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Escherichia coli mutants induced by multi-ion irradiation

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Wild-type *Escherichia coli* K12 strain W3110 was irradiated by 10 keV nitrogen ions. Specifically, irradiation was performed six times by N^+ ions, followed by the selection of *lac* constitutive mutants, and each time a stable S55 mutant was produced. By sequencing the whole genome, the fine map of S55 was completed. Compared with reference sequences, a total of eighteen single nucleotide polymorphisms (SNPs), two insertions and deletions (Indels), and nine structural variations (SVs) were found in the S55 genome. Among the 18 SNPs, 11 are transversional from A, T or C to G, accounting for 55.6% of point mutations. GCCA insertion occurs in the target gene *lacI*. Four SNPs, including three in *rlpB* and one in *ygbN*, are connected with cell envelope and transport. All nine structural variations of S55 are deletions and contain insertion sequence (IS) elements. Six deleted SVs contain disrupted ISs, nonfunctional pseudogenes, and one more 23 252 bp SV in the Rac prophage region. Overall, our results show that deletion bias observed in *E. coli* K12 genome evolution is generally related to the deletion of some nonfunctional regions. Furthermore, since ISs are unstable factors in a genome, the multi-ion irradiations that caused these deleted fragments in S55 turn out to be beneficial to genome stability, generating a wider mutational spectrum. Thus, it is possible that the mutation of these genes increases the ability of the *E. coli* genome to resist etch and damage caused by ion irradiation.

Keywords: ion irradiation; multiple mutagenesis; stable mutants; whole genome sequences

INTRODUCTION

Low-energy ion beam biotechnology was developed in the 1980s by Chinese scientists. A low-energy ion beam can cause strong mutagenesis in microbes and plant seeds and has been introduced as a useful tool for microbial and plant breeding [1–3]. However, the biological effects of such low-energy ion beam irradiation remain unclear.

Previously, researchers compared the base-pair substitutions of the genomic *rpoB* gene induced by nitrogen ion irradiation *in vivo* with those induced by γ-rays and found that the preferential substitutions are transitional from pair CG to TA, pair AT to GC and transversional from pair AT to TA, accounting for 92.13% (82/89) of the total mutational type. Particularly, they found that the AT to TA transversions, as induced by low-energy ion irradiation, are about 2-fold greater than those induced by γ-rays [4]. The mutational spectrum of the *lacI* gene in *Escherichia coli*, as induced by the low-energy ion beam, has also been studied. Results have indicated that the ratios among three kinds of mutations, namely base substitution, one-base addition/deletion, and + GCCA/ – GCCA, as induced by the low-energy ion beam, are close to those induced by glyoxal and menadione. These authors have suggested that the intracellular free-radicals and adducts produced by low-energy ion radiation are responsible for the mutation of *lacI* [5].

However, to the best of our knowledge, no study has yet reported the effects of low-energy ion irradiation on whole genome sequences. The newly developed DNA sequencing technology provides an approach to solving this problem. Using lllumina whole genome sequence technology, we have sequenced the whole genome of stable mutant S55 of *E. coli* and completed the fine genome map. Our results reveal the overall picture of the genomic mutation of *E. coli* K12 induced by multiple low–energy ion irradiations and indicate the general trend of deletion bias existing in this stable mutant.

MATERIALS AND METHODS

E. coli strains

E. coli wild-type strain W3110 (F-λ-), a gift from Prof. Kaj Frank Jensen, was used for mutation research. *E. coli* DH5α was used as the *lacZ*$^-$ strain for the scavenging of alternative carbon sources from the minimal agar plate several hours in advance and was stored in our laboratory.

Reagents and media

LB medium and minimal A salts were prepared as described by Miller [6, 7]. Vogel–Bonner salts [8] were modified and they contained 0.2 g $MgSO_4 \cdot 7H_2O$, 2.0 g $(NH_4)H_2PO_4$, 10.0 g K_2HPO_4, and 2.8 g sodium citrate per liter. Minimal glucose plates contained minimal A salts at standard concentration, 1mM $MgSO_4$, 5 μg/ml thiamine, 0.2% glucose and 1.5% agar. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) minimal plates consisted of minimal glucose plates supplemented with 45 μg/ml X-gal. Phenyl-β-D-galactopyranoside (P-gal) plate used for the selection of *lac* constitutive mutants consisted of modified Vogel–Bonner salts supplemented with 75 mg/l P-gal and 15 g purified agar/l.

Mutagenesis of W3110 strain via multi-ion irradiations

The sample was prepared and irradiated according to the method given by Tang *et al*. [5]. Briefly, a white colony of *lacI*$^+$ genotype from the X-gal plate was cultured in LB medium overnight at 37°C; 0.1 ml mixture of this culture was spread onto a glass Petri dish (75 mm) and dried. The samples were then irradiated with nitrogen ions, using 10 keV and a dosage of 31.2×10^{14} ions/cm^2. The irradiated cells were cultured on P-gal plates and LB plates, respectively. The mutant colonies (*lacI*$^-$ and *lacO*^{c}) that grew on the P-gal plates for 48–72 h were scored and cultured in 0.1 ml P-gal medium overnight at 37°C. Small portions of the culture were grown on minimal P-gal and X-gal plates by overnight incubation at 37°C. A deep blue colony on the minimal X-gal plate indicated *lacI*$^-$; its corresponding colony on the minimal P-gal plate was inoculated in 5 ml of LB medium, and the culture was incubated overnight at 37°C. For multiple mutation experiments, the above procedure was repeated. Briefly, 1 ml of the *lacI*$^-$ mutant culture was washed twice and diluted to appropriate concentration with 0.2M PBS (pH 7.0), and 0.1 ml of this mixture was spread onto a glass petri dish and dried. These *lacI*$^-$ mutant colonies were then irradiated with nitrogen ions again, using 10 keV and a dosage of 31.2×10^{14} ions/cm^2. Thus, in multi-ion irradiation experiments, the process of irradiation and inoculation on the P-gal plate was repeated 5–6 times. Moreover, to study evolutionary stability, after single or multiple ion irradiations, the *lacI*$^-$ mutant colonies on the minimal P-gal plate were selected and propagated several times.

Stable mutant selection through multiple mutations

In order to select a stable mutant, we defined the P-gal selectivity (not mutation rate) of low-energy ion irradiation as

$$K(n, m) = \frac{\text{the colony number on P} - \text{gal plates}}{\text{the colony number on LB plates}} = \frac{P(n, m)}{P(n, m) + Q(n, m)} \quad (1)$$

where *P(n, m)* represents the number of *lacI*$^-$ mutants on P-gal plates, i.e. the number of mutants of P-gal type, *Q (n, m)* represents the number of mutants of non-P-gal type (including the number of the possible wild-type mutant reversals), *n* is the number of ion irradiations, and *m* is the number of passages after irradiation (each passaged time is 72 h).

The *lacI*$^-$ mutant colonies on the minimal P-gal plate under multiple mutations were selected and propagated for the passaged stability test. The *lacI*$^-$ mutant colony was diluted to appropriate concentration with 0.2M PBS. A total 0.1 ml of the resulting mixture was cultured on minimal plates containing P-gal and LB, respectively, and the number of colonies on each plate was counted to calculate *K*. We observed the change of *K* with *m* (for given *n*). If the fluctuations of *K* were small and *K* tended towards 1, then the mutants were considered stable.

Whole-genome sequencing of mutant and detection of SNPs, Indels and SVs

The stable mutant S55 was repeatedly mutated and passaged five times each from the wild-type W3110. After sequencing in BGI using lllumina, the fine genome map was completed. By comparing the fine genome map of S55 with the reference genome, we obtained all mutational sites of the genome, including single-nucleotide polymorphisms (SNPs), insertions and deletions (Indels) and structural variations (SVs).

RESULTS

Law of multiple mutations on *E. coli* K12 by ion irradiations

Figure 1 illustrates P-gal selectivity of mutants vs passaged time *m* after ion irradiation once or six times, respectively. Figure 2 gives P-gal selectivity of mutants vs the number of times of ion irradiation *n*. From Figs 1 and 2, we found that the law of mutation of *E. coli* under multi-ion irradiations can be expressed as follows. First, both for single and multiple mutations, *K*(1, *m*) and *K*(6, *m*) increase with *m* and take a value near 1 for $m \geq 1$ (see Fig. 1 and Table S4

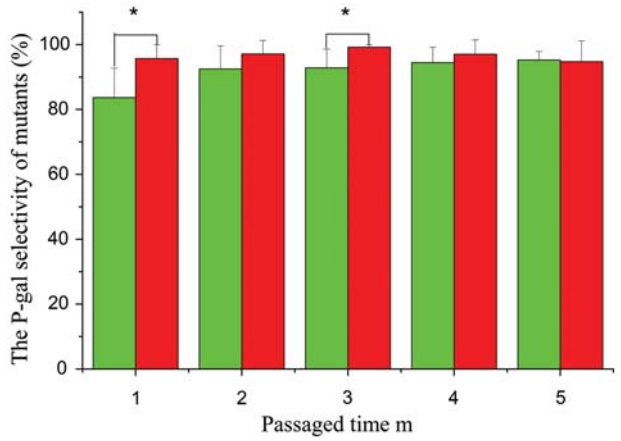

Fig. 1. The relation of P-gal selectivity of mutants with passaged time. The averaged P-gal selectivity $K(1, m)$ after ion-beam irradiation once is plotted in green, and the averaged P-gal selectivity $K(6, m)$ after ion-beam irradiation six times is plotted in red. The asterisk indicates that the difference between $K(6, m)$ and $K(1, m)$ is significant, $P < 0.05$.

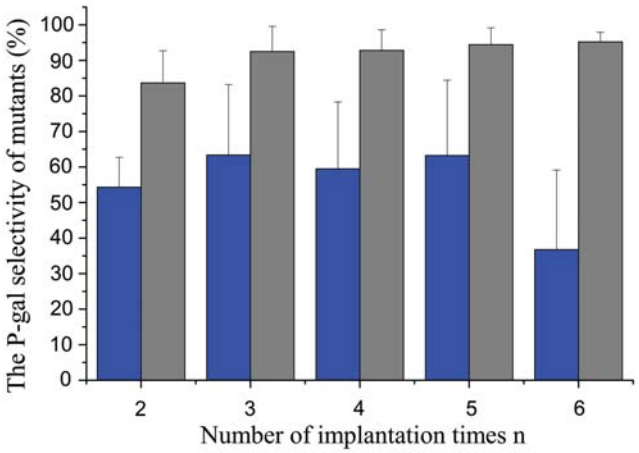

Fig. 2. The relationship between P-gal selectivity of mutants and frequency of ion irradiation. The averaged P-gal selectivity $K(n, m = 0)$ vs number of times for ion implantation n $(n \geq 2)$ is plotted in blue, and the averaged P-gal selectivity $K(1, m)$ in the contrast set under the same number $(= n + m)$ of P-gal selections is plotted in grey.

and S6 in the Supplementary Material), but the fluctuations of K were small and tended toward 1. This result means that hereditary stability increased under P-gal selection, since K toward 1 means that the number of non-P-gal mutant colonies remains a small fraction of the mutants during the passaged process. Moreover, the genetic stability of mutants was attained with the passaged number of times more quickly in multiple mutation, as seen from the comparison of $K(6, m)$ to $K(1, m)$ in Fig. 1. Second, the P-gal selectivity of mutants with multi-ion irradiations is significantly lower than that with single ion irradiation, i.e. $K(n \geq 2, m = 0)$ is much lower than $K(n = 1, m)$ in the contrast set under the same number $(n + m)$ of P-gal selections, as shown in Fig. 2. The large $Q(n \geq 2Q(m = 0)$ means the number of non-P-gal mutant colonies increases after repeated irradiations. Interestingly, the colony polymorphisms of mutants on P-gal plates were observed directly in our experiments. The shape and size of colonies are quite different between single and multi-ion irradiation experiments. The relationship between the number of mutant types and the number of ion irradiations is nonlinear. Therefore, the periodic change of environment through ion irradiation could effectively increase mutagenesis, and such multi-mutagenesis may generate more abundant mutational spectrum than single mutagenesis.

Stable mutants mutagenized by multi-ion irradiation selection

As shown in Fig. 2, after multiple mutagenesis with five rounds of ion irradiation, we found that the average P-gal selectivity K is minimal, i.e. for most of the mutants $K(6, 0) < K(n \neq 6, 0)$ (Table S5). Thus, after six rounds of ion irradiation and P-gal selection, a mutant with more abundant mutational spectrum may be obtained. On the other hand, based on the stability law deduced above, a mutant strain of higher genetic stability can be obtained by using longer passaged time. Using these data, we selected a stable mutant obtained from wild type implanted by N^+ ions six times and passaged five times as the target strain for the sequence investigation. This strain was called S55.

Detection of mutations in S55

A total of 18 SNPs, 2 Indels, and 9 SVs were found in the S55 genome, as summarized in Table 1 and detailed in Table S1 (Supplementary Material). Among the 18 SNPs, 15 (84.3%) are located in coding regions, and 3 (16.7%) are in intergenic sequences. Among the 15 SNPs in coding regions, 3 are synonymous and 12 are nonsynonymous. Indels are summarized in Table S2 (Supplementary Material). There are 2 Indels detected in S55. One is the deletion of AA in the intergenic region, and another is the insertion of GCCA at target gene *lacI*. The mutation ±GCCA at the hot spot has been reported many times in the literature [8, 9]. Finally, SVs detected in S55 are summarized in Table 2 of the text and Table S3 of the Supplementary Material. A total of 9 SVs were detected in S55, all of which are deletions with no insertions or complexes found. The longest deletion of 23 252bp occurs in the Rac prophage region. Another 8 deletions are 1446 bp, 1530 bp, 1645 bp, 1426 bp, 1524 bp, 1563 bp, 1383 bp and 1418 bp in length, respectively. All nine deletions contain insertion sequences.

Table 1. Mutations detected in S55

SNP		Indel		SV		
transition	**transversion**	**insertion**	**deletion**	**insertion**	**deletion**	**complex**
8	10	1	1	0	9	0

Table 2. Structural variations (SVs) detected in the S55 genome

SV No	Length	Ref-gene-name
Deletion 1	1446	*dcuC, insH, dcuC*
Deletion 2	23 252	*ydaO, intR, ydaQ, ydaC, lar, recT, recE, racC, ydaE, kil, sieB, ydaF, ydaG, racR, ydaS, ydaT, ydaU, ydaV, ydaW, rzpR, rzoR, trkG, ynaK, ydaY, ynaA, lomR, insH, lomR, stfR, tfaR, pinR, ynaE*
Deletion 3	1530	*gatA, insH, gatA*
Deletion 4	1645	*rcsC, insD, insC, rcsC*
Deletion 5	1426	*tdcE, insH, tdcD*
Deletion 6	1524	*insD, insC, yidZ*
Deletion 7	1563	*tnaB, insH, tnaB*
Deletion 8	1383	*insH*
Deletion 9	1418	*insH alsK*

The repetitive occurrence of a gene in one SV means different fragments of the gene; *ins* means gene contains IS.

DISCUSSION

High-energy ionizing radiation typically has energies ranging in the millions of electron volts (eV). However, it has been reported that damage from such irradiation in living cells is actually induced by multiple low-energy events [10, 11]. After keV ions are implanted into organisms, secondary electrons may be generated by the interaction of ions with biomolecules. This secondary electron emission causes DNA strand breaks or base damage [1]. The degree of the damage to DNA bases caused by secondary electrons is controlled by the dissociative electronic attachment cross-section on DNA bases. The cross-sections on A, T, G and C were reported as $\sigma_A = 9.01 \times 10^{-16}$ cm^2, $\sigma_T = 1.085 \times 10^{-15}$ cm^2, $\sigma_C = 2.2741 \times 10^{-16}$ cm^2, and $\sigma_G = 7.1124 \times 10^{-17}$ cm^2, respectively [12]. Since σ_G is the least among these four dissociative electronic attachment cross-sections, guanine is more stable under secondary electron interactions. By the same reasoning, the base pair A:T is more easily damaged than the base pair G:C [12]. Therefore, among the 18 SNPs we observed, only the substitutions of A, T or C, but not G, have already been found by others. The observed relative frequencies of SNPs through A, T or C substituted by G are 5/11:4/11:2/11, which are roughly proportional to the cross-section ratio of $(\sigma_A - \sigma_G) : (\sigma_T - \sigma_G) : (\sigma_C - \sigma_G)$.

Out of 18 SNPs, 8 are transitions (G:C A:T(2/8) and A:T G:C(6/8)), and 10 are transversions (G:C T:A(3/10), G:C C:G(2/10), A:T C:G(4/10), and A:T T:A(1/10)). Meanwhile, 15 SNPs were found in the coding region: 3 are synonymous, 12 are nonsynonymous, and 3 are in intergenic sequences. The higher nonsynonymous mutation rates are consistent with the observation of predominant beneficial substitutions in other *E. coli* experiments [13, 14]. Among the 15 SNPs in the coding region, two genes are mutated three times, respectively, namely *rlpB* (T G(2/3) and A G (1/3)) and *malP* (A G, T G, and C G). RlpB encoded by the *rlpB* gene is an essential outer membrane (OM) lipoprotein that interacts with inner membrane protein (IMP) to effect lipopolysaccharide (LPS) biogenesis [15]. *malP* is a gene which encodes maltodextrin phosphorylase.

It has been reported many times in the literature that keV ion irradiation can etch and damage the cell envelope [16, 17]. When Phanchaisri *et al.* [18] used Ar ions implanted into *E. coli* with an energy of 26 keV and dosage of 1×10^{15} ions/cm^2, the ion bombardment created novel crater-like structures on the surface of the bacterial cell envelope, as observed by scanning electron microscopy (SEM).

However, in this paper, we found 4 SNPs (3 SNPs in *rlpB* and 1 in *ygbN*) connected with cell envelope and transport, but these SNPs were nonsynonymous and thus may be beneficial [13]. In fact, the mutations of these genes may enhance the ability of *E. coli* to resist DNA etch and damage upon ion irradiation.

The 9 large SVs detected in S55 are summarized in Table 2 and detailed in Table S3 of the Supplementary Material. To the best of our knowledge, no similar results have been reported in previous studies. The novelty of the present findings can be described as follows. First, all 9 SVs are deletions with no insertions or complexes in S55. Second, all 9 deletions contained insertion sequences. Third, apart from the functionality of an individual deleted gene (*yidZ* in the 6th deletion, Table 2), nearly all of the deletions are IS-disrupted, nonfunctional pseudogenes. For example, *dcuC, gatA, rcsC, tdcD, tnaB* and *alsK* in the first, third, fourth, fifth, seventh and ninth SVs, respectively, are pseudogenes [19] (Table 2). Meanwhile, the second SV occurs in the Rac prophage region. While the prophage is not indispensable for bacterial function, it can be regarded as a dangerous 'molecular time bomb' that can kill cells upon their eventual induction [20].

Recent research has indicated that deletional bias is a major force shaping bacterial genomes. Based on statistical studies on Indel events, it was shown that deletions outnumbered insertions in most prokaryotes [14, 21, 22]. Our experiments supported the view of deletional bias in prokaryotes and give more evidence for the presence of large deletions (longer than a thousand bp spanning multiple loci) in bacterial genome evolution. At the same time, we demonstrated that the pervasive bias towards segmental deletion, as observed in this experiment, is connected with the deletion of pseudogenes and other nonfunctional insertion sequences. Because of frequent horizontal gene transfers, the bacterial genome contains many foreign genes and insets, and these foreign DNAs, often useless or even deleterious, will be eliminated under evolution. The direction of genome evolution is an interesting problem in theory. It has been proposed that the function-coding information quantity of a genome always grows in the course of evolution through sequence duplication, expansion of code, and horizontal gene transfer [23]. In the context of the growth of function-coding information quantity, even though deletions happen as insertions in stochastic evolution, it is more likely that the deletion of nonfunctional sequences is selected. The SVs observed in *E. coli* evolution are consistent with this point. Of course, the deletion of functional genes may sometimes occur, as in the case of DNA damage under strong external forces. Although functional elements in old species may have been deleted, they must have been replaced by a compensatory functional network in the surviving mutant. That the individual deleted gene (*yidZ*) is functional in the S55 mutant could be interpreted according to this theory. The *yidZ* gene is a predicted DNA-binding transcriptional regulator. Meanwhile, an SNP occurring in the intergenic region is close to *yidP* (Table S1). One may therefore assume that such a deletion event of a functional gene is accompanied by a compensatory change in the gene regulatory network. The point needs to be studied further. It has been shown that transposons and insertion sequences play an important role in the organization of the *E. coli* genome [24]. Hence, while IS elements could be potentially toxic during irradiation based on stress-induced recombinational repair, resulting in lethal rearrangements between long repeats [25], their deletion increases the stability of the *E. coli* genome [26]. The S55 mutant is such a stable mutational strain, implanted by nitrogen ions six times and passaged five times. In fact, the observed 9 deletions in S55 may have enhanced the stability of the genome.

A long-term experiment with *E. coli* was made where the population evolved with glucose as a limiting nutrient [13, 14, 27]. There were no mutator phenotypes observed; only 29 SNPs and 16 DIPs (deletions, insertions and other polymorphisms) were detected through the first 20 000 generations [14]. A hypermutable phenotype appeared by about generation 26 500. The population exhibited a greatly elevated rate of genomic evolution only in later generations. However, in our experiments with *E. coli* by repetitious ion irradiation, the colony polymorphisms of mutants on P-gal plates were observed in the very early stage. Therefore, ion irradiation can effectively accelerate the genome evolution of *E. coli*. We found 18 SNPs, 2 Indels and 9 SVs in S55. The identified SNPs and DIPs in our experiment are different from those identified in Barrick's long-term experiments [14], except 1 SNP. S55 contained an SNP in *ycdT*, while Barrick *et al.* found an SNP mutation of T G in the intergenic region neighboring gene *ycdT* (a predicted diguanylate cyclase). The genome size of the 40K clone in Barrick's experiments is 4.57×10^6 bp, representing a reduction of 1.2% from the ancestor, while the reduction of S55 genome is about 0.76%, as compared to the wild-type W3110. Both works support the idea that bacterial genome evolution is biased toward deletions, but the present paper has furnished more information on large (each longer than 1300 bp) structural variations of the deletional type.

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SUPPLEMENTARY DATA

Supplementary data is available at the *Journal of Radiation Research* online.

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