Renewal
Assessment Report

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

Volume 3 – B.1 Identity

July 2018

Rapporteur Member State: The Netherlands

Co-Rapporteur Member State: Germany

Version history

When	What
July 2018	Initial RAR

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B Summary of the data and information

Introduction

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

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

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

B.1 Identity of the micro-organism

B.1.1 Applicant

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B.1.2 Producer

See Volume 4/Confidential Volume

B.1.3 Name and species description, strain characterisation

B.1.3.1 Accession number in culture collection

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

Bacillus thuringiensis ssp. *aizawai*, strain GC-91, is deposited in the National Collection of Type Cultures (NCTC), at the Health Protection Agency, Centre for Emergency Preparedness and Response, Porton Down, Salisbury, Wiltshire, SP4 0JG, UK (formerly Central Public Health Laboratory, Colindale Avenue, London NW9 5HT, UK) under the reference number NCTC 11821.

B.1.3.2 Scientific name and taxonomic grouping, i.e. family, genus, species, strain, serotype, pathovar or any other denomination relevant to the micro-organism

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

Section:	Endospore-forming Gram positive Rods and Cocci
Family:	Bacillaceae
Genus:	*Bacillus*
Species:	*Bacillus thuringiensis*
Subspecies :	*aizawai*
Serotype:	H7
Strain:	GC-91

First description: Burges et al. (1991) (US Patent) N.5,063,055.

Strain GC-91 is described as a trans-conjugant of the Btk strain HD 191-A2 and the Bta strain HD 135-S4, both derived from indigenous wild type strains and described by Burges et al. (1991) in the US patent number 5,063,055.

Strain GC-91 is not a genetically modified micro-organism according to the Directive 2001/18/EC

B.1.3.3 Test procedures and criteria used for identification at strain level

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

Strain GC-91 is a product of a natural crossing or conjugation (i.e. transconjugant) between two parental strains that differ in their flagella serotype as well as in their δ-endotoxin genes, using the conjugation-like plasmid transfer system. The actual event was the transfer of two plasmids carrying a Cry1Ac and a Cry2A δ-endotoxin gene, respectively, from the strain HD-191-A2 (flagella serotype kurstaki) into the strain HD-135-S4 (flagella serotype aizawai), which already carried δ-endotoxin genes of the Cry1C and Cry1D types. So the strain GC-91 has the aizawai flagella serotype (H 7) and carries the Cry1Ac, Cry1C, Cry1D and Cry2A genes

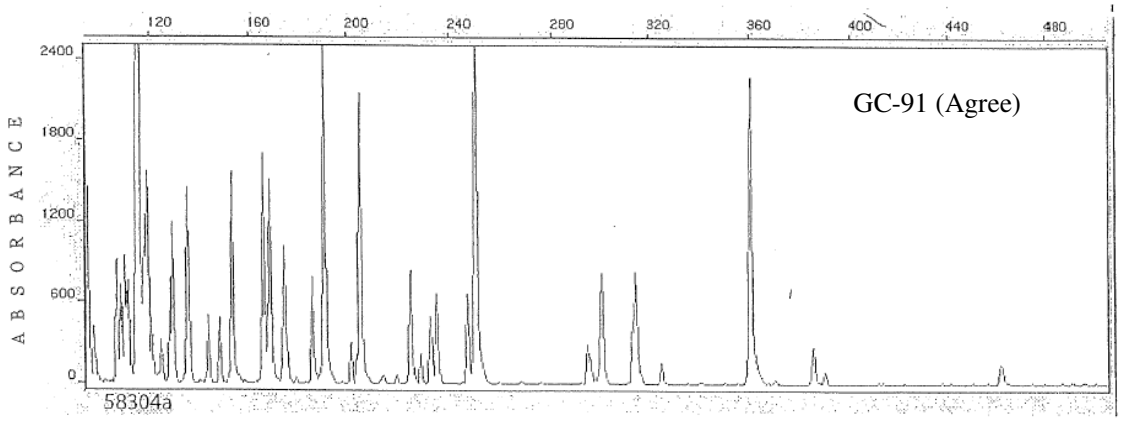
The morphology of the Bta strain can be studied microscopically. The biochemical characteristics of Bta strain GC-91 were determined according to the methods prescribed in Bergey's Manual of Systematic Bacteriology, Vol. 2 (Sneath et al., 1986) Bta strain GC-91 is a spore-forming, gram+ bacillus, generating bipyrimidal and cuboidal parasporal crystal proteins. The flagella antigen serotyping of the Bta strain, indicating the kind of sub-species, was determined using the standardized analytical methods described by deBarjac (1981). According to Verma (1991), the strain GC91 can be classified as serotype H:7.

Due to the transconjugant character of strain GC-91, the genetic properties of this *Bacillus thuringiensis* ssp. *aizawai* / *kurstaki* are similar to commercially used *Bacillus thuringiensis* ssp. *kurstaki* strains SA-11, SA-12 and the most frequently commercially used strain HD-1.

The analysis of the fatty acid composition of GC-91 was conducted by FAME-GC using the identification soft-ware Sherlock Version 4.5.The strains GC-91, SA-11, SA-12 and the reference strain HD-1 show an almost identical fatty acid profile. Differences between the chromatograms, i.e. fatty acid pattern and retention times of GC-91 and the Btk strains SA-11 (Bt09703), SA-12 (Bt30791) and HD-1, particularly between the retention times of the fatty acids, could hardly be detected (Strauss, 2005a-d), implicating the affiliation of Bta strain GC-91 to the serovar *B. thuringiensis* ssp. *kurstaki*.

The data generated by AFLP analysis of DNA (Jackson et al., 1999; Hill et al., 2004) contain fragment sizes with each fragment having a peak height. The chromatograph shows a high similarity of the strains regarding the average of the DNA fragments sizes and their intensity (Chen, 2005a; see Fig B.1.3.3)

Figure B.1.3.3 DNA fragment sizes, randomly amplified after enzymatic digestion of genomic DNA



A phylogenetic tree was generated by Jackson et al. (2005) at the Lawrence National Laboratory, Livermore CA and Los Alamos National Laboratory, Los Almos, NM, USA, based on the analytical methods of Hill et al. (2004) and including the commercially important serovars of *B. thuringiensis* and strains of *B. cereus* and *B. anthracis* isolates. The cluster analysis shows that strains of commercially used *B. thuringiensis* cluster on branches of the tree that are well away from the *B. anthracis* isolates and pathogenic *B. cereus* isolates. The strain GC-91, as well as the strains SA-11 (B-30790) and SA-12 (B-30791), are very close to each other based on the AFLP comparison analysis of 40 DNA fragments with a maximum genetic distance of 2%. All strains appear on the mapping tree to be in one homogeneous cluster.

According to AFLP analysis, the various Bta strains considered are scattered throughout various groupings falling within the same similarity range.

Another method to characterize GC-91 strain is the analysis of the plasmid profile. The plasmid profile of Bta strain GC-91 was analysed in comparison to several other strains using agarose gel electrophoresis in 0.5 % agarose with a horizontal submarine apparatus in electrophoresis buffer. The analyses were conducted in accordance with the methods of Gonzalez & Carlton (1980).

The electrophoretic analysis of the DNA of strain GC-91, including the reference strain HD-1 and the parental strains of Bta strain GC-91 shows 8 plasmid bands between 4 and 60 mega Dalton. The plasmids of GC-91 only differ from those of the parental strain HD-135-S4 in the replacement of the native 53 mDa and 8 mDa plasmids by an analogous but different plasmid from HD-191-A2 (Verma, 1991). The plasmids comprise different genes expressing δ-endotoxins that are responsible for the specific insecticidal activity of the *B. thuringiensis* strains. Different sizes and numbers of plasmid fragments encoding different entomocidal proteins may therefore result in varying entomocidal efficacy.

However, the molecular mass of the plasmids carrying cry1Ac is so small, relative to the whole chromosome of GC-91, that it cannot be used for differentiation of GC-91 from other Bta strains in an AFLP experiment as described above. This differentiation (for example from strain Bta HD-860 (falling within the same similarity AFLP group as GC-91) can be achieved using specific primers for the cry1Ac gene in a PCR test (the cry 1Ac gene has been acquired after the conjugation event and characterize the GC-91 strain). In the case of strain HD847, also falling within the same AFLP similarity group as GC-91, the differentiation is due to the fact that HD847 is a Bt with a serotype belonging to the sub species *colmen*, different from the serotype of Bta GC-91.

In summary the Applicant has provided the characteristics for the identification of strain GC91 summarized in the Table B.1.3.3.

Table B.1.3.3 Characteristics of GC-91

Test	**Result Bta GC-91**
Strain	GC-91
Serotype	H: 7
Serovar	*aizawai*
Morphology	bacillar

	motile
	gram +
Culture conditions	
Temperature	30°C
Growth medium	Meat peptone and soyflour
Entomocidal crystal proteins	Cry1Ac, Cry1C, Cry1D, Cry2A
Mode of action	Ingestion is followed by crystal solubilization and proteolytic activation of protoxin in the midgut of the insect. Activated toxin binds to the receptors in the midgut epithelial membrane and inserts into the membrane, leading to cell lysis and death of the insect.
Fatty acid analysis	High similarity with Btk strains SA-11, SA-12 and HD-1
Plasmid analysis	8 plasmid bands, between 4 and 60 mega Dalton
DNA Fingerprinting	Strains GC-91 is very similar to SA-11 and SA-12 based on AFLP comparison analysis of 40 DNA fragments, maximum difference 2%..
Test	GC-91
Gram Reaction	gram+
Motility	+
Gaseous conditions	aerobe
Koser's Citrate	-
Indole	-
Methyl Red	+
Voges-Proskauer Test	+
Hydrogen Sulfide Production	-
Nitrate Reduced to nitrite	+
Nitrite reduced	-
Catalase	+
Gelatin Liquified	+
Anaerobic Growth	-
Acid from Carbohydrates: D-glucose L-Arabinose D-Xylose Lactose Sucrose Maltose D-Mannitol Dulcitol Sorbitol Salicin	 + - NT - - + - - - +
Hydrolysis of: Casein Urea Starch Aesculin	 + + + NT
Hough and Leifson Reaction	NIL
Oxidase	+
Gluconate	-
Malonate	-
Phenylalanine	-
Decarboxylases: Arginine Lysine Ornithine	 + - -
Growth on MacConkey	+
Growth on Nutrient Agar at: 22°C 37°C	 + +

42°C	+
60°C	-

NT not tested
\+ positive
\- negative
NIL no reaction

DNA fingerprinting generated by AFLP and PCR analysis shows that strain GC91 is very similar to Btk strains SA11 and SA12, and fatty acid analysis confirms the high similarity with Btk strain SA11 SA12 and HD1, the plasmid analysis shows that the plasmids of GC91 differ from those of the parental strains. Therefore a combined biochemical and molecular approach can be used to differentiate this active substance at strain level, with respect to its parental strains and from other Bta strains falling within the same AFLP similarity group.

New data

Data submitted during original approval of Bta GC-91 is still valid. In addition, to allow an unequivocal identification of Bta GC-91 an attempt was made to develop strain specific markers based on the sequences of the whole genome and plasmids of the strain. The developed marker has been tested for specificity with a set of *Bacillus* reference strains including different Bt and potentially pathogenic *B. cereus* strains. In addition, reproducibility of the results was assessed. A set of four highly specific primers is available allowing a clear identification of Bta GC-91. The study is briefly summarised below. For more details, please refer to C.1.4.1 in Volume 4.

Reference:	KMA 1.3/01
Report:	Brader, G. (2016) Strain identification study with the Btk strains SA-11, SA-12, EG 2348 and Bta GC-91. Part 1 and 2 Unpublished Report No: CEU09361_AIT151
Guideline(s):	-
Deviations:	No
GLP:	No
Acceptability:	Acceptable
Duplication: **(if vertebrate study)**	No

Executive summary

Sequencing of *B. thuringiensis* serovar *aizawai* (Bta) GC-91resulted in 150 assembled contigs and 10 plasmids. The genome of Bta GC-91 has >99% identity to *B. thuringiensis* serovar *aizawai* draft genomes and to the assembled *B. thuringiensis* serovar *galleriae* HD-29 (Btg) genome available at the NCBI. Genome comparison was used to find regions specific for Bta GC-91. Despite a high similarity to published genome sequences distinguishable areas to all sequenced *B. thuringiensis* strains both in the plasmid and on the chromosome were found. Four primers sets were developed and validated for amplification specificity and efficacy. All four primers of Bta GC-91 (on plasmid and chromosome) gave characteristic amplification only in the specific strain. The primers can be used as markers for an unequivocal identification of the strain.

B.1.3.4 Common name or alternative and superseded names and code names used during the development

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

Code no.	The period for which it was used	Remarks
CGA-237218	From 1989-present	The code is given as technical name of GC-91 as well as of NCTC 11821

B.1.4 Specification of the material used for manufacturing of formulated products

B.1.4.1 Content of the micro-organism

Information from DAR and DAR addendum (May 2007, February 2013)
The potency of the strain is derived from the 5-batch analysis (Chen, 2005b). The CFU / g is evaluated by Iqbal & Chen (2005). The potency (average of five batches) is 53,436 IU/mg and the average of three batches 6.1 x 10^{10} CFU/g of Bta Technical (Agree 50WP / GC-91).

Table 1.4.1-1. Potency and Colony

New data

The content of *B. thuringiensis aizawai* in the technical grade are $6.2x10^{10}$ CFU/kg to $7.9x10^{10}$ CFU/kg (see Volume 3 B.1 MA).

Based on the batch data for Agree 50 WG a maximum of 3.3×10^{10} CFU/g can be established. Available product data indicate a minimum content of 2.0×10^{10} CFU/g. However, according to the manufacturer a minimum of 8.5×10^{9} CFU/g is required to reach the target biopotency in the end-use product. This value is therefore considered as the minimum CFU value for Agree 50 WG.

B.1.4.2 Identity and content of impurities, additives, contaminating micro-organisms

Contaminating microorganisms

Measurements are carried out to test for microbial contaminants in accordance with the working document SANCO/12116/2012 rev. 0 (see further B.5 Volume 3 for the analytical methods and Volume 4 for the 5-batch analysis).

Metabolites:

Measurements on potential metabolites are reported in Volume 4.

Additives and impurities:

There are no additives and no impurities.

B.1.4.3 Analytical profile of batches

For the confidential information concerning the contaminating microorganisms and potential metabolites , see Volume 4.

B.1.5 References relied on

Data point CADDY (ongoing numbering)	Author(s)	Year	Title Owner, Report No. Source (where different from owner) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously submitted Y/N* If Y => old data point
KMA 1.3/01	Burges et al.	1991	UNITED STATES PATENT FOR GC-91 not available, 5063055 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 1.3.6
KMA 4.1/05	de Barjac, H.	1981	Identification of H-serotypes of Bacillus thuringiensis not available, not applicable Microbial control of pests and plant diseases, 35-43 GLP/GEP: no Published: yes	no	no	not protected	-	Y

KMA 1.3/03	Verma, M.	1991	CONFIDENTIAL APPENDIX TO VOLUME OF SUB-MISSION TECHIN-CAL CGA-237218 PRODUCT CHEM-ISTRY Certis USA LLC, PC-91-005 Agricultural Division Ciba-Geigy Corpora-tion, Greensboro, NC GLP/GEP: no Published: no	no	yes	protected	CEU	Y KIIM 1.3.3
KMA 1.3/04	Strauss, S.	2005 a	FATTY ACID COM-POSITION AND CHROMATOGRAM OF GC-91 Certis USA LLC, not applicable not available GLP/GEP: no Published: no	no	yes	protected	CEU	Y KIIM 1.3.3
KMA 1.3/05	Strauss, S.	2005 b	FATTY ACID COM-POSITION AND CHROMATO-GRAMM OF SA11 Certis USA LLC, not applicable not available GLP/GEP: no Published: no	no	yes	protected	CEU	Y KIIM 1.3.3
KMA 1.3/06	Strauss, S.	2005 c	FATTY ACID COM-POSITION AND CHROMATOGRAM OF SA12 Certis USA LLC, not applicable not available GLP/GEP: no Published: no	no	yes	protected	CEU	Y KIIM 1.3.3
KMA 1.3/07	Strauss, S.	2005 d	FATTY ACID COM-POSITION AND CHROMATOGRAM OF HD-1 Certis USA LLC, not applicable not available GLP/GEP: no Published: no	no	yes	protected	CEU	Y KIIM 1.3.3

KMA 4.1/06	Jackson, P.J., Hill, K.K., Laker, M.T., Ticknor, L.O., Keim, P.	1999	Genetic comparison of Bacillus anthracis and its close relatives using amplified fragment length polymorphism and polymerase chain reaction analysis not available, not applicable Journal of Applied Microbiology, 87, 263-269 GLP/GEP: no Published: yes	no	no	not protected	-	Y
KMA 4.1/03	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., Keim, P., Jackson, P.J.	2004	Fluorescent Amplified Fragment Length Polymorphism Analysis of Bacillus anthracis, Bacillus cereus, and Bacillus thuringiensis isolates not available, not applicable Applied and Environmental Microbiology, 70, 1068-1080 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 4.3.1
KMA 4.1/10	González, J.M.,Carlton, B.C.	1980	Pattern of plasmid DNA in crystalliferous and acrystalliferous strains of Bacillus thuringiensis not available, not applicable Plasmid, 3, 92-98 GLP/GEP: no Published: yes	no	no	not protected	-	Y

KMA 1.3/01 (KMA 1.3/17)	Brader, G.	2016 a	STRAIN IDENTIFICATION STUDY WITH THE BTK STRAINS SA-11, SA-12, EG 2348 AND BTA GC-91 Certis USA LLC, CEU09361_AIT151 Austrian Institute of Technology GmbH GLP/GEP: no Published: no	no	yes	New data for active ingredient, not previously submitted nor evaluated	CEU	N
KMA 2.2.2/10	Dulmage, H.T., Boening, O.P., Rehnborg, C.S., Hansen, G.D.	1971	A PROPOSED STANDARDIZED BIOASSAY FOR FORMULATIONS OF BACILLUS THURINGIENSIS BASED ON THE INTERNATIONAL UNIT not available, not applicable Journal of invertebrate Pathology, 18, 240-245 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 2.3.2