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Effect on Genetic Mutation Induction from Nano-Ranged Low-Energy Plasma Ion Bombardment of DNA and Gene Fragment

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This study was aimed to investigate relevant aspects of effect from nano-ranged low-energy plasma ion bombardment of DNA on mutation induction for fundamental understanding of low-energy ion effect on life. Samples of extracellular plasmid DNA pUC19 and DNA fragment containing *lacZ* gene (encoding ß-galactosidase) were directly bombarded using the nitrogen plasma immersion ion implantation (PIII) method with varied bias in an order of kV and varied fluences in an order of 10^{15} ions/cm^2. The PIII-treated DNA and DNA fragment were transferred using the electroporation into bacteria *Escherichia coli* (*E. coli*) to observe mutation induction. Mutant DNA was sequenced. The mutation frequencies as a function of the bias voltage at a fixed fluence and as a function of the fluence at a fixed bias were found to increase linearly as increasing of the bias or the fluence. Damage in the *lacZ* gene was thereafter identified as being responsible for the bacterial mutation induced by PIII of DNA. DNA sequencing confirmed the *lacZ* gene damage and revealed the damage types dominated by the base substitution and cytosine having the highest radiation-sensitivity. Mutation could certainly be induced by nano-ranged keV-ion bombarded DNA and damage, dominated by the base substitution, in the marker gene fragment was the key mutation source. The mutation frequency was linearly proportional to the ion energy and fluence.

Keywords: Plasma immersion ion implantation (PIII), Bombardment, DNA, Gene fragment, Mutation, *Escherichia coli* (*E. coli*).

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1. INTRODUCTION

Long-ranged high-energy (MeV – GeV) ion irradiation effect on DNA has long been studied and understood, however, nano-ranged low-energy (keV or lower) ion bombardment effect on DNA has not yet profoundly studied. Study on low-energy ion interaction with DNA is important in understanding relevant fundamentals of ion beam biotechnology, radiation life science and plasma sterilization. The key reasons are that the energy deposition from irradiating ions in the irradiated matter, no matter with what ion energy, dominantly occurs around the Bragg peak which is immediately before the ion comes to the rest, and the irradiating ions recoil low-energy atoms which further interact with target atoms. In these cases, the interacting energy is around the order of kilo-electron-volts (keV; 1 eV = 1.6×10^{-19} J) or lower. DNA has a complex structure which is frail to external action and thus easily damaged in certain extents which may subsequently cause genetic mutation. The key interest is then how DNA is damaged under low-energy ion interaction. There have recently been some studies on direct low-energy-ion effect on DNA damage [1-8]. The studies have confirmed that ions with energy of keV or lower can induce certain damage in DNA such as single strand break (SSB), double strand break (DSB) and fragments. Our previous investigations demonstrated that transferring of the damaged DNA which was caused by direct low-energy ion bombardment in bacteria could induce bacterial mutation [8,9]. Low-energy ion beam bombardment induced DNA changes have been found to have certain characteristics. In 30-keV carbon ion bombarded double-stranded (ds) M13mp18 DNA, induced mutation in *lacZa* gene (encoding ß-galactosidase) had preferential base changes mostly involving GC base pairs, especially GC→TA transversions and GC→AT transitions [10]. The result was similar to the mutation spectra induced by γ-ray in the same dsM13 DNA [11]. In 10-keV nitrogen ion bombarded bacteria *Escherichia coli* (*E. coli*) containing *rpoB* gene, induced mutation was found to have predominant base-pair substitution of CG → TA transition, AT → GC transitions and AT → TA transversion [12]. But, also in 10-keV N-ion bombarded *E. coli* containing *lacI* gene was found the mutation spectra having relative low-level base substitutions but high-level addition/deletion ±TGGC [13]. These previous studies applied either higher ion energy (> 10 keV) or indirectly ion bombardment of DNA, and thus the results seemed inconsistent. To further understand the relations between the low-energy ion bombardment conditions and the induced mutation, we carried out this study using keV plasma ions to directly bombard extracellular DNA and its marker gene frag-

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ment, then transfer the DNA and the fragment into bacteria *E. coli* to observe mutation, and finally analyzing the mutated gene sequence to identify the mutation locations and analyze mutational spectra. Induced mutations could be in many types, but for a concentrated and convenient study we focused on one phenotype, namely color, which was the expression of one of the key genes in the DNA that we used. Potential significance of our research could be in fields such as plasma sterilization, life science and ion-beam/plasma biotechnology.

2. MATERIALS AND METHODS

2.1 Sample Preparation

Two types of DNA samples were prepared, plasmid DNA and *lacZ* gene fragment from the DNA. An initial sample of plasmid DNA pUC19 (2686 base pairs, plasmid in the name of University of California [14]) was purchased from Clonetech (Mountain View, CA, USA). The plasmid pUC19 contains three main components, one amp^R gene (ampicillin-resistant gene), an N-terminal fragment of ß-galactosidase (*lacZ*) gene of bacterium *E. coli*, and the *ori* (origin) site or replicon [15], as shown in Figure 1. The *lacZ* gene product can hydrolyze an artificial chemical called X-gal (abbreviated for 5-bromo-4-chloro-indolyl-ß-D-galactopyranoside) to release a blue pigment, and hence a cell containing the pUC19 DNA can express blue color under natural light in X-gal added medium. This DNA was smaller than pGFP (plasmid DNA containing green fluorescence protein, 3344 base pairs), which was used in our previous study, for a more sensitive detection of ion bombardment effect. The initial sample was replicated by transferring the DNA into *E. coli* (strain DH5α) and subsequently extracted and purified using the High-Speed Plasmid Mini Kit (Geneaid, Taipei, Taiwan). The plasmid DNA produced by this procedure was dissolved in sterile, elution buffer resulting in a plasmid concentration of 0.1 µg/µl. Aliquots of 3 µl plasmid DNA solution (containing 300 ng plasmid DNA) were deposited in holes of a special DNA sample disk holder (Sarapirom et al. 2010), which was made from stainless steel and had 9 holes with each of 5 mm in diameter and 5 mm in depth. This sample arrangement guaranteed a monolayer of DNA in the sample hole to be ion-bombarded so that the mutation frequency could be correctly obtained†. The samples were then dried in laminar flow for 1 hr and then placed in the chamber of our plasma immersion ion implantation (PIII) facility [16] for plasma ion bombardment (Figure 2). To detect ion bombardment effect on this gene and identify the DNA damage location responsible for the DNA character expression, *lacZ* gene fragment samples were also prepared. The *lacZ* gene fragment was prepared by digesting the plasmid pUC19 with the restriction enzymes, *Nde*I/*Hind*III which covered the entire section of the *lacZ* gene in pUC19, as shown in Figure 1, to obtain a size of 264 base pairs. The fragment was detected and confirmed by gel electrophoresis in a comparison with the DNA marker (Lambda/PstI). The *lacZ* gene fragment was eluted from gel and purified using the Silica Bead DNA Gel Extraction Kit (Fermentas, Glen Burnie, Maryland, USA) and ready for ion bombardment.

To control the DNA layer to be mono, the layer thickness was calculated as described below. The DNA concentration was 300 ng in 3 µl DNA solution. After drying, the DNA mass was 300 ng. The A-DNA mass density was about 0.7 g/cm^3[17]. So, the DNA sample volume was

$$(300 \times 10^{-9}\ \text{g})/(0.7\ \text{g/cm}^3) \approx 4 \times 10^{-4}\ \text{mm}^3.$$

If this volume was full of the bottom of the sample holder hole which was 5 mm in diameter, the height h of this volume was calculated from

$$4 \times 10^{-4}\ \text{mm}^3 = (2.5)^2\ \pi h\ \text{mm}^3 \approx 20\ h\ \text{mm}^3,$$

to be $h \approx 20$ nm.

The DNA had nearly 2700 base pairs (as memtioned above in Part 2.1). The volume of a DNA chain that contained one base pair was about 1.6 nm^3 [17]. So, the total volume of the DNA was

$$V = 1.6 \times 2700 = 4320\ \text{nm}^3.$$

DNA was originally in the supercoiled form. It was assumed that the supercoiled form DNA was in a sphere shape. Then the sphere diameter D was calculated from

$$4320 = 1/6\ \pi D^3$$

to be

$$D \approx (4320 \times 2)^{1/3} \approx 20\ \text{nm}.$$

Comparing this D with the DNA sample height h, we saw the same, which indicated that the supercoiled DNAs prepared in the sample hole were in a monolayer.

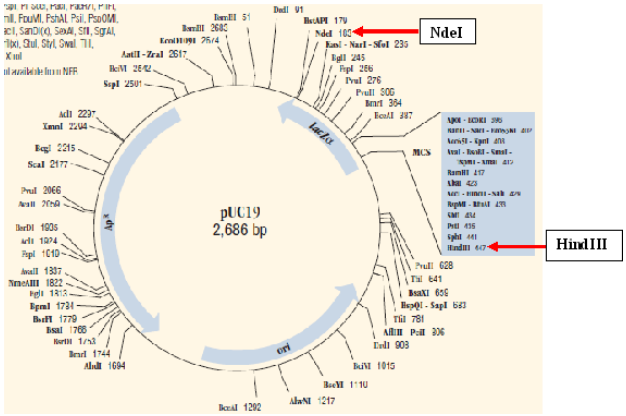

Fig. 1 – Map of plasmid DNA pUC19

2.2 Plasma Ion Bombardment

After the samples of either the whole plasmid DNA or the *lacZ* gene fragment solution were placed in the PIII multicasp chamber [16], the chamber was pumped down to a pressure of 10^{-3} Pa. Nitrogen (N) was used as the plasma source material for treatment. The plasma was generated with 50-W radiofrequency (RF) power and operated with a frequency of 50 Hz and a pulse length of 10 µs including a rise time of about 2 µs. Fig-

ure 2 shows the PIII chamber and the DNA sample holder used in this experiment. The PIII conditions of the applied bias and ion fluence were designed to investigate the bias and fluence dependences of PIII effects with the bias varied from -2.5 kV to -9 kV and the ion fluence varied around the order of 10^{15} ions/cm^2. The bias was a negative voltage provided to the sample holder to attract and accelerate positively charged ions in the plasma to bombard the samples in the holder that was immersed in the plasma. If no bias was applied, the plasma ion energy was in the thermal energy order, eV. When a bias, -*U*, was applied, a singly charged ion then obtained energy of *U*eV to bombard the sample (in normal plasma singly charged ions are dominantly in majority). Therefore, the applied bias determined the ion energy. The ion fluence was the number of the atomic ions bombarding at a unit area, controlled by the total PIII operating time with the measured pulse peak current, pulse length and frequency, as well as taking into account for the measured molecular ion concentration and the tabulated secondary electron emission coefficient for the case of N-PIII. The ion fluence could be estimated by

$$Fluence = \frac{It\eta(1+\varepsilon)}{zA(1+\gamma)},$$

where I is the measured ion current in Amp, t is the total operating time in second, η is the duty cycle, η = frequency (Hz) × pulse width (sec), ε is the molecular nitrogen ion concentration, $\varepsilon = N_2^+/(N_2^+ + N^+)$, z is the number of the ion charge (for a singly charged ion, z = 1), A is the total area bombarded in cm^2, and γ is the secondary electron emission coefficient, depending on many parameters (such as material, energy, ion species etc.), which is normally tabulated in some handbooks.

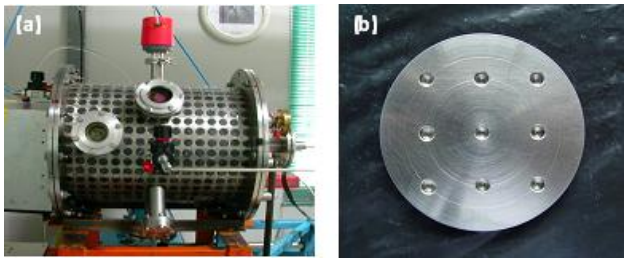

Fig. 2 – Photographs of (a) the PIII multicasp chamber and (b) the DNA sample holder. The chamber is about 40 cm long and 30 cm in diameter. The sample holder is about 5 cm in diameter. The samples are put inside the holes on the sample holder

The ion fluence used was equivalent to radiation dose in an order of 10^8 Gy. There were two reasons for using the high dose. In ion beam biotechnology, the ion beam fluence applied to induce mutation was around 10^{16} – 10^{17} ions/cm^2 [3], and our study was aimed at investigating mechanisms involved. From our experience, fluences of the order of 10^{15} ions/cm^2 could result in clear observable and countable mutation [8,9]. Plasma in the PIII system was characterized using a Langmuir probe (Hiden Analytical, Warrington, UK) and optical emission spectroscopy (OES) (Ocean Optics, Dunedin, FL, USA) to be reliable with negligible impurities for experiments [16].

2.3 Gene Transfer, Mutation Analysis and DNA Sequencing

After the plasma ion bombardment, the samples were recovered in 20 µl of de-ionized water and divided into six parts for gene transfer (3 µl used for transfer each time). The plasmid pUC19 was transferred into *E. coli* (DH5α) competent cells using the standard electroporation. The *lacZ* gene fragment was first ligated into plasmid DNA pUC19 (digested by *Nde*I/*Hind*III) and then the DNA was transferred into *E. coli* (DH5α) competent cells. In the transferring process, the sample was first mixed with pure water, and then the DNA solution was put into 100 µl of Lysogeny broth (LB) medium with a high concentration of the bacterial cells, followed by electroporation at 1.8 kV. After the electroporation, the solution was put into 3-ml LB medium, shaken for two hours, then added with ampicillin (100 µg/ml) and shaken for overnight. The DNA-transferred *E. coli* cells were incubated at 37°C for overnight and spread on plates containing reagent IPTG (Isopropyl ß-D-1-thiogalactopyranoside) with X-gal. After the incubation, bacterial mutation was selected in terms of phenotypical changes. One of the easily identified phenotypical changes was the cell color. "White", or the original color of the *E. coli*, indicated the cell without the gene transferred or with the transferred DNA but damaged, whereas the normal DNA transferred bacteria would express blue color because of the presence of the *lacZ* gene. But, due to ampicillin added in the culture medium and the ampicillin resistance of the plasmid DNA, original bacterial cells without transferred DNA would be killed and thus, in the final medium, white colonies, if any, should be only mutants. Therefore, observation of the cell color was the first step to identify mutation and select mutants of the bacteria. White colonies were streaked in 5 generations on plates again to check for their purities and stability of the phenotype with PCR (polymerase chain reaction) used for identification. The number of the white colonies was counted compared with the total number of the tested bacterial colonies, normally thousands, for calculation of the mutation frequency.

After the transferring of the plasmid pUC19 or the *lacZ* gene fragment into the bacteria and selection of mutants, DNA was extracted from the white colonies or mutants and the extracted DNA samples were sequenced at the First Base Laboratories Sdn. Bhd., Malaysia. The specific primers used to cover the promoter and complete the *lacZ* gene sequence were LacZF1 primer (5'- GCT TGT CTG TAA GCG GAT GC -3') and LacZR1 primer (5' -GCG GGC AGT GAG CGC AAC GC -3'). The DNA sequencing result was compared with the original pUC19 sequence for analysis of the base changes. The mutation frequency of a certain base was calculated as a ratio between the number of the base changes and the total number of the changes in all bases. The total changes of each base for all types of mutations was summarized to result in the radiosensitivity of the base, which was defined as a ratio between the total changes of a base and the total number of the changes in all bases.

3. RESULTS

3.1 Effect of Ion Bombardment of Whole DNA on Mutation

After overnight incubation of the DNA-transferred bacteria at 37°C, it was observed that blue and white bacterial colonies were produced, as shown in Figure 3(a). White colonies indicated that these colonies were mutants because the transferred DNA inside the bacterial cells was damaged and thus the characteristic blue color expression could not function. The white colonies were then picked out, cultured and plated again to check their purity, as shown in Figure 3(b). The fully white color confirmed no contamination on the colonies and mutation heritage to new generations, and hence the white colonies were indeed the mutants. The numbers of the blue and white colonies were recorded and studied for the PIII bias and fluence dependences. Figure 4 shows the PIII bias dependence of the mutation frequency which is defined as a ratio of the number of the white colonies or mutants over the total number of the examined colonies at a fixed fluence of 2×10^{15} N^+/cm^2. It is clearly seen that the bacterial mutation frequency increases almost linearly with increasing of the bias applied. Figure 5 shows the ion fluence dependence of the mutation frequency at a fixed bias of -2.5 kV. It is also seen that the bacterial mutation frequency increases almost linearly with increasing of the ion fluence.

3.2 Effect of Ion Bombardment of Gene Fragment on Mutation

After overnight incubation of the *lacZ*-gene-ligated-DNA-transferred bacteria at 37 °C, the colony color was observed. As shown in Figure 6(a), many white colonies were observed together with blue colonies.

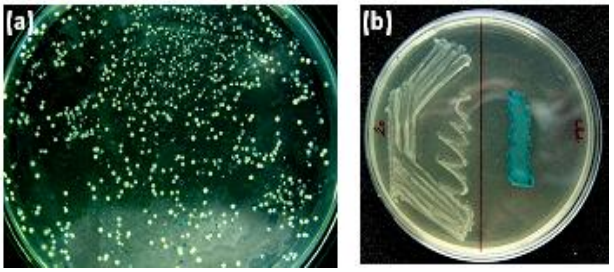

Fig. 3 – Observation of the plasma-ion-bombarded whole pUC19-DNA transferred *E. coli* (DH5α). (a) The bacterial blue and white colonies observed under natural light. (b) The re-streaked bacterial white colony (left) compared with blue colony (right, control)

The white colonies were the mutants while the blue colonies were the non-mutants. White colonies were then picked out and plated again to check their purification. As shown in Figure 6(b), the restreaked bacterial white colonies showed pure white, indicating no contamination on the white colonies which were then the only consequence of mutation. The numbers of the total colonies, blue colonies and white colonies were recorded. The PIII bias and ion fluence dependences of the mutation frequencies of the *lacZ* gene fragment transferred *E. coli* were analyzed, as shown in Figures 4 and 5. It is seen that the mutation frequency as a function of the bias or the fluence is nearly linear.

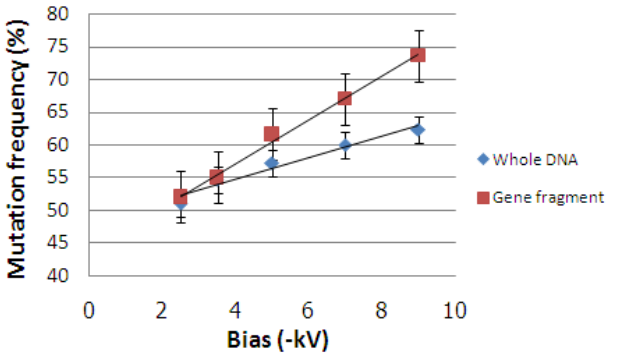

Fig. 4 – Mutation frequencies of *E. coli* transferred with nitrogen-plasma-ion-bombarded whole DNA and gene fragment as a function of the bias applied at a constant ion fluence of 2×10^{15} ions/cm^2. The straight lines are trendlines. The error bars indicate the standard error of mean for three replicates

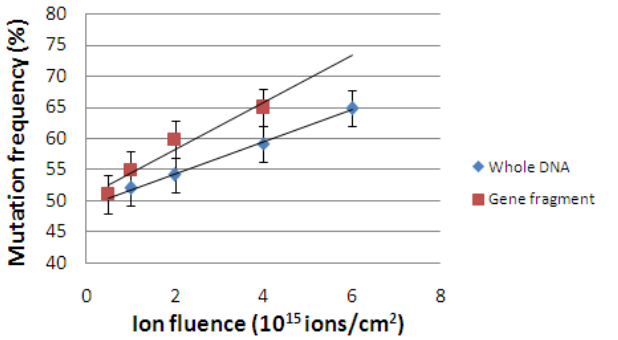

Fig. 5 – Mutation frequencies of *E. coli* transferred with nitrogen-plasma-ion-bombarded whole DNA and gene fragment as a function of the ion fluence at a constant bias of -2.5 kV. The straight lines are trendlines. The error bars indicate the standard error of mean for three replicates

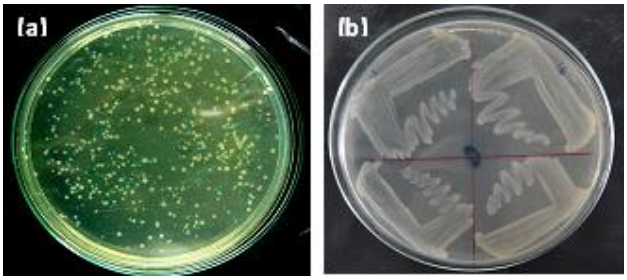

Fig. 6 – Observation of the bacteria *E. coli* (DH5α) transformed with *lacZ* gene fragment ligated DNA. (a) The bacterial blue and white colonies observed under UV. (b) The re-streaked bacterial white colony compared with the blue colony at the center of the dish

3.3 Sequence Analysis

To reveal changes in the *lacZ* gene fragment base sequence due to PIII, the sequences of the mutant clones from the ion-bombarded whole DNA pUC19 and *lacZ* gene fragment were compared with the homology database using ClustalW program [18], as shown in Figure 7. The base sequence changes are summarized in Table 1. The result showed that all types of the base mutations induced by N-PIII were possible but having different frequencies. The base substitution including transversion and transition was dominant with

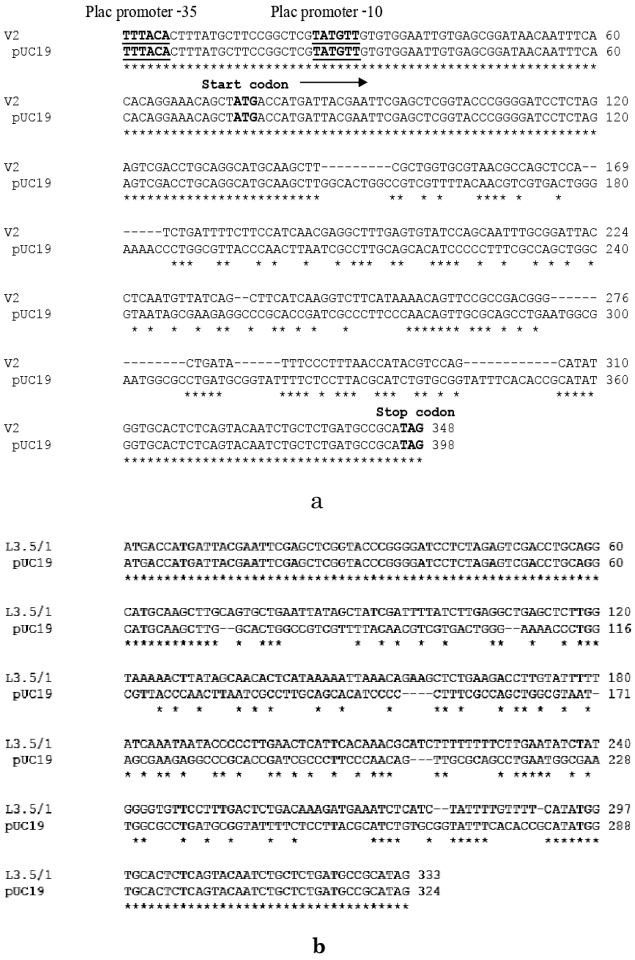

Fig. 7 – Comparison of the base sequence between pUC19 (control) with mutant clones from the ion bombarded (a) whole DNA pUC19, named V2, and (b) *lacZ* gene fragment, named L3.5/1. The stars mark the same bases, but the bases without star marking are different between the mutant and the control

high frequencies, while the other two types of changes, insertion and deletion, were minor with low frequencies. In transition, C→T had the highest frequency, about twice those of G→A and T→C and four times that of A→G, which had the lowest frequency. In transversion, AG→CT and CT→AG were in the similar probabilities. Table 2 shows the radiosensitivities of each base to the N-PIII. It is seen that the radiosensitivity of the base from high to low follows the order of cytosine (C), guanine (G), thymine (T) and adenine (A).

4. DISCUSSION

The result from the plasma ion bombardment of the whole DNA indicated that PIII of the DNA created certain damage to the DNA to cause malfunction of the DNA character color expression after the DNA was transferred into the bacteria. Although the DNA samples were dried and exposed to vacuum, our previous studies showed the dehydration and low pressure had negligible effects on DNA damage and consequent bacterial mutation [9,19], thus the observed phenotypic change in color was indeed consequence of the PIII of DNA. The low-energy PIII induced DNA conformation change or damage included changes from the

Table 1 – Observed (from Figure 6) types and numbers of occurrence of mutations in the mutant clones from the ion bombarded (a) whole DNA pUC19 and (b) *lacZ* gene fragment.

Mutation type	Number of occurrence		Frequency (%)	
	(a)	(b)	(a)	(b)
Base substitution	81	128	62	90
Transition	36	53	28	37
A → G	4	6	3	4
G → A	7	14	5	9
C → T	17	25	13	17
T → C	8	8	7	7
Transversion	45	75	34	53
AG → CT	21	37	16	26
CT → AG	24	38	18	27
Deletion	50	3	38	2
-A	12		9	
-G	16	3	12	2
-C	13		10	
-T	9		7	
Insertion		12		8
+A		4		3
+G		2		1
+C		3		2
+T		3		2
Total number of occurrence	131	143	100	100

Table 2 – Observed (from Table 1) total base changes in the mutant clones from the ion bombarded (a) whole DNA pUC19 and (b) *lacZ* gene fragment and their radiosensitivities to N-PIII

Base	Number of base change occurrences		Radio-sensitivity (%)	
	(a)	(b)	(a)	(b)
A	24	25	18	17
G	36	41	27	29
C	43	47	33	33
T	28	30	22	21
Total numberof base change occurrences	131	143	100	100

natural supercoils to the relaxed form and even the linear form due to either single strand break or double strand break [9]. These conformation changes could be repaired wrongly after the DNA was transferred in the bacteria to result in mutation.

As the plasma bias voltage is directly related to the ion energy, the result demonstrates that the higher the ion energy, the higher the mutation frequency, and this relationship is linear. The low-energy (keV or lower) ion range in DNA has been studied to be in the order of nanometers [17]. In ion implantation, normally the higher the ion energy, the more the radiation damage is created. This is particularly true when the ion energy is low as in this case the nuclear stopping dominates the interaction between the ions and the target atoms. Although for biological materials the electronic stopping can be also important, SRIM (The Stopping and Range of Ions in Matter) simulation [20] showed the

nuclear stopping several times the electronic stopping in our cases where N-ions at a few keV were implanted in DNA. Moreover, damage in DNA refers to atoms of DNA displaced or removed, and this can be only the result of nuclear-stopping-caused atomic collision. In PIII, as a negative bias normally in orders of 1 kV to 10 kV is applied to the sample holder, positively charged ions are attracted and accelerated to the sample with energy in orders of 1 – 10 keV (for singly charged ions) to implant perpendicularly to every surface area of the sample. PIII is different from only plasma bathing of samples, but a non-line-of-sight ion implantation process and hence basic ion implantation theories can be applied to it. Thus, when a low-energy ion bombarded DNA, the number of the atomic collisions between the ion and the DNA atoms could be considered to be proportional to the ion range, namely the longer the ion travels, the more the atomic collisions. From the study of the low-energy ion range in DNA, it is known that the ion range is linearly proportional to the ion energy when the nitrogen ion energy is higher than the low-energy upper limit, about 1 keV [17]. In the experiment, the nitrogen ion energy was higher than 1 keV for all of the bias applied (low-energy target atom recoils were negligible in all cases applied from SRIM simulation [20]) and hence the number of the atomic collisions should be linearly proportional to the ion energy or the bias. Assuming each atomic collision being able to create an atomic displacement or a damage site for DNA in an equal certain probability and the damage hardly being correctly repaired, the result of the linear relations shown in Figure 3 could be reasonably interpreted. As the ion fluence is directly related to the number of the ions implanted and thus the number of the atomic collisions, the result in Figure 5 demonstrates that the mutation frequency is linearly proportional to the number of the atomic collisions. This should be true because of the low-energy ion implantation feature, in which the nuclear interaction dominates the ion stopping process.

The result from the plasma ion bombardment of the gene fragment confirmed that PIII of the *lacZ* gene fragment could indeed induce some damage to the *lacZ* gene fragment, which in turn caused mutation in the phenotype of the bacteria. The culture media contained ampicillin and thus the bacteria which had mutation of losing the antibiotic property which came from damaging the amp^R gene in the DNA were already eliminated. Furthermore, the grown colonies already indicated that they were not mutants from damaging the *ori* site or replicon gene. Therefore, the finally grown bacterial colonies expressing white should be those only having *lacZ* gene damaged but the amp^R and replicon genes remaining intact. The *lacZ* fragment, whose synthesis can be induced by reagent IPTG, is capable of intra-allelic complementation with a defective form of ß-galactosidase enzyme encoded by host chromosome. In the presence of IPTG in growth medium, the bacteria synthesize both fragments of the enzyme. Both the fragments can together hydrolyze X-gal and form blue colonies on media with X-gal. Therefore, the experiment identified the location of the bacterial mutation source really coming from the damage in the *lacZ* gene. The same physics as that for the case of the ion-bombarded whole DNA can also interpret the linear relationships between the mutation frequency and the bias and fluence for the case of ion bombardment of the gene fragment.

Comparing the mutation frequencies between the cases of bombarding the whole DNA and the gene fragment in Figures 4 and 5, we found that the bacterial mutation frequency in the case of bombarding gene fragment was generally higher than that in the case of bombarding the whole DNA. The whole DNA pUC19 contains not only the *lacZ* gene but also other components, the *ori* or replicon and amp^R gene, as mentioned above. When ions bombarded the whole DNA, the ions were distributed homogeneously to every component, and hence the *lacZ* gene was bombarded by a part of the total ions. But, when ions bombarded the *lacZ* gene fragment, the gene component was bombarded by all of the ions. So, if the ion fluence was the same, the *lacZ* gene was bombarded with more ions when the only gene fragment was bombarded than when the whole DNA was bombarded. Therefore, the mutation frequency for the case of bombarding only the gene fragment was higher than that for the case of bombarding the whole DNA. This result indicates damage in the *lacZ* gene indeed acting as the dominant mutation source.

It should be noted that when the bias or the fluence was zero, no bias applied meaning a plasma control and no fluence meaning a vacuum control, no mutation was found [9], or the mutation frequency was zero. Therefore, in the very low bias or fluence range, the mutation frequency should have a logrithmic increase as increasing of the bias or fluence.

The results of the sequencing are basically similar to those reported using 30-keV N-ion beam implantation in plasmid DNA M_{13}mp18, which had also the base substitution dominant and cytosine the most radiosensitive [21]. By noting some differences in physics between our experimental method and that used in Song's experiment, keV ion energy from PIII in our case but multiply high energy of ions from ion beam in their case, the similar results indicate certain intrinsic nature of ion bombardment effect on DNA and mutation induction.

Some differences are noticed between the sequencing data obtained from the ion-bombarded whole DNA and the ion-bombarded gene fragment as shown in Table I. The detected base substitutions in the case of the bombardment of the whole DNA are generally less than those for the case of the bombardment of the gene fragment, while the other types of mutations for the former are more than for the latter. This is because the gene fragment after ion bombardment was ligated to the intact plasmid DNA and then transferred into the bacteria, and so certain damages were repaired by DNA repairing mechanisms in the cell. But, in the case of the bombardment of whole DNA, ion bombardment would induce damage everywhere in DNA so that repairing the damage could be more difficult and misrepairing could be in higher probabilities. It is seen that the type of deletion, which has a high frequency when the whole DNA was bombarded but very low frequency when the only gene fragment was bombarded, could be mostly repaired. In the contrast, some insertions oc-

curred when the gene fragment was bombarded but did not occur when the whole DNA was bombarded. Although errors in the experiment could not be ruled out, a speculation could be that both deletion and insertion occurred in ion-bombarded gene fragment, but the repairing mechanisms recovered the deletion by using the insertion. As for mechanisms of the mutation bias, either a chemical one [e.g. 22,23,24] or a biochemical one [e.g. 25,26], or both, are possible. A chemical bias may cause some bases more likely to be damaged by our low-energy plasma-ion radiation. A biochemical bias may play a role in the repairing mechanisms to cause some changes more readily repaired than others leaving the detected ones behind to be observed.

5. CONCLUSION

This was the first study on low-energy PIII of extracellular plasmid DNA and marker gene fragment to investigate the relevant effects on mutation of the DNA- or fragment-transferred bacteria. Mutation of the bacteria could indeed be induced by nano-ranged keV-ion bombardment of DNA. In the ranges of a few keV of the N-ion energy and 0.5-6 × 10^{15} ions/cm^2 in fluence, the bacterial mutation frequency was linearly proportional to the deposited ion energy and ion fluence for the DNA-transferred *E. coli* and the *lacZ*-gene-fragment-transferred *E. coli*. Damage to the *lacZ* gene was identified as the dominant mutation source. The DNA mutation types were dominated by the base substitution and cytosine had the highest radiation-sensitivity. It is noted that this study was only focused on one phenotype change in mutations, that is, the color change, but as the applied DNA contained three marker genes, three phenotype changes in induced mutation could all occur. It should be interesting to see how low-energy ion bombardment affects all these mutational changes in different frequencies and this could be a future research topic.

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REFERENCES

1. S. Lacombe, C. Le Sech, V.A. Esaulov, *Phys. Med. Biol.* **49**, N65 (2004).
2. Z.W. Deng, I. Bald, E. Illenberger, M.A. Huels, *Phys. Rep.* **95**, 153201 (2005).
3. Z.L. Yu (original), L.D. Yu, T. Vilaithong, I. Brown (Eng. eds.), *Introduction to Ion Beam Biotechnology* (New York: Springer Science & Business Media: 2006).
4. Z.L. Yu, *Surf. Coat. Tech*. **201**, 8006 (2007).
5. F.A. Chacon, *Ion induced radiation damage on the molecular level*, Ph.D. Thesis (University of Groningen: 2007).
6. C.A. Hunniford, D.J. Timson, R.J.H. Davies, R.W. McCullough, *Phys. Med. Biol.* **52**, 3729 (2007).
7. W. Wang, Z.L. Yu, W.H. Su, eprint, arXiv:0807.0079v1 </abs/0807.0079v1> (2008).
8. R. Norarat, N. Semsang, S. Anuntalabhochai, L.D. Yu, *Nucl. Instrum. Methods B* **267**, 1650-1653 (2009).
9. S. Sarapirom, K. Sangwijit, S. Anuntalabhochai, L.D. Yu, *Surf. Coat. Tech.* **204**, 2960-2965 (2010).
10. Q. Wang, G. Zhang, Y. Du, Y.H. Zhao, G.Y. Qiu, *Mutat. Res.* **528**, 55 (2003).
11. B. Hoebee, J. Brouwer, P.V.D. Putte, H. Loman, J. Retel, *Nucleic Acids Res.* **16**, 8147 (1988).
12. C.X. Xie, A. Xu, L.J. Wu, J.M. Yao, J.B. Yang, Z.L. Yu, *Genet. Mol. Biol.* **27**, 284 (2004).
13. M.L. Tang, S.C. Wang, T. Wang, S.G. Zhao, Y.J. Wu, L.J. Wu, Z.L. Yu, *Mutat. Res.* **602**, 163 (2006).
14. J. Vieira and J. Messing, Gene **19** (3), 259 (1982).
15. LabLife, Cambridge, MA, USA, *http://www.lablife.org* (2010).
16. P. Chaivan, N. Pasaja, D. Boonyawan, P. Suanpoot, T. Vilaithong, *Surf. Coat. Tech.* **193**, 356-360 (2005).
17. L.D. Yu, T. Kamwanna, I.G. Brown, *Phys. Med. Biol.* **54**, 5009 (2009).
18. European Bioinformatics Institute (EBI), *http://www.ebi.ac.uk/Tools/msa/clustalw2/* (2011).
19. S. Sarapiroma, P. Thongkumkoon, S. Anuntalabhochai, L.D. Yu, *Vacuum* **86**, 374 (2011).
20. J.F. Ziegler, *Particle Interaction with Matter*, www.srim.org (2011).
21. D.J. Song, X. Yu, Z.L. Yu, *Chinese High Technology Letters* **1999-01**, 47 (1999).
22. P.O. Löwdin, *Rev. Mod. Phys*. **35**, 724 (1963).
23. K.C. Cheng, D.S. Cahill, H. Kasai, S. Nishimural, A. Loeb, *J. Biol. Chem*. **267**, 166 (1992).
24. V. Thiviyanathan, A. Somasunderam, D.E. Volk, T.K. Hazra, S. Mitra, D.G. Gorenstein, *Biochem. Biophys. Res. Commun*. **366**, 752 (2008).
25. E.C. Friedberg, G.C. Walker, W. Siede, R.D. Wood, R.A. Schultz, T. Ellenberger, *DNA Repair and Mutagenesis* (Washington DC, ASM Press, 2005).
26. E.C. Friedberg, *Cell Res.* **18**, 3 (2008).