

RAISE: a simple and novel method of generating random insertion and deletion mutations

Ryota Fujii, Motomitsu Kitaoka* and Kiyoshi Hayashi

National Food Research Institute, 2-1-12 Kannondai, Tsukuba, Ibaraki, 305-8642, Japan

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ABSTRACT

Although proteins may be artificially improved by random insertion and deletion mutagenesis methods, these procedures are technically difficult, and the mutations introduced are no more variable than those introduced by the introduction of random point mutations. We describe here a three-step method called RAISE, which is based on gene shuffling and can introduce a wide variety of insertions, deletions and substitutions. To test the efficacy of this method, we used it to mutate TEM β -lactamase to generate improved antibiotic resistance. Some unique insertion or deletion mutations were observed in the improved mutants, some of which caused higher activities than point mutations. Our findings indicate that the RAISE method can yield unique mutants and may be a powerful technique of protein engineering.

INTRODUCTION

Through several billion years, living things have evolved to achieve an enormous diversity. The driving force in creating this diversity is random mutations in their genes. In recent decades, the evolution of proteins by random mutation has been reproduced *in vitro* by using random mutagenesis and screening and selection techniques (1–6). These methods can yield mutants with evolved properties, similar to those created through natural evolution by random mutations. This process, which is called directed evolution, is one of the most potent methods for artificially improving protein properties.

The creation of mutant libraries are important in directed molecular evolution (7). The most popular methods for library construction involve random point mutagenesis, such as error-prone PCR (8). These methods are easy to perform, since they consist of only a few steps that involve standard techniques, making them quite useful for improving proteins.

Recently, random mutagenesis methods have been developed to introduce fixed length of insertions or deletions in DNA sequences (7). The introduction of insertions and deletions (indels) can change the structures and properties of proteins more drastically than do point mutations (9). However, none of these methods have been shown to surpass the efficacy of traditional point mutations. This may be due to their introduction of a limited variation of mutations, since the possibility of the mutations are limited with insertions of fixed sequences or deletions of fixed length (10–14). Thus, a novel method to generate random indels with flexible sequences and length has been desired.

To enhance the ability to introduce random mutations containing indels into proteins, we have developed a simple method, the RAndom Insertional-deletional Strand Exchange mutagenesis (RAISE) method based on gene shuffling (15). The protocol of RAISE consists of only three steps that do not require any specific skills. We utilized terminal deoxynucleotidyl transferase (TdT), a DNA polymerase (16,17) found to be a key enzyme to introduce random insertions into the antibody hyper-variable regions in mammals (18), to introduce random nucleotides at the 3' terminus of the digested DNA before the self-priming PCR step. By adding this step, we succeeded in introducing various lengths of random insertions, deletions and substitutions into an entire target gene.

TEM β -lactamase is an enzyme that cleaves β -lactam antimicrobial agents such as ampicillin. This enzyme is clinically important because it accounts for antibiotic resistance of bacteria. Substrate specificity of this enzyme is changed easily by point mutation and has been studied by isolating naturally occurring antibiotic-resistant bacteria (19) (<http://www.lahey.org/Studies/temtable.asp>), by site-directed mutagenesis (20) and by random point mutagenesis (21,22).

We used RAISE to mutate TEM β -lactamase to generate enzymes with improved activity against another β -lactam antibiotic, ceftazidime. Although mutations in this enzyme have been studied extensively, we found that RAISE generated many novel mutations. Some of the deletion mutations generated caused higher activities than point mutations.

*To whom correspondence should be addressed. Tel: +81 29 838 8071; Fax: +81 29 838 7321; Email: mkitaoaka@affrc.go.jp

Present address:

Ryota Fujii, Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, 1479 Gortner Avenue, Saint Paul, MN 55108, USA

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MATERIALS AND METHODS

Materials

Escherichia coli strains DH5 α [F⁻ ϕ 80*dlacZ* Δ M15 Δ (*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17*(r_K⁻ m_K⁺) *phoA supE44* λ ⁻ *thi-1 gyrA96 relA1*], as well as RNase-free DNase I and restriction enzymes were purchased from TaKaRa (Otsu, Japan). TdT, KOD Dash and KOD plus DNA polymerases were purchased from Toyobo (Osaka, Japan). Deep Vent (exo⁻) DNA polymerase was purchased from New England Biolabs (Beverly, MA). Ampicillin sodium salt, ceftazidime pentahydrate and tetracycline were purchased from Nacalai Tesque (Kyoto, Japan) and Sigma (St. Louis, MO). The MinElute Reaction Cleanup and Gel Extraction Kits were purchased from QIAGEN (Hilden, Germany). Plasmid vectors, pUC19 [possessing TEM β -lactamase gene (*bla*)] pBR322 (possessing *bla* gene and a tetracycline-resistant gene) and were purchased from TaKaRa.

RAISE method

TEM β -lactamase was examined for a target of RAISE. The *bla* gene was amplified by PCR using the primers 5'-TATATGAGTAAACTTGGTCTGACAG-3' (primer A) and 5'-AGGGCCTCGTGATACGCCTATTTTATAGG-3' (primer B) using pUC19 as a template and the PCR product (1080 bp) was purified by using a MinElute Kit. Twenty micrograms of the PCR product were digested with 0.5 U RNase-free DNase I at 16°C for 10 min in 1 ml of buffer containing 50 mM Tris-HCl (pH 7.0) and 10 mM MnCl₂. MnCl₂ was used as a DNase I co-factor to control the size of the digestion products in order to avoid introduction of too many point mutations in the ensuing self-priming PCR step (23). When the average size of the fragments was 100–300 bp, as determined by agarose gel electrophoresis, the reaction was terminated by adding 40 μ l of 0.5 M EDTA. The fragments were purified with a MinElute Reaction Cleanup Kit. The concentrations (by weight) of the fragments were determined by measuring their absorbance at 260 nm, and their molar concentrations were estimated from the concentration by weight and the average size. The fragments (135 ng, 2 pmol) were 3'-tailed by the addition of 2.5 U of TdT in a 20 μ l solution containing buffer and dNTPs at 37°C for 1 h. The concentrations of the dNTPs were adjusted to 10 times the molar concentrations of the fragments (20 pmol), which yielded tails of average 5 bases at each 3' terminus of the fragments. The 3'-tailed fragments were purified with a MinElute Reaction Cleanup Kit and reassembled by self-priming PCR with a DNA polymerase possessing no proofreading activity. The fragments (72 ng) were reassembled with 2 U of Deep Vent (exo⁻) DNA polymerase or 2.5 U of KOD Dash in 10 μ l containing 200 μ M of each dNTP and buffer. The amplification protocol consisted of an initial denaturation at 96°C for 2 min, followed by 40 cycles of denaturation at 96°C for 30 s; annealing at 60°C for 30 s; and extension at 75°C for 30 s.

Then the full-length of the mutated *bla* gene (RAISE product) was amplified by PCR as following. A 1 μ l aliquot of the reaction mixture of the self-priming PCR was mixed with 1 U KOD Plus in a 50 μ l solution containing 200 μ M of each dNTP, 1 mM MgSO₄, buffer and 0.3 pmol/ μ l of

primers A and B, followed by the amplification using a protocol consisting of an initial denaturation at 96°C for 2 min, followed by 10 cycles of denaturation at 96°C for 15 s, annealing at 56°C for 30 s, and extension at 68°C for 1 min. The 1.1 kb RAISE product was separated by 2% agarose gel electrophoresis and extracted with a MinElute Gel Extraction Kit.

Cloning and *in vitro* selection

The RAISE product was inserted into pBR322 or pUC19 by overlap extension PCR (24). The pBR322 vector was used to clone the mutant and to determine mutation frequency, whereas the pUC19 vector was used to select mutants with improved β -lactamase activity. The linear fragments of the vectors excluding the *bla* gene were produced by PCR of each plasmid with primers complementary to primers A and B. One picomole (710 ng) of RAISE product was mixed with 1 pmol of each vector fragment in a 10 μ l solution containing 200 μ M of each dNTP, 1 mM MgSO₄, 0.2 U KOD Plus and buffer, followed by self-priming PCR using the amplification protocol (96°C for 2 min, followed by 10 cycles of 96°C for 15 s; 56°C for 30 s; and 68°C for 1 min). The product was purified with a MinElute kit.

E. coli DH5 α competent cells were electroporated with the PCR product (100–400 ng), and the transformants were plated on Luria-Bertani (LB) plates containing 20 μ g/ml tetracycline (for cloning) or an arbitrary concentration of ceftazidime (for selection). After 24 h of incubation at 37°C, colonies were picked, and their plasmid sequences were determined.

Determination of minimum inhibitory concentration (MIC) of ceftazidime

A colony of *E. coli* harboring pUC19 variants was diluted 1:10³, 1:10⁴, 1:10⁵ and 1:10⁶ in sterilized water. A 2 μ l aliquot of each was spotted onto a fresh LB plate containing ceftazidime, which was incubated for 24 h at 37°C. A spot that generated 10–100 colonies was used to determine the MIC.

RESULTS

Theory of the RAISE method

This method is composed of only three steps: DNA fragmentation, attachment of a random short sequence and reconstruction (Figure 1). First, the target DNA was fragmented randomly by DNase I. Second, several random nucleotides were attached to the 3' terminus of the fragment using TdT. Finally, each fragment with a tail of random nucleotides was reconstructed into a full-length sequence by self-priming PCR (15). The experimental protocol of RAISE is similar to that of the DNA shuffling (15) except the tailing by TdT before the self-priming PCR step. The series of manipulations of DNase I digestion, TdT modification and self-priming PCR was defined as RAISE.

We suppose that the additional random sequences caused the random mutations by the following mechanism. The single strand fragment (primer) having a flanking tail produced by TdT (random sequence) was annealed with one of its complementary fragments (template) at the proper position of the primer, where the 3' terminus of the random sequence

was annealed randomly close to the position to be extended by DNA polymerase in the self-priming PCR step as shown in Figure 2A. Then, the region of the template between the primer and the random sequence (strand X) was substituted with the random sequence (strand Y) by extending the primer with DNA polymerase. If the tailed random sequence was longer than the replaced region, an insertion was included (Figure 2B). A deletion was included if the tailed sequence was shorter. If the lengths were the same, it was a simple substitution. We call these mutations 'region-exchanged mutations'.

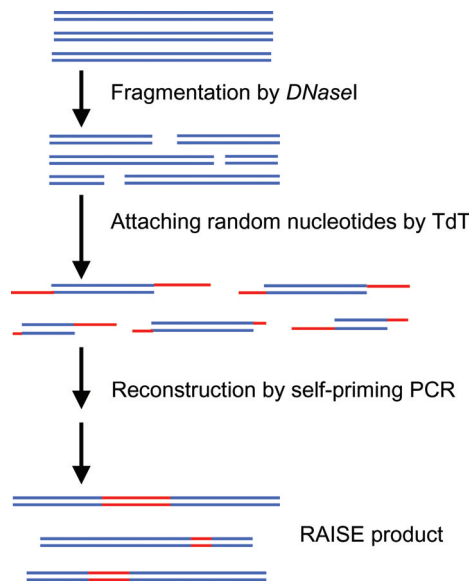


Figure 1. Schematic diagram of the RAISE method.

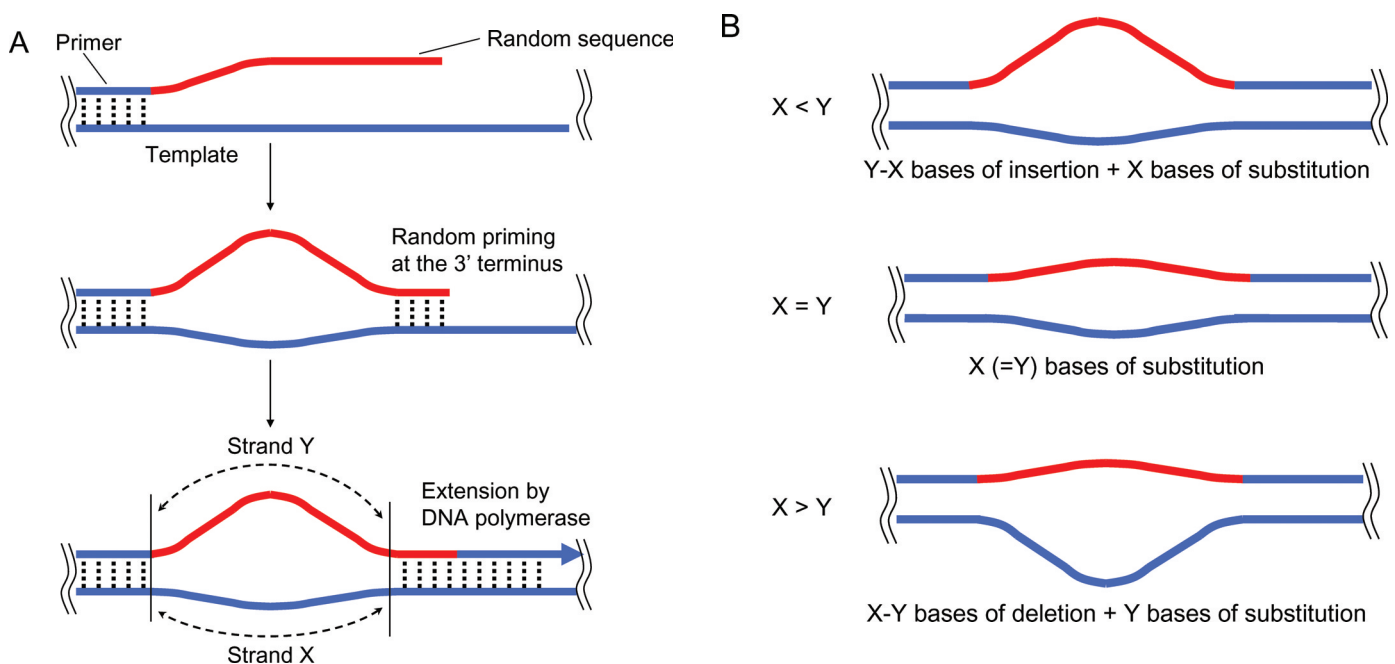


Figure 2. Region-exchanged mutation. (A) Mechanism to form region-exchanged mutation. (B) Types of region-exchanged mutation.

Mutation frequency and mutation length

We estimated the mutation frequency with the RAISE method by reading some DNA sequences of RAISE product. The mutated β -lactamase gene was inserted into pBR322 and was cloned on a tetracycline plate. Approximately 2000 colonies were grown on the plate and 41 colonies were randomly picked. The region of the RAISE product in each clone (1 kb) was sequenced. We detected two types of mutations, region-exchanged mutations and point mutations. Point mutations can be introduced automatically in the self-priming PCR step as the DNA shuffling (15,23). We found 29 region-exchanged mutations (Table 1) located independently in 19 clones. Number of the region-exchanged mutations in each clone was 1 (12 clones), 2 (6 clones), or 5 (1 clone). We also found 79 point mutations widely spread over the sequences in 34 clones, 15 of which had both mutations. Three clones did not possess mutations. Their mutation frequencies were 0.7 ± 0.1 /kb and 2.1 ± 0.2 /kb, respectively (the standard errors were calculated assuming that the values follow a Poisson distribution: square root of the number of mutation/sequenced length). The region-exchanged mutations included frameshifts, such as 1 or 2 bp indels, whereas the point mutations included silent mutations. Approximately two-thirds of the region-exchanged mutations included frameshifts, and almost two-thirds of the point mutations were silent; therefore, in terms of amino acids, the frequencies of significant region-exchanged mutations and point mutations were 0.2 ± 0.1 and 0.6 ± 0.1 amino acids/kb DNA, respectively.

Improving ceftazidime-hydrolyzing activity of TEM β -lactamase by RAISE

To verify that RAISE could be used for *in vitro* evolutionary experiments, we tested its ability to enhance the activity

of TEM β -lactamase in the hydrolysis of the antibiotic ceftazidime. The lactamase gene was inserted into a plasmid and mutated by RAISE, and the plasmid was used to transform *E. coli* DH5 α cells. The transformants were cultured on plates containing ceftazidime to select mutants with improved antibiotic resistance. The mutant showing the greatest

enhancement of TEM β -lactamase activity was used as a parent for the next generation of RAISE. Totally 10^3 – 10^5 mutants were selected in each generation. This selection cycle was repeated for three generations.

The selected mutants were classified as those improved by region-exchanged mutations and those improved by point mutations. Region-exchanged mutations are specific to the RAISE method, whereas point mutations can be reproduced by other conventional random point mutagenesis methods, such as error-prone PCR (8). Therefore, region-exchanged mutations can be regarded as representative of RAISE, and point mutations can be regarded as representative of traditional random point mutagenesis methods. The effects of region-exchanged mutations were compared with those of point mutations.

We characterized the DNA sequences and MICs of the selected mutants. Those improved by each generation of region-exchanged and point mutations are shown in Figure 3. We found that the best region-exchanged mutations showed higher activity than point mutations in the second and third generations. To our knowledge, this is the first method showing greater efficacy than point mutagenesis.

The region-exchanged mutations generated by this method are shown in Figure 4. These mutations consisted of insertions, deletions and substitutions. Many deletion mutations were observed at amino acids 173–179 and 240–242, indicating that these positions are hot spots for deletion mutations. The amino acid substitution L21T and C77V, which were accompanied by DNA substitutions such as CT(55,56)AC and TGT(223–225)GTG, respectively, are impossible to be generated by the traditional random mutagenesis methods such as the error-prone PCR, because substitutions of at least two bases in a codon are necessary to change Leu to Thr or Cys to Val.

Table 1. Examples of mutations introduced by RAISE

Length of strand X ^a (bp)	Length of strand Y ^a (bp)	Sequence
20	10	ATGAACGAAATAGACAGATC-CTGGTTTATT
10	16	GTGGGTCTCG-CGCTGCCGTC
8	2	ATCTCAAC-CT
7	3	TATTGAC-GGA
7	3	AACAATT-CTC
6	6	TACACG-GCTCCT
6	6	ATGAAC-GTGGAA
5	6	ACGTT-TGGAAG
2	2	GC-AA
2	2	GA-AT
2	2	TA-CC
2	2	AC-GT
2	1	AG-C
2	1	GT-C
1	2	T-AA
1	0	G deletion (2) ^b
1	0	C deletion
1	0	A deletion (4) ^b
1	0	T deletion (3) ^b
0	1	G insertion (2) ^b
0	1	T insertion (2) ^b

^aSee Figure 2A.

^bNumber of each mutation found.

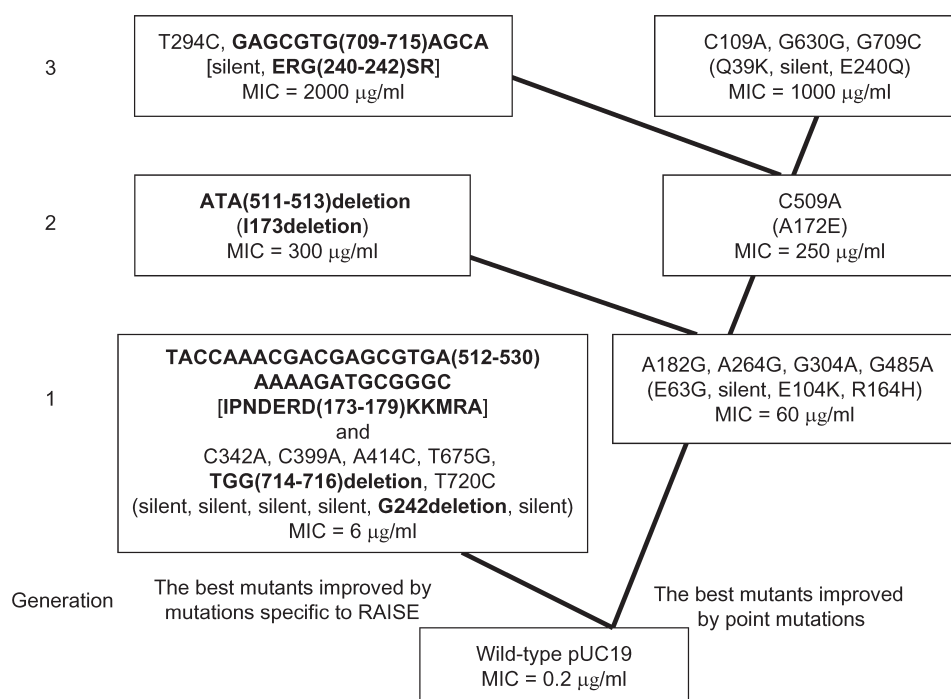


Figure 3. Mutants with the best MICs for ceftazidime, improved by region-exchanged and point mutations. The amino acid positions are based on the standard numbering for class A β -lactamase (30).

Table 2. Probabilities of finding mutants with improved ceftazidime resistance

Generation	Ceftazidime in selection plate ($\mu\text{g/ml}$)	DNA amount for transformation (ng)	Library size	Mutants improved by region-exchanged mutation		Mutants improved by point mutation	
				Number of mutants	Probability to find mutants	Number of mutants	Probability to find mutants
1	1	27	4×10^4	6	2×10^{-4}	108	3×10^{-3}
2	120	4	6×10^3	2	4×10^{-4}	7	1×10^{-3}
2	140	12	2×10^4	2	1×10^{-4}	10	6×10^{-4}
2	150	18	2×10^4	1	4×10^{-5}	3	1×10^{-4}
3	300	10	1×10^4	9	7×10^{-4}	18	1×10^{-3}
3	500	68	9×10^4	2	2×10^{-5}	2	2×10^{-5}

TEM_{pUC19} β -lactamase was mutated by RAISE and inserted into pUC19, which was used to transform *E.coli* DH5 α cells. Clones were selected on a ceftazidime plate. The parent of each generation is shown in Figure 3. The probabilities of finding improved mutants were determined by dividing the number of improved mutants by the total number of transformants.

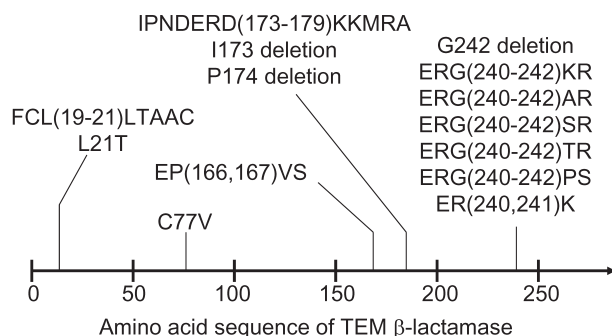


Figure 4. Region-exchanged mutations identified from the mutants with improved ceftazidime resistance. The amino acid positions are based on the standard numbering for class A β -lactamase (30).

We found that the probabilities of generating region-exchanged and point mutations were $1:10^4$ – $1:10^5$ and $1:10^3$ – $1:10^5$ per transformant, respectively (Table 2).

DISCUSSION

We here demonstrate a novel random mutation method, RAISE, consisting in simple procedures. RAISE has an advantage over conventional mutation methods in that it can produce a wide variety of mutations, including insertions, deletions and substitutions. The sequences of the mutations are random, and the length of these mutations can vary from one to several amino acids. In addition, indels and substitutions are often combined. For example, the replacement of IPNDERD at amino acids 173–179 by KKMRA consists of two amino acid deletions and five amino acid substitutions. These long substitutions can greatly expand the variation of mutations. For example, 5 amino acid substitutions can yield 20^5 varieties.

Short indels within a few amino acids were frequently produced by this method (Table 1). Because a few amino acid indels are often found during natural protein evolution (9,25), the variation of indels by RAISE seems appropriate for the evolution of proteins.

The RAISE method is suitable for high-throughput screening/selection methods because of its ability to introduce an almost infinite variety of mutations. The more mutants are assayed, the better mutants will be found. In addition, high-throughput screening/selection will make it easier to identify

mutations in what is an inefficient method, generating a low frequency of region-exchanged mutations (0.3 amino acids/kb) and a high frequency of frameshifts (0.7 amino acids/kb), which cause a catastrophic effect on a protein. The probability of finding efficient region-exchanged mutations was about $1:10^4$ per transformant, indicating that significant mutants will be found by assaying more than 10 000 mutants. This number of assays is now commonplace in protein engineering (26–29).

This method will not only improve enzyme properties but also will constitute a unique prospect for protein engineering. We found hot spots for deletions around amino acids 173–179 and 240–242, positions close to hot spot for point mutations at amino acids 173, 179 and 240 (22), indicating that region-exchanged mutations are extensions of point mutations. When the 3D structure of lactamase is examined, these deletions are found to be located near the bulky side-chain of ceftazidime (20), indicating that these deletions enlarge the cleft to accommodate the bulky substrate. These findings have never been produced by any other mutagenesis methods, although TEM β -lactamase is among the enzymes most studied for mutations. Our findings thus indicate that the RAISE method will give unique information on proteins.

The RAISE method can introduce not only indels but also point mutations and long substitutions. Furthermore, it can be utilized for DNA recombination because it includes DNA fragmentation and reconstruction steps as well as DNA shuffling (15).

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Conflict of interest statement. None declared.

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