A Genetic Toxicology Study of the Rapid Detection of Nitrosamine Compounds by the rpsL Gene Mutation Assay

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Abstract: In a rpsL gene mutation experiment, the mutagenicity of the nitrosamine compounds N-diethylnitrosamine (NDEA) and N-dipropynitrosamine (NDPA) was investigated at the cellular level, as well as with PCR (polymerase chain reaction) and RCA (rolling-circle amplification) amplification systems. The experiments were set up with 10 ppm, 100 ppm, and 1000 ppm concentration gradients of NDEA and NDPA, and ethidium bromide (EB) was used as a positive control group. The results demonstrated that the mutagenic frequency of NDEA and NDPA was significantly higher than the spontaneous mutation frequency of the rpsL gene under the same conditions, but lower than the mutagenic rate of EB in the positive control, and there was a dose-effect relationship, indicating that NDEA and NDPA could induce rpsL gene mutation. The rpsL mutation system has a low spontaneous mutation background and high sensitivity, thus the system is expected to become an effective tool for the rapid detection of carcinogens in the field of food.

Keywords: genotoxicity test; nitrosamine; rpsL; safety assessment

1. Introduction

Nitrosamines are a class of compounds with the structural formula R2 (R1) N-N=O. They are one of the four major food contaminants and an important class of strong chemical carcinogens [1,2]. Nitrite, nitrate, and amines, the precursors for the formation of nitrosamines, are ubiquitous in food, so the toxicology of nitrosamines has attracted extensive attention from researchers [3].

The toxicity of nitrosamines to humans and animals mainly includes acute toxicity and carcinogenicity [4]. The entry of nitrosamines into humans and animals mainly causes hepatic lobular damage and central necrosis, manifested as dizziness and fatigue, liver lesions and necrosis, pleural and ascites, and jaundice. In addition to acute toxicity, nitrosamines have broad-spectrum and strong carcinogenicity. Studies have found that preserved fish, vegetables, and smoked foods are rich in nitrite and nitrosamines [5,6], and the intake of such foods is closely related to gastric and esophageal cancer [7].

Nitrosamines need metabolic activation in the body to play a carcinogenic role. Its carcinogenicity has obvious organotropism, and long-term or excessive ingestion of nitrosamines can induce tumors in animals and humans [8]. Nitrosamine is a relatively stable compound, and its carcinogenic mechanism is as follows: the hydrogen on the carbon atom connected to ammonia nitrogen in the compound is subjected to the action of liver microsome P450, and the hydrogen on the carbon is oxidized to form a hydroxyl group, further decomposes, and undergoes isomerization into alkyl azohydroxyl compounds; it is a highly active carcinogen. It should be noted that its carcinogenicity is related to its chemical structure, physicochemical properties, and metabolic processes in vivo [4,9].

In the toxicological evaluation for food safety, the Ames genotoxicity test is often used to screen for mutagenic chemicals [10]. However, the Ames test has its inherent...
limitations: there are few types of mutations that can be detected, and in the Ames test, the revertant generation is random. At present, there is still a lack of research on the mutagenic mechanism of nitrosamines from the perspective of molecular biology, so it is necessary to use gene mutation experiments to study the genetic toxicology of nitrosamines.

The rpsL gene mutation detection system designed and used in this paper is a highly sensitive and simple genotoxicity detection method, that can provide a fast and effective way to detect potential carcinogens generated during food processing. The rpsL (ribosomal protein small subunit) gene encodes the S12 protein in the 30 S subunit of the ribosome. The S12 protein acts at the beginning of protein translation, and the action site of antibiotics such as streptomycin is the S12 protein in the 30 S subunit of the ribosome [11]. Under normal circumstances, the combination of streptomycin and S12 protein makes the protein biosynthesis unable to proceed, and the cell stops growing; the mutation of the rpsL gene makes the streptomycin loose effective binding with S12 protein, so that the strain with this gene mutation has resistance to streptomycin. It can grow on plates containing streptomycin, so mutant strains were selected [12,13]. The host strain MF101 used in this experiment has streptomycin resistance, and the plasmid pMOL21 contains ampicillin resistance and the rpsL gene sequence [14]. Due to the recessive genetic expression of streptomycin resistance, only when the rpsL gene segment of the plasmid is mutated will the host cell have streptomycin resistance, to achieve the purpose of forward screening of the mutant rpsL gene.

In this experiment, we selected two representative nitrosamines, NDEA and NDPA [15], as the research objects. Using the rpsL gene mutation detection system, through the change in resistance caused by gene mutation, the detection system of mutant strains was positively selected to realize the rapid detection of the genotoxicity of nitrosamine compounds and, according to the results, to study the genetic toxicology of nitrosamine compounds.

2. Materials and Methods

2.1. Strains and Plasmids

The strains and plasmids used in this experiment were donated by Professor Hisaji Maki of the Nara University of Science and Technology, Japan [16,17].

The mutagenicity detection of nitrosamine compounds was carried out using the rpsL gene on plasmid pMOL21 as a selectable marker. The ribosomal protein S12 encoded by the rpsL gene can form a complex with streptomycin and enhance the ability of streptomycin to bind to 16 S rRNA, thereby preventing the initiation of transcription. When the rpsL gene is mutated, the interaction between 16 S rRNA and streptomycin is disrupted, resulting in cell resistance to streptomycin.

We adopted Escherichia coli MF101, whose rpsL gene is mutated, so this strain has streptomycin resistance. The plasmid pMOL21 contains ampicillin as a selection marker and the correct rpsL gene. When the pMOL21 plasmid is transformed into the host cell, due to the recessive genetic expression of streptomycin, only when the rpsL gene on the plasmid is mutated will the host cell have streptomycin resistance, to achieve the purpose of forward screening of the mutant rpsL gene. For specific information on strains and plasmids, please refer to [14].

2.2. Determination of the Mutation Rate of the rpsL Gene at the Cellular Level

E. coli MF101 + pMOL21 were cultured with different concentrations (10 ppm, 100 ppm, or 1000 ppm) of NDEA or NDPA. Ten hours after growth, the strains were collected and spread on plates containing ampicillin (Amp\(^+\), 50 µg/mL) to determine the total number of transformed cells and spread on plates containing ampicillin and streptomycin (Amp\(^+\), Str\(^+\), 50 µg/mL) to determine the number of colonies with rpsL gene mutations. The mutation rate is the ratio of the number of mutant colonies to the total number of colonies. Each group of experiments was repeated five times, and the total number of colonies was not less than 10\(^6\). The experimental data and images were statistically analyzed by Origin2018 software.
2.3. Determination of the rpsL Gene Mutation Rate in the PCR Amplification System

The PCR system was as follows: 10 × PCR buffer (MgCl2) 5 μL, dNTP 2 μL, pMOL21 plasmid template 1 μL, upstream and downstream primers 1 μL, Pfu DNA polymerase 0.5 μL, ddH2O supplemented to 20 μL (nitrosamine compounds were directly added to the PCR system).

The PCR program was as follows: predenaturation at 95 °C for 5 min, denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 120 s, 25 cycles, full extension at 72 °C for 10 min, and storage at 16 °C.

The PCR products were recovered and ligated with restriction enzymes (MscI, BamHI), transformed into MF101 strains, plated on ampicillin-containing plates to determine the total number of transformed cells, and plated on ampicillin- and streptomycin-containing plates to identify rpsL gene-mutated colonies. A flow chart of the treatment of PCR products is provided in Figure 1.

![Figure 1. Flow chart of the treatment of PCR products.](image1)

2.4. Determination of the rpsL Gene Mutation Rate in the RCA Reaction System

Heat denaturation of samples and annealing reaction system of primers and plasmids: 1 μL of 10 × phi29 DNA polymerase reaction buffer, 2.5 μL of 100 μM random primers, 1 μL of plasmid pMOL21 to be amplified, and 4.3 μL of ddH2O. The above system was heated at 95 °C for 3 min and then placed on ice for 15 min.

Amplification reaction system: 10 mM dNTP 0.5 μL, 100 × BSA 0.2 μL, phi29 DNA polymerase 0.5 μL to the above reaction solution (nitrosamine compounds or EB are added to the amplification reaction system), and then incubated at 30 °C overnight.

Heat-inactivated phi29 DNA polymerase: heat at 65 °C for 10 min.

A flow chart of the treatment of RCA products is provided in Figure 2.

![Figure 2. Flow chart of the treatment of RCA products.](image2)
3. Results and Discussion

3.1. Determination of the rpsL Gene Replication Spontaneous Mutation Rate in E. coli MF101

E. coli was grown without a mutagen and screened by Amp\(^+\) and Amp\(^+\)&Str\(^+\) resistance plates, and the colonies were counted. The spontaneous mutation rate of the rpsL gene was \(0.70 \times 10^{-6}\). The above experiments were repeated four times, and the average number of strains in each group of parallel controls reached \(10^8\). The data are provided in Table 1.

### Table 1. Spontaneous mutation rate of rpsL.

| Total Strain \((\times 10^6)\) | Mutant Strain | Ratio \((\times 10^{-6})\) |
|-------------------------------|--------------|---------------------|
| 121                           | 98           | 0.81                |
| 257                           | 153          | 0.60                |
| 94                            | 102          | 1.09                |
| 205                           | 67           | 0.33                |

### Table 2. Statistics of spontaneous mutation types of rpsL.

| Types                        | Mutant Strains | Ratio (%) |
|------------------------------|----------------|-----------|
| Base substitution            | 120            | 24        |
| Sequence replacement         | 56             | 11.2      |
| Single base frameshift       | 27             | 5.4       |
| Double base frameshift       | 1              | 0.2       |
| Large segment deletion       | 280            | 56.1      |
| Fragment insertion           | 15             | 3         |

Studies have demonstrated that, during the replication process of the rpsL of E. coli, the proportion of spontaneous mutation types of large fragment deletion is the highest, reaching 56%, followed by base substitution, sequence substitution, frameshift mutation, and large fragment deletion, the four main types of gene mutation, as well as insertion mutation (the data are provided in Figure 3). Among them, gene mutation types that Ames cannot detect (large fragment deletion, sequence substitution, fragment insertion, etc.) accounted for more than 70%. This indicates that the rpsL detection system avoids the limitation of mutation types in the Ames test and is suitable for the analysis and detection of trace mutagenic substances.

3.2. Statistics of Spontaneous Mutation Types of rpsL Gene Duplication

Through large-scale sequencing, we determined the rpsL gene sequence in a total of 500 mutant strains, compared them with WT, and obtained the type of spontaneous mutation in the process of rpsL gene replication. The statistical results are found in Table 2.

3.3. Detection of the Induced Mutation Rate of rpsL Gene Replication at the Cellular Level

EB was used as a mutagenic positive control, and the rpsL mutation system was used to detect the mutation rate of rpsL induced by it at the cellular level. The experimental data obtained are provided in Table 3. At concentrations of 10 ppm, 100 ppm, and 1000 ppm EB, the mutation rates of the rpsL gene in E. coli were \(3.71 \pm 0.502 \times 10^{-6}\), \(5.78 \pm 0.728 \times 10^{-6}\), and \(7.87 \pm 1.842 \times 10^{-6}\), respectively. The above results demonstrated that EB had strong mutagenicity at lower concentrations, and this mutagenicity increased with increasing concentration.
The results of the rpsL gene mutation induced by nitrosamine compounds at the cellular level demonstrated that NDEA and NDPA had high genotoxicity at a concentration of 10 ppm, and the induced rpsL gene mutation rates were $2.42 \pm 0.717 \times 10^{-6}$ and $2.67 \pm 0.909 \times 10^{-6}$, respectively, which were significantly higher than the spontaneous mutation rate of rpsL under the same conditions ($0.70 \pm 0.322 \times 10^{-6}$). The mutation rates of the rpsL gene induced by NDEA and NDPA at 100 ppm and 1000 ppm were $3.76 \pm 0.852 \times 10^{-6}$ and $4.75 \pm 1.417 \times 10^{-6}$, $4.82 \pm 1.738 \times 10^{-6}$ and $5.14 \pm 1.433 \times 10^{-6}$, respectively. This result illustrates that the genotoxicity of NDEA and NDPA to E. coli DNA replication increases with increasing concentration. The higher the concentrations of NDEA and NDPA were, the stronger the mutagenicity, and the difference in the mutation rate between the two was greater. These results indicate that the mutagenicity of NDPA at the cellular level is higher than that of NDEA. The detailed experimental data are provided in Table 3; Figure 4 illustrates the statistics of the data in Table 3.

| Concentration | Mutagen | Mutant Strains | Total Strains | Mutation Rate ($10^{-6}$) |
|---------------|---------|----------------|---------------|--------------------------|
| 10 ppm        | NDEA    |                |               |                          |
|               | 120     | 832            | 1.44          |                          |
|               | 129     | 552            | 2.34          |                          |
|               | 104     | 352            | 2.95          |                          |
|               | 40      | 135            | 2.96          |                          |
|               | NDPA    |                |               |                          |
|               | 125     | 735            | 1.70          |                          |
|               | 182     | 650            | 2.80          |                          |
|               | 265     | 687            | 3.86          |                          |
|               | 200     | 856            | 2.34          |                          |
| 10 ppm        | EB      |                |               |                          |
|               | 307     | 840            | 3.65          |                          |
|               | 310     | 708            | 4.38          |                          |
|               | 224     | 616            | 3.64          |                          |
|               | 245     | 775            | 3.16          |                          |
Table 3. Cont.

| Concentration | Mutagen | Mutant Strains (10^5) | Total Strains | Mutation Rate (10^−6) |
|---------------|---------|-----------------------|---------------|-----------------------|
|               | NDEA    |                       |               |                       |
| 100 ppm       |         | 250                   | 752           | 3.32                  |
|               |         | 159                   | 566           | 2.81                  |
|               |         | 208                   | 492           | 4.23                  |
|               |         | 304                   | 648           | 4.69                  |
|               | NDEA    | 332                   | 580           | 5.72                  |
|               |         | 213                   | 800           | 2.66                  |
|               |         | 344                   | 676           | 5.09                  |
|               |         | 420                   | 760           | 5.53                  |
| 1000 ppm      | NDPA    | 330                   | 508           | 6.50                  |
|               |         | 184                   | 304           | 6.05                  |
|               |         | 260                   | 544           | 4.78                  |
|               |         | 272                   | 470           | 5.79                  |
|               | EB      | 140                   | 484           | 2.89                  |
|               |         | 140                   | 364           | 3.85                  |
|               |         | 272                   | 452           | 6.02                  |
|               |         | 280                   | 428           | 6.54                  |
|               | NDPA    | 289                   | 410           | 7.05                  |
|               |         | 136                   | 324           | 4.20                  |
|               |         | 160                   | 296           | 5.41                  |
|               |         | 218                   | 560           | 3.89                  |
|               | EB      | 110                   | 112           | 9.82                  |
|               |         | 51                    | 76            | 6.71                  |
|               |         | 76                    | 128           | 5.94                  |
|               |         | 109                   | 121           | 9.01                  |

3.4. Detection of the Spontaneous Mutation Rate of rpsL Gene Replication in the PCR System

Compared with Taq DNA polymerase, Pfu DNA polymerase has excellent thermal stability and proofreading properties, and its amplification error rate is stable at approximately 10^−6 [18]. The rpsL detection system in this experiment uses Pfu DNA polymerase to amplify the rpsL gene in the PCR system. The results demonstrated that the spontaneous
mutation rate of the rpsL gene in the PCR system was $1.30 \pm 0.635 \times 10^{-6}$ (the detailed experimental data are provided in Table 4), which was basically consistent with the previously reported $Pfu$ DNA polymerase amplification error rate.

Table 4. Spontaneous mutation rate of rpsL in PCR.

| Total Strains ($10^6$) | Mutant Strains ($\times 10$) | Mutant Rate ($10^{-6}$) |
|-------------------------|-------------------------------|--------------------------|
| 87                      | 70                            | 0.80                     |
| 27                      | 41                            | 1.52                     |
| 23                      | 18                            | 0.78                     |
| 19                      | 40                            | 2.11                     |
|                         |                               | 1.30 $\pm$ 0.635         |

3.5. Detection of the Induced Mutation Rate of rpsL Gene Replication in the PCR System

We measured the mutation rate of mutagen-induced rpsL gene mutations in the PCR system. The mutagen was added directly to the PCR system to determine the mutation rate of the rpsL gene during DNA amplification. The results are as follows: the mutation rates of rpsL induced by NDEA and NDPA at concentrations of 10 ppm, 100 ppm, and 1000 ppm were $2.83 \pm 1.197 \times 10^{-6}$ and $2.93 \pm 1.430 \times 10^{-6}$, $4.51 \pm 1.314 \times 10^{-6}$ and $4.98 \pm 1.330 \times 10^{-6}$, and $5.02 \pm 1.890 \times 10^{-6}$ and $6.85 \pm 1.316 \times 10^{-6}$, respectively. The mutation rates of the rpsL gene induced by positive control EB with the same concentration gradient were $4.06 \pm 0.471 \times 10^{-6}$, $6.01 \pm 1.319 \times 10^{-6}$, and $10.35 \pm 1.143 \times 10^{-6}$, respectively (the detailed experimental data are provided in Table 5; Figure 5 illustrates the statistics of the data in Table 5). In the PCR system, the mutagenic rate of NDEA was lower than that of NDPA, but it was lower than that of EB in the positive control group.

Table 5. Mutation rate of rpsL induced by mutagens in PCR.

| Concentration | Mutagen | Mutant Strains | Total Strains ($10^5$) | Mutation Rate ($10^{-6}$) |
|---------------|---------|----------------|------------------------|---------------------------|
| 10 ppm        | NDEA    | 90             | 705                    | 1.28                      |
|               |         | 163            | 614                    | 2.65                      |
|               |         | 173            | 421                    | 4.11                      |
|               |         | 60             | 182                    | 3.30                      |
|               | NDPA    | 148            | 769                    | 1.92                      |
|               |         | 204            | 668                    | 3.05                      |
|               |         | 340            | 693                    | 4.91                      |
|               |         | 175            | 954                    | 1.83                      |
|               | EB      | 356            | 841                    | 4.23                      |
|               |         | 346            | 774                    | 4.47                      |
|               |         | 275            | 662                    | 4.15                      |
|               |         | 273            | 807                    | 3.38                      |
| 100 ppm       | NDEA    | 315            | 851                    | 3.70                      |
|               |         | 185            | 599                    | 3.09                      |
|               |         | 272            | 497                    | 5.47                      |
|               |         | 403            | 699                    | 5.77                      |
|               | NDPA    | 387            | 628                    | 6.16                      |
|               |         | 258            | 839                    | 3.08                      |
|               |         | 391            | 717                    | 5.45                      |
|               |         | 427            | 819                    | 5.21                      |
|               | EB      | 363            | 606                    | 5.99                      |
|               |         | 273            | 355                    | 7.69                      |
|               |         | 281            | 629                    | 4.47                      |
|               |         | 334            | 568                    | 5.88                      |
|               |         |                |                        | 4.98 $\pm$ 1.330          |
|               |         |                |                        | 6.01 $\pm$ 1.319          |
Table 5. Cont.

| Concentration | Mutagen | Mutant Strains | Total Strains ($10^5$) | Mutation Rate ($10^{-6}$) |
|---------------|---------|----------------|------------------------|---------------------------|
|               | NDEA    | 168            | 531                    | 3.16                      |
|               |         | 156            | 424                    | 3.68                      |
|               |         | 343            | 551                    | 6.23                      |
|               |         | 307            | 437                    | 7.03                      |
| 1000 ppm      | NDPA    | 321            | 416                    | 7.72                      |
|               |         | 342            | 421                    | 8.12                      |
|               |         | 191            | 302                    | 6.32                      |
|               |         | 310            | 590                    | 5.25                      |
|               | EB      | 206            | 172                    | 11.98                     |
|               |         | 64             | 176                    | 9.32                      |
|               |         | 137            | 135                    | 10.15                     |
|               |         | 169            | 170                    | 9.94                      |

Figure 5. Comparison of rpsL mutation rates induced by NDEA, NDPA, and EB by PCR.

3.6. Determination of the Spontaneous Mutation Rate of rpsL in the RCA System

Unlike traditional DNA amplification techniques, such as the PCR system, RCA is an isothermal nucleic acid amplification technique in which the polymerase continuously adds nucleotides to a circular template, resulting in a long tandem repeat (ssDNA) containing tens to hundreds of tandem repeats (complementary to the circular template) [19]. The DNA polymerases used in RCA are phi29, Bst, and Vent (exo-), but since phi29 DNA polymerase has the best processivity and strand displacement ability among the above polymerases, it is commonly used in the RCA reaction process [20–22]. Unlike PCR systems, RCA can be performed at a constant temperature (from room temperature to 37 °C) [23]. In addition, RCA can amplify a single molecule binding event a thousand-fold, making it suitable for the detection of ultralow abundance targets [24]. Currently, RCA, as a general signal amplification tool, has a wide range of applications in genomics, proteomics, diagnostics, and biosensing [25,26].

We used the RCA system to determine the spontaneous mutation rate of the rpsL gene, and the result, provided in Table 6, was $1.09 \pm 0.456 \times 10^{-6}$. The amplification mutation rate of the rpsL gene under the action of Pfu DNA polymerase was $1.30 \pm 0.635 \times 10^{-6}$, which was one order of magnitude lower than that of Taq DNA polymerase, which was $10^{-5}$. 
### Table 6. Spontaneous mutation rate of rpsL in the RCA reaction.

| Total Strains ($10^6$) | Mutant Strains | Mutation Rate ($10^{-6}$) |
|-------------------------|----------------|---------------------------|
|                         |                |                           |
| 113                     | 74             | 1.53                      |
| 61                      | 45             | 1.36                      |
| 42                      | 84             | 0.50                      |
| 60                      | 62             | 0.97                      |

1.09 ± 0.456

3.7. Detection of the Induced Mutation Rate of the rpsL Gene in the RCA System

We then determined the mutation rate of mutagen-induced rpsL gene mutations in the RCA system. In this experiment, we adopted the method of directly adding a mutagen to the RCA amplification reaction solution to provide a direct mutagenic environment for the amplification of the rpsL gene. This method avoids the possibility of affecting the mutagenicity of NDEA, NDPA, or EB during thermal denaturation.

The experimental results were as follows: the rpsL gene mutation rates induced by NDEA and NDPA at concentrations of 10 ppm, 100 ppm, and 1000 ppm were $2.49 \pm 0.692 \times 10^{-6}$ and $2.98 \pm 0.585 \times 10^{-6}$, $3.62 \pm 0.316 \times 10^{-6}$ and $5.59 \pm 2.287 \times 10^{-6}$, and $5.54 \pm 2.444 \times 10^{-6}$ and $6.20 \pm 1.110 \times 10^{-6}$, respectively. The mutation rates of the rpsL gene induced by the positive control EB with the same concentration gradient were $3.81 \pm 0.335 \times 10^{-6}$, $6.38 \pm 0.915 \times 10^{-6}$, and $7.35 \pm 2.615 \times 10^{-6}$, respectively (the detailed experimental data are provided in Table 7; Figure 6 illustrates the statistics of the data in Table 7). In the RCA process, the mutagenicity of NDEA was lower than that of NDPA, and both were lower than that of EB in the positive control group.

### Table 7. Mutation rate of rpsL induced by mutagens in the RCA reaction.

| Concentration | Mutagen | Mutant Strains | Total Strains ($10^5$) | Mutation Rate ($10^{-6}$) |
|---------------|---------|----------------|-------------------------|---------------------------|
| 10 ppm        | NDEA    | 158            | 881                     | 1.79                      |
|               |         | 174            | 863                     | 2.02                      |
|               |         | 191            | 597                     | 3.20                      |
|               |         | 212            | 716                     | 2.96                      |
|               | NDPA    | 264            | 735                     | 3.59                      |
|               |         | 251            | 823                     | 3.05                      |
|               |         | 305            | 985                     | 3.10                      |
|               |         | 187            | 856                     | 2.18                      |
|               | EB      | 317            | 876                     | 3.62                      |
|               |         | 343            | 796                     | 4.31                      |
|               |         | 242            | 669                     | 3.62                      |
|               |         | 303            | 821                     | 3.69                      |
| 100 ppm       | NDEA    | 261            | 753                     | 3.47                      |
|               |         | 231            | 683                     | 3.38                      |
|               |         | 210            | 592                     | 3.55                      |
|               |         | 240            | 588                     | 4.08                      |
|               | NDPA    | 354            | 722                     | 4.90                      |
|               |         | 268            | 697                     | 3.85                      |
|               |         | 489            | 546                     | 8.96                      |
|               |         | 351            | 751                     | 4.67                      |
|               | EB      | 393            | 541                     | 7.26                      |
|               |         | 263            | 395                     | 6.66                      |
|               |         | 283            | 555                     | 5.10                      |
|               |         | 310            | 478                     | 6.49                      |

5.59 ± 2.287

6.38 ± 0.915
Table 7. Cont.

| Concentration | Mutagen | Mutant Strains | Total Strains (10^5) | Mutation Rate (10^-6) |
|---------------|---------|----------------|----------------------|-----------------------|
| 1000 ppm      | NDEA    | 132            | 421                  | 3.14                  |
|               |         | 141            | 356                  | 3.96                  |
|               |         | 191            | 290                  | 6.59                  |
|               |         | 270            | 319                  | 8.46                  |
|               | NDPA    | 301            | 387                  | 7.78                  |
|               |         | 187            | 361                  | 5.18                  |
|               |         | 242            | 406                  | 5.96                  |
|               |         | 257            | 438                  | 5.87                  |
|               | EB      | 127            | 185                  | 6.86                  |
|               |         | 59             | 145                  | 4.07                  |
|               |         | 152            | 147                  | 10.34                 |
|               |         | 159            | 196                  | 8.11                  |

Figure 6. Comparison of rpsL mutation rates induced by NDEA, NDPA, and EB in the RCA reaction.

4. Conclusions

We determined the type of mutation in the rpsL gene in E. coli. From the sequencing results, it can be observed that there are many types of spontaneous mutations in the process of rpsL gene replication in E. coli, but the Ames test cannot detect nearly 70% of the gene mutations, and the rpsL detection system avoids the limitations of this detection method. This result demonstrates that the rpsL detection system is suitable for the analysis and detection of microbial mutagens.

We determined that the spontaneous mutation rate of the rpsL gene in E. coli was 0.70 × 10^-6, while the mutation rate of the rpsL gene induced by EB was 3.71 ± 0.502 × 10^-6; the mutagenicity results demonstrated that the mutation rate of the rpsL gene reached 2.42 ± 0.717 × 10^-6 and 2.67 ± 0.909 × 10^-6 when 10 ppm NDEA and NDPA were added, respectively.

In the PCR system, the amplification error rate of Pfu DNA polymerase was stable at approximately 10^-6 [27], while the spontaneous mutation rate of the rpsL gene in the PCR system we determined was 1.30 ± 0.635 × 10^-6, which is consistent with a previous report. The amplification error rate of Pfu DNA polymerase is basically the same. When 10 ppm EB was added to the PCR system, the mutation rate of the rpsL gene was 4.06 ± 0.471 × 10^-6; when 10 ppm nitrosamine compound NDEA or NDPA was added, the mutation rate of the rpsL gene was 2.83 ± 1.197 × 10^-6 and 2.93 ± 1.430 × 10^-6, respectively.
The spontaneous mutation rate of the rpsL gene in the RCA reaction system was $1.09 \pm 0.456 \times 10^{-6}$, which was lower than the amplification mutation rate of the rpsL gene in the PCR system ($1.30 \pm 0.635 \times 10^{-6}$) and one order of magnitude lower than the mutation rate of the rpsL gene by Taq DNA polymerase [27]. In the RCA system, when EB was added at a concentration of 10 ppm, the mutation rate of the rpsL gene reached $3.81 \pm 0.335 \times 10^{-6}$; when 10 ppm NDEA and NDPA were added, the mutation rate of the rpsL gene reached $2.49 \pm 0.692 \times 10^{-6}$ and $2.98 \pm 0.585 \times 10^{-6}$, respectively.

The above results illustrated that the mutagenic rate of NDEA and NDPA increased with increasing concentration, indicating that nitrosamines could induce mutation of the rpsL gene. At the same time, it was found that the mutagenicity of nitrosamine at a concentration of 10 ppm was still significantly higher than the spontaneous mutagenicity of the rpsL gene, but it was still lower than the mutagenicity of EB, indicating that the system has the characteristics of a low spontaneous mutation background and high sensitivity.

Previous studies have proposed that the determination of NDPA and NDEA in drinking water and beer can be performed using solid-phase micro-extraction (SPME) along with gas chromatography (GC) and mass spectrometry (MS), and the process can be completed within 70 min [28]. However, the disadvantage is that the requirements for instruments and equipment are relatively high, and some laboratories may not have this equipment. In addition, studies have proposed the use of host-mediated assays (HMAs) for the toxicological analysis of this compound [29]. However, this method involves mouse experiments. First, the experimental period is long. In addition, it also considers the limitations of mouse feeding conditions in some laboratories. There are also experiments using the classical Ames test to measure the mutagenicity of five N-nitrosamines (N-nitrosodimethylamine (NDMA), NDEA, NDPA, N-nitrosopyrrolidine (NPYR), and N-nitrosodiphenylamine (NDPhA)), but their results indicate that the formation of mutagens during UV photolysis was detected only in the case of NDPhA in strain TA98. The oxidation products of NDMA, NDEA, and NDPhA did not indicate any significant mutagenicity in the strains used, whereas the oxidation of NDPA and NPYR by hydroxyl radicals seems to lead to the formation of direct mutagens in YG7108 and TAMix [30].

Previous studies have applied rpsL gene mutation to the detection and analysis of chromosomal DNA mutations [31] and to detect the mutagenicity of compounds in the aquatic environment [32]. However, there is no relevant report on the quantitative determination of the mutagenicity of compounds using this system. Commonly, mutagenicity evaluation systems mainly use mammalian cell forward mutation experiments, namely hypoxanthine guanine phosphoribosyl transferase (HPRT) gene and thymidine kinase (TK) gene mutation experiments [33]. However, these experiments all require complex mammalian culture conditions, and the experimental process is mostly more than 10 days. The rpsL system was used for detection, and the whole analysis process was realized within 24 h. At the same time, the spontaneous mutation of the rpsL is low. For example, the spontaneous mutation rate of the TK gene is $10^{-5}$, while the spontaneous mutation rate of rpsL is as low as $10^{-6}$, which is much lower than that of similar screening systems. In addition, using the rpsL positive selection system to evaluate the mutagenicity caused by nitrosamine compounds does not require complicated experimental conditions, saving manpower and material resources. Therefore, the rpsL gene mutation assay is expected to become an effective tool for the rapid detection of carcinogens in the food industry.

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