

Analysis of Genetic Variation by Methyl Orange in Rhizobium Using RAPD – PCR

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Abstract: In this study, we analyzed the induced genetic variability in *Rhizobium* using azo dye (methyl orange) which are commercially used in textile industries. We isolated and characterized the *Rhizobium* from root nodules of *Arachis hypogaea* by using colonial, morphological and biochemical methods. Inoculation of *Rhizobium* strain in different concentrations of methyl orange. Then isolated the DNA from control and mutated samples. Analyzed the DNA quantitatively in agarose gel electrophoresis. After estimating the DNA from control and mutated samples is amplified using Random primers by polymerase chain reaction. The amplification of DNA was confirmed by agarose gel electrophoresis. Determine the variation in molecular weight of amplified bands by gel documentation system. Then constructed the dendrograms according to genetic variations.

Key words: *Rhizobium*, Methyl Orange, RAPD, PCR.

Introduction

India is the second largest exporter of dyestuff and intermediates developing countries, after China. The textile industry accounts for the largest consumption of dyestuffs, at nearly 80%. The textile industries are to satisfy the ever-growing demands in terms of quality, variety, fastness and other technical requirements. The Indian textile industries now predominantly use synthetic organic dyes like direct dyes, processing dyes, reactive dyes, etc. The large variety of dyes and chemical used in an attempt to make more attractive popular shades of fabrics for a competitive market render them very complex¹. During the last decade, environmental issues associated with dyestuff production and applications have grown significantly and

indisputably among the major driving forces affecting the textile dye industry today. Considerable amounts of dyes have been noticed in these textile waste waters, due to their incomplete use and washing operations. The dyes disposed off, can be found in dissolved state or in suspension in the waste water. These dye stuffs are highly structured polymers and are very difficult to decompose biologically². Chemicals such as surfactants, chelating agents, pH regulators, densifiers etc, are the common contaminant compounds present in waste waters in great variety and concentration range³. Dyes are used in large quantities in many industries including textile, leather, cosmetics, paper, printing, plastic, pharmaceuticals, food, etc., to color their products⁴. The most obvious impact of the discharge of dye colored effluent is the persisting

nature of the color. It is stable and fast, difficult to degrade, toxic, rendering the water unfit for its intended use. Further, the color removal is also not adequate by the conventional chemical and biological treatment. Such dyestuffs can reach the aquatic environment, primarily dissolved or suspended in water, since the conventional treatment of waste water from textile mills and dyestuff factories are unable to remove most of the azo and other dyes effectively. Since large quantities of dyes are used, such pollution due to dyes may occur on a significant scale. The International Agency for Research on Cancer (IARC) has classified various dyes like benzidine as being associated with cancer in humans. Benzidine is known to be carcinogenic to a variety of mammalian species, including humans⁵. A number of dyes have been tested for mutagenicity using *salmonella* assay. Several of them have been found to be carcinogenic^{6,7,8}. Azo dyes are one of the oldest industrially synthesized organic compounds used in textile industry, food and cosmetics. Under anaerobic conditions can be converted to aryl amines which are potentially more toxic than the parent compounds^{9,10}. Azo dyes are designed to be recalcitrant under typical product service conditions it is this property, allied with their toxicity to microorganisms that makes biological treatment difficult¹¹. Various synthetic azo dyes have been shown to induce a variety of tumors in mice and rats and to exhibit inhibitory effects on the biosynthesis of proteins. It is prepared by coupling diazotized sulphanilic acid with dimethylaniline. This acid dye is used for wool and silk. It imparts an orange colour, as the name suggests though the colour, is not fast to light or washing. It is used in acid-alkali titrations, as an indicator and gives a yellow colour with alkali and pink with acid. The heterogeneous photo catalytic system has been used for performing the oxidation of two common and very stable azo-dyes Methyl orange (Meo) $C_{14}H_{11}N_3SO_3Na$, and orangeII (OII) $C_{16}H_{11}N_2SO_4Na$. Meo and OII, these are characterized by sulphoic groups, which are responsible for the high solubility of these dyes in water. Bleaching of Meo and of several azo-dyes has been carried out by using ozone as oxidant agent^{12,13}. While the oxidation of orange II has been carried out by using a photo-fenton type reaction whereas the reduction has been carried out by photo-oxidation process^{14,15}. The photo catalytic oxidation of Methyl-orange (Meo) and OII was carried out in aqueous suspensions of polycrystalline TiO_2 (anatase) irradiated by sunlight. The abatement of substrate, of total organic carbon content and of colour was monitored. The dye photo-oxidation rate

depends on the following parameters: i) Substrate concentration, ii) catalyst amount

Materials and Methods

Collection and Isolation of samples

The sample was collected from *Arachis hypogaea* in near by areas of Thanjavur and plant material was authenticated by a Botanist Prof. Dr. K. Singaravadivel, Department of Microbiology, Indian Institute of Crop Processing Technology (IICPT), Thanjavur, Tamil Nadu, India. From a young leguminous plant healthy root nodules were selected and made into pieces with the help of sterile blade. The nodules were thoroughly washed with tap water and then with sterile distilled water over the nylon mesh under aseptic conditions so as to remove contaminants and adhering soil particles. Thereafter, nodules were immersed in 0.1% acidified mercuric chloride for 5 minutes. The nodules were transferred into sterile beaker containing 10ml 95% ethanol and waited for 2-3 minutes. Then the nodules were thoroughly washed for five times with sterile tap water, and blot dried by using sterile blotting paper. Aseptically crushed the nodules with glass rod or the nodules were dissected by using nichrome blade and the dilutions were prepared. 1ml suspension was poured on YEM agar plates and the suspension was spreaded using L-rod. The inoculated plates were incubated at 28°C for 48 hours. Thereafter the bacterial colonies were observed, they are gummy, translucent or white opaque. A discrete colony was picked up and streaked on a second YEM agar plates for better separation. The inoculated plates were incubated at 28°C for 48 hours.

Identification of Microorganism:

The cultures were morphologically and biochemically identified by GRAM'S staining, motility test, IMVIC Test, methyl red test, voges proskauer test, catalase test, sugar fermentation test and biochemical procedures.

Cultivation of pure culture:

The microorganism was noted as mass culture and so they were separated and pure culture isolation was done. The culture of organism was streaked on freshly prepared YEM agar plates. The broth was then incubated for about 48 hours for the growth of microorganism and also dye induced broth culture was prepared.

Isolation of DNA and Quantification of DNA

The phenol – chloroform extraction method was used for DNA extraction from specimen. 2ml of

culture sample was taken in test tubes and it was centrifuged at 10,000rpm for 10 minutes. After recovering DNA by ethanol precipitation method the DNA fragments were resolved by agarose gel electrophoresis method .The DNA was also estimated quantitatively by colorimetric method.

RAPD-PCR analysis of Genomic DNA:

Ten oligonucleotide OPI-06 primer (AAGGCGGCAG) was used in RAPD-PCR . The concentration of each dNTP in the reaction mixture is usually 200 μ M. The four dNTPs (dATP, dCTP, dGTP, dTTP) should be in equal concentration. The PCR reaction mixture (50 μ l) contained 1 μ l of 50 μ g genomic DNA, 0.3 mM

dNTPs, 2.0 mM $MgCl_2$, 1.5 μ l of Taq DNA polymerase and 1 μ M primer were used in 50 μ l of reaction mixer and rest of the volume was adjusted by using sterile nuclease free water. The samples were placed in a thermocycler (Biometra, Germany). The amplification reaction performed upto 40 cycles which are composed of the following steps: Initial step of denaturation was performed at 94 $^{\circ}$ C over an interval of 1-3 min , annealing at 55 $^{\circ}$ C for 1 minutes and elongation at 73 $^{\circ}$ C for 1 min and final extension step was carried out at 72 $^{\circ}$ C for 10 min for newly synthesized PCR products . After amplification of PCR products by using agarose gel electrophoresis were visualized under UV trans-illuminator lamp. 1 Kb DNA ladder was used as molecular marker.

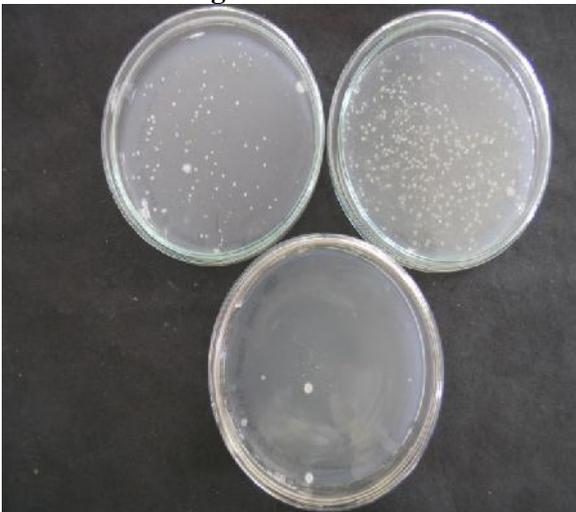
Figure 1 (a)
Groundnut Plant



Figure 1 (b)
Dissected Root Nodules



Figure 2(a)
Isolated microorganisms from root nodules



Figures 2(b)
Grams staining

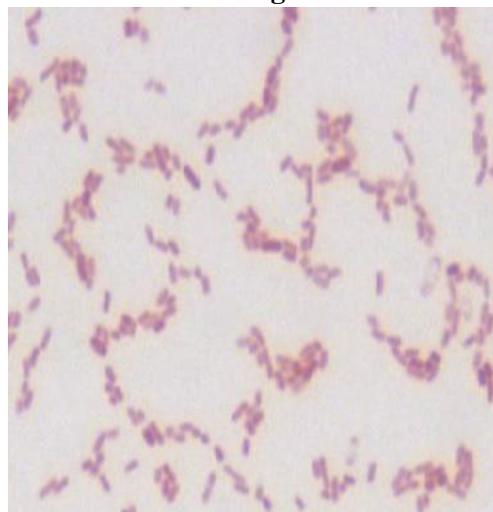
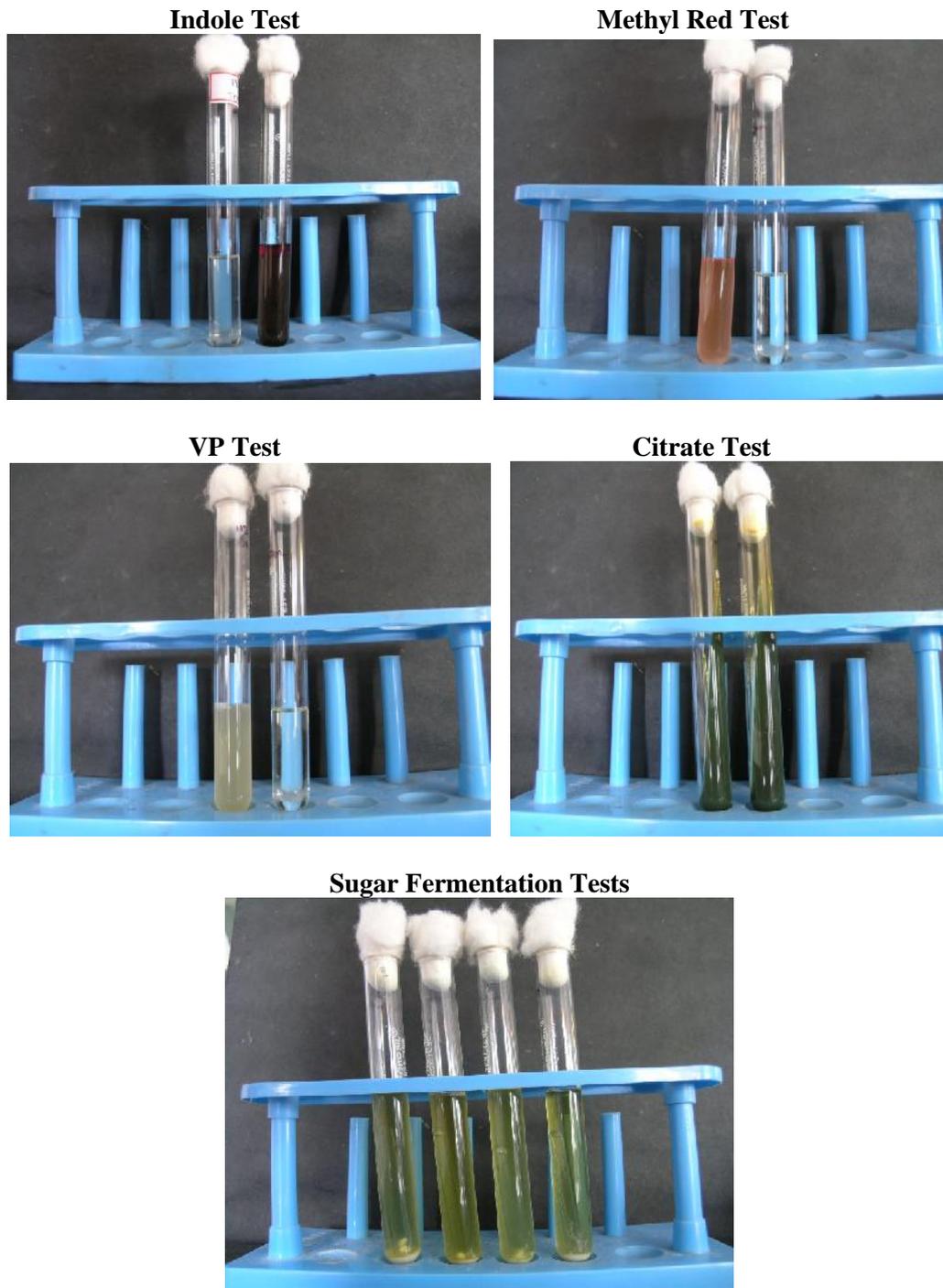


Figure 3
Biochemical tests



Results and discussion

Plant and soil samples were collected from agricultural fields from Thanjavur field areas. Soil samples and groundnut roots were collected in sterilized polythene bags with the help of sterile spatula as described by Reddy et al., (1986)¹⁶ was shown in figure-1(a). The root nodules were surface

sterilized by mercuric chloride was shown in figure-1(b) and the crushed nodules were mixed with sterile distilled water. It was serially diluted and spreaded over the Yeast Extract Mannitol (YEM) agar plates after incubation time mucoid colony growth were observed on the agar plates was shown in figure-2, the grown colonies were identified as *Rhizobium* by morphological and biochemical test results shown in

figure-3 and table-1. In an another study A.K Chandra have isolated a wild strain. BU/S4 of *Rhizobium japonicum* was isolated from the nodules of a locally grown soybean. The isolated *Rhizobium* culture was pure cultured (figure-4) and grown in YEM broth at different concentrations (1ppm, 2ppm, 3ppm, 4ppm) of methyl orange and a control was maintained without adding the dye was shown in figure-5. Finally growth was observed in control and the growth of *Rhizobium* got decreased when the concentration of dye was increased. The DNA was isolated by phenol chloroform extraction method and it was confirmed by agarose gel electrophoresis method (figure-6).In most of the previous studies the

DNA was isolated using readymade kits (Abushady *et al.*, 2005, DNA purification kit, USA)^{17,18}. In another study the DNA of 15 isolates was extracted from overnight grown culture by using prepman ultra kit from ABI according to manufactures protocol and sent for sequencing Poulomi Nandy AR *et al.* Similarly the chromosomal DNA of Staphylococcus cultures isolates were extracted by the mini- preparation method by Williams *et al*¹⁹. But in the present study the sufficient amount of DNA was isolated by cell- lysis method using buffer. The applied method was so cost effective and reliable that it resulted is relatively high DNA concentrations.

Table 1: Biochemical Characterization for Identification of Rhizobium.

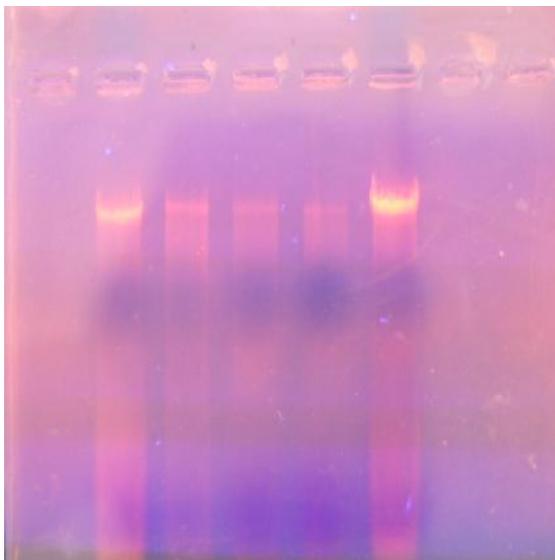
SNO	TEST	OBSERVATION	RESULTS
1.	Gram's staining	Gram Negative rods	Positive
2.	Motility	Motile	Positive
3.	Indole	Cherry red ring was formed	Positive
4.	Methyl red	Orange colour was formed	positive
5.	Voges Proskauer	No colour change remains yellow colour	Negative
6.	Citrate Utilization	No colour change remain green colour	Negative
7.	Sugar Fermentation	Only acid production, No gas production was seen.	Acid positive
8.	Catalase	Bubbles appears with in 20 minutes	Positive

Figure 4
Pure Culture of Rhizobium



Figure 5
Rhizobium cultures in different Concentration of methyl Orange Dyes



Figure 6**Confirmation of DNA on agarose gel Electrophoresis****Figure 7****Confirmation of PCR amplified DNA on agarose gel Electrophoresis****Table 2: Estimation of DNA from Azo dye induced mutants.**

SNO	SAMPLES	OD AT 595nm	CONCERNTRATION
1.	Blank	0.000	00
2.	Standard	1	0.24
3.		2	0.32
4.		3	0.40
5.		4	0.48
6.		5	0.56
7.	Organism	Control	0.69
8.		1ppm	0.81
9.		2ppm	0.77
10.		3ppm	0.82
11.		4ppm	0.80

Quantification of DNA

The amount of DNA was quantitatively estimated by spectrophotometer analysis and the results were given in Table-2.

Amplification of DNA by PCR for induced genetic variation analysis

The DNA samples were amplified using decamer primers which were complementary to short tandem repeats present in DNA. After amplification the

samples were loaded in 1.5% agarose gel in order to check the amplified product. Discrete amplified bands were observed under UV-transilluminator as shown in figure-7. The gel was photographed and the image was applied in non-linear dynamic software and the variation between amplified bands was observed by presence and absence of DNA fragments. A dendrogram was constructed based on UPGMA cluster analysis according to dice square co-efficient method was shown in figure-8.

From the dendrogram results it was observed that from the control strain 1ppm dye have altered the DNA at an average of 0.53 nucleotides, similarly from the 2ppm dye the culture exposed to 3ppm dye have got altered 0.36 nucleotides from the control *Rhizobium* and at the highest 4ppm dye have altered 0.91 nucleotides from the control. From the results it was clearly observed that the increasing in concentration of dye will leads to high rate of mutation in bacteria. In the previous study stated that radiation is one of the best known physical mutants was it dissipates the atoms of water molecules and causes the generation of hydroxyl radicals that are the most reactive. They react with most of the biomolecules by the radicals damage their structure and biological activity. By the way genetic alterations occur on the DNA molecules. This is the cause of mutations depend on radiation several workers have shown the RAPD markers, which can quickly detect a large number of genetic maps in a number of woody fruits crops and the detection of mutation in sunflower and grapes including changes due to DNA damage.

Few studies analyzing induced mutations at molecular level have been carried out in Arabidopsis, Lettuce, Maize and Tomato. In Arabidopsis, mutations at abscisic acid insensitive *abi3-6* auxin, ethylene and abscisic acid resistance *axr2-1* ; chlorate resistance *CHL3* and *NAI2* , gibberellin responsive dwarfs *gal* ; and trichrome differentiation gene *gl-1-1* involved deletions ranging from 17bp to 19CM. A chalcone flavanone

isomerase (*CHI*) mutation obtained following fast neutron irradiation revealed a 1.4kb inversion with in the *CHI* gene and a 272-bp insertion adjacent to another inversion from the same chromosome. A Dihydroflavanol-4- reductase (*DFR*), have revealed complicated alterations . RAPD analysis can provide the molecular markers microsatelite or simple sequence repeats which detect simple sequence repeats length polymorphism (SSLP) may reveal substantial variation ²⁰

Many of the dyes used by textile industries are known carcinogens and teratogens. Dyes are introduced into the environment through industrial effluents of these industries. There are sample evidences of their harmful effects. Triple primary cancer involving kidney, urinary bladder and liver in a dye worker have been reported ²¹. Unlimited and uncontrolled use of such dyes can lead to grave consequences in terms of human health and ecological balance. Further all the three tested processing dyes or cremazoles dyes were so toxic that they inhibited the growth of bacteria, at higher dose levels. Again although orange 3R and Brown GR were moderately mutagenic (1200-1400 induced revertants, per 100µl of dye) while blue S1 turned out to be extremely mutagenic (15,000 induced revertants per 100µl of dye) strain TA 100 of *Salmonella typhimurium*, concluded that all of these six dyes cause genetic damage through base pair substitution mutations . The presence of impurities in the commercially available dyes has been reported to contribute to the mutagenicity of these dyes .

Figure 8. Analysis of Genetic variation by dendrogram

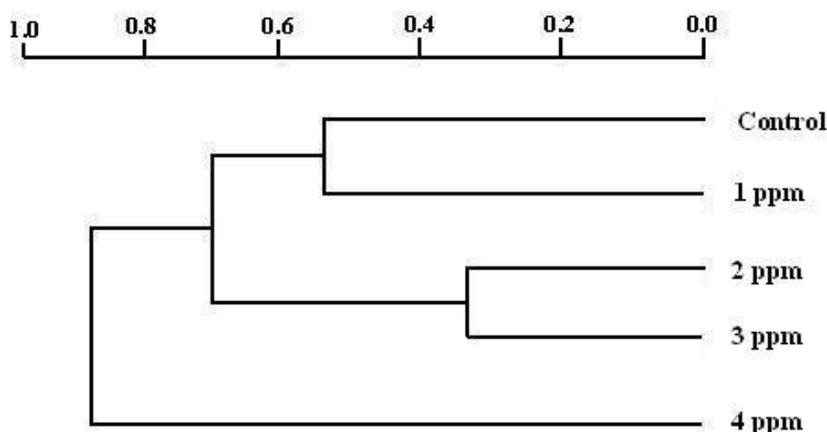
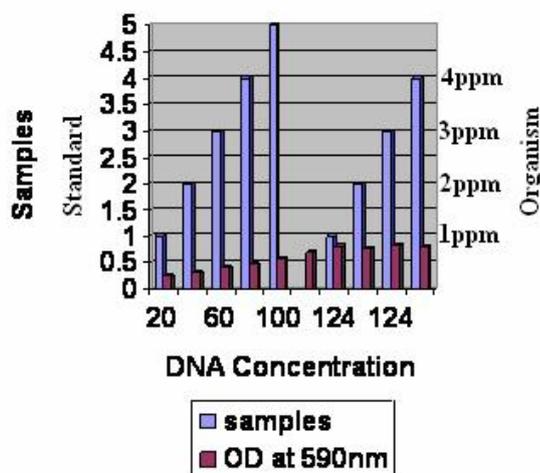


Figure. 9. The amount of DNA was quantitatively estimated by spectrophotometer analysis

Conclusion

This study led to the analysis of induced genetic variation by methyl orange in *Rhizobium* using RAPD (Random Amplified Polymorphic DNA). The *Rhizobium* was isolated and grown in YEM broth. To the culture different concentrations (1ppm, 2ppm, 3ppm, 4ppm) of methyl orange was added and a control was maintained without adding the dye. The growth was observed in the control and the growth of *Rhizobium* got decreased when the

concentration of the dye increased. Then the dye induced *Rhizobium* was subjected for DNA analysis. The result shows that the mutation was caused in the *Rhizobium* due to the action of dye in various concentrations, as it got compared with control. Therefore pretreatment is necessary for the disposal of such toxic dyes. Before performing pretreatment for the dyes it is necessary to monitor the dyes and it should be used in great caution.

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