

Mutagenicity of Pure and Altered Tobacco in *Salmonella typhimurium* Strain TA100

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ABSTRACT

The mutagenicity of homegrown tobacco and consumer brand cigarette tobacco extracts were tested and compared using the Ames Test. Both of the tobacco extracts were tested in *Salmonella typhimurium* TA100, and the mutagenicity was determined by the number of revertant colonies that were able to grow in the presence of the potential mutagen. Because the store brand tobacco contains added chemicals, it was predicted that this tobacco would cause more revertants than the homegrown tobacco. Because the homegrown tobacco does not have added chemicals, the mutagenicity of the store brand tobacco should also be higher. After a 48-hour incubation period, it was found that the store brand tobacco caused significantly more revertants than the homegrown tobacco when both were tested in the absence of the S9 rat liver enzymes. However, in the presence of S9 liver enzymes, there was not a significant difference in the mutagenicity of either type of tobacco. Also, within each tobacco type, the S9 did not appear to effect the number of revertant colonies found.

INTRODUCTION

As approximately 400,000 people in the United States die each year from tobacco related deaths, it is important to research the effects of tobacco (Puglia, 1998). In consumer brand cigarettes, the tobacco companies put many additives into their tobacco, including ammonia, a substance thought to speed the delivery of nicotine (Dutch, 1999). In addition, humectants, such as propylene glycol and glycerol are added to cigarette tobacco in order to retain moisture (Wynder et al., 1967). These humectants are precursors to acrolein, a compound that is toxic to cilia (Wynder et al., 1967). Also, tobacco companies use insecticides, herbicides, and fungicides on the tobacco, and add flavoring to the tobacco before it is rolled into a cigarette (Anti Cancer, 1990 and Wynder et al., 1967).

Ammonia emissions lead to the formation of ammonium nitrate and ammonium sulfate, two compounds that may contribute to health problems, such as premature mortality, chronic bronchitis, and asthma attacks (McCubbin et al., 2002). Moreover, ammonia has been found to be very toxic to the plant pathogen, *Verticillium dahliae* (Tenuta and Lazarovits, 2002). In addition, pesticides, when exposed to the mother during the first trimester, have been shown to have an effect on the developmental transposition of the great arteries in newborn children (Loffredo et al., 2001). Specifically, herbicides and rodenticidal chemicals were found to have the greatest effect on heart formation (Loffredo et al., 2001). Also, infant acute leukemia may be caused when the mixed-lineage leukemia oncogene (MLL) is broken and recombined with another unknown gene (Freda et al., 2001 and Armstrong et al., 2002). Possibly, this breakage occurs when the fetus is exposed to chemicals that have an effect on topoisomerase II (Freda et al., 2001). Mothers using cigarettes are exposed to pesticides, a topoisomerase II inhibitor, and these pesticides have been found to be linked to the fusion of the MLL gene causing infant acute leukemia (Freda et al., 2001). Acrolein, an unsaturated aldehyde, has been found to inhibit the activation of caspase-3 thereby leading to increased neutrophil recruitment and decreased neutrophil apoptosis (Finkelstein et al., 2001). When neutrophils are recruited and prevented from undergoing apoptosis, inflammatory diseases of the respiratory tract are enhanced (Finkelstein et al., 2001).

Although cigarette companies include many additives in their cigarettes, my grandfather's homegrown tobacco does not contain any additives, and pesticides are not used in the growing process (C. Berry, pers. Comm). It is possible that the lack of additives will have a profound effect on the ability of homegrown tobacco to cause genotoxic effects, such as the ability to cause mutations.

In determining mutagenicity of chemicals, the Ames Test has shown a variety of chemicals to be either mutagenic or anti-mutagenic, and has been shown to be over 90% accurate in predicting genotoxicity (Weisburger, 2001). In the Ames Test, *Salmonella typhimurium* strains that have a mutation in the *his*-operon are used to detect the mutagenicity of chemicals (Maron and Ames, 1983). In addition, the histidine requiring strains also contain mutations, such as the *rfa*, *uvr*, and *R-factor* that increase the capability of finding mutagens (Maron and Ames, 1983). Mutagenic chemicals cause the mutant strains to revert back to the wild-type; therefore, the number of growing revertant colonies is related to the mutagenic potency (Maron and Ames, 1983). For example, mainstream cigarette condensates were found to be no more

mutagenic than Kentucky Reference cigarette condensates (Steele et al., 1995). Moreover, removal of protein and peptides from flue-cured tobacco showed that mutagenicity decreases by 80% after removal, suggesting that protein pyrolysis products are a major contributor to the mutagenicity of smoke condensates (Clapp et al., 1999). Furthermore, smoker's urine has been tested for promutagens using the Ames Test, and it was found that cigarette consumption and tobacco type were the main factors in the degree of mutagenicity (Kuenemann et al., 1996). Black tobacco (cigar tobacco) had a higher genotoxicity than blond tobacco (cigarette tobacco) and the higher the daily intake of smoke, the higher the mutagenicity (Kuenemann et al., 1997). Also, the N-nitrosamines in tobacco were shown to be mutagenic using the *Salmonella typhimurium* assay (Fujita and Kamataki, 2001). In addition, four alkylacroleins were found to be mutagenic in the TA100 mutated strain (Eder and Deininger, 2001).

Many of the chemical additives in store brand cigarettes have been shown to be toxic and/or cause defects and health problems, and previous research has found some of those compounds to be mutagenic using the Ames Test. Because of these factors, it seems that store brand cigarette extracts should cause more of the mutant *Salmonella typhimurium* TA100 strain to revert back to the wild-type phenotype than my grandfather's tobacco. The mutagenic potency of the store brand cigarette extracts should be higher than that of my grandfather's homegrown tobacco. Without the addition of S9 liver enzymes, the store brand tobacco was significantly more mutagenic than my grandfather's tobacco in *Salmonella typhimurium* TA100. However, my grandfather's tobacco and the store brand tobacco did not show significant difference in mutagenicity in the presence of the S9 liver enzyme mix.

MATERIALS AND METHODS

To prepare the tobacco extracts, the tobacco was removed from 20 store brand cigarettes. It was then weighed and ground to a fine powder. Equal amounts of both types of tobacco (12.75g) were dissolved in 300mL of distilled water, and each was heated on a plate at a power level of 9.5 and a stirring position of 9.5. After 20 minutes, my grandfather's tobacco was dissolved, and after 37 minutes, the store brand tobacco was in liquid form. Both tobacco types were filtered three times using gravity filtration with fluted filter paper. The tobacco extracts were put into covered beakers and stored at 4 degrees C for future use.

To determine when the bacteria had grown to a density of $1-2 \times 10^9$ cells/mL, 5mL of Oxoid Nutrient Broth No. 2 was combined with one disc of *Salmonella typhimurium* TA100 and incubated (gyrotory) at 37 degrees C for 12hrs. To obtain the bacteria, forceps were covered with ethanol and heated. Then, another 7mL of Oxoid broth was added to the bacteria solution. Using a Petroff-Hausser counting chamber, four squares with bacteria were counted and averaged, and the bacteria/cm³ was calculated using the following equation: (bacteria/square) (25squares) (1/chamber depth) (10^3).

Using the information above, bacteria were grown to a density of $1-2 \times 10^9$ cells/mL, and the mutagenicity test was used to determine the number of spontaneous revertants and positive control revertants. Spontaneous revertants are determined without the addition of test chemical and are used as a baseline to compare with the test chemicals. Sodium azide is used for a positive control to confirm the reversion properties and specificity of the bacteria (Maron and Ames, 1983). In the spontaneous revertant assay, 2mL of L-Histidine/D-Biotin top agar was heated in the microwave until liquified and added to a minimal glucose agar plate. 0.1 mL of the fresh overnight bacteria culture and 20uL of S9 liver cell extracts were mixed and added to the plate. According to the Ames Test instructions, 0.1mL of the test chemical and bacteria as well as 20uL of the enzyme are recommended amounts (Maron and Ames, 1983). Another plate was made with only 0.1mL of the bacteria. In the positive control assay, 2mL of the heated top agar, 0.1mL of sodium azide (1mL water added to 15ug sodium azide), and 0.1mL of the bacteria were mixed and added to a minimal glucose agar plate. Another plate was made with the same ingredients, but 20uL of S9 liver cell extracts were mixed and added as well. All plates were incubated at 37C for 48hr, and the number of bacteria colonies was determined. S9 liver cell extracts contain enzymes that may activate the potential mutagen.

To determine the mutagenicity of the test chemicals, bacteria cultures were grown to a density of $1-2 \times 10^9$ cells/mL. 2mL of heated top agar, 0.1mL of store brand tobacco extracts, 0.1mL of bacteria, and 20uL of S9 were mixed and added to three different minimal glucose agar plates. In addition three plates were made without S9. Also, 2mL of heated top agar, 0.1 mL of my grandfather's tobacco, 0.1mL of bacteria, and 20uL of S9 were mixed and poured into three separate minimal glucose agar plates. Again, three more plates were assembled with my grandfather's tobacco, but without the addition of S9. All of these plates were incubated at 37C for 48hr, and the number of bacteria colonies was found. The entire

experiment was replicated again on a different day for a total of six plates with store brand tobacco extracts + S9, six plates with only store brand extracts, six plates with my grandfather's tobacco + S9, and six plates with only my grandfather's tobacco. A two-factor analysis of variance (ANOVA) and four independent sample t-tests were used to determine the effect of tobacco type and enzyme presence ($p = 0.05/4$).

RESULTS

In determining the density of the bacteria cells, a spectrophotometer was blanked with 1mL of Oxoid broth, and the absorbance of 1mL of the *S. typhimurium* solution was determined at OD 600 at $t=0$ as 0.012. At the same time, the bacteria cells were counted with a Petroff-Hauser counting chamber, and found to be at a density of 4.72×10^8 cells/mL [(37.75)(25)(50)(10³)]. Then, the sample was incubated at 37 degrees C for 30min, and the absorbance was 0.03 at OD 600. Using the counting chamber, the density was 5.8×10^8 cells/mL [(46.75)(25)(50)(10³)]. The bacteria was incubated again for 30min (total 60min), and the absorbance was 0.08 while the density was 7.8×10^8 cells/mL [(62.75)(25)(50)(10³)]. After incubating for 30min (total 90min), the bacteria had an absorbance of 0.174, and the density was 1.54×10^9 cells/mL [(123.5)(25)(50)(10³)]. Again, the cells were incubated for 30min (total 120min), and the bacterial absorbance was 0.350, and the density was at 2.4×10^9 cells/mL [(173.5)(25)(50)(10³)]. Therefore, the cells needed to be incubated with 12mL of nutrient broth between 90 and 120min with an absorbance between 0.174 and 0.350 to grow to a density of $1-2 \times 10^9$ cells/mL as advised by the Ames Test (Figure 1; Maron and Ames, 1983).

Because tobacco type and enzyme presence showed a significant interaction (ANOVA with type and enzyme as univariates: $F = 6.388$, $p = 0.020$, mean square = 1666.667), four independent sample t-tests were used to evaluate differences in tobacco type and differences within tobacco types. Store bought (SB) tobacco, in the absence of the S9 activation enzymes, elicited a significantly higher mutagenic response compared with my grandfather's tobacco ($t = 3.754$, $df = 10$, $p = 0.004$; Figure 2; Table 1). Moreover, with the addition of S9 liver enzymes, my grandfather's tobacco and the SB tobacco did not show significant difference in mutagenicity ($t = 1.037$, $df = 10$, $p = 0.324$; Figure 2; Table 1), although the mean of the SB colonies was slightly higher than that of my grandfather's tobacco (mean = 41.5, mean = 33.8). Within the SB tobacco treatments, there was not a significant difference in mutagenicity with the addition or absence of the S9 mix although the mean of the store bought colonies without S9 was higher ($t = -2.935$, $df = 10$, $p = 0.015$; Figure 3; Table 2). Comparing between my grandfather's tobacco, no significant difference was found with or without S9 ($t = 0.614$, $df = 10$, $p = 0.553$; Figure 3; Table 2).

In addition, the number of spontaneous revertants was less than all of the different treatment averages for the first trial, as a control was not used in the second trial (Figure 4). Also, the number of sodium azide positive control colonies was much higher than the spontaneous revertants as well as all of the treatment averages in the first trial (Figure 4).

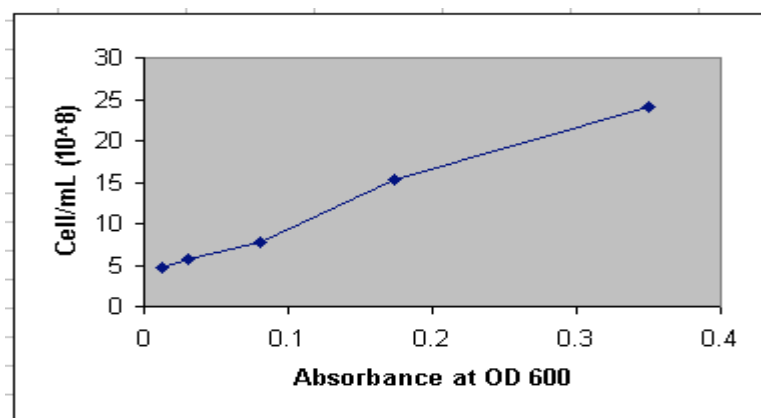


Figure 1. Absorbance of bacteria (*S. typhimurium* TA100) at OD 600 versus the cells/mL as determined by the Petroff-Hauser method. Each point on the graph represents a 30 minute incubation time increment starting at $t=0$. Between an absorbance of 0.174 and 0.350, the bacteria had grown to a density of $1-2 \times 10^9$ cells/mL. This corresponded to an incubation time between 90 and 120 minutes.

Table 1. Effect of my grandfather's tobacco and SB tobacco on the number of revertant colonies in *Salmonella typhimurium* TA100. Numbers are revertant colonies from each test plate.

	Gramp's Tobacco (-S9)	SB Tobacco (-S9)
	Number of Colonies	Number of Colonies
Trial One	18	41
	21	56
	15	67
Trial Two	46	80
	16	69
	53	102

Table 2. Effect of my grandfather's tobacco with S9 and SB tobacco with S9 on the number of revertant colonies in *S. typhimurium* TA100. Numbers are revertant colonies from each test plate.

	Gramp's Tobacco (+S9)	SB Tobacco (+S9)
	Number of Colonies	Number of Colonies
Trial One	26	36
	47	50
	44	24
Trial Two	28	44
	48	45
	10	50

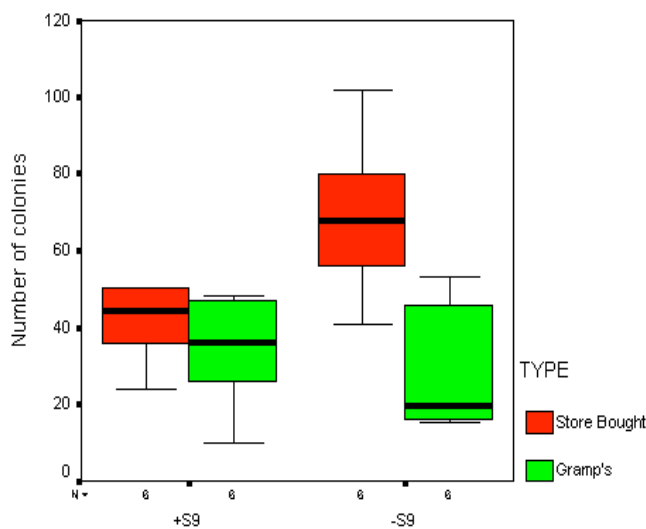


Figure 2. Box-plots of the number of revertant Colonies in *S. typhimurium* TA 100 for each of the treatments comparing my grandfather's tobacco to the SB tobacco with and without S9. The line inside the box represents the median. The box edges are at the standard deviation. The whiskers show the range of values for the data.

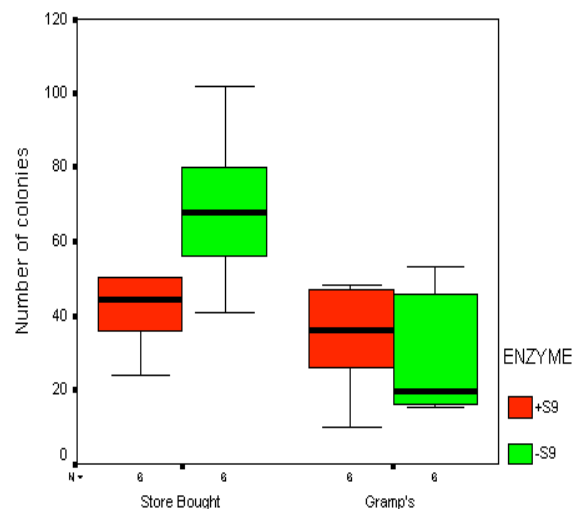


Figure 3. Box-plots of the number of revertant colonies in *S. typhimurium* TA100 for each of the treatments comparing the presence of S9 within each tobacco type. The line inside box represents the median. The box edges are at the standard deviation. The whiskers show the range of values for the data.

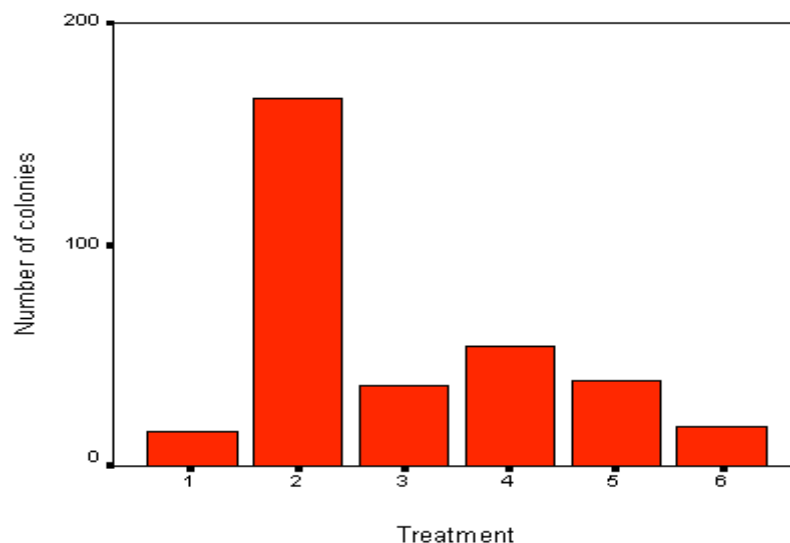


Figure 4. Comparison of the number of revertant colonies in *S. typhimurium* TA100 with each treatment for trial one (Table 1 and Table 2). Numbers are averages. Case number 1 shows the spontaneous revertants (16), case number 2 represents the sodium azide positive control (166), case number 3 is SB + S9 (36.7), case number 4 depicts the SB tobacco (54.7), case number 5 displays my grandfather's tobacco + S9 (39), and case number 6 shows my grandfather's tobacco (18).

DISCUSSION

This study suggests that homegrown tobacco may be significantly less mutagenic than consumer tobacco. A possible explanation for this observed difference is the lack of chemical additives in my grandfather's homegrown tobacco. Chemicals, such as ammonia, humectants (acrolein), insecticides, herbicides, fungicides, and flavoring may increase the mutagenicity of store bought tobacco. Specifically, the pesticides, captan and captafol were found to be mutagenic (with and without S9) in the Ames *Salmonella* test and the SOS chromotest (Ruiz and Marzin, 1997). These chemicals, found in the store bought tobacco, may influence the mutagenicity. Ammonia and acrolein have also been shown to cause adverse health effects; consequently, these chemicals may have had an impact on the higher mutagenicity in the store bought tobacco. In order to determine which of these chemicals has an effect on mutagenicity, each separate chemical should be tested with the Ames Test assay.

In addition, more spontaneous reversion control plates need to be examined, so that there is an adequate baseline to compare the degree of mutagenicity for each of the individual tobacco types. Control plates were only included in trial one, and only one plate was made to determine the spontaneous revertants and positive control (sodium azide). Therefore, when conducting the statistical analyses, these plates could not be included. Rather, the controls could only be used to show that the number of revertants (averages) from the potential mutagens were in between the number of spontaneous revertants and positive control revertants. In the second trial controls were not used, so there was no baseline to compare with the potential mutagens. Moreover, subsequent testing should be carried out to determine if there is a dose-dependent relationship in the mutagenicity of both tobacco types. In each experiment, 100uL of the test chemical was used, but it is suggested that different concentrations need to be tested, as there is usually a certain concentration range that produces a linear dose-response curve (Maron and Ames, 1983). The number of revertants per plate in this concentration range gives the most accurate measure of chemical mutagenicity (Maron and Ames, 1983). However, the tobacco extracts were already fully concentrated, and the mutagenic response was weak. Thus, testing different dilutions would be difficult, as the response would not be mutagenic.

Furthermore, the results using the S9 mix were not expected, as the liver enzymes are supposed to metabolically activate the mutagen chemicals. In the store bought tobacco test plates, the S9 appeared to decrease the mutagenicity, as the mean number of revertants was less than the treatments without S9.

Possibly, the S9 enzymes actually inhibit the activation of one of the chemicals in the store bought tobacco. Although this finding does not seem to occur, some antimutagenic chemicals, such as the plant extracts of *Phyllanthus obicularis*, have been shown to be more effective in the Ames Test without the addition of S9 (Ferrer et al., 2001). When captan and captafol were each tested with S9, they were mutagenic; however, it is possible that the interactions of these and other chemicals in the complex mixture of tobacco and S9 mix negates the mutagenicity. The S9 seems to inhibit the antimutagenic activity in these extracts (Ferrer et al., 2001). However, the results using my grandfather's tobacco suggest that the S9 mix had a weak activating effect, as the mean number of revertants was slightly higher with the addition of the S9 mix. Although this result was as expected, the overall effect of the S9 liver enzymes on the number of revertants in any of the test conditions was not significant. The S9 enzymes may not be strong activators of the mutagens in any tobacco type. Moreover, different amounts of S9 mix should be tested for effect on the revertants, as only one dose of 20uL was used for each treatment. It is recommended that 50uL per plate of S9 be used if the initial response is negative with 20uL (Maron and Ames, 1983). In addition, too much or too little of the S9 mix can drastically reduce the mutagenic response (Maron and Ames, 1983). Also, rodent kidney extracts have been shown to be more efficient activators of some chemicals, including the mushroom constituent, hydrazine; therefore, these extracts should be tested with the tobacco to develop more accurate results (Walton et al., 1997).

Moreover, the genotypes of the tester strains (TA100) should be tested more thoroughly, as contamination or absence of certain mutations in the strain may decrease the sensitivity of the bacteria to some mutagens (Maron and Ames, 1983). In *Salmonella* mutants, the *rfa* mutation allows larger molecules to pass through the cell wall thereby increasing its ability to detect mutants (Maron and Ames, 1983). In addition, the *uvrB* mutation allows for an increase in detection capability, as it deletes the gene that codes for the DNA excision repair system (Maron and Ames, 1983). Without these mutations, the bacteria would not efficiently or accurately detect mutagens. In subsequent experiments, it may be advisable to use a nitroreductase derivative of TA100 to test the mutagenicity of tobacco. These derivatives are more efficient in determining the mutagenicity of nitro-carcinogens (Maron and Ames, 1983).

Lastly, additional mutagenicity tests should be conducted to validate mutagenicity detection in the Ames Test. It has been suggested that certain compounds may increase the number of revertants in *Salmonella* tester strains via mechanisms that would correspond to multicellular eukaryotes (Gocke and Albertini, 1996). For example, the umu test may be used in conjunction with the Ames Test, as it indicates carcinogens as those chemicals that induce the expression of the umu operon (Reifferschied and Heil, 1996). Furthermore, an in vivo genotoxicity test should be conducted, such as the comet assay that can measure DNA damage of a single cell in any organ (Sasaki et al., 2000). These tests would help confirm the findings of the Ames Test and establish predictions for the chemical's effect in an organism. In addition, the mutagenicity of the smoke condensates from the homegrown tobacco and store bought tobacco should be tested.

ACKNOWLEDGMENTS

I would like to thank Dr. Andrew Ray for all of his help, patience, and time in completing this project. I would like to give my grandfather, Charles Berry, a special thanks for donating his tobacco. I would also like to thank Dr. Linda Swift, Dr. Mark Kuhlmann, and Rob Hunt for taking the time to help me as well.

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