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Genotoxicity of Graphene in Escherichia coli

Ananya Sharma
University of Arkansas, Fayetteville

Genotoxicity of Graphene in *Escherichia coli*

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Ananya Sharma
Chaudhary Charan Singh University
Bachelor of Science in Biotechnology, 2010
Mississippi State University
Master of Science in Biochemistry and Molecular Biology, 2013

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University of Arkansas

This thesis is approved for recommendation to the Graduate Council.

_____
Dr. Ravi D. Barabote
Thesis Director

_____
Dr. Douglas D. Rhoads
Committee Member

_____
Dr. Young Min Kwon
Committee Member

_____
Dr. Ralph L. Henry
Committee Member

ABSTRACT

Rapid advances in nanotechnology necessitate assessment of the safety of nanomaterials in the resulting products and applications. One key nanomaterial attracting much interest in many areas of science and technology is graphene. Graphene is a one atom thick carbon allotrope arranged in a two-dimensional honeycomb lattice. In addition to being extremely thin, graphene has several extraordinary physical properties such as its exceptional mechanical strength, thermal stability, and high electrical conductivity. Graphene itself is relatively chemically inert and therefore pristine graphene must undergo a process called functionalization, which is combination of chemical and physical treatments that change the properties of graphene, to make it chemically active. Functionalization of graphene is of crucial importance as the end application of graphene depends on proper functionalization. In the field of medicine, graphene is currently a nanomaterial of high interest for building biosensors, DNA transistors, and probes for cancer detection. Despite the promising applications of graphene in several areas of biomedicine, there have been only few studies in recent years that focus on evaluating cytotoxicity of graphene on cells, and almost no studies that investigate how graphene exposure affects cellular genetic material. Therefore, in this study we used a novel approach to evaluate the genotoxicity, i.e., the effects of graphene on DNA, using *Escherichia coli* as a prokaryotic model organism.

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INTRODUCTION

Graphene is a one-atom-thick planar sheet consisting of sp2 carbon atoms that are densely packed in a honeycomb crystal lattice. Graphene has many unique properties such as high surface area, high electrical conductivity, high thermal conductivity and high optical transmittance. Due to these unique chemical and structural properties, graphene has been attracting interest in several commercial fields. Specifically, in the field of medicine, graphene is currently a nanomaterial of interest for building biosensors, DNA transistor and even biosensor for cancer detection (Sun *et al.* 2008; Park *et al.* 2009; Shao *et al.* 2010; Feng and Liu 2011; Kuila *et al.* 2012; Wang *et al.* 2012; Chung *et al.* 2013; Liu *et al.* 2013; Chabot *et al.* 2014).

Even though scientists had theorized graphene since the 1980s it was produced and isolated in the lab for the first time in 2004. Andre Geim and Konstantin Novoselov, at the University of Manchester were the first ones to isolate pristine graphene from graphite and their contribution towards the isolation and characterization of graphene (Novoselov *et al.* 2005) won them the Nobel Prize in Physics in 2010. Pristine graphene, which is composed of only sp2 carbon atoms, is a zero-gap semiconductor which is why it is chemically inert and needs to be functionalized in order for graphene to have the desired chemical and physical attributes to be used in the development of graphene based devices.

Functionalization of graphene is one of the key topics in graphene research (Georgakilas *et al.* 2012, Kulia *et al.* 2012; Chabot *et al.* 2014). Generally, there are two main categories of functionalization: chemical and nonchemical. Chemical functionalization is carried out through the formation of new covalent bonds between the atoms native to graphene and different functional groups (such as -O, –COOH, and -OH). In contrast, nonchemical functionalization is

mainly based on non-covalent interaction between functional molecules and graphene. Both types of functionalization change the properties of pristine graphene, but the chemical routes are more effective (Hu and Sun 2008). However, the addition of certain chemical groups to graphene has been shown to cause oxidative stress and toxicity in bacterial cells (Akhavan 2010; Sanchez 2011; Yue *et al.* 2012; Yang *et al.* 2013). Hence, characterization of the bioactivity of various graphene derivatives is very important so that we can develop graphene based materials and devices that have minimum risk of toxicity to living organisms and also so that these materials can be disposed and degraded effectively without causing any alteration to the ecological balance (Bussy *et al.* 2012).

Since its discovery and successful isolation, technology for the use of graphene and its derivatives is being developed actively (Chung *et al.* 2013). Due to its excellent properties, there is a growing interest in use of graphene based nanomaterials in biomedical devices. This means that the interaction of graphene with human cells and other living cells will increase with the increased use of this nanomaterial. This is the very reason which is driving the study of its biological activity as well. It is necessary to evaluate environmental risks of graphene-containing technological objects to biological systems (Akhaven 2010; Bussy *et al.* 2012). Studies so far have evaluated the effects of graphene on living cells, most importantly its effect on cell viability and proliferation. Graphene toxicity studies show that its number of layers, lateral size, stiffness, hydrophobicity, surface functionalization, and dose are important factors that determine its effects on cells (Adams and Jia 2005, Georgakilas *et al.* 2012, Katz and Hershberg 2013, Keseler *et al.* 2013). However, the toxicity and biocompatibility of graphene are debated (Bianco 2013). Evaluating the activity of graphene against bacteria is an important first step to understanding graphene's bioactivity. Prokaryotic model organisms provide the basis for understanding of

toxicity mechanisms of graphene on a simpler scale due to their relatively simple physiological manifestation (Efremova *et al.* 2015).

In 2010, Akhavan and Ghaderi first described the toxic effect of graphene against several bacterial species and also showed that graphene oxide (produced through functionalization process) was more toxic to cells when compared to pristine graphene. Since then, the toxicity of different forms of graphene against bacteria has been studied extensively, but the results in these studies are somewhat contradictory. Most studies have used some form of functionalized graphene and compared the effects of functionalized graphene to the effects of pristine graphene on cells. In addition to the research on properties of functionalized graphene, there have been several studies that have linked the toxicity of graphene based materials on the presence of rough edges of graphene which cause physical damage to cells (Liu 2011).

There have also been several studies that have looked at the interactions between graphene based nanoparticles and human tissues and cells. Most specifically, these studies have focused on the uptake and cellular response of macrophages to graphene nanoparticles and histopathological response to deposition of these nanoparticles (Liao *et al.* 2011; Bussy *et al.* 2012). All studies that have been published so far have focused only on the cytotoxic effects of graphene.

Previous unpublished work in our lab has characterized the cytotoxicity of oxidized graphene to *Escherichia coli* cells and the data suggests that concentrations greater than 160 µg/ml of the functionalized graphene (FG) is toxic to *E. coli*. Cultures that were exposed to commercial graphene (CG), i.e., pristine graphene, at these concentrations, however, did not show decreases in cell density. This work served as the foundation for genotoxic analyses

described here as the graphene concentrations used in the present study were based on the above cytotoxicity analyses.

Since there are no studies to date that investigate the effects of graphene on the DNA of cells we devised a novel approach to do so. The first step in this study was to evaluate the mutagenic potential of graphene by using Fluctuation Assay. Fluctuation Assay, also known as a Luria-Delbruck experiment, was first proposed and demonstrated by Nobel laureates Max Delbrück and Salvador Luria in 1943 (Luria and Delbrück, 1943). This assay was developed to assess the random nature of mutations that arise in a population of cells in the absence of any selection pressure. For these experiments, small numbers of cells are used to inoculate several parallel cultures (C) of bacteria that are grown to saturation in a non-selective growth medium. Several dilutions are then plated onto selective media (e.g. antibiotic containing media) to get an estimate of the number of mutants in each culture (*r*). Based on the number of mutants that grow on selective media, mutation rates can be estimated using several mathematical equations (described in the Results and Discussion chapter). Mutation rates are more reliable than merely calculating the frequency of mutants as mutation frequency varies greatly between the parallel cultures. This is because mutations are random in nature and the mutations that arise in earlier generations will be more prevalent than the mutations arising in later generations. To normalize for this variation, it is important to calculate mutation rate. The methods we have used for mutation rate calculation are based on either mean or median mutation frequency (see Results and Discussion section).

Next, we assessed the nature of mutations that graphene exposure causes to the *E. coli* cells by sequencing the whole genome of representative rifampicin resistant mutants isolated in the Fluctuation Assay (Katz and Hershberg 2013). Mutations are changes in the DNA

sequencing that are inherited through generations. If a mutation causes an alteration in the amino acid sequence of protein then it is termed as nonsynonymous substitution and if the mutation does not produce any change in the protein sequence then these changes are called synonymous mutations. We used the Illumina MiSeq platform for sequencing the genomic DNA of these mutants. MiSeq is a next-generation sequencing platform which produces reliable highthroughput data (Quail *et al.* 2012). Paired-end reads produced from a MiSeq sequencer were assembled to get the genome sequence of the mutants and parent, which were subsequently used to analyze the patterns of mutations. Preprocessing of the DNA sequence data was performed on Galaxy server (https://usegalaxy.org/) and the assemblies were generated and analyzed using software package DNASTAR (DNA Star Inc.). We then used EcoCyc and UniProt to understand the function of the genes that were affected by the mutations in the genome of the mutants.

Lastly, we conducted growth analysis of the mutants (Davison *et al.* 2007, Wehrli 1983) to evaluate if the mutations confer any physiological advantage to *E. coli* cells for growth in presence of graphene.

MATERIALS AND METHODS

Media and Culture Conditions

The *Escherichia coli* strain used in this study is DH5 alpha [F- Φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rk-, mk+) phoA supE44 λ-thi-1 gyrA96 relA1]. The *E. coli* DH5α cells were revived from glycerol stocks by scraping off some cells from the frozen glycerol stock and streaking them on Luria-Bertani agar plate followed by incubating the plate at 37°C for 12 hours. The liquid media used for culturing *E. coli* DH5α cells in this study is Luria-Bertani broth (Sezonov *et al.* 2007), which is a rich medium for *E. coli* growth. The composition of Luria-Bertani media per liter is 10.0 g Tryptone, 5.0 g Yeast extract, 5.0 g Sodium chloride. While performing Fluctuation Assay, Luria-Bertani broth was supplemented with 100 μg/ml of Rifampicin. Rifampicin stock was prepared using the protocol suggested by Sambrook *et al*. (2012). Solid media used in the study was Luria-Bertani agar (Luria-Bertani broth + 10.0 g/L agar). Luria-Bertani agar was also supplemented with 100 μg/ml Rifampicin, where appropriate.

Graphene Source and Properties

Graphene used in this study was provided by the Arkansas Research Alliance. From the dry graphene stock received, 2 mg each of either commercial (pristine) graphene (CG) or functionalized graphene (FG) were weighed and were resuspended in 1 ml double autoclaved water to make stock solutions of concentration 2 mg/ml. Final concentration of commercial and functionalized graphene used in the Fluctuation Assay experiments was 80 μg/ml, so 120 μl of the stock graphene solution was added to each 3 ml liquid culture.

Fluctuation Assay

We used Fluctuation Assay (Rosche and Foster, 2000) to estimate the frequencies of rifampicin resistance in *E. coli* DH5α cells in order to estimate the spontaneous mutation rates in *E. coli* DH5α cells. Fluctuation Assay was performed in two rounds using either shaken or unshaken cultures of *E. coli*. In the first round, we used ten parallel, shaken cultures of *E. coli* DH5α to estimate the intrinsic spontaneous mutation rate in the absence of any exposure to graphene. Briefly, ten each 14 ml culture tubes containing 5 ml Luria-Bertani broth were inoculated with ~1000 *E. coli* DH5α cells from a common starter culture. These ten parallel *E. coli* DH5α cultures were then incubated overnight at 37°C with 250 rpm shaking. After incubation, 1 ml of culture was taken in a cuvette to measure the OD_{600} value using a spectrophotometer to quantitate the cell density of each culture. Out of the remaining 4 ml of culture, 100 μl was spread directly on LB-agar plates containing Rifampicin, and 1 ml was concentrated 10-fold before being spread on LB-agar plates containing Rifampicin. For concentrating the cultures, 1 ml was taken from each of the ten culture tubes in ten 1.5 ml microcentrifuge tubes and centrifuged at 10,000 rpm at 23°C for 10 minutes. 900 μl of supernatant was decanted from the microcentrifuge tube and the cell pellet was resuspended in the remaining 100 μl broth in the tube by vortexing. These 10-fold concentrated cultures (100 μl in volume) were spread on LB-agar plates containing Rifampicin. All 20 plates from this experiment were incubated at 37°C for 36 hours before counting the number of colony forming units (CFUs) on each plate.

In the second round of Fluctuation Assay, we set up 5 parallel, non-shaken cultures each for three different treatments. Fewer parallel cultures were used because the amount of graphene received was limited, and cultures were not shaken in order to minimize the shearing effects on

cells caused by graphene (Liu 2011). Cells were grown in 3 ml of either LB broth alone (control), LB broth containing 80 μg/ml of commercial graphene (CG), or LB broth containing 80 μg/ml of functionalized graphene (FG). Five 14 ml culture tubes (per treatment) were inoculated with ~1000 *E. coli* cells from a common starter culture. The-cultures were incubated for 14 hours at 37°C without shaking. After incubation, 100 μl was spread directly on LB-agar plates containing Rifampicin, and also 1 ml from the culture was concentrated 10-fold before being spread on LB-agar plates containing Rifampicin. We spread three dilutions (10^{-5}, 10^{-6}, 10^{-7}) of the cultures on LB-agar plates to obtain an estimate of total viable cells per culture. We incubated these plates at 37°C for 12-36 hours.

After 12 hours of incubation, CFUs on the LB agar plates were counted to estimate the total number of viable cells per culture. After 36 hours of incubation, CFUs on the Rifampicin supplemented LB agar plates were counted to estimate the total number of Rifampicin resistant mutants.

Mutation Frequency and Mutation Rate calculation

Frequency of spontaneous mutations to Rifampicin resistance was estimated based on the second round of Fluctuation Assay by comparing the number of mutant colonies that grew on Rifampicin supplemented plates against the total number of cells in the culture (estimated based on the OD_{600} values). For estimating the mutation rate we used three independent methods described previously (Roshe and Foster, 2000), namely the Luria-Delbrück's Method of the Mean (Luria and Delbrück 1943), the Lea-Coulson's Method of the Median (Lea and Coulson 1949) and, the Drake Formula using the median (Drake 19991).

Genomic DNA Isolation

We isolated genomic DNA (gDNA) from six representative rifampicin resistant *E. coli* mutants and the parent strain (WT) for sequencing using the Illumina MiSeq platform. Procedure for DNA isolation is described below.

After growth on Rifampicin supplemented LB agar plates we randomly picked six individual colonies from independent plates for sequencing. Only one colony was picked from any given plate, and each colony originated from a different sample within each treatment. Genomic DNA extraction of each of these mutants was performed using Qiagen DNeasy Blood and Tissue Kit. Selected colonies were grown overnight in 5 ml LB broth supplemented with Rifampicin at 37°C with shaking at 250 rpm. The bacterial cells were then harvested by centrifuging the cultures for 10 minutes at 7500 rpm, supernatant was discarded and cell pellet was resuspended in 180 µl enzymatic lysis buffer and incubated at 37°C for 1 hour. The composition of the lysis buffer was 20 mM Tris-HCl, 2 mM Na-EDTA, 1.2% Triton X-100, and 20 mg/ml of lysozyme from chicken egg white (Sigma-Aldrich). After 1 hour of incubation, 180 µl buffer ATL was added to the cell lyaste followed by addition of 20 µl of proteinase K and then the tubes were incubated at 56°C for 30 minutes to ensure complete lysis of the bacterial cells. Following cell lysis, the tubes were briefly vortexed and-200 µl of buffer AL was added to the samples and the samples were vortexed briefly. Then, 200 µl of 99% ethanol was added to the samples followed by brief vortexing. The mixture was carefully transferred to DNeasy Mini spin column and centrifuged at 8000 rpm for 1 minute after which the flow-through was discarded. Buffer AW1 (500 µl) was added to the column and the column was then centrifuged at 8000 rpm for 1 minute following with the flow-through was discarded along with the collection tube. Spin column was placed in a new collection tube and 500 µl of buffer AW2 was

added to the column. The column was centrifuged at 14,000 rpm for 3 minutes for the DNeasy membrane to dry. Flow-through was discarded along with the collection tube and the DNeasy Mini spin column was placed in a clean 1.5 ml Eppendorf tube and 50 µl of pre-warmed Qiagen elution buffer water was added directly to the DNeasy membrane. The column was incubated for 3 minutes at room temperature and then we centrifuged the column at 8000 rpm to elute the DNA. We repeated the elution step one more time by transferring the column to a clean 1.5 ml Eppendorf tube and adding 50 µl of pre-warmed Qiagen elution buffer, incubating the column for 3 minute at room temperature, and then centrifuging the column at 8000 rpm to elute the DNA.

For extraction of genomic DNA from *E. coli* DH5α, the protocol described above was used with some minor modification; we used RNase treatment in the protocol since the initial gDNA preparations were contaminated with ribosomal RNA. We added 4 µl of RNase A (100 mg/ml) to the cell lysate and the microcentrifuge tube was incubated at room temperature for 2 minutes which was followed by the addition of buffer AL (neutralization buffer) and ethanol. The DNA extraction steps following the RNase treatment were the same as described above.

Genomic DNA Quantitation

We used NanoDrop (Thermo Scientific) and Qubit assay (Thermo Fisher) to evaluate the quality of our genomic DNA samples and also to estimate the concentration of genomic DNA. For quantifying the genomic DNA, we used Qubit dsDNA BroadRange Assay kit (Life Technologies Inc). For each sample quantified, we diluted the assay reagent 200x in the buffer solution provided in assay kit. We also prepared Standards 1 and 2 from the assay kit to calibrate the Qubit fluorometer before quantifying the samples. As per manufacturer's protocol, 10 µl each

of standard 1 and standard 2 were mixed with 190 µl of diluted reagent in two Qubit assay tubes, followed by brief vortexing and centrifugation of these standard solutions. We then mixed 1 µl of the genomic DNA into 199 µl of diluted reagent in Qubit assay tubes and mixed the sample by vortexing the tubes briefly and then centrifuging the tubes briefly. The tubes were then incubated for 1 minute at room temperature before placing the tubes in Qubit fluorometer to get the concentration values of the sample. We used the 'calculate stock concentration' option on the fluorometer to get the total concentration of the sample. We used NanoDrop to get Abs_{260}/Abs_{280} ratio of the samples so we could be sure that the samples were free of contamination from proteins. In doing so, we used 1µl from each of the sample and measured its absorbance using NanoDrop.

Agarose Gel Electrophoresis

We separated 1 µl of each of the rifampicin mutant genomic DNA samples and 5 µl of *E. coli* DH5α genomic DNA sample on an agarose gel to check the quality of extracted genomic DNA. Agarose gel (1% concentration) was prepared by dissolving 0.5 g agarose in 50 ml 0.5X TBE buffer by heating in a microwave for 1 minute. The liquid agarose gel was then allowed to cool for 5 minutes before adding 5 µl SYBR Safe DNA gel stain and the liquid agarose gel was poured in gel tray with the well comb in place and was left for 30 minutes for solidifying. After 30 minutes, the well comb was removed and the gel was transferred to electrophoresis chamber filled with 0.5X TBE and samples were loaded in gel wells. We ran the gel at 80V for 90 minutes. DNA imaging protocol for SYBR safe (ThermoFisher Scientific) was used on a Bio-Rad Molecular Imager Gel Doc (Bio-Rad) to visualize DNA.

Genome Sequencing

After checking the quality and quantity of purified genomic DNA from the six Rifampicin resistant mutants (3 mutants from commercial graphene (CG) treatment and 3 mutants from functionalized graphene (FG) treatment) and the *E. coli* DH5α genomic DNA, we provided approx. 250 ng of genomic DNA of each sample to the UAMS DNA Sequencing Core Facility for shotgun whole genome sequencing. The gDNA samples were sequenced on Illumina MiSeq platform. DNA samples from the six mutants were bar coded and pooled together prior to being run on a single MiSeq cartridge. A total of 23,346,132 paired-end reads (2 x 250 bp) were obtained for the six pooled mutant genomes, and 33,857,684 paired-end reads (2 x 250 bp) were obtained for *E. coli* DH5α gDNA, which was sequenced separately to obtain a higher coverage reference sequence.

Sequence Data Preprocessing

The Fastq sequence read files obtained from the sequencing facility were uploaded on Galaxy server (https://usegalaxy.org/). Fastq files were first groomed using FASTQ groomer package followed by trimming of low quality reads using FASTQ quality trimmer package. Sequences with a quality score less than 30 were trimmed from 5' and 3' ends using sliding window (Window size 1, Step size 1), as well as reads with zero length were excluded from the files. Quality of filtered and trimmed reads was assessed through FASTQC and the sequence read files were then downloaded for subsequent analyses.

Genome Sequence Analysis

After downloading the filtered and trimmed read files, the two files containing the left and right paired-end sequences were concatenated in one file. After this step, we had 7 files, one each for the six mutants and one for the parent strain. The genome of *E. coli* strain K-12 MG1655 was downloaded from NCBI and was used as template for assembly of all seven genomes. The reads were first assembled using SeqMan NGen software (DNAStar, Inc.) using the Templated Assembly Workflow using *E. coli* strain K-12 MG1655 as template. High SNP Filter stringency was used while assembling the reads.

For each assembly generated through SeqMan NGen, SNP reports were created using SeqMan Pro. SNPs were filtered using High SNP Filter stringency (%SNP >= 75% and coverage depth >= 40). After filtering out the ambiguous SNPs, the remaining SNPs were confirmed and exported as tab delimited files. We compared the nucleotide changes between the parent strain (WT) and the rifampicin resistant mutants. Downstream analyses of these SNPs were done manually by comparing the position of nucleotide change in reference sequence and nucleotide change occurring at each position in the mutants. We used EcoCyc (Keseler *et al.*) and UniProt (UniProt Consortium 2015) to find the SNP containing genes and to find the pathways that these genes are involved in.

Growth Analysis of Mutants

We performed growth analysis (Davison *et al.* 2007, Wehrli 1983) of the rifampicin resistant mutants that were isolated from Fluctuation Assay performed in the presence of graphene in the growth medium. For doing so, we revived glycerol stocks of two CG and two FG mutants on LB-agar plates containing rifampicin (100 µg/ml). We used the parent *E. coli* DH5α

as the control (wildtype, WT) for this experiment which was revived on LB-agar plates. We then picked a single colony from each plate and inoculated starter culture. For wildtype *E. coli* DH5α we used 2 ml LB as growth medium, whereas 2 ml LB broth supplemented with rifampicin (100 µg/ml) was used for growth of CG and FG mutants. All culture tubes were incubated for 12 hours at 37°C with 250 rpm shaking. OD_{600} for each culture was measured for each culture after incubation. The cell density for each culture was estimated. Approximately 10^6 cells from each culture of the mutants and wildtype *E. coli* DH5α were used to inoculate LB broth (supplemented with rifampicin for CG and FG mutants) in a 96-well plate. Commercial graphene (CG) or functionalized graphene (FG) was added at concentrations 0 µg/ml (control), 80 µg/ml, or 320 µg/ml. The 96-well plate was then incubated at 37°C for 6 hours. Following incubation, dilution plates were made for each treatment using LB broth as medium with seven successive 10-fold dilutions. From these dilution plates, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} dilutions were plated on LB agar plates and the plates were incubated at 37°C for 14 hours before counting the number of colony forming units (CFUs) on each plate in order to estimate the number of viable cells in each treatment.

RESULTS AND DISCUSSION

Frequency of spontaneous mutations to rifampicin resistance in *E. coli*

To estimate the intrinsic rate of spontaneous mutations to rifampicin resistance in *E. coli* DH5α cells we set up Fluctuation Assay (Rosche and Foster 2000) with 10 parallel, shaken cultures. Cultures were grown in LB broth for 12 hours. Cell density was measured using OD_{600} and cell count was estimated using an online calculator (http://www.genomics.agilent.com/biocalculators/calcODBacterial.jsp). A complete list of OD_{600} values and the corresponding cell densities is given in Table I. As seen from the data, the OD_{600} values ranged from 1.099, which corresponds to about 8.79×10^8 cells/ml, to 1.237, which corresponds to about 9.9×10^8 cells/ml.

To determine the frequency of spontaneous mutations to rifampicin resistance (Rif^R), aliquots from each culture were plated on LB-agar plates supplemented with rifampicin. The numbers of Rif^R colonies per culture are given in Table II. From this experiment we estimated that the frequency of spontaneous mutations to rifampicin resistance (Rif^R) in our Fluctuation Assay cultures was about 4.76 (± 2.71 standard deviations) mutants per 10^8 cells. Frequency of spontaneous mutations to rifampicin resistance has been previously reported to be 2.6×10^{-8} in *E. coli* strain K12 MG1655 (Katz and Hershberg, 2013) which is comparable to the mutation frequency we observe in our experiment. The Rif^R frequency we determined through this preliminary experiment helped us plan the following experiment as the Rif^R frequency was neither too high nor too low for a 5 ml culture volume to be used in our subsequent Fluctuation Assay.

Effect of graphene exposure on the rate of spontaneous mutations to rifampicin resistance in *E. coli*

In order to estimate the rate of spontaneous mutations to rifampicin resistance in *E. coli* cells upon exposure to graphene, we performed the Fluctuation Assay with five parallel cultures containing either commercial graphene (CG) or functionalized graphene (FG) or no graphene (control). Table III lists the number of colonies formed on each plate and the cell number calculated based on the number of colony forming units (CFUs) on plates. We estimated cell numbers in each culture by counting the colonies on LB agar plate as we could not measure the OD_{600} for cultures containing graphene. We could not rely on OD_{600} values for these cultures as graphene particles in media would interfere with measurement of the optical density of cultures. We excluded the cultures where no colonies grew on rifampicin plates from these analyses (indicated as No Data (ND) in Table IV). We estimated average cell number in the cultures grown in LB broth, LB broth containing commercial graphene, and LB broth containing functionalized graphene. The cultures that were grown in the presence of graphene (either commercial or functionalized) on average have 5 times less cells than the cultures grown in LB broth without graphene. The cell number in the previous experiment (Fluctuation Assay in the absence of graphene) was higher than in this experiment as the culture growth conditions (shaking vs. non-shaking, respectively) were different between the two experiments. Cultures tubes were incubated without shaking to prevent any potential physical damage to cells by the rough edges of graphene (Liu 2011).

From this experiment, we estimated the frequency of Rif^R mutations (Table IV). The Rif^R frequency was found to be around 3.98 mutants per 10^8 cells in the control (LB alone). The commercial graphene (CG) and functionalized graphene (FG) treatment had Rif^R frequencies of

3.93 and 8.74 per 10^8 cells, respectively. These data indicate an increase in the frequency of Rif^R mutants in cultures exposed to functionalized graphene. The value of Rif^R in CG treatment is comparable to that of LB alone (control). On the other hand, in FG treatment, the Rif^R frequency is 2-fold higher than that of control (LB alone) or CG treatments.

After determining the frequency of Rif^R mutations, we wanted to calculate the mutation rate under each treatment. For calculating the mutation rate for each treatment (LB control, CG, and FG) we used three methods; the Luria- Delbrück (Luria and Delbrück 1943) method of the mean ($r=m$ ln(mC)), the Lea-Coulson (Lea and Coulson 1949) method of the median ((r/m) – ln(m) = 1.24), and the Drake (Drake 19991) formula ((r/m) – ln(m) = 0). In these equations, r is the observed number of mutants in a culture, C is the number of cultures in the experiment, and m is the number of mutants per culture. The values for mutation rates calculated using these methods are given in Table V. As the data suggests, all three methods give us a different value for the mutation rate but if we compare the three mutation rates values for each treatment we see that there is an increase in the mutation rate in cultures exposed to functionalized graphene. The mutation rate values for CG treatment was higher than the untreated control (LB) when Luria-Delbrück method of the mean was used to calculate mutation rate, but lower when we used the methods of median (Lea-Coulson and Drake formula). This could be due to the fact that data was available for only three CG-treated cultures (see Table IV), which may have skewed the calculation of the mean. Mutation rate values for FG treatment were higher than for either control (LB alone) or CG treatment regardless of the mutation rate calculation method used. The increased mutation rate values for FG treatment suggests that functionalized graphene is genotoxic to *E. coli*.

Whole genome sequencing and analysis of *E. coli* mutants and parent (WT)

After determining the mutation rates for cells grown in the presence of graphene, we wanted to investigate the nature of mutations in the genomic DNA of mutants obtained in the presence of CG or FG treatment. For this, we sequenced the genomic DNA of random Rif^R mutants. We picked a total of six Rif^R mutant colonies, three each from CG and FG treatments. These mutants were grown overnight in LB broth supplemented with Rifampicin and genomic DNA was extracted using Qiagen DNeasy blood and tissue kit. To assess the quality of the samples, 1 µL from each genomic DNA sample was run on agarose gel (Figure 1). For comparing the size of extracted genomic DNA, we ran all samples against 1 kb ladder from NEB with a range of 10,000 bp-500 bp. All samples ran as a single band >10,000 kb without any smear or accessory band which indicates that the extracted genomic DNA was of high quality. We then quantitated the samples using Qubit BR DNA assay and NanoDrop. Concentration and Abs_{260}/Abs_{280} ratios for each sample is given in Table VI. Values of Abs_{260}/Abs_{280} ratio of between 1.8-2.0 are indicative of pure DNA that is free of protein contamination. Approximately 250 ng from each sample was then sent for sequencing on Illumina MiSeq at UAMS DNA sequencing facility.

We also sequenced the parent strain in order to get more reliable insight on the changes in DNA that may have been caused by graphene exposure. We extracted genomic DNA from parent (WT) cells and sent approx. 250 ng to UAMS DNA sequencing facility for Illumina MiSeq sequencing. Qubit concentration and Abs_{260}/Abs_{280} ratio obtained from Nanodrop for the WT sample are given in Table VI. Figure 2 is the agarose gel image of the 5 µL of genomic DNA ran against 1 kb ladder. The WT gDNA ran as single band >10,000 kb which confirms the quality of DNA extracted. For sequencing we used DNA from first elution as labeled in Figure 2.

Sequence reads that we acquired were first processed on Galaxy server (https://usegalaxy.org/) and after filtering out the low quality reads we imported the sequence reads in DNASTAR software (DNAStar, Inc.). A summary of reads from Illumina MiSeq NextGen sequencing is shown in Table VII. The paired-end reads for each mutant sample were aligned to the reference *E. coli* genome K-12 MG1655 and the reads assembled into a single contig of 4,639,675 bp without gaps which is the same length as the reference genome. The length of each contig with gaps along with the median coverage values for each contig is listed in Table VIII. All assembled contigs had N50 value of 4641k.

Sequence reads of the genomic DNA of the parent strain (WT) were also processed and assembled in the exact same way. Table VII lists the number of reads for the sample and the number of low quality reads that were filtered out using the Galaxy server. The number of reads and coverage for the parent strain were substantially higher than those of the mutants as the six mutant samples were multiplexed whereas the parent strain was not. The median coverage and contig length for parent strain are listed in Table VIII.

We used SeqManPro in the DNASTAR software to generate SNP reports for each of the seven assembled genomes (parent and six mutants). From the SNP reports generated we identified SNPs using very high stringency parameters, namely read depth of 40 or higher and %SNP of 75% or higher. SNPs with low coverage score were rejected from further analysis. Numbers of SNPs for each genome are given in Table IX and the number of shared/unique SNPs is represented in a Venn diagram in Figure 3. A total of 22 mutations were common across all six mutant genomes. In addition to the shared mutations, there are changes that are exclusive to either CG or FG mutants which are shown in Figure 4. There are two mutations that are shared within the three CG mutants and one mutation that is shared within the three FG mutants.

Among the 22 mutations shared by all six mutants, 6 mutations were synonymous (Henaut and Danchin 1996), i.e. these mutations do not cause changes in the amino acid sequence of the protein, 6 mutations were found in non-coding portions of the genome, 3 of these mutations occurred in the *rRNA* gene sequences. The non-synonymous mutations (Henaut and Danchin 1996) were found in genes *insB* (insertion element), *lacZ* (part of lac operon which hydrolyses lactose to galactose and glucose), *mhpD* (calalyzes conversion of 2-hydroxypentadienoic acid to 4-hydroxy-2-ketopentanoic acid), *ymfE* (uncharacterized membrane protein), and *wbbK* (putative glycosyltransferase, part of outer membrane biogenesis). We could not find any evidence from literature on the association between these genes and bacterial stress response.

The mutations that are not shared by all six mutants are given in Table X. The table has information about the mutants which have the mutation, position of nucleotide change, nucleotide base change, and the gene which has that mutation. There are 4 different non-synonymous mutations in the *rpoB* gene. Mutations in *rpoB* gene are known to confer resistance to Rifampicin (Reynolds 2000). Gene *yghO* also contains a non-synonymous mutation and this gene is involved in biofilm production (Beloin *et al.* 2004). The other two non-synonymous changes are present in the promoter regions of genes *fixA* and *yhhW*. Gene *fixA* is predicted to play a role in electron transport in *E. coli* (Eichler *et al.* 1995) and gene *yhhW* is proposed to be involved in a mechanism that prevents inhibition of DNA gyrase by quercetin (Adams and Jia 2005). None of these genes, however, have previously been shown to be directly involved in pathways related to stress response in *E. coli*.

Functional characterization of genomic mutations induced by graphene exposure

We performed growth analysis (Davison *et al.* 2007, Wehrli 1983) of the FG and CG mutants from Fluctuation Assay to assess whether mutations towards rifampicin confers any selective advantage to these mutants when compared to wildtype *E. coli* DH5α cells. We picked two CG and two FG mutants (which we had already sequenced) for this test and included wildtype *E. coli* DH5α cells as control. Each strain was revived from frozen glycerol stocks on LB agar plates and then grown in liquid medium to be used in the experiment. CG and FG mutants were grown in medium supplemented with rifampicin and the wildtype cells were grown in non-selective media. OD_{600} values were measured and cell densities of the cultures were estimated (Table XI) to ensure each treatment has comparable number of cells at the beginning of experiment. The CG, FG mutants and the wildtype cells were each exposed to either commercial or functional graphene at concentrations 0 µg/ml (control), 80 µg/ml, and 320 µg/ml. After incubation, five dilutions (10^{-3}, 10^{-4},10^{-5}, 10^{-6}, and 10^{-7}) from each treatment were spread on LB agar plates to estimate the number of viable cells in each culture.

Plates where 10^{-3} and 10^{-4} dilutions were plated had a lawn of bacteria in each treatment, and therefore it was not possible to get a cell count from these dilutions. Table XII contains the cell count for all the other dilutions. Mutant FG1 dilution plates had lawns even on the 10^{-5}, 10^{-6}, and 10^{-7} dilutions and hence that data has been marked as TNTC (too many to count) in the table. Based on the number of colony forming units on each plate, we calculated the average number of viable cells. The average cell density for CG1 and CG2 mutants, FG2 mutant, and WT *E. coli* DH5α cells is represented in Figure 5 along with the standard error bars.

Since these mutants were originally obtained upon exposure of *E. coli* to commercial or functionalized graphene at concentration 80 µg/ml, we expected that CG mutants will have higher viability when re-exposed to commercial graphene at this concentration and FG mutants will have higher viability when re-exposed to 80 µg/ml of functionalized graphene.

Results from the CG mutants did not show a consistent trend. CG2 grew better in the presence of either commercial or functionalized graphene compared to the control. CG1 grew slightly better in the presence of commercial and functionalized graphene compared to control, except at 80 µg/ml of functionalized graphene. FG2 also showed better growth in the presence of commercial graphene, but not in the presence of functionalized graphene, compared to the control. From previous work in our lab, we know that functionalized graphene is cytotoxic to WT *E. coli* DH5α cells. Data from the WT cultures are in general agreement with our previous data. However, data obtained for WT exposed to 320 µg/ml of functionalized graphene was surprising and may represent an error in data acquisition in this study. Additional experiments are needed to clarify if the mutations isolated from graphene exposure confer any physiological advantage to *E. coli*.

CONCLUSION

This study used a novel approach to study the genotoxic effects of graphene on the *E. coli* genome. By performing Fluctuation Assay we calculated and compared the rate of mutation to rifampicin resistance (Rif^R) in the presence and absence of graphene. From this experiment we concluded that there is an increase in Rif^R mutation rate when cells are exposed to functionalized graphene. We then selected and sequenced the genomes six Rif^R mutants from Fluctuation Assay to analyze mutations in the genomes of the mutants. Using very high stringency parameters, we discovered 25-27 nucleotide changes in the genomes of the mutants when compared to the parent out of which 22 changes were shared by all mutants. This was very interesting as these mutants came from different plates, samples, and treatments. If mutations were truly random we would not have seen the same position and nucleotide change in all six mutants. Finally, since the mutations arose in the presence of either commercial or functionalized graphene, we wanted to test if the mutants had any growth advantage in the presence of either graphene. Results from this experiment revealed that in general, all mutants grew better in the presence of commercial graphene compared to the control, while only the CG mutants grew better in the presence of functionalized graphene compared to control. Additional experiments are needed to understand the physiological relevance of these mutations in *E. coli*.

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Tables and Figures

Table I

OD_{600} and cell density values for ten untreated, shaken cultures of *E. coli* used for Fluctuation Assay

Culture number	OD_{600} values after 12 hours	Estimated cell density (per ml)
1	1.099	8.79×10^8
2	1.156	9.25×10^8
3	1.133	9.06×10^8
4	1.195	9.56×10^8
5	1.131	9.05×10^8
6	1.115	8.92×10^8
7	1.14	9.12×10^8
8	1.129	9.03×10^8
9	1.237	9.9×10^8
10	1.158	9.26×10^8

Table II

Calculation of the frequency of spontaneous mutations to rifampicin resistance (Rif^R) in untreated, shaken cultures of *E. coli*

Culture number	Number of colonies on Rif plates (100 μl)	Number of colonies on Rif plates (100 μl) x10	Number of colonies on Rif plates (1ml)	Average number of Rif^R mutants	Estimated cell density (per ml)	Rif^R frequency per 10^8 cells
1	3	30	127	78.5	8.79×10^8	8.93
2	1	10	61	35.5	9.25×10^8	3.84
3	2	20	26	23	9.06×10^8	2.54
4	1	10	18	14	9.56×10^8	1.46
5	1	10	22	16	9.05×10^8	1.77
6	2	20	73	46.5	8.92×10^8	5.21
7	3	30	93	61.5	9.12×10^8	6.74
8	2	20	49	34.5	9.03×10^8	3.82
9	ND	ND	43	43	9.9×10^8	4.34
10	ND	ND	83	83	9.26×10^8	8.96
Average Rif^R mutation frequency per 10^8 cells						4.76
Standard deviation						2.71

Note: ND (no data) indicates that no colonies grew on the corresponding plate.

Table III

Cell density values for five treated, non-shaken cultures of *E. coli* used for Fluctuation Assay

Culture number	Number of colonies on LB-agar plates (10^{-5} dilution)	Number of colonies on LB-agar plates (10^{-6} dilution)	Number of colonies on LB-agar plates (10^{-7} dilution)	Cell density per culture
LB1	TNTC	215	23	2.23×10^8
LB2	TNTC	153	37	2.62×10^8
LB3	TNTC	197	35	2.74×10^8
LB4	TNTC	235	36	2.98×10^8
LB5	TNTC	328	96	6.44×10^8
Average cell density for LB cultures				3.40×10^8
CG1	612	48	2	0.43×10^8
CG2	564	54	7	0.60×10^8
CG3	572	53	8	0.63×10^8
CG4	556	63	2	0.46×10^8
CG5	456	57	4	0.48×10^8
Average cell density for CG-treated cultures				0.52×10^8
FG1	656	50	7	0.62×10^8
FG2	560	72	3	0.53×10^8
FG3	724	62	6	0.65×10^8
FG4	372	59	9	0.62×10^8
FG5	592	59	11	0.76×10^8
Average cell density for FG-treated cultures				0.63×10^8

Note: TNTC (too many to count) indicates that there was a bacterial lawn on the corresponding plate. LB indicates control (untreated) cultures; CG indicates CG-treated cultures; FG indicates FG-treated cultures of *E. coli*.

Table IV

Calculation of the frequency of spontaneous mutations to rifampicin resistance (Rif^R) in treated, non-shaken cultures of *E. coli*

Culture number	Number of colonies on Rif plates (100µl)	Number of colonies on Rif plates (100µl) x10	Number of colonies on Rif plates (1ml)	Average number of Rif^R mutants	Rif^R frequency per 10^8 cell
LB1	1	10	9	9.5	4.27
LB2	2	20	19	19.5	7.46
LB3	ND	ND	3	3	1.10
LB4	ND	ND	4	4	1.34
LB5	4	40	34	37	5.75
Average Rif^R mutation frequency for LB cultures					3.98
CG1	ND	ND	ND	ND	ND
CG2	ND	ND	3	3	2.78
CG3	ND	ND	ND	ND	ND
CG4	ND	ND	2	2	3.12
CG5	ND	ND	4	4	5.89
Average Rif^R mutation frequency for CG-treated cultures					3.93
FG1	ND	ND	1	1	0.87
FG2	ND	ND	2	2	2.41
FG3	2	20	6	13	10.32
FG4	1	10	7	8.5	7.35
FG5	5	50	29	39.5	22.76
Average Rif^R mutation frequency for FG-treated cultures					8.74

Note: ND, no data was available as no colonies grew on the corresponding plate. LB indicates control (untreated) cultures; CG indicates CG-treated cultures; FG indicates FG-treated cultures of *E. coli*.

Table V

Calculation of the rate of spontaneous mutations to rifampicin resistance in treated, non-shaken cultures of *E. coli*

Treatment	LD mean	LC median	Drake formula
Control (LB alone)	1.807	2.122	3.164
CG exposure	2.134	1.737	3.097
FG exposure	3.448	2.915	4.729

Table VI

Concentration and Abs_{260}/Abs_{280} ratio of genomic DNA extracted from Rif^R mutants and parent (WT)

Sample	Concentration (ng/µl)	A_{260}/A_{280} ratio
CG1	72.8	1.80
CG2	72.2	1.82
CG3	61.6	1.96
FG4	98.6	1.77
FG5	40.2	1.78
FG6	52.2	1.99
WT	44.2	1.97

Table VII

Summary of reads from Illumina MiSeq NextGen sequencing of the *E. coli* genomic DNA

Sample	Total reads obtained	Low-quality reads (filtered out)	Final number of high-quality reads used for genome assembly
CG1	4,754,534	1,444	4,753,090
CG2	3,445,746	771	3,444,975
CG3	4,564,014	2,658	4,561,356
FG4	4,239,402	1,120	4,238,282
FG5	3,504,652	622	3,504,030
FG6	2,837,784	2,705	2,835,079
WT	33,857,684	27,893	33,829,791

Table VIII

Summary of genome assembly statistics using DNASTAR software

Sample	Contig length with gaps	Median coverage
CG1	4,640,974	213.30
CG2	4,640,676	166.28
CG3	4,641,042	200.99
FG4	4,641,046	203.28
FG5	4,640,972	175.00
FG6	4,640,599	136.92
WT	4,645,702	1411

Table IX

Number of nucleotide mutations in each genome

Sample	Number of nucleotide mutations
CG1	25
CG2	27
CG3	27
FG4	25
FG5	27
FG6	27

Table X

Summary of nucleotide mutations not shared by all mutants

Mutant	Nucleotide position	Nucleotide change	Gene or region
CG1,CG2,CG3,FG4,FG5	42108	A>G	*fixA* promoter
CG3,FG5,FG6	2302544	GG>TC	
FG6	3128183	G>A	*yghO*
CG2	3205570	G>-	*ttdB*
FG4,FG5,FG6	3579873	A>G	*yhhw* promoter
CG2,CG3	3742392	T>C	*yiaM*
FG5	3939897	C>T	*rRNA*
CG1,CG2,CG3,FG5,FG6	4169804	G>A	*rRNA*
CG1,FG6	4179710	A>C	*rpoB*
CG2,CG3	4180852	C>T	*rpoB*
FG5	4180954	A>C	*rpoB*
FG4	4180981	A>C	*rpoB*

Table XI

OD_{600} values of the cultures of *E. coli* mutants used for growth analysis

Sample	OD_{600}	Cell density
CG1	1.327	1.06×10^9
CG2	1.355	1.08×10^9
FG1	0.813	6.5×10^8
FG2	1.196	9.57×10^8
WT	1.456	1.16×10^9

Table XII

Number of colony forming units (CFUs) on dilution plates for growth analysis

Sample	Graphene conc. (µg/ml)	Commercial Graphene			Functionalized graphene		
		10^{-5} dilution	10^{-6} dilution	10^{-7} dilution	10^{-5} dilution	10^{-6} dilution	10^{-7} dilution
CG1	0	67	46	6	624	24	6
	80	880	59	4	528	53	1
	320	368	69	6	444	100	7
CG2	0	376	48	8	336	52	7
	80	724	81	9	788	52	8
	320	784	114	7	534	81	11
FG1	0	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
	80						
	320						
FG2	0	178	24	4	406	55	11
	80	556	61	4	894	94	7
	320	508	33	5	702	90	8
WT	0	250	58	3	212	26	4
	80	430	57	3	228	35	1
	320	328	40	8	296	44	5

Note: TNTC (too many to count) indicates that there was a bacterial lawn on the corresponding plate.

Figure 1

Agarose gel electrophoresis of genomic DNA isolated from CG and FG mutants for genome sequencing

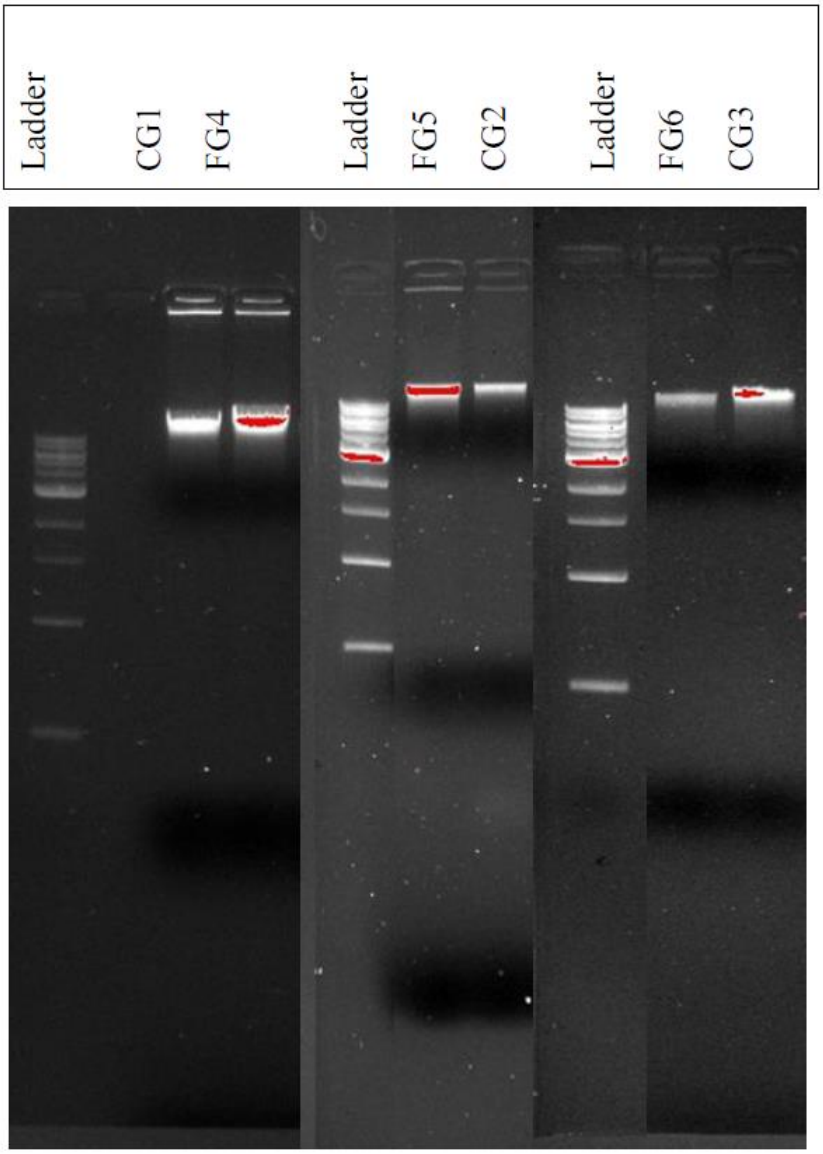


Figure 2

Agarose gel electrophoresis of genomic DNA isolated from *E.* coli DH5α for genome sequencing

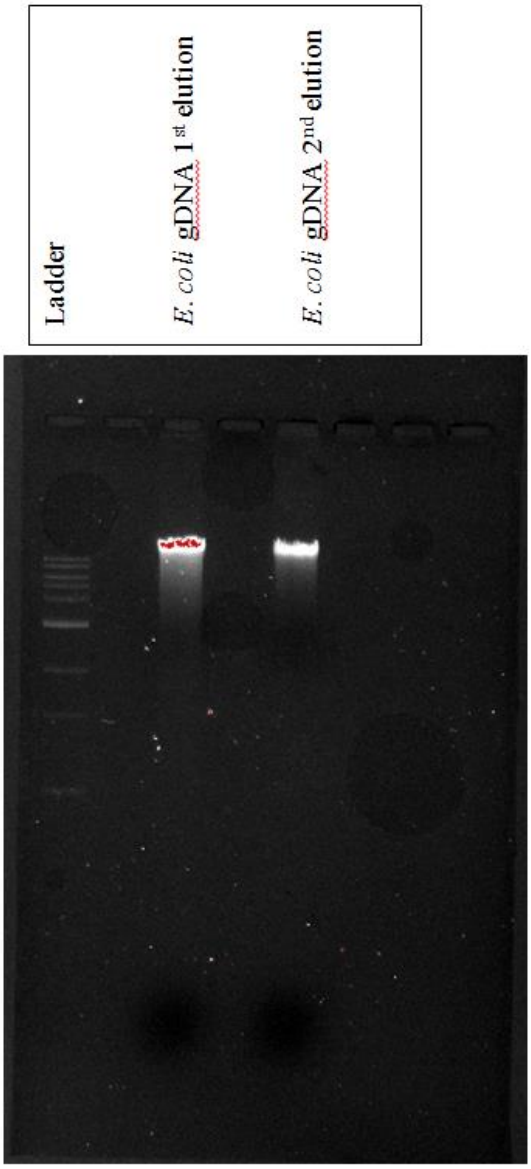


Figure 3

Analysis of shared and unique mutations in the genomes of CG and FG mutants

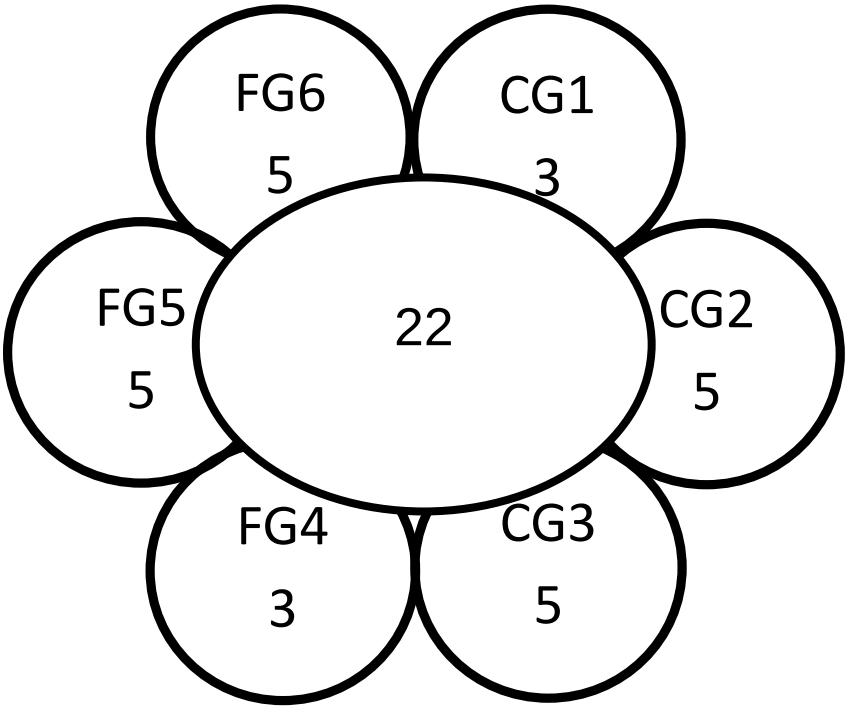


Figure 4

Analysis of shared and unique mutations within the genomes of either CG or FG mutants

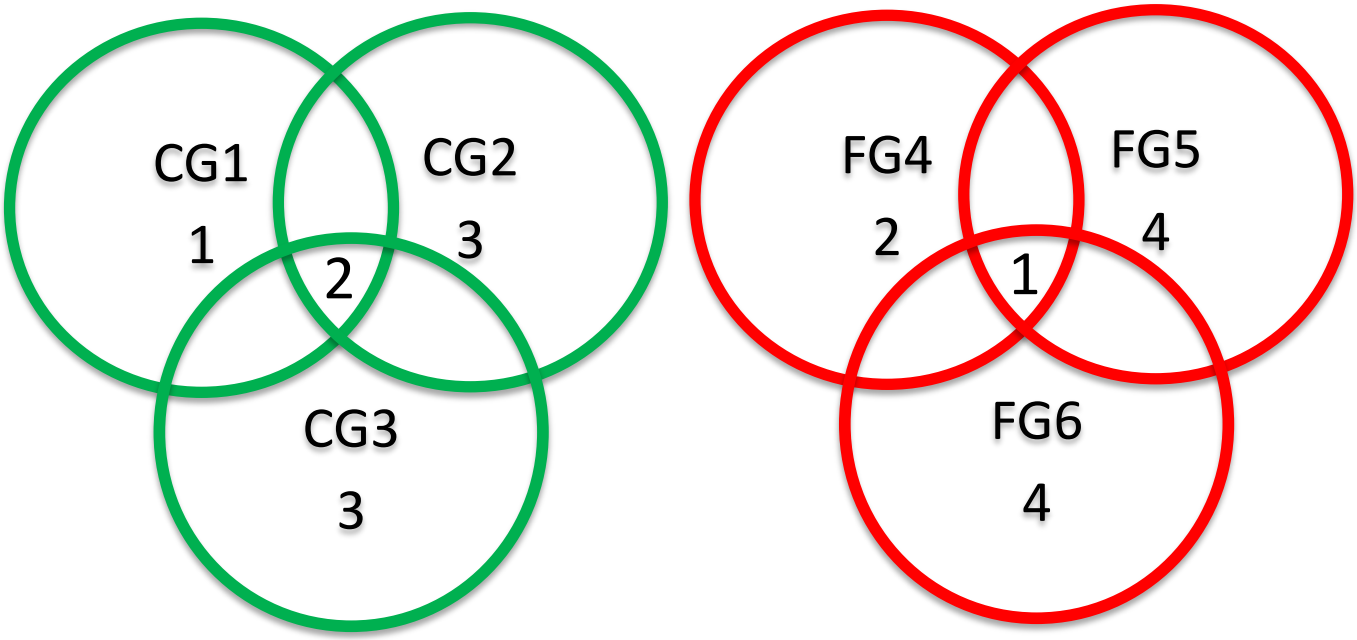
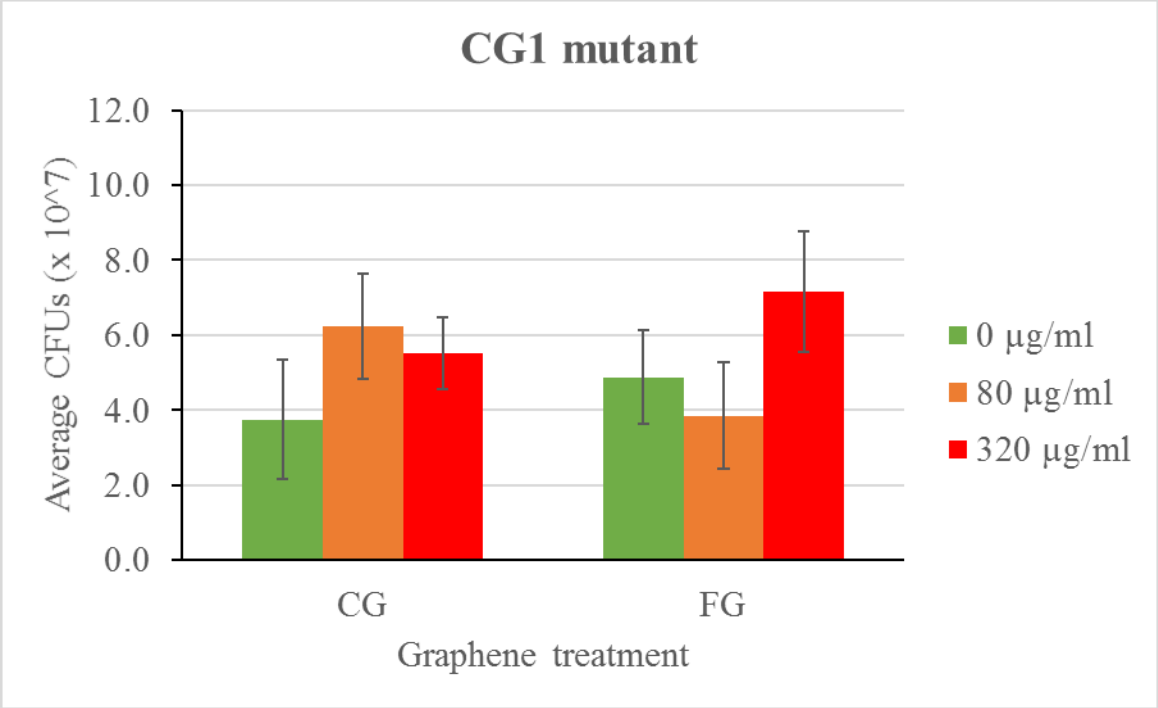
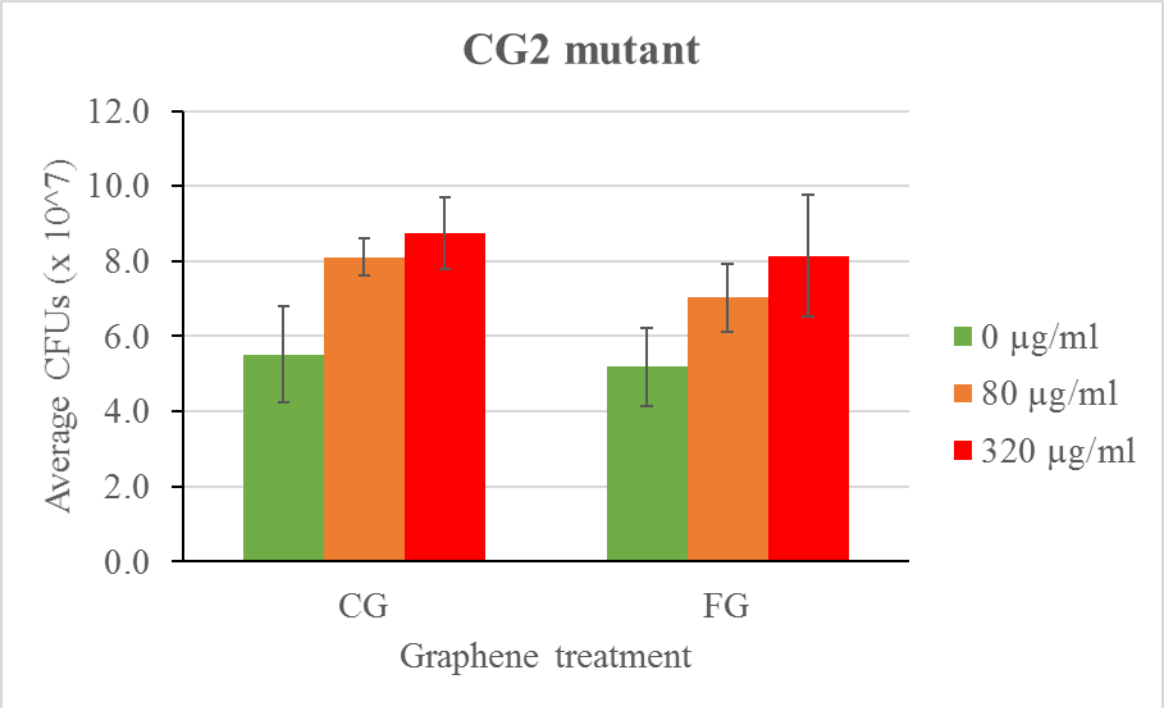
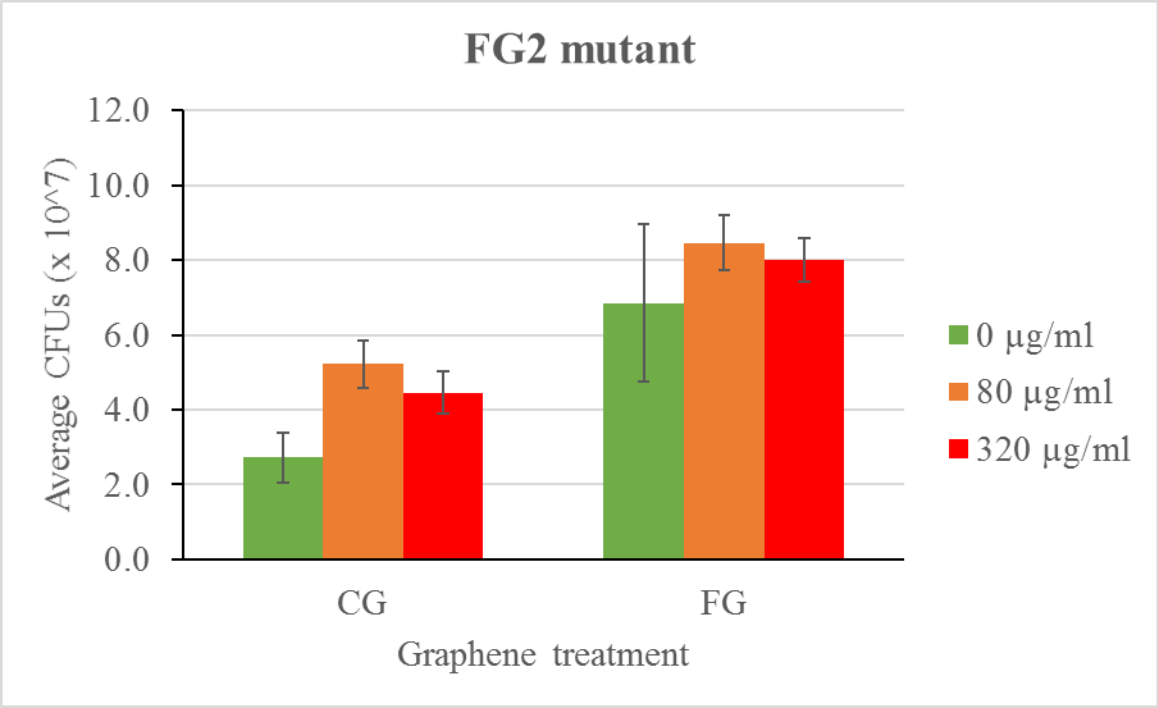


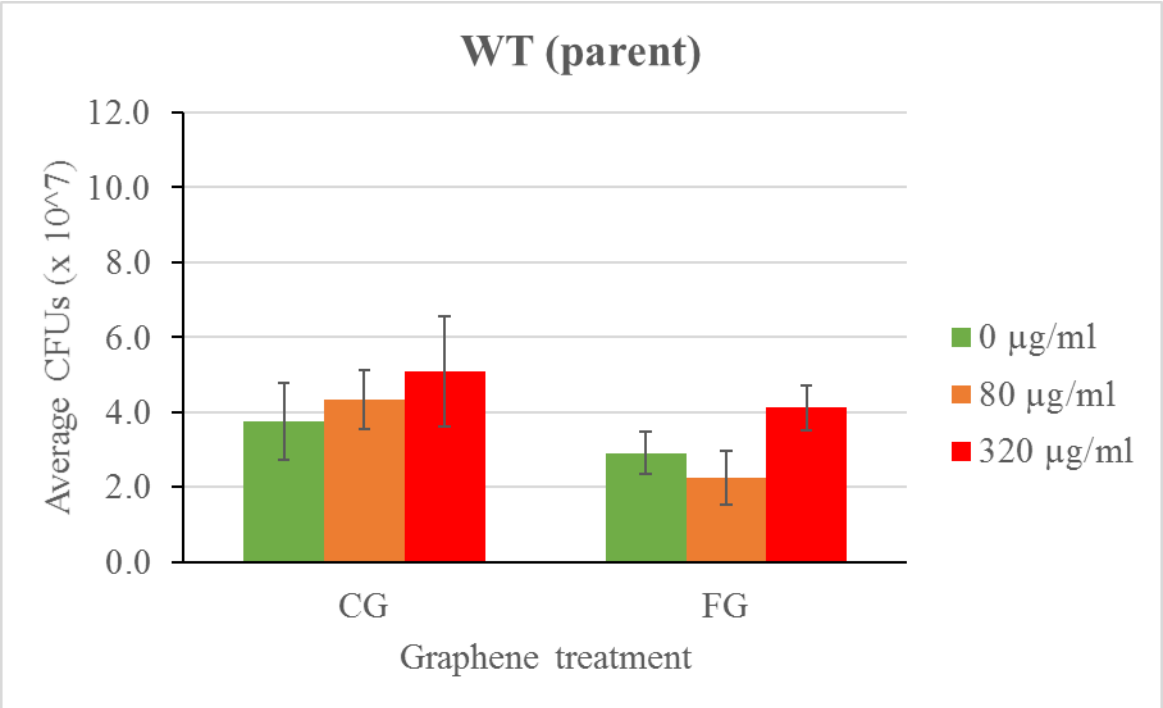
Figure 5

Effect of graphene on the growth of CG and FG mutants as well as parent (WT)






FG2 mutant
12.0
10.0
8.0
6.0
4.0
2.0
0.0
Average CFUs (x 10^7)
CG
FG
Graphene treatment
0 µg/ml
80 µg/ml
320 µg/ml


WT (parent)
12.0
10.0
8.0
6.0
4.0
2.0
0.0
Average CFUs (x 10^7)
CG
FG
Graphene treatment
0 µg/ml
80 µg/ml
320 µg/ml