The Ames mutagenicity testing – mutations in reverse gear

Luka Opašić, Marina Zajc, Charlotte Virapin, Erika Petelin and Anamarija Štafa

Introduction

In our everyday life we are surrounded by different chemicals and compounds that we eat and breathe or use to cure us from different diseases. The aim of our project was to determine mutagenic effects of different substances in everyday environment (in later text *potential mutagens*) because mutagenic properties of some of them were discovered years after they were brought in use**.** The Ames mutagenicity assay is simple bacterial assay that allows us to determine mutagenic potential (Maron and Ames, 1980) defined as the ability to cause DNA mutations that may lead to cancers. Due to mutation in one of *his* genes in *his* operon all Ames strains are histidine auxotrophs. Mutations in *his* genes can revert leading to the formation of histidine prototrophs.

Materials and methods

In this research four different strains of bacteria *Salmonella enterica subsp. enterica serovar Typhimurium str. LT2* were used*:* G46, TA1950, TA100, TA98 and these strains differ in characteristic mutation in *hisG* or *hisD* gene. Additionally, these strains have same other mutations that make them more susceptible to potential mutagens (Table 1.).

Table 1. List of bacterial strains used in this work

Strain	Genotype	Type of mutation in *his* gene
G46	*hisG46*	'base pair' mutation
TA1950	*hisG46 ΔuvrB*	'base pair' mutation
TA100	*hisG46 ΔuvrB rfa pKM101 Amp^R*	'base pair' mutation
TA98	*hisD3052 ΔuvrB rfa pKM101 Amp^R*	'frame-shift' mutation

Genotype testing: To confirm the genotype of selected *Salmonella* strains we performed UV and crystal violet sensitivity test and we have determined the sensitivity to antibiotic ampicillin. UV sensitivity test was performed by inoculating 15 μL of overnight culture of all strains in straight lines on solid NB media containing histidine and half of the inoculated Petri dish was exposed to UV light. To confirm ampicillin resistance and the *rfa* mutation, 100 μL of overnight culture of each strain was inoculated in top agar and spread over the solid NB medium**.** 5 μL of antibiotic ampicillin (20 μg/μL) and crystal violet (20 μg/μL) were placed on top of top agar containing Ames strains. Also, to confirm the genotype of Ames strains we put 100 μL of overnight cultures in top agar and spread them on the solid, minimal VB medium lacking histidine. Only cells in which the retromutation in *hisG* or *hisD* gene occurred were able to grow on media lacking histidine. To test the inducibility of the retromutations we put 5 μL of standard mutagens N- methyl-N'-nitro-N-nitrosoguanidine (MNNG), 4-nitroquinoline 1-oxide (4-NQO) and sodium azide (NaN_3) on top of top agar. The induction of retromutations by standard mutagens is shown as increased number of colonies in media lacking histidine in comparison to control. For control water was tested in the same way. All solid plates were incubated in incubator at 37 °C.

Growth curve: 10 mL of complete liquid NB media was inoculated with 100 μL of overnight culture of four *Salmonella* strains and incubated in the dark on shaker at 27 °C. Serial dilutions were prepared and plated every hour on solid NB media and the generation time was calculated according to the formula:

$$N_t = N_o \, 2^{t/g} \quad \text{(formula 1)}$$

N_t – number of bacteria after time t

N_o - number of bacteria at the beginning

t - time of growth

g - generation time

Citotoxicity testing was perfomed with strain TA100 (Mortelmans and Zeiger, 2000) to determine the concentration of potential mutagen that does not kill more than to 50% of bacteria (LD_{50}). This was done by plating serial dilutions of bacteria after one hour of incubation with different dilutions of potential mutagens that were sterilised by filtration or boiling.

Ames testing: First Ames test was done by adding a 5 μL of potential mutagen on top of top agar containing bacteria (Ames spot test; Mortelmans and Zeiger, 2000). Incubation lasted for two days at 37 °C after which number of colonies was counted. As a controls, we used water and DMSO to determine the number of spontaneous retromutations and with these results all other were compared. Substance is considered mutagen if the number of bacteria on a plate with potential mutagen is at least twice of the number of bacteria on a plate containing respective solvent (water or

DMSO). First Ames Test was done with original amount of potential mutagens from our environment while second Ames test (plate incorporation assay) was preformed after calculating LD_{50}. Substances that were found to be toxic were diluted to concentration which kills less than 50% of bacteria.

DNA isolation: Isolation of DNA was done from 6 ml of overnight culture of *Escherichia coli* and *Salmonella enterica* culture. Cultures were centrifuged for 5 minutes at 3000 rpm, supernatant was removed and cells were resuspended in water. Cultures were centrifuged for 5 minutes at 3000 rpm and resuspended in TE buffer (pH 8). Lysosim was added to break cell wall along side with 50 µL of SDS which brakes cellular membrane and 20 µL of proteolytic enzyme pronase which degrades proteins to oligopeptides. Proteins were precipitated by addition of 200 µL of potassium acetate (3 M) so they can be extracted by centrifuging. After centrifuging supernatant was transferred to new microtube and 50 µL of sodium acetate and 330 µL of isopropanol were added to pellet DNA.

Results

Growth curve

Using serial dilutions we were able to make growth curves of Ames strains listed in Table 1 (Figure 1.) and from logarithmic part of the curve we calculated what is the generation time of *Salmonella* strains incubated at 27 °C

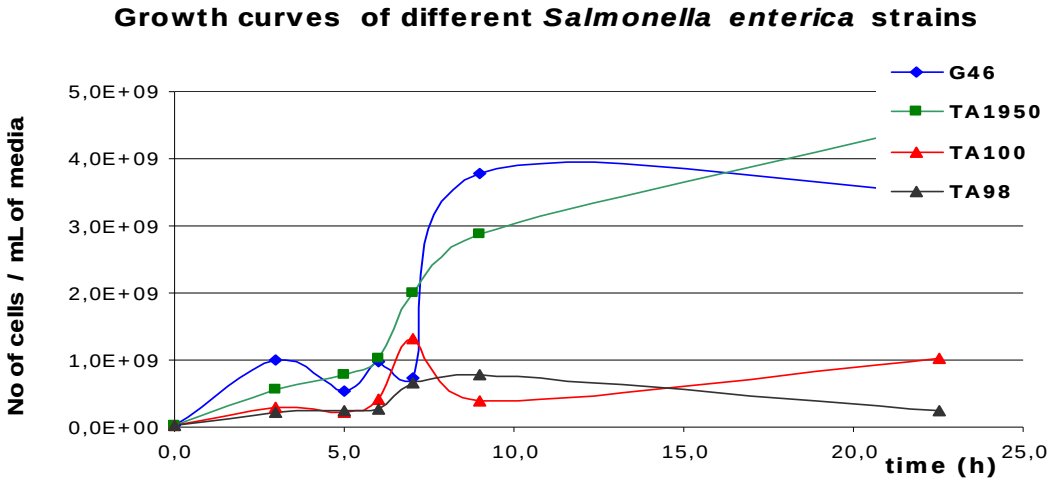

Figure 1. Growth curves of *Salmonella* strains used in this work.

Strain G46 shows big increase in number of cells 6 hours after platting on complete media suggesting that exponential phase started. Cell number begun to decrease 13 hours after inoculation in liquid media which implies on stationary phase of culture. TA1950 shows lesser increase in

reproduction in exponential phase than G 46 strain after which it continue to grow. TA100 shows beginning of small increase at the same time as upper strains but after two hours decresement is the same. After that there is small increase in population. TA98 enters in phases in same time as G46 but increase of number of cells is very small. Calculations of reproduction rate based on formula 1 showed that G46 in our conditions divided every 51, 94 minutes; TA1950 every 64, 66 minutes, TA100 every 44, 55 minutes and TA98 every 59, 89 minutes.

Citotoxicity testing: After plotting growth curves citotoxicity test of potential mutagens was perfomed on strain TA100 to find out if the original concentration of potential mutagen kills more than 50% of bacteria. Toxicity tests showed that some potential mutagens (painkiller tablets, energy drink, one component of two component glue and one body milk) kill more then 50% of bacteria so it was necesarry to dilute them.

The Ames testing (genotype control + our chemicals) At the beginning, we tested ampicillin resistance, UV sensitivity and the presence of *rfa* mutation of selected strains. We confirmed the genotype of every Ames strain used in this work and some of the results were shown in figure 2.

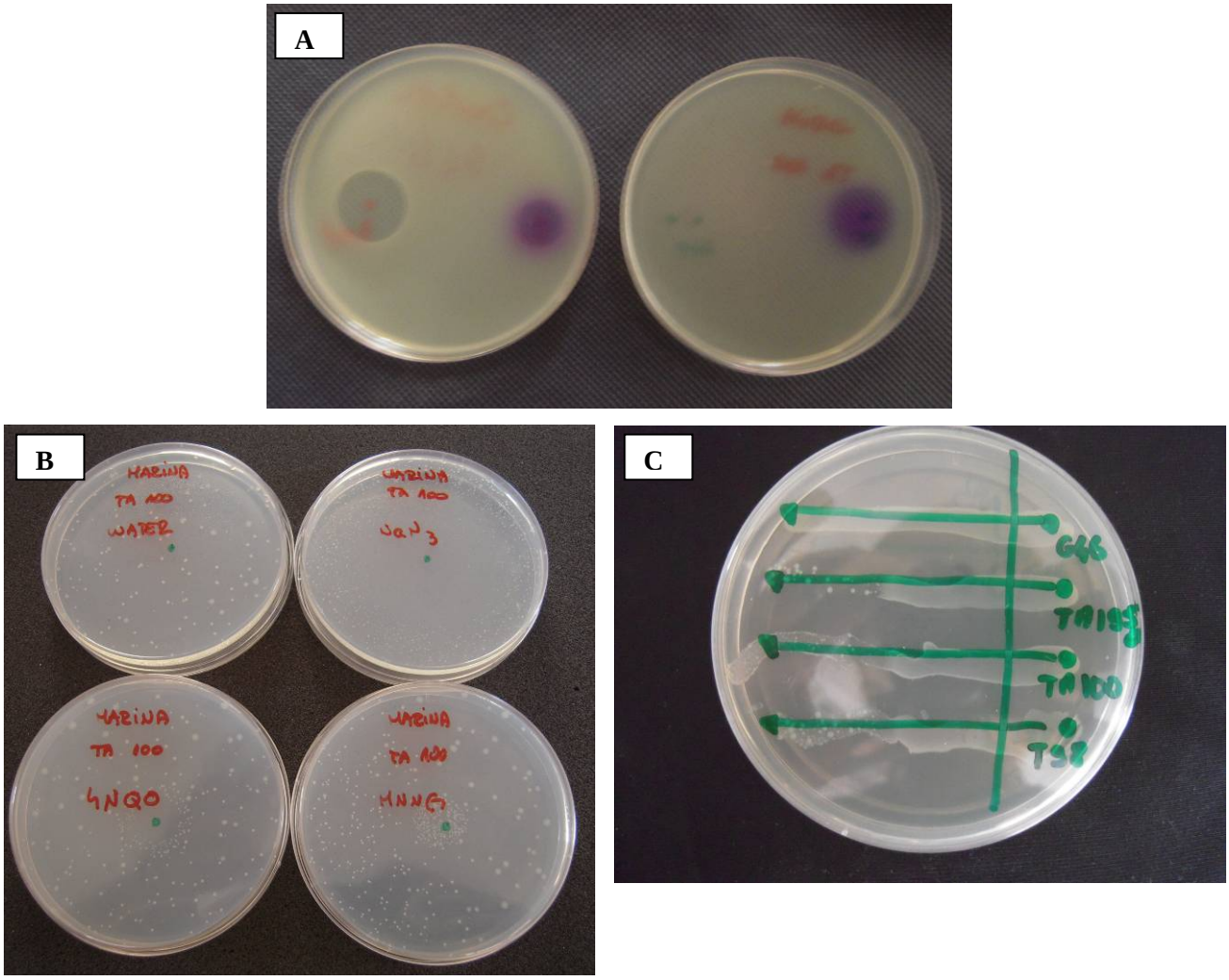

Figure 2. Results of genotype testing of Ames strains. A) Ampicillin resistance and crystal violet sensitivity test of strains G46 and TA100; B) retromutation testing of strain TA100 using standard mutagens sodium azide (NaN_3), 4-nitroquinoline 1-oxide (4-NQO), N- methyl-N'-nitro-N-nitrosoguanidine (MNNG); C) UV sensitivity test.

To find out if potential mutagen has mutagenic features, the number of bacteria incubated with specific chemical is divided by the number of bacteria incubated with respective solvent and our results were shown in table 2.

$$Q_m = \frac{N_m \text{ (retromutants on the plates with potential mutagens)}}{N_o \text{ (retromutants on the plates with solvent (water or DMSO))}}$$

Table 2. Q_m values of different compounds tested in this work

Compound	Q_m			
	Strain G46	Strain TA1950	Strain TA100	Strain TA98
Painkiller tablets	2; 1,5			
Energy drink	2; 2,75			
Body milk 1	11*; 0,25			
Body milk 2	1; 0,5			
Estradiole		1,67; 1; 0,97		
Sunscrean cream		1; 1,06; 1,45		
Soft drink 1		0,54; 1		
Facial mask		0,46; 1,27		
Barbeque			1,2; 0,9; 0,8	
Water from plastic tank			1,33; 1,25	
Mayonaisse			1,57; 1,93; 2,6	
Soft drink 2			0,90; 1,05	
Pain killer cream			1,33; 1,01; 1,8	
Water from plastic bottle 1				1; 1,16; 0,87
Water from plastic bottle 2				1; 0,87
Component 1 (glue)				0,86; 1,92; 0,76
Component 2 (glue)				1,77; 0,71
Soft drink 3				0,17; 0,96; 0,95
Smoke				1,45

* due to experimental mistake

DNA isolation and electrophoreses

At the end, we successfully isolated DNA from bacteria *E. coli* and *Salmonella enterica,* cut the DNA with restriction enzyme *Hin*dIII and made gel electrophoresis (figure not shown).

Discussion

Four *Salmonella* strains were used in this research due to their genetic differences. Those differences included deletion of *uvrB* gene and presence of plasmid pKM101 and were confirmed by experiments.

Growth curves: Growth curves of strains G46 and TA1950 are similar to ideal growth curve but ones of TA100 and TA98 are quite different which implies that there were some mistakes during plating. Usually in the ideal laboratory conditions it takes 30 minutes for the bacteria to divide on temperature of 37°C, but as the results has shown it took from 44,55 to 64,66 minutes as they were growing on 27°C.

Citotoxicity testing: During citotoxicty testing we discovered that some compounds that are in every day use are toxic to *Salmonella* strains and had to be diluted in water or DMSO. After dilution, these compounds (painkiller tablets, energy drink, one component of two component glue and one body milk) were not toxic to Ames strains anymore.

The Ames testing: (genotype control and chemicals) Genotype testing confirmed the genotype of Ames strains suggesting that strains could be used for mutagenicity assay. Substances which we found to be mutagenic (Q_m higher than 2) such as one type of pain killer tablets, one energy drink and mayoinasse are usually used daily. Therefore it would be necessary to test all compunds, that are mixed in this products individually, to detect the compound that raises Qm. Also, it is of outmost importance to test this chemicals on eukaryotic cells that metabolyse chemicals via cytochrome P450.

Conclusions

Ames test is simple assay to determine mutagenic effects of different substances (Goodson-Gregg and De Stasio, 2008). Two substances which we found to be mutagenic are closely related with people everyday life so it is crucial to continue further investigation on eukaryotic systems such as yeast and mammalian cell culture.

References:

Maron DR and Ames BN (1980) Revised methods for the *Salmonella* mutagenicity test. *Mutation Research* 113: 173-215.

Mortelmans K and Zeiger E (2000) The Ames *Salmonella*/microsome mutagenicity assay. *Mutation Research* (455) 29-60.

Goodson-Gregg N and De Stasio EA (2009) Reinventing the Ames Test as a Quantitative Lab that Connects Classical and Molecular Genetics. *Genetics* 181: 21-30.