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UV intensity and duration of exposure affects the reversion frequency of *hisG46* in *Salmonella typhimirium* TA100

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Ultraviolet (UV) radiation has been shown to be a potent mutagen of DNA. Photocarcinogenesis is caused largely by mutations at sites of incorrectly repaired DNA photoproducts, of which the most common is the thymine dimer. Whether the total dose is the critical causative agent or if the conditions of exposure play a role in the mutagenesis has not been established. Total dose is dependent on the total time of exposure and the distance from the source of radiation. The purpose of this experiment was to determine whether UV radiation delivered with high intensity for a short duration is more mutagenic than that delivered with a low intensity for a long duration if both conditions result in the same total dose. The reversion frequency of *hisG46* in *Salmonella typhimurium* TA100 was used as a model to determine the mutagenic effects of different UV conditions. A range of total doses from 36 Jm^{-2} to 158 Jm^{-2} of UVC (254 nm) were compared by varying conditions of intensity and duration. The results of these comparisons show that UV doses of high intensity, short durations are more mutagenic than those of low intensity, long duration doses.

The carcinogenic potential of the sun's energy has been understood for over a hundred years (5). More recently the study of the potentially harmful rays has focused on the ultraviolet (UV) spectrum. UV light is further separated into three divisions of ascending energy: UVA (315-400nm), UVB (280-315nm), and UVC (100-280nm). Higher energy light, such as UVC, has greater penetrating power and is more mutagenic (6).

There are two principal methods used in mutational studies: forward mutation and back mutation. Forward mutation systems often provide comprehensive information, but are more complex and can be cumbersome (6). Back mutation systems can be performed easily using any of several readily available auxotrophic bacterial strains. Auxotrophic histidine-requiring strains of *Salmonella typhimurium* LT2 became the standard in prokaryotic carcinogen testing after being used in the extensively validated assay developed by McCann and Ames (10) in the late 1970s. The principle of the assay is to expose the histidine auxotroph to a mutagen, which will revert bacteria to a prototrophic state. Revertants are then counted to establish the mutagenicity of a chemical or condition. Mutations are possible in many forms such as mismatch (substitution) or frameshift mutations. UV light (non-ionizing radiation) is able to produce mutations by physically altering the structure of DNA with its high energy. The most common form of DNA alteration induced by UV light is the formation of cyclobutane pyrimidine dimers such as thymine-thymine, thymine-cytosine, or cytosine-cytosine dimers (2,11). Other possible UV-induced damage includes 6-4 pyrimidine-pyrimidone photoproducts, pyrimidine hydrates, purine photoproducts, strand breaks and DNA cross-linking (2,11).

However, all organisms possess mechanisms to repair DNA mutations. There are several error-proof DNA repair mechanisms such as photoreactivation, excision repair, and recombination repair (11); these are able to effectively remove pyrimidine dimers in the DNA. However, most mutations that arise are actually derived from the cell trying to repair itself using the error-prone repair mechanism called the SOS repair (reviewed in 1,7,9,12). This system is well characterized in *Escherichia coli.* A system very similar to the one in *E. coli* also exists in *S. typhimurium* (3,4). The SOS system is activated in response to pyrimidine dimer blockades and generates a cascade of genes that ultimately modifies the DNA polymerase so that it is able to replicate across unreadable UV lesions, but by doing so the polymerase loses its proofreading function. The gene *umuDC* (*MucA/B*) is part of this SOS gene cascade and functions in binding the DNA polymerase, causing the DNA polymerase to by-pass UV lesions (1). As a result, random bases are incorporated at the site leading to mutation in the genetic sequence.

The aim of this experiment is to examine the relationship between the rate of reversion in *S. typhimurium* (TA100), the intensity of the UV light, duration of exposure, and the total dose. It is postulated that for the same total dose of 254 nm UVC light, a shorter, more intense exposure will generate more revertants than a longer, less intense exposure. *Salmonella typhimurium* (TA100) is an auxotrophic model with a point mutation in the first gene of histidine biosynthesis, *hisG46* (10). This point mutation is easily affected by the damage associated with UV light. In addition, this Ames strain has other genetic modifications that make it more susceptible to UV mutations. These include deletion of the *uvrB*, and *rfa* genes. The *rfa* mutation causes the membrane to be more permeable to

UV. Whereas, the *uvrB* deletion causes *S. typhimurium* to be defective in error-proof repair essentially leaving only the error-prone SOS repair system. The TA100 strain also carries the plasmid pKM101 which increases the mutation frequency because it encodes *umuDC*, enhancing the error-prone SOS DNA repair mechanism.

Our findings may have implications towards recommendations for human sun tanning. Previous research has shown that UV causes the same class of base substitutions in *S. typhimurium* TA100 as in the p53 mutations seen in human carcinomas (4). If high intensity exposure is more hazardous, longer tanning in the morning or evening would be recommended over shorter midday exposure to sunlight. "The results of several independent large-scale studies have shown that there is a good correlation between mutagenicity in *Salmonella* and carcinogenicity in mammals" (http://embryo.ib.amwaw.edu.pl/invittox/prot/30.htm).

MATERIALS AND METHODS

Growth of Salmonella typhimurium strain. The strain used in the experiment was kindly provided by Dr. Ramey as a liquid culture. Tester strain *Salmonella typhimurium* TA100 is a histidine auxotroph with a point mutation in *hisG46* and contains the R-factor plasmid pKM101 that encodes the *MucA/B* operon and ampicillin resistance gene. The presence of this plasmid serves to enhance the rate of reversion of TA100 over its genetically identical but plasmid-lacking sister-strain, TA1535. Deletion of *uvrB* in the majority of the Ames (10) strains (including TA100 and TA1535) extends into the bio gene thus necessitating a requirement for biotin. Tests were done to determine ampicillin resistance and histidine requirement as described by Maron and Ames (10). A "master plate" was streaked from an overnight culture of *S. typhimurium* and stored at 4°C, as per Maron and Ames (10). The master plate was used in all subsequent inoculations of liquid media for this experiment.

Control strains. *Escherichia coli* strain B23 with and without the plasmid pBR322 (which confers ampicillin resistance) was used to determine the efficacy of our ampicillin stock. Cultures of *E. coli* B23 + pBR322 and B23 were grown both in the presence and absence of ampicillin and then compared after overnight growth.

Preparation of reagents. All reagents used, except Luria-Bertani (LB) broth and plates, were prepared as described by Ames, et al. (10) with slight modifications to the Vogel-Bonner minimal media recipe. Detailed recipes are included in Appendix A. All reagents were autoclaved and stored at room temperature.

Mutagenicity assay. The mutagenicity assay was performed according to Maron and Ames (10). In brief, an overnight culture of *S. typhimurium* was prepared by inoculating LB media with a colony from our master plate and incubating overnight with aeration at 37°C. The overnight culture was used to inoculate 50 ml of LB and the new culture grown to an OD_{600} = 0.81-0.84. Liquid Top Agar (TA) supplemented with 10% (v/v) top agar reagents was inoculated with 100 µl of culture and poured onto minimal media Bottom Agar (BA) plates. The TA/BA plates were incubated at 37°C right-side up for 2 hours. Mutagenesis was carried out in triplicate in a Chromato-vue UV cabinet (model CC-10) under a 6 watt 254 nm UV bulb (Model UVGL-58 mineralight lamp). Uncovered plates were placed either 11 cm (intensity = 39.5 $Js^{-1}m^{-2}$) away from the bulb and mutagenized for 1, 2, 3, or 4 sec or 20 cm (intensity = 11.9 $Js^{-1}m^{-2}$) away from the bulb and mutagenized for 3, 6, 9, or 12 sec. Time was started with the activation of the bulb, not the turning on of the switch. Plates, including positive controls (i.e. not exposed to UV light) were wrapped in aluminum foil immediately following mutagenization to prevent spontaneous back mutations and the plates were incubated at 37°C. Colonies were counted after 48 hours of incubation. The counted revertants were multiplied by a Kill Factor (KF) to compensate for UV induced mortality. The KF was calculated by taking the reciprocal of percent survival as determined by the survival curve assay.

The following equations were used to calculate UV dosage:

Luminosity of UV light tube = 6 Js^{-1} = 6 W

Distance from UV light tube (d) = 0.11 m or 0.20 m

Intensity of UV light ($Js^{-1}m^{-2}$) = luminosity (Js^{-1})/ $4\pi * d^2$

Dosage (Jm^{-2}) = Intensity * time

Survival curve assay. Bacteria were prepared in the same manner as for the mutagenicity assay but with the following modifications: bacteria were serially diluted (10^{-4} to 10^{-7}) before being added to the TA and poured onto LB plates instead of BA plates so that all the cells could grow. UV exposure was carried out under the same parameters as for the mutagenicity assay and colonies counted after 48 hours of incubation at 37°C.

RESULTS

Salmonella typhimurium TA100 was tested for both histidine auxotrophy and ampicillin resistance. Briefly, TA100 grew on minimal media supplemented with both histidine and biotin but failed to grow without histidine or when ampicillin was added (Table 1).

Table 1: Phenotypic determination of *S. typhimurium* TA100

Strains	Required Supplements			
	His, Bio, Amp	His, Bio	Bio	None
S. typhymurium TA100	-	+	-	-
E. coli B23	-	+	+	+
E. coli B23 + pBR322	+	+	+	+

All plates made with VB minimal media and described supplements
His: L-histidine Bio: D-biotin Amp: ampicillin
"+" growth, "-" no growth

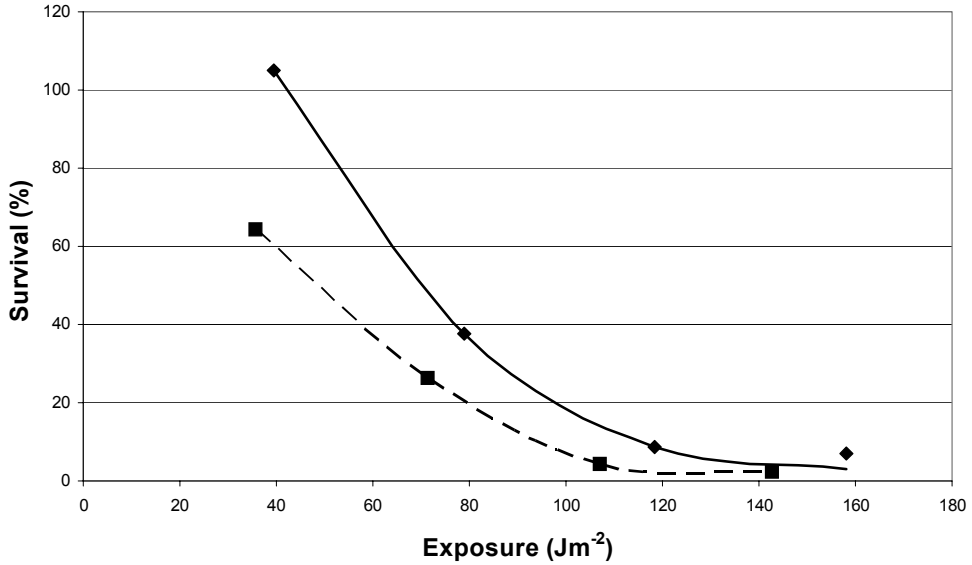

Fig. 1: Survival Curve for *S. typhimurium* exposed to 254 nm UV light. Solid line and diamond shaped data points represent the 39.5 $Js^{-1}m^{-2}$ exposure. Dashed lines and square data points represent the 11.9 $Js^{-1}m^{-2}$ exposure.

The control bacteria, *E. coli* B23 with plasmid pBR322, grew under all conditions whereas *E. coli* B23 without the plasmid pBR322 only grew in the absence of ampicillin (Table 1).

The survival curve assay data (Table 2) clearly shows that as UV total dose increases there is a corresponding decrease in survival (Fig. 1).

Table 2: Survival Data for *S. typhimurium* exposed to 254 nm UV light

Distance (cm)	Total Dose (Jm^{-2})	CFU	FPD	Survival (%)	KF
N/A	0	101	10^{-7}	N/A	N/A
11	40	106	10^{-7}	105	1.0
11	79	38	10^{-7}	38	2.7
11	119	88	10^{-6}	9	11.5
11	158	70	10^{-6}	7	14.4
20	36	65	10^{-7}	64	1.6
20	71	153	10^{-6}	26	3.8
		38	10^{-7}		
20	107	45	10^{-6}	4	22.4
20	143	240	10^{-6}	2	42.1

KF: kill factor

There was a significant difference between the 39.5 $Js^{-1}m^{-2}$ and the 11.9 $Js^{-1}m^{-2}$ survival curves with the 11.9 $Js^{-1}m^{-2}$ having a lower survival throughout the dose range.

Although the colony counts per plate were high (Table 3), the colonies observed were discrete and countable. The spontaneous reversions seen without exposure to UV are shown as the 0 dose data points (Fig. 2). The total numbers of reversions per plate (Fig. 2) for each exposure clearly demonstrates a higher number of revertants at 39.5 $Js^{-1}m^{-2}$.

The number of revertants was normalized using the KF to correct for UV mortality (Fig. 3). After normalization the 39.5 $Js^{-1}m^{-2}$ exposure remained more mutagenic than the 11.9 $Js^{-1}m^{-2}$ exposure.

Table 3: Mutagenicity of *S. typhimurium* exposed to 254 nm UV light

Distance (cm)	Total Dose (Jm^{-2})	Reversions	Reversions x KF
N/A	0	180	N/A
11	40	951	734
11	79	1,351	3,114
11	119	1,314	13,017
11	158	680	7,213
20	36	335	240
20	71	392	805
20	107	329	3,347
20	143	309	5,433

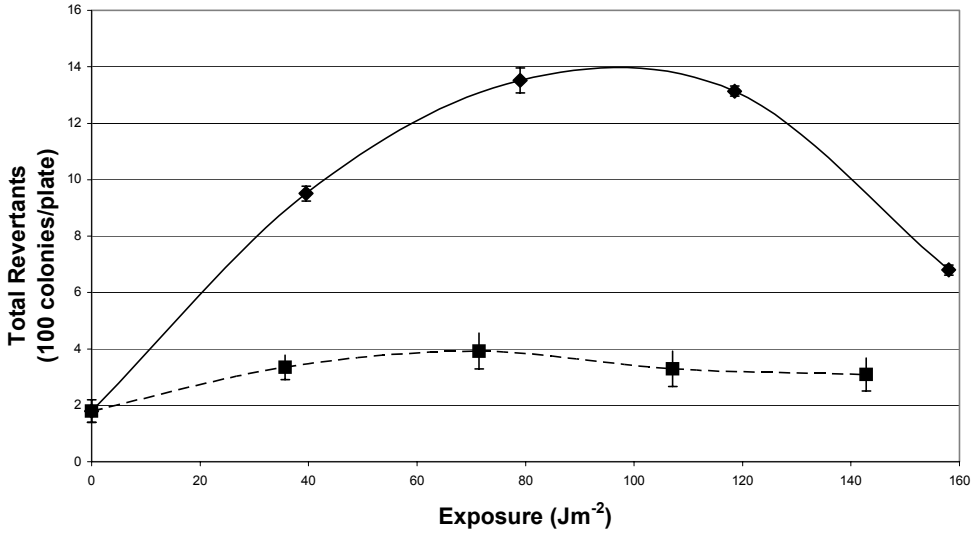

Fig. 2: Mutagenicity of S. typhimurium TA100 exposed to 254 nm UV light. Solid line and diamond shaped data points represent revertants at 39.5 $Js^{-1}m^{-2}$ exposure. Dashed lines and square data points represent revertants at 11.9 $Js^{-1}m^{-2}$ exposure.

DISCUSSION

Based on the observation that there was no growth of *Salmonella typhimurium* TA100 on the minimal media plates supplemented with histidine, biotin, and ampicillin and growth of the control strain *E. coli* B23 + pBR322 in the same media, we suggest the following: the supplied strain was not *S. typhimurium* TA100, the *S. typhimurium* TA100 lost the plasmid pKM101 with its ampicillin resistance gene, or a mutation in the pKM101 plasmid affected its ampicillin resistance gene rendering it non-functional. However, based on the observation by Ames et al. (10) that the plasmid pKM101 is easily lost, we conclude that its loss is the most parsimonious explanation for the lack of ampicillin resistance. Therefore, the master plate of *S. typhimurium* TA100 used for subsequent inoculations contained minimal media supplemented with histidine and biotin but no ampicillin.

The loss of the plasmid pKM101 encoding the *MucA/B* operon could have affected our model system for measuring DNA mutation rates. The *MucA/B* operon functions to increase the proportion of transversions to transitions and results in an approximately 11-fold increase in the number of revertants in the Ames tester strains (8). However, this was not addressed in this study.

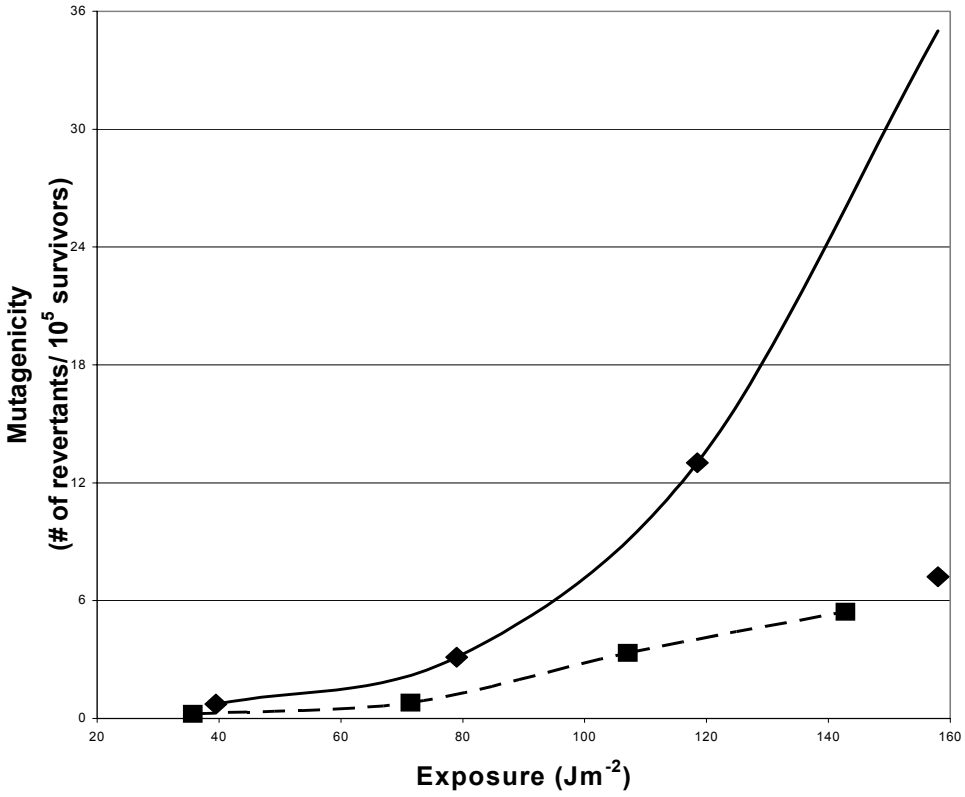

Fig. 3: Kill Corrected Mutagenicity for *S. typhimurium* TA100 exposed to 254 nm UV light. Solid line and diamond shaped data points represent 39.5 $Js^{-1}m^{-2}$ revertants times KF. Dashed lines and square data points represent 11.9$Js^{-1}m^{-2}$ revertants times KF.

The difference observed between the 39.5 $Js^{-1}m^{-2}$ and the 11.9 $Js^{-1}m^{-2}$ survival curves (Fig. 1) was due to either the length of exposure and/or the intensity of exposure. Some of the possible causes of greater cell death in the 11.9 $Js^{-1}m^{-2}$ intensity exposure could have been due to an irreparable amount of DNA damage, such as two strand chromosomal breaks, or damage to other cellular components such as membranes. Since the model used measures DNA damage and repair, more revertants were expected if mortality was due to DNA damage. A higher mutation rate occurred under the 39.5 $Js^{-1}m^{-2}$ exposure conditions (Fig. 2). Therefore, increased DNA damage was excluded as a possibility for the increased cell mortality under the 11.9 $Js^{-1}m^{-2}$ conditions.

An alternative possibility is that the SOS repair mechanism is incapable of a sustained response. Therefore, with longer doses, DNA repair may remain incomplete. However, this aspect is not addressed in this study.

As seen in the survival curve (Fig. 1) the different UV exposures resulted in different surviving population sizes. Therefore, we may not be able to directly compare total numbers of revertants without normalizing the surviving population. The number of revertants per number of survivors (Fig. 3) still demonstrates a higher number of revertants at the 39.5 $Js^{-1}m^{-2}$ exposure conditions. The 39.5 $Js^{-1}m^{-2}$ exposure trendline (Fig. 3) correlates with the expected pattern of UV induced mortality (Fig. 1). The inconsistent data point at the dose of 158 Jm^{-2} for the 39.5 $Js^{-1}m^{-2}$ exposure condition may have been due to an abnormally high value in the corresponding data point of the survival curve (Fig. 1). It is hypothesized that at this total dose less survival should have been observed. This translates into an abnormally low kill factor and the correspondingly low value for this data point.

In addition to high cell mortality, the deficient SOS response may contribute to the low mutation rates seen at 11.9 $Js^{-1}m^{-2}$. A deficiency in the SOS repair mechanism would account for both the lower survival rate and lower reversions observed in the 11.9 $Js^{-1}m^{-2}$ condition. However, nothing in the current research indicates that the SOS repair mechanism declines in activity over a defined period of time.

From the data it is our conclusion that a high intensity, short duration dose of UV is more mutagenic than a low intensity, long duration dose in our model. In addition, the low intensity, long duration doses resulted in a higher mortality rate in our model.

FUTURE EXPERIMENTS

The anomalous survival curve warrants further investigation. The higher mortality observed during low intensity, long duration doses may be attributed to the loss of plasmid pKM101 with its SOS genes. Therefore, *S typhimurium* TA100 containing plasmid pKM101 should be tested for this phenomenon. In addition, it would be useful to determine the optimal exposure for revertant survival.

Although UVC is highly mutagenic, it is normally filtered out by the atmosphere and is not considered a significant naturally occurring photocarcinogen (5). UVB is another potent solar mutagen that, unlike UVC, penetrates the atmosphere to reach the Earth's surface (5). Consequently, to increase the relevance of these results to the natural environment, different exposures of UVB light should be tested for the same pattern of responses observed in this study.

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APPENDIX A:

Complete recipes of reagents used in experiment.

Vogel-Bonner 10X – 100 ml

Sterile distilled H_2O	400 ml
Magnesium sulfate	1.0 g
Citric acid	10.0 g
K_2HPO_4	50 g
NH_4Cl	4.5 g
$NaH_2PO_4 \cdot 2H_2O$	11 g

Add salts in order indicated to warm water. Allow each salt to dissolve completely before adding next salt. Adjust volume to 500 ml with distilled H_2O. Autoclave, loosely capped, for 20 min at 121ºC.

40% glucose – 200 ml

Glucose	80 g
Sterile distilled H_2O	200 ml

Add glucose to distilled H_2O and autoclave for 20 min at 121ºC.

0.5% Histidine (0.5% His) – 5 ml

L-histidine · HCl	25 μg
Sterile distilled H_2O	5 ml

Add glucose to distilled H_2O and autoclave for 20 min at 121ºC.

0.5 mM Biotin (0.5 mM Bio) – 2 ml

D-biotin	247 μg
Sterile distilled H_2O	2 ml

Dissolve biotin by heating the distilled H_2O to 100ºC. Filter sterilize.

Ampicillin (Amp) – 10 ml

Ampicillin	80 mg
Sterile distilled H_2O	10 ml

Dissolve ampicillin in distilled H_2O and filter sterilize through a 22 μm filter.

Histidine/Biotin/Ampicillin agar (HBA agar) – 500 ml

Agar	7.5 g
Sterile distilled H_2O	410 ml
VB 10X	50 ml
40% glucose	25 ml
0.5% L-Histidine	5 ml
0.5 mM D-Biotin	3 ml
Ampicillin (8 mg/ml)	1.575 ml

Autoclave agar and distilled H2O. Add sterile glucose, 10 VB salts, and histidine to hot solution. Allow to cool to 50ºC and add ampicillin. Swirl to mix and pour plates.

Top agar reagents – 50 ml

D-biotin	6.18 mg
L-histidine	4.8 mg
Sterile distilled H_2O	50 ml

Dissolve biotin by heating the distilled H_2O to the bioling point. Add histidine and filter sterilize.

Top Agar – 200 ml

Agar	1.2 g
NaCl	1.0 g
Sterile distilled H_2O	200 ml

Add salt and agar to distilled H_2O and autoclave for 20 min at 121ºC.

Bottom Agar Plates– 3 L

Agar	45 g
Sterile distilled H_2O	2.55 L
VB 10X	300 ml
40% glucose	150 ml

Add agar, salts, and glucose to distilled H2O and autoclave for 20 min at 121ºC. Allow to cool to 50ºC and pour plates.

Luria Broth – 500 ml

Tryptone	5.0 g
Yeast extract	2.5 g
NaCl	2.5 g
Glucose	1.0 g
Sterile distilled H_2O	500 ml

Add reagents in order listed to distilled H2O and autoclave for 20 min at 121ºC.