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Altered 16S rRNA Gene Size in Agrobacterium Tumefaciens at IC50 of Heavy Metals

Ayman Younus Fathi[1], Najwa Ibrahim Khaleel Al-Barhawee[2]
1, 2. Department of Biology, College of Education for Pure Sciences, University of Mosul, Mosul, Iraq

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Corresponding author:
Name: Ayman Younus Fathi
Affiliation: Department of Biology, College of Education for Pure Sciences, University of Mosul, Mosul, Nineveh, Iraq
Email: ayman.21esp49@student.uomosul.edu.iq

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A B S T R A C T:

Agrobacterium tumefaciens is a soil-endemic bacterial species that causes crown galls disease on a range of medicinal and economically important plants. In this study, the diagnosis of this bacterium was confirmed by its formation of crown galls on the discs of carot. And when treated in a laboratory with eight different heavy metals (Mo, Fe, Zn, Cu, Cr, Cd, Ni, and Pb), and with ten concentrations (40, 20, 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078 mM/ Metal), it was found to have an inhibitory effect on the growth of these bacteria by microtiter plate method, especially when the concentration was increased. The size of the 16S rRNA gene in bacteria resistant to these metals varied at the half inhibitory concentration IC50 from the comparison sample with volume values of about 1500, 1000, 1000, 1500, 1500 , 1500, 1500, 1500, and 1000 base pairs, respectively.

Introduction

Plants that are exposed to the soil-borne, gram-negative bacterium Agrobacterium tumefaciens develop crown gall disease. The disease's molecular pattern has been researched for the past 20 years, Agrobacterium has also been utilized as a crucial model to comprehend how plants defend themselves during the interaction between plants and Agrobacterium. However, there is still a chance for Agrobacterium-mediated crown gall disease, and it is associated with a serious decrease in the yield of cultivated plants [1]. Heavy metal pollution poses many risks to the ecosystem and humans and affects the safety of the food chain, food quality, and the ability to use land for agricultural production, which in turn affects food security and exacerbates land tenure problems, and the contamination of arable land through agricultural practices with heavy metals Especially due to chemical fertilizers, pesticides, animal manure, compost, biosolid waste based on wastewater, and irrigation indirectly affect the growth, spread, and diversity of soil microbes [2]. Heavy metals are known to limit or impede the activity of soil enzymes, disrupt the transformation of carbon, nitrogen, and organic matter, and reduce the biodiversity and biomass of microorganisms [3], resulting in the predominance of particular HM-tolerating bacteria in soils [4]. The growth and reproduction of microorganisms are directly impacted by heavy metals, which lowers the production and metabolism of microbial enzymes [5]. Due to the established capacity of microorganisms, particularly bacteria, to sequester and convert toxic chemicals, microbial bioremediation has emerged as a viable method to lower the concentration of heavy metals in the environment [6]. Several studies have been conducted to understand how microbial communities in HMs-contaminated areas form and function [7], In order to develop the best and most efficient bioremediation strategy [8]. However, the published information has not been comprehensively analyzed so far to recommend the most efficient microbial resources for application in bioremediation or phytoremediation strategies with the help of bacteria that may help improve plant growth in contaminated soil. Agrobacterium as the most efficient based on several criteria:being part of the plant microbiome and being closely interacting with plant species, it is expressed for resistance to metals, especially heavy metals, at MIC, as well as susceptibility to metal resistance by biochemical or molecular mechanisms [9].

The focus of this laboratory study was on the extent of resistance of Agrobacterium tumefaciens, which causes crown galls disease, on agricultural crops, to eight types of heavy metals and ten concentrations for each type, with the determination of the concentration equal to or close to the half inhibitory concentration IC50 and its effect on obtaining variation in the size of the 16S rRNA gene for these bacteria.

Material and Methods

The bacteria used in this study:

The Central Environment Laboratory/University of Baghdad/Al-Jadriya provided the wild strain of *Agrobacterium tumefaciens*.

Testing the ability of bacteria to form crown galls in the laboratory:

Preparation of A. tumefaciens inoculm:

According to the methods of [10], [11]. carrot plants were cut into discs and sterilized with sodium hypochlorite solution at a concentration of 2% and 70% ethyl alcohol for some time. They were then washed with sterile distilled water each time to remove traces of the sterile substance. The pathogenicity test was conducted using carrot plant discs (*Daucus carota*). The surface of the carrot discs was inoculated by injecting it with a fine syringe needle containing the previously prepared bacterial suspension. The discs were transferred using sterile forceps to a sterile petri dish containing Water-Agar medium, incubated under lighting conditions of 16 hours of light and 8 hours of darkness, and were examined periodically to detect the development of tumors on it.

Testing the inhibitory effect of heavy metals salts on the growth of A. tumefaciens:

Preparation of the bacterial suspension:

The bacterial suspension was prepared by inoculating Luria Bertani (LB) liquid medium with several colonies of the bacteria under study, then incubated for 24 hours at a temperature of 28±2°C. The optical density of the lamp was measured until it reached a value of 0.01 using a UV-visible spectrophotometer.

Microplate preparation:

A twelve-row, eight-column microplate, equivalent to 96 wells, was used. The first column, one through eight, is named after the eight heavy metals used in the study: Mo, Fe, Zn, Cu, Cr, Cd, Ni and Pb. Grades one to

twelve were marked as follows: negative control, positive control, 40, 20, 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078 mM/metal.

Method of work:

The use of a Microtiter Plate can be summarized according to the method of [12], as follows:

1- In the first wells of the first column, 100 µl of medium and 100 microliters of metal were placed, and these pits were considered negative control. In the second pits of the second column, the medium was placed with a suspension of bacteria, and these pits were considered positive control.
2- In the third pits of the third column, 50 µl of each mineral were placed at a concentration of 40 mM, then a series of half-dilutions were made: 20, 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078 mM.
3- 40 µl of medium and 10 microliters of bacterial suspension were added from the third hole to the last hole and for all minerals in all their concentrations, then the Micotiter Plate was covered with its cover and incubated at a temperature of 28 ± 2 °C for 24 hours.
4- After measuring the optical density at a wavelength of 630 nm using an Eliza reader.

Molecular detection of mutations in the 16S rRNA gene affected by heavy metals:

A.tumefaciens was grown on solid LB media, fortified with heavy metals, at a concentration close to the half inhibitory concentration (IC50) for each metal, and incubated at 28 ± 2 °C for 24 hours.

DNA extraction from A.tumefaciens bacteria:

The DNA extraction process was carried out from the Agrobacterium bacteria that were grown in the previous paragraph with the comparison sample, which is the sample not treated with heavy metals, using solutions prepared by Promega as follows:

1- A carrier with a loop was used by transferring 2-3 campaigns from the young bacterial culture to an Eppendorf tube of 1.5 ml volume, and EDTA buffer solution was added to it and mixed well with the bacteria, then centrifuged at a speed of 13000-16000 rpm for 2 minutes.
2- After the buffer solution was removed, 600 µl of Nuclei Lysis Solution was added for the analysis of the cell wall of the bacterial cells, they were mixed well using the Vortex mixer for 10 seconds.
3- The samples were placed in the oven at a temperature of 80 °C for 5 minutes and left to cool at room temperature.
4- 3 µl of RNase Solution were added to it, and mixed well several times to get rid of RNA. Then the samples were incubated at a temperature of 37°C for 15-60 minutes and cooled to room temperature.
5- 200 µl of Protein Precipitation Solution was added, and mixed well for 20 seconds, then the samples were placed in the refrigerator for 5 minutes until the protein precipitated, then a centrifugation process was conducted at a speed of 13000-16000 rpm for 3 minutes.
6- Transfer the supernatant to a new sterile 1.5 mL Eppendorf tube containing 600 µl of Isopropanol. The tubes were placed in a centrifuge at a speed of 13000-16000 rpm for 2 minutes.
7- The filtrate and Isopropanol were discarded, and ethanolic alcohol was added at a concentration of 70% to make a wash for the DNA from the remains of Isopropanol and others, then centrifugation was carried out at a speed of 13000-16000 rpm for 2 minutes.
8- The ethanol was carefully removed, then left the Eppendorf tubes to air dry for 10-15 minutes.
9- 100 µl of DNA Rehydration Solution were added to the Eppendorf tubes, and the samples were incubated at a temperature of 65 °C for one hour. The solution was mixed periodically by gently tapping the tubes. The DNA was separated and kept at a temperature of -4 °C.

Prepare the agarose gel and Electrophoresis of the DNA:

The agarose gel was prepared at a concentration of 1.5% for electrophoresis of the extracted DNA and detecting it, by dissolving 1.5 g of agarose powder in 150 ml of TBE (1X) solution. Then it was placed in the microwave for 1-2 minutes and left to cool to a degree of 50 °C, add 3 microliters of red-safe dye, stirring to mix the dye with the solution well. Pour the agarose gel solution into the tray of the electrophoresis device, after the comb of Wells formation has been placed at the tip of the gel, taking into account that the process of pouring the gel is quiet to prevent the formation of bubbles. Room temperature for half an hour, then gently lift the comb. 5 microliters of genomic DNA sample and 3 microliters of DNA Loading Dye were used to create the electrophoresis samples. Following the loading of these samples into the agarose gel wells, the mold was moved to an electrophoresis tank filled with buffer solution (1X) TE. Turn on the relay by passing the samples at a voltage of 5 V/cm for 1.5-2 hours. Exposing the gel to ultraviolet light with a UV-transilluminator and at a wavelength of 260 nm, the visible bands were photographed with a system of Gel Documentation [13].

Amplification of DNA:

Adjust the concentration of DNA to complete the PCR reactions at 50 ng/µl using TE Buffer. The master reaction mixture was prepared by mixing the 16S rRNA primer and its forward and reverse parts by 1 µl each (Table 1) with 4 µl of extracted DNA sample as a template and added 5 µL of the Master mix components: dNATPS, MgCl, TE Buffer DNA polymerase [14], into a 0.2 mL Eppendorf tube supplied by Promega, bring the reaction volume to 20 µl using deionized water, and discard. Mix using a Microcentrifuge for 5 seconds to mix the reaction components. The tubes were inserted into a thermocycler to perform DNA amplification, depending on the optimal temperature conditions for the reaction cycles [15], which are shown in Table 2. The samples were loaded into the well inside the agarose gel prepared in advance at a concentration of 1%, with the DNA Ladder size guide of 100-200 base pairs and prepared by the same company (Promega) loaded into another well. The surface of the gel was immersed in 1X TE buffer, and the samples were analyzed using an electrophoresis device according to a voltage difference of 5 V / cm for 1.5 - 2 hours, after which the gel was exposed to ultraviolet light with a UV-transilluminator at a wavelength of 260 nm and the gel was photographed using a Gel Documentation device for detection About the beams and determining their molecular weights by comparing them with the volumetric guide.

Table 1. Primer of the 16S rRNA gene. [16].

Primer	Sequens
Forward	’5 AGAGTTTGATCMTGGCTCAG 3’
Revers	’5 AAGGAGGTGATCCARCCGCA 3’

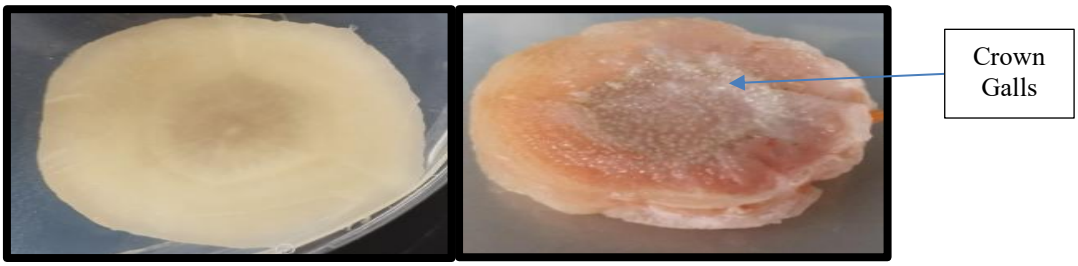
Table 2. Optimum conditions for thermocycler operation stages

No.	Stage	Temperature	Time	The number ofcourses
1	Start Copying	95	6 Minutes	1
2	The Freak	95	4 Second	
3	Primer Fusion with DNA	58	1 Minutes	5
4	Elongation	72	1 Minutes	
5	Final Elongation	72	5 Minutes	1

Results:

Pathogenicity test of A.tumefaciens :

The results showed when infection of carrot tablets with pathogenic bacteria A. tumefaciens by acupuncture method, the appearance of tumors on the surface of carrot discs after more than 30 days of infection, when compared to a negative control sample injected with sterile distilled water only, which did not show any pathological symptoms as shown in the figure below.



A:Control sample **B:Treated sample**

Effect of the heavy metals salts on the rate of optical density of A.tumefaciens:

The results presented in Table (3), regarding the effect of heavy metal salts on the growth of A.tumefaciens bacteria, showed the stimulating effect of Mo metal with an optical density index of 0.29 and 0.30 at the two highest concentrations of 20 and 40 mM, respectively, compared to the optical density of 0.28 for the comparison sample. As for the rest of the elements, it had a different inhibitory effect, reaching its maximum when using the element Cd, then the following elements in the order Cr, Ni, Cu, then Zn and Pb, which had an equal inhibitory effect, while the concentration was 40 mM of the strongest effect, according to the results of the statistical analysis and indicative of the letters indicated by the average concentrations of each metal.

Table 3. Effect of heavy metal salts on the average optical density of A.tumefaciens.

Mean	Heavy metals								Conc.Mm
	Pb	Ni	Cd	Cr	Cu	Zn	Fe	Mo	
0.10 k	0.09	0.08	0.03	0.05	0.07	0.08	0.10	0.30	**40**
0.11 j	0.12	0.09	0.04	0.07	0.09	0.10	0.12	0.29	**20**
0.12 i	0.13	0.08	0.06	0.09	0.10	0.11	0.14	0.27	**10**
0.14 h	0.14	0.12	0.09	0.11	0.12	0.13	0.17	0.27	**5**
0.15 g	0.15	0.13	0.10	0.13	0.13	0.14	0.18	0.25	**2.5**
0.17 f	0.17	0.14	0.12	0.13	0.14	0.16	0.20	0.24	**1.25**
0.18 e	0.19	0.16	0.13	0.15	0.17	0.18	0.22	0.26	**0.625**
0.20 d	0.20	0.17	0.15	0.18	0.19	0.21	0.24	0.26	**0.312**
0.22 b	0.22	0.20	0.17	0.21	0.22	0.23	0.26	0.27	**0.156**
0.25 a	0.24	0.23	0.20	0.24	0.24	0.26	0.28	0.29	**0.078**
0.28 c	0.28	0.28	0.28	0.28	0.28	0.28	0.28	0.28	**Control**
0.18	**0.18 c**	**0.15 e**	**0.13 g**	**0.15 f**	**0.16 d**	**0.17 c**	**0.20b**	**0.27 a**	**Mean**

The different letters indicate the presence of significant differences, the value of the least significant difference (LSD) for the interaction between minerals and concentration (0.003018), * Absorbance values are represented at a concentration close to or equal to the concentration IC50, which was dependent on the effect on the *16S rRNA* gene.

Molecular detection of bacteria under study after treatment with heavy metals salts:

Isolation and characterization of the genomic DNA of selected bacterial isolate under study:

Electrophoresis was carried out for DNA samples extracted from A.tumefaciens growing in a medium supplemented with concentrations close to or equal to the half inhibitory concentration IC50 and for each of the eight metals used under study, as well as the comparison sample. Using an agarose gel at a concentration of 1%, and after the bands were exposed to ultraviolet light, they were photographed, and it was found that the resulting bands varied in size with and between the comparison sample, according to the concentration and type of each mineral, as seen in Figure 2.

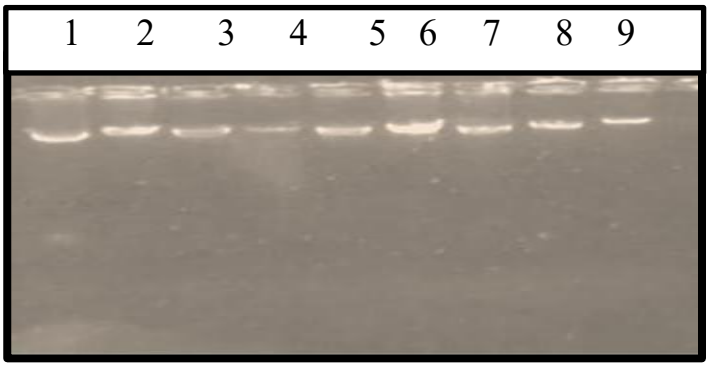


Figure 2. Electrophoresis of DNA content in 1% agarose gel isolated from A.tumefaciens. 1 (comparison sample), 2 (Cd), 3 (Mo), 4 (Pb), 5 (Fe), 6 (Zn), 7 (Cu), 8 (Ni) and 9 (Cr).

Results of specialized replication interaction for 16S rRNA gene of genomic DNA samples:

This reaction was performed for genomic DNA samples extracted from A.tumefacines bacteria as well as for the entire primer 16S rRNA gene in the presence of a DNA Ladder as shown in Figure 3. It is noted from the aforementioned figure the clarity of one inflated band of the bacteria under study, as a result of the double interaction of the general and specialized initiator of the 16S rRNA gene with DNA samples that appeared in different sizes and estimated their total size (1500, 1000, 1000, 1500, 1500, 1500, 1500, and1500 bp), in terms of its measurement with the DNA Ladder Volumetric Index, for each of the following minerals marked with numbers from 2-9: Cd, Mo, Pb, Fe, Zn, Cu, Ni and Cr, and at a concentration close to or equal to that of IC50/metal, respectively, as estimated by volume This gene in comparison samples and indexed by about 1000 bp.

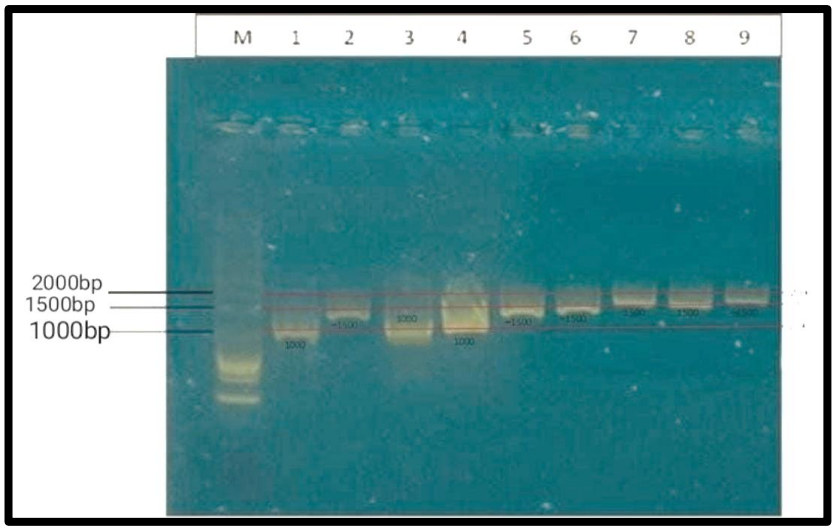


Figure 3. The product of the PCR reaction of Agrobacterium tumefaciens treated with heavy metals, the letter M represents the genetic marker.

Discussion:

The results of the formation of crown galls by the bacterium A.tumefaciens on the surface of carrot discs agreed with the results of the study of the researcher [17], and the researcher [18] in which the pathogenicity of this bacterium was tested on carrot discs. Coronary tumors appeared on discs Carrots are bumpy and have different colors between green and white 34 days after infection.

The results of the microtiter plate in this study showed that high concentrations of metals had a clear effect on the absorbance rate, except for molybdenum. And they found that copper, for example, had a negative effect represented by a decrease in the optical density value from 1.3, 1.03, and 0.500 when increasing the concentration of this metal from 300, 400, and 500 µg / ml, respectively [19]. Also, the study of researchers [20], showed, through the results of their study, a decrease in the optical density of bacteria by increasing the concentration of nickel metal from 0.5 to 1 and 2 mM, and it also decreased at a concentration of 0.5 and 1 mM of cadmium metal to 2.4 and 1. 5 respectively, compared to the comparison sample.

The size of the 16S rRNA gene in the comparator isolates in this study was estimated at about 1000 base pairs, which is close to what the researcher [21] found when studying the genetic diversity of ten isolates in Egypt, as they produced bundles with a size ranging between 850-1000 bp.

The results also showed that there is a difference in the size of this gene between the isolates treated with close or equal concentrations of the half inhibitory concentration IC50 of heavy metals. Therefore, this simple change may indicate the occurrence of a mutation of the type of spontaneous mutations that occur at a rate of 1 in 105 to 108, which has a role in the random variation that occurs. in soil endemic microorganisms [22].

In this regard, [23], mild mutations have a medium to strong effect in preventing the translation process, and the majority of these mutations tend to accumulate in rRNA sites of

high functional importance and are found in nucleotides that show >98% conservation in 16S rRNA of different types of bacteria. The mutations observed in conserved rRNA sites, including those conserved across evolutionary domains, had only weak or even no developmental defects [24], and this is identical to what we observed in this study. It resisted the presence of minerals and grew well on the nutrient medium. On the other hand, researchers' results varied about the effect of mutations on the function of this gene [25] indicated that they did not affect the function of this gene, because its structural composition did not change. While [23] confirmed that twelve of the identified mutations occurred in the target sites of this gene, about half of the mutations coincided with known functional sites in the ribosome, while the remaining mutations affected ribosomal sites of less clear functional significance. On the other hand, the 16S rRNA gene has been the mainstay of molecular technologies for decades, but the use of a universal primer that represents parts of the 16S rRNA gene does not give the desired results, because sometimes highly variable regions may not be affected, but at the same time highly conserved regions may be affected. overexpression [26], [27], so an initiator representing the entire gene was used in this study, which has been adopted recently in the past few years [28]. It is a common axiom that genotyping methods, such as comparing the nucleotide sequence of the bacterial 16S rRNA gene, have become the preferred genetic method because it allows diagnosis at the level of the genus, species, and even subspecies

[28]. While the isolate used did not show a sufficient level of homology due to insufficient sequencing data on the full-length 16S rRNA gene [29].

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Competing Interests

The authors declare the following competing interests, the authors declare no competing interests.

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