BASELINE DOSSIER

Bacillus subtilis QST 713

Microbial pest control agent against plant pathogenic fungi and bacteria

Dossier according to OECD guidance for industry data submissions for microbial pest control products and their microbial pest control agents – August 2006

Summary documentation, Tier II

Annex IIM, Section 3

Point IIM 5: Toxicological and Exposure Data and Information on the Microbial Pest Control Agent

Revised
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Applicant

Bayer CropScience AG

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Introduction

This document summarizes all data submitted for the initial evaluation of *Bacillus subtilis* QST 713 as an active substance under Directive 91/414. Data provided in the initial dossier and in subsequent additional submissions are listed chronologically under the respective data point according to the OECD dossier guidance (2006).
This document is further named as **"Baseline Dossier"** since it presents all data previously submitted.

IIM 5 Toxicological and Exposure Data and Information on the Microbial Pest Control Agent

Please refer to Point 5.2

IIM 5.1 Summary: potential of microbial pest control agent to be hazardous to humans with consideration of its pathogenic potential, its ability to infect and pattern of clearance, and its toxicological effects

Report:	KIIM 5.1/01; [redacted]; 1989; M-484919-01-1
Title:	Introduction to the biotechnology of Bacillus
Report No.:	M-484919-01-1
Document No.:	M-484919-01-1
Guideline(s):	not specified
Guideline deviation(s):	not specified
GLP/GEP:	**no**

Please refer to Point 5.6

IIM 5.2 Occupational health surveillance report on workers during production and testing of MCPA

Report:	KIIM 5.2/01; [redacted] V; 1989; M-477486-01-1
Title:	Safe biotechnology - III. Safety precautions for handling microorganisms of different risk classes
Report No.:	M-477486-01-1
Document No.:	M-477486-01-1
Guideline(s):	not applicable
Guideline deviation(s):	not applicable
GLP/GEP:	**no**

Report:	KIIM 5.2/02; [redacted]; [redacted]; 1991; M-486912-01-1
Title:	On the safety of Bacillus subtilis and B. amyloliquefaciens: a review
Report No.:	M-486912-01-1
Document No.:	M-486912-01-1
Guideline(s):	not applicable
Guideline deviation(s):	not applicable
GLP/GEP:	**no**

Report:	KIIM 5.2/03; [redacted]; 1997; M-528163-01-1
Title:	Final decision document: TSCA section 5 (H) (4) exemption for Bacillus subtilis
Report No.:	M-528163-01-1
Document No.:	M-528163-01-1
Guideline(s):	-
Guideline deviation(s):	-
GLP/GEP:	**no**

The following references were submitted during the Annex I review and were not included in the Baseline dossier. They have been added at the request of the RMS.

KIIM 5.2 / 06 Tae? • J§o(. >,; 2001; 2nd draft - 4-week repeated dose inhalation toxicity study of bacillus subtilis (QST 713) to sprague-dawley rats; M-595981-02-1

EU-Dossier: Doc M-IIB, Point 5.4.1

No medical observations on plant personel were conducted.

Exposure to *Bacillus subtilis* in the manufacturing plant will be minimal due to rigorous application of Good Manufacturing Practise (GMP), quality controls and due to protective equipment worn by the plant workers (Document Submission Template to U.S. BPPD – Biopesticides and Pollution Prevention Division, 1998)

The EPA (1997) states that the only human health concern for workers in the fermentation facility is the potential of *B. subtilis* to elicit allergic reactions in individuals repeatedly exposed to subtilisin (a proteinaceous compound produced by *B. subtilis*). This risk is minimised by appropriate limits set by the U.S. OSHA (Occupational Safety and Health Administration) for subtilisin in the industrial setting.

B. subtilis is characterised as non-pathogenic in the literature (BOER & DIDERICHSEN, 1991; EPA, 1997) and does not require containment to protect workers since it is a harmless micro-organism with a long history of safe use in enzyme production (FROMMER et al., 1989). Furthermore, this species falls under Class 1 Containment of European Federal Law of Biotechnology (EPA, 1997).

The low human health risk of *B. subtilis* therefore does not require a special medical surveillance programme.

EU-Dossier: Doc M-IIB, Point 10

Handling of the technical product, QST 713 Technical, will only be relevant for workers at the producing facilities which are restricted to the U.S. territory. No production of Serenade™ WP will occur in any country of the EC. Thus, there is no need for a label meeting the EC legal requirements. The US label complies with the US legal provisions.
Notwithstanding, the submitted study reports prove that the active substance, strain QST 713 of *B. subtilis*, is non-hazardous to human and animal health in compliance with the relevant EC directives 67/548/EEC and 91/414/EEC.
With regard to environmental fate and behaviour this micro-organism is not expected to impose any environmental risk. Therefore, the technical product, QST 713 Technical, would not have to be classified as a harmful or dangerous substance and would not require any safety or risk phrase.

IIM 5.2.1 Sensitisation and allergenic response of workers

EU-Dossier: Doc M-IIB, Point 5.4.2

No observational data are available. The potential for allergic reactions elicited by the proteinaceous compound subtilisin is reported by the EPA (1997).

IIM 5.2.2 Details on any occurrence of hypersensitivity and chronic sensitisation

Please refer to Point 5.2.

IIM 5.2.3 Any significant clinical findings related to exposure, with special attention to those whose susceptibility may be affected

Report:	KIIM 5.2.3/01; [redacted], D. C.; [redacted]; 1973; M-153632-01-1
Title:	Clinical spectrum of infection die to Bacillus species
Report No.:	A81036
Document No.:	M-153632-01-1
Guideline(s):	--
Guideline deviation(s):	--
GLP/GEP:	**no**

Report:	KIIM 5.2.3/02; [redacted]; [redacted]; 1991; M-486912-01-1
Title:	On the safety of Bacillus subtilis and B. amyloliquefaciens: a review
Report No.:	M-486912-01-1
Document No.:	M-486912-01-1
Guideline(s):	not applicable
Guideline deviation(s):	not applicable
GLP/GEP:	**no**

EU-Dossier: Doc M-IIB, Point 5.4.3

Clinical cases have been investigated by IHDE & ARMSTRONG (1973) over a 6-year period. In twelve patients *Bacillus* species were determined to be present. They report that disseminated bacterial infections by *B. subtilis* and other bacteria developed in two patients with acute leukemia who were under intense chemotherapy and finally died of their infections. *B. subtilis* isolates from the remaining ten patients were locally restricted to surgical wound or tumor drainages and did not appear to affect wound healing. Other pathogenic bacteria were sometimes present in such culture material as well.

The authors conclude that the presence of *Bacillus* species seems to indicate the infection of a wound or tumor mass. With the exception of the two immuno-compromised patients no colonization of other organs or tissues took place.

From a review of additional and more recent references on clinical cases which partly were related to GRAS petitions published by the U.S. Food and Drug Administration BOER & DIDERICHSEN (1991) conclude that no case demonstrating invasive properties of *B. subtilis* was described and that the 50 reported *B. subtilis* infections (covering a 20-year period) were associated with drug abusers or severely debilitated patients. Under safety aspects they assume that due to the ubiquitous distribution of *B. subtilis* it is inevitable that sometimes it may be found in association with other bacteria in infected humans.

IIM 5.2.4 Published reports of adverse effects, especially reports of clinical cases and follow-up studies; list databases and key words used in a literature search

Report:	KIIM 5.2.4/01; [redacted]; [redacted]; 1991; M-486912-01-1
Title:	On the safety of Bacillus subtilis and B. amyloliquefaciens: a review
Report No.:	M-486912-01-1
Document No.:	M-486912-01-1
Guideline(s):	not applicable
Guideline deviation(s):	not applicable
GLP/GEP:	**no**

EU-Dossier: Doc M-IIB, Point 5.4.4

The general population already is exposed to *B. subtilis* since it is an ubiquitous micro-organism which inhabitats primarily the soil environment and plant residues but has also been reported to occur in the immediate environment of humans, such as the kitchen (BOER & DIDERICHSEN, 1991). *B. subtilis* is not pathogenic or toxic as proved by the submitted toxicological studies and e.g. has been shown to clear the body (of tested rats) after oral intake within 14 days (see [redacted], 1998a). Based on these findings no epidemiological studies have been performed, nor are corresponding reports available from the open literature.

IIM 5.2.5 Proposed first aid measures and medical treatment

EU-Dossier: Doc M-IIB, Point 5.4.5

In case direct contact to *B. subtilis* material occurs the following first behavioural steps are to be carried out according to the applicant:

- If inhaled: move to fresh air
- If in contact with eyes: flush eyes with plenty of water

- If in contact with skin, open cuts or wounds: wash skin with soap and water
- If swallowed: immediately give large amounts of water

Specific medical treatment following inhalative, oral or eye exposure is not required since *B. subtilis* is not pathogenic or toxic. Following direct contact of *B. subtilis* to open cuts or wounds preventively the relevant sites should be disinfected.

Handling of the Technical Product and any first aid measures or medical treatment are only relevant for workers at the production facilities, which are located in the U.S.A. and not in Europe. Thus, only U.S. legal requirements are applicable, and concerning QST Technical, no information is needed for registration in Europe.

IIM 5.3 Basic studies

IIM 5.3.1 Sensitisation properties

EU-Dossier: Doc M-IIB, Point 5.1.1.6

A skin sensitization test has been performed with the wettable powder formulation Serenade™ WP.

IIM 5.3.2 Acute oral infectivity, toxicity and pathogenicity

EU-Dossier: Doc M-IIB, Point 5.1.1.1

Report:	[redacted]; [redacted]A. (1998a): Toxicity / pathogenicity testing of QST 713 following acute oral challenge in rats; [redacted]2; unpublished, Laboratory Project ID L08726 SN4; dates of experimental work: Apr. 13, 1998 – May 6, 1998
Document No:	M-474035-01-2
Guidelines:	EPA-Pesticide Assessment Guidelines, Subdivision M – Section Series 152A-10 (Microbial Pesticide Test Guidelines OPPTS 885.3050); Corresponds generally to EEC B1 - Directive 92/69/EEC (limit-test), and to OECD guideline 401 (Deviations: dose level not applicable to microbial preparations)
GLP:	Yes (self certification by the laboratory)
Material and methods:	QST 713 Technical (dried *Bacillus subtilis* with residual fermentation media); Lot No. 8AQ07C2; Titer: 4.3 × 10^{10} cfu/g, no bacterial or fungal contamination; homogeneity Test (10 mL): positive The test substance was suspended in sterile water and administered to groups of male and female CD® rats (3 rats per group/sacrifice day/ sex) at a dose level of 1.13 × 10^{8} cfu/ test animal (1 mL orally). One group received the heat-killed test substance to elucidate a potential stimulation of germination. Rats were sacrificed at day 0, 3, 7, and 14. Clearance of *B. subtilis* in rat tissues was determined by plating analysis.
Findings:	No mortality occurred, no toxic or pathogenic effects, no adverse clinical signs or gross lesions in necropsy, and no treatment-related effects on body or organ weight were observed during the observation period of 21 days. **Infectivity/ persistence**: Test substance (viable *Bacillus subtilis*) was detected in stomach and intestines, caecum and feces of treated rats (male and female), plus in the lungs, liver and mesenteric lymph nodes of female rats at the day of administration only. Highest numbers of cfu were found in the stomach. Within 14 days (after dosing) test substance was cleared from all tissues tested in accordance with the oral administration. **NOEL**: >1.13 × 10^{8} cfu/test animal; ~ 5 × 10^{8} cfu/kg b.w. **LD_{50}**: >1.13 × 10^{8} cfu/test animal The LD_{50} could not be calculated because no mortality occurred.

Conclusion:	The absence of any clinical signs show that the active substance, *Bacillus subtilis*, can be classified as non-toxic (no labelling requirements according to EC directive 67/548/EEC).

IIM 5.3.3 Acute intratracheal/inhalation infectivity, toxicity and pathogenicity

EU-Dossier: Doc M-IIB, Point 5.1.1.2

Report:	[redacted], [redacted] A. (1998b): Toxicity/ pathogenicity testing of QST 713 following acute intratracheal challenge in rats; [redacted].; unpublished; Laboratory Project L08726 SN6; dates of experimental work: Apr. 13, 1998 – May 29, 1998
Document No	M-474038-01-1
Guidelines:	EPA-Pesticide Assessment Guidelines, Subdivision M – Section Series 152A-12 (Microbial Pesticide Test Guidelines OPPTS 885.3150) No OECD guideline applicable
GLP:	Yes (self certification by the laboratory)
Material and methods:	QST 713 Technical (dried *Bacillus subtilis* with residual fermentation media; Lot No. 8AQ07C2); Titer: 4.3 × 10^{10} cfu/g; no bacterial or fungal contamination; homogeneity Test (10 mL): positive The test substance was suspended in sterile water and intratracheally administered to groups of male and female CD® rats (5 rats per group/sacrifice day/sex) at a dose level of approximately 1.2 × 10^{8} CFU/ test animal (0,1 mL). Control groups: naïve control, shelf control and a control group receiving the heat-killed test substance. Rats were sacrificed at day 0, 7, 21 and 35. Clearance of *B. subtilis* in rat tissues was determined by plating analysis, for the treatment group both without and with heat treatment of the tissue samples (to inactivate vegetative/heat intolerant spores).
Findings:	No deaths occurred, and except for one male rat (of 40) showing rough hair coat on Day 0 following dosing, no adverse clinical signs were observed. Sole necropsy findings were: mottled lung parenchyma in animals examined on Day 0 following test substance administration. Body weight gain significantly decreased during the first week after administration of test substance, during the 4th week male rats compensated this decrease. Increased relatively lung weights were found on Day 0 and Day 7 (males only). **LD_{50}:** >1.2 x 10^{8} cfu/ test animal, ~ 5 × 10^{8} cfu/ kg b.w. The LD_{50} could not be calculated because no mortality occurred. **Infectivity/ persistence**: No test substance was detected in any tissue of the control groups, including the group that received the heat-killed test substance. Decreasing titer values of test substance were detected in the lungs and associated lymph nodes of treated rats, up to Day 35 (end of observation period), both pre- and post-heat-treatment of tissues. Post *heat-treatment of tissues* test substance was also detected in liver, kidney and spleen (up to Day 7). **Clearance:** By Day 21 test substance numbers were significantly decreased or below detection limit from all tissues tested. There was no evidence of germination or vegetative growth of *B. subtilis* in the rats. Clearance from all tissues was estimated to occur within approximately 108 days from challenge.

Summary table of results:
Recovery of *B. subtilis* (cfu) from rat tissues of different treatment groups on Day 0/ Day 35 of test

Tissue/ body fluid	Naive Control Group	Shelf Control Group	Killed Test Substance Group	Test Substance Group[a)]
Blood	BDL[b)] /BDL	BDL /BDL	BDL /BDL	BDL /BDL
Lungs & lymph nodes	BDL /BDL	BDL /BDL	BDL /BDL	6.3×10^7/ 8.7×10^3
Spleen	BDL /BDL	BDL /BDL	BDL /BDL	2.3×10^{10}/ BDL
Liver	BDL /BDL	BDL /BDL	BDL /BDL	7.9×10^2/ BDL
Kidneys	BDL /BDL	BDL /BDL	BDL /BDL	1.2×10^3/ BDL
Brain	BDL /BDL	BDL /BDL	BDL /BDL	BDL /BDL
Caecum	BDL /BDL	BDL /BDL	BDL /BDL	BDL /BDL

a) determination of titer post-heat treatment of tissues (see Table 10 of submitted study report/male rats; geometric mean of cfu/ tissue or mL blood))

b) BDL = below detection limit (< 30 cfu/tissue or mL blood)

Conclusion: The generally minor and short-termed clinical signs and necropsy findings show that intratracheally applied *Bacillus subtilis* can be evaluated as a low health risk (no labelling requirements according to EC directive 67/548/EEC).

IIM 5.3.4 Acute intravenous/intraperitoneal infectivity

EU-Dossier: Doc M-IIB, Point 5.1.3

This test was not performed in consistence with the slight dermal irritation symptoms caused by *B. subtilis* (see [redacted] 1998b (This was a typographical error- the study name is [redacted] 1998a), Primary dermal irritation in rabbits with QST 713 TP). Further, the conducted intratracheal challenge in rats can be considered as appropriate compensation, since intravenous exposure presents more severe test conditions (see [redacted] 1998c).

EU-Dossier: Doc M-IIB, Point 5.3.1

Toxicity tests conducted under STEP I did not show any health effects. An additional acute percutaneous study also did not reveal any significant toxic effects on exposure to the test substance QST 713 TP. Strain QST 713 of *Bacillus subtilis* does not produce any toxins. Thus, any specific toxicity, pathogenicity or infectivity studies were not conducted. In addition to studies referred to under Step I and II, an acute intravenous toxicity test was conducted as basically required by the U.S. EPA. The test results are reported below.

Report: [redacted]. K. A. (1998c): Toxicity/ pathogenicity testing of QST 713 following acute intravenous challenge in rats; [redacted].; Laboratory Project ID L08726 SN5; unpublished; dates of experimental work: Apr. 13, 1998 – May 28, 1998.

Document No.: M-474033-01-1

Guidelines: EPA-Pesticide Assessment Guidelines, Subdivision M – Section Series 152A-13; Microbial Pesticide Test Guidelines OPPTS 885.3200.

No OECD guideline applicable

GLP: Yes (self certification by the laboratory)

Material and methods: QST 713 Technical (dried *Bacillus subtilis* with residual fermentation media; Lot No.: 8AQ07C2; reported titer: ~ $4{,}3 \times 10^{10}$ cfu/g).

Groups of male and female CD®rats (3 rats per group/sacrifice day/ sex) were dosed intravenously with ~ $9{,}4 \times 10^6$ cfu in a 0,5 mL volume (suspended in sterile water). Control groups: naive control, shelf control and a control group receiving the heat-killed test substance. Rats were sacrificed at day 0, 7, 21 and 35.

Clearance of *B. subtilis* in rat tissues was determined by plating analysis, for the treatment group both without and with heat treatment of the tissue samples (to inactivate vegetative/heat intolerant spores).

Findings: Neither deaths nor adverse clinical signs or gross lesions at necropsy were observed during the study. No treatment-related effects on body weight or body weight gain were observed during the observation period of 35 days.

NOEL: > $9{,}4 \times 10^6$ cfu/ animal, ~4×10^7 cfu/kg b.w.
LD_{50}: > $9{,}4 \times 10^6$ cfu/ animal
The LD_{50} could not be calculated because no mortality occurred.

Infectivity/ persistence: Test substance (viable *Bacillus subtilis*) was detected in the blood, liver, lungs, spleen and kidneys of treated rats. Clearance of test substance occurred in most tissues within the observation period (by Day 35), reduced levels of test substance were found in spleen and liver (post-heat treatment) of dosed rats. There was no evidence of germination or vegetative growth of QST 713 Technical in the rats. Clearance from all tissues was estimated to occur within approximately 80 days.

Summary table of results:
Recovery of *B. subtilis* (cfu) from rat tissues of different treatment groups on Day 0/ Day 35 of test

Tissue/ body fluid	Naive Control Group	Shelf Control Group	Killed Test Substance Group	Test Substance Group[a)]
Blood	BDL[b)] /BDL	BDL /BDL	BDL /BDL	4.4×10^2 /BDL
Lungs	BDL /BDL	BDL /BDL	BDL /BDL	5.4×10^5 /BDL
Spleen	BDL /BDL	BDL /BDL	BDL /BDL	4.4×10^5 /1.7×10^3
Liver	BDL /BDL	BDL /BDL	BDL /BDL	3×10^6 /1.9×10^2
Kidneys	BDL /BDL	BDL /BDL	BDL /BDL	4.7×10^3 /BDL
Brain	BDL /BDL	BDL /BDL	BDL /BDL	BDL /BDL
Mesenteric lymph nodes	BDL /BDL	BDL /BDL	BDL /BDL	BDL/ BDL
Caecum	BDL /BDL	BDL /BDL	BDL /BDL	BDL /BDL

a) determination of titer post-heat treatment of tissues (see table 10 of submitted study report: male rats; geometric mean of cfu/ tissue or ml blood))

2 BDL = below detection limit (< 30 cfu/tissue or ml blood)

Conclusions: QST 713 Technical caused no toxic or pathogenic effects when administered intravenously to rats. Detection of *B. subtilis* from blood and organs was consistent with the intravenous route. The results show that the active substance, *B. subtilis*, is non-toxic by the intravenous route (no labelling requirements according to EC directive 67/548/EEC).

IIM 5.3.5 Genotoxic potential, especially for fungi and actinomycetes: a discussion of the potential for genotoxin production based on the relationship of the microorganism to a genus/species known to produce genotoxins. If a related fungus/ actinomycete produces a genotoxin, either an appropriate and sensitive analytical test (e.g. HPLC) must be done to detect its presence in the MPCA (for Canada), or genotoxicity testing is required (for EC).

The following references were submitted during the Annex I review and were not included in the Baseline dossier. They have been added at the request of the RMS.

KIIM 5.3.5 / 03 [redacted]; 2004; Serenade WP {Bacillus subtilis, strain QST713} - Annex IIB, Point 5.3.2: Genotoxicity and cytotoxicity Testing expert statement; M-595984-01-1

IIM 5.3.6 Cell culture study, for viruses and viroids or specific bacteria and protozoa with intracellular replication

EU-Dossier: Doc M-IIB, Point 5.1.3

No cell culture studies were performed since *B. subtilis*, as a natural soil inhabitant, does not enter the cytoplasm to replicate intracellularly. The members of the species *B. subtilis* do not show specific attachment mechanisms typically found in organisms capable of colonizing humans. (EPA, 1997).

IIM 5.3.7 Short-term toxicity (including inhalatory short-term toxicity), pathogenicity, infectivity

Report:	KIIM 5.3.7/01; [redacted]; [redacted]; [redacted]; [redacted]; [redacted]; 1989; M-528282-01-1
Title:	Production of pneumolysin, a pneumococcal toxin, in Bacillus subtilis
Report No.:	M-528282-01-1
Document No.:	M-528282-01-1
Guideline(s):	--
Guideline deviation(s):	--
GLP/GEP:	**no**

Report:	KIIM 5.3.7/02; [redacted]; 2001; M-528849-01-1
Title:	US OSHA limits for subtilisin
Report No.:	M-528849-01-1
Document No.:	M-528849-01-1
Guideline(s):	not specified
Guideline deviation(s):	not specified
GLP/GEP:	**no**

Report:	KIIM 5.3.7/03; [redacted]; [redacted]; [redacted]; 1973; M-529218-01-1
Title:	Clearance and inactivation of the vegetative and spore forms of Bacillus subtilis Var Niger in rat lungs
Report No.:	M-529218-01-1
Document No.:	M-529218-01-1
Guideline(s):	--
Guideline deviation(s):	--
GLP/GEP:	**no**

Report: KIIM 5.3.7/04; [redacted]; [redacted]; [redacted]; 1983; M-153625-01-1
Title: Pulmonary clearance of Bacillus subtilis spores in pigs
Report No.: A81029
Document No.: M-153625-01-1
Guideline(s): --
Guideline deviation(s): --
GLP/GEP: **no**

Report: KIIM 5.3.7/05; [redacted]; [redacted]; [redacted]; 1997; M-529064-01-1
Title: Clearance and effects of intratracheal instillation to spores of Bacillus thuringiensis or Metarhizium anisopliae in rats
Report No.: M-529064-01-1
Document No.: M-529064-01-1
Guideline(s): --
Guideline deviation(s): --
GLP/GEP: **no**

Report: KIIM 5.3.7/06; [redacted]; 1966; M-529076-01-1
Title: Causes of the failure of antibiotic prophylaxis of inhalation anthrax and clearance of the spores from the lungs
Report No.: M-529076-01-1
Document No.: M-529076-01-1
Guideline(s): --
Guideline deviation(s): --
GLP/GEP: **no**

Report: KIIM 5.3.7/07; [redacted]; [redacted]; [redacted]; A.; 1984; M-529217-01-1
Title: Mucormycotic infection in mice following prolonged incubation of spores in vivo and the role of spore agglutinating antibodies on spore germination
Report No.: M-529217-01-1
Document No.: M-529217-01-1
Guideline(s): --
Guideline deviation(s): --
GLP/GEP: **no**

Report: KIIM 5.3.7/08; [redacted]; 1977; M-529221-01-1
Title: Germination of Aspergillus fumigatus conidia in the lungs of normal and cortisone-treated mice
Report No.: M-529221-01-1
Document No.: M-529221-01-1
Guideline(s): --
Guideline deviation(s): --
GLP/GEP: **no**

Report:	KIIM 5.3.7/09; [redacted]; [redacted]; [redacted]; [redacted]; 1980; M-528896-01-1
Title:	SEM studies on the in vivo uptake of Aspergillus terreus spores by alveolar macrophages
Report No.:	M-528896-01-1
Document No.:	M-528896-01-1
Guideline(s):	--
Guideline deviation(s):	--
GLP/GEP:	**no**

Report:	KIIM 5.3.7/10; [redacted]; 1984; M-528898-01-1
Title:	Interaction of Aspergillus fumigatus spores and pulmonary alveolar macrophages of rabbits
Report No.:	M-528898-01-1
Document No.:	M-528898-01-1
Guideline(s):	--
Guideline deviation(s):	--
GLP/GEP:	**no**

Report:	KIIM 5.3.7/11; [redacted]; [redacted]; [redacted]; [redacted]; [redacted]; [redacted]; 1971; M-529211-01-1
Title:	Etude comparative du pouvoir d'epuration pulmonaire du cobaye vis-a-vis d'Aspergillus fumigatus, de Candida albicans et de Micropolyspora faeni
Report No.:	M-529211-01-1
Document No.:	M-529211-01-1
Guideline(s):	--
Guideline deviation(s):	--
GLP/GEP:	**no**

Report:	KIIM 5.3.7/12; [redacted]; [redacted]; 2001; M-528965-01-1
Title:	Organic dust-related respiratory and eye irritation in norwegian farmers
Report No.:	M-528965-01-1
Document No.:	M-528965-01-1
Guideline(s):	- not applicable -
Guideline deviation(s):	- not applicable -
GLP/GEP:	**no**

EU-Dossier: Doc M-IIB, Point 5.1.4

No relating study was performed in consistence with the results of the above cited study reports proving the absence or minor significance of clinical signs.

An overall low health risk imposed by *B. subtilis* may also be derived from the safe application of *B. subtilis* in the industrial setting, e.g. large scale enzyme production, and regarding its use in vaccine production (see TAIRA et al., 1989).

An appropriate compensation of this study may be presented by the performed intravenous challenge with 35-Day observation period, which caused no adverse impacts (see [redacted], 1998c)

Included in 1st additional submission (September 2001)

Further data requirements on toxicity of *B. subtilis* are primarily addressed to the clearance capacity of rats for spores of strain QST 713 of *B. subtilis* following repeated inhalative exposure (Volume 1, Point 4.1.3).

The toxicological concern regarding the potential sensitizer subtilisin, as expressed in the monograph, (see Vol. 1, Point 4.1.3) is not regarded as relevant by the German BgVV (National Agency for Consumer's Health Care and Veterinary Medicine) in view of the lack of valid exposure limits for subtilisin in the US ([redacted] FOX, 2001). Therefore no further data or information has been generated on this topic.

A first draft of a protocol for a relevant study has been submitted in April 2001 ([redacted], 2001a), but major changes have been implemented after discussions with experts from the BgVV (Consument's Health and Veterinary Agency). The new protocol has been submitted for review to the BgVV in September 2001 ([redacted], 2001b).

To date the study still has not been initiated, because of new evidence provided by a literature search. The below mentioned references have been submitted to both the German BBA and the BgVV (Consument's Health and Veterinary Agency) to re-evaluate the data request as outlined in the monograph.

Relevant studies have been reported by WATSON et al. (1973) and SAUNDERS et al. (1983), applying *B. subtilis* spores on rats (intratracheally) and pigs (inhalative exposure), respectively. Rats received a single dose of 8 × 10^7 CFU viable spores, and clearance from lungs was monitored over a 48h period (WATSON et al., 1973). At 48 h post-exposure no viable spore was detected, and clearance was achieved to 85% (15% of injected dose remaining). In preliminary tests inactiviation of viable spores was demonstrated to occur in lung tissue due to bactericidal substance(s) found naturally in the lungs. Pigs were exposed to a an aerosol generated by an ultrasonic nebulizer for 15 minutes (SAUNDERS et al., 1983). This technique implies that no viable spores were applied, but for determining clearance of spores this was not required. Clearance was monitored during a 12-h period based on the initial deposition of spores in lungs determined immediately after exposure. No dose rate per animal was given, since this is hard to be exactly defined for the route of inhalative exposure. Conclusively, the results of both references indicate a fast clearance of *B. subtilis* spores from exposed tissues.

Further publications address to other *Bacillus* species or fungal pathogens, indicating that respiratory tract and lungs own specific defense mechanisms to eliminate even pathogenic spores.

One reference on pulmonary clearance relates to *B. thuringiensis* (TSAI et al., 1997) after intratracheal injection of a single dose of 1 × 10^8 CFU/rat. Behaviour and toxicological effects of this species cannot be compared to the species *B. subtilis*, which does not produce exotoxins.

Clearance of Bacillus anthracis was studied following inhalative exposure to guinea pigs (VANCURIK, 1965). The authors calculated the dose rate per animal from the aerosol concentration by a special mathematical formula. The employed dose rates ranged from 1.32 × 10^5 cfu/ animal (for mice), to 2.9 × 10^5 (small guinea-pigs), and 2.43 × 10^6 (larger guinea-pigs).

Results of the toxicological investigation are not relevant for *B. subtilis*, since *B. anthracis* is a known pathogen and *B. subtilis* is innocuous to humans. Clearance of *B. anthracis* spores from lungs was determined to occur fast, with a half-life of not more than 2 days, and to be complete within 36 days. Spores infiltrating the tracheobronchial nodes were cleared less rapidly and suggested to be a cause of the noted relapse having discontinued antibiotic prophylaxis.

Clearance of fungal spores from lungs of mice was determined to be 30 days following intranasal inoculation at 5 × 10^6 cfu/ animal (WALDORF et al., 1984). Spores of the employed fungal species (*Rhizomucor pusillus*) extracted from the tissues were found to be viable and infectious, however, this result is not applicable to the non-pathogenic spores of *B. subtilis*. The studies of WALDORF et al. (1984) and more specifically WHITE (1977) indicate the importance of an active defense mechanism of the exposed tissue, since Cortison treatment did impair the defense profoundly and resulted in markedly higher germination of fungal spores.

The fate of metal stained *Aspergillus terreus* spores following inhalation was monitored by microscopy (GREEN et al., 1980). The uptake of spores by alveolar macrophages was demonstrated to be rapid virtually completed within 3 hours after exposure. This reference gives an insight into the defense mechanisms of the respiratory system towards spores in general.

Inactivation of fungal spores upon intratracheal installation in rabbit lung was demonstrated by KURUP (1984), who examined the ability of macrophages to destroy pathogenic fungal spores of different species under different conditions. The significance of the species was clearly shown.

VOISIN et al. (1971) also address to the immune system response towards pathogenic fungi, following inhalation and intratracheal inoculation.

Evidence for a low health impact of bacterial spores in general can be delineated from an epidemiological study on 8482 farmers and spouses performed in Norway (MELBOSTAD & EDUARD, 2001). Exposure to bacterial and fungal spores is accompanying manifold tasks carried out in farms. The National Institute of Occupational Health in Norway concluded from the vast data generated that work related symptoms are common in farmers and are associated with exposure to total dust, fungal spores and endotoxins. No statistical correlation was determined for bacterial spores.

In conclusion, there is an effective defense mechanism of lungs towards inhaled spores, and there is no epidemiological evidence for an inhalative health risk for farmers who are exposed to bacterial spores.

The current status of the official evaluation process is, that the BgVV experts offered to evaluate the data request newly, based on the submitted literature, and referred to the discussion on member state level at a future ECCO meeting to ultimately decide upon this data request.

Conclusions:

Considering the presented scientific evidence of a fast and efficient clearance of spores by the exposed respiratory tissues, the applicant concludes that the data requirement, as outlined in the monograph (Volume 1, Point 4.1.3; repeated dose inhalation toxicity study) is adequately addressed to by submitted references and information and therefore the performance of a repeated dose inhalation toxicity study is not justified.

Therefore, the applicant did not initiate the corresponding study (see [redacted], 2001b) (this and applies for an exemption from this data request.

In addition, it has to be taken into account that the non-pathogenic and non-infectious character of strain QST 713 of *B. subtilis* has been proven in the toxicological and ecotoxicological studies submitted within the EU Dossier. The relevant studies showed that this strain of *B. subtilis* does not produce toxins, and does not germinate or proliferate in tissues of mammals following oral, or intratracheal, or inhalative exposure.

Further, the performance of the repeated inhalative toxicity study itself is a critical point, since there is no specific OECD guideline for testing micro-organisms yet, which act basically different than chemicals. The relevant test guideline OECD 412, addressing to chemical active ingredients, states a daily 6-h interval for a period of 4 weeks for a repeated inhalative exposure. This exposure scenario will under no circumstances reflect real conditions, under which applicants may be exposed to the dust when preparing the spray.

So far two study protocols have been developed in an extensive discussion process with the German officials at the BgVV ([redacted], 2001a and 2001b) to meet all required data demands, especially the main task of assessing clearance. Still the protocol would require some discussion and adjustments, since it is technically almost impossible to ensure a pre-set dose rate per animal by inhalative exposure, which only employs a given concentration of spores in the air. Regarding determination of clearance a pre-set concentration of spores would allow the monitoring of tissue spore content as well.

Finally, determining complete clearance of spores from lungs requires a long post-exposure observation period and a high number of test animals, without yielding necessary toxicological information.

IIM 5.3.7.1 Short-term toxicity, pathogenicity, infectivity (28-day minimum)

Please refer to Point 5.3.7

IIM 5.3.7.2 Inhalatory short-term toxicity

Included in 3rd additional submission (April 2005)

A study on sub-acute inhalation toxicity of Serenade Biofungicide (technical powder) was conducted by [redacted] (2004) and submitted in November 2004.

Report 5.1.4/01: [redacted] (2004): SUB-ACUTE (4-WEEK) INHALATION TOXICITY STUDY, INCLUDING AN 8-WEEK RECOVERY STUDY, WITH SERENADE BIOFUNGICIDE IN RATS
TNO, Location [redacted]
– published: no, report No. V 5435 (Dates of work: 12/18/2003 to 04/29/2004)

Document No: M-474026-01-1

Guideline: OECD Guideline for Testing of Chemicals, No. 412 (May 1981);
EC Guideline B.8, EEC Directive 92/69/EEC;
OPPTS Guideline No. 885.3600

Deviations: none

GLP: Yes

Materials and methods: Test item: Serenade Biofungicide (technical powder, approx. 1.4×10^{10} cfu/g), batch 8AQ07D29.

The inhalation toxicity of Serenade Biofungicide was studied in Sprague Dawley rats (groups of 16 male, 16 female each), exposed to control air or a target concentration of 0.35 mg/L test item for six hours a day on five days a week during 28 days, with a total of 20 exposure days. Two male and two female rats of each group were necropsied prior to exposure, on day 1 after the last exposure and every four weeks later up to 8 weeks after exposure. As *B. subtilis* was not detected in any organs/tissues eight weeks after the last exposure, the recovery period lasted eight instead of 24 weeks in deviation of the study plan.

Clinical sings, body weights, food consumption and food conversion efficiency were determined prior, during and after exposure. In addition, a full necropsy was performed, lung weights determined and a selection of organs/tissues was analysed to determine the presence of viable bacteria.

Observations: The concentration level used during the 28-day exposure period was based on a preceding 5-day pilot test with a total of 5 exposure days. In this study one group of 2 male and 2 female rates was exposed to 0.65 mg/L. Animals were necropsied one week after the last exposure. Changes consisted of reduced body weight gain, changes in breathing pattern during exposure, and clinical signs after the first exposure. Based on these results it was decided in consultation with the sponsor to reduce the target concentration in the main study to 0.35 mg/L. By generating the concentration of 0.35 mg/L (or 350 mg/m³) and taking into account the concentration of the test substance (i.e. approximately 1.4×10^{10} cfu/g) in theory, a number of 0.49×10^{10} cfu/m³ air was generated. In 6 hours an animal will inhale about 1000 litres of air (taking into account a ventilation of 250 mL/min), indicating a number of about 5 E8 cfu (external dose) per day. This is above the required dose of 1×10^8 units of MCPA (microbial pest control agent) administered per day according to the OPPTS 885.3600 guideline.

In the main study, the mean actual concentration (± standard deviation) of the test item in the test atmospheres was 350 ± 28 mg/m³. The mean nominal concentration was 769 ± 73 mg/m³, indicating a generation efficiency of 46%. The average mass Median Aerodynamic Diameter and the geometrical standard deviation of the particles was 2.6 µm ± 2.1.

Findings: No treatment-related abnormalities were observed before, during and after exposure.

Mean body weight gain was significantly reduced in male animals during the 4-week exposure period, but recovered thereafter. Such a decrease was not seen in females.

Food consumption was significantly increased in males on day 49; food conversion efficiency was significantly decreased in males on day 7 and 14 but

significantly increased on days 35 and 42. Such changes were not observed in females.

The day after the last exposure, absolute and relative weight of the mediastinal lymph nodes were significantly higher in the test group than in the control group. Similarly, the absolute weight of the lungs was significantly increased in males and females compared to the control group and the relative weight of the lungs was significantly increased in the female animals of the test group. Four weeks after the last exposure, the absolute weight of the lungs was still significantly increased; however the relative weight was not. The absolute and relative weight of the mediastinal lymph node was similar in control and test animals, but the absolute weight of the cervical lymph nodes was significantly increased in males of the test group. Finally, eight weeks after the 28-day exposure period a significant difference in the absolute and relative weight of the lungs and lymph nodes was no longer seen.

No treatment-related macroscopic changes were observed at any of the necropsy periods.

No *B. subtilis* was detected in organs or tissues of the control or test animals before the start of exposure. On the day after the 4-week exposure period, the numbers of *B. subtilis* in the lungs of the test animals were ca. 1 E5 – 1 E6 cfu/mL organ suspension (Table 5.1.4-1, as the total volume was 6 mL, the total number was ca. 6 E5-6 E6 cfu/lung). In the mediastinal lymph nodes, numbers of *B. subtilis* were ca. 100 cfu/mL tissue suspension. In the remaining organs/tissues (cervical, internal jugular plus posterior, superior mesenteric lymph nodes, and spleen and thymus), no or low numbers of colonies of *B. subtilis* were detected. In control animals, colonies with the typical colony morphology of *B. subtilis* were also observed, but those were considerably lower than the numbers found in the lungs of the test group animals at that time.

Four weeks after the last exposure, a low number of *B. subtilis* colonies (ca. 20 cfu/mL organ suspension) were detected in the lungs of only one of the four test group animals (Table 5.1.4-2). *B. subtilis* was not detected in any of the other organs/tissues.

Eight weeks after the last exposure *B. subtilis* was not detected in any of the organs/tissues of the test group animals (Table 5.1.4-3). Therefore, no measurements were carried out at the other time points after the last exposure.

Figure 5.1.4-1: The presence of viable micro-organisms* (*Bacillus subtilis* QST 713) in organ suspensions of the control and test group after the 4-week exposure period (<u>Wk 4</u>).

TNO Codes Animals	Colony counts (cfu [1]/mL organ suspension)							Colony counts (cfu[1]/mL organ suspension) after pasteurization [3]						
	SPL [2]	THY	MED	IJP	MES	CER	LL	SPL	THY	MED	IJP	MES	CER	LL
Group A – control group														
5	< 10	< 10	< 10	< 10	< 10	< 10	< 10	Not determined						
6	< 10	< 10	< 10	< 10	ca. 10-200	< 10	< 10							
7	< 10	< 10	< 10	< 10	< 10	< 10	10^2-10^3							
8	< 10	< 10	< 10	< 10	< 10	< 10	+ [4]							
Group B – test group														
37	+ [5]	+ [5]	1.4×10^2	< 10	< 10	+ [5]	5.7×10^5	< 10	+ [5]	ca. 80	< 10	< 10	ca. 40	7.0×10^5
38	+ [5]	< 10	1.5×10^2	ca. 10	+ [5]	ca. 20	4.4×10^5	ca. 20	< 10	+ [5]	ca. 20	< 10	ca. 20	5.2×10^5
39	+ [5]	ca. 20	2.8×10^2	ca. 20	< 10	+ [5]	1.5×10^5	ca. 20	ca. 50	1.8×10^2	ca. 10	ca. 10	ca. 60	6.6×10^5
40	< 10	< 10	1.0×10^2	+ [5]	< 10	ca. 20	7.2×10^5	ca. 40	+ [5]	1.5×10^2	ca. 20	ca. 10	ca. 10	5.6×10^5

* viable micro-organism: counts of colonies which had the typical colony morphology of the *Bacillus subtilis* test substance

1) cfu = colony forming units
2) SPL = Spleen, THY = Thymus, MED = Mediastinal lymph nodes, IJP = Internal jugular plus posterior lymph nodes, MES =Mesenteric lymph nodes, CER = Cervical lymph nodes. LL = Lung lobes
3) Pasteurization at 65°C for 30 minutes
4) + = colonies with typical colony morphology of the *Bacillus subtilis* test substance were present, but the outcome of the decimal dilution series was irregular, possibly caused by clumping factors. Therefore, no (range of) colony count(s)could be calculated, but the numbers found in the lungs of the animal of the control group were considerably lower than the numbers found in lung lobes of the animals from the test group.
5) + = colonies with typical colony morphology of the *Bacillus subtilis* test substance were present, but the outcome of the decimal dilution series was irregular, possibly caused by clumping factors. Therefore, no (range of) colony count(s)could be calculated, but the numbers found in the examined organ were considerably lower than the numbers found in lung lobes of the animal.

Figure 5.1.4-2: The presence of viable micro-organisms* (*Bacillus subtilis* QST 713) in organ suspensions of the control and test group four weeks after the last exposure (Wk 8).

TNO Codes Animals	Colony counts (cfu [1]/mL organ suspension)							Colony counts (cfu[1]/mL organ suspension) after pasteurization [3]						
	SPL [2]	THY	MED	IJP	MES	CER	LL	SPL	THY	MED	IJP	MES	CER	LL
Group A – control group														
9	< 10	< 10	< 10	< 10	< 10	< 10	< 10							
10	< 10	< 10	< 10	< 10	< 10	< 10	< 10							
11	< 10	< 10	< 10	< 10	< 10	< 10	< 10	Not determined						
12	< 10	< 10	< 10	< 10	< 10	< 10	< 10							
Group B – test group														
41	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
42	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
43	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
44	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	ca. 20

* viable micro-organisms: counts of colonies which had the typical colony morphology of the *Bacillus subtilis* test substance

1) cfu = colony forming units
2) SPL = Spleen, THY = Thymus, MED = Mediastinal lymph nodes, IJP = Internal jugular plus posterior lymph nodes, MES =Mesenteric lymph nodes, CER = Cervical lymph nodes, LL = Lung lobes
3) Pasteurization at 65 °C for 30 minutes

Figure 5.1.4-3: The presence of viable micro-organisms* (*Bacillus subtilis* QST 713) in organ suspensions of the control and test group eight weeks after the last exposure (Wk 12).

TNO Codes Animals	Colony counts (cfu [1]/mL organ suspension)							Colony counts (cfu[1]/mL organ suspension) after pasteurization [3]						
	SPL [2]	THY	MED	IJP	MES	CER	LL	SPL	THY	MED	IJP	MES	CER	LL
Group A – control group														
13	< 10	< 10	< 10	< 10	< 10	< 10	< 10	Not determined						
14	< 10	< 10	< 10	< 10	< 10	< 10	< 10							
15	< 10	< 10	< 10	< 10	< 10	< 10	< 10							
16	< 10	< 10	< 10	< 10	< 10	< 10	< 10							
Group B – test group														
45	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
46	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
47	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
48	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10

* viable micro-organism: counts of colonies which had the typical colony morphology of the *Bacillus subtilis* test substance

1) cfu = colony forming units
2) SPL = Spleen, THY = Thymus, MED = Mediastinal lymph nodes, IJP = Internal jugular plus posterior lymph nodes, MES =Mesenteric lymph nodes, CER = Cervical lymph nodes, LL = Lung lobes
3) Pasteurization at 65°C for 30 minutes

Conclusions: The presence of viable spores in lungs and draining lymph nodes and the increases in weight of these organs indicated a physiological, local response in the absence of treatment-related clinical signs. Eight weeks after the last exposure, viable spores and organ weight increases could no longer be observed in lungs and draining lymph nodes, which indicated that a full 24-week recovery period was not necessary. The concentration given to the animals (i.e. 350 mg/m³) induced slight toxicity only (decreased body weight gain in males) and was, therefore, considered an optimal concentration to test toxicity and clearance of the test substance. The dose administered exceeded the required administered dose according to the OPPTS 885.3600 guidelines.

IIM 5.4 Toxicity studies on metabolites (especially toxins)

Report:	KIIM 5.4/02; [redacted]; [redacted]; [redacted]; [redacted]; [redacted]; [redacted]; [redacted]; [redacted]; [redacted], E.H.; [redacted], M.T.; [redacted]; [redacted]; [redacted]; [redacted], J.L.; [redacted]; [redacted]; [redacted]; V; 1989; M-477486-01-1
Title:	Safe biotechnology - III. Safety precautions for handling microorganisms of different risk classes
Report No.:	M-477486-01-1
Document No.:	M-477486-01-1
Guideline(s):	not applicable
Guideline deviation(s):	not applicable
GLP/GEP:	**no**

Report:	KIIM 5.4/03; [redacted]; [redacted]; 1991; M-486912-01-1
Title:	On the safety of Bacillus subtilis and B. amyloliquefaciens: a review
Report No.:	M-486912-01-1
Document No.:	M-486912-01-1
Guideline(s):	not applicable
Guideline deviation(s):	not applicable
GLP/GEP:	**no**

Report:	KIIM 5.4/04; [redacted]; [redacted], H.O.; 1998; M-473465-01-2
Title:	Bacillus subtilis - A 48-hour static acute toxicity test with the cladoceran (Daphnia magna)
Report No.:	M-473465-01-2
Document No.:	M-473465-01-2
Guideline(s):	FIFRA Subdivision E, Series 72-2
Guideline deviation(s):	not specified
GLP/GEP:	**yes**

Included in 2nd additional submission (July 2004)

Bacillus subtilis produces several different secondary metabolites. Detailed information on secondary metabolites formed by the strain QST 713 of *B. subtilis* has been submitted to all Member States in October 2001 for the evaluation for the Annex I inclusion (MANKER, 2001).

A well known class of such secondary metabolites includes the lipopeptide surfactin and iturin compounds, which are amphiphilic membrance-active biosurfactants and peptide antibiotics with potent antimicrobial activities.

The surfactin and iturin compounds are cyclic lipoheptapeptides, which contain a ß-hydroxy fatty acid and a ß-amino fatty acid, respectively, as lipophilic moiety and a heptapeptide as hydrophilic component.

No genotoxicity tests have been conducted with the metabolites of *Bacillus subtilis.* However, it is deduced from the structure of the surfactin and iturin lipopeptides, that there are no structural moieties, which suggest that these lipopeptides may induce direct mutagenicity, e.g. point mutations, frameshift mutations, or clastogenicity. Regarding a possible genotoxic activity, it is, thus, reasonable to assume that these metabolites of *Bacillus subtilis* do not represent a direct genotoxic hazard. Therefore, genotoxicity testing with surfactin and iturin compounds is not regarded as necessary.

Moreover, there is no public literature report available on this well-studied species indicating a genotoxic or carcinogenic harzard by *B. subtilis*. According to the U.S. EPA (1997) *B. subtilis* does not appear to have specialised attachment mechanisms typically found in organisms capable of colonizing the human body. The information provided under Point 5.4 (medical data) confirms the low human health risk attributed to *B. subtilis*: e.g. FROMMER et al. (1989) state a long history of safe use of *B. subtilis* in enzyme production and that worker protection does not require containment of *B. subtilis.* Furthermore, this species falls under Class 1 Containment of European federal Law of Biotechnology (EPA, 1997).

Reviewing clinical cases and referring to GRAS petitions, which in no case demonstrated invasive properties of *B. subtilis*, BOER & DIDERICHSEN (1991) concluded that *B. subtilis* is a safe host for the production of harmless products.

Furthermore, the results of the submitted toxicological studies prove that *B. subtilis* does neither act toxic nor pathogenic against higher organisms. The absence of secreted toxins acting against invertebrates was proved by exposing daphnids to a cell-free, spray-dried filtrate which did not cause any effect (DROTTAR & KRUEGER, 1998b).

IIM 5.5 Other/special studies

IIM 5.5.1 Specific toxicity, pathogenicity and infectiveness studies

Report: KIIM 5.5.1/01; [redacted]; [redacted]; 1991; M-486912-01-1
Title: On the safety of Bacillus subtilis and B. amyloliquefaciens: a review
Report No.: M-486912-01-1
Document No.: M-486912-01-1
Guideline(s): not applicable
Guideline deviation(s): not applicable
GLP/GEP: no

Report: KIIM 5.5.1/02; Ihde, D. C.; [redacted]; 1973; M-153632-01-1
Title: Clinical spectrum of infection due to Bacillus species
Report No.: A8103a
Document No.: M-153632-01-1
Guideline(s): --
Guideline deviation(s): --
GLP/GEP: no

Report: KIIM 5.5.1/03; [redacted]; 1997; M-528163-01-1
Title: Final decision document: TSCA section 5 (H) (4) exemption for Bacillus subtilis
Report No.: M-528163-01-1
Document No.: M-528163-01-1
Guideline(s): --
Guideline deviation(s): --
GLP/GEP: no

<u>EU-Dossier: Doc M-IIB, Point 5.1.5</u>

Bacillus subtilis can grow at temperatures higher than 32°C, as given in human bodies, but it is known as usually non-pathogenic. Therefore, no epidemiological studies were performed and no medical surveillance programme was conducted. Following statements can be inferred from literature:

B. subtilis has a low virulence and low risk potential for human health and is regarded as non-pathogenic and non-toxic (EPA, 1997; BOER & DIDERICHSEN, 1991).

B. subtilis does have a potential in eliciting allergic reactions in individuals repeatedly exposed to the secreted proteinaceous compound *subtilisin* (EPA, 1997).

Publications on clinical cases suggest no invasive properties of *B. subtilis*: in some cases *B. subtilis* was isolated from surgical wound or tumor drainages, but it remained locally restricted and did not influence the course of wound healing; only highly immuno-suppressed patients were reported to have suffered from dissipating bacterial infections caused by *B. subtilis* (and other species) (IHDE & ARMSTRONG, 1973; BOER & DIDERICHSEN, 1991).

These findings suggest that under normal health conditions no pathogenicity and infectivity of *B. subtilis* is expected to occur, esp. in view of the given ambient exposure towards this ubiquitous bacteria.

<u>EU-Dossier: Doc M-IIB, Point 5.1.1.4</u>

Primary dermal irritation

Report:	[redacted] (1998b) (This was a typographical error- the study name is [redacted], [redacted] 1998a): Primary dermal irritation in rabbits with QST 713 TP; [redacted]; unpublished; Study Number: 0420XA54.004; dates of experimental work: June 25– June 28, 1998.
Document No:	M-473980-01-1
Guidelines:	EPA-Pesticide Assessment Guidelines, Subdivision F, (No. 81-5) Corresponds to EEC B4 – Directive 92/69/EEC, and to OECD guideline 404 (applying to chemical substances)
GLP:	Yes (self certification by the laboratory)
Material and methods:	QST 713 Technical (dried *Bacillus subtilis* with residual fermentation media; Lot No.: 8AQ07C2). Titer of this lot (determined in [redacted] 1998a) is ~ 4,3 × 10^{10} cfu/g, conclusively 500 mg contain ~ 2,1 × 10^{10} cfu, as dose level/ animal. 500 mg of moistened (0,3 ml saline) test substance was applied to the shaved skin of 3 male and 3 female New Zealand [redacted] rabbits and held in place with an occlusive wrapping for 4 hours. Observations were recorded at ~30 minutes, 24, 48 and 72 hours after unwrapping.
Findings:	No mortality was observed. Slight erythema symptoms appeared within 24 h following application, symptoms cleared by 48 h. The primary irritation index was calculated to be 0,3 (on a scale 0 to 4). No significant effects on body weights were noticed. **Dermal responses**: very slight erythema **Other clinical signs**: none
Conclusions:	QST 713 TP caused very slight irritation symptoms after 4 h of dermal exposure. According to EC directive 67/548/EEC QST 713 TP is classified as **non-irritant**.

EU-Dossier: Doc M-IIB, Point 5.1.1.5

Primary Eye Irritation

Report:	[redacted]. (1998c) (This was a typographical error- the study name is [redacted], V.T. 1998b): Primary eye irritation in rabbits with QST 713 TP; [redacted].; unpublished; Study Number: 0421XA54.004, dates of experimental work: July 10 – July 14, 1998.
Document No:	M-474019-01-2
Guidelines:	Dose selection according to EPA Pesticide Assessment Guidelines, subdivision F No. 81-4 (1984), partly also applying to scale for scoring ocular lesions. Tabulating of test article according to Addendum 2 on EPA Pesticide Assessment Guidelines – Eye Irritation (1988) Corresponds to EEC B5 - Directive 92/69/EEC, and to OECD guideline 405 (applying to chemical substances)
GLP:	Yes (self certification by the laboratory)
Material and methods:	QST 713 Technical (dried *Bacillus subtilis* with residual fermentation media; Lot No.: 8AQ07C2). Titer of this lot (determined in [redacted] 1998a) is ~ 4,3 × 10^{10} cfu/g, conclusively 81 to 100 mg contain ~ 3,5 – 4,3 × 10^{9} cfu, as dose level/animal. Packed into a 1cc syringe to a 0,1 ml volume (sample weight 81-99,9 mg) the test substance was instilled into the conjunctivae sac of the right rabbit eye (3 males, 3 females). Reactions were recorded: initially, at 1, 24, 48 and 72 hours and on day 4 following administration.
Findings:	Main affected area was the conjunctivae predominantly showing redness; slight swelling (chemosis) mainly occurred within the first hour following application; the iris exhibited only lowest grades considered positive. All symptoms had ceased within the 4-day observation period. The reported calculation and evaluation of the Draize scores is based upon the U.S. provisions which differ fundamentally from the relevant EC directive 67/548/EEC. The adopted calculation gives following values, referring to each symptom separately:

Symptom	Mean score value	Classification
Cornea opacity	0	none
Iris lesions	<1	none
Redness of conjunctivae	<1	none
Chemosis of conjunctivae	<1	none

Conclusions:	QST 713 Technical was determined to be **non-irritant** according to the relevant EC directive 67/548/EEC.

EU-Dossier: Doc M-IIB, Point 5.2.1

Report:	[redacted]. (1998): Acute dermal toxicity/ pathology study of QST 713 in rabbits; [redacted].; Laboratory Project ID L08726 SN7; unpublished; dates of experimental work: Apr. 1 – April 29, 1998.
Document No:	M-474031-01-1
Guidelines:	EPA - Pesticide Assessment Guidelines, Subdivision M – Section Series 152A-

10 (Microbial Pesticide Test Guidelines OPPTS 885.3100)

Corresponds generally to EEC B3 - Directive 92/69/EEC (limit-test) and to OECD guideline 402 applying to chemical substances

GLP: Yes (self certification by the laboratory)

Material and methods: QST 713 Technical (dried *Bacillus subtilis* with residual fermentation media; Lot No.: 8AQ07C2; reported titer: 4,7 × 10^{10}cfu/g).
Individual test substance doses ranged between 2,3 – 2,73 × 10^{11} cfu

Individual doses of 2 g/kg body weight, suspended in 3 mL sterile water, were administered in paste form to the shaved backs of five male and five female New Zealand White rabbits and left in contact for 24 hours. Daily observations were recorded for 14 days.

Findings: No deaths and no systemic toxicity signs occurred during the study:
All rabbits showed varying degrees of *dermal irritation* (erythema, edema, eschar formation, sores and necrosis). Superficial flaking of the skin appeared frequently, partly persisting at the study termination. In most cases symptoms had disappeared at the end of the observation period, new or repaired skin appeared at the application site.
LD_{50}: > 2 g/kg body weight
The LD_{50} could not be calculated because no mortality occurred.

Conclusions: The results show that the active substance, *B. subtilis*, can be classified as non-toxic (no labelling requirements according to EC directive 67/548/EEC).

IIM 5.5.2 Genotoxicity- in vivo studies in somatic cells

Report: KIIM 5.2/01; [redacted]; [redacted]; [redacted]; [redacted]; [redacted]; [redacted]; [redacted]; [redacted]; [redacted], E.H.; [redacted], M.T.; [redacted]; [redacted]; [redacted]; [redacted], J.L.; [redacted]; [redacted]; [redacted]; V; 1989; M-477486-01-1
Title: Safe biotechnology - III. Safety precautions for handling microorganisms of different risk classes
Report No.: M-477486-01-1
Document No.: M-477486-01-1
Guideline(s): not applicable
Guideline deviation(s): not applicable
GLP/GEP: no

Report: KIIM 5.5.2/02; [redacted]; [redacted]; 1991; M-486912-01-1
Title: On the safety of Bacillus subtilis and B. amyloliquefaciens: a review
Report No.: M-486912-01-1
Document No.: M-486912-01-1
Guideline(s): not applicable
Guideline deviation(s): not applicable
GLP/GEP: no

Report:	KIIM 5.5.2/03; [redacted] 1997; M-528163-01-1
Title:	Final decision document: TSCA section 5 (H) (4) exemption for Bacillus subtilis
Report No.:	M-528163-01-1
Document No.:	M-528163-01-1
Guideline(s):	--
Guideline deviation(s):	--
GLP/GEP:	**no**

Report:	KIIM 5.5.2/04; [redacted]; 1989; M-484919-01-1
Title:	Introduction to the biotechnology of Bacillus
Report No.:	M-484919-01-1
Document No.:	M-484919-01-1
Guideline(s):	not specified
Guideline deviation(s):	not specified
GLP/GEP:	**no**

Report:	KIIM 5.5.2/05; [redacted]; [redacted], H.O.; 1998; M-473465-01-1
Title:	Bacillus subtilis - A 48-hour static acute toxicity test with the cladoceran (Daphnia magna)
Report No.:	489A-103
Document No.:	M-473465-01-1
Guideline(s):	FIFRA Subdivision E, Deries 72-2
Guideline deviation(s):	not specified
GLP/GEP:	**yes**

<u>EU-Dossier: Doc M-IIB, Point 5.1.2</u>

The genotoxic potential of the ubiquitous bacteria B. subtilis has not been determined since there is strong evidence for a very low, or non-existent genotoxic potential of this species in the literature, specified by submitted study reports on QST 713 strain of B. subtilis, which does not produce any toxins.

In no reference has B. subtilis been associated with cancerogenesis as the causative agent or merely with entry into mammalian cells. According to the U.S. EPA (1997) B. subtilis does not appear to have specialised attachment mechanisms typically found in organisms capable of colonizing the human body. The information provided under Point 5.4 (medical data) confirms the low human health risk attributed to *B. subtilis*: e.g. FROMMER et al. (1989) state a long history of safe use of B. subtilis in enzyme production and that worker protection does not require containment of B. subtilis. Furthermore, this species falls under Class 1 Containment of European Federal Law of Biotechnology (EPA, 1997).

Reviewing clinical cases and referring to GRAS petitions, which in no case demonstrated invasive properties of *B. subtilis*, BOER & DIDERICHSEN (1991) concluded that B. subtilis is a safe host for the production of harmless products.

The only secondary metabolite of *B. subtilis* with health concern is the proteinaceous compound subtilisin, which has merely been attributed to allergic reactions of exposed individuals, such as workers in fermentation facilities (EPA, 1997).

Furthermore, the results of the submitted toxicological studies prove that *B. subtilis* does neither act toxic nor pathogenic against higher organisms (see toxicological summary report, Doc. K-IIB, P. 5.5 and ecotoxicological risk assessment, Doc. K-IIB, Sec. 6, P. 9). The absence of secreted toxins acting against invertebrates was proved by exposing Daphnids to a cell-free, spray-dried filtrate

which did not cause any effect (DROTTAR & KRUEGER, 1998b, Doc. K-IIB, Section 6, Point 8.2.2/01).

Finally, this micro-organism is regarded as non-pathogenic in the literature (BOER & DIDERICHSEN, 1991; EPA, 1997; FROMMER et al., 1989; HARWOOD, 1989a).

Considering the low risk potential of *B. subtilis* and its ubiquitous distribution, even in foods, genotoxicity testing appeared to be dispensable.

Report: KIIM 5.5.2/06; [redacted]; [redacted]; [redacted]; 2002; M-352553-01-1
Title: Cytotoxic potential of industrial strains of Bacillus sp.
Report No.: M-352553-01-1
Document No.: M-352553-01-1
Guideline(s): --
Guideline deviation(s): --
GLP/GEP: **no**

Included in 2nd additional submission (July 2004)

The cytotoxic potential of selected strains of *B., subtilis* has been assessed. Cytotoxicity was determined in Chinese hamster ovary (CHO-K1) cells. The *B.s subtilis* strains tested were nontoxic to CHO-K1 cells. Additionally it was demonstrated that industrially used strains of *Bacillus subtilis* did not react with antibodies against *B. cereus* enterotoxins (PEDERSEN et al., 2002).

Recently, the structural closely related lipopeptide daptomycin (Cubicin) was approved by US FDA as a human therapeutic for the treatment of complicated skin and skin structure infections.

Finally, this micro-organism is regarded as non-pathogenic in the literature (BOER & DIDERICHSEN, 1991; EPA, 1997; FROMMER et al., 1989; HARWOOD, 1989a).

Considering the low risk potential of *B. subtilis* and its ubiquitous distribution, even in foods, genotoxicity and cytotoxicity testing appeared to be dispensable.

IIM 5.5.3 Genotoxicity – in vivo studies in germ cells

The following references were submitted during the Annex I review and were not included in the Baseline dossier. They have been added at the request of the RMS.

KIIM 5.5.3 / 01[redacted]; 2004; Serenade WP (Bacillus subtilis strain QST713) - Annex IIB, Point 5.3.2: Genotoxicity and cytotoxicity Testing expert statement; M-595984-01-1

IIM 5.6 Summary of mammalian toxicity and overall evaluation

Report: KIIM 5.6/01; [redacted] C.; 2000; M-497565-01-1
Title: Bacillus subtilis (150 g/kg technical powder) & Serenade WP (AI:100g/kg, formulation: Wettable powder) - Summary of mammalian toxicity, pathogenicity and infectivity, exposure risk assessments and overall evaluation
Report No.: M-497565-01-1
Document No.: M-497565-01-1
Guideline(s): --
Guideline deviation(s): --
GLP/GEP: **no**

EU-Dossier: Doc M-IIB, Point 5.5

Summary table of acute toxicity and primary irritation studies

Oral				
Species	Vehicle	Sex	NOEL (cfu/animal)	LD_{50} (cfu/animal)
Rat	Sterile water	3 per group/ sacrifice day/ sex	> 1.13×10^8	> 1.13×10^8
Intratracheal				
Species	Vehicle	Sex	NOEL	LD_{50} (cfu/animal)
Rat	Sterile water	5 per group/ sacrifice day/ sex	Not determined	> 1.2×10^8
Primary dermal irritation				
Species	Vehicle	Sex	NOEL	LD_{50}
Rabbit	0,3 ml saline	3 males/3 females	Not relevant (non-irritant)	Not relevant (non-irritant)
Primary eye irritation				
Species	Vehicle	Sex	NOEL	LD_{50}
Rabbit	Moistened with sterile water	3 males/3 females	Not relevant (non-irritant)	Not relevant (non-irritant)
Acute dermal				
Species	Vehicle	Sex	NOEL	LD_{50} (cfu/animal)
Rabbit	Sterile water	5 males/5 females	Not determined	> $2.3\text{-}2.7 \times 10^{11}$
Acute intravenous				
Species	Vehicle	Sex	NOEL (cfu/ animal)	LD_{50} (cfu/animal)
Rat	Sterile water	3 rats per group/ sacrifice day/ sex	> 9.4×10^6	> 9.4×10^6

The active substance, *Bacillus subtilis*, has no toxic or clinical effects after oral, intravenous or dermal administration to rats. Very slight irritating effects were recorded after skin exposure and following application to the eye of rabbits but symptoms did not imply a classification according to the relevant EC directive 67/548/EEC. This also applies to the intratracheal challenge, which caused generally minor and mostly short-termed symptoms but no deaths or gross lesions at final necropsy.

According to the above mentioned results and to the all in all low risk potential of *B. subtilis* further studies concerning the

- Short-term toxicity
- Genotoxicity potential
- Long-term toxicity and carcinogenicity
- Reproductive toxicity
- Teratogenicity potential and
- Neurotoxicity potential

were not performed. A skin sensitization test was performed with the preparation, Serenade™ WP.
In addition, no clinical cases relating to strain QST 713 of *B. subtilis* were reported to occur in the laboratories and production facilities of the applicant. In the literature incidents of progressive *B. subtilis* infections were only reported for immuno-deficient patients suffering e.g. from leukemia. No specific clinical signs or poisoning symptoms can be attributed to strain QST 713 of *B. subtilis*, accordingly no special therapeutic regimes can be recommended for this non-toxic and non-pathogenic micro-organism.
In addition, any protection and precaution measures in handling QST 713 Technical are only relevant for workers at the producing facilities which are restricted to the U.S. territory and thus handling QST 713 Technical is under U.S. legal requirements. The producer, AgraQuest Inc., states that worker exposure is minimised due to rigorous application of Good Manufacturing Practise (GMP), quality control and protective clothing worn by workers at the facilities.