Ames assay cytotoxic assessment using bacterial lawn integrity with 35 mm plate spread technique

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ENTHALPY ANALYTICAL

BACKGROUND

The Ames assay is commonly used to evaluate the mutagenic potential of tobacco products for regulatory purposes. A significant increase in the number of His+ revertant colonies observed in agar containing Salmonella bacteria and a test chemical indicates mutagenicity (Ames, 1975). To assess cytotoxicity in this assay, thinning of the background bacterial lawn is microscopically determined. With the development of new *in vitro* exposure systems that permit continuous cellular exposure to smoke or aerosol, the Ames assay method required modification by spreading the bacterial solution on top of agar in a 35 mm plate. However, studies detailing the use of bacterial lawn thinning as an indicator of cytotoxicity are lacking. The Salmonella tester strains used in this study are TA98, TA100, TA102, TA1535, TA1537 and TA97a.

OBJECTIVES

1. To determine if microscopic analysis can detect thinning of the bacterial background lawn on top of the agar (spread technique) in 6 different TA Salmonella strains (+/- S9) fractions.
2. To develop a technique to visualize bacterial lawn thinning in 6 different bacterial strains by limiting the concentration of histidine/biotin.

METHODS

Ames assay was performed using a 35 mm agar plate spread technique (Thorne et al., 2015). The dosing solution contained ~ 1-2 x 10^7 bacteria, 40 µg/ml histidine, 48.8 µg/ml of biotin, presence and absence of S9 and treatment. The assays were performed with six tester strains (obtained from Moltox, Inc.). The treatment included spontaneous revertants (not treated), vehicle control (1% DMSO), 30 µg of TPM (3R4F) and relevant amount of positive control chemicals (1% v/v) in the absence and presence of S9. The relevant positive control chemicals are listed in figure 4 legend. The effect of 5% and 10% S9 on bacterial lawn integrity was also studied. To study bacterial background lawn thinning, varying amounts of His-Bio solutions were used in the assay. The mean number of revertants and bacterial background lawn thinning were determined after 48 hours of incubation at 37 °C.

Mutagenic Response:- more than 2-fold significant increase in mean number of revertants in treatment group as compared to vehicle control.

Cytotoxic Response:- is determined by significant decrease in mean number of revertants and/or observing thinning or absence of bacterial lawn using Olympus inverted light microscope. Scoring for background lawn integrity- Score 1- Normal and healthy background lawn; Score 2- Thinning in background lawn (cytotoxic); Score 3- A complete absence of background lawn (cytotoxic).

RESULTS

1. Bacterial background lawn thinning with varying amounts of His-Bio (-S9 fraction)

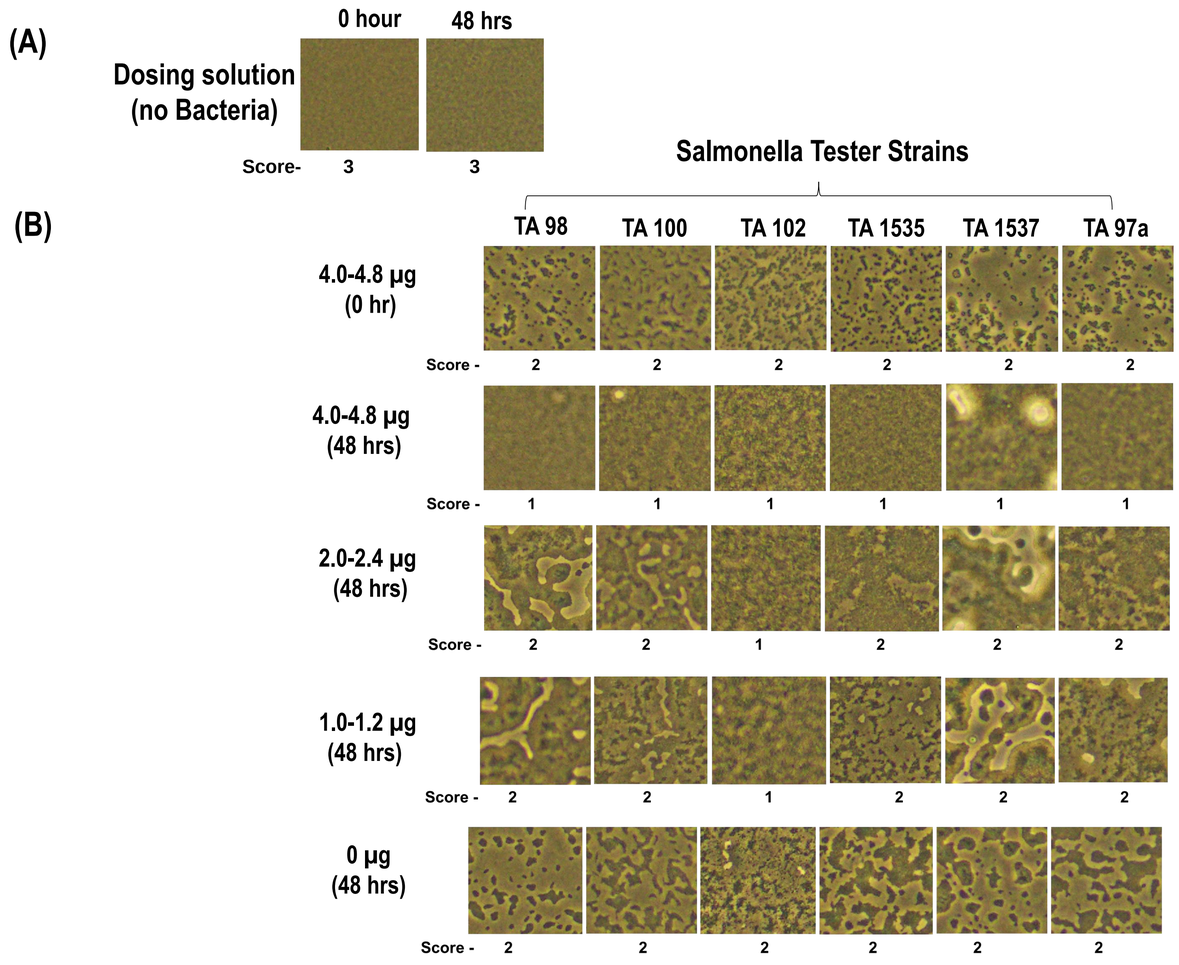

Figure 1: Effect of [His-Bio] on bacterial lawn thinning. (A) Photomicrographs of 35 mm VBX plate without bacteria at 0 hr and 48 hrs time points. (B) Ames strains were grown in the presence of varying ug concentrations of his-bio (in the absence of S9 fractions). Photomicrographs are bacterial lawns from spontaneous revertant plates captured with light microscope (10 X). Bacterial lawn integrity Scores are provided underneath each for 0 hr and 48 hrs time points. Score- 1- Normal lawn; 2- Thin lawn; 3- Absence of lawn

2. Bacterial background lawn thinning in the presence of 3R4F TPM (-S9 fraction)

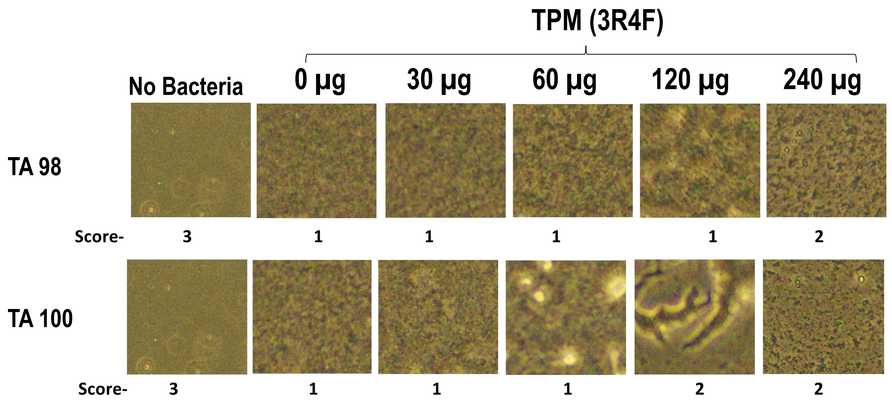

Figure 2: TPM 3R4F- induced background lawn cytotoxicity assessment after 48 hrs incubation. Ames assay was performed in the presence of increasing doses of TPM (in the absence of S9) using TA98 and TA100 strains. Photographs of bacterial lawns are provided with the lawn integrity score. Scoring: 1- Normal lawn, 2- Thin lawn, 3- Absence of lawn.

3. Bacterial background lawn in the presence of 5% S9 and 10% S9

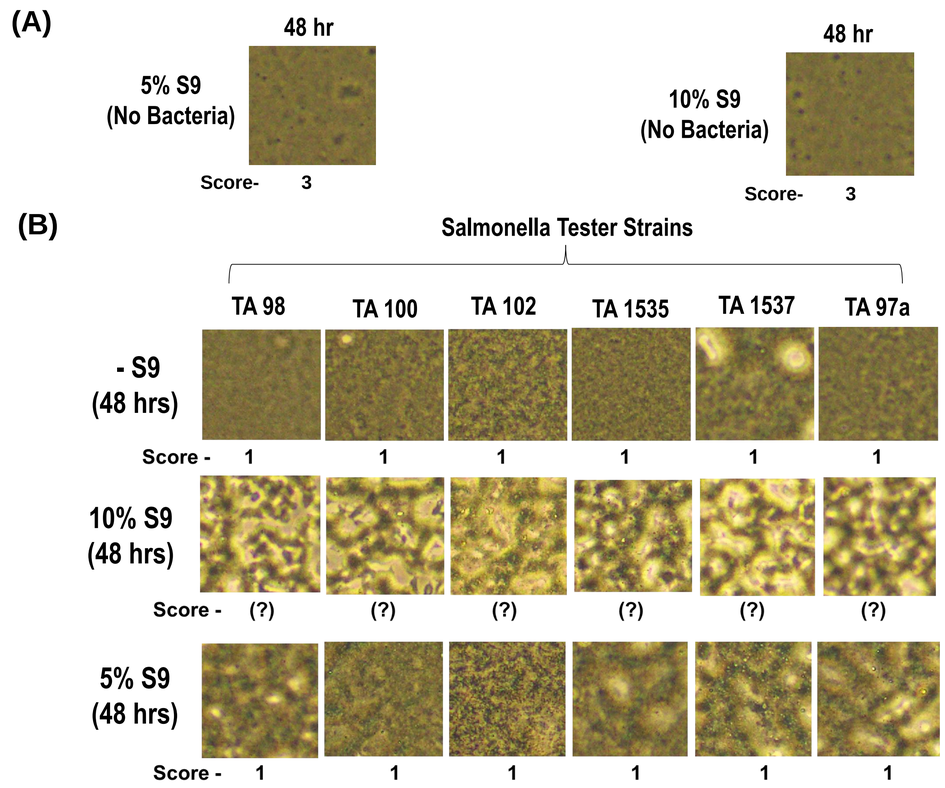

Figure 3: Effect of % S9 (5 % *vs* 10 % S9) on bacterial lawn thinning. (A) Background of 35 mm VBX plate spreading with dosing solution containing 5% S9 and 10% S9 fractions without bacteria at 48 hrs incubation. (B) Bacterial background lawn from spontaneous revertants plates in the presence of no S9 (-S9), 5% S9 and 10% S9. Photomicrographs of bacterial lawns captured by inverted light microscope (10 X) 48 hr time points. Scoring: 1- Normal lawn, 3- Absence of lawn, (?)- difficult to assess.

4. Effect of % S9 fraction on number of revertants in presence of positive and negative controls

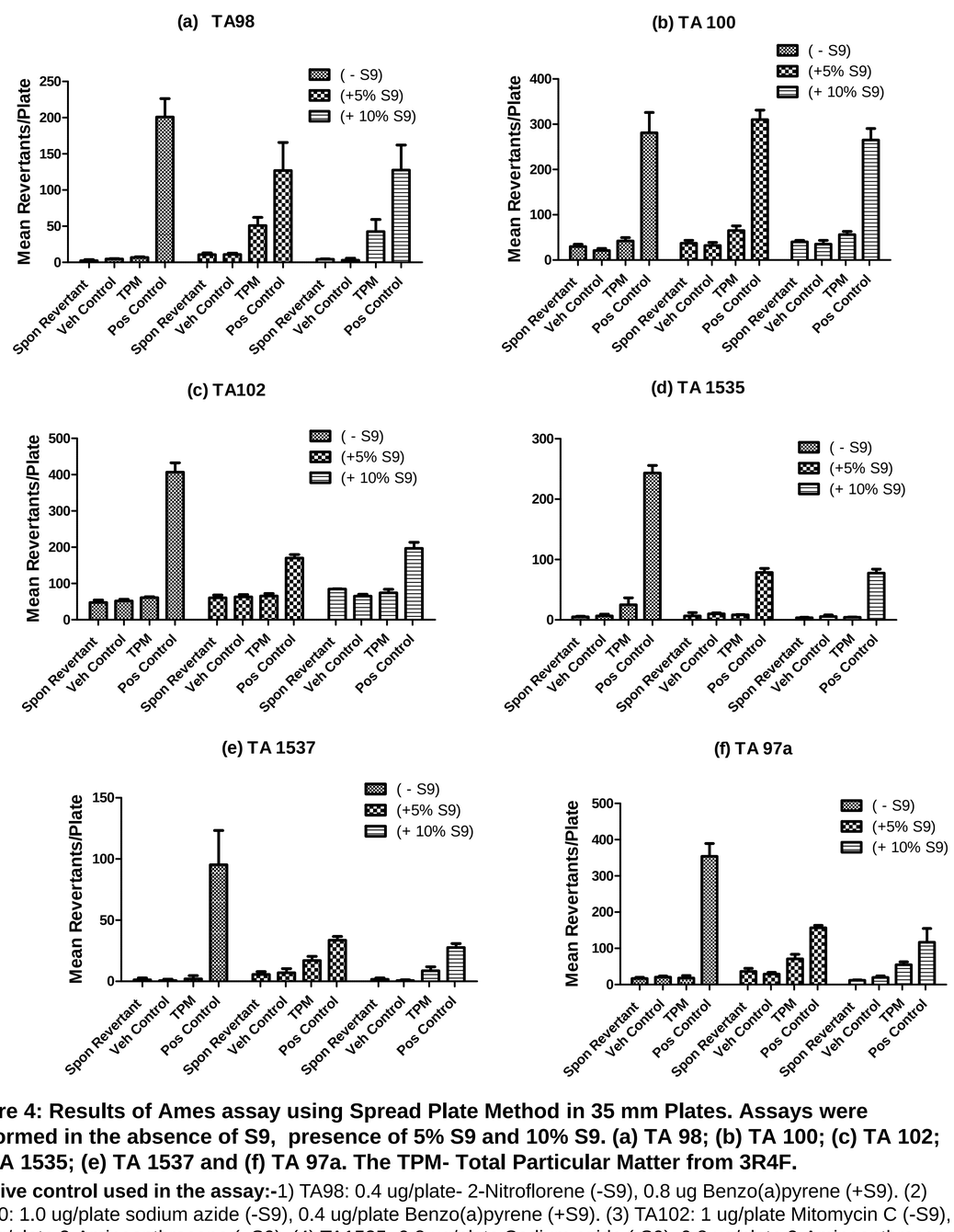

Figure 4: Results of Ames assay using Spread Plate Method in 35 mm Plates. Assays were performed in the absence of S9, presence of 5% S9 and 10% S9. (a) TA 98; (b) TA 100; (c) TA 102; (d) TA 1535; (e) TA 1537 and (f) TA 97a. The TPM- Total Particular Matter from 3R4F.

Positive control used in the assay:-1) TA98: 0.4 ug/plate- 2-Nitroflorene (-S9), 0.8 ug Benzo(a)pyrene (+S9). (2) TA100: 1.0 ug/plate sodium azide (-S9), 0.4 ug/plate Benzo(a)pyrene (+S9). (3) TA102: 1 ug/plate Mitomycin C (-S9), 3.2 ug/plate 2-Aminoanthracene (+S9). (4) TA1535- 0.8 ug/plate Sodium azide (-S9), 0.8 ug/plate 2-Aminoanthracene (+S9). TA97a and TA1537- 0.3 ug/plate ICR-191 (-S9), 0.4 ug/plate Benzo(a)pyrene (+S9).

CONCLUSION

We developed a method to investigate bacterial background lawn thinning (cytotoxicity) by depleting his-bio concentration (-S9 fractions). Thinning of the background bacterial lawn was clearly observed in all strains (TA98, TA100, TA102, TA1535, TA1537 and TA97a) using the Ames assay with spread technique in the absence S9 fractions. However, the presence of 10% S9 compromised the lawn assessment but was improved by reducing the S9 to 5%. Using 5% S9, the positive and negative control values for revert colonies were within historical control ranges (similar to 10% S9) for all strains. Using TPM as an example of treatment known to induce cytotoxicity, we showed that a high dose of TPM exhibited cytotoxicity in TA98 and TA100 using spread technique. Additionally, we compared TA97a and TA1537 and observed that TA97a responded very similar to TA1537 in the presence of positive control chemicals.

REFERENCES

1. Ames B.N., McCann, J., and Yamasaki, E. (1975). Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. Mutation research, 31:347-364.

2. Thorne, D., Kilford, J., Hollings, M., Dalrymple, A., Ballantyne, M., Meredith, C., & Dillon, D. (2015). The mutagenic assessment of mainstream cigarette smoke using the Ames assay: A multi-strain approach. Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 782, 9-17.