
Reverse Mutation Test of *Bacillus subtilis* DB9011
with Bacteria

FINAL REPORT

Prepared: March 17, 2005

Hashima Laboratory, Nihon Bioresearch Inc.

Statement of Quality Assurance Unit

Study No.: 901524

Title: Reverse Mutation Test of Bacillus subtilis DB9011 with Bacteria

With respect to the above study, Quality Assurance inspections were conducted from January 25, 2005 to March 17, 2005. Findings of the inspections were reported to the Management and Study Director.

This statement certifies that the above study was conducted in compliance with the Ordinance on Standards for Implementation of Nonclinical Studies on Safety of Drugs (Ordinance No. 21 of the Japanese Ministry of Health and Welfare, March 26, 1997). It also certifies that this report accurately describes the methods of the study, and the reported results accurately reflect the raw data obtained during the course of the study.

(Details of the inspections are shown on Attached sheet 1.)

March 17, 2005

Hashima Laboratory, Nihon Bioresearch Inc.

Manager of Quality
Assurance Unit:

(signed)

(seal)

Yoshiaki Hongo

Attached sheet 1

Item inspected	Date of inspection	Date on which findings were reported
1. Protocol and master schedule sheet	January 25, 2005	January 25, 2005
2. Amendment to protocol (No. 1)	February 2, 2005	February 2, 2005
3. Dose-finding test	February 2, 2005	February 2, 2005
4. Amendment to protocol (No. 2)	February 10, 2005	February 10, 2005
5. Preincubation of bacterial strains (mutagenicity test)	February 14, 2005	February 17, 2005
6. Management of the test article	February 15, 2005	February 17, 2005
7. Formulation	February 15, 2005	February 17, 2005
8. Mutagenicity test	February 15, 2005	February 17, 2005
9. Evaluation (mutagenicity test)	February 17, 2005	February 17, 2005
10. Raw data	February 24 and 25, 2005	February 25, 2005
11. Draft report (1st draft)	February 25, 2005	February 25, 2005
12. Draft report (1st draft) (2nd draft)	March 1, 2005	March 1, 2005
13. Final report	March 17, 2005	March 17, 2005

Signature of Person Who Prepared Interim Report

Study No.: 901524

Title: Reverse Mutation Test of Bacillus subtilis DB9011 with Bacteria

March 17, 2005

Hashima Laboratory, Nihon Bioresearch Inc.

Study Director: _____ (signed) (seal)
Yoshihisa Miwa

Test Article: Bacillus subtilis DB9011

Test Systems: *Salmonella typhimurium*: TA100, TA98, TA1535, and TA1537
Escherichia coli: WP2uvrA

Sponsor: AHC Co., Ltd.
343-1 Koaigimachi, Maebashi, Gunma, Japan

Testing Facility: Hashima Laboratory, Nihon Bioresearch Inc.
104, 6-chome, Majima, Fukuju-cho, Hashima, Gifu, Japan

Objective: A reverse mutation test of Bacillus subtilis DB9011 using bacteria was performed, and its mutagenic potential was examined.

Guidelines Followed: Guidelines for Genetic Toxicity Studies of Drugs (Notification No. 1604 of the Pharmaceutical Affairs Bureau, Japanese Ministry of Health and Welfare, November 1, 1999)

GLP Followed: Ordinance on Standards for Implementation of Nonclinical Studies on Safety of Drugs (Ordinance No. 21 of the Japanese Ministry of Health and Welfare, March 26, 1997)
Neither the stability of the test article or of test article preparations nor the concentrations of the test article in the test preparations were examined in the present study.

Start of the Study: January 25, 2005

Completion of the Study: March 17, 2005

Study Schedule:

Dose-finding test		
Pre-incubation of bacterial strains		February 1, 2005
Implementation of the test		February 2, 2005
Evaluation		February 4, 2005
Mutagenicity test		
Pre-incubation of bacterial strains		February 14, 2005
Implementation of the test		February 15, 2005
Evaluation		February 17, 2005

Archives: All data generated in the present study in the testing facility will be stored in the data storage room of Hashima Laboratory, Nihon Bioresearch Inc. for 5 years after completion of the study. Subsequent disposition of the data will be determined by mutual agreement with the sponsor.

Deviations from the Protocol: There were no deviations from the protocol.

Unforeseeable Circumstances: There were no unforeseeable circumstances which might have adversely affected the reliability of the study during the study period.

Study Personnel and Work Responsibility

Study Director:

Yoshihisa Miwa

Preparation of the protocol, directions of test implementation, verification of the data obtained, and preparation of the final report

Study Personnel:

Koji Goto

Formulation, test implementation, compilation of the data obtained, and verification of the data obtained

Risa Kobayashi

Test implementation, compilation of the data obtained, and verification of the data obtained

Keiji Iwata, Kikuko Fujii, and Yumiko Yoneyama

Management of the test article, positive control articles, and negative control article

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Summary

The mutagenic potential of *Bacillus subtilis* DB9011 was examined by a reverse mutation test using *Salmonella typhimurium* TA100, TA98, TA1535, and TA1537, and *Escherichia coli* WP2uvrA. The study was performed by the pre-incubation method in the absence and presence of S9 mix.

The number of revertant colonies/plate treated with *Bacillus subtilis* DB9011 was not greater than twice the number of colonies/plate treated with the negative control article in any bacterial strain either in the absence or in the presence of S9 mix.

The positive control articles caused marked increase in the number of revertant colonies, and the number of revertant colonies treated with the negative control article and that with the positive control article were within the background data of the testing facility.

The results of the dose-finding and mutagenicity tests were confirmed to be reproducible.

From the above results, *Bacillus subtilis* DB9011 is judged to have no mutagenic potential under the conditions of the present study.

2) Sodium azide (abbreviated name: NaN₃)

Lot No. M3H9553; purity: 99.8%; Nacalai Tesque, K.K.; expiry: September 1, 2008 (in-house regulation); storage conditions: stored under refrigerated (set at 4°C; refrigerator used: MPR-311D, Sanyo Co., Ltd.), desiccated, light-shielded conditions.

3) 9-Aminoacridine hydrochloride (abbreviated name: 9AA)

Lot No. M3N4147; purity: 97.6%; Nacalai Tesque, K.K.; expiry: October 13, 2008 (in-house regulation); storage conditions: stored under refrigerated (set at 4°C; refrigerator used: MPR-311D, Sanyo Co., Ltd.) conditions.

4) 2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide (abbreviated name: AF-2)

Lot No. SEL1402; a yellowish red crystalline powder; purity: 99.0%; Wako Pure Chemical Industries, Ltd.; expiry: February 4, 2007 (in-house regulation); storage conditions: stored under refrigerated (set at 4°C; refrigerator used: MPR-311D, Sanyo Co., Ltd.), light-shielded conditions.

(4) Negative Control Article

DMSO, which served as the vehicle for the test article, was used as the negative control article.

2. Test Solutions

(1) Test Article Solutions

To prepare the solutions of *Bacillus subtilis* DB9011 at the highest concentrations (50 mg/mL for the dose-finding test and 3.125 mg/mL for the mutagenicity test), the required amount of *Bacillus subtilis* DB9011 was measured out (electronic balance used: AT261, Mettler-Toledo GmbH) and suspended in DMSO. Test article solutions at lower concentrations were prepared by serial dilution of the test article solution at the highest concentration. The test article

solutions were prepared when they were needed. Test article solutions remaining after use were discarded.

Neither the stability of the test article solutions nor the concentrations of the test article in the test article solutions were examined in the present study.

(2) Positive Control Article Solutions

2AA, 9AA, and AF-2 were each dissolved in DMSO (for ultraviolet absorption spectrum; Lot No. NJ151 for 2AA and AF-2 and Lot No. PU140 for 9AA, Dojin Chemical Laboratory), and NaN_3 was dissolved in water for injection (Lot No. K2K75, Otsuka Pharmaceutical Factory, Inc.) (prepared on April 9, 2004 for 2AA, on July 2, 2004 for 9AA and NaN_3 , and on August 6, 2004 for AF-2; expiry: 1 year after preparation for all positive control solutions). The concentrations of the positive control article are shown below.

The positive control article solutions thus prepared were dispensed in a volume of 0.5 mL each into tubes (2-mL Serum Tubes, Sumitomo Bakelite Co., Ltd.) and stored frozen (set at -85°C ; values by actual measurement: $-87 - -79^\circ\text{C}$) in a freezer (test preparation storage container No. 8) (MDF-291AT, Sanyo Co., Ltd.). The positive control article solutions were thawed prior to the test, and those remaining after use were discarded.

	Bacterial strain	Positive control article	Prepared concentration ($\mu\text{g/mL}$)	Test concentration ($\mu\text{g/plate}$)
S9 mix (+)	TA100	2AA	10	1
	TA1535	2AA	20	2
	WP2 <i>uvrA</i>	2AA	100	10
	TA98	2AA	5	0.5
	TA1537	2AA	20	2
S9 mix (-)	TA100	AF-2	0.1	0.01
	TA1535	NaN_3	5	0.5
	WP2 <i>uvrA</i>	AF-2	0.1	0.01
	TA98	AF-2	1	0.1
	TA1537	9AA	800	80

3. Bacterial Strains

S. typhimurium TA100, TA98, TA1535, and TA1537, and *E. coli* WP2uvrA were used in compliance with the Guidelines for Genetic Toxicity Studies of Drugs. We obtained TA100 and TA98 on October 18, 1996, and TA1535, TA1537, and WP2uvrA on February 25, 1995, all from Japan Bioassay Laboratory, Japan Industrial Safety and Health Association.

The bacterial strains were examined for amino acid requirements, ultraviolet radiation sensitivity, membrane specificity due to *rfa* gene mutation, and presence or absence of drug resistance R-factor plasmid in accordance with the Mutagenicity Tests in the Industrial Safety and Health Law of Japan—Testing Guidelines and GLP—¹⁾ (TA100 and TA98 were tested from February 25 to 27, 2004, and TA1535, TA1537, and WP2uvrA were tested from October 29 to 31, 2003). Bacterial colonies which met the standards of the testing facility were selected (Attachment 1).

The bacterial colony selected on the basis of the results of the characterization tests was incubated, and a bacterial suspension was obtained. A mixture of the bacterial suspension thus prepared and DMSO at a volumetric ratio of 0.8 mL to 0.07 mL was dispensed in a volume of 200 μ L each into tubes (2-mL Serum Tubes, Sumitomo Bakelite Co., Ltd.) and stored in a freezer [BFV-130 (LR), Espec Co., Ltd.] set at -80°C (TA100 and TA98 were dispensed on March 23, 2004, and TA1535, TA1537, and WP2uvrA were dispensed on December 26, 2003; expiry: 2 years after dispensation).

A mixture of OXOID NUTRIENT BROTH No. 2 (Lot No. 298714, OXOID Ltd.) and water for injection at a ratio of 2.0 g and 80 mL was autoclaved (at 121°C for 15 minutes) and used for pre-incubation of the bacteria. The medium was dispensed in a volume of 10 mL each into sterilized (at 180°C for 1 hour; the same conditions were used throughout the test) L-shaped tubes (capacity: about 40 mL) with a molten cap. After being thawed, 20 μ L of the bacterial suspension was inoculated into the medium, and the medium was incubated at 37°C for 9 hours in a reciprocating shaker (M-100^N, Taitec Co., Ltd.; frequency of shaking: 90 times/minute; amplitude: 4 cm).

After incubation, the turbidity of the bacterial suspension was determined with a spectrophotometer (Novaspec II, Amersham Biosciences Ltd.), and the number of bacteria in the suspension was determined from the O.D. values of its turbidity (Attachment 2). The prepared bacterial suspensions were stored at room temperature until use. Test operations were performed in an air-conditioned room for mutagenicity tests (Bldg. A).

4. S9 Mix

S9 (Lot No. 04100107) manufactured by Oriental Yeast Co., Ltd. on October 1, 2004 under the conditions specified in Attachment 3 was purchased on November 2, 2004 and stored in a freezer [BFV-130 (LR), Espec Co., Ltd.] set at -80°C until use.

To prepare S9 mix, cofactors (trade name: Cofactor-I, Lot No. 999402, Oriental Yeast Co., Ltd.) for S9 mix were dissolved in water for injection, and the prepared solution was decontaminated by filtration with a membrane filter (pore size $0.2\ \mu\text{m}$, Nalge). S9 was added to the solution immediately before use. The composition of the S9 mix thus prepared is shown in Attachment 4.

5. Culture Media

Tesmedia[®] AN agar (Lot No. ANI990KT; manufactured by Oriental Yeast Co., Ltd. on November 16, 2004) was used as the minimum glucose agar medium. The composition of Tesmedia[®] AN agar is shown in Attachment 5.

The top agar was prepared as follows: Bacto Agar (Lot No. 2120028, DIFCO) and sodium chloride were added to water for injection at concentrations of 0.6% and 0.5%, respectively, and autoclaved (at 121°C for 20 minutes; the same conditions were used throughout the test). The mixture and an amino acid solution were mixed at a volumetric ratio of 10 to 1; the amino acid solution used for *S. typhimurium* was an aqueous mixture of 0.5 mmol/L L-histidine and 0.5 mmol/L D-biotin, and that for *E. coli* was an aqueous solution of 0.5 mmol/L L-tryptophan. Prior to use, these amino acid solutions were filtered using Vacuum Filter Systems (pore size 0.22

µm, CORNING).

6. Sterility Tests

Sterility tests of the test article solutions at the highest concentrations (50 mg/mL for the dose-finding test and 3.125 mg/mL for the mutagenicity test) and of S9 mix were performed by the plate method using 2 plates each in the dose-finding and mutagenicity tests as described below.

The top agar was warmed to 45°C, and a mixture of 2 mL of the top agar and 0.1 mL of the test article solution at the highest concentration or a mixture of 2 mL of the top agar and 0.5 mL of S9 mix was poured over the minimum glucose agar medium plates and incubated at 37°C for about 48 hours in an incubator (IA-81, Yamato Scientific Co., Ltd.) with the plates upside down. After incubation, the plates were checked for the presence of bacterial colonies.

7. Study Methods

(1) Test Operation

The conditions under which the present study was performed are shown in Attachment 6.

The study was performed by the pre-incubation method without metabolic activation (in the absence of S9 mix) and with metabolic activation (in the presence of S9 mix) as follows: (a) 0.1 mL of the test solution, (b) 0.5 mL of autoclaved 0.1 mol/L Na-phosphate buffered solution (pH 7.4) (without metabolic activation) or 0.5 mL of S9 mix (with metabolic activation), and (c) 0.1 mL of the bacterial suspension were dispensed into sterilized test tubes (15.5 x 100 mm LARUBO disposable test tubes, Terumo Corporation) in that order and incubated using a reciprocating shaker at 37°C for 20 minutes. Then 2 mL of the top agar maintained at 45°C was added to each tube. After being mixed, the contents of the tube were poured over the minimum glucose agar medium plates. The plates were inverted and incubated at 37°C for about 48 hours in an incubator.

The presence or absence of precipitation on the plates was observed macroscopically on completion of incubation. Since precipitation was noted in the plates treated with the test article in the dose-finding test [it was concluded that the number of revertant colonies/plate treated with the test article would not be counted with a colony analyzer (CA-11D, System Science Co., Ltd.)], the number of revertant colonies/plate treated with the negative control article and that with the test article were counted with a handy colony counter (CC-1, AS ONE Corporation) in the dose-finding test. The number of revertant colonies/plate treated with the positive control article was counted with the colony analyzer in the dose-finding test. In the mutagenicity test, the number of revertant colonies/plate treated with the negative control article, that with the test article, and that with the positive control article were counted with the colony analyzer.

Then the presence or absence of bacterial growth inhibition was examined using a microscope at 100-fold magnification. The presence or absence of precipitation was also observed macroscopically at the start of incubation.

Two plates were used for each combination of bacterial strains, test procedures (with or without metabolic activation), and concentrations. Test tubes and plates were individually identified by coloring with oil-based ink of a different color for each bacterial strain.

(2) Dose-finding Test

According to the Guidelines for Toxicity Studies of Drugs, the highest concentration of *Bacillus subtilis* DB9011 was set at 5000 µg/plate, and lower concentrations were calculated by decreasing geometrically by a factor of 4 and set at 1250, 312.5, 78.1, 19.5, 4.88, 1.22, and 0.305 µg/plate, 8 concentrations in total, for all bacterial strains both in the absence and in the presence of S9 mix. A negative control and a positive control were employed as controls for each bacterial strain.

(3) Mutagenicity Test

Based on the results of the dose-finding test, the mutagenicity study was performed at the following 6 concentrations, which were calculated by decreasing by a factor of 2 and determined in such a way as to include at least 4 concentrations at which bacterial growth inhibition was not expected to occur: 9.77, 19.5, 39.1, 78.1, 156.3, and 312.5 $\mu\text{g}/\text{plate}$ for TA100, TA1535, WP2*uvrA*, and TA98, and at 2.44, 4.88, 9.77, 19.5, 39.1, and 78.1 $\mu\text{g}/\text{plate}$ for TA1537. A negative control and a positive control were employed as controls for each bacterial strain.

8. Criteria for Judging the Validity of the Study

The present study can be considered valid if it meets the following conditions: there is no bacterial contamination either in the test article solutions at the highest concentration or in S9 mix in the sterility tests, the number of revertant colonies in the negative control and that in the positive control are within the range of the background data of the testing facility (Attachment 7), and no factors that might affect the test system exist.

9. Statistical Methods

The mean number of revertant colonies was calculated for each concentration; no statistical analysis was performed.

10. Evaluation

Results of the test were considered positive when the number of revertant colonies treated with the test article was twice or greater than the number of colonies in the negative control and increased concentration-dependently.

Results

1. Dose-finding Test (Tables 1-1 and 1-2; Figures 1-1 and 1-2)

(1) Precipitation on the Plates

Fine brown precipitation was noted on the plates treated with the test article at 78.1 $\mu\text{g}/\text{plate}$ and higher both in the absence and in the presence of S9 mix at the start and completion of incubation.

(2) Bacterial Growth Inhibition

Bacterial growth inhibition was noted on the plates treated with the test article at 312.5 $\mu\text{g}/\text{plate}$ and higher for TA100, TA1535, WP2uvrA, and TA98, and at 78.1 $\mu\text{g}/\text{plate}$ and higher for TA1537 both in the absence and in the presence of S9 mix.

(3) Number of Revertant Colonies

The number of revertant colonies treated with the test article was less than twice the number of colonies treated with the negative control article in all bacterial strains both in the absence and in the presence of S9 mix.

(4) Sterility Test

No bacterial contamination was noted in the solution of the test article at the highest concentration (50 mg/mL) or in the S9 mix.

(5) Negative Control and Positive Controls

The positive control articles caused marked increase in the number of revertant colonies. The number of revertant colonies treated with the negative control article and that with the positive control articles were within the background data of the testing facility.

2. Mutagenicity Test (Tables 2-1 and 2-2; Figures 2-1 and 2-2)

(1) Precipitation on the Plates

Fine brown precipitation was noted on the plates treated with the test article at 39.1 $\mu\text{g}/\text{plate}$ and higher both in the absence and in the presence of S9 mix at the start and completion of incubation.

(2) Bacterial Growth Inhibition

Bacterial growth inhibition was noted on the plates treated with the test article at 156.3 $\mu\text{g}/\text{plate}$ and higher for TA100, TA1535, and TA98, at 312.5 $\mu\text{g}/\text{plate}$, the highest concentration, for WP2*uvrA*, and at 39.1 $\mu\text{g}/\text{plate}$ and higher for TA1537 in the absence of S9 mix. Bacterial growth inhibition was noted on the plates treated with the test article at 312.5 $\mu\text{g}/\text{plate}$, the highest concentration, for TA100, TA1535, WP2*uvrA*, and TA98, and at 78.1 $\mu\text{g}/\text{plate}$, the highest concentration, for TA1537 in the presence of S9 mix.

(3) Number of Revertant Colonies

The number of revertant colonies treated with the test article was less than twice the number of colonies treated with the negative control article in all bacterial strains both in the absence and in the presence of S9 mix.

(4) Sterility Test

No bacterial contamination was noted in the solution of the test article at the highest concentration (3.125 mg/mL) or in the S9 mix.

(5) Negative Control and Positive Controls

The positive control articles caused marked increase in the number of revertant colonies. The number of revertant colonies treated with the negative control article and that with the positive control articles were within the background data of the testing facility.

Discussion

The mutagenic potential of *Bacillus subtilis* DB9011 was examined by a reverse mutation test using bacteria.

The number of revertant colonies treated with *Bacillus subtilis* DB9011 at all concentrations was less than twice the number of colonies treated with the negative control article in all bacterial strains both in the absence and in the presence of S9 mix.

No bacterial contamination was noted in the solution of *Bacillus subtilis* DB9011 at the highest concentration or in S9 mix in the sterility tests.

The positive control articles caused marked increase in the number of revertant colonies, and the number of revertant colonies treated with the negative control article and that with the positive control article were within the background data of the testing facility.

The results of the dose-finding and mutagenicity tests were confirmed to be reproducible.

From the above results, *Bacillus subtilis* DB9011 is judged to have no mutagenic potential under the conditions of the present study.

Data and Literature

- 1) Mutagenicity Tests in the Industrial Safety and Health Law of Japan. —Testing Guidelines and GLP—. Edited by Chemical Substance Investigation Division, Industrial Safety and Health Department, Labour Standards Bureau, Ministry of Labour and issued by Japan Industrial Safety Health Association on March 25, 1991.

Attachment 1. Specificity of test strains

		TA100	TA1535	WP2 _{uvrA}	TA98	TA1537
Amino acid requirement	His ^{a)}	32 ^{b)} 44	0 0	/	3 6	0 2
	Trp	/	/	6 7	/	/
Sensitivity to UV radiation		+ ^{c)}	+	+	+	+
rfa specificity		11.3 mm ^{d)}	13.0 mm	0.0 mm	10.2 mm	15.8 mm
R-factor presence		0.0 mm ^{e)}	11.8 mm	17.8 mm	0.0 mm	19.7 mm

a): D-biotin was contained in plates.

b): Number of revertant colonies/plate.

c): +; Present of sensitivity.

d): Diameter of inhibition zone induced by paper disk (diameter: 6 mm) containing crystal violet (10 µg/10 µL).

e): Diameter of inhibition zone induced by paper disk (diameter: 6 mm) containing ampicillin sodium (10 µg/10 µL).

Attachment 2. Number of surviving cells in bacterial suspension

	Number of surviving cells ($\times 10^9$ cells/mL)				
	TA100	TA1535	WP2 _{uvrA}	TA98	TA1537
Dose-finding test	3.1	4.2	5.2	3.6	2.6
Mutagenicity test	3.1	4.2	5.2	3.6	2.6

Attachment 3. Preparation of S9

Animal used		Inducing substance	
Species, strain	Rat, Sprague-Dawley rat(CRJ)	Name	Phenobarbital (PB)
Sex	Male		5,6-Benzoflavone (BF)
Age	7 weeks	Administration method	i.p.
Body weight	210.5 ± 8.8 g* (n=63)	Day of administration and dose	Day 1 PB 30 mg/kg
			Days 2,3 and 4 PB 60 mg/kg
			Day 3 BF 80 mg/kg

*: Mean ± S.D.

Attachment 4. Composition of S9 mix

Constituents	Quantity in 1 mL S9 mix	Constituents	Quantity in 1 mL S9 mix
S9	0.1 mL	NADPH	4 μ mol
MgCl ₂	8 μ mol	NADH	4 μ mol
KCl	33 μ mol	Na-phosphate buffer (pH 7.4)	100 μ mol
Glucose-6-phosphate	5 μ mol	Distilled water	0.9 mL

Attachment 5. Composition of minimum glucose agar plate medium

Amount in 1000 mL minimum glucose agar plate		
A	MgSO ₄ • 7H ₂ O	0.2 g
	Citric acid • H ₂ O	2 g
	K ₂ HPO ₄	10 g
	NaNH ₄ HPO ₄ • 4H ₂ O	1.92 g
	NaOH	0.66 g
	Distilled water	200 mL
B	Glucose	20 g
	Distilled water	100 mL
C	Agar	15 g
	Distilled water	700 mL

Each medium A, B, C was autoclaved, refrigerated at 60°C and mixed with each other.

The mixed medium in a volume of 30 mL was dispensed into a petri plate sterilized by gamma rays and then solidified.

Attachment 6. Test conditions

Method		Pre-incubation method
Test condition		
Test substance solution		0.1 mL
0.1mol/L Na-phosphate buffer (pH 7.4) (in case of non-metabolic activation method)		0.5 mL
S9 mix (in case of metabolic activation method)		0.5 mL
Bacteria suspension		0.1 mL
Pre-incubation	Temperature	37°C
	Time	20 minutes
Top agar solution		2 mL
Incubation	Temperature	37°C
	Time	48 hours

Attachment 7. Background data of reverse mutation test with bacteria

Supplier of bacteria: Japan Bioassay Laboratory, Japan Industrial Safety and Health Association.

Date of procurement of bacteria: 1995. 2. 25 (TA1535, TA1537, WP2*uvrA*) and 1996. 10. 18 (TA98, TA100).

Period of accumulation of data: 2003.1 ~ 2004. 12.

Culture media: Minimum glucose agar plate medium.

Bacteria strains		Revertant colonies/plate				
		TA100	TA1535	WP2 <i>uvrA</i>	TA98	TA1537
S9 mix (-)	Negative control	73 ~ 176 (n=167)	3 ~ 23 (n=159)	16 ~ 58 (n=157)	10 ~ 32 (n=167)	3 ~ 20 (n=157)
		114 ± 40.9 (73 ~ 155)	10 ± 7.6 (2 ~ 17)	33 ± 17.7 (15 ~ 51)	18 ± 9.8 (8 ~ 28)	9 ± 7.2 (2 ~ 16)
	Positive control	AF-2 0.01 µg/plate	NaN ₃ 0.5 µg/plate	AF-2 0.01 µg/plate	AF-2 0.1 µg/plate	9AA 80 µg/plate
		320 ~ 618 (n=165)	421 ~ 660 (n=159)	103 ~ 200 (n=155)	357 ~ 586 (n=167)	167 ~ 665 (n=157)
		514 ± 106.6 (407 ~ 620)	539 ± 96.1 (443 ~ 636)	148 ± 42.1 (106 ~ 190)	441 ± 95.4 (346 ~ 537)	409 ± 227.9 (181 ~ 637)
S9 mix (+)	Negative control	68 ~ 193 (n=161)	3 ~ 24 (n=155)	17 ~ 61 (n=151)	12 ~ 46 (n=159)	5 ~ 30 (n=153)
		121 ± 44.5 (76 ~ 165)	10 ± 8.0 (2 ~ 18)	36 ± 18.4 (18 ~ 55)	27 ± 12.1 (15 ~ 39)	15 ± 9.8 (5 ~ 25)
	Positive control	2AA 1 µg/plate	2AA 2 µg/plate	2AA 10 µg/plate	2AA 0.5 µg/plate	2AA 2 µg/plate
		583 ~ 2372 (n=161)	231 ~ 473 (n=155)	720 ~ 1200 (n=151)	265 ~ 859 (n=159)	93 ~ 372 (n=153)
		987 ± 485.7 (501 ~ 1473)	338 ± 106.5 (232 ~ 444)	920 ± 210.5 (710 ~ 1131)	420 ± 171.6 (249 ~ 592)	172 ± 105.3 (66 ~ 277)

Upper row: Minimum-maximum (n=number of plates).

Lower row: Mean±2×S.D. [calculated by Microsoft (R) Excel 97].

Hashima Laboratory, Nihon Bioresearch Inc.

Table 1-1. Reverse mutation test of *Bacillus subtilis* DB9011 with bacteria(dose-finding test)

With (+) or without (-) S9 mix	Test substance concentration ($\mu\text{g}/\text{plate}$)	Number of revertants (number of colonies/plate)									
		Base-pair substitution type						Frameshift type			
		TA100		TA1535		WP2 <i>uvrA</i>		TA98		TA1537	
S9 mix (-)	Negative control	101	110	14	20	38	56	18	19	8	10
		(106)		(17)		(47)		(19)		(9)	
	0.305	97	112	15	22	55	59	19	20	8	9
		(105)		(19)		(57)		(20)		(9)	
	1.22	109	112	14	26	53	55	13	20	9	13
		(111)		(20)		(54)		(17)		(11)	
	4.88	110	129	20	25	50	55	22	25	7	14
		(120)		(23)		(53)		(24)		(11)	
	19.5	89	99	24	27	46	56	20	27	8	13
	(94)		(26)		(51)		(24)		(11)		
78.1#	91	97	23	26	50	62	21	21	8*	11*	
	(94)		(25)		(56)		(21)		(10)		
312.5#	114*	117*	18*	21*	47*	56*	21*	27*	4*	14*	
	(116)		(20)		(52)		(24)		(9)		
1250#	85*	92*	17*	18*	46*	48*	19*	23*	5*	10*	
	(89)		(18)		(47)		(21)		(8)		
5000#	78*	83*	12*	17*	36*	39*	24*	26*	5*	8*	
	(81)		(15)		(38)		(25)		(7)		
Positive control not requiring S9 mix	Name	AF-2		NaN ₃		AF-2		AF-2		9AA	
	Concentration ($\mu\text{g}/\text{plate}$)	0.01		0.5		0.01		0.1		80	
	Number of colonies/plate	495	560	520	603	162	167	403	425	541	619
	(528)		(562)		(165)		(414)		(580)		

Negative control : Dimethylsulfoxide.

AF-2 : 2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide; NaN₃ : sodium azide;

9AA : 9-aminoacridine hydrochloride.

() : Mean.

* : Bacterial growth inhibition was observed.

: Brown fine precipitations were observed on the surface of agar plate.

Table 1-2. Reverse mutation test of *Bacillus subtilis* DB9011 with bacteria(dose-finding test)

With (+) or without (-) S9 mix	Test substance concentration ($\mu\text{g}/\text{plate}$)	Number of revertants (number of colonies/plate)									
		Base-pair substitution type						Frameshift type			
		TA100		TA1535		WP2uvrA		TA98		TA1537	
S9 mix (+)	Negative control	106	109	14	22	45	61	30	32	17	18
		(108)		(18)		(53)		(31)		(18)	
	0.305	100	108	19	26	52	53	22	37	10	21
		(104)		(23)		(53)		(30)		(16)	
	1.22	112	114	18	20	42	45	43	45	13	17
		(113)		(19)		(44)		(44)		(15)	
	4.88	106	114	17	19	54	57	29	34	15	20
		(110)		(18)		(56)		(32)		(18)	
	19.5	118	126	19	21	53	63	32	36	18	26
	(122)		(20)		(58)		(34)		(22)		
78.1#	107	115	14	21	52	58	34	36	21*	22*	
	(111)		(18)		(55)		(35)		(22)		
312.5#	120*	149*	23*	24*	46*	63*	29*	40*	15*	20*	
	(135)		(24)		(55)		(35)		(18)		
1250#	137*	143*	26*	26*	55*	63*	28*	30*	13*	22*	
	(140)		(26)		(59)		(29)		(18)		
5000#	101*	120*	10*	18*	34*	43*	21*	24*	10*	14*	
	(111)		(14)		(39)		(23)		(12)		
Positive control requiring S9 mix	Name	2AA									
	Concentration ($\mu\text{g}/\text{plate}$)	1		2		10		0.5		2	
	Number of colonies/plate	814	821	346	353	771	917	348	420	125	129
	(818)		(350)		(844)		(384)		(127)		

Negative control : Dimethylsulfoxide.

2AA: 2-Aminoanthracene.

() : Mean.

* : Bacterial growth inhibition was observed.

: Brown fine precipitations were observed on the surface of agar plate.

Table 2-1. Reverse mutation test of *Bacillus subtilis* DB9011 with bacteria (mutagenicity test)

With (+) or without (-) S9 mix	Test substance concentration ($\mu\text{g}/\text{plate}$)	Number of revertants (number of colonies/plate)				
		Base-pair substitution type			Frameshift type	
		TA100	TA1535	WP2uvrA	TA98	TA1537
S9 mix (-)	Negative control	119 131 (125)	8 13 (11)	22 23 (23)	12 24 (18)	6 9 (8)
	2.44	/	/	/	/	7 11 (9)
	4.88	/	/	/	/	8 13 (11)
	9.77	124 125 (125)	10 12 (11)	16 31 (24)	12 13 (13)	5 9 (7)
	19.5	131 138 (135)	5 11 (8)	14 25 (20)	13 13 (13)	7 11 (9)
	39.1#	123 140 (132)	8 9 (9)	25 40 (33)	16 20 (18)	6* 7* (7)
	78.1#	124 145 (135)	15 15 (15)	32 33 (33)	12 18 (15)	6* 14* (10)
	156.3#	112* 123* (118)	11* 12* (12)	27 35 (31)	11* 16* (14)	/
	312.5#	141* 143* (142)	9* 10* (10)	21* 30* (26)	16* 16* (16)	/
Positive control not requiring S9 mix	Name	AF-2	NaN ₃	AF-2	AF-2	9AA
	Concentration ($\mu\text{g}/\text{plate}$)	0.01	0.5	0.01	0.1	80
	Number of colonies/plate	570 586 (578)	594 625 (610)	159 170 (165)	464 507 (486)	448 640 (544)

Negative control : Dimethylsulfoxide.

AF-2 : 2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide; NaN₃ : sodium azide;

9AA : 9-aminoacridine hydrochloride.

() : Mean.

* : Bacterial growth inhibition was observed.

: Brown fine precipitations were observed on the surface of agar plate.

Table 2-2. Reverse mutation test of *Bacillus subtilis* DB9011 with bacteria (mutagenicity test)

With (+) or without (-) S9 mix	Test substance concentration ($\mu\text{g}/\text{plate}$)	Number of revertants (number of colonies/plate)									
		Base-pair substitution type						Frameshift type			
		TA100		TA1535		WP2uvrA		TA98		TA1537	
S9 mix (+)	Negative control	126	127	7	9	22	40	20	26	15	16
	2.44	/		/		/		/		13	13
	4.88	/		/		/		/		11	14
	9.77	120	132	8	10	18	32	20	30	12	15
	19.5	119	165	9	10	18	24	27	42	14	15
	39.1#	142	152	5	8	25	31	28	41	11	13
	78.1#	127	146	6	12	23	31	33	35	15*	25*
	156.3#	143	151	11	13	30	30	28	35	/	
	312.5#	132*	159*	4*	12*	25*	28*	33*	34*	/	
Positive control requiring S9 mix	Name	2AA									
	Concentration ($\mu\text{g}/\text{plate}$)	1		2		10		0.5		2	
	Number of colonies/plate	867	898	295	304	827	961	353	397	156	166

Negative control : Dimethylsulfoxide.

2AA: 2-Aminoanthracene.

() : Mean.

* : Bacterial growth inhibition was observed.

: Brown fine precipitations were observed on the surface of agar plate.

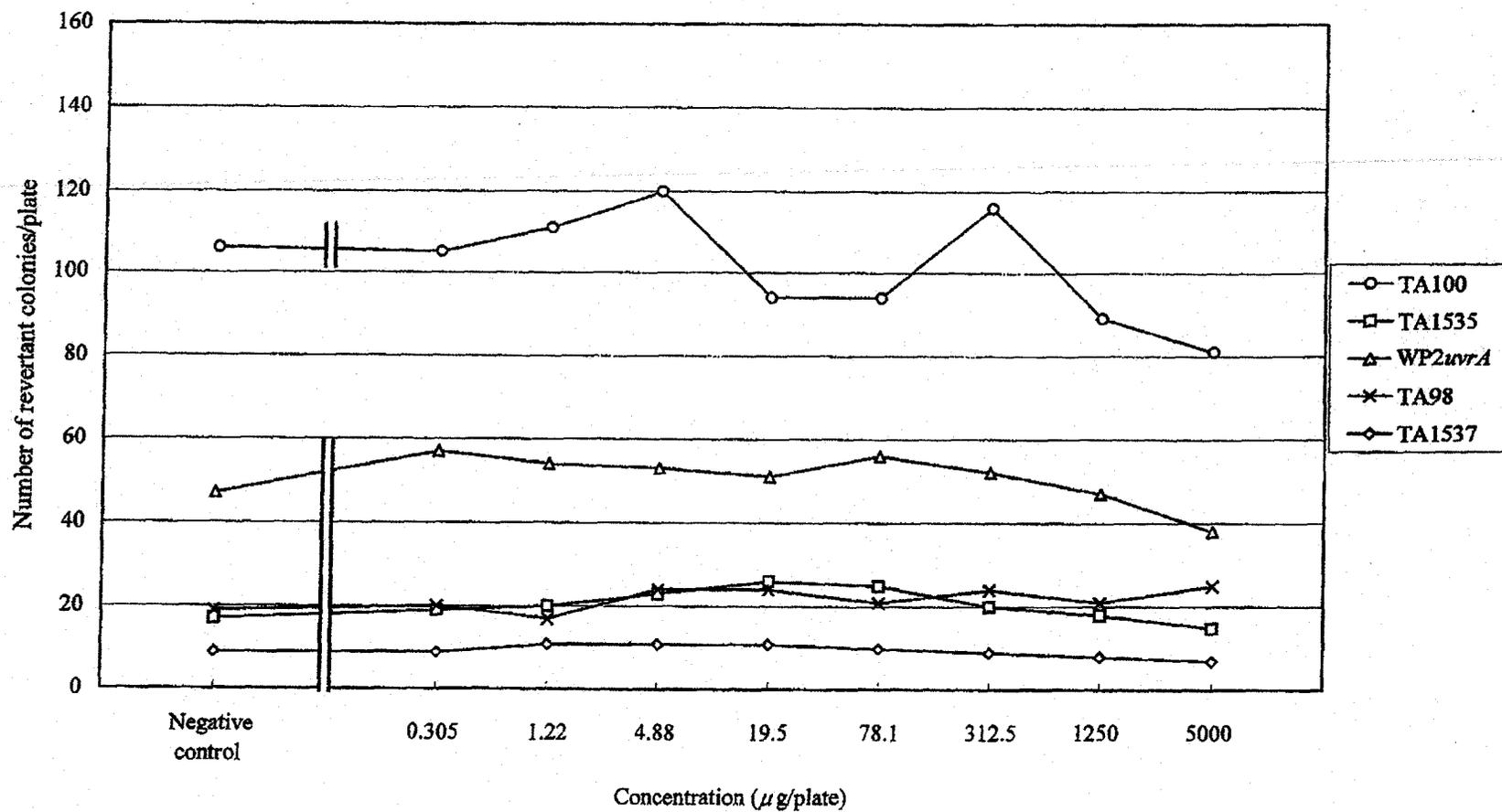


Figure 1-1. Reverse mutation test of *Bacillus subtilis* DB9011 with bacteria (dose-finding test: without S9 mix).

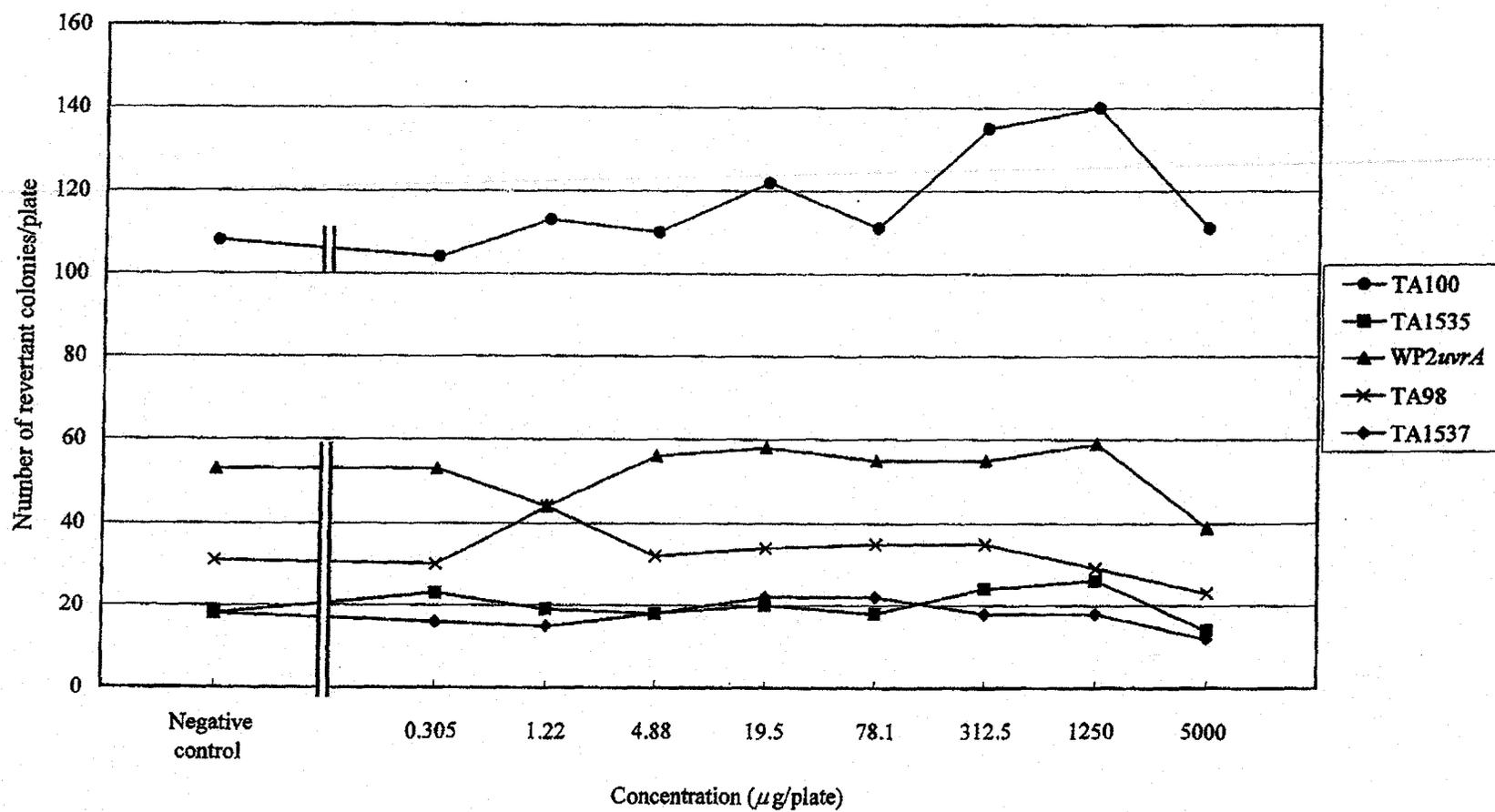


Figure 1-2. Reverse mutation test of *Bacillus subtilis* DB9011 with bacteria (dose-finding test: with S9 mix).

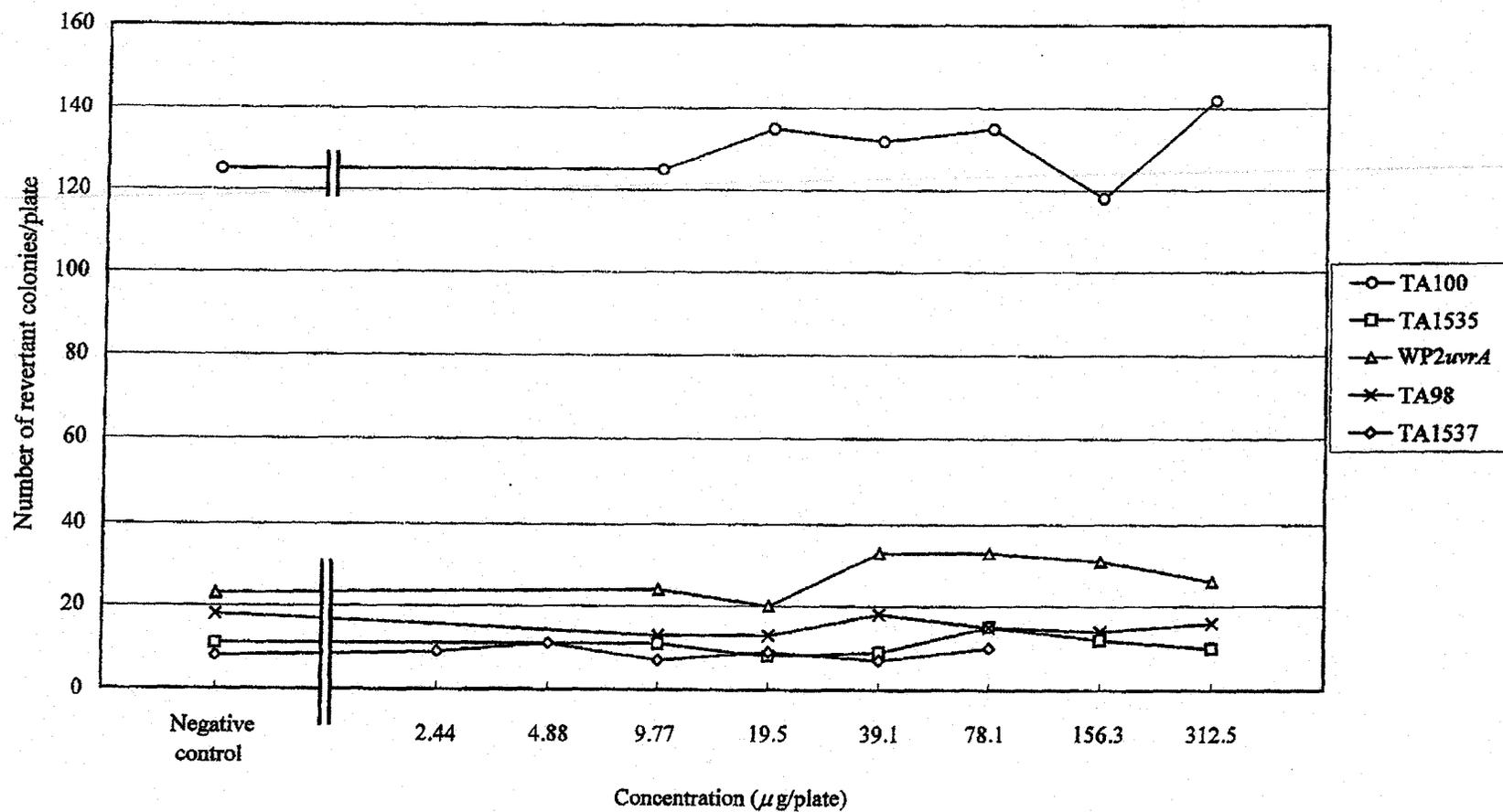


Figure 2-1. Reverse mutation test of Bacillus subtilis DB9011 with bacteria (mutagenicity test: without S9 mix).

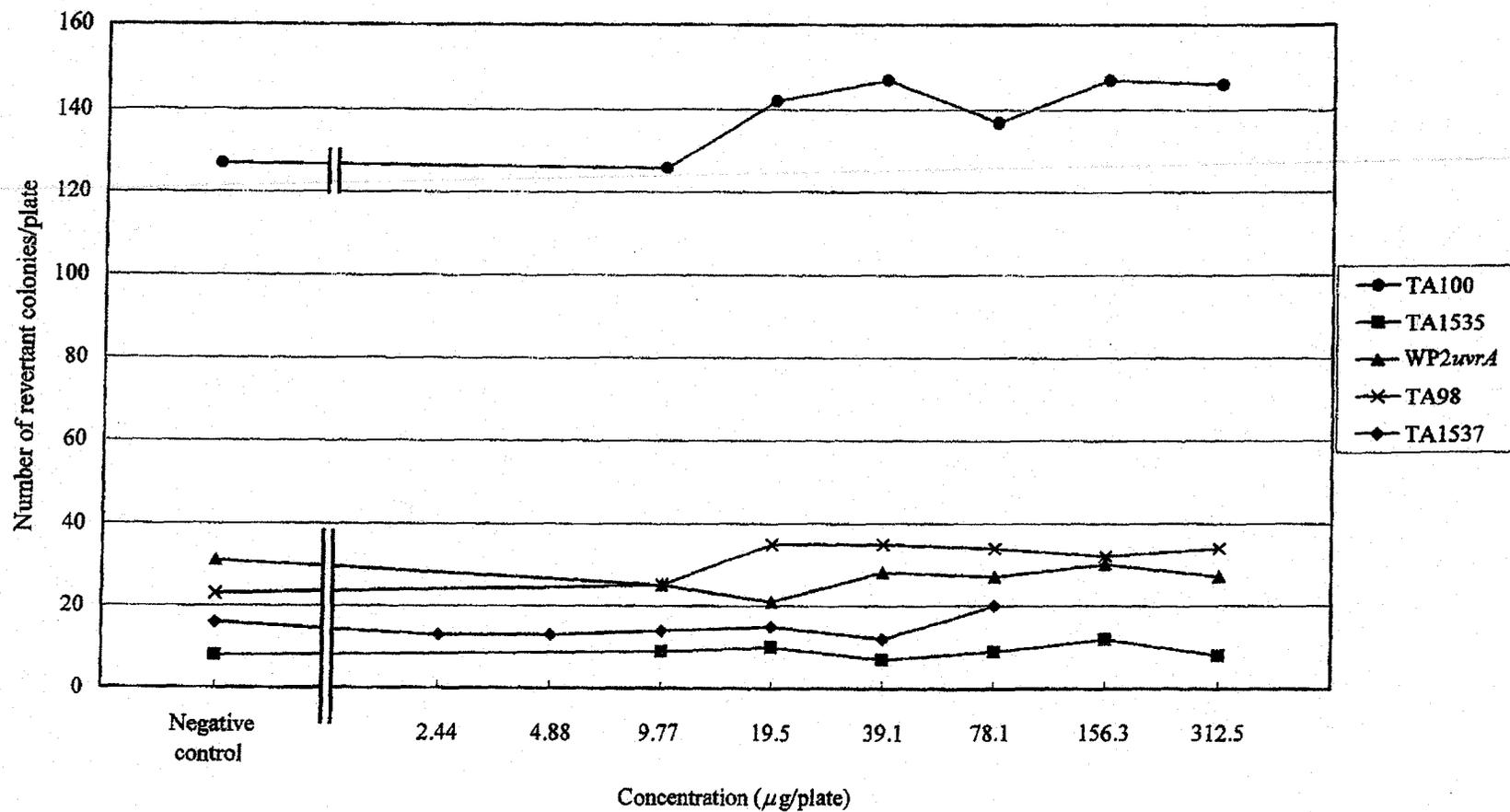


Figure 2-2. Reverse mutation test of Bacillus subtilis DB9011 with bacteria (mutagenicity test: with S9 mix).