillus* spores are considerably more resistant to UV light than are the vegetative cells (Donnellan & Stafford, 1968). Pusztai et al. (1991) confirmed that sunlight-mediated inactivation is believed to be caused by UV damage to the spores and their δ-endotoxins.

There are several factors influencing the UV sensitivity of *Bacillus* species. The presence of several small acid soluble proteins (SASP), which are responsible for the conformation of the DNA, strengthen the UV resistance (Mason & Setlow, 1986; Setlow, 1988) as well as dipicolinic acid (DPA) deposited in the spore (Germaine & Murrell, 1973).

Ignoffo (1992) summarized data for the reduction of spore viability and ICP activity on leaves of various plants in sunlight: Bt spore viability was reduced by 80% in one day on red cedar leaves and 8% on soy bean leaves, while the ICP activity declined by 20% on red cedar leaves but 65% on soy bean leaves. Dent (1993) reported that Bt formulations on foliage frequently have half-lives of up to 10 days. However, unformulated Bt may have a half-life of only a few hours. Pedersen et al. (1995) found that the initial spore half-life was 16 h during the first week after spraying cabbage with unformulated Btk. For further information regarding half-life please refer to Annex B7.

There is also evidence that plant chemicals can inactivate Bt or influence infectivity. Lüthy (1986) demonstrated that extracts prepared from cotton leaves could inactivate ICPs.

Commercially applied *Bt* may persist at low levels for considerable periods of time. Reardon & Haissig (1983) reported that Btk was still present on balsam fir (*Abies balsamea*) one year after applications to control spruce budworm. The proliferation of spores through infection of susceptible insects should not be discounted as a source of low levels of Bt in treated areas. Several studies have demonstrated that *Bt* is able to grow and sporulate in insect cadavers (Pedersen et al., 1995). From dead Egyptian cotton leafworm (*Spodoptera littoralis*), Jarrett & Stephenson (1990) isolated between 5.0×10^5 and 9.2×10^7 spores per larva. *Bt* may be lost to the soil by overspray during application or by the action of rain on plant surfaces. Further losses arise from *in situ* degradation by environmental factors, such as ultraviolet (UV) radiation and microbial activity (Griego & Spence, 1978, submitted in Point IIM 2.3.2; Sorenson & Falcon, 1980; Beegle et al., 1981; West et al., 1984a, b). Pedersen et al. (1995) found that *Bt* was dispersed by rain splash from the soil to the lower leaves of cabbage (reviewed in WHO, EHC 217, 1999).

Regarding all the different environmental factors affecting the survival, growth and infectivity of *B. thuringiensis* it has to be stated that it is the combined action of multiple environmental factors (e.g. sunlight, leaf temperature and vapour pressure deficit) rather than the action of a single factor that contributes significantly to *B. thuringiensis* endospore decay, especially during the early (0-48 h) exposures (Leong et al., 1980).

Aquatic environment

Furlaneto et al. (2000) showed that cells and spores of *B. thuringiensis* can survive for 10 days in water, without altering their number. The sporulation process began 12-15 hours after inoculation in water.

Menon & De Mestral. (1985) has in laboratory studies indicate that spores of *B. thuringiensis kurstaki* can survive for relatively long period in fresh water and marine environment. It was shown that *B. thuringiensis kurstaki* can survive in fresh water and sea water for more than 70 and 40 days, respectively at 20°C. A higher percentage of *B. thuringiensis kurstaki* was found surviving in lake water than in tap and distilled water for an extended period. This is most probably attributable to the presence of higher concentrations of available nutrients in the lake water which may enhance the growth of bacteria. Whereas seawater is generally considered to be bactericidal to non-marine bacteria, the growth of *B. thuringiensis* is favoured by more available nutrients in the lake water. *B. thuringiensis* has not been isolated from any drinking water supplies (Menon & deMestral, 1985).

B. thuringiensis kurstaki has been recovered from rivers and public water distribution systems after an aerial application of Thuricide 16B (Btk strain SA-12). Standard water treatment processes are not adequate to destroy *B. thuringiensis kurstaki* spores (Menon & deMestral, 1985). However, *Bti* spores and crystals have been shown to bind readily to sediments in the water column (Menon & deMestral, 1985; Ohana et al., 1987) which reduces their efficacy by making them inaccessible to mosquito and blackfly larvae. *B. thuringiensis* spores may persist for at least 22 days in sediments and the spores may be mobilized with such sediments during floods (Ohana et al., 1987).

RMS evaluation of section from the DAR 2008	We have no remarks to the information and references referred to in the original DAR of Btk strains SA-11, SA-12 and EG2348. We find the information relevant and still valid for renewal of Btk SA-12. The information submitted previously is still considered adequate to cover current requirements in particular for general information for Btk and the subspecies *kurstaki*.

New information

A literature review aiming to assess the fate of Btk 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. On foliage, 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 carried out according to EFSA guidance[5] and presented in Vol 3 MA, B.8.3 (Cornelese, 2016b).

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 (Vol. 3 MA, B.8). 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, rainfall and plant growth (dilution effects), lack of nutrients and low humidity.

RMS evaluation	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. Available information on Btk in general and strain specific data confirm that non-target organisms or humans are not infected by Btk SA-12.
Endpoint: Infectivity, dispersal and	Spores are the form of Bt that assures survival. They can survive in soil for months and it was shown that cells and spores of Bt can also survive for 10 days

[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

colonisation ability:	in water, without altering their number. Neither cells nor spores of Bt are mobile, so their dispersal is limited. It is generally agreed that Bt is a poor competitor and does not germinate and grow extensively in the environment. Except for target insects, Btk SA-12 is not expected to colonize any non-target organism and is not infective in humans.

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

General

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., 2000a). *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, pXO1 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 lack parasporal inclusions as well as *B. anthracis* strains. *B. anthracis* is additionally distinguishable from *B. thuringiensis* due to its sensitivity to ampicillin, non-mobility and its requirement of thiamine for growth.

DNA from over 300 strains of *Bacillus thuringiensis*, *Bacillus cereus* and *Bacillus anthracis* have been analysed by amplified fragment length polymorphism (AFLP). This technique produces a visual fingerprint of the DNA and the data obtained have been used to construct a phylogenetic tree which shows that the strains could be placed into 3 clusters, each of which contained 3 or 4 branches (Hill et al, 2004). The DNA of *Bacillus thuringiensis* subsp. *kurstaki* strain SA-11, SA-12 and EG2348 have been analysed by the same technique and the results placed them into Cluster 1 Branch C (Chen, 2005a**).** This is a phylogenetic grouping consisting of many *Bacillus thuringiensis* strains that are not toxigenic to vertebrates. It is distinctly separate from pathogenic and toxigenic *Bacillus* isolates which are included in a different cluster. The analysis also showed that *Bacillus thuringiensis* is only distantly related to *Bacillus anthracis* and there is consequently no likelihood that *Bacillus thuringiensis* will produce toxins similar to those which cause anthrax (Hill et al, 2004).

The conclusion that pathogenic strains present their genotypes in one cluster has been reached by various other researchers (Helgason et al., 2000b). Pathogenic strains, including *B. anthracis*, have been shown to be more closely related to one another than to environmental isolates (Ticknor et al., 2001). All emetic strains, as well as *B. anthracis*, seem to represent a single clone. Nakamura (1994) considered the levels of DNA relatedness between *B. cereus* and *B. thuringiensis* strains to range between 65 and 70%. This result suggests that many of the *B. thuringiensis* serovars are genetically distinct from *B. cereus* but closely related.

Plant pathogenicity

Bacillus thuringiensis subsp. *kurstaki*, strain SA-11, SA-12 and EG2348 is toxic specifically to insects of the *Lepidopteran* order and no effects on aquatic plants from applications of Btk in insecticidal formulations targeted specifically at these insects is expected or envisaged. Furthermore, the lack of reports of negative effects on plants from numerous studies on the persistence and fate of Btk on plants, as well as lack of reported negative effects from decades of use in agricultural and forestry environments indicates that no adverse effect occurs on plants. However, the notifier has submitted two studies on the effects of Btk on seedling emergence and vegetative vigour. These are evaluated in Vol 3 MP, B.9.7.

Animal and human pathogenicity

Bacillus anthracis

Anthrax is primarily a disease of herbivore 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 it is 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 the following three forms, depending on the route of infection.

1. Cutaneous anthrax,

which accounts for more than 95 % of cases worldwide, results from infections through skin lesions. About 20% of untreated cases of cutaneous anthrax will result in death. Deaths are rare with appropriate antimicrobial therapy.

2. Intestinal anthrax

results from ingestion of spores, usually in infected meat and is characterized by an acute inflammation of the intestinal tract. Intestinal anthrax results in death in 25% to 60% of cases.

3. Pulmonary anthrax

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.

Bacillus cereus

B. cereus is frequently isolated as a contaminant of various foods and can occasionally be an opportunistic human pathogen (Drobniewski, 1993; Helgason et al., 2000b, all submitted in Point IIM 2.7). The consumption of foods that contain more than 10^5 CFU *B. cereus* per gram can result in food poisoning (Kramer & Gilbert, 1989; EFSA, 2005). However, in some outbreaks, lower numbers in the food (3–4 log per g) were reported (EFSA, 2005) and discussed by 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 or more to 6 log per g).

B. cereus causes two types of food borne intoxications, diarrhoea and emesis, which are described below. 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 preparation and consumption of foodstuffs (EFSA, 2005).

Diarrhoea

It 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)

This 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, three endotoxins produced by *B. cereus* have been described: haemolysin (HBL), non-haemolysin (NHE) and cytotoxin-K.

Emesis

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 and 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.

The emetic toxin or vomiting factor is a highly stable peptide of less than 10kDa, 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. 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. It has been suggested that it may be a breakdown product from food stuffs supporting the growth of *B. cereus* (Turnbull, 2005), but now it is known that it is produced by a non-ribosomal protein synthetase, which is plasmid encoded (Hoton et al., 2005; Ehling-Schulz et al., 2005). Mikkola et al. (1999) demonstrated that cereulides induce the formation of K+ channels, disturbing the osmotic balance and permeability of the membrane.

Bacillus thuringiensis

Occasionally *B. thuringiensis* strains are involved 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 can vary from strain to strain. However, there is no case report in medical literature associating *B. thuringiensis* with food poisoning (Siegel, 2001).

What distinguish 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, and *B. thuringiensis* will be indistinguishable to *B. cereus* by genetic methods. The reverse process is also possible; i.e. a *B. cereus* gaining a *B. thuringiensis* plasmid and thus becoming a *B. thuringiensis* (González et al., 1982).

There are several methods to verify microorganisms on strain level. Molecular determination of *B. thuringiensis* ssp. *kurstaki* strain SA-11, SA-12 and EG2348 can be done by a combination of three techniques: 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, the three *B. thuringiensis* ssp. *kurstaki* strains might be distinguished from each other and from other *B. thuringiensis* subspecies, as well as from *B. cereus* strains.

RMS evaluation of section from the DAR 2008	The information and references referred to in the original DAR of Btk strains SA-11, SA-12 and EG2348 are only partly valid for renewal of Btk SA-12 because the knowledge about phylogenetic relationships within the *B. cereus* group evolved considerably during the last 10 years. Twenty years ago identification relied mostly on morphological and biochemical methods and loss of the plasmid makes both of *B. anthracis* and *B. thuringiensis* indistinguishable to *B. cereus.* Today there is 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.

New data

As a member of the *B. cereus*-group, Btk 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 Btk SA-12. For more details, please refer to Point B.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. weihenstephanensis, B. mycoides, B. pseudomycoides and have not yet been described to have the potential to cause foodborne diseases. *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.

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 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).

[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

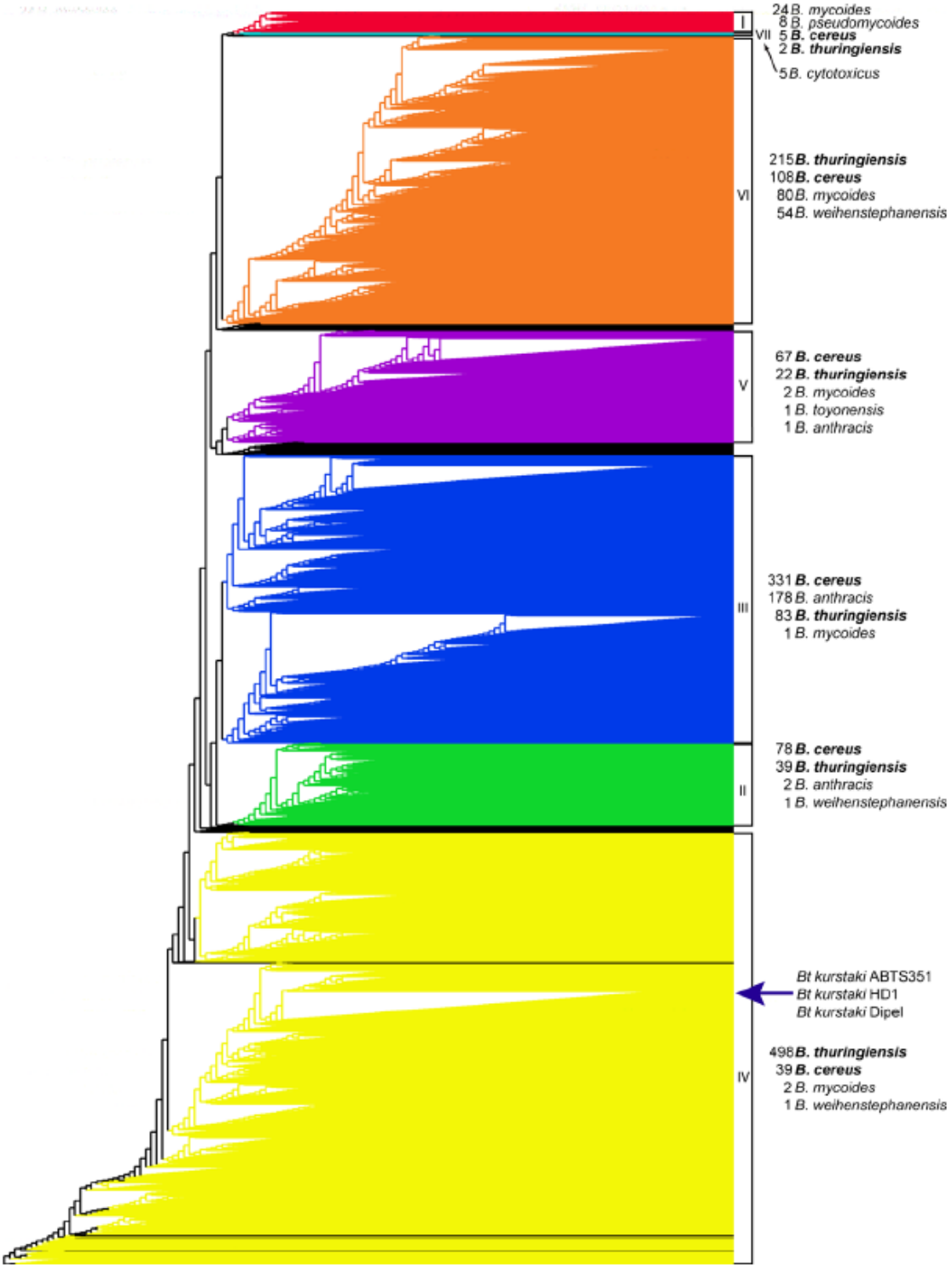

Figure 2.6-1 Phylogenetic relationship between 3193 *B. cereus* group isolates (http://mlstoslo.uio.no/) based on MLST and AFLP typing data. Strains from each group defined in Guinebretiere et al. (2008) are given different colours. Those outside any group are in black. Picture obtained from EFSA Scientific 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]).

RMS evaluation	The knowledge about phylogenetic relationships within the *B. cereus* group evolved considerably during the last 10 years. All strains of the *B. cereus* group should be considered a single species with the currently defined species being

[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

	regarded as subspecies. Sequence data available for the closely related Btk strain ABTS-351 currently registered in Europe are placed 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.
Endpoint: Relationships to known plant, animal or human pathogens	As a member of the *B. cereus*-group, Btk is closely related to *B. anthracis* and *B. cereus*. Btk strains are however phylogenetically distinguishable from *B. cereus* and *B. anthracis.*

Cited literature abstracts:

Report: KMA 2.6/31 - 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.

Evaluation RMS	The reference is applicable and acceptable.

Report:	KMA 2.6/32 - 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.

Evaluation RMS	The reference is applicable and acceptable.

Report:	KMA 2.6/33 - 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.

Evaluation RMS	The reference is applicable and acceptable.

B.2.7 Genetic stability and factors affecting it

A "culture maintenance programme" and a "quality check programme" ensure genetic and phenotypic stability of the bacterial strains (Hargrove et al., 2003; Hargrove 1990; Chen and Hargrove, 2003). The extent and the frequency of the exchange of genetic material between *B. thuringiensis* strains and species are still under discussion and depend on several environmental factors and growth conditions.

Bacterial conjugation is a mechanism of genetic exchange that requires cell-to-cell contact and which is not susceptible to DNase present 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. the loss of one or more plasmids. These characteristics facilitate the generation of strains that contain more potent ICP combinations or those having broadened insecticidal activity spectra (Carlton, 1993).

There is no significant evidence of the horizontal plasmid transfer between *Bacillus* species and the extent of genetic exchange between strains in realistic natural conditions. Although gene exchange between *B. thuringiensis* and *B. cereus* could be demonstrated under laboratory conditions (González et al., 1982). Vilas-Boas et al. (2002) could not find evidence that such transfer occurs randomly in sympatric natural isolates of *B. cereus* and *B. thuringiensis*.

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. Hu et al. (2004) succeded in transfer of the *kurstaki* plasmid pHT73 to other members of the *B. cereus* group. However, the transferred plasmid was unstable, except in other *Bt. kurstaki* isolates

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 which prevents plasmid exchange. Genetic exchange occurs only in vegetative phase of the bacterium. *B. thuringiensis* products mainly consist of endospores and spore germination only occurs under favourable conditions.

Favourable conditions are not given due to surface application (as mentioned earlier *B. thuringiensis* spores are very sensistive to UV radiation).

The insertion of a *B. thuringiensis* plasmid into a *B. cereus* strain implicates no competitive advantage for *B. cereus* by producing insecticidal crystal proteins.

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

The identification of a cross-contaminated fermentation is done utilizing the Fermentation Monitoring Program (FMP). (Chen 2005b; 2005c; 2005d). 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.

The manufacture of the Technical Powder of SA-11, SA-12 and EG2348 is proved to be stable by regular quality control checks. Human pathogens have never been associated with its manufacture, however indigenous bacteria have been found, albeit at a low titer, which is why the FMP is utilized to prevent or minimize their occurrence. The spontaneous loss of plasmids, carrying the information for the insecticidal active crystal proteins, would be detected in bioassays; changes in physiology would be detected by HPLC, fly assay and mice injection. Furthermore, the transfer of genetic material in the fermentation broth is very unlikely due to the absence of microbial impurities and the continuous stirring movement and aeration of the fermentation broth. Conjugation requires a stable unity between mating bacteria which is broken by mechanical disruption. The quality control of the production batches confirms the stability of the three *B. thuringiensis kurstaki* strains.

RMS evaluation of section from the DAR 2008	The information and references referred to in the original DAR of Btk strains SA-11, SA-12 and EG2348 are still valid for renewal of Btk SA-12. Although it was concluded that an exchange of genetic material after field or greenhouse use of the strain is unlikely and will not lead to any adverse effects EFSA concluded the following: *No information has been provided on the potential transfer of genetic material from Bacillus thuringiensis to other organisms. However, bacilli are known to have the capacity to transfer genetic material trough exchange of plasmids. Therefore, data or assessment to demonstrate that this transfer does not occur or in case of occurring will not lead to unacceptable effects on the environment has to be provided (See specific Annex VI decision making criteria in Directive 2005/25/EC).*

New data

As already stated during first evaluation of Btk SA-12, the possibility of exchange of genetic material before and during production of the technical material/end-use product is very unlikely. For manufacturing of Btk SA-12 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. As already described in the DAR 2008, the potential for altering Btk SA-12 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 Btk SA-12 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 Btk SA-12 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 in May 2016 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. Of the 64 obtained references by the seach for "genetic stability" 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 2.7/12).

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 Btk SA-12.

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

- Maximum application rate/min water volume = 1.0 kg CoStar WG in 200 L water (use in tomato)

- Considering 5.7×10^{13} CFU/kg this would result in 2.9×10^{11} CFU/L = 2.9×10^8 CFU/mL in the spraying liquid

- Assuming a density of 1 g/mL this would correspond to 2.9×10^8 CFU/g for the potential donor strain what is still 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 Btk SA-12.

[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

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 as 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 SA-12 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 Btk SA-12 as plant protecting agent in vegetable 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 mono-associated 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.

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 SA-12 are provided below.

RMS evaluation and conclusion.	There exists no reasonable experimental approach and no specific study guideline is available to assess whether Btk SA-12 may transfer genetic material to other microorganisms after field application. From the references obtained by the literature search for Btk SA-12 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 extremely low because the event requires germination and growth of the applied SA-12 spores at a high level and the presence of competent recipient vegetative cells at a similar high level. In more details: A successful horizontal transfer would require stable insertion of chromosomal genes sequences into another bacterial genome and a selective advantage to be conferred on the transformed recipient cell. The only mechanism known to facilitate horizontal transfer of non-mobile, chromosomal DNA

[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.

	fragments among bacterial genomes is homologous recombination. This requires the presence of stretches of DNA sequences that are similar in the donor's DNA molecules that, in addition to substitutive gene replacement, would facilitate the insertion of non-homologous DNA sequences if their flanking regions share sequence similarity with the bacterial sequences in the recipient. Since the process requires actively growing, i.e. dividing cells, and cannot occur among dormant spores (which are the most frequent physiological status of *Bt* in soil), the likelihood of this kind of recombination can be expected to be negligible. In addition to homology-based recombination processes, non-homologous (illegitimate) recombination that does not require similarity between the recombining DNA molecules is theoretically possible. Non-homologous recombination has rarely been described in bacteria. However, transformation rates for illegitimate recombination are considered to be 10^{10}-fold lower than for homologous recombination. Since actively dividing cells are required, this process, compared with homologous recombination, is considered not to contribute significantly to horizontal gene transfer events for Bt spores. In comparison with the above-described homology-facilitated recombination processes, the contribution of illegitimate recombination would be extremely low. Even if the rare event of DNA transfer occurs the risk is acceptable as SA-12 is a wild type strain and does not have the capacity to produce any other compounds than indigenous Btk´s already present in the environment. In addition, Btk SA-12 is not a multi-resistant strain. This means that a transfer of genetic material would not be related to a spread of antibacterial resistance.
Endpoint: Genetic stability	Culture maintenance programs ensure that only genetically unchanged and pure cultures of Btk SA-12 are used for manufacturing of the strain and the end-use product. After field or greenhouse application genetic exchange is unlikely to occur and will not lead to any adverse effects. From the literature search for Btk SA-12 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 SA-12 spores at a high level and the presence of competent recipient vegetative cells at a high level. Even under these conditions, rates of genetic exchange were shown to be extremely low. In addition, Btk SA-12 is a wild type strain and does not have the capacity to produce any other compounds than indigenous Btks already present in the environment and it is not multi-resistant. Hence, in the unlikely case that genetic material would be transferred from SA-12 to indigenous bacteria, there is no risk that any unwanted properties are spread in the environment.

Summary/abstracts of cited literature references:

Report:	KMA 2.7/12 - Süß, J. (2016)
Title:	Literature review on *Bacillus thuringiensis* subsp. *kurstaki* SA-12 Biological properties
Document No:	2281384-MA-02-01_SA-12
Abstract	Not available

Evaluation RMS	The literature search was accepted as valid, both regarding inclusion of databases and use of search terms. Please refer to Vol 3, point B.2.10.

Report: KMA 2.7/13–Bizzarri, M. F., Bishop, A. H. (2008), published report

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

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 and 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 10^6 CFU/mL and sprayed onto the plants until run-off. It is noteworthy that spraying was 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**.**

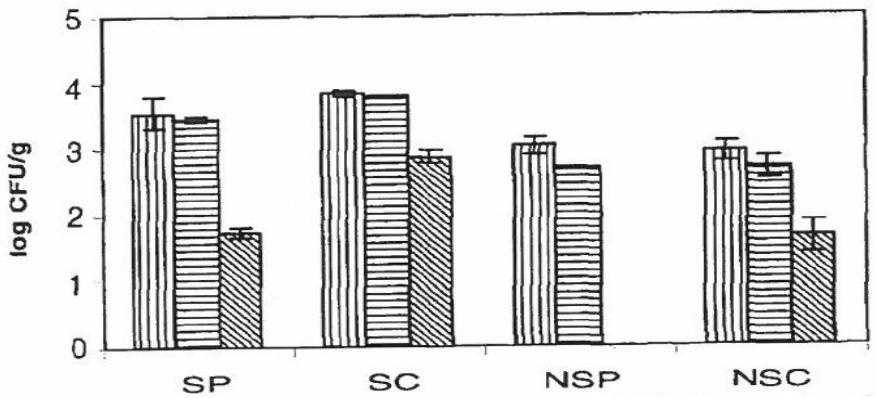

Figure 2.7-1 Transfer of plasmids between Btk strains in the phylloplane on pak choi (SP) and clover (SC) grown on sterile and non-sterile (NSP and NSC) soils. Recipient strain (vertical stripes), donor strain (horizontal stripes), transconjugant strain (diagonal stripes).

Discussion and 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.

Evaluation RMS	The reference is applicable and acceptable.

Report: KMA 2.7/14–Yuan, Y.M., Hu, X.M., Liu, H.Z., Hansen, B.M., Yan, J.P., Yuan, Z.M., (2007), published report

Kinetics of plasmid transfer among *Bacillus cereus* group strains within lepidopteran larvae

Arch Microbiol.,187(6):425-431

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 and 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-hole-plates. The third instar larvae of the different colonies were first infected with the donor and then with the respective recipient strain for 2 hours each via the diet. The infection procedure was repeated until 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 points 0, 24 and 48 hours during the infection and then every 4th hour post infection. Sampled larvae were weighed, 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 *cry1Ac* genes. Spore crystal mixtures of sporulated cultures were collected by centrifugation and submitted to SDS page pro-

tein 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/reference
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. (1995)
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 four 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.

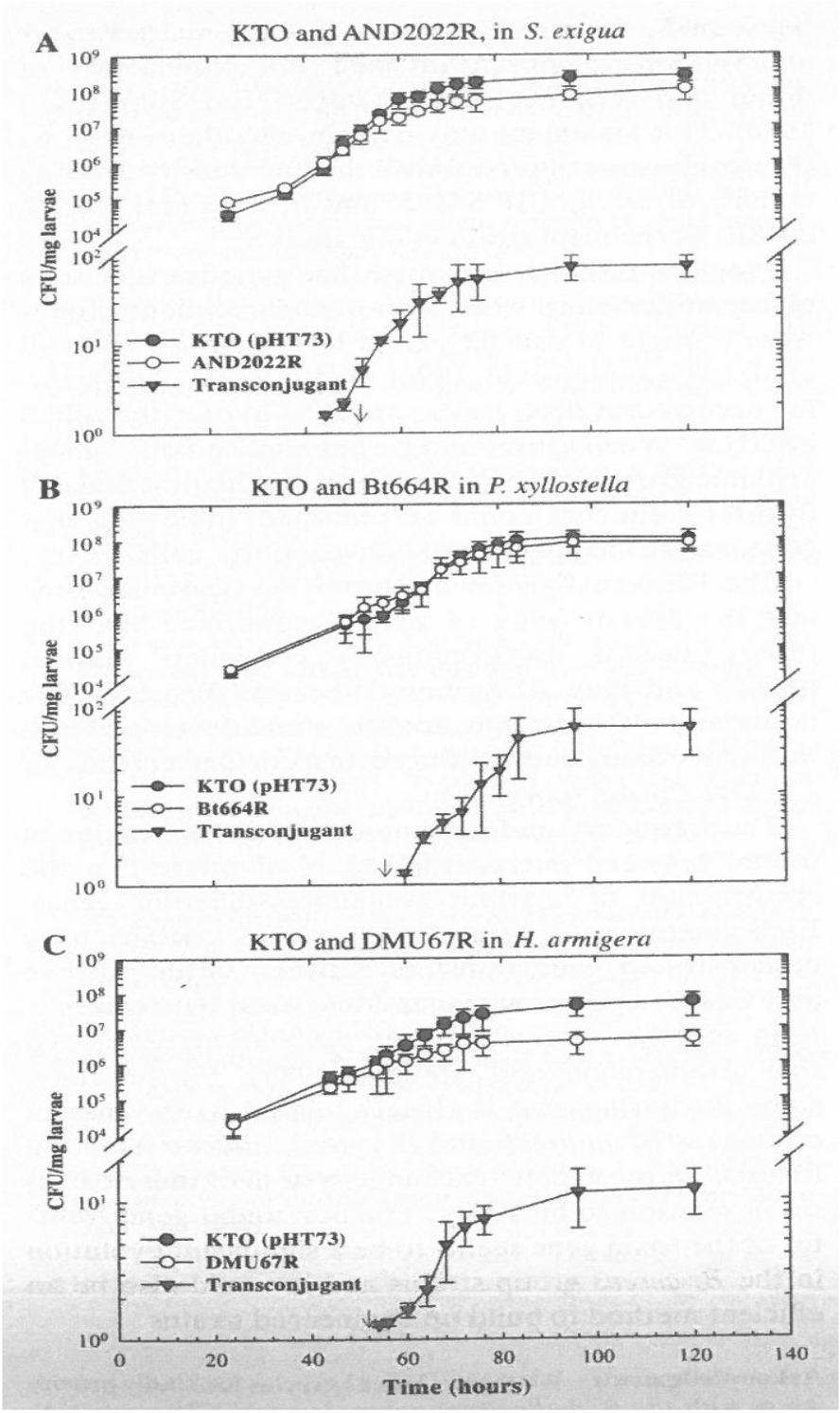

Figure 2.7-2 Population development of donor, recipients and transconjugant strains in three insect species. Infection took place via artificial diet containing 10^9 spores/g of the donor (KTO) and recipient strains. The arrow indicates the time point of spore germination.

Discussion and 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.

Evaluation RMS	The reference is applicable and acceptable.

Report: KMA 2.7/15– Santos, C.A., Vilas-Bôas, G.T., Lereclus, D., Suzuki, M.T., Angelo, E.A., Arantes, O.M.N., (2010), published report

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

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^R) 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 and Methods: *B. thuringiensis* subsp. *kurstaki* strain KT0 (pHT37-Em^R) 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 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 boxes, 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 except for the donor strain), they 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 ± 0.50 × 10^2
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 ± 0.40 × 10^3
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 ± 0.53 × 10^3
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 ± 0.40 × 10^1
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 ± 0.44 × 10^2
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 ± 0.62 × 10^3
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 ± 1.1 × 10^3
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 = EC/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]					Stability of pHT73-Em^R (%)
	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}	99.3
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}	98.3
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}	89.3
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}	98.5
B. cereus D1 4430	NC	NT	3.7 ± 0.58 × 10^{-9}	2.6 ± 0.37 × 10^{-4}	NT	91.0
B. cereus ATCC 10987	NC	NT	9.7 ± 0.15 × 10^{-6}	NC	NT	88.5
B. cereus 569	NC	NT	1.7 ± 0.40 × 10^{-8}	1.8 ± 0.21 × 10^{-3}	NT	99.0
B. cereus ATCC 14579	NC	NT	1.1 ± 0.90 × 10^{-9}	NC	NT	94.8

±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 and 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.

Evaluation RMS	The reference is applicable and acceptable.

Report: KMA 2.7/16– Donnarumma, F., Paffetti, D., Stotzky, G., Giannini, R., Vettori, C. (2010), published report

Potential gene exchange between *Bacillus thuringiensis* subsp. *kurstaki* and *Bacillus* spp. in soil *in situ*

Soil Biology & Biochemistry, 42:1329-1337

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 and 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. Isolates 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 Quickstix 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 from 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*) were 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 Quickstix. 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 and 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.

Evaluation RMS	The reference is applicable and acceptable.

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

Vegetative growth

B. thuringiensis 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 (Hansen & Salamitou, 2000).

During vegetative growth, certain strains of certain *B. thuringiensis* subspecies like *Bt thuringiensis*, *Bt galleriae* and *Bt darmstadiensis*, produce ß-exotoxins, 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, EHC 217, 1999).

Commercial *B. thuringiensis* products

From the data provided on the product and production process of the *B. thuringiensis kurstaki* strain SA-11, SA-12 and EG2348 there is no indications of presence of metabolites toxic for human health or the environment in the product (see Annex C). 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 and fly test (Chen, 2005, a, b, c, d). 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 (Hargrove et al., 2003).

Report :	(Chen, 2004) Inability of [redacted] *Bacillus thuringiensis* production cultures to produce beta-exotoxin, Unpublished Report, December 2004.
Guideline:	not stated
GLP:	No

Materials and Methods: The study was conducted during the period 20.09.2004 – 15.12.2004, by Benzon Research. The test substance SA-11, SA-12 and EG2348 was produced by [redacted]

The fermentation broth of each production culture was obtained by growing the cultures in their respective production substrates. A ß-exotoxin producing strain of *B. thuringiensis* ssp. *Thuringiensis (Btt)* 579-16, fermented in each of the production substrates served as positive control. A bioassay with house fly larvae was used to detect the production of ß-exotoxins.

Findings: No ß-exotoxin could be determined in the production substrates of the three strains. The detection limit of ß-exotoxin by fly larvae assay is 0.028 µg/mL. The positive control, *Btt* produced 191µg/mL to 378 µg/mL of ß-exotoxins

Conclusions: The strains SA-11, SA-12 and EG2348 do not produce detectable amount of ß-exotoxin in their respective production substrate.

RMS evaluation of section from the DAR 2008	In a laboratory study it was shown that Btk strain SA-12 do not produce detectable amount of ß-exotoxin in the production substrate. The information and references referred to in the original DAR of Btk strains SA-11, SA-12 and EG2348 are still valid for renewal of Btk SA-12. However, the information in the DAR was very limited.

New data

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

Available information will be provided following the three categories:

- Metabolites involved in the mode of action

- Metabolites of possible concern for human health

- Other metabolites potentially produced by Btk

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 Btk metabolites, metabolites identified during the EFSA peer review of SA-12 and metabolites identified for Btk 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 sub-species specific 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 Literature Review Report provided in Vol. 3 MA, Section B.6, Point B.6.3 (Seehase, 2016, KMA 6.1/01).

[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

Metabolites involved in the mode of action

Relevant information about insecticidal proteins produced by *Bacillus thuringiensis* including Btk are presented in a review paper of Palma et al. (2014) which is summarised in B.2.2.2 above. Spore bound toxins (Cry, Cyt) as well as secreted insecticidal toxins produced during vegetative growth (Vip, Sip) are already known for decades and not assumed to be of concern for human health or the environment.

No risk assessment is performed for metabolites. Cry and Cyt proteins are spore bound and therefore only biologically active in the presence of the microorganism. As such, the environmental risk assessment of the Cry and Cyt proteins are covered by the risk assessment of the microorganism itself. Consequently, no risk assessment is needed for metabolites.

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)[11]. Based on available literature information the authors concluded that Cry-toxins are not of concern for human health. Two references referring to potential side effects of another insecticidal protein are mentioned but the references refer to other Bt subsp. namely *israelensis* and *aizawai*, and are therefore not further considered here. Apart from that, no further information was provided.

Beta-exotoxins, also called thuringiensins, are considered to have toxic properties but were shown not to be produced by commercial Btk strains, including strain SA-12. Please see the information and references already referred to in the original DAR above.

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 Btk (Seehase, 2016, KMA 5.1/01). The obtained references confirm the absence of toxicity to humans and mammals. Onose et al. (2008) for example studied the sub-chronic toxicity of the Cry1Ab protein in rats and did not observe any toxic effect. Shimada et al. (2006) tested whether Cry proteins can bind and adversely affect bovine, porcine and human intestine cells and concluded that this is not the case. 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$^-$ strain with insecticidal activity. The study suggests that insecticidal δ-endotoxins of certain Btk strains may have hemolytic properties. However, it was shown already that the potential to bind to human intestine cells and to negatively affect their membrane potential is rather low. Production of ß-exotoxins was widespread among the tested isolates and always accompanied by hemolytic activity. The absence of production of ß-exotoxins has been proven for all commercial Btk strains and thus, is not a matter of concern for registered Btk strains including Btk SA-12.

Two additional references referring to the potential of Btk to produce *B. cereus*-enterotoxins are summarized in the sub-point *Metabolites of concern for human health* below (Kim et al., 2015 and Wilcks et al., 2006a).

In conclusion, confirming information provided previously, there is no indication in the published literature that metabolites involved in insecticidal activity of Btk SA-12 pose a risk for human health or the environment.

Summary/abstracts of literature references:

Report:	KMA 2.8/11 - 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.

Evaluation RMS	The reference is applicable and acceptable.

Report: KMA 2.8/12 – Onose, J-I., Imai, T., Hasumura, M., Ueda, M., Ozeki, Y., Hirose, M. (2008), published report

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

Food Chem Toxicol., 46(6), 2184-2189

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 (A)
Cry1Ab from *Bacillus thuringiensis* var. *kurstaki* HD1 (= Bt) (B)
Indomethacin and famotidine to induce Gastro-intestinal impairment (= GI) (C)
Bt + GI (D)

Study design:

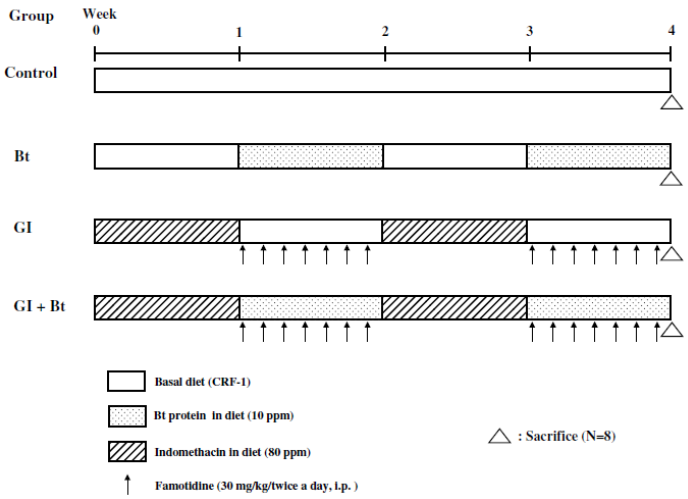

Figure 2.8-1 Experimental design

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. Interpretation 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.

Evaluation RMS	No significant toxicological effect of refined Bt protein Cry1Ab was observed with and without the present gastrointestinal impairment. The reference is acceptable.

Report: KMA 2.8/13 – Shimada, N., Miyamoto, K., Kanda, K., Murata, H. (2006), published report

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

In Vitro Cellular & Developmental Biology - Animal, 42(1):45-49

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. Determina-

tion 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 in 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 does not have the potential to damage intestine cells of cattle, pigs or human.

Evaluation RMS	The results strongly indicate that Cry1Ab toxin does not have the potential to damage intestine cells of cattle, pigs or human. The reference is acceptable.

Report: KMA 2.8/14 – Obeidat, M., Khyami-Horani, H., Al-Momani, F. (2012), published report

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

African Journal of Biotechnology, 11(46):10504-10512

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, 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 were submitted to hemolytic assays. Out of these, 16 strains produced non-hemolytic δ-endotoxins. These strains belonged to the serovars *aizawai, israelensis, kurstaki pakistani* and *tohokuensis*. 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 ± 11.1^{def}	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. Based on additional phylogenetic analysis the authors concluded that production of insecticidal toxins with hemolytic activity is a strain specific property.

Evaluation RMS	Based on additional phylogenetic analysis the authors concluded that production of insecticidal toxins with hemolytic activity is a strain specific property. The reference is acceptable.

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 strain SA-12 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]. Therein, a complete overview about current knowledge on virulence factors potentially produced by members of the *B. cereus* group including Bt is provided. 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 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 2.8-4 Potential virulence factors produced by members of the *B. cereus* group

Toxin	**Biological activity**	**Remarks**	**Produced by some *B. thuringiensis* strains**
Cereulide	Emetic syndrome	-	No
Enterotoxins			
Non-hemolytic enterotoxin	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
Hemolysin	Hemolytic and dermonecrotic activity	Involved in non-GI infections	Yes
PlcR	Global regulator for transcription of enterotoxin genes	-	Yes
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
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
InhA1	Escaping of *B. cereus* from macrophages	-	Not yet observed
NprA	Metalloprotease, immune evasion and tissue degradation by *B. anthracis*	-	Not yet observed

[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

The literature search on possible toxic effects of metabolites potentially produced by Btk 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 Literature Review Report provided in Doc M-MA, Section 5, Point MA 5.1 (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. Wilks 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 viv*o 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 is well known that commercial Bt strains 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 biological 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 Btk strains including strain SA-12, 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 Btk. 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 thus very unlikely that high amounts of Bt will be present on food commodities at the time of consumption.

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, rainfall 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 (Du & Nickerson, 1996).

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). See for more details and references the environmental fate section Vol.3 MA, B.8.1. In

the table below data on persistence of Btk in foliage/crops is summarised. Both data already provided for Annex I inclusion of Btk SA-12 and new data is provided.

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

Com-pound	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]
	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 8

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 contrast, in the presence of specific insect receptors, they germinated 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 they pay for higher insect pathogenicity with a decreased spore resistance. Extremely low germination rates in the absence of any activator (heat, alkaline conditions, germinant) in particular when compared to it´s 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 Btk strains. Taken together it can be concluded, that insecticidal Bt strains, such as commercial strains including Btk SA-12, 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 2.8-6: 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 from Cry-deficient Bt and *B. cereus*
[#] BBMV: Brush Border Membrane Vesicles obtained from the gut of *M. sexta*

RMS remark	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 it 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 will 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. 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 the stomach passage, germination and growth in the intestine, adhesion to and invasion into 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). There was a clear tendency that survival and growth of the tested Bt strains was negatively affected by low pH (2.0), bile acid at 0.3 g/L, as well as by microaerobic conditions. 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 gastrointestinal 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 for approved Btk strains, including Btk SA-12. One reference however, also focusses on commercial Bt strains (Wilcks, et al. 2006a; obtained by the literature search according to EFSA guidance). In this study, rats with associated human-intestinal-microflora, were fed with commercial Bt strains, 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 5 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, the two strains were not able to survive the gastrointestinal passage and where not detectable in intestine samples and also not in faeces.

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, in the study by Wilcks et al (2006a), the density of the two commercial Bt strains, one of which was a Btk and the other one a Bti strain, was assessed in intestine samples and faeces upon oral gavage of. In 5/6 animals, Btk but not Bti, was detectable two weeks post administration in faeces samples. 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 revealed the presence of a high percentage of living cells. The authors concluded that Btk germinated and grew in the rat intestine, at least in this animal. As mentioned previously, when administered as living cells and not as spores, both strains quickly disappeared from intestine and faeces.

The same authors 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 well survived the gastric barrier, 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. 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 of vegetative bacterial cells to cell surfaces is positively correlated with the presence of an S-layer (Auger et al., 2009). The S-layer, a regularly ordered protein layer, is the outermost cell envelope component of bacterial and archeal cells. The authors assessed the presence of the S-layer in 102 pathogenic (diarrheal, emetic, and oral diseases) and non-pathogenic strains of *B. cereus* and Bt by means of Western blot analysis. Notably, none of the 24 Bt strains tested in the study possessed an S-layer; in the contrary, all of the 12 *B. cereus* oral disease strains and all 11 of the periodontal *B. cereus* strains, had one. The differences were significant, showing that the presence of the S-layer correlated with the strain origin. Further assays targeted the ability to form biofilms and it turned out that this trait (measured in PVC microtiter plates at 30°C in LB medium) is strongly dependent on the strain origin in the *B. cereus* group with *B. cereus* strains involved in gut colonization being better biofilms formers.

Adhesion and invasion experiments were also performed by Hansen et al. (2011) using vegetative bacterial cells of commercial Bt 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 two of the commercial Bt strains (Bta and Bti), independent from the dosage tested, always acted rather slowly. One of the commercial Bt strains (Btk) switched from an aggressive phenotype at high dosages 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 transepithelial electrical resistance (= TEER) it turned out that the strains acting less aggressive against Caco-cells (commercial Bta and Bti strain) also showed a low potential to cause damage to 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)).

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.

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, the density of attached cells was too low to extract sufficient amounts of rRNA for the analysis and expression of housekeeping genes was 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. (2012b) 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.

From the available information it can 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 diagnostic tools 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:

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 Btk SA-12, 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 Btk strains, including SA-12, 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 SA-12, 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 another 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 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)

[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

[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.

- 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

- 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).

- Pathogenic *B. cereus* strains produce much higher amounts of enterotoxins under optimal laboratory conditions than commercial Bt strains do (e.g. 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 the paper below titled written by B. Raymond and B.A. Federici in 2017:

Report:	Raymond B, Federici BA, 2017. FEMS Microbiology Ecology, Jun 22. doi: 10.1093/femsec/fix084
Title:	In defence of *Bacillus thuringiensis*, the safest and most successful microbial insecticide available to humanity—a response to EFSA
Abstract	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.

RMS agrees with the conclusion of the paper that the recent controversial case of food poisoning in Germany presents no convincing evidence that Bt was the causative agent, since individuals with food poisoning had also consumed a dose of Bc sufficient to cause the observed level of infection. Overall, the MLST databases, the epidemiological studies and safety testing literature present a well-informed and coherent view of the biology and ecology of the Bc group. The arguments in the EFSA report, that we do not understand the risks of consuming Bt spores, are therefore unfounded and overly cautious. 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. 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 (Guinebretiere 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 *an-*

[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

thracis 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*.

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. This, together with the proven absence of pathogenicity of Btk SA-12 indicates that the risk for consumers following use of the strain 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 2.8/14 – 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 2.8-7.**

Five of the commercial strains harboured one and three harboured 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 were very similar.

Table 2.8-7: 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 2.8-8**. 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 2.8-9**).

Table 2.8.-8 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 2.8-9 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.

Evaluation RMS	The reference is applicable and acceptable.

Report: KMA 2.8/15 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 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, 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, 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, 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 2.8-10**). In the same animal, passage of Bti. HD567 was found at a much 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 2.8-11**).

Table 2.8-10 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 2.8-11 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.

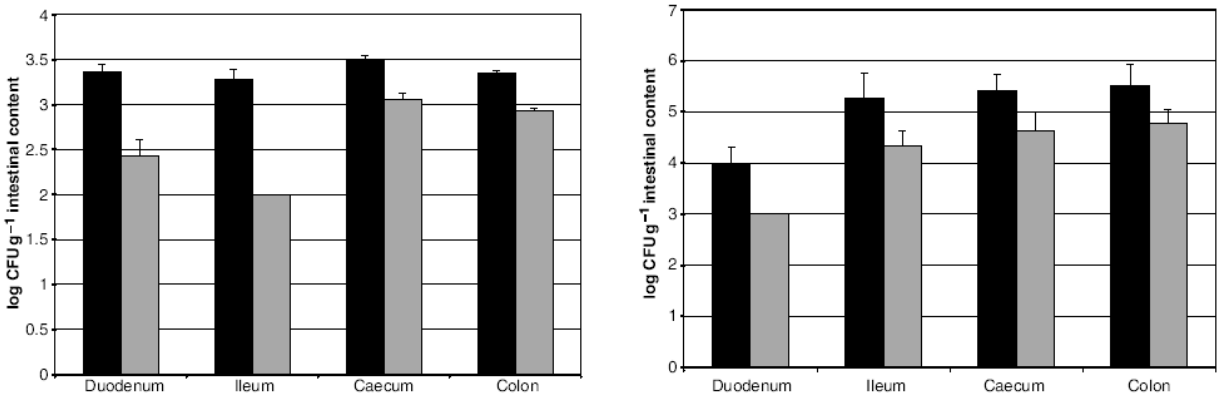

Figure 2.8-2 Heat-treatment of intestinal samples from animal 15 fed Btk DMU67R spores (left) and Bti HD567 (right) at sacrifice on day 18 of the experiment. Black columns represent the untreated samples, whereas grey columns represent the heat-treated (80°C, 15 min) samples. The experiment was performed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

Evaluation RMS	The reference is applicable and acceptable.

Report:	KMA 2.8/16 - Du, C., Nickerson, K.W. (1996b), 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.

Evaluation RMS	The reference is applicable and acceptable.

Report:	KMA 2.8/17 - 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.

Evaluation RMS	The reference is applicable and acceptable.

Report:	KMA 2.8/18 - 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.

Evaluation RMS	The reference is applicable and acceptable.

Report: KMA 2.8/19 Hansen, B.M., Thorsen, L., Nielsen-LeRoux, C., Wilcks, A., Hendriksen, N.B. (2011), published report

New experimental approaches for human risk assessment of microbial pest control agents – exemplified by the bacterium *Bacillus thuringiensis*

Pesticides Research 136 2011 Danish Ministry of the Environment, Environmental Protection Agency

Abstract/Summary: 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.

The bacterium *Bacillus thuringiensis* has been used as a model for microorganisms used in pest control agents. This bacterium was chosen because it is an active ingredient in several plant protection products, which are the most successful worldwide. Further it is known that some strains are potential pathogens. Four different strains were chosen as primary objects for the investigations; these strains originate from the products Agree, Vectobac, Dipel and Novodor. The strains are from four different subspecies of *B. thuringiensis*, and can be used for control of lepidopteran, dipteran and coleopteran larvae. Mainly Dipel and Vectobac are in use in Denmark.

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). Further two strains supposed to be avirulent were selected. The choice of positive control strains from our own collection of *B. thuringiensis* / *B. cereus* strains was done on the basis of their origin from cases of illness and/or their relationship to other strains isolated from such casesThe relationship to other strains was investigated by the use of a method known as "multiple locus sequence typing" (MLST). In addition, were strains able to grow at 43°C selected *B. cereus* is very closely related to *B. thuringiensis*, and several researchers is of the meaning that these two species should be regarded as one species; some strains of *B. thuringiensis* is in a phylogenetic context more related to some *B. cereus* strains than to other *B. thuringiensis* strains. As negative controls were a *B. thuringiensis* strain mutated in the regulator of the most common virulence factors and a *B. cereus* strain from a probiotic chosen.

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. However, to make a firm conclusion, it is necessary to study more strains.

Caenorhabditis elegans model:

Caenorhabditis elegans is a nematode, which consumes bacteria. This nematode has during the last ten years been used as a model-organism in a number of different connections. Its significance as a model-organism depends on its easy culturability, that it starts reproduction within 3 - 4 days and have a lifespan on 2 - 3 weeks, further has its full genome been sequenced and it exists as a high number of types (defined mutants); e.g. is the experiments in this project carried out with a mutant unable to reproduce at 25°C. *C. elegans* has increasingly been used to study interactions between microorganisms and eukaryotes, and especially to investigate human pathogens and their virulence factors. According to our knowledge has it not previously been used for studies on the virulence of *B. thuringiensis* / *B. cereus* for humans. In the project a method, which in a relatively easy and reproducible way can be used for investigations on the effects of *B. thuringiensis* / *B. cereus* strains on *C. elegans*. The developed methodology is most likely also applicable with other bacteria. The results obtained with the develop 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 from Agree, followed by the strains Bc11, Bc14, Bt48, Bt52 and Bt53, a mixture of the negative control strain, strains from products and positive control strains; the next group consist of the strains Bc25 and Bc38, two positive control strains, while the fourth group only contains one strain Bt55, from the product Novodor. The *C. elegans* model is, thus, able to distinguish between different *B. thuringiensis/B. cereus* strains with regard to virulence. The model is relatively easy to handle, and to use with more strains, the data is relatively easy to analyse. It can be used for investigations on the potential virulence of strains, however a simple relationship between pathogenicity towards humans and the virulence towards nematodes has not been found.

Galleria model

Galleria mellonella insect larvae are often used to identify virulence and infection of both human pathogens and insect pathogens. An advantage of such a simple model is that it contains some of the physical barriers as well as innate immunity similar to those of man and higher animals. For instance the intestinal cells posses a protective gel-like layer, immune cells can ingest micro-organism and particular compounds can attack the surface of pathogens. And beside to this, larvae supports well human body temperatures and are easy to rear. *Galleria* is already used for virulence analysis of *B. cereus* group bacteria.

The analysis is conducted with two routes of infection: oral and by injection into the hemocoel (equivalent to blood/lymphe). Our tests were run with both spores (the dormant stage of the bacteria, the one which is present in the commercial *B. thuringiensis*-based products) and vegetative bacteria (the form which can multiply). Using spores for infection is relevant to situations where the Bt based products may contaminate food, and vegetative bacteria can both be part of consumed food and be in this growth stage when in contact with the host cells (intestinal or immune cells). 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 except for strain Bt53 (*kurstaki*) that was most virulent while using spores. All strains were equivalent virulent with vegetative bacteria except the negative control Bt48 (PlcR mutant). Meanwhile, we found a more pronounced strain difference when the strains were used for infection by injection into the hemocoel. Indeed, strains were found more or less virulent especially at lower dose.

From our results we may conclude that Galleria can probably be used as a model to estimate virulence potential and that such estimation is more easily obtained with vegetative bacteria. It is interesting to note that the Bt strains are both in low and high virulent groups. Meaning that some strains are potentially more virulent than others, if they are tested a particular situation. Thus, although we only have analysed a few strains it was possible to identify a positive and a negative control strain, which might be used in the development of a new risk model protocol. However, in order to validate the model, it is important to make similar studies on a larger number of strains.

In vitro/in vivo gene expression analysis

One of the big challenges of this project was to analyse *in vivo* gene expression when the bacteria are in con-

tact with the host using Q-RT-PCR. This aspect was analysed in the insect *Galleria* and in the intestinal cell culture $Caco_2$. Upon optimisation we were finally able to measure expression of several genes in a few strains, while the bacteria were located inside the larval gut and in contact with the $Caco_2$ cells. In summary it is very interesting results which highlights our knowledge to which genes are expressed *in vivo*. We found for instance that the enterotoxins (Hbl, NhE, CytK) were expressed at different levels in the analysed strains. These results are especially of interest for further *in vivo* studies both to analyse other bacterial factors but also to analyse host responses. But we do not recommend that for routine analysis, because of the complexity to standardize Q-RT-PCR.

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 is it possible to measure the electrical resistance across a cell layer (trans-epithelial 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). Further as a measure of bacterial virulence, it is possible to study adhesion to and invasion of the epithelial layer as well as to observe morphological changes by microscopy and measure the effect on the viability of immune cells. All these aspects have been analyzed with most of the selected strains in the current work. 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 bc49 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. Thus, in this work, by performing infections with low bacterial numbers it was observed that bacteria are differentiated in terms of how quickly TEER was impaired, and how quickly epithelial cells were damaged (observed by microscopy). By use of the functional mammalian cell models, two product strains (bt50 and bt52) could be placed primarily in the group of bacteria with low potential for virulence (were "slower" than the probiotic bacterium). By comparison, there was one product strain (bt53) that independent of type of model positioned itself as more "rapid" than the pathogenic bacteria (but not by very low infectious dose). One product strain (bt55) positioned itself in some models in line with the pathogenic bacteria (bc25 and bc38) and in other models among those with low potential for virulence. We suggest that the rate at which the different bacteria performed "damage" may be used as a target for determining the potential to effectively cause damage during the passage of the intestine, interaction and competition with many other bacteria will be of great importance. "Speed" was previously used to differentiate the product strain *Bacillus* subtilis (natto) used for fermentation of Natto (a Japanese dining that is considered to be beneficial to health) from various pathogenic bacteria (Hosoi et al. 2003).

In conclusion TEER measurements seem to be more objective than the adhesion / invasion model that includes subjective observations by microscopy. The model which examines the effect of the dose on the time for injury also contributes to good information about individual differences in the bacterial pathogenic potential. It is possible that the results we have achieved in the models were influenced by the choice of atmosphere (5% CO_2, 95% atmospheric air) affecting the growth rate and gene expression, and perhaps hence the virulence as compared to if conducted under anaerobic conditions. It could therefore be interesting in the future to work with models with reduced oxygen, which is more comparable to the conditions in the gut. The results for gene expression during infection in the cell models showed that genes associated with diarrhea (*cytK*, *nheA*) were expressed in bacteria that were categorized as having high and low potential for virulence.

The results obtained for the functional mammalian cell models in this study suggest that if we want to assess other types of biological control agents it will be necessary to develop new models for these types. The results for the product strains in relation to pathogenic potential are similar to the results obtained with *G. mellonella* and *C. elegans* models.

The results and the experience with the three different approaches are discussed in relation to their applicability

in relation to risk assessment of microbial pest control agents.

Material and Methods:

RT-PCR detection of virulence expression

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

Overnight (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 number 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 numerous 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 2.8-12, 2.8-13 and 2.8-14.**

Table 2.8-12 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 2.8-13 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 2.8-14 Product strains

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 2.8-15**).

Table 2.8-15 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.

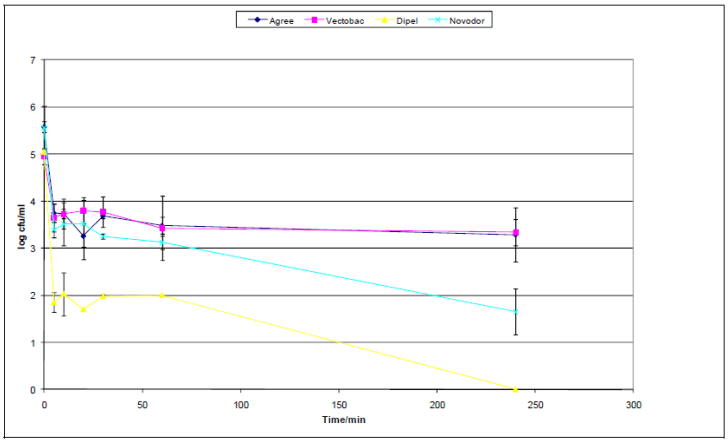

Figure 2.8-3 Persistence of the selected commercial strains in simulated gastric fluid at pH 2.0.

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.

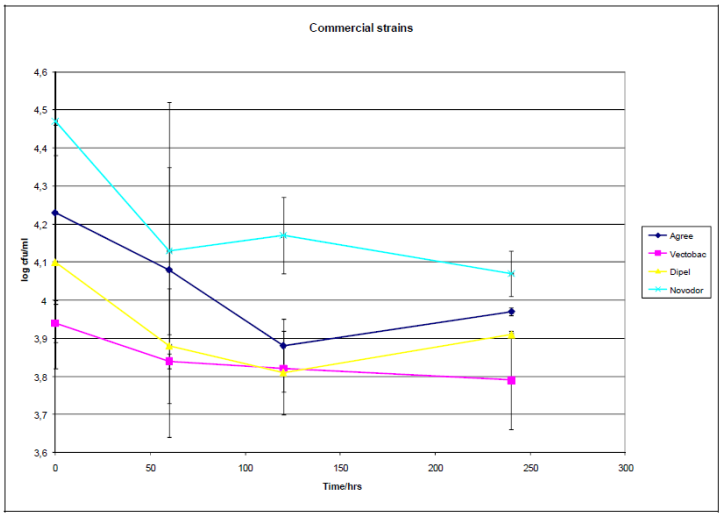

Figure 2.8-4 Persistence of the selected commercial strains in 0.3 g/L bile acids

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 2.8-16**).

Table 2.8-16 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 nonpatho-

genic to humans.

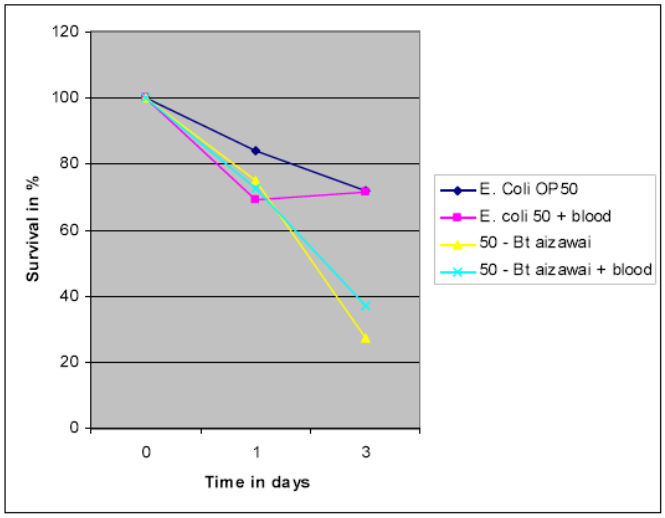

Figure 2.8-5 Nematode survival after exposure to *E. coli* and *B. thuringiensis* GC-91

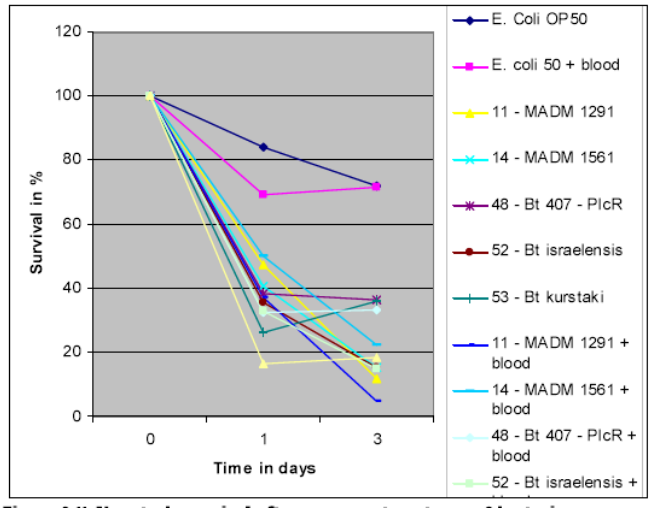

Figure 2.8-6 Nematode survival after exposure to category 2 bacteria

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 2.8-17 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 *PlcR*)	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.

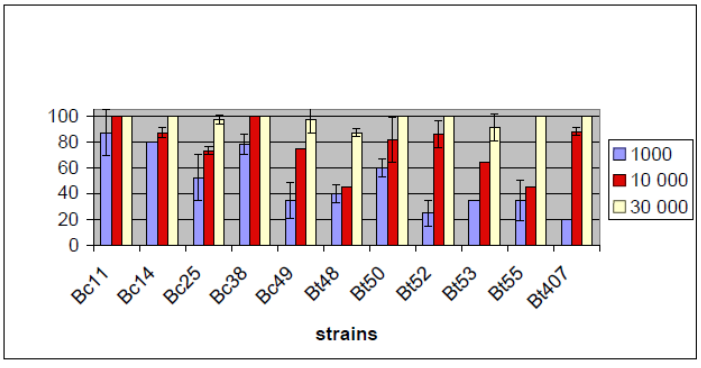

Figure 2.8-7 Mortality in % at 48 h post injection of *Bacillus* vegetative cells (10^3, 10^4 and 3×10^4) into the *Galleria mellonella* hemocoel.

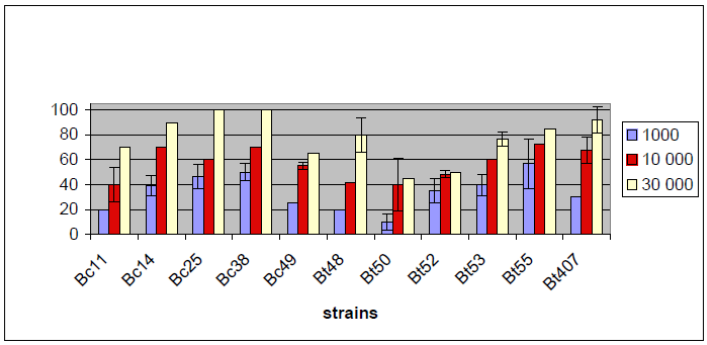

Figure 2.8-8 Mortality in % at 48 h post injection of *Bacillus* spores (10^3, 10^4 and 3×10^4) into the *Galleria mellonella* hemocoel.

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 2.8-18**). 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 2.8-18 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.

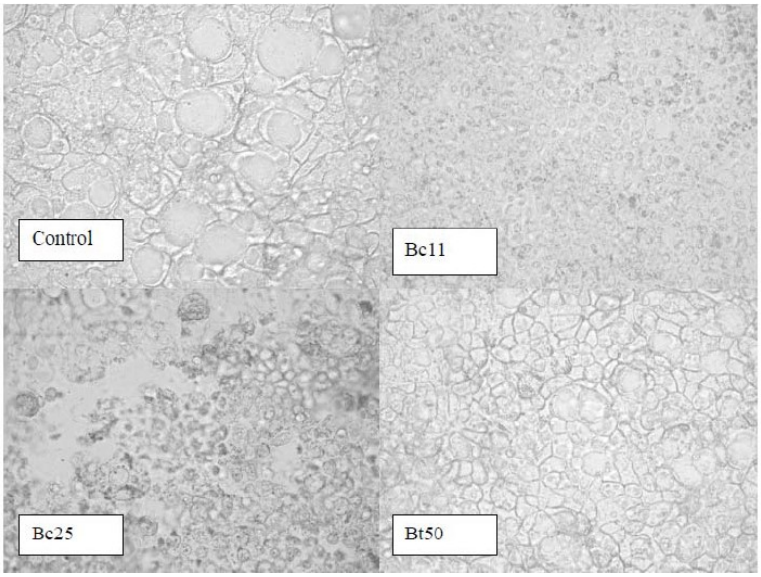

Figure 2.8-9 Morphological changes to Caco-2 cells infected with various *Bacillus* spp. (vegetative cells) For 4 hours at 37°C, 5% CO2.

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.

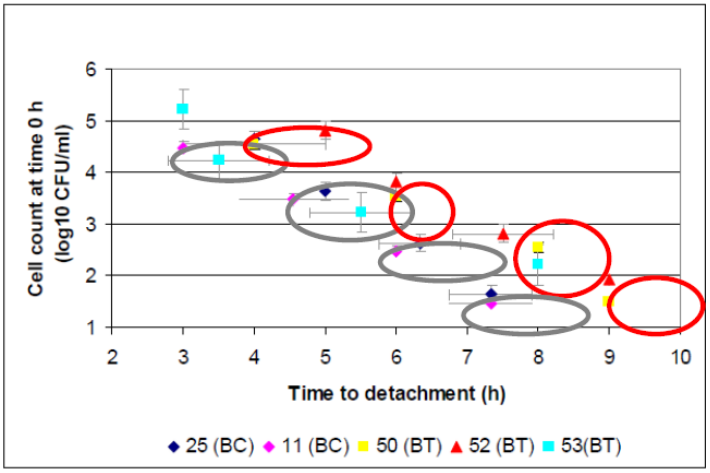

Figure 2.8-10 Effect of five different bacteria on the physiology of Caco-2 monolayers as observed by bright field microscopy. The bacteria were added at different concentrations, and the monolayer was observed for a maximum of nine hours. Results are shown as averages of 2 - 3 independent experiments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

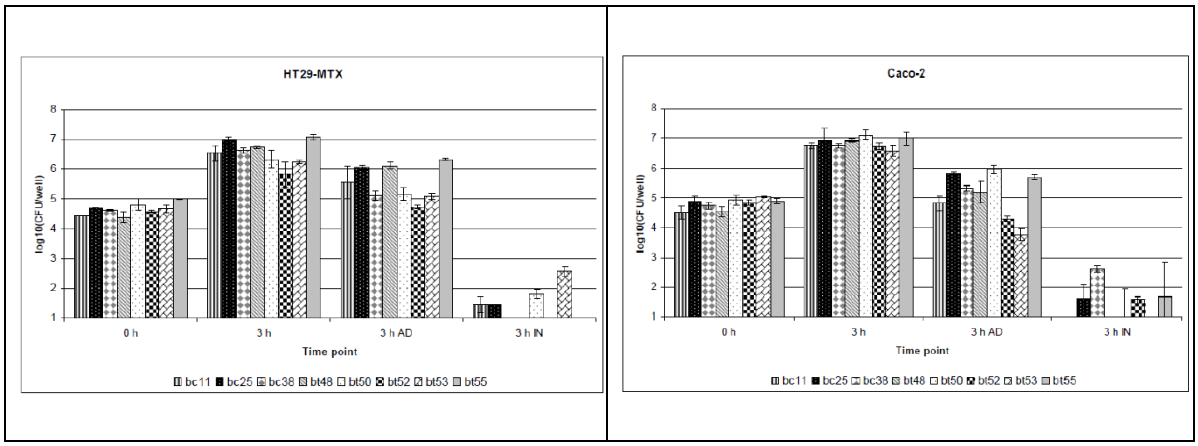

Figure 2.8-11 HT29-MTX cells (28 - 29 Days) and Caco-2 cells (17 - 18 days) infected with eight different bacteria (vegetative cells) for 3 hours at 37°C, 5% CO_2. Data is the average of 2 independent experiments performed in duplicate. Data are shown with standard deviations. AD = Adhesion, IN = Invasion.

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.

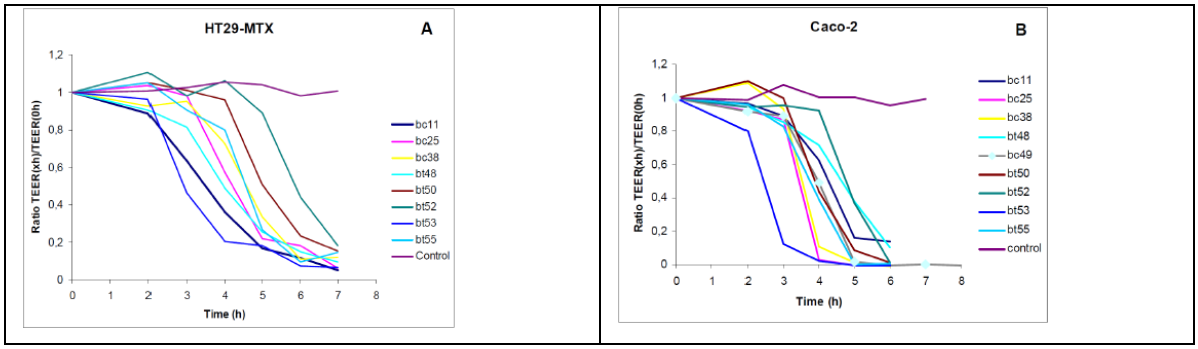

Figure 2.8-12 TEER of polarized HT29-MTX and Caco-2 monolayers exposed to various *Bacillus* spp. at a oncentration of 1 × 10^4 CFU/well (bc25, bc38, bt50, bt52, bt53, bt55) and 3 × 10^3 CFU/well (bc11, bt48). TEER (Ω/cm^2) is expressed as the ratio of TEER at time t in relation to the initial value (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 monocyte/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). Non adherent Bt50 cells expressed the virulence associated genes *cytK, ilsA, nheB*, while the gene expression levels of the adherent cells were too low to be detected. 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 2.8-19 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 detach				+				+	+
	Bc 11/4 Ad	$9,7*10^4$					-				-	-
9/3-10**	Bt48/3h	$2,8*10^6$	none				?				?	+
	Bt48/3h Ad	$6,5*10^3$					-				?	-
	Bt48/4h	$1,3*10^7$					-				-	-
	Bt48/4h Ad	$6,5*10^5$					-				+	+
8/10-09	Bt50/0h	$8.5*10^4$	none	+	+	+	+	-	(+?)	-	+	(+)
	Bt50/3h	$1.4*10^7$		+	+	-	+	+	(+?)	-	+	
	Bt50/3h AD	$9.6*10^5$		(+?)	(+?)	-	+	-	?	-	+	
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	$1.1*10^5$	none	+	+	+	+	+	-	-	+	-
	Bt53/3h	$4.1*10^6$	Strong detach	+/nd	+/+	+/(+)	+/+	+/nd	(+?)/nd	-/nd	+/+	(+)/+
	Bt53/3h AD	$6.4*10^3$		-	-	-	-	-	-	-	(+)	-
8/10-09	Bt55/0h	$7.9*10^4$	none	+/+	+/+	+/+	+/+	+/-		+/+	+/+	(+)/-
	Bt55/3h	$1.1*10^7$	detach	-/-	-/+	-/-	-/-	-/-		-/-	-/-	-/-
	Bt55/3h AD	$5.2*10^5$		-/-	-/-	-/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 survive 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 2.8-20 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*	*isralensis*	*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 2.8-21 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*	*isralensis*	*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

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

Evaluation RMS	The reference is applicable and acceptable.

Report:	KMA 2.8/20 - 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.

Evaluation RMS	The reference is applicable and acceptable.

Report:	KMA 2.8/21 - 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.

Evaluation RMS	The reference is applicable and acceptable.

Report:	KMA 2.8/22 - 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*.

Evaluation RMS	The reference is applicable and acceptable.

Report:	KMA 2.8/23 - 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.

Evaluation RMS	The reference is applicable and acceptable.

Report:	KMA 2.8/24 - 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.

Evaluation RMS	The reference is applicable and acceptable.

Report: KMA 2.8/25 Damgaard, P.H. (1995), published report

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

FEMS Immunology and Medical Microbiology 12(3-4):245-250

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 2.8-22**).

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 2.8-22**).

Table 2.8-22 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	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 2.8-23**). 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 2.8-23 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.

Evaluation RMS	The reference is applicable and acceptable.

Other metabolites produced by *Bacillus thuringiensis* subsp. *kurstaki*

In the External Scientific Report on Literature review and data collection on microbial active substances which can be used for the environmental risk characterisation[18], 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 Literature Review Report provided in Doc M-MA, Section 5, Point MA 5.1 (Seehase, 2016, KMA 5.1/01).

RMS evaluation and conclusion.	**Metabolites involved in the mode of action** Spore bound toxins (Cry, Cyt) as well as secreted insecticidal toxins produced during vegetative growth (Vip, Sip) are already known for decades and not assumed to be of concern for human health or the environment. Beta-exotoxins are considered to have toxic properties but were shown to be not produced by commercial Btk strains, including strain SA-12. No risk assessment is performed for metabolites. Cry and Cyt proteins are spore bound and therefore only biologically active in the presence of the microorganism. As such, the environmental risk assessment of the Cry and Cyt proteins are covered by the risk assessment of the microorganism itself. Consequently, no risk assessment is needed for metabolites. There is no indication in the published literature that Cry-toxins/metabolites involved in insecticidal activity of Btk SA-12 pose a risk for human health or the environment. **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 strain SA-12 and is still under discussion by the European authorities. In the EFSA Scientific Opinion (2016) 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. It is well known that commercial Bt strains harbour the genetic material of *B. cereus* enterotoxin genes. However, studies elucidating the very complex expression kinetics of potential *B. thuringiensis* enterotoxin genes in the human gastro-intestinal tract can neither be designed nor performed easily. Indeed, scientific studies related to this subject have been on-going for decades and have so far not given conclusive answers but indicative anwers. Overall, it is unlikely that biopesticidal Btk strains are able to produce enterotoxin at biologically relevant levels in the human intestine. Plasmid-encoded high expression of Cry toxins in biopesticidal Btk strains is very likely to reduce competitive ability and infectious potential in the human gut. In comparison with pathogenic *B. cereus* strains, biopesticidal Btk strains differ significantly in their toxigenic potential, but also in their physiology and their environmental behaviour. This, together with the proven absence of pathogenicity of Btk SA-12 indicates that the risk for consumers following use of the strain for pest control in agricultural settings is acceptable. Since the authorisation of microorganisms is by strains level, no MRL should be set on a link to another species (*B. cereus*) and inclusion in Annex IV of Reg. (EC) No. 396/2005 is strongly supported. Please refer to Vol 3 MA, section B.7, point B.7.3.

[18] 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

	Other metabolites produced by *Bacillus thuringiensis* subsp. *kurstaki* In the External Scientific Report on Literature review and data collection on microbial active substances which can be used for the environmental risk characterisation[19], several potential metabolites were identified for *B. thuringiensis* without any consideration of the subspecies. Since the literature search did not provide any references on any other metabolites than those already described above and Btk is an extensively studied subspecies "other metabolites" are most likely not of any importance.
Endpoint: Information on the production of relevant metabolites (especially toxins)	Btk SA-12 produces Cry1A and Cry2A insecticidal proteins. Apart from the Cry proteins several other insecticidal proteins are produced by Bt (vegetative insecticidal proteins VIP, cytolytic proteins Cyt etc.). Absence of toxicity to humans and mammals from all metabolites involved in the mode of action was confirmed by a literature search. Beta-exotoxins, are considered to have toxic properties but were shown not to be produced by commercial Btk strains. The ability to produce *B. cereus*-enterotoxins and possible consequences for consumers is discussed since first evaluation of the strain. However, based on available knowledge on Btk including Btk SA-12, there is no hint that the strain has the ability to cause foodborne disease as it will not fulfil prerequisites required for pathogenic action in humans.

B.2.9 Antibiotics and other anti-microbial agents

The pattern of sensitivity to 34 antibiotics was determined for the SA-11 and SA-12 strain using the Kirby-Bauer Disc Diffusion Method (Smith & Regan, 1989a and b).

The susceptibility of the strain EG2348 to six clinically used antimicrobials was determined using the National Committee for Clinical Laboratory Standards (NCCLS) disk susceptibility test (Barbera, 1990). In both studies, the *Staphylococcus aureus* strain ATTC 25923 was used as positive control organism.

[19] 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

Table 2.9-1 shows the sensitivity to antibiotics of the three strains.
The larger the inhibition zone, the more susceptible is the strain, and contrary the smaller the inhibition zone, the more resistant is the strain.

Antibiotic	**Zone Diameter (mm) [(2)]**			
	***S. aureus*[(3)]**	**SA-11**	**SA-12**	**EG2348[(4)]**
Amikacin	22-24 (22.7)	24-26 (25.3)	22-24 (23.3)	NR
Ampicillin	30-32 (31.2)	9-10 (9.7)	8-10 (8.7)	NR
Augmentin	28-30 (28.7)	6-16 (11.3)	12-12 (12.0)	NR
Cefamandole	28-30 (29.3)	10-12 (10.7)	9-10 (9.7)	NR
Cefazolin	30-34 (31.3)	8-10 (9.3)	8-9 (8.4)	NR
Cefonicid	24-26 (25.3)	8-8 (8.0)	8-9 (8.4)	NR
Cefoperazone	28-32 (29.3)	20-22 (21.3)	18-21 (19.3)	NR
Cefotaxime	26-28 (26.7)	8-8 (8.0)	8-8 (8.0)	NR
Cefoxitin	24-27 (25.7)	8-10 (8.7)	9-10 (9.7)	NR
Cefuroxime	28-30 (29.0)	6-6 (6.0)	6-6 (6.0)	NR
Ceftazidime	18-19 (18.7)	6.7 (6.0)	6-6 (6.0)	NR
Ceftizoxime	28-30 (28.7)	6-6 (6.0)	6-6 (6.0)	NR
Cephalothin	34-36 (35.0)	10-10 (10.0)	8-10 (9.3)	NR
Chloramphenicol	24-26 (25.0)	24-26 (25.30	23-28 (25.0)	23
Clindamycin	26-30 (28.0)	18-21 (19.7)	20-22 (20.7)	24
Doxycycline	29-32 (30.2)	24-24 (24)	23-26 (24.3)	NR
Erythromycin	14-28 (24.0)	24-25 (24.3)	24-26 (27.7)	26
Gentamicin	23-27 (25.0)	18-22 (20)	20-24 (21.3)	NR

Antibiotic	Zone Diameter (mm) [2]			
	***S. aureus*[3]**	**SA-11**	**SA-12**	**EG2348[4]**
Kanamycin	24-26 (24.5)	20-22 (21.3)	22-22 (22.0)	NR
Methicillin	18-22 (20.0)	6-8 (6.7)	6-6 (6.0)	NR
Minocycline	28-30 (29.5)	18-19 (18.3)	18-20 (19.3)	NR
Moxalactam	22-23 (22.5)	14-15 (14.70	13-15 (14.0)	NR
Nafcillin	16-20 (18.2)	6-6 (6.0)	6-6 (6.0)	NR
Netilmicin	27-30 (28.75)	24-25 (24.7)	23-28 (25.0)	NR
Nitrofurantoin	22-24 (23.0)	25-26 (25.70	20-26 (22.7)	NR
Oxacillin	18-20 (18.7)	6-6 (6.0)	6-6 (6.0)	NR
Penicillin G	32-36 (34.7)	8-9 (8.3)	6-10 (7.7)	0
Streptomycin	18-18 (18.0)	22-22 (22)	22-22 (22.0)	20
Sulfisoxazole	27-30 (28.3)	30-31 (30.3)	28-28 (28.0)	NR
Tetracycline	26-29 (27.7)	20-22 (21.0)	20-20 (20.0)	17
Tobramycin	24-25 (24.3)	20-22 (20.7)	18-20 (18.7)	NR
Trimethoprim	21-22 (21.7)	6-6 (6.0)	6-6 (6.0)	NR
Trimethoprim-sulfamethoxazole	30-30 (30.0)	22-24 (22.70	20-22 (20.7)	NR
Vancomycin	18-19 (18.3)	21-26 (23.7)	20-25 (22.3)	NR

[1] Tests for SA-11 and SA-12 were performed using the Kirby-Bauer disc diffusion method as described in the American Society for Microbiology's *Manual of Clinical Microbiology*. Test of EG2348 was performed using he NCCLS Standard Disk Susceptibility Test of *Bacillus thuringiensis* strains.

[2] Top number for each antibiotic is the range of zone diameter values, bottom number in parenthesis is the average value for all tests. In general, at least three tests were run for each antibiotic.

[3] Control Strain, *Staphylococcus aureus* (ATCC 25923)

[4] Average of two plates

NR: not reported

New data

Available data have not been fully compliant with the EFSA guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance[20]. Therefore, a new study on antibiotic resistance including MIC determination is provided for renewal of Btk SA-12 under Regulation (EC) No 1107/2009. Btk SA-12 has been shown to be sensitive to all relevant antibiotics as provided in the new EFSA FEEDAP Panel guidance document on the characterisation of microorganisms used as feed additives or as production organisms (EFSA, 2018)[21]. The summary is presented below. The strain is not multi-resistant and can be proposed for approval as low risk active substance.

Report:	KMA 2.9/01; Shaw, G. (2019) Antibiotic resistance and sensitivity testing of the Microbial Pest Control Agent *Bacillus thuringiensis* subsp. *kurstaki* strain SA-12 Unpublished Report No: APIS-016-002
Guideline(s):	EFSA FEEDAP Panel. Guidance on the characterisation of microorganisms used as feed additives or as production organisms. EFSA Journal 2018; 16(3):5206 CLSI Methods for dilutional antimicrobial susceptibility test for bacteria that grow aerobically: Approved standard – tenth edition.
Deviations:	None
GLP:	Yes
Acceptability:	Acceptable
Duplication: **(if vertebrate study)**	No

Executive summary

This study was conducted to determine the antibiotic resistance and sensitivity profile of *Bacillus thuringiensis* subsp. *kurstaki* strain SA-12. *Bacillus thuringiensis* subsp. *kurstaki* strain SA-12 showed sensitivity to all eight antibiotics tested. All the validity criteria in the study were met and the study was deemed to be valid.

Material and Methods

Test Item

Designation	*Bacillus thuringiensis* subsp. *kurstaki* strain SA-12
Formulation type	Live culture on nutrient agar slant
Batch no.	10-12-18
Expiry date	12th Oct 2023
Storage temperature	4ºC

Study Design and Methods

[20] 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

[21] EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), Rychen G, Aquilina G, Azimonti G, Bampidis V, Bastos ML, Bories G, Chesson A, Cocconcelli PS, Flachowsky G, Gropp J, Kolar B, Kouba M, Lopez-Alonso M, Lopez Puente S, Mantovani A, Mayo B, Ramos F, Saarela M, Villa RE, Wallace RJ, Wester P, Glandorf B, Herman L, Kärenlampi S, Aguilera J, Anguita M, Brozzi R and Galobart J, 2018. Guidance on the characterisation of microorganisms used as feed additives or as production organisms. EFSA Journal 2018;16(3):5206, 24 pp. https://doi.org/10.2903/j.efsa.2018.5206

Microdilution assays have been used to determine the Minimum Inhibitory Concentration (MIC) of relevant antibiotics as stated in EFSA Guidance on the characterisation of microorganisms used as feed additives or as production organisms. Microdilution plates were prepared with Cation Adjusted Mueller Hinton Broth (CAMHB). The test was set up in triplicate. Each triplicate included:

a) Blank control (uninoculated CAMHB), to ensure sterility of the medium

b) Positive growth control (test item in CAMHB), to assure optimal growth conditions for Btk strain SA-12

c) Dilution series of each antibiotic inoculated with QC organism (*Bacillus subtilis* ATCC 6633) to ensure activity of the antibiotics tested

d) Dilution series of each antibiotic inoculated with test item.

The QC organism and test item were inoculated at 5 x 10^5 cfu/mL. The antibiotics (all obtained from Sigma Aldrich) have been tested at 1024, 512, 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5, and 0.25 µg/mL. The plates were inoculated at 35°C for 19.5 hours under aerobic conditions. Microdilution plates were assessed to ascertain the antimicrobial concentration at which growth was inhibited as observed by the unaided eye. This concentration was stated as the MIC and used to determine bacterial resistance or sensitivity according to EFSA guidance.

The following criteria were used to assess the validity of the study.

- No growth in any blank control wells
- Satisfactory growth of a bacterial button ≥2mm in all positive growth control wells
- The quality control strains shows sensitivity as outlined in the EFSA guidance document

Results

Table 2.9-1. Result summary for the Btk strain SA-12 antibiotic resistance and sensitivity profile

Antibiotic	QC organism ATCC 6633 MIC [µg/mL]	*Bacillus thuringiensis kurstaki* strain SA-12 MIC [µg/mL]	EFSA Microbiological cut-off values [µg/mL]	Sensitivity / Resistance
Chloramphenicol (Macrolide group)	4	2	8	Sensitive
Clindamycin (Lincosaminde group)	2	0.25	4	Sensitive
Erythromycin (Macrolide group)	0.125	0.125	4	Sensitive
Gentamycin (Aminoglycoside group)	1	1	4	Sensitive
Kanamycin (Aminoglycoside group)	4	4	8	Sensitive
Streptomycin (Aminoglycoside group)	4	4	8	Sensitive
Tetracycline (Tetracycline group)	2	1	8	Sensitive
Vancomycin (Glycopeptide group)	1	1	4	Sensitive

Conclusions

Bacillus thuringiensis subsp. *kurstaki* strain SA-12 was observed to be sensitive to all eight antibiotics tested in this study. As all the validity criteria in the study were met, the study was deemed to be valid.

RMS evaluation and conclusion.	In the DAR 2008 the pattern of sensitivity to 34 antibiotics was determined for Btk strains SA-11 and SA-12 using the Kirby-Bauer Disc Diffusion Method. The susceptibility of the strain EG2348 to 10 clinically important antimicrobials was determined using the National Committee for Clinical Laboratory Standards (NCCLS) disk susceptibility test and BD BBL Sensi-Disc Antimicrobial Susceptibility Test Discs from VWR. In all studies, the *Staphylococcus aureus* strain ATTC 25923 was used as positive control organism. These data demonstrate that Btk strains SA-11, SA-12 and EG 2348 show sufficient susceptibility to a wide range of clinically important antibiotics of different groups. However, these data are not in compliance with the EFSA guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance[22] because results are not given as cut-off values (mg/L). According to EFSA FEEDAP guidance 2018 (2.2.1) (EFSA, 2018)[23] "Qualitative or semi-quantitative methods to determine MIC indirectly, such as diffusion methods, are not acceptable except in specific and justified circumstances (e.g. when the antimicrobial is not otherwise available)". Therefore, a new study on antibiotic resistance including MIC determination is provided for renewal of Btk SA-12 under Regulation (EC) No 1107/2009. Btk strain SA-12 was observed to be sensitive to all eight antibiotics relevant for *Bacillus* sp. as mentioned in the new EFSA FEEDAP Panel guidance document on the characterisation of microorganisms used as feed additives or as production organisms (EFSA, 2018). As all the validity criteria in the study were met, the study was deemed to be valid. According to Commission Regulation (EU) 2017/1432 amending Regulation (EC) No 1107/2009 criteria for the approval of low-risk active substances are laid down as follows: "*A micro-organism may be considered to be of low-risk unless at strain level it has demonstrated multiple resistance to antimicrobials used in human or veterinary medicine*." At the moment no guidance exists on how to demonstrate "*multiple resistance to antimicrobials used in human or veterinary medicine"*. The EFSA feedstuff guidance document aims at assisting applicants in the preparation and presentation of dossier for feed additives containing microorganisms or produced with microorganisms by fermentation. It has to be noted that recommendations and requirements laid down in the EFSA guidance refer to strains used in food and feed and the intent is to avoid that these strains add to the pool of antimicrobial resistance genes already present in the **gut bacterial population.** This could contribute to the spread of antimicrobial resistance to pathogens presenting a serious safety concern. However, use of a *Bacillus* strain as food and feed additive leads to high exposure to gut microbes and eventually also to human and animal pathogens, while possible ways of contact and interaction of a biocontrol strain with gut microbiota and pathogens are incomparable to that. Therefore, the risk related to antibiotic resistance found in a biocontrol strain should not be considered the same as for feed and food additives. Nevertheless, the EFSA guidance document as such provides useful information about antimicrobials which are relevant for use in humans and animals (critically important antimicrobials (CIAs) or highly important antimicrobials (HIAs), last revision WHO, 2016) which could be also assessed in phenotypic testing of biocontrol strains (if affiliating with the species/genus mentioned in the guidance document). It is proposed that *Bacillus thuringiensis* subsp. *kurstaki* strain SA-12 shall be considered of low risk. Btk SA-12 has been shown to be sensitive to a broad range of antibiotics commonly used in human and veterinary medicine. Data on

[22] 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

[23] EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), Rychen G, Aquilina G, Azimonti G, Bampidis V, Bastos ML, Bories G, Chesson A, Cocconcelli PS, Flachowsky G, Gropp J, Kolar B, Kouba M, Lopez-Alonso M, Lopez Puente S, Mantovani A, Mayo B, Ramos F, Saarela M, Villa RE, Wallace RJ, Wester P, Glandorf B, Herman L, Kärenlampi S, Aguilera J, Anguita M, Brozzi R and Galobart J, 2018. Guidance on the characterisation of microorganisms used as feed additives or as production organisms. EFSA Journal 2018;16(3):5206, 24 pp. https://doi.org/10.2903/j.efsa.2018.5206

	the antibiotic sensitivity tests of Btk strain SA-12 which are compliant with the EFSA feedstuff guidance document are considered acceptable to cover current requirements. Btk SA-12 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). In conclusion, the strain is not multi-resistant to antimicrobials used in human or veterinary medicine and can be proposed for approval as low risk active substance.
Endpoint: Resistance/ sensitivity to antibiotics / anti-microbial agents used in human or veterinary medicine	Btk SA-12 has been shown to be sensitive to a broad range of antibiotics commonly used in human and veterinary medicine. The strain is not multi-resistant and can be proposed for approval as low risk active substance.

B.2.10 References relied on

Several literature review reports have been provided according to the guidance of EFSA (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). The aim of these reports was to provide a global overview of peer-reviewed literature concerning potential side effects of *B. thuringiensis* subsp. *kurstaki* strain SA-12.

Overview of literature reports provided according to the guidance of EFSA

Data point	Author	Year	Title	Section of RMS evaluation
KMA 2.7/12 & 3.5/06	Süß, J.	2016	Literature review on *B. thuringiensis* subsp. *kurstaki* strain SA-12: Biological properties	Vol. 3MA, B.2.10
KMA 6.1.1/07	Seehase, S.	2016	Literature review on *B. thuringiensis* subsp. *kurstaki* strain SA-12: Toxicology	Vol. 3MA, B.6.3
KMA 7/01	Cornelese, A.	2016a	Literature review on *B. thuringiensis* subsp. *kurstaki* strain SA-12 and metabolites: Residues in or on treated products, food and feed	Vol. 3MA, B.7.3
KMA 8.1/10	Cornelese, A.	2016b	Literature review on *B. thuringiensis* subsp. *kurstaki* strain SA-12: Fate and behavior in the environment	Vol. 3MA, B.8.3
KMA 9/01	Schöbinger, U.	2016	Literature review on *B. thuringiensis* subsp. *kurstaki* strain SA-12: Effects on non-target organisms	Vol. 3MA, B.9.8

Literature review on *Bacillus thuringiensis* ssp. *kurstaki* SA-12, Section 1: Biological properties" (Süß, 2016)

RMS comments on the literature search: "Literature review on *B. thuringiensis* subsp. *kurstaki* strain SA-12: Biological properties". (Süß, J., 2016; submitted in Point KMA 2.7/12 and 5.1/01)	The review was made in order to identify scientific peer-reviewed open literature on the active substance *Bacillus thuringiensis* subsp. *kurstaki* SA-12 with respect to the genetic stability and the possible development of resistances in the target pests. The search strategy was based on a multi-concept approach. **Relevance criteria** • Property investigated was relevant for data requirements of Regulation (EC) 1107/2009 • Identification of the species and subspecies referred to as *Bacillus thuringiensis* subsp. *kurstaki* • Relevant information on the development of resistance in the target pests • Relevant information on the genetic stability of *Bacillus thuringiensis* subsp. *kurstaki* **Database searched** A search was conducted using the DIMDI database provided by the German Institute of Medical Documentation and comprised of searches in MEDLINE, BIOSIS, CAB and SCISEARCH databases **Search methods** Search strategy aimed to find all recent (from 2006 onwards) references relevant for each of the topics: genetic stability and development of resistance. The following keywords were used in the searches: Genetic stability: *Bacillus thuringiensis 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: *Bacillus thuringiensis* AND *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. **Results of the study selection process** In total 305 references were retrieved and 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 total 21 references were identified as being potentially relevant. These references were subjected to a full-text assessment in Step 2. 16 references were finally classified as relevant and supportive and are included in the dossier. From a total 64 obtained references, by the seach for genetic stability, five were subjected to full text assessment and four were considered relevant and reliable and are included in the dossier. By the seach for development of resistance a total of 241 hits were found, 16 were submitted to full text analysis and 12 were finally considered relevant and reliable and are included in the dossier.
Conclusion	The literature search regarding data of biological properties was accepted as valid, both regarding inclusion of databases and use of search terms. By the seaches for “genetic stability” and “development of resistance” four and 12 references were finally considered relevant and reliable and are included in the dossier, respectively.

Data point	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	Previous evaluation
KMA 2.1.1 /01	Ishiwata S,	1901	ON A KIND OF SEVERE FLACHERIE (SOTTO DISEASE). Dainihon Sanshi Kaiho 9, 1-5 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.1.1 /02	Martin, P.A.W., Travers, R.S.	1989	WORLDWIDE ABUNDANCE AND DISTRIBUTION OF BACILLUS THURINGIENSIS ISOLATES Applied and Environmental Microbiology, 55(10), 2437-2442 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.1.1 /03	Delucca, A.J., Simonson, J.G., Larson, A.D.	1981	BACILLUS THURINGIENSIS DISTRIBUTION IN SOILS OF THE UNITED STATES Can. J. Microbiol., 27: 865-870 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.1.1 /04	Ohba, M., Aizawa, K.	1986	DISTRIBUTION OF BACILLUS THURINGIENSIS ON SOILS OF JAPAN Journal of Invertebrate Pathology, Volume 47, pp. 277-282 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.1.1 /05	Brownbridge, M., Margalit, J.	1986	NEW BACILLUS THURINGIENSIS STRAINS ISOLATED IN ISRAEL HIGHLY TOXIC TO MOSQUITO LARVAE Journal of Invertebrate Pathology 48, p. 216-222 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.1.1 /06	Asimeng, E.J, Mutinga, M.J.	1992	ISOLATION OF MOSQUITO-TOXIC BACTERIA FROM MOSQUITO-BREEDING SITES IN KENYA Journal of the American Mosquito Control Association, 8:86-88 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008

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KMA 2.1.1 /07	Dulmage, H.T.	1970	INSECTICAL ACTIVITY OF HD-1, A NEW ISOLATE OF BACILLUS THURINGIENSIS VAR. ALESTI Journal of Invertebrate Pathology, Volume 15, pp. 232-239 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.1.1 /08	Delucca, A.J., Palmgren, M.S., Ciegler, A.	1982	BACILLUS THURINGIENSIS IN GRAIN ELEVATOR DUSTS Can. J. Microbiol., 28:452-456 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.1.1 /09	Smith, R.A., Couche, G.A.	1991	THE PHYLLOPLANE AS A SOURCE OF BACILLUS THURINGIENSIS VARIANTS Applied and Environmental Microbiology, 57(1): 331-315 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.1.1 /10	Feitelson, J.F., Payne, J., Kim, L.	1992	BACILLUS THURINGIENSIS: INSECTS AND BEYOND Biotechnology Vol. 10 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.1.1 /11	de Castilhos-Fortes, R., Matsumura, A.T.S., Diehl E., Fiuza, L.M.	2002	SUSCEPTIBILITY OF NASUTITERMES EHRHARDTI (ISOPTERA: TERMITIDAE) TO BACILLUS THURINGIENSIS SUBSPECIES Braz. J. Microbiol. 2002, 33(3): 219-222 Report-no.: not applicable GLP/GEP: no Published:	no	no	not protected	-	DAR 2008
KMA 2.1.1 /12	Glare, T.R., O'Callaghan, M.	2000	BACILLUS THURINGIENSIS: BIOLOGY, ECOLOGY AND SAFETY in Bacillus thuringiensis: Biology, Ecology and Safety, Glare, T.R., O'Callaghan, M. (eds.) John Wiley & Sons, Ltd. Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.1.1 /13	Travers, R.S., Martin, P.A.W., Rewichelderfer, C.F.	1987	SELECTIVE PROCESS FOR EFFICIENT ISOLATION OF SOIL BACILLUS SPP. Applied and Environmental Microbiology, 53(6): 1263-1266 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008

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KMA 2.1.1 /14	Chilcott, C. N., Wigley, P. J.	1988	TECHNICAL NOTE: AN IMPROVED METHOD FOR DIFFERENTIAL STAINING OF BACILLUS THURINGIENSIS CRYSTALS. Lett. Appl. Microbiol., 7: 67-70 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.1.1 /15	Bravo, A., Likitvivatanavong, S., Gill, S.S., Soberon, M.	2011	BACILLUS THURINGIENSIS: A STORY OF A SUCCESSFUL BIOINSECTICIDE. Insect Biochemistry and Molecular Biology 41(7):423-431 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	New data for active ingredient, not previously submitted nor evaluated
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 Journal of Invertebrate Pathology, Volume 70, pp. 59-68 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.1.2 /02	Chaufaux, J., Marchal, M., Gilois, N., Jehanno, I., Buisson, C.	1997	RECHERCHE DE SOUCHES NATURELLES DU BACILLUS THURINGIENSIS DANS DIFFERENTS BIOTOPES, A TRAVERS LE MONDE Report-no.: not applicable GLP/GEP: no Published: no	no	no	not protected	-	DAR 2008
KMA 2.1.2 /03	Martin, P.A.W., Travers, R.S.	1989	WORLDWIDE ABUNDANCE AND DISTRIBUTION OF BACILLUS THURINGIENSIS ISOLATES Applied and Environmental Microbiology, 55(10): 2437-2442 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected		DAR 2008

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KMA 2.1.2 /04	Landén, R., Bryne, M., Abdel-Hameed, A.	1994	DISTRIBUTION OF BACILLUS THURINGIENSIS STRAINS IN SOUTHERN SWEDEN World Journal of Microbiology & Biotechnology, Vol 10, pp. 45-50 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.1.2 /05	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 System. Appl. Microbiol. 21, 97-106 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.1.2 /06	Bel, Y., Granero, F., Alberola, T.M., Martínez-Sebasdtián, M., Ferré, J.	1997	DISTRIBUTION, FREQUENCY AND DIVERSITY OF BACILLUS THURINGIENSIS IN OLIVE TREE ENVIRONMENTS IN SPAIN System. Appl. Microbiol. 20, 652-658 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.1.2 /07	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 J. Gen. Appl. Microbiol., 44, 133-138 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.1.2 /08	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) Neotropical Entomology 32(4): 639-644 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008

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KMA 2.1.2 /09	Vásquez, M., Parra, C., Hubert, E., Espinoza, P., Theoduloz, C., Meza-Basso, L.	1995	SPECIFICITY AND INSECTICIDAL ACTIVITY OF CHILEAN STRAINS OF BACILLUS THURINGIENSIS Journal of Invertebrate Pathology 66, 143-148 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.1.2 /10	Ohba, M., Aizawa, K.	1978	SEROLOGICAL IDENTIFICATION OF BACILLUS THURINGIENSIS AND RELATED BACTERIA ISOLATED IN JAPAN Journal of Invertebrate Pathology, Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.1.2 /11	Damgaard, P.H., Hansen, B.M., Pedersen, J.C., Eilenberg, J.	1997a	NATURAL OCCURRENCE OF BACILLUS THURINGIENSIS ON CABBAGE FOLIAGE AND IN INSECTS ASSOCIATED WITH CABBAGE CROPS not applicable Journal of Applied Microbiology, 82, 253-258 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.1.2 /12	Damgaard, P.H., Abdel-Hameed, A., Eilenberg, J., Smits, P.H.	1998	NATURAL OCCURRENCE OF BACILLUS THURINGIENSIS ON GRASS FOLIAGE World Journal of Microbiology & Biotechnology, 14: 239-242 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.1.2 /13	Smith, R.A., Couche, G.A.	1991	THE PHYLLOPLANE AS A SOURCE OF BACILLUS THURINGIENSIS VARIANTS Applied and Environmental Microbiology, 57(1): 331-315 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
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KMA 2.1.2 /16	Dulmage, H.T.	1970	INSECTICAL ACTIVITY OF HD-1, A NEW ISOLATE OF BACILLUS THURINGIENSIS VAR. ALESTI Journal of Invertebrate Pathology, Volume 15, pp. 232-239 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.2.2 /01	Höfte, H., Whiteley, H.R.	1989	INSECTICIDAL CRYSTAL PROTEINS OF BACILLUS THURINGIENSIS Microbiological Reviews, June 1989, p. 242-255 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.2.2 /02	Feitelson, J.F., Payne, J., Kim, L.	1992	BACILLUS THURINGIENSIS: INSECTS AND BEYOND Biotechnology Vol. 10 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.2.2 /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 Proc Natl Acad Sci USA, 100: 2760-2765 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.2.2 /04	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 Report-no.: not applicable GLP/GEP: no Published: no	no	no	not protected	-	DAR 2008

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KMA 2.2.2 /05	Hofmann, C., Vanderbruggen, H., Höfte, H., van Rie, J., Jansens, S., van Mellaert, H.	1988a	SPECIFICITY OF BACILLUS THURINGIENSIS ENDOTOXINS IS CORRELATED WITH THE PRESENCE OF HIGH-AFFINITY BINDING SITES IN THE BRUSH BORDER MEMBRANE OF TARGET INSECT MIDGUTS Proc. Natl. Acad. USA Vol. 85, pp. 7844-7848 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.2.2 /06	Hofmann, C., Lüthy, P., Hütter, R., Pliska, V.	1988b	BINDING OF THE DELTA ENDOTOXIN FROM BACILLUS THURINGIENSIS TO BRUSH-BORDER MEMBRANE VESICLES OF THE CABBAGE BUTTERFLY (PIERIS BRASSICAE) Eur. J. Biochem. 173, 85-91 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.2.2 /07	Schwartz, J.L., Garneau, L., Masson, L., Brousseau, R.	1991	EARLY RESPONSE OF CULTURED LEPIDOPTERAN CELLS TO EXPOSURE TO ENDOTOXIN FROM BACILLUS THURINGIENSIS: INVOLVEMENT OF CALCIUM AND ANIONIC CHANNELS Biochimica et Biophysica Acta, 1065, 250-260 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.2.2 /08	Broderick, N.A., Raffa, K.F., Handelsman J	2006	MIDGUT BACTERIA REQUIRED FOR BACILLUS THURINGIENSIS INSECTICIDAL ACTIVITY Proc Natl Acad Sci USA 103(41): 15196-15199 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.2.2 /09	Yamamoto T., Chen, C.-Y.	2006	DELTA-ENDOTOXIN COMPONENT ANALYSIS OF DELFIN, COSTAR AND CONDOR CERTIS USA LLC Report-no.: not applicable GLP/GEP: no Published: no	no	yes	protected	Certis USA	DAR 2008

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KMA 2.2.2 /10	Yamamoto, T., Iizuka, T.	1983	TWO TYPES OF ENTOMOCIDAL TOXINS IN THE PARASPORAL CRYSTALS OF BACILLUS THURINGIENSIS KURSTAKI Archives of Biochemistry and Biophysics Vol. 227, No.1, pp. 233-241 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.2.2 /11	Luo, K., Banks, D., Adang, M.J.	1999	TOXICITY, BINDING AND PERMEABILITY ANALYSES OF FOUR BACILLUS THURINGIENSIS CRYL ENDOTOXINS USING BRUSH BORDER MEMBRANE VESICLES OF SPODOPTERA EXIGUA AND SPODOPTERA FRUGIPERDA Applied and Environmental Microbiology, 65(2): 457-464 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.2.2 /12	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 Applied and Environmental Microbiology, 66(4): 1553-1558 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.2.2 /13	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 Applied and Environmental Microbiology, 68(4): 1509-1515 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.2.2 /14	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 Journal of Invertebrate Pathology Volume 18, pp. 240-245 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008

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KMA 2.2.2 /15	Aronson, A.I.	1993	THE TWO FACES OF BACILLUS THURINGIENSIS: INSECTICIDAL PROTEINS AND POST-EXPONENTIAL SURVIVAL Molecular Microbiology 7: 489-496 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.2.2 /16	Burges, H.D.	1982	CONTROL OF INSECTS BY BACTERIA. Parasitology 84: 79-117 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.2.2 /17	Griego, V.M., Spence, K.D.	1978	INACTIVATION OF BACILLUS THURINGIENSIS SPORES BY ULTRAVIOLET AND VISIBLE LIGHT Applied and Environmental Microbiology, 35(5): 906-910 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.2.2 /18	Pusztai, M., Fast, P., Gringorten, L., Kaplan, H., Lessard, T., Carey, P.R.	1991	THE MECHANISM OF SUNLIGHT-MEDIATED INACTIVATION OF BACILLUS THURINGIENSIS CRYSTALS Biochem. J. 273, p. 43-47 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.2.2 /19	Broderick, N.A., Robinson, C.J., McMahon, M.D., Holt, J., Handelsman, J., Raffa, K.F.	2009	CONTRIBUTIONS OF GUT BACTERIA TO BACILLUS THURINGIENSIS-INDUCED MORTALITY VARY ACROSS A RANGE OF LEPIDOPTERA BMC Biology, 7(11): 1-9 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	New data for active ingredient, not previously submitted nor evaluated
KMA 2.2.2 /20	Palma, L., Munoz, D., Berry, C., Murillo, J., Caballero, P.	2014	BACILLUS THURINGIENSIS TOXINS: AN OVERVIEW OF THEIR BIOLOGICAL ACTIVITY Toxins, 6(12), 3296-3325 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	New data for active ingredient, not previously submitted nor evaluated
KMA 2.3 /01	Höfte, H., Whiteley, H.R.	1989	INSECTICIDAL CRYSTAL PROTEINS OF BACILLUS THURINGIENSIS Microbiological Reviews, p. 242-255 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008

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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 Report-no.: not applicable GLP/GEP: no Published: no	no	no	not protected	-	DAR 2008
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 Applied and Environmental Microbiology, 57(4): 981-986 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.3 /04	Jaquet, F., Hütter, R, Lüthy, P.	1987	SPECIFITY OF BACILLUS THURINGIENSIS DELTA-ENDOTOXIN Appl. and Environ. Microbiol. 53(3): 500-504 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.3 /05	McGaughey, W.H., Whalon, M.E.	1992	MANAGING INSECT RESISTANCE TO BACILLUS THURINGIENSIS TOXINS Science 258, pp. 1451-1455 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.3 /06	Haider, M.Z., Knowles. B.H., Ellar, D.J.	1986	SPECIFITY OF BACILLUS THURINGIENSIS VAR. COLMERI INSECTICIDAL Eur. J. Biochem. 156, p. 531-540 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.3 /07	Honée, G. and Visser, B.	1993	THE MODE OF ACTION OF BACILLUS THURINGIENSIS CRYSTAL PROTEINS Entomol. exp. appl. 69, p. 145-155 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008

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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 J. Exp. Biol. 161: 519-525 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.3 /09	Cerf, D.C.	1990	SUSCEPTIBILITY OF FOUR ORDERS OF INSECTS (LEPIDOPTERA, DIPTERA, COLEOPTERA, AND ORTHOPTERA) TO TECHNICAL GRADE ACTIVE INGREDIENTS (TGAI'S), MANUFACTURING PRODUCTS (MP'S) AND END-USE PRODUCTS (EP'S) PRODUCED FROM FERMENTATION OF BACILLUS THURINGIENSIS Analyt. Services, [redacted] Palo Alto, CA Certis USA LLC, Columbia Report-no.: 90/03/12 GLP/GEP: yes Published: no	no	no	not protected	Certis USA	DAR 2008
KMA 2.3 /10	WHO-EHC	1999	ENVIRONMENTAL HEALTH CRITERIA 217 – MICROBIAL PEST CONTROL AGENT BACILLUS THURINGIENSIS World Health Organization. Geneva Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.3 /11	Siegel, J.P.	2001	THE MAMMALIAN SAFETY OF BACILLUS THURINGIENSIS-BASED INSECTICIDES Journal of Invertebrate Pathology 77, 13-21 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008

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KMA 2.4 /01	Griego, V.M., Spence, K.D.	1978	INACTIVATION OF BACILLUS THURINGIENSIS SPORES BY ULTRAVIOLET AND VISIBLE LIGHT Applied and Environmental Microbiology, 35(5): 906-910 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
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 Biochem. J. 273, p. 43-47 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
2.4 /03	WHO-EHC	1999	ENVIRONMENTAL HEALTH CRITERIA 217 – MICROBIAL PEST CONTROL AGENT BACILLUS THURINGIENSIS World Health Organization. Geneva Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.4 /04	Bravo, A.	1997	PHYLOGENETIC RELATIONSHIPS OF BACILLUS THURINGIENSIS Journal of Bacteriol. 179, p. 2793-2801 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.4 /05	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 Proc. Natl. Acad. Sci. 93, p. 5389-5394 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.4 /06	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 Appl. and Environ. Microbiol. 63(2): 532-536 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008

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KMA 2.4 /07	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 Appl. and Environ. Microbiol. 67(12): 5855-5858 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.4 /08	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 Microbiol Mol Biol Rev, 62(3): 775-806 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.5 /01	Aronson, A.I.	1993	THE TWO FACES OF BACILLUS THURINGIENSIS: INSECTICIDAL PROTEINS AND POST-EXPONENTIAL SURVIVAL Molecular Microbiology 7: 489-496 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.5 /02	Burges, H.D.	1982	CONTROL OF INSECTS BY BACTERIA. Parasitology 84: 79-117 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.5 /03	Griego, V.M., Spence, K.D.	1978	INACTIVATION OF BACILLUS THURINGIENSIS SPORES BY ULTRAVIOLET AND VISIBLE LIGHT Applied and Environmental Microbiology, 35(5): 906-910 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.5 /04	Pusztai, M., Fast, P., Gringorten, L., Kaplan, H., Lessard, T., Carey, P.R.	1991	THE MECHANISM OF SUNLIGHT-MEDIATED INACTIVATION OF BACILLUS THURINGIENSIS CRYSTALS Biochem. J. 273, p. 43-47 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008

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KMA 2.5 /05	Hendriksen, N.B., Hansen, B.M.	2002	LONG-TERM SURVIVAL AND GERMINATION OF BACILLUS THURINGIENSIS VAR. KURSTAKI IN A FIELD TRIAL Canadian Journal of Microbiology 48: 256-261 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.5 /06	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 Journal of Invertebrate Pathology, Vol. 70, pp. 59-68 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.5 /07	West, A.W., Burges, H.D., Dixon, T.J, Wyborn, C.H.	1985	SURVIVAL OF BACILLUS THURINGIENSIS AND BACILLUS CEREUS SPORE INOCULA IN SOIL: EFFECTS OF PH, MOISTURE, NUTRIENT AVAILABILITY AND INDIGENOUS MICROORGANISMS Soil Biol. Biochem, Vol. 17, p. 657-665 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.5 /08	Saleh, S.M., Harris, R.F., Allen, O.N.	1970	FATE OF BACILLUS THURINGIENSIS IN SOIL: EFFECT OF SOIL PH AND ORGANIC AMENDMENT Can. J. Microbiol. 16, p. 677-680 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.5 /09	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 MICROORGANISM FEMS Microbiology Ecology 31, p. 255-259 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008

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KMA 2.5 /10	West, A.W., Burges H.D.	1985	PERSISTENCE OF BACILLUS THURINGIENSIS AND BACILLUS CEREUS IN SOIL SUPPLEMENTED WITH GRASS OR MANURE Plant and Soil 83, p. 388-398 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.5 /11	Pruett, C.J.H., Burges, H.D., Wyborn, C.H.	1980	EFFECT OF EXPOSURE TO SOIL ON POTENCY AND SPORE VIABILITY OF BACILLUS THURINGIENSIS Journal of Invertebrate Pathology 35, p. 168-174 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.5 /12	Petras, S.F., Casida, L.E.	1985	SURVIVAL OF BACILLUS THURINGIENSIS SPORES IN SOIL Appl. And Environ. Microbiol. P. 1496-1501 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.5 /13	Pedersen, J.C., Damgaard, P.H., Eilenberg, J., Hansen, B.M.	1995	DISPERSAL OF BACILLUS THURINGIENSIS VAR. KURSTAKI IN AN EXPERIMENTAL CABBAGE FIELD Can. J. Microbiol. 41, p. 118-125 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.5 /14	Addison, J.A.	1993	PERSISTENCE AND NONTARGET EFFECTS OF BACILLUS THURINGIENSIS IN SOIL – A REVIEW Canadian Journal of Forest Research - Revue Canadienne De Recherche Forestiere 23 (11): 2329-2342 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.5 /15	West, A.W. Burges, H.D., Wyborn, C.H.	1984a	EFFECT OF INCUBATION IN NATURAL AND AUTOCLAVED SOIL UPON POTENCY AND VIABILITY OF BACILLUS THURINGIENSIS Journal of Invertebrate Pathology 44, p. 121-127 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008

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KMA 2.5 /16	West, A.W., Burges, H.D., White, R.J., Wyborn, C.H.	1984b	PERSISTENCE OF BACILLUS THURINGIENSIS PARASPORAL CRYSTAL INSECTICIDAL ACTIVITY IN SOIL Journal of Invertebrate Pathology 44, p. 128-133 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.5 /17	Akiba, Y.	1986	MICROBIAL ECOLOGY OF BACILLUS THURINGIENSIS VI. GERMINATION OF BACILLUS THURINGIENSIS SPORES IN THE SOIL Appl. Ent. Zool. 21 (1): 76-80 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.5 /18	Donnellan, J.E., Stafford, R.S.	1968	THE ULTRAVIOLET PHOTOCHEMISTRY AND PHOTOBIOLOGY OF VEGETATIVE CELLS AND SPORES OF BACILLUS MEGATERIUM Biophys. J. 8:17-27 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.5 /19	Mason, J.M., Setlow, P.	1986	ESSENTIAL ROLE OF SMALL, ACID.SOLUBLE SPORE PROTEINS IN RESISTANCE OF BACILLUS SUBTILIS SPORES TO UV LIGHT J. of Bacteriology 167, p. 174-178 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.5 /20	Setlow, P.	1988	SMALL, ACID-SOLUBLE SPORE PROTEINS OF BACILLUS SPECIES: STRUCTURE, SYNTHESIS, GENETICS, FUNCTION AND DEGRADATION Ann. Rev. Microbiol. 42, p. 319-383 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008

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KMA 2.5 /21	Germaine, G.R., Murrell, W.G.	1973	EFFECT OF DIPICOLINICACID ON THE ULTRAVIOLET RADIATION RESISTANCE OF BACILLUS CEREU SPORES. Photochem. Photobiol. 17:145-154 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.5 /22	Ignoffo, C.M.	1992	ENVIRONMENTAL FACTORS AFFECTING PERSISTENCE OF ENTOMOPATHOGENS The Florida Entomologist, 75(4), 516-525 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.5 /23	Dent, D.R.	1993	THE USE OF BACILLUS THURINGIENSIS AS AN INSECTICIDE In: Exploitation of Microorganisms, D.G. Jones (ed.), Chapman & Hall, London Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.5 /24	Lüthy, P.	1986	INSECT PATHOGENIC BACTERIA AS PEST CONTROL AGENTS Fortschr. Zool., 32:201 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.5 /25	Reardon, R.C., Haissig, K.	1983	SPRUCE BUDWORM (LEPIOPTERA:TORTRICIDAE) LARVAL POPULATIONS AND FIELD PERSISTENCE OF BACILLUS THURINGIENSIS AFTER TREATMENT IN WISCONSIN Journal of Eco. Entomol. 76, p. 1139-1143 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.5 /26	Jarret, P., Stephenson, M.	1990	PLASMID TRANSFER BETWEEN STRAINS OF BACILLUS THURINGIENSIS INFECTING GALLERIA MELLONELLA AND SPODOPTERA LITTORALIS Appl. And Environ. Microbiol. 56(6): 1608-1614 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008

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KMA 2.5 /27	Sorensen, A.A., Falcon, L.A.	1980	COMPARISON OF MICRODROPLET AND HIGH VOLUME APPLICATION OF BACILLUS THURINGIENSIS ON PEAR: SUPPRESSION OF FRUIT TREE LEAFROLLER (ARCHIPS ARGYROSPILUS) AND COVERAGE ON FOLIAGE AND FRUIT Environ. Entomol. 9, p. 350-358 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.5 /28	Beegle, C.C., Dulmage, H.T., Wolfenbarger, D.A., Martinez, M.	1981	PERSISTENCE OF BACILLUS THURINGIENSIS BERLINER INSECTICIDAL ACTIVITY ON COTTON FOLIAGE Environ. Entomol. 10, p. 400-401 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.5 /29	WHO-EHC	1999	ENVIRONMENTAL HEALTH CRITERIA 217 – MICROBIAL PEST CONTROL AGENT BACILLUS THURINGIENSIS World Health Organization. Geneva Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.5 /30	Leong, K.L.H., Cano, R.J., Kubinski, A.M.	1980	FACTORS AFFECTING BACILLUS THURINGIENSIS TOTAL FIELD PERSISTENCE Environmental Entomology, Volume 9, pp. 593-599 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.5 /31	Furlaneto, L., Saridakis, H.O., Arantes, O.M.N.	2000	SURVIVAL AND CONJUGAL TRANSFER BETWEEN BACILLUS THURINGIENSIS STRAINS IN AQUATIC ENVIRONMENT Brazilian Journal of Microbiology, Volume 31, No. 4, pp. 233-238 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.5 /32	Menon, A.S., De Mestral, J.	1985	SURVIVAL OF BACILLUS THURINGIENSIS VAR. KURSTAKI IN WATERS Water, Air, and Soil Pollution 25, p. 265-274 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008

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KMA 2.5 /33	Ohana, B., Margalit, J., Barak, Z.	1987	FATE OF BACILLUS THURINGIENSIS SUBSP. ISRAELENSIS UNDER SIMULATED FIELD CONDITIONS Appl. And Environ. Microbiol. 53(4), 828-831 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.6 /01	Drobniewski, F.A.	1993	BACILLUS CEREUS AND RELATED SPECIES Clinical Microbiology Reviews, 4, p. 324-338 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.6 /02	Anonymous	2004	ANTHRAX FACT SHEET Report-no.: not applicable GLP/GEP: no Published: no	no	no	not protected	-	DAR 2008
KMA 2.6 /03	Carlson, C.R., Johansen, T., Kolsto, A.-B.	1996	THE CHROMOSOME MAP OF BAC. THUR. SUBSP. CANADENSIS HD224 IS HIGLY SIMILAR TO THAT OF THE BAC. CEREUS TYPE STRAIN ATCC 14579 FEMS Microbiology Letters 141, pp. 163-167 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.6 /04	Helgason, E., Okstad, O.A., Caugnat, D.A., Johansen, H.A., Fouet, A., Mock, M., Hegna, I., Kolsto	2000a	BACILLUS ANTHRACIS, BACILLUS CEREUS, AND BACILLUS THURINGIENSIS-ONE SPECIES ON THE BASIS OF GENETIC EVIDENCE Appl. And Environ. Microbiol. 66(6): 2627-2630 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
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 Microbiol Mol Biol Rev 62(3): 775-806 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
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 Appl. And Environ. Microbiol. 65(12): 5436-5442 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008

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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 FACTOR FEMS Microbiology Letters 188, pp. 7-13 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.6 /08	Ivanova, N., Sorokin, A., Anderson, I., Galleron, N., Candelon, B., Kapatral, V., Bhattacharyya, A., Reznik, G., Mikhallova, N., Lapidus, A., Chu, L., Mazur, M., Goltsman, E., Larsen, N., D	2003	GENOME SEQUENCE OF BACILLUS CEREUS AND COMPARATIVE ANALYSIS WITH BACILLUS ANTHRACIS Nature, 423, p. 87-91 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.6 /09	Hill, K.K., Ticknor, L.O., Okinaka, R.T., Asay, M., Blair, H., Bliss, K. A,, Laker, M., Pardington, P.E., Richardson, A.P., Tonks, M., Beecher, D.J., Kemp, J.D., Kolsto, A.-B., Wong, A.C.L., Keim, P., Jackson, P.J.	2004	FLUORESENT AMPLIFIED FRAGMENT LENGTH POLYMORPHISM ANALYSIS OF BACILLUS ANTHRACIS, BACILLUS CEREUS AND BACILLUS THURINGIENSIS ISOLATES Applied and Environmental Microbiology, 70(2), 1068-1080. Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.6 /10	Chen, C.Y.	2005a	FINGERPRINTS OF BACILLUS THURINGIENSIS Certis USA LLC, Columbia Report-no.: not applicable GLP/GEP: no Published: no	no	no	not protected	Certis USA	DAR 2008
KMA 2.6 /11	Helgason, E., D. A. Caugant, I. Olsen, A.-B. Kolstø	2000b	GENETIC STRUCTURE OF POPULATION OF BACILLUS CEREUS AND B. THURINGIENSIS ISOLATES ASSOCIATED WITH PERIODONTIS AND OTHER HUMAN INFECTIONS J. Clin. Microbiol. 38:1615-1622 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008

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KMA 2.6 /12	Ticknor, L.O., Kolstø, A.-B., Hill, K.K., Keim P., Laker, M.T., Tonks, M., Jackson, P.J.	2001	FLUORESENT AMPLIFIED FRAGMENT LENGTH POLYMORPHISM ANALYSIS OF NORWEGIAN BACILLUS CEREUS AND BACILLUS THURINGIENSIS SOIL ISOLATES Applied and Environmental Microbiology, 67(10), 4863-4873. Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.6 /13	Nakamura, L.K.	1994	DNA RELATEDNESS AMONG BACILLUS THURINGIENSIS SEROVARS International Journal of Systematic Bacteriology 44(1): 125-129 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.6 /14	Kramer, J.M., Gilbert, R.J.	1989	BACILLUS CEREUS AND OTHER BACILLUS SPECIES In: Foodborne Bacterial Pathogens,Ch. 2, MP Doyle (ed.), pp. 21-70 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.6 /15	EFSA	2005	OPINION OF THE SCIENTIFIC PANEL OF BIOLOGICAL HAZARD ON BACILLUS CEREUS AND OTHER BACILLUS SPP IN FOODSTUFFS The EFSA Journal 175, p. 1-48 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.6 /16	Rosenquist, H., Smidt, L., Andersen, S.R., Jensen, G.B. and Wilcks, A.	2005	OCCURRENCE AND SIGNIFICANCE OF BACILLUS CEREUS AND BACILLUS THURINGIENSIS IN READY-TO-EAT FOOD FEMS Microbiol Lett., 250(1):129-36 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008

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KMA 2.6 /17	Shinagawa et al	1984	SURVEILLANCE OF RAW MEAT PRODUCTS AND MEAT-PRODUC ADDITIVES FOR CONTAMINATION WITH BACILLUS SEREUS AND ENTEROTOXIGENI-CITY OF THE ISOLATED STRAIN J. Fac. Agric. Iwate Univ. 17:175-182 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not pro-tected	-	DAR 2008
KMA 2.6 /18	Granum, P.E., Lund, T.	1997	BACILLUS CEREUS AND ITS FOOD POISONING TOXINS FEMS Microbiology Letters 157: 223-228 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not pro-tected	-	DAR 2008
KMA 2.6 /19	Andersson, A., Granum, P.E., Römer, U.	1998	THE ADHESION OF BACILLUS CEREUS SPORES TO EPITHELIAL CELLS MIGHT BE AN ADDITIONAL VIRULENCE MECHANISM International Journal of Food Microbiol. 39, p. 93-99 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not pro-tected	-	DAR 2008
KMA 2.6 /20	Granum, P.E.	2001	FOOD MICROBIOLOGY: FUNDAMENTALS AND FRONTIERS Food Microbiology: fundamentals and frontiers, 2nd Ed., M.P. Doyle (ed.), pp. 373-381, ASM Press, Washington, D.C Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not pro-tected	-	DAR 2008
KMA 2.6 /21	Turnbull, P.C.B.	2005	BACILLUS Medimicro Chapter, http://gsbs.utmb.edu/microbook/ch015.htm, 2005 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not pro-tected	-	DAR 2008
KMA 2.6 /22 NOT SUB-MIT-TED	Hoton FM, Andrup L, Swiecicka I, Mahillon, J.	2005	THE CEREULIDE GENETIC DETERMINANTS OF EMETIC BACILLUS CEREUS ARE PLASMID-BORNE Microbiology-SGM 151: 2121-2124 Report-no.: not applicable GLP/GEP: no Published:	no	no	not pro-tected	-	DAR 2008

Data point	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	Previous evaluation
KMA 2.6 /23	Ehling-Schulz, M., Vukov, N., Schulz, A., Shaheen, R., Andersson, M., Martlbauer, E., Sherer, S.	2005	IDENTIFICATION AND PARTIAL CHARACTERIZATION OF THE NONRIBOSOMAL PEPTIDE SYNTHETASE GENE RESPONSIBLE FOR PRODUCTION IN EMETIC BACILLUS CEREUS Applied and Environmental Microbiology 71(1): 105-113 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.6 /24	Mikkola, R., Saris, N.-E.L., Grigoriev, P.A., Andersson, M.A., Salkinoja-Salonen, M.S.	1999	IONOPHORETIC PROPERTIES AND MITOCHONDRIAL EFFECTS OF CEREULIDE Eur. J. Biochem. 263, p. 112-117 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.6 /25	Jackson, S.G., Goodbrand, R.B., Ahmed, R., Kasatiya, S.	1995	BACILLUS CEREUS AND BACILLUS THURINGIENSIS ISOLATED IN A GASTROENTERITIS OUTBREAK INVESTIGATION Letters in Appl. Microbiol. 21, p. 103-105 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.6 /26	Damgaard, P.H., Granum, P.E., Bresciani, J., Torregrossa, M.V., Eilenberg, J., Valentino, L.	1997b	CHARACTERIZATION OF BACILLUS THURINGIENSIS ISOLATED FROM INFECTIONS IN BURN WOUNDS FEMS Immunology and Medical Microbiology 18, p. 47-53 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.6 /27	Siegel, J.P.	2001	THE MAMMALIAN SAFETY OF BACILLUS THURINGIENSIS-BASED INSECTICIDES Journal of Invertebrate Pathology 77, 13-21 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.6 /28	Gonzáles, J.M., Brown, B.J., Carlton, B.C.	1982	TRANSFER OF BACILLUS THURINGIENSIS PLASMIDS CODING FOR Proc. Natl. Acad. Sci. 79, p. 6951-6955 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008

Data point	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	Previous evaluation
KMA 2.6 /29	Hansen, B.M., Damgaard, P.H., Eilenberg, J., Pedersen J.C.	1998	MOLECULAR AND CLASSICAL CHARACTERIZATION OF BACILLUS THURINGIENSIS ISOLATED FROM LEAVES AND INSECTS Journal of Invertebrate Pathology 71, 106-114 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.6 /30	Valadares de Amorim, G., Whittome, B., Shore, B., Levin D.B.	2001	IDENTIFICATION OF BACILLUS THURINGINSIS SUBSP. KURSTAKI STRAIN HD1-LIKE BACTERIA FROM ENVIRONMENTAL AND HUMAN SAMPLES AFTER AERIAL SPRAYING OF VICTORIA, BRITISH COLUMBIA, CANADA, WITH FORAY 48 Applied and Environmental Microbiology, 67(3): 1035-1043 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.6 /31	Guinebretiére, 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 DIVERSIFICA-TION IN THE BACILLUS CEREUS GROUP Environ Microbiol., 10(4):851-865 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	New data for active ingredient, not previously submitted nor evaluated
KMA 2.6 /32	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 J Clin Microbiol., 48(9):3388-3391 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	New data for active ingredient, not previously submitted nor evaluated

Data point	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	Previous evaluation
KMA 2.6 /33	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 Food Microbiol., 28(2):236-244 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	New data for active ingredient, not previously submitted nor evaluated
KMA 2.7 /01	Hargrove, J.L., Iqbal, M., Chen, C.Y., Gouker, T.	2003	**Confidential information Vol.** 4	no	no	not protected	Certis USA	DAR 2008
KMA 2.7 /02	Hargrove, J.L.	1990	**Confidential information Vol.**	no	no	not protected	Certis USA	DAR 2008
KMA 2.7 /03	Chen, C.Y., Hargrove, J.L.	2003	**Confidential information Vol.**	no	no	Not protected	Certis USA	DAR 2008
KMA 2.7 /04	Gonzáles, J.M., Brown, B.J., Carlton, B.C.	1982	TRANSFER OF BACILLUS THURINGIENSIS PLASMIDS CODING FOR Proc. Natl. Acad. Sci. 79, p. 6951-6955 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.7 /05	Carlton, B.C.	1993	GENETICS OF BT INSECTICIDAL PROTEINS AND STRATEGIES FOR THE CONSTRUCTION OF IMPROVED STRAINS American Chemical Society, pp. 326-334 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.7 /06	Vilas-Boas, G., Sanchis, V., Lereclus, D., Lemos, M.V., Bourguet, D.	2002	GENETIC DIFFERENTIATION BETWEEN SYMPATRIC POPULATIONS OF BACILLUS CEREUS AND BACILLUS THURINGIENSIS Appl. And Environ. Microbiol., 68(3): 1414-1424 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.7 /07	Battisti, L., Green, B.D., Curtis, B.T.	1985	MATING SYSTEM FOR TRANSFER OF PLASMIDS AMONG BACILLUS ANTHRACIS, BACILLUS CEREUS AND BACILLUS THURINGIENSIS Journal of Bacteriology, pp. 543-550 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008

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KMA 2.7 /08	Hu, X.M., Hansen, B.M., Eilenberg, J., Hendriksen, N.B., Smidt, L., Yuan, Z.M., Jensen, G.B.	2004	CONJUGATIVE TRANSFER, STABILITY AND EXPRESSION OF A PLASMID ENCODING A cry1Ac GENE IN BACILLUS CEREUS GROUP STRAINS FEMS Microbiology Letters 231(1): 45-52 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.7 /09	Chen, C.Y.	2005b	**Confidential information Vol.**	no	yes	not protected	Certis USA	DAR 2008
KMA 2.7 /10	Chen, C.Y.	2005c	**Confidential information Vol.**	no	yes	not protected	Certis USA	DAR 2008
KMA 2.7 /11	Chen, C.Y.	2005d	**Confidential information Vol.**	no	yes	not protected	Certis USA	DAR 2008
KMA 2.7 /12	Süß, J.	2016	LITERATURE REVIEW ON BACILLUS THURINGIENSIS SUBSP. KURSTAKI SA-12 BIOLOGICAL PROPERTIES Certis USA LLC Report-no.: 2281384-MA-02-01_SA-12 GLP/GEP: no Published: no	no	yes	Protected	Certis USA	New data for active ingredient, not previously submitted nor evaluated
KMA 2.7 /13	Bizzarri, M.F., Bishop, A.H.	2008	THE ECOLOGY OF BACILLUS THURINGINSIS ON THE PHYLLOPLANE: COLONIZATION FROM SOIL, PLASMID TRANSFER, AND INTERACTION WITH LARVAE OF PIERIS BRASSICAE Microb Ecol, 56, 133-139 Report-no.: not applicable GLP/GEP: no Published: yes.	no	no	not protected	-	New data for active ingredient, not previously submitted nor evaluated
KMA 2.7 /14	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 Archives of Microbiology, 187, 425-431 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	New data for active ingredient, not previously submitted nor evaluated

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KMA 2.7 /15	Santos, C.A., Vilas-Bôas, 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 Journal of Invertebrate Pathology, 105, 171-175 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	New data for active ingredient, not previously submitted nor evaluated
KMA 2.7 /16	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 and Biochemistry, 42, 1329-1337 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	New data for active ingredient, not previously submitted nor evaluated
KMA 2.7 /17	Zhang, Q., Sun, M., Xu, Z., Yu, Z.	2007	CLONING AND CHARATERIZATION OF pBMB9741, A NATIVE PLASMID OF BACILLUS THURINGIENSIS SUBSP. KURSTAKI STRAIN YBT-1520 Current Microbiolgy, 55, 302-307 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	New data for active ingredient, not previously submitted nor evaluated
KMA 2.7 /18	Yuan, Y., Zheng, D., Hu, X., Cai, Q., Yuan, Z.	2010	CONJUGATIVE TRANSFER OF INSECTICIDAL PLASMID pHT 73 FROM BACILLUS THURINGIENSIS TO B. ANTHRACIS AND COMPATIBILITY OF THIS PLASMID WITH pXO1 AND pXO2 Applied and Environmental Microbiology, 76, 468-473 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	New data for active ingredient, not previously submitted nor evaluated
KMA 2.7 /19	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 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	Initial New data for active ingredient, not previously submitted nor evaluated

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KMA 2.7 /20	Wilcks, A., Jacobsen, B.B.	2010	LACK OF DETECTABLE DNA UPTAKE BY TRANSFORMATION OF SELECTED RECIPIENTS IN MONO-ASSOCIATED RATS BMC research notes, 3, 49 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	New data for active ingredient, not previously submitted nor evaluated
KMA 2.8 /01	Hansen, B.M., Salamitou, S.	2000	VIRULENCE OF BACILLUS THURINGINSIS In: Charles, J.-F., Delécluse, A. & Nielsen-Le Roux, C. (eds.): Entomopathogenic Bacteria: From Laboratory to Field Application Kluwer Academic Publishers. Chapter 1.3 pp. 41-64 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.8 /02	WHO-EHC	1999	ENVIRONMENTAL HEALTH CRITERIA 217 – MICROBIAL PEST CONTROL AGENT BACILLUS THURINGIENSIS World Health Organization. Geneva Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 2.8 /03	Chen, C.Y.	2005a	FINGERPRINTS OF BACILLUS THURINGIENSIS Certis USA LLC, Columbia Report-no. not applicable GLP/GEP: no Published: no	no	yes	not protected	Certis USA	DAR 2008
KMA 2.8 /04	Chen, C.Y.	2005b	**Confidential information Vol.**	no	yes	not protected	Certis USA	DAR 2008
KMA 2.8 /05	Chen, C.Y.	2005c	**Confidential information Vol.**	no	yes	not protected	Certis USA	DAR 2008
KMA 2.8 /06	Chen, C.Y.	2005d	**Confidential information Vol.**	no	yes	not protected	Certis USA	DAR 2008
KMA 2.8 /07	Hargrove, J.L., Iqbal, M., Chen, C.Y., Gouker, T.	2003	**Confidential information Vol.**	no	yes	not protected	Certis USA	DAR 2008
KMA 2.8 /08	Chen, C.-Y.	2004	**Confidential information Vol.**	no	yes	not protected	Certis USA	DAR 2008

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KMA 2.8 /09	Seehase, S.	2016	LITERATURE REVIEW ON BACILLUS THURINGINSIS SUBSP. KURSTAKI SA-12: TOXOCOLOGY Certis USA LLC, 2281384-MA-05-01_SA-12 GAB Consulting GmbH, Stade, Germany Report-no.: not applicable GLP/GEP: no Published: no	no	yes	protected	Certis USA	New data for active ingredient, not previously submitted nor evaluated
KMA 2.8 /10	Palma, L., Munoz, D., Berry, C., Murillo, J., Caballero, P.	2014	BACILLUS THURINGIENSIS TOXINS: AN OVERVIEW OF THEIR BIOCIDAL ACTIVITY Toxins, 6(12), 3296-3325 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	New data for active ingredient, not previously submitted nor evaluated
KMA 2.8 /11	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 IMPARIMENT Food Chem. Toxicol., 46(6), 2184-2189 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	New data for active ingredient, not previously submitted nor evaluated
KMA 2.8 /12	Shimada, N., Miyamota, 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 In Vitro Cellular & Developmental Biology – Animal, 42(1), 45-49 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	New data for active ingredient, not previously submitted nor evaluated

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KMA 2.8 /13	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 African Journal of Biotechnology, 11(46), 10504-10512 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	New data for active ingredient, not previously submitted nor evaluated
KMA 2.8 /14	Kim, M.J., Han, J.K., Park, J.S., Lee, J.S., Lee, S.H., Cho, J.I., Kim, K.S.	2015	VARIOUS ENTEROTOXIN AND OTHER VIRULENCE FACTOR GENES WIDESPREAD AMONG BACILLUS CEREUS AND BACILLUS THURINGIENSIS STRAINS J. Microbial Biotechnology, 25(6), pp. 872-879 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	New data for active ingredient, not previously submitted nor evaluated
KMA 2.8 /15	Wilcks, A., Hansen, B.M., Hendriksen, N.B., Licht, T.R.	2006a	PERSISTENS OF BACILLUS THURINGIENSIS BIOINSECTICIDES IN THE GUT OF HUMAN-FLORA-ASSOCIATED RATES FEMS Immunol. Med. Microbiol., 48(3), 410-418 Report-no.: not applicable GLP/GEP: no Published: yes	yes	no	not protected	-	New data for active ingredient, not previously submitted nor evaluated
KMA 2.8 /16	Du, C., Nickerson, K.W.	1996b	BACILLUS THURINGIENSIS HD-73 SPORES HAVE SURFACE-LOCALIZED CryAc TOXIN: PHYSIOLOGICAL AND PATHOGENIC CONSEQUENCES Applied and Environmental Microbiology, 62(10): 3722-3762 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	New data for active ingredient, not previously submitted nor evaluated
KMA 2.8 /17	Abdoarrahem, M.M., Gammon, K., Dancer, B.N., Berry, C.	2009	GENETIC BASIS FOR ALKALINE ACTIVATION OF GERMINATION IN BACILLUS THURINGIENSIS SUBSP. ISRAELENSIS Applied and Environmental Microbiology, 75(19), 6410-6413 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	New data for active ingredient, not previously submitted nor evaluated

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KMA 2.8 /18	King, P.J.H., Ong, K.H., Sipen, P., Mahadi, N.M.	2012	TOXICITY OF LOCAL MALAYSIAN BACILLUS THURINGIENSIS SUBSPECIES KURSTAKI AGAINST PLUTELLA XYLOSTELLA African Journal of Biotechnology, 11(56), 11925-11930 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	New data for active ingredient, not previously submitted nor evaluated
KMA 2.8 /19	Hansen, B.M., Thorsen, L., Nielsen-LeRoux, C., Wilcks, A., Hendriksen, N.B.	2011	NEW EXPERIMENTAL APPROACHES FOR HUMAN RISK ASSESSEMENT OF MICROBIAL PEST CONTROL AGENTS – EXEMPLIFIED BY THE BACTERIUM BACILLUS THURINGIENSIS Danish EPA, Pesticides Research, 136 2011, 1-128 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	New data for active ingredient, not previously submitted nor evaluated
KMA 2.8 /20	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 FEMS Immunol. Med. Microbiol., 46, 70-77 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	New data for active ingredient, not previously submitted nor evaluated
KMA 2.8 /21	Ceuppens, S., Uyttendaele, M., Drieskens, K., Heyndrickx, M., Rajkovic, A., Boon, N., Van de Wiele, T.	2012a	SURVIVAL AND GERMINATION OF BACILLUS CEREUS SPORES WITHOUT OUTGROWTH OR ENTEROROXIN PRODUCTION DURING IN VITRO SIMULATION OF GASTROINTESTINAL TRANSIT Applied and Environmental Microbiology, 78(21), 7698-7705 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	New data for active ingredient, not previously submitted nor evaluated

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KMA 2.8 /22	Ceuppens, S., Van de Wiele, T., Rajkovic, A., Ferrer-Cabaceran, T., Heyndrickx, M., Boon, N., Uyttendaele, M.	2012b	IMPACT OF INTESTINAL MICROBIOTA AND GASTROINTESTINAL CONDITIONS ON THE IN VITRO SURVIVAL AND GROWTH OF BACILLUS CEREUS Int. J. Food Microbiology, 155(3), 241-246 Report-no.: not applicable GLP/GEP: no Published: yes	no	yes	not protected	-	New data for active ingredient, not previously submitted nor evaluated
KMA 2.8 /23	Auger, S., Ramarao, N., Faille, C., Fouet, A., Aymerich, S,, Gohar, M.	2009	BIOFILM FORMATION AND CELL SURFACE PROPERTIES AMONG PATHOGENIC AND NON-PATHOGENIC STRAINS OF THE BACILLUS CEREUS GROUP Applied and Environmental Microbiology, 75(20), 6616-6618 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	New data for active ingredient, not previously submitted nor evaluated
KMA 2.8 /24	Phelps, R.J., McKillip, J.L.	2002	ENTEROTOXIN PRODUCTION IN NATURAL ISOLATES OF BACILLACEAE OUTSIDE THE BACILLUS CEREUS GROUP Applied and Environmental Microbiology, 68(6), 3147-3151 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	New data for active ingredient, not previously submitted nor evaluated
KMA 2.8 /25	Damgaard, P.H.	1995	DIARRHOEAL ENTEROTOXIN PRODUCTION BY STRAINS OF BACILLUS THURINGIENSIS ISOLATED FROM COMMERCIAL BACILLUS THURINGIENSIS-BASED INSECTICIDES FEMS Immunology and Medical Microbiology, 12(3-4), 245-250 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	New data for active ingredient, not previously submitted nor evaluated

Data point	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	Previous evaluation
KMA 2.9 /01	Smith, R.W., Regan, K.M.	1989a	ANTIBIOTIC SENSITIVITY PATTERNS FOR BACILLUS THURINGIENSIS SUBSP. KURSTAKI STRAIN SA11001C98-1-1 [redacted] Palo Alto, CA, USA Certis USA, LLC, Columbia Report-no. 89/12/12 GLP/GEP: no Published: no	no	no	not protected	Certis USA	DAR 2008
KMA 2.9 /02	Smith, R.W., Regan, K.M.	1989b	ANTIBIOTIC SENSITIVITY PATTERNS FOR BACILLUS THURINGIENSIS SUBSP. KURSTAKI STRAIN SA-12 [redacted] Palo Alto, CA, USA Certis USA, LLC, Columbia Report-no. 89/12/12F GLP/GEP: no Published: no	no	no	not protected	Certis USA	DAR 2008
KMA 2.9 /03	Barbera, P.W.	1990	NCCLS STANDARD DISK SUSCEPTIBILITY TEST OF BACILLUS THURINGIENSIS STRAINS Ecogen Incorporated, Langhorne, PA 19047 Certis USA LLC, Columbia Report-no.: EC-02 GLP/GEP: no Published: no	no	no	not protected	Certis USA	DAR 2008
KMA 2.9/04	Shaw, G.	2019	Antibiotic resistance and sensitivity testing of the Microbial Pest Control Agent *Bacillus thuringiensis* subsp. *kurstaki* strain SA-12 Certis USA LLC APIS-016-002 Applied Insect Science Ltd. GLP/GEP: yes Published: no	no	yes	Protected	Certis USA	New data for active ingredient, not previously submitted nor evaluated