10-20-93

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

OCT 20 1993

MEMORANDUM

OFFICE OF
PREVENTION, PESTICIDES AND
TOXIC SUBSTANCES

SUBJECT: SAB Review of Data Submitted by Gustafson, Incorporated to Support the Registration of GUS 376 Concentrate Biological Fungicide, Bacillus subtilis (I.D. No.: 007501-RUT; Submission No.: S446779; DP Barcode No.: D194490).

TO: Clarence Lewis/Julie Fairfax (PM-21)
Fungicide-Herbicide Branch
Registration Division (H7505C)

FROM: Cindy Schaffer, Microbiologist
Biological Pesticide Section
Science Analysis Branch
Health Effects Division (H7509C)

THROUGH: Roy Sjoblad, Ph.D, Section Head
Biological Pesticide Section
Science Analysis Branch
Health Effects Division (H7509C)

DATA REVIEW RECORD

Product Name:	GUS 376 Concentrate Biological Fungicide	
Trade Name:	Bacillus subtilis	
ID No:	007501-RUT	
Caswell No:	066C	
MRID No:	419074-01	Product Chemistry Data.
	419074-02	Acute Oral Toxicity and Infectivity /Pathogenicity to Rats of MBI 600.
	419074-03	Acute Dermal Toxicity Study to Rabbits of MBI 600.
	419074-04	Acute Pulmonary Toxicity and Infectivity/Pathogenicity to Rats of MBI 600.
	419074-05	Acute Intravenous Toxicity and Infectivity/Pathogenicity to Rats of MBI 600.
	419074-06	Primary Eye Irritation and Infectivity of MBI 600.
	419074-07	Delayed Contact Hypersensitivity in the Guinea-Pig with MBI 600.

ACTION REQUESTED: SAB has been asked to review product analysis and toxicology data in support of the registration of GUS 376 Concentrate Biological Fungicide, Bacillus subtilis, for use as a seed treatment alone or with other seed treatment chemicals to act as a rapid colonizer of root surfaces to protect seedlings from fungal root rot. SAB has also been asked to review a petition for the exemption from the requirement for a tolerance.

CONCLUSION: SAB found one deficiency in the Product chemistry portion of this package. The certificate of analysis submitted for the salmonella assay was for *B. subtilis* GBO7 not MBI 600. SAB will upgrade this package to acceptable pending the submission of the correct certificate of analysis.

SAB supports the exemption from tolerance based on *B. subtilis* natural occurance and lack of pathogenicity or infectivity.

SUMMARY OF DATA SUBMITTED:

Product Identification (151A-10 - 151A-16):

SAB found one deficiency in the Product chemistry portion of this package. The certificate of analysis submitted for the salmonella assay was for *B. subtilis* GBO7 not MBI 600. SAB will upgrade this package to acceptable pending the submission of the correct certificate of analysis.

CLASSIFICATION: UNACCEPTABLE - May be upgraded with the submission of the correct certificate of analysis.

Acute Oral Toxicity/Pathogenicity (152A-10):

Bacillus subtilis was not toxic, infective nor pathogenic to rats at a dose of $2 \times ^{8}$ CFU per animal.

CLASSIFICATION: ACCEPTABLE - TOX CATEGORY IV

Acute Dermal Toxicity (152A-11):

MBI 600 was not toxic for rats when a single 5×10^{10} CFU/animal (2 ml/kg body weight) dose was administered dermally. Irritation dissipated by day 3.

CLASSIFICATION: ACCEPTABLE- TOX CATEGORY IV

Acute Pulmonary Toxicity/Pathogenicity (152A-12):

Although a high mortality was observed (24% in ♂, 15% in ♀) , death was not attributed to the microorganism tested, it was attributed to the dose administered. A distinct clearance pattern was noted throughout the study in all organs. Based on the submitted data, MBI 600 was neither toxic, pathogenic nor infective for rats when dosed intratracheally with 3.4×10^{8} CFU of the test material.

CLASSIFICATION: ACCEPTABLE - TOX CATEGORY IV

Acute Intravenous Toxicity/Pathogenicity (152A-13):

MBI 600 was not infective, pathogenic or toxic for rats when dosed intravenously with approximately 4×10^{7} CFU of the test material.

CLASSIFICATION: ACCEPTABLE

Primary Eye Irritation (152A-14):

MBI 600 produced a slight irritation when a single 0.1 g ocular dose was administered. Ocular irritation dissipated 4 days post dosing.

CLASSIFICATION: ACCEPTABLE - TOX CATEGORY IV

Skin Sensitization Study (152A-15):
An overall moderate skin sensitization reaction was noted in the treated guinea pigs 24 to 72 hours post test challenge with the GMPT.
CLASSIFICATION: ACCEPTABLE
Hypersensitivity Incidents:
None reported. The registrant must notify the Agency of any hypersensitivity incidents.

DATA EVALUATION REPORT

Reviewed by: Cindy Schaffer, Microbiologist, SAB/HED
Secondary Reviewer: John Kough, Microbiologist, SAB/HED JK

STUDY TYPE:	Product Chemistry Data
MRID NO:	419074-01
CASWELL NO:	066C
TEST MATERIAL:	GUS 376 Concentrate Biological Fungicide
SYNONYMS:	Bacillus subtilis, MBI 600
PROJECT NO:	91339A, 0347-51; MBT - 186-102
SPONSOR:	Gustafson, Inc., Dallas, TX
TESTING FACILITY:	Gustafson, Inc., McKinney, TX MicroBioTest, Inc., Chantilly, VA
TITLE OF REPORT:	Product Chemistry Data
AUTHOR(S):	Donald S. Kinney, Fred C. Rosa, Mary Kay Bruch
STUDY COMPLETED:	23 February, 1993
CONCLUSION:	SAB found one deficiency in the Product chemistry portion of this package. The certificate of analysis submitted for the salmonella assay was for *B. subtilis* GB07 not MBI 600. SAB will upgrade this package to acceptable pending the submission of the correct certificate of analysis.
CLASSIFICATION:	UNACCEPTABLE - May be upgraded with the submission of the correct certificate of analysis.

PRODUCT ANALYSIS

151A-10 Product Analysis and Disclosure of Ingredients

Identity: The active ingredient of GUS 376 Concentrate Biological Fungicide is identified as Bacillus subtilis MBI 600 (ATCC No.: SD 1414).

Confidential Statement of Formula has been submitted. GUS 376 contains the active ingredient Bacillus subtilis MBI 600 (2.75%) [redacted]

INERT INGREDIENT INFORMATION IS NOT INCLUDED

Information on Active Ingredient:

General Taxonomy: Bacillus subtilis is approximately 0.7 to 0.8 μm by 2.0 to 3.0 μm in length, has a marginally swollen sporangium, ellipsoidal and central to subterminal spore, colonies are cream colored, have a dull luster and a crinkly edge.

History: Bacillus subtilis MBI 600 was isolated from faba bean growing in Nottingham University School of Agriculture at Sutton Boningham, U.K. Bacillus subtilis is considered ubiquitous and non-pathogenic.

Active ingredient characterization:

Biochemical/Nutritional Characteristics:

Positive(+): Voges Proskauer, hydrolysis of gelatin, hydrolysis of starch, citrate utilization, growth at 50 C, hydrolysis of esculin, glycerol, L-arabinose, ribose, D-xylose, galactose, D-glucose, D-fructose, D-mannitol, sorbitol, ∝-methyl D-glucose, amygdalin, esculin, salicin, cellobiose, maltose, lactose, melibiose, saccharose, trehalose, D-raffinose, glycogen, D-turanose, ONPG, glucose.

Negative(-): Hydrolysis of casein, nitrate reduced to nitrite, fermentation of indole, growth at 55 C.

These characteristics are consistent with the Bergey's Manual method of identification for B. subtilis.

Inert Ingredients: GUS 376 Concentrate Biological Fungicide contains [redacted] as its only inert ingredients.

Manufacturing Process(151A-11):

GUS 376 Concentrate Biological Fungicide is manufactured [redacted]

Unintentional Ingredients (151A-12):

[redacted]

INERT INGREDIENT INFORMATION IS NOT INCLUDED

MANUFACTURING PROCESS INFORMATION IS NOT INCLUDED

Analysis of Samples (151A-13):

The registrant used standard plate count methods to determine the number of CFUs per pilot batch. The results of analysis of five pilot batches of GUS 376 ranged from 92.1 x 10^9 CFU/gm to 13.7 x 10^{10} CFU/gm.

Produduc Stabity:

When the active ingredient Bacillus subtilis MBI 600, at a concentration of 1.5 x 10^{11} spores per gram, was exposed to sunlight, pH, temperature, or metal ions the viability of the spore was affected (as measured by bioassays) in the following manner:

Sunlight exposure:

Twenty hours at 100,000 - 145,000 lux for 24 hours reduced the viability of a dry film of spores by 89%.

pH:

When exposed to a pH of 1.0 and 9.0 for 6 hours the spore viability was reduced by 95% and 33% respectively.

A 14 day exposure to pH 3.0 and 5.0 reduced spore viability by 75% and 39% respectively.

Temperature:

Spore viability was reduced by >95% and 97% when aqueous suspensions were exposed to temperatures of 100 C for 15 minutes and 80 C for 30 minutes respectively.

Metal ions:

When spores were exposed to 0.5N $CaCl_2$, Na_2SO_4, $CuSO_4$ and $Al_2(SO_4)_3$ for 14 days the viability was reduced by 93%, 72%, 92% and 90% respectively. The registrant contributes these losses to the pH of the solutions.

A 4 day exposure to 0.25N $FeCl_3$ elicited a greater than 99% loss in spore viability. The registrant contributed this loss to low pH.

Overall Storage Stability:

The spore viability was tested at temperatures of -20°C, 40°C, 40°C (high pressure), and 50°C for a period of 30 days. The optimal temperature for storage stability of Bacillus subtilis spores under these conditions was 40°C ± 2°C for 30 days.

Quality Control: QUALITY CONTROL PROCEDURE INFORMATION IS NOT INCLUDED

The presence of microbial contaminants is the major concern. The microbiological testing performed during each step of the

[redacted]

Microbiology Laboratory Guidebook. A sample of this product is registered with the American Type Tissue Collection (#SD-1414).

MANUFACTURING PROCESS INFORMATION IS NOT INCLUDED

Certification of Limits (151A-15): INERT INGREDIENT INFORMATION IS NOT INCLUDED

[redacted]

Physical/Chemical Properties (151A-16):

Color	Buff/Yellow cream Munsell notation - 5Y 8/2
Physical State	A free flowing solid powder
Odor	Musty, cheese-like, typical of fermentation products
Bulk Density	26 - 29 lbs. per cubic foot
pH	5.10 for a 1% weight/unit volume suspension at 25°C
Storage Stability	30 days at 40°C ± 2°C

DISCUSSION

SAB found one deficiency in the Product chemistry portion of this package. The certificate of analysis submitted for the salmonella assay was for *B. subtilis* GB07 not MBI 600.

DATA EVALUATION REPORT

Reviewed by: Cindy Schaffer, Microbiologist, SAB/HED CS
Secondary Reviewer: John Kough, Ph.D., Biologist, SAB/HED JLK

STUDY TYPE:	Acute Oral Toxicity/Pathogenicity-Rat (152A-10)
MRID NO:	419074-02
TEST MATERIAL:	*Bacillus subtilis* (NCIB 12376) Spore/Metabolite suspension.
SYNONYMS:	MBI 600
PROJECT NO:	89396/AGC 1/0/AC
SPONSOR:	Gustafson, Inc., Dallas, TX
TESTING FACILITY:	Huntingdon Research Centre, LTD. Huntingdon, Cambridgeshire, England
TITLE OF REPORT:	Acute Oral Toxicity and Infectivity /Pathogenicity To Rats of MBI 600.
AUTHOR(S):	David J.N. Hossack; Sarah A. Allan; Martin N. Baker
STUDY COMPLETED:	2 June 1989
CONCLUSION:	Although the microbe was found in the stomach, small intestines, caecum, feces and urine of the treated rats, the test material was cleared from all organs by day 21 of the study. Based on the submitted data, Bacillus subtilis was not toxic, infective nor pathogenic to rats.
CLASSIFICATION:	ACCEPTABLE - TOX CATEGORY IV

I. STUDY DESIGN

Test Material: The microbial pest control agent is Bacillus subtilis (NB 12376) Spore/Metabolite suspension in distilled water. The potency, homogeneity and stability was not determined by the testing facility. Each test animal received 20 ml/kg dose (approximately 2 x 10^8 CFU) by oral gavage.

Test Animals: Twenty-one male and twenty-one female CD rats, approximately four to six weeks old, were obtained from Charles River, France. The male rats weighed between 96g and 116g and females weights ranged from 114g to 137g at the beginning of the study.

Methods: The animals were assigned as follows: The undosed control, group F, and the autoclaved control, group E, consisted of five male and five female rats each. Eleven female and eleven male rats were treated with the test substance. The rats were randomly weighed on the day of initial dosing, day 2, 8, 15 and 22. An electronic thermometer was used to record rat body temperature on day 1 prior to dosing, and 2, 4 and 24 hours post dosing. Treated animals were observed for signs of toxicity at frequent intervals post dosing, day 1 and twice

daily thereafter. Feces and urine were collected on days 2, 3, 4 and 22. All rats of each sex in the treatment groups were sacrificed by cervical dislocation. The treated animals were divided into groups A through E based on sacrifice times as follows: Groups A - C were comprised of two rats of each sex and were sacrificed at days 1, 3, 7 and 14; and group D, containing 5 males and 5 females, were sacrificed 22 days post dosing. Five rats per sex from Group F were sacrificed on day 22, the last day of the study. The animals were examined by necropsy for any macroscopic abnormalities. Samples of the brain, kidneys, liver, lungs, mesenteric lymph nodes, spleen, as well as contents of the stomach, and 1st and 7th loop of the small intestine and caecum were analyzed using cut tissue smear plating. If viable CFU were observed in the initial dose, the organs, blood, feces, stomach, small intestine and caecum content CFUs were determined by suspending 1 gm of B. subtilis (or sample material; i.e. caecum contents) in 9 ml phosphate buffered saline (PBS), then serially diluting this mixture 1:10 in PBS. [NOTE: The fecal material was heat treated at 65°C for 30 minutes to kill any vegetative organisms normally found in the feces.] One ml aliquots of each dilution [urine was added to molten agar undiluted] were added to molten Tryptone Soy Agar (TSA), plated out in triplicate, and incubated at 32°C for 48 hours. The plates were then examined for typical B. subtilis colonies.

II. RESULTS

A. Body Weights:

All treated animals gained weight throughout the study.

B. Clinical observations:

Piloerection was noted in all animals except the undosed controls during the first day of the study. No clinical signs were noted from day 2 through the end of the study.

C. Body temperature:

The treated rats demonstrated a slight decrease (<1°) in body temperature during the first four hours post dosing. Body temperatures remained consistant with the control groups, untreated and autoclaved MBI 600) throughout the rest of the study.

D. Necropsy observations:

No abnormalities were noted upon necropsy.

E. Microbial clearance/infectivity:

Clearance and infectivity were evaluated in the brain, blood, lymph nodes, kidney, liver, spleen,

lungs and stomach, 1st and 7th loop of the small intestine, caecum, feces and urine. The submitted data (see below) demonstrated that isolated incidences of the organism were detected in the stomach, 1st and 7th loop of the small intestine, caecum, feces and urine. In all instances, the test material was cleared by day 21.

ORGAN	DAY	# rats*	MEAN CFU/GM OF TISSUE
Stomach	1	3/4	2.0×10^3
Small intestine 1st loop	1	2/4	3.2×10^4
Small intestine 7th loop	1	1/4	1.5×10^2
Caecum	1	4/4	5.5×10^5
	7	2/4	1.0×10^2
Feces	1	10/10	1.57×10^7
	2	10/10	2.34×10^6
	3	6/6	5.21×10^4
Urine	1	5/10	1.87×10^3
	2	1/10	57
Lymph nodes	7	1/4	•5 colonies found
Blood	7	1/4	•29 colonies found

* # of (+) treated rats/ total # of treated rats examined
• Taken from organ smear plates; actual CFU/gm not enumerated.

III. SAB DISCUSSION:
Although the microbe was found in the stomach, small intestines, caecum, feces and urine of the treated rats, the test material was cleared from all organs by day 21 of the study. Based on the submitted data, Bacillus subtilis was not toxic, infective nor pathogenic to rats.

DATA EVALUATION REPORT

Reviewed by: Cindy Schaffer, Microbiologist, SAB/HED
Secondary Reviewer: John Kough, Ph.D., Biologist, SAB/HED JK

STUDY TYPE:	Acute Dermal Toxicity-Rabbit(152A-11)
MRID NO:	419074-03
TEST MATERIAL:	*Bacillus subtilis* (NCIB 12376) spore/metabolite suspension
SYNONYMS:	MBI 600
PROJECT NO:	89270D/AGC 1/1/AC
SPONSOR:	Gustafson, Inc., Dallas, TX
TESTING FACILITY:	Huntington Research Centre, Ltd., Cambridgeshire, England
TITLE OF REPORT:	Acute Dermal Toxicity Study to Rabbits of MBI 600.
AUTHOR(S):	Michael P. Liggett, Sarah Allan
STUDY COMPLETED:	4 January, 1989
CONCLUSION:	MBI 600 produced a mild dermal irritation when a single 5 x 10^{10} CFU (2 ml/kg body weight) dose was administered. Dermal irritation dissipated by day 3. Note: This study is not required by the Agency for TGAI's of microbial pesticides.
CLASSIFICATION:	ACCEPTABLE- TOX CATEGORY IV

I. STUDY DESIGN

Test Material: The microbial pest control agent (MPCA) is Bacillus subtilis (NCIB 12376) spore/metabolite suspension. The stability and absorption was not determined by the testing facility. The administered concentration was determined to be approximately 5 x 10^{10} CFU (2 ml/kg body weight).

Test Animals: Five male and five female New Zealand White rabbits were obtained from Froxfield Rabbits, Petersfield, Hampshire, England. The male rabbits weighed between 2.44 kg and 2.84 kg and females weights ranged from 2.30 kg to 2.79 kg the day of dosing.

Methods: Approximately 24 hours prior to testing, no less than 10% of the trunk fur was clipped. The MPCA was administered over the prepared skin followed by gauze, an elastic bandage and "sleek" plaster. Approximately 24 hours following application, all wrappings were removed and the skin was washed with water. The animals were observed frequently for signs of toxicity on day 1 and twice daily thereafter. The animals were evaluated for dermal irritation, using the Draize method, 30 minutes after the removal of the skin patches, then daily

through the end of the study . Body weights were recorded on day 1, day 8 and day 15.

II. RESULTS

A. Body Weights:
No abnormalities were noted in body weights or body weight gain.

B. Clinical Signs:
No abnormalities were noted in clinical signs throughout the study. One male rabbit was found dead 24 hours post dosing (see section D).

C. Dermal Irritation Scoring:
Erythema: All males and females showed very slight redness on day 2.

Edema: Three out of four males and two out of five females displayed very slight swelling on day 2.

D. Animal Death:
One male died within 24 hours post dosing. The results of necropsy exhibited congested blood vessels in the heart, lungs, liver kidneys and stomach; a gaseous and distended stomach; liquid filled small intestine and colon; and large intestine contained loose feces and red mucosa. The testing facility did not consider this death to be treatment related.

III. SAB DISCUSSION:

Although one animal died within 24 hours post dosing, the test material did not appear to be the causative agent. Overall, MBI 600 displayed a very slight irritation when a single 5 x 10^{10} CFU dose was administered dermally. Note: This study is not required by the Agency for TGAI's of microbial pesticides.

DATA EVALUATION REPORT

Reviewed by: Cindy Schaffer, Microbiologist, SAB/HED CS
Secondary Reviewer: John Kough, Ph.D., Biologist, SAB/HED JLK

STUDY TYPE:	Acute Pulmonary Toxicity/Pathogenicity-Rat(152A-10)
MRID NO:	419074-04
TEST MATERIAL:	*Bacillus subtilis* (NCIB 12376) Spore/Metabolite suspension.
SYNONYMS:	MBI 600
PROJECT NO:	89397/AGC 1/2/AC
SPONSOR:	Gustafson, Inc., Dallas, TX
TESTING FACILITY:	Huntingdon Research Centre, LTD. Huntingdon, Cambridgeshire, England
TITLE OF REPORT:	Acute Pulmonary Toxicity and Infectivity/Pathogenicity To Rats of MBI 600.
AUTHOR(S):	David J.N. Hossack; Sarah A. Allan; Martin N. Baker
STUDY COMPLETED:	2 February 1989
CONCLUSION:	Although a high mortality was observed (24% in ♂, 15% in ♀), death was not attributed to the microorganism tested. A distinct clearance pattern was noted throughout the study in all organs. Based on the submitted data, *Bacillus subtilis* was not toxic, infective nor pathogenic to rats when dosed intratracheally.
CLASSIFICATION:	ACCEPTABLE - TOX CATEGORY IV

I. STUDY DESIGN

Test Material: The microbial pest control agent is Bacillus subtilis (NB 12376) Spore/Metabolite suspension in distilled water. The potency, homogeneity and stability was not determined by the testing facility. Each test animal received 1.2 ml/kg dose (approximately 3.4 x 10^8 CFU) by intratracheal instillation.

Test Animals: Twenty-six male and twenty-five female CD rats, approximately four to six weeks old, were obtained from Charles River, France. The male rats weighed between 281g and 320g and females weights ranged from 270g to 300g at the beginning of the study.

Methods: The animals were assigned as follows: The undosed control, group G, and the autoclaved control, group F, consisted of five male and five female rats each. Fifteen female and sixteen male rats were treated with the test substance. The rats were randomly weighed on the day of initial dosing, day 2, 8, 15 and 22. An electronic thermometer was used to record rat body temperature on day 1 prior to dosing, and 2, 4 and 24 hours post dosing.

Treated animals were observed for signs of toxicity at frequent intervals post dosing, day 1 and twice daily thereafter. Feces and urine were collected on days 2 and 22. All rats of each sex in the treatment groups were sacrificed at scheduled times by ether inhalation. The treated animals were divided into groups A through E based on sacrifice times as follows: Groups A - D were comprised of two rats of each sex and were sacrificed at 1 hour, 2, 8 and 15 days post dosing; and group E, containing 8 males and 7 females, were sacrificed 22 days post dosing. Five rats per sex from Group F, dosed with autoclaved test material, were sacrificed on day 22, the last day of the study. The animals were examined by necropsy for any macroscopic abnormalities. Samples of the brain, heart, kidneys, liver, lungs, mesenteric lymph nodes, spleen and blood were analyzed by plate smears, then homogenized if microorganisms were present. The number of viable CFU observed in the initial dose, organs, blood, feces, and caecum was determined by suspending 1 gm of B. subtilis (or sample material; i.e. macerated lung) in 9 ml phosphate buffered saline (PBS), then serially diluting this mixture 1:10 in PBS. [NOTE: The fecal material was heat treated at 65°C for 30 minutes to kill any vegetative organisms normally found in the feces.] One ml aliquots of each dilution were added to molten Tryptone Soy Agar (TSA), [urine was added undiluted] plated out in triplicate, and incubated at 32°C for 48 hours. The plates were then examined for typical B. subtilis colonies.

II. RESULTS

A. Body Weights:

All treated animals gained weight throughout the study.

B. Clinical observations:

Animals receiving heat-killed and live test material: Gasping and collapse was observed in the majority of animals within the first hour post dosing. Pilo-erection, abnormal body carriage, abnormal gait, lethargy and pallor of the extremities was displayed in virtually all animals through day 6. Increased respiration was noted in one animal on day 3. All clinical signs disappeared by day 8.

C. Body temperature:

Body temperatures dropped significantly, 4°C for live *B.s.*, 3.7°C for autoclaved, within 2 hours post dosing but approached normal temperatures as compared with the control rats within 24 hours.

D. Animal Mortality:

Twenty four percent (5/21) of the males and fifteen percent (3/20) of the female rats treated with the test substance were found dead from day 2 to day 4 of the study.

E. Necropsy observations:

No abnormalities were noted upon necropsy.

F. Microbial clearance/infectivity:

Clearance and infectivity were evaluated in the brain, blood, lymph nodes, kidney, liver, spleen, lungs and stomach, feces and urine. The submitted data (see below) demonstrated that the organism was detected in all the organs. In all instances, a distinct clearance pattern was observed by day 21.

ORGAN	DAY	# rats*	MEAN CFU/GM OF TISSUE
Lungs	1 hr	4/4	4.4×10^6
	1	4/4	4.8×10^5
	7	4/4	2.2×10^6
	14	2/2	7.2×10^5
	21	9/9	4.7×10^4
Brain	1 hr	3/4	246
	1	2/4	70
Heart	1 hr	2/4	222
	1	2/4	<10
	7	2/4	<15
Liver	1 hr	2/4	<10
	1	2/4	62
	7	3/4	55
Kidney	1 hr	3/4	<10
	1	2/4	<11
Spleen	1 hr	2/4	<10
	1	3/4	380
Lymph nodes	1 hr	2/4	840
	1	2/4	<10
	7	1/4	<10
Blood	1	1/4	<10
	7	1/4	<10
Urine	1	10/10	1.57×10^3
	21	9/10	<10
Feces	1	7/10	6.03×10^3
	21	9/10	1.54×10^3
Caecum	1 hr	4/4	<10
	1	4/4	4.16×10^5
	7	4/4	3.76×10^3
	14	2/2	5.25×10^3
	21	9/9	1.70×10^3

* # of (+) treated rats/ total # of treated rats

III. SAB DISCUSSION:

Although a high mortality was observed (24% in ♂, 15% in ♀), death was not attributed to the microorganism tested, but to the dose administered. A distinct clearance pattern was noted throughout

the study in all organs. Based on the submitted data, *Bacillus subtilis* was not toxic, infective nor pathogenic to rats when dosed intratracheally.

DATA EVALUATION REPORT

Reviewed by: Cindy Schaffer, Microbiologist, SAB/HED
Secondary Reviewer: John Kough, Ph.D., Biologist, SAB/HED JCK

STUDY TYPE:	Acute Intravenous Toxicity/Pathogenicity-Rat (152A-13)
MRID NO:	419074-05
TEST MATERIAL:	*Bacillus subtilis* (NCIB 12376) Spore/Metabolite suspension.
SYNONYMS:	MBI 600
PROJECT NO:	89398D/AGC 1/3/AC
SPONSOR:	Gustafson, Inc., Dallas, TX
TESTING FACILITY:	Huntingdon Research Centre, LTD. Huntingdon, Cambridgeshire, England
TITLE OF REPORT:	Acute Intravenous Toxicity and Infectivity /Pathogenicity To Rats of MBI 600.
AUTHOR(S):	David J.N. Hossack; Sarah A. Allan; Martin N. Baker
STUDY COMPLETED:	2 June 1989
CONCLUSION:	Although the microbe was found in all the organs of the treated rats, the test material displayed a pattern of clearance from all organs during the study. Based on the submitted data, Bacillus subtilis was not toxic, infective nor pathogenic to rats when dosed intravenously.
CLASSIFICATION:	ACCEPTABLE - TOX CATEGORY IV

I. STUDY DESIGN

Test Material: The microbial pest control agent is Bacillus subtilis (NB 12376) Spore/Metabolite suspension in distilled water. The homogeneity and stability was not determined by the testing facility. Each test animal received 3 ml/kg dose (approximately 4 x 10^7 CFU) by intravenous injection.

Test Animals: Thirty-three male and thirty-three female CD rats were obtained from Charles River, France. The male rats weighed between 136g and 160g and females weights ranged from 146g to 160g at the beginning of the study.

Methods: The animals were assigned as follows: The undosed control, group G, and the autoclaved control, group F, consisted of five male and five female rats each. Thirteen female and thirteen male rats were treated with the test substance. The rats were randomly weighed on the day of initial dosing, day 8, 15 and 22. An electronic thermometer was used to record rat body temperature on day 1 prior to dosing, and 2, 4 and 24 hours post dosing. Treated animals were observed for signs of toxicity at

frequent intervals post dosing on day 1 and twice daily thereafter. Feces and urine were collected on days 2 and 22. All rats of each sex in the treatment groups were sacrificed by ether inhalation. The treated animals were divided into groups A through E based on sacrifice times as follows: Groups A - E were comprised of two rats of each sex and were sacrificed at one hour, days 2, 8 and 15 and 22 days post dosing. Five rats per sex from Group F and G were sacrificed on day 22, the last day of the study. The animals were examined by necropsy for any macroscopic abnormalities. Samples of the brain, heart, kidneys, liver, lungs, mesenteric lymph nodes, spleen, as well as contents of the caecum were analyzed by tissue smear plates. If colonies were found, macerated tissue would be plated. The number of viable CFU observed in the initial dose, organs, blood, feces, stomach, small intestine and caecum contents was determined by suspending 1 gm of B. subtilis (or sample material; i.e. macerated lung) in 9 ml phosphate buffered saline (PBS), then serially diluting this mixture 1:10 in PBS. [NOTE: The fecal material was heat treated at 65°C for 30 minutes to kill any vegetative organisms normally found in the feces.] One ml aliquots of each dilution [urine samples plated undiluted] were added to molten Tryptone Soy Agar (TSA), plated out in triplicate, and incubated at 32°C for 72 hours. The plates were then examined for typical B. subtilis colonies.

II. RESULTS

A. Body Weights:

All treated animals gained weight throughout the study.

B. Clinical observations:

Piloerection was noted in all animals except the undosed controls during the first day of the study. No clinical signs were noted from day 2 through the end of the study.

C. Body temperature:

No abnormalities were noted in body temperature during the study.

D. Necropsy observations:

No abnormalities were noted upon necropsy.

E. Microbial clearance/infectivity:

Clearance and infectivity were evaluated in the brain, blood, lymph nodes, kidney, liver, spleen, lungs, caecum, feces and urine. The submitted data (see below) demonstrated that the organism was detected in all organs, feces and urine. The mean CFU/g tissue is only noted for days the organism was detected. In all instances, a clearance pattern

was demonstrated by the test material through the end of the study.

ORGAN	DAY	# rats*	MEAN CFU/GM OF TISSUE
Brain	1 hr	2/4	135
	1	3/4	116
Heart	1 hr	4/4	643
	1	4/4	295
	7	2/4	<10
	14	1/4	<10
Lungs	1 hr	4/4	3.96×10^4
	1	4/4	5.44×10^3
	7	3/4	1.35×10^3
	14	3/4	52
Liver	1 hr	4/4	5.21×10^5
	1	4/4	1.25×10^5
	7	4/4	8.60×10^4
	14	4/4	5.28×10^3
	21	5/5	3.95×10^3
Kidney	1 hr	4/4	1.13×10^3
	1	3/4	1.79×10^3
	7	4/4	64
Spleen	1 hr	4/4	5.38×10^5
	1	4/4	1.12×10^5
	7	4/4	5.41×10^4
	14	4/4	1.98×10^4
	21	6/6	3.10×10^3
Lymph nodes	1 hr	1/4	1.60×10^3
	1	1/4	<10
	7	3/4	1.67×10^3
Blood	1 hr	3/4	306
	1	2/4	86
	7	1/4	2.70×10^3
Feces	1	10/10	2.25×10^4
	21	10/10	<10
Urine	1	10/10	78
	21	10/10	<10

* # of (+) treated rats/ total # of treated rats

III. SAB DISCUSSION:

Although the microbe was found in all the organs of the treated rats shortly after dosing, the test material displayed a pattern of clearance from all organs during the study. Based on the submitted data, Bacillus subtilis was not toxic, infective nor pathogenic to rats when dosed intravenously.

DATA EVALUATION REPORT

Reviewed by: Cindy Schaffer, Microbiologist, SAB/HED
Secondary Reviewer: John Kough, Ph.D., Biologist, SAB/HED JLK

STUDY TYPE:	Primary Eye Irritation-Rabbit(152A-14)
MRID NO:	419074-06
TEST MATERIAL:	MBI 600
SYNONYMS:	Bacillus subtilis (NCIB 12376) spore/metabolite suspension
PROJECT NO:	89399D/AGC 1/4/SE
SPONSOR:	Gustafson, Inc., Dallas, TX
TESTING FACILITY:	Huntingdon Research Centre, LTD., Huntingdon, Cambridgeshire, England
TITLE OF REPORT:	Primary Eye Irritation and Infectivity of MBI 600.
AUTHOR(S):	Michael P. Liggett, Sarah A. Allan, David J. Hossack
STUDY COMPLETED:	2 June 1989
CONCLUSION:	MBI 600 produced a slight ocular irritation when a single 0.1 mg ocular dose was administered. Ocular irritation dissipated by day 4. NOTE: This study is not currently required for TGAI's of microbial pesticides.
CLASSIFICATION:	ACCEPTABLE- TOX CATEGORY IV

I. STUDY DESIGN

Test Material: The microbial pest control agent (MPCA) is Bacillus subtilis (NCIB 12376) spore/metabolite suspension. The purity of the test material was determined to be 1.0×10^{10} CFU/ml. The dosing material was 1.0×10^{9} CFU/gm.

Test Animals: Six female New Zealand White rabbits, obtained from Froxfield Rabbits, Petersfield, Hampshire, weighed between 2.6 and 3.1 kg.

Methods: A single dose of 0.1 g (1.0×10^{9} CFU) of the MPCA was administered into the conjuntival sac of one of the lower eyelids of each animal. The eye lids were gently held together for a second to prevent a loss of material. The other eye served as the control for each animal. The Draize Method was used to score ocular lesions at 1 hour, 1, 2, 3, 4, 7, 14 and 21 days post application.

II. RESULTS

A. Clinical Observations/Eye Irritation Scoring:

CONJUNCTIVAE:

Discharge:

All six females showed signs of slight discharge up to 3 days post dosing.

Swelling:

All six animals demonstrated mild chemosis within 72 hours post dosing starting at day 1.

Redness:

All six females displayed slight redness through day 2 and two females displayed slight redness through day 3 post dosing.

No abnormalities were observed in any control eye during the study.

B. Infectivity:

The organism was detected in the fluid or eyelid of each animal up to 21 days post dosing.

III. SAB DISCUSSION:

The microorganism was detected in the fluid or eyelid of each animal through the end of the study; and Bacillus subtilis produced a slight ocular irritation when a single 0.1 mg ocular dose was administered. Ocular irritation was no longer present by day 4. NOTE: This study is not currently required for TGAI's of microbial pesticides.

DATA EVALUATION REPORT

Reviewed by: Cindy Schaffer, Microbiologist, SAB/HED CS
Secondary Reviewer: John Kough, Ph.D., Biologist, SAB/HED JK

STUDY TYPE:	Skin Sensitization Study-Guinea Pig (152A-15)
MRID NO:	419074-07
TEST MATERIAL:	Bacillus subtilis (NCIB 12376) spore/metabolite suspension
SYNONYMS:	MBI 600
PROJECT NO:	89429D/AGC 2/SS
SPONSOR:	Gustafson, Inc., Dallas, TX
TESTING FACILITY:	Huntingdon Research Centre, Ltd., Huntingdon, Cambridgeshire, England
TITLE OF REPORT:	Delayed Contact Hypersensitivity in the Guinea-Pig with MBI 600.
AUTHOR(S):	Sheena R. Kynoch, Brenda I. Parcell
STUDY COMPLETED:	11 July, 1989
CONCLUSION:	An overall moderate skin sensitization reaction was noted in the treated guinea pigs 24 to 72 hours post test challenge.

I. STUDY DESIGN

Test Material: The microbial pesticide control agent is Bacillus subtilis (NCIB 12376) spore metabolite suspension. The absorption, stability and potency of the test article was not determined. A preliminary study was performed to determine the highest non-irritant concentration and threshold irritation concentration to be used for the topical induction and challenge applications. Based on the results of this study, a concentration of 5% v/v in distilled water was used for the intradermal induction and 50% v/v in distilled water was used for the topical induction and challenge applications.

Test Animals: Thirty female albino Dunkin-Hartley guinea pigs were obtained from D. Hall, Newchurch, Staffordshire, England. The guinea pigs weighed between 362 and 488 grams at dosing.

Methods: The guinea pig maximization test consists of two stages. The induction phase, which consists of a series of intradermal injections potentiated by a simultaneous injection of Freunds Complete Adjuvant (50% v/v in distilled water), followed 7 days later by a topical application of the test material. Three sets of intradermal injections were made on either side of a 4cm x 6cm clipped area on the guinea pigs shoulder as follows:

1. 0.1 ml Freunds Complete Adjuvant (50% v/v in water)
2. 0.1 ml 5.0% v/v MBI 600 in distilled water

3. 0.1 ml 5.0% w/v MBI 600 in distilled water plus 0.1 ml Freunds Complete Adjuvant (50% v/v in water).

Twenty guinea pigs in the control group received a similar set of injections at either side of the midline as follows:

1. 0.1 ml Complete Adjuvant (50% v/v in water)
2. 0.1 ml Distilled water
3. 0.1 ml Complete Adjuvant (50% v/v in water).

The same shoulder area was clipped one week following the intradermal induction. A 2 cm x 4 cm patch of Whatman No. 3 filter paper saturated with test material, as supplied, (0.4 ml) was applied to the clipped area and fastened by an overlapping occlusive tape. The control animals received a similar treatment using "vehicle" only for the intradermal injections and topical application. The test and control guinea pigs left flanks were shaven on day thirteen after the induction phase. The next day (day 14), these animals were challenged with an application of 0.2 ml of MBI 600 was applied to an anterior site on the left flank of each guinea pig. MBI 600, 50% v/v in distilled water was applied to the posterior flank in the same fashion. After a contact period of twenty four hours, the occlusive dressings were removed. The skin reactions at the challenge site were assessed twenty four, forty eight and seventy two hours after the dressings were removed and graded according to the following scale:

REACTION	VALUE
ERYTHEMA AND ESCHAR FORMATION:	
No reaction	0
Slight erythema	1
Well defined erythema	2
Moderate erythema	3
Severe redness to slight eschar formation	4
EDEMA FORMATION:	
No reaction	0
Slight edema	1
Well-defined edema	2
Moderate edema	3
Severe edema	4

II. RESULTS

A. Skin Reactions in Test Animals after challenge application at 24 hrs:

Anterior site: One control animal demonstrated slight redness; all twenty test animals displayed well defined

redness: nineteen had well defined swelling, one had slight swelling.
Posterior site: Nine treated guinea pigs showed signs of slight to well defined redness and three experienced slight to moderate edema.

B. Skin Reactions in Test Animals after challenge application at 48 hours:
Anterior site: Four control animals had slight redness and two were observed with slight swelling. Eight treated guinea pigs experienced moderate edema with some form of necrosis at the challenge site while two experienced slight to well defined redness. All ten test animals showed slight (1/10) to severe (1/10) edema with the majority demonstrating moderate swelling at the test site.
Posterior site: Three treated animals displayed moderate redness with some necrosis at the test site; six test animals had slight to well defined erythema. One treated animal exhibited severe edema, three showed signs of moderate swelling and three animals had slight to well defined swelling.

C. Skin reactions in Test Animals after challenge application at 72 hours:
Anterior site: Three control guinea pigs showed signs of localized to slight redness. Three treated animals displayed necrosis; six had well defined to moderate erytherma with thickening, drying and sloughing of the epidermis; and one showed signs of slight redness.
Posterior site: Four treated guinea pigs displayed slight to well defined edema and four had moderate to severe swelling. Four treated animals had dryness and sloughing of the epidermis with well defined edema; one guinea pig had moderate swelling with thickening, dryness and sloughing of the epidermis; and four animals were observed with slight to well defined edema.

D. Body weights:
No abnormalities were noted in body weight or body weight gain throughout the study.

II. SAB DISCUSSION:
At 24 hours post test material challenge, 100% of the animals showed a mild to moderate sensitization reaction; at 48 and 72 hours, a moderate to severe skin sensitization reaction was noted in 50% of the guinea pigs; while. Mild reactions were noted in a few of the control animals. An overall moderate skin sensitization reaction was noted in the treated guinea pigs 24 to 72 hours post test challenge. NOTE: Although this study is not required for MPCA's; incident reports must be submitted to the Agency.

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