Bacillus amyloliquefaciens QST 713

Microbial Pest Control Agent against plant parasitic fungi and bacteria

Dossier according to OECD dossier guidance for microbial agents and microbial pest control products –August 2006

Summary documentation, Tier II

Annex IIM, Section 5

Point IIM 7: Fate and Behaviour Studies on the Microbial Pest Control Agent in the Environment

Date: September 2015

Applicant

Bayer CropScience AG

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OWNERSHIP STATEMENT

Introduction

The company Bayer CropScience AG is submitting a dossier for the re-approval of the microorganism *Bacillus amyloliquefaciens* QST 713 as an active substance under regulation (EC) 1107/2009, previously designated as *Bacillus subtilis* QST 713. Due to most current information on taxonomy, *B. subtilis* QST 713 is classified as a member of *B. amyloliquefaciens* group. As a consequence, the active substance is now named as *B. amyloliquefaciens* subsp. *plantarum* QST 713, hereinafter named as *B. amyloliquefaciens* QST 713.

The initial evaluation of *Bacillus subtilis* QST 713 was performed under Directive 91/414. Data provided in the initial dossier and in subsequent additional submissions according to the OECD dossier guidance (2006) are submitted as a "Baseline Dossier", separately.

Here we submit all new data and information basing on previous literature searches and studies.

IIM 7 Fate and Behaviour Studies on the Microbial Pest Control Agent in the Environment

IIM 7.1 Sufficient information on the origin, properties, survival and residual metabolites of the microorganism to assess its fate and behaviour in the environment. Viability/population dynamics, persistence, multiplication and mobility

B. subtilis and *B. amyloliquefaciens* naturally occur ubiquitous in the environment. For the background information, please refer to the baseline dossier.

To gain sufficient information on the fate and behaviour of *B. amyloliquefaciens* QST 713, an intensive literature search was conducted on the DIMDI database provided by the German Institute of Medical Documentation ([redacted], 2015). Four databases were considered in this search: MEDLINE, CAB Abstracts, SCISEARCH and BIOSIS. To consider also close related strains of the old designation, search on *Bacillus amyloliquefaciens* was expanded to *B. subtilis*. After full text assessment, 5 articles were identified as relevant and supportive and are considered in this dossier under Point IIM 7.1.1. However, no additional information was identified regarding persistence and mobility in water or air. Hence, please refer to the baseline dossier for the background information.

Cited references (abstracts):

Report: KIIM 7.1/01 – [redacted] (2015), Literature review on *Bacillus amyloliquefaciens* QST 713 and metabolites: Fate and behaviour in the environment
Unpublished report.
Owner: Bayer CropScience AG
Report No. 6791109-A2-07-01
M-535711-01-1

Abstract: A detailed literature research review on the fate and behaviour of *Bacillus amyloliquefaciens* QST 713, using DIMDI engine from German Institute of Medical Documentation and comprised of searches in MEDLINE, BIOSIS, CAB Abstracts and SCISEARCH databases.

IIM 7.1.1 Persistence and mobility in soil

B. subtilis and *B. amyloliquefaciens* naturally and ubiquitously occur worldwide in soil and other environmental compartments and are even food sources for soil living organisms like earthworms ([redacted] et al, 2007). For background information, please refer to the baseline dossier.

[redacted] et al. (2008) developed a strain-specific genomic marker for *B. subtilis* 101 to study the bacterial fate in rhizosphere after application onto tomato seeds in two different soil types (sandy-loam soil and peat-based substrate). After application, *B. subtilis* population was about 1.6-5.5 × 10^6 CFU/seed. Bacterial population decreased after inoculation of seeds in both soil types. However, there were significant differences between soil and time. In sandy-loam soil vegetative cells of bacteria decreased drastically under the detection limit at days 28 and 35, whereas cells decreased more gradually in the other soil type. Although, *B. subtilis* may behave different from *B. amyloliquefaciens* QST 713, general findings may be transferrable.

This was later confirmed by [redacted] et al. (2009) who studied an AFLP (amplified fragment length polymorphism)-derived tracking systems for *Bacillus* and *Paenibacillus* in soil. For *B. amyloliquefaciens* DSL 13563-0, the authors report a strong decrease in sandy-loam soil under the detection limit (1 × 10^2 CFU/g soil) at 25 days after application. Similar was observed for *B. subtilis* strain ATCC 55405. *B. subtilis* strains ATCC 6051A and NRRL B-949 decreased too, but detection limit was reached between 88 and 110 days after application. These findings indicate that environmental fate may differ between species and strains. Nevertheless for all tested microorganisms, reduction of population densities in rhizosphere was reported.

[redacted] et al. (2005) studied the transport of *B. subtilis* under different water contents in an intact soil column. Under unsaturated conditions a very low leakage of *B. subtilis* trough the soil matrix was observed. On the other hand, at saturation breakthrough rates of 51% were recorded suggesting that

detachment processes happen when hydrodynamic shearing stress exceeds the attachment strength. Bacterial retention was largest in the first few centimetres of the top soil and strongly decreased at 20 cm depth. It was assumed that this happened due to a higher content of organic matter, a high fraction of macro-pores and the presence of grass roots at the soil surface layers. However, only 32% of the applied bacteria were detected along the soil column at the end of the 3 month leaching experiment. The authors concluded that a low mass balance appears to be common for microbial transport in soils and attributed the population loss to die-off and predation. These findings were confirmed by [redacted] et al. (2009). However, transportation through soil column was only studied for *B. circulatans*. Nevertheless, the authors found that limited vertical dispersal of cells occurred, since most cells were detected in the top 2 cm soil layer.

By use AFLP, the persistence of ten different introduced bacterial strains in microcosms was studied as well ([redacted] et al., 2010). These strains were applied onto soils, including three *B. subtilis* strains. It was shown, that persistence behaviour in soil differed between the bacterial strains. Moreover, [redacted] et al. (2010) were able to differentiate three types of bacteria, basing on their persistence. While *Pseudomonas stutzeri* showed long term persistence, population densities of *B. subtilis* declined much faster. The number of *B. subtilis* strains either dropped dramatically after about 10 days (*B. subtilis* 6051), or decreased gradually below the detectable level ($\sim 8 \times 10^2$ CFU/mL) within 140-180 days (*B. subtilis* 13933 and 14579). This is in concordance with previous findings, stated above.

Taken together, it is assumed that *B. amyloliquefaciens* QST 713 populations decline strongly by time in soil after application. Transportation through soil may happen, but it was shown for close related *B. subtilis* strains, that vertical dispersal is limited.

<u>Cited references (abstracts):</u>

Report: KIIM 7.1.1/15 – [redacted], K., [redacted], L.S., [redacted], [redacted] (2007)
Diversity of microflora in the gut and casts of tropical composting earthworms reared on different substrates, published report.
J Environ Biol 28, 87-97
M-518911-01-1

Abstract: The diversity of fungi, bacteria, yeast, actinomycetes and protozoa were analysed in the gut and casts of *Eudrilus eugeniae, Lampito mauritii, Eisenia fetida* and *Perionyx excavatus*, both qualitatively and quantitatively as influenced by different feed substrates like clay loam soil, cowdung and pressmud. While actinomycetes (*Streptomyces albus, S. somaliensis, Nocardia asteroides, N. caviae* and *Saccharomonosporia*) were not digested by any of these species of worms, protozoa (*Amoeba proteus, A. terricola, Paramecium trichium, Euglena viridis, E. orientalis, Vorticella picta* and *Trichomonas hominis*) and yeast (*Candida tropicalis, C. krusei C. albicans* and *Cryptococcus neoformans*) were totally digested. Certain species of fungi (*Saksenae vasiformis, Mucor plumbeus, Cladosporium carrionii, C. herbacium, Alternaria sp., Cunninghamella echinulata, Mycetia sterila, Syncephalostrum racemosum, Curvalaria lunata, C. geniculata* and *Geotrichum candidum*) and bacteria (*Pseudomonas aeruginosa, Bacterium antitratum, Mima polymorpha, Enterobacter aerogenes, E. cloacae, Proteus vulgaris, P. mirabilis, P. rettgeri, Escherichia coli, Staphylococus citreus, Bacillus subtilis, B. cereus, Enterococci* and *Micrococci*) were completely digested. Certain other species were not digested fungi like *Aspergillus fumigatus, A. flavus, A. ochraceous, Trichoderma koningii* (except by *Eeugeniae*), *Fusarium moniliforme* (except by *E. eugeniae*) and *Rhizopus* sp., and bacteria like *Klebsiella pneumoniae* and *Morganella morganii*) and these were multiplied during the transit of the organic residues through the gut of worms. The microbial proliferation was more in the casts, due to the environment prevailing--rich in nutrient supply and large surface area available for growth and reproduction of the microbes that lead to enhanced microbial activity and humic acid contents in the casts.

Report: KIIM 7.1.1/16 – [redacted], [redacted], [redacted], [redacted] (2008), Development of a strain-specific genomic marker for monitoring a *Bacillus subtilis* biocontrol strain in the rhizosphere of tomato
published report
FEMS Microbiol Ecol, 65, 289-298
M-518863-01-1

Abstract: A strain-specific molecular marker enabling the detection and tracking of the biological control agent *Bacillus subtilis* 101, when released into the environment, was developed. Random amplified polymorphic DNA (RAPD) technique was used to differentiate this from other *B. subtilis* strains. A differentially amplified fragment obtained from RAPD profiles was sequenced and characterized as sequence-characterized amplified region (SCAR) marker, and four primer pairs were designed and evaluated for their specificity towards this strain. The sensibility of the selected SCAR primer pair was evaluated by qualitative PCR and Southern blotting, and the detection limit was assessed around 10(2) CFU (g dry wt soil)(-1), thus providing a reliable tool for the traceability of this *B. subtilis* strain in greenhouse or field trials. A plating assay coupled to PCR with the SCAR primer pair was then used as a detection method in microcosm experiments for monitoring the population of *B. subtilis* 101 in the rhizosphere of tomato, grown under two different soil conditions, i.e. nonsterile peat-based substrate and sandy-loam agricultural soil, respectively. The data of rhizosphere colonization indicated that the soil conditions significantly affected the rhizosphere establishment of strain 101.

Report: KIIM 7.1.1/17 – [redacted], M.A., [redacted], [redacted], [redacted], [redacted], [redacted], [redacted], L.A., [redacted], [redacted], M.L (2009), Identification and application of AFLP-derived genetic markers for quantitative PCR-based tracking of *Bacillus* and *Paenibacillus* spp. released in soil.
published report
Canadian Journal of Microbiology, 55, 1166-1175.
M-520076-01-1

Abstract: In this study noncoding sequences from amplified fragment length polymorphisms (AFLPs) can provide robust and sensitive genetic markers suitable for PCR-based discrimination of closely related strains of *Bacillus* and *Paenibacillus*, and quantitative PCR (qPCR)-based tracking of the strains in complex natural systems like soil. Quantitative PCR was accurate in the approximately 1×10^9 to approximately 1×10^4 colony forming units (CFU)/g soil range. The detection limit was improved to approximately 1×10^2 CFU/g when amplicons were analyzed by gel electrophoresis. Studies with laboratory-contained intact soil-core microcosms indicated that environmental persistence trends vary among different strains. For example, *Bacillus circulans* ATCC 9500, *Bacillus amyloliquefaciens* DSL 13563-0, *Bacillus licheniformis* ATCC 12713, *Paenibacillus polymyxa* NRRL B-4317, and 3 *Bacillus subtilis* strains (ATCC 6051A, ATCC 55405, and NRRL B-941) died down to below the 1×10^2 CFU/g detection limit by days 28-105. In contrast, over a 105-day period, *B. licheniformis* ATCC 55406, *Bacillus megaterium* NRRL B-14308, and *P. polymyxa* strains ATCC 55407 and DSL 13540-4 died down but persisted at levels just above the detection limit, whereas *Bacillus thuringiensis* ATCC 13367 experienced a less than 10-fold decrease in cell numbers.

Report: KIIM 7.1.1/18 – [redacted], [redacted], M., [redacted], G.D., [redacted] (2005), Transport and deposition of *Bacillus subtilis* through an intact soil column
published report
Australian Journal of Soil Research, 43, 695-703
M-530182-01-1

Abstract: Bacterial transport in unsaturated soils is much less well understood than in saturated conditions, especially for intact soils. This paper aims to investigate the fate and transport of bacteria in intact soils with different water saturations, and particularly the effect of low suction (and hence removal of water flow in the largest macropores). An intact soil column (0.50 m diameter by 0.70 m depth) with a tension infiltrometer was used to investigate the transport and deposition of *Bacillus subtilis* endospores (i.e. dormant and persistent bacteria) during saturated and unsaturated flows. Soil porosity and pore size distribution were measured. Porosity decreased with depth and macropores were concentrated in the topsoil. Three tensiometers and a temperature sensor were installed along the soil column to monitor matric suction and temperature. Breakthrough curves for bacteria and chemical tracer Br^- at 0 and 0.5 kPa suction were obtained during the 3-month leaching experiment. Bacterial breakthrough occurred earlier than the inert chemical tracer, which is consistent with effects of pore size exclusion. Also, saturated flow gave a significantly higher concentration and recovery ratio of leached bacteria, i.e. 51% *v.* 0.88%. Recovery of Br^- in leachate at both suctions reached >85%. The column was destructively sampled for deposited endospores at the completion of leaching. Bacterial deposition was concentrated in the top 0.10 m, then decreased abruptly and was relatively constant with column depth, although showing some irregularity at the bottom of the column.

Report: KIIM 7.1.1/19 – [redacted] R., [redacted], [redacted], [redacted], [redacted], [redacted], [redacted], L.A. (2010), Development of amplified fragment length polymorphism-derived functional strain-specific markers to access the persistence of 10 bacterial strains in soil microcosms
published report
Appl Environ Microbiol, 76, 7126-7135
M-518902-01-1

Abstract: To augment the information on commercial microbial products the persistence patterns of high-priority bacterial strains from the Canadian Domestic Substance List (DSL) was investigated. Specific DNA markers for each of the 10 DSL bacterial strains were developed using the amplified fragment length polymorphism (AFLP) technique, and the fates of DSL strains introduced in soil were assessed by real-time quantitative PCR (qPCR). The results indicated that all DNA markers had high specificity at the functional strain level and that detection of the target microorganisms was sensitive at a detection limitation range from 1.3×10^2 to 3.25×10^5 CFU/g of dry soil. The results indicated that all introduced strains showed a trend toward a declining persistence in soil and could be categorized into three pattern types. The first type was long-term persistence exemplified by *Pseudomonas stutzeri* (ATCC 17587) and *Pseudomonas denitrificans* (ATCC 13867) strains. In the second pattern, represented by *Bacillus subtilis* (ATCC 6051) and *Escherichia hermannii* (ATCC 700368), the inoculated strain populations dropped dramatically below the detection threshold after 10 to 21 days, while in the third pattern there was a gradual decrease, with the population falling below the detectable level within the 180-day incubation period. These patterns indicate a selection effect of a microbial community related to the ecological function of microbial strains introduced in soil. As a key finding, the DSL strains can be quantitatively tracked in soil with high sensitivity and specificity at the functional strain level. This provides the basic evidence for further risk assessment of the priority DSL strains.

IIM 7.1.2 Water

A literature search was conducted to identify peer reviewed open literature providing information on fate and behaviour of *B. amyloliquefaciens* QST 713 in water (please refer to the literature review report submitted under Point IIM 7.1). No additional relevant articles were identified compared to information presented in the baseline dossier. From this information, it is known that *Bacillus* species occur worldwide in freshwater, estuarine and marine waters (please refer to Annex II, Doc IIM, Point IIM 8.2).

For the detailed background information, please refer to the baseline dossier.

IIM 7.1.3 Air

B. amyloliquefaciens QST 713 spores may occur in areal samples due to transportation via drift. However, due to lack of nutrients and stress factors as UV-radiation or desiccation, survival of living cells is limited. Air is not the natural habitat of *B. amyloliquefaciens*. Thus, no relevant publications where identified by literature search (please refer to the literature review report submitted under Point IIM 7.1.1).

For the background information, please refer to the baseline dossier.

IIM 7.2 Other/special studies

No further studies are considered to be necessary.