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New mutation assay using a next generation DNA sequencer - characteristics of mutation spectra -

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ABSTRACT

Ames 試験は、**DNA に対する化学物質のハザードを検出する**簡便で有用な方法であり、現在も広く使用されている。試験に 5 菌株を用いる理由は、1 菌株では異なる種類の突然変異を同時に検出できないからであるが、試験で得られる復帰変異コロニーの数から 6 種類ある塩基置換を特定することはできない。そこで、**次世代シークエンサー**を用いて被験物質で処理したエームス試験菌株の**ゲノム全体の塩基配列を解析**すれば、1 つの菌株でもより多くの情報が得られると考え、次の実験を行った。アルキル化剤に高感受性である YG7108 株で Ethyl nitrosourea（ENU; 50, 250 μg/plate）についてのAmes試験を実施し、得られた復帰変異コロニー 4 個ずつのゲノムをMiseq (Illumina)で調べ、溶媒対照と比較した。溶媒対照のゲノムでは 4 クローンでゲノム当たり平均 0.25 個、50 μg/plateでは平均 9 個, 250 μg/plateでは平均 58 個の突然変異が検出され、スペクトラムは GC から AT がほとんどで、残りはAT から GC（全体の 5% 程度）で、ENU が誘発する突然変異の特徴を反映していた。最終的には、**被験物質処理後に表現型によるセレクションをせずにコロニーを得てゲノムを解析するという簡便な手法**が可能になると考えている。

METHODS

Selection of mutant clones: The conventional Ames test was carried out using YG7108 for a tester strain and ENU for a test chemical, using two different doses. Four revertant colonies were randomly selected from the plates for each dose and subjected to DNA preparation.

DNA preparation: The genomic DNA was prepared for overnight culture. Cell pellet was lysed by SDS and proteinase K for one hour at 37 ˚C, mixed thoroughly with 5M NaCl, added CTAB/NaCl solution, then incubated the solution for 10 min at 65˚C. Extraction with $CHCl_3$/isoamyl alcohol, and phenol/$CHCl_3$/isoamyl alcohol was carried out. The aqueous phase was mixed with 2-propanol to precipitate the genomic DNA in it. The genomic DNA was treated with RNase, then the RNase was removed by phenol extraction. The average amount of the obtained genomic DNA was about 6.2 μg.

DNA sequencing: Whole-genome sequencing of each of the 12 clones was carried out in a single run by using a high-throughput DNA sequencer, MiSeq (Illumina, San Diego, CA). The sequencing data compiled to fastaq files were analyzed using CLC Genomics Workbench ver.5 software (CLC bio A/S, Aarhus, Denmark). First, the multiplex raw data were divided by sample name, and low-quality sequencing data were trimmed, depending on quality scores. The cleaned-up sequencing data were then mapped to the following reference sequences: NC_003197 (*S. typhimurium* str. LT2 chromosome, complete genome, 4,857,432bp), AY046276 (IncN plasmid R46, complete sequence, 50,969bp) and CP003387 (*S. typhimurium* str. 798 plasmid p798_93, complete sequence, 93,877bp).

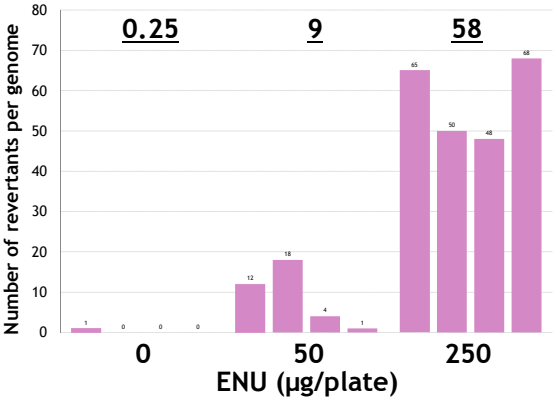
BACKGROUND

- Using a next-generation DNA sequencer, we analyzed whole genome of His+ revertants of YG7108 which lacks two O^6-methylguanine methyl transferases, and determined how much is the frequency, which is typical spectra and in which locus the mutations are accumulated.
- The size of *S. typhimurium* genome is about 5 Mb, and the cells are roughly divided every 30 minutes, that is, 50 generations a day.

RESULTS

Dose (μg/plate)	rev/plate	ave±SD	GC to AT	AT to GC	total
0	18 15 19	17±2	1	0	1
50	4,003 2,671 3,077	3,250±683	33	2	35
250	9,698 8,799 6,361	8,953±682	217	12	229

✓ Most of the mutations were GC to AT, and the remaining is AT to GC, about 5% of the total.



✓ The average number of mutation per genome for four clones for each dose was as follows: 0.25 for solvent control, 9 for 50 μg/plate, and 58 for 250 μg/plate.

DISCUSSION

- Ninety five percent of the mutation was G:C to T:A, which reflected the characteristics of mutations induced by ENU.
- The reversion occurs at GC pairs in principle of Ames test, but actual mutation occurred at AT pairs, too, in this study. Whole genome sequencing can detect any mutations in one strain.
- Only nine plates were used for the assay this time while a usual Ames test requires nearly 100 plates for the equivalent assay using standard five strains even without a dose-finding test.
- There might be a limit of the numbers of mutation that can be kept in one genome for generating a colony. In this study, it was revealed that one genome can keep at least 50 - 60 mutations.
- It must be necessary to decide how many mutations against dose are enough to judge whether the chemical is genotoxic.

FUTURE PERSPECTIVE

- The whole genome sequencing can provide more comprehensive information regarding the genotoxic effects of chemicals. So, this can be a new tool for mutation assay in chemical hazard assessment.
- Our goal is a simple method, i.e., DNA sequencing analyses of whole genome derived from colonies with no phenotypic selection after treatment of test chemicals.
- Considering the remarkable progress in the performance of the DNA sequencer, it would be possible to determine whole genome of rodents or cultured human cells exposed to chemicals.

Cf. Matsuda et al., submitted to Genes and Environment.

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New mutation assay without any phenotypic selections using a next generation DNA sequencer

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ABSTRACT

Ames試験は、DNAに対する化学物質の**ハザードを検出する**簡便で有用な方法であり、現在も広く使用されている。しかし、データはコロニーの数のみであり、5菌株用いても得られる情報は限られる。我々は昨年に引き続き、次世代シークエンサーを用いて被験物質の変異原性を解析する新しい方法を検討した。今回は、一菌株、TA1535をエチルニトロソ尿素（ENU）、250 μg/plateで処理し、プレインキュベーションの時間を、20、40、60分取って、Ames試験のように最小培地に重層する条件と、重層前の溶液の一部を希釈してLB培地に塗抹する条件でそれぞれコロニーを得て、条件ごとに3個のころにーの全ゲノムの塩基配列をMiseq(Illumina)で調べた。LB培地の溶媒対照のゲノムではゲノム当たり平均1.3個の突然変異、ENU処理時間を20，40分，60分と時間を延ばした場合、40、12、30個と、ばらつきはあったが、時間を延ばしても特に突然変異の数は増えなかった。スペクトラムはGCからATへの変化がほとんどで、ENUが誘発する突然変異の特徴を反映していた。最小培地の場合は、時間を変えても20個程度で変化が小さく、LB培地の方がスペクトラムの多様性が見られるなどの特徴があった。**表現型によらない変異原性試験**としての本試験法の利用が期待される。

BACKGROUND

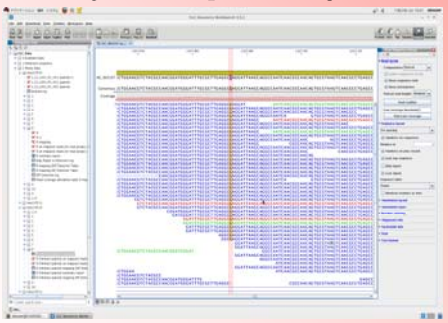
- The conventional bacterial reverse mutation assay, Ames test, cannot specify mutation spectra even using five strains.
- Many days and plates are required to carry out Ames test for several chemicals.
  - Making progress with its performance remarkably, the DNA sequencer may have a possibility to resolve such problems.

METHODS

Chemical treatment of cells: As the conventional Ames test, overnight culture of TA1535, 100 μl, was mixed with ENU (250 μg/ml), 100 μl, and buffer, 500 μ l, for 20, 40, 60 min at 37˚C. Then, one μl was diluted in 10^6 times, then 100 μl was spread onto an LB plates (A). The remaining culture was mixed with two ml of soft agar and poured onto minimal plates (B). The LB plates and the minimal plates were incubated at 37˚C for 16 and 48 hours, respectively. Three colonies for each condition were randomly selected from the plates and subjected to DNA preparation.

DNA preparation: The genomic DNA was prepared for overnight culture, 10 ml. Cell pellet was lysed by SDS and proteinase K for one hour at 37 ˚C, mixed thoroughly with 5M NaCl, added CTAB/NaCl solution, then incubated the solution for 10 min at 65˚C. Extraction with $CHCl_3$/isoamyl alcohol, and phenol/$CHCl_3$/isoamyl alcohol were carried out. The aqueous phase was mixed with 2-propanol to precipitate the genomic DNA in it. The genomic DNA was treated with RNase, which was removed by phenol extraction later. The average amount of the obtained genomic DNA was 101 μg.

A part of the screen for DNA sequence analysis



Data analysis: The sequencing data compiled to fastaq files were analyzed using CLC Genomics Workbench ver.5 software (CLC bio A/S, Aarhus, Denmark).

Reference Sequences

NC_003197: *Salmonella typhimurium* str. LT2 chromosome, complete genome, 4,857,432bp

AY046276: *IncN* plasmid R46, complete sequence, 50,969bp

CP003387: *S. typhimurium* str. 798 plasmid p798_93, complete sequence, 93,877bp

RESULTS

A: The number of mutations occurring on the genome of colonies on LB plates

Pre-incubation time (min)	x 10^6 cells	Mutation per genome	Ave.	GC to AT	GC to TA	GC to CG	AT to GC	AT to CG	AT to TA	Insertion	Deletion
0	295	1	1.3	1	0	0	0	0	0	0	0
		2		0	0	0	1	0	0	1	0
		1		0	0	0	0	0	0	1	0
20	47*	28	40	23	2	0	2	0	0	1	0
		43		37	0	0	6	0	0	0	0
		50		46	1	0	1	2	0	0	0
40	46	7	12	5	0	0	0	0	0	1	1
		29		27	0	0	2	0	0	0	0
		1		1	0	0	0	0	0	0	0
60	43	26	30	23	0	0	1	0	0	2	0
		18		10	0	0	5	0	1	2	0
		46		42	0	0	3	0	0	1	0

*Decrease of the number of colonies toward the solvent control indicates influence of the test chemical to cells.

B: The number of mutations occurring on the genome of reversion colonies on minimal plates

Pre-incubation time (min)	rev/plate	Mutation per genome	Ave.	GC to AT	GC to TA	GC to CG	AT to GC	AT to CG	AT to TA	insertion
0	38	32**	2**	26**	0	0	6**	0	0	0
		2		0	0	0	0	0	0	2
		2		0	0	0	1	0	0	1
20	2064	22	25	19	0	0	2	0	0	1
		28		25	0	0	3	0	0	0
		25		21	0	0	4	0	0	0
40	2360	10	22	4	0	0	3	0	0	2
		37		31	0	0	6	0	0	0
		18		15	0	0	2	0	0	1
60	2604	27	24	23	0	0	3	0	0	1
		21		16	0	0	4	0	1	1
		23		19	0	0	3	0	0	1

** The clone indicating 32 mutations is neglected.

DISCUSSION

- About ten plates were used for the assay this time while a usual Ames test requires nearly 100 plates for the equivalent assay using standard five strains even without a dose-finding test.
- The reversion occurs at GC pairs in the case of TA100 according to the principle of Ames test, but mutations occurred at various sites. Even insertions/deletions were detected in this study. Whole genome sequencing can detect any mutations in a single strain.
- The whole genome sequencing can provide more comprehensive information regarding the genotoxic effects of chemicals. So, this can be a new tool for mutation assay in chemical hazard assessment.
- Thinking of this method as a genotoxic test for risk assessment, it must be necessary to find how many mutations against dose are enough to judge whether the chemical is genotoxic.
- Considering the remarkable progress in the performance of DNA sequencers, it would be possible to determine whole genome of rodents or cultured human cells exposed to chemicals.

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