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Transposon-directed base-exchange mutagenesis (TDEM): a novel method for multiple-nucleotide substitutions within a target gene

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In this report we describe transposon-directed base-exchange mutagenesis (TDEM), an efficient and controllable method for introducing a mutation into a gene. Each round of TDEM can remove up to 11 base pairs from a randomly selected site within the target gene and replace them with any length of DNA of predetermined sequence. Therefore, the number of bases to be deleted and inserted can be independently regulated providing greater versatility than existing methods of transposon-based mutagenesis. Subsequently, multiple rounds of mutagenesis will provide a diverse mutant library that contains multiple mutations throughout the gene. Additionally, we developed a simple frame-checking procedure that eliminates nonfunctional mutants containing frameshifts or stop codons. As a proof of principle, we used TDEM to generate mutant *lacZα* lacking α -complementation activity and recovered active revertants using a second round of TDEM. Furthermore, a single round of TDEM yielded unique, inactive mutants of *ccdB*.

Introduction

Many different methods of mutagenesis have been developed to alter protein function or activity (1,2). UV (3–5), chemical mutagenesis (6,7), or site-directed mutagenesis (8) have traditionally been used to introduce mutations. Current mutagenesis methods can be categorized into two groups: controlled or random mutagenesis (9).

PCR-based site-directed mutagenesis (10,11) is the method of choice for controlled mutagenesis. Alanine (12–16) or cysteine scanning mutagenesis (17–19) has been widely used to investigate protein function or structure. Additionally, mutator bacterial strains (20–23), gene shuffling (24), and error-prone PCR (epPCR) (25) have been widely used for random mutagenesis. While these methods have been used for many studies, they have several disadvantages (9,26). Site-directed mutagenesis can be costly and labor-intensive, as different mutations require the use of different oligonucleotides, PCR, and cloning. Although random mutagenesis methods can provide

mutant libraries using simple procedures, the rate and the site of mutations cannot be controlled. Therefore, there is a risk of high wild-type background and if a gene contains multiple mutations, each mutation must be individually analyzed to identify the amino acid change(s) responsible for the mutant phenotype (24). Although the random insertion and deletion (RID) method was developed to surmount these shortcomings, its use is limited to those who are technically sophisticated (27). As an alternative approach, transposons have recently been used for pentapeptide scanning (28) and linker scanning mutagenesis (29), but these methods are also limited and not appropriate for protein evolution because they only insert extra sequences into the target gene.

With an optimal mutagenesis method, it should be possible to (i) control the number of mutations per round of mutagenesis and the sequence to be inserted or substituted, (ii) have low wild-type background, and (iii) have flexibility so that different types of mutations—including insertions, deletions, or substitutions—can be obtained.

None of the currently available mutagenesis methods satisfies all of these requirements. In order to achieve these characteristics, we developed transposon-directed base-exchange mutagenesis (TDEM), which can substitute up to 11 bases of the target gene with a predetermined sequence of any length into a site selected by the random integration of a transposon (30,31). TDEM introduces a mutation into a single site of the target gene so that the diversity of the mutant library can be easily evaluated. Furthermore, because it can substitute ≥ 3 bases, TDEM can be used for amino acid scanning mutagenesis using up to 3 consecutive amino acids providing a novel approach for directed evolution.

As a proof of principle, we used TDEM to substitute 3 random bases of the target genes *lacZα* (α fragment of β -galactosidase) and *ccdB* (controller of cell division protein B) with three bases determined by the mutation insert (M1). In both target genes, we showed that (i) one round of TDEM generates a mutation at a single site with no wild-type background, (ii) TDEM can be used to generate a predetermined

Figure 1. Schematic diagram of TDEM method. The transposon was introduced into the target gene in a plasmid vector (A) and transformed into *E. coli* by electrotransformation. Five bases of the transposon integration site are duplicated and a single A or T added at both ends of the transposon as depicted (B). The plasmid vectors containing the transposon were digested with *NotI* which cuts at both ends of the transposon (C). The mutation insert (MI) containing the Zeo^r resistance gene was cloned into the target gene. The plasmid containing the MI (D) was digested with *BspI* and self-ligated (E). The plasmid was then digested with *BpmI* that cuts between -4 and -3 base of the left end and at the 5' end of the NNN sequence that is to be introduced into the gene. The final plasmid (F) has a three base substitution at a random position within the target gene. The NNN sequences used were GCC, CGC, AAC, GAT, TGC, CAG, GAA, GGC, CAT, ATC, CTG, AAA, ATG, TTT, CCA, AGC, ACC, TGG, TAC, or GTG.

3-base substitution at a random site, and (iii) repeated rounds of TDEM accumulate mutations within the gene. Therefore, TDEM exhibited characteristics of both controlled and random mutagenesis. Thus, we propose that the use of TDEM will dramatically facilitate structural studies and directed evolution of proteins.

Materials and methods

Plasmid constructs and transposons

The pCompact-Kana vector was constructed by ligating an origin of replication with kanamycin resistance gene (Supplementary Figure 1 and Supplementary Table 1). All inserts were cloned into pCompact-Kana, which had been amplified by PCR with primers Kan-up and Ori-lo (Supplementary Table 1) to make a linear plasmid. The detailed methods are in the Supplementary Materials.

Transposition

For TDEM of *lacZa* (150 bp), transposition was carried out using commercial Tn7 transposase (TnsABC* transposase; New England Biolabs, Ipswich, MA, USA) and Mu-transposase (Finnzymes, Espoo, Finland) according to the manufacturers' protocol. The mixture of Tn7 and Mu transposition reactions was used in order to increase the spectrum of possible

mutations. For TDEM of *ccdB* (300 bp), N-terminally deleted MuA prepared in the laboratory was used in the modified buffers that altered site preference (A: 25 mM Tris-Cl (pH 7.0), 5 mM MgCl₂, 0.05% (v/v) Triton X-100, 10 mM NaCl and 40% (v/v) glycerol; B: same as A without NaCl; C: same as A with 15% (v/v) DMSO; and D: same as A with 10% (v/v) glycerol) (32) and transposition reactions carried out using these buffers were mixed. Tn7 was not used for *ccdB* mutagenesis because it showed batch to batch variation in enzyme activity. Following transposition, the DNA mixture was electrotransformed into *Escherichia coli*. The transformants were inoculated into LB broth containing 50 µg/mL kanamycin and 3 µg/mL chloramphenicol and cultured overnight at 37°C with shaking (Excelsa E24, New Brunswick Scientific, Edison, NJ, USA). All transposition reactions in this study yielded >20,000 transposition events. The plasmid DNA from the pool was harvested and digested with the restriction enzymes that flank the target gene: *NcoI*/*AgeI* (New England Biolabs) for *lacZa* or *BsaI* (New England Biolabs) for *ccdB*. The target gene carrying the transposon was purified from agarose gels after electrophoresis, ligated back into the parental vector digested with compatible restriction enzymes and electrotransformed again yielding ~1 × 10⁷ colonies. This step elimi-

nated plasmids having the transposon in the vector sequence and resulted in a plasmid population with the transposon only in the target gene.

When blunt ends were required, 3 µg of purified plasmid DNA containing sticky ends was suspended in 0.5 mM dNTP and 1× T4 DNA polymerase buffer (New England Biolabs) with 6 units of T4 DNA polymerase (New England Biolabs) and incubated at 12°C for 25 min.

Evaluation of transposon integration sites

The pool of plasmids containing the transposon only in the target gene was harvested and digested with *NotI* (New England Biolabs), which cuts both ends of Tn7 (New England Biolabs) and M1-Cam^R (Finnzymes), resulting in a linear plasmid opened at the integration site in the target gene. After agarose gel purification, the linearized plasmid was end-labeled with γ-³²P ATP and then digested with *AgeI* (New England Biolabs) (*lacZa*) or *PvuII* (New England Biolabs) (*ccdB*), which cuts the vector sequence outside of the target gene. The resulting DNA mixture then contains various sized ³²P-labeled DNA fragments depending on the transposon integration site. The digested DNA fragments were analyzed by 6% (w/v) polyacrylamide DNA gel electrophoresis in the presence of 5 M urea.

Mutant Screening

The α-complementation activity of mutant *lacZa* was identified using LB agar containing 50 µg/mL kanamycin, 60 µg/mL X-gal and 1 mM IPTG after overnight incubation at 37°C. To isolate inactive *ccdB*, the mutated *ccdB* was harvested from pCompact-Kana-CcdB using *BsaI* digestion and ligated into the pFinalScreen vector (Supplementary Figure 1) prepared by removing stuffer DNA with *BsaI* digestion. The ligation mixture was then electro-transformed into Top10 electrocompetent cells (Invitrogen, Carlsbad, CA, USA). Surviving colonies were observed on LB agar plates containing 50 µg/mL kanamycin and 1 mM IPTG after overnight incubation at 37°C.

Eliminating mutants containing frameshifts or stop codons using pFrameCheck

The mutated *ccdB* gene was harvested from pCompact-Kana-CcdB by digesting with *BsaI* and cloned into pFrameCheck prepared by digestion with *BsaI*. The ligated mixture was transformed into DB3.1 electrocompetent cells (Invitrogen) and grown in LB containing 50 µg/mL carbenicillin and 50 µg/mL kanamycin overnight at 37°C with shaking. The plasmid was purified from the

E. coli lysate and the mutant *ccdB* gene was purified from the agarose gel after digestion with *Bsa*I. The harvested mutant *ccdB* was cloned into pFinalScreen and screened as described above.

Results

General procedure of TDEM

The procedure for producing a 3-base substitution using TDEM is outlined in Figure 1 and the Supplementary Materials. The key to the success of the method is the design of the MI. We choose to use *Bpm*I and *Bsg*I for constructing the MI after testing the activity of several type II restriction enzymes including *Bpm*I, *Bpu*EI, *Bsg*I, *Mme*I and *Eco*PI5I. As shown in Figure 1D, the MI consists of three components, (i) distal *Bsg*I and *Bpm*I sites to remove bases from the target gene, (ii) nucleotides to be inserted into the target gene (incorporating bases, IB) between the proximal *Bsg*I and *Bpm*I sites and (iii) a Zeocin resistance gene (*zeo*^r). *Bpm*I and *Bsg*I, type II restriction endonucleases, cut 16 bp outside their recognition sequence. Therefore, by adjusting the relative position of the distal restriction enzyme sites, the number of bases to be deleted from the target can be altered (Supplementary Figure 2). In Figure 1D, the distal *Bpm*I and *Bsg*I are positioned so that three bases are removed from the target; however, it should be stressed that it is possible to position the enzyme sites so that up to 11 bases are deleted from the target (Supplementary Figure 2). On the other hand, the sequence to be inserted is determined by the IB that is placed between the proximal *Bpm*I and *Bsg*I sites. The IB can be of any length. Thus, the number of bases to be deleted and the length of the DNA to be inserted can be separately determined so that many types of mutant libraries can be generated. Lastly, the *zeo*^r gene confers resistance to Zeocin so that only plasmids containing the MI are recovered following selection with Zeocin and kanamycin.

To carry out TDEM, the Mu and/or Tn7 transposons are first integrated into random sites within the target gene. These transposons contain *Not*I sites at their ends (Figure 1A). As a result of transposition, the five bases of the integration site are duplicated with the transposon located between the five duplicated bases (33) (Figure 1B). The transposon is then removed by digesting with *Not*I (Figure 1C). Subsequently, the MI is ligated into the linearized vector (Figure 1D) and transformed into *E. coli*. The plasmids containing the MI are then digested with *Bsg*I to remove the right hand side of the MI (Figure 1E). After making the plasmid ends blunt with T4 DNA

Table 1. Position and Sequence of Mutations within Inactive *ccdB*

Mutations	DNA substitutions	No. of colonies ^a	
		(-) Frame checking	(+) Frame checking
Q2L	CAG→CTG	2	0
Q2A	CAG→GCT	2	3
Q2E	CAG→GAA	1	2
Q2R+F3L	CAG TTT→CGG CTT	3	0
V18D+D19H	GTG GAT→GAC CAT	1	1
V20Q	GTA→CAG	1	3
Q21W	GTA CAG→GTG TGG	0	4
Q21P	CAG AGT→CCG AGT	3	2
Q21P+S22C	CAG AGT→CCA TGT	3	0
Q21P+S22G	CAG AGT→CCT GGT	5	0
Q21P+S22R	CAG AGT→CCG CGT	4	0
Q21H+S22R	CAG AGT→CAC CGT	2	4
Q21H+S22G	CAG AGT→CAT GGT	0	5
Q21R+S22R	CAG AGT→CGC CGT	0	3
Q21R+S22G	CAG AGT→CGT GGT	0	4
S22R+D23T	AGT GAT→AGA ACT	3	0
D23E+I24C	GAT ATT→GAG TGT	0	2
R31G	CGG→GGC	0	1
L83P+S84C	CTC AGC→CCA TGC	0	1
M97R	ATG→CGC	0	2
F98N	TTC→AAC	2	2
F98G	TTC→GGC	0	2
F98Q	TTC→CAG	0	3

^aThe number of colonies recovered from TDEM without (-) or with (+) frame checking.

polymerase, the linearized plasmid is self-ligated and transformed into *E. coli*. The resulting plasmid is then digested with *Bpm*I to remove the left hand side of MI (Figure 1F). Following treatment with T4 DNA polymerase to create blunt ends, self-ligation and electrotransformation are performed to finally obtain the mutant library containing a substitution of three bases at random positions in the target. The number of colonies recovered after each step was $\sim 1 \times 10^7$, and always exceeded the number of colonies recovered in the previous step.

In this study, MIs that substitute three bases were used so that the resulting mutants would have a single amino-acid substitution, or, at most, two. The MI can, of course, generate various mutations depending on the frame and orientation of the MI (Supplementary Figure 3). Thus, it is not possible to completely control the mutations obtained using TDEM. However, this can be an advantage because multiple mutants can be generated using one MI, increasing the diversity of the library. If two codons are affected, it is possible to generate two consecutive amino acid changes, an event that rarely occurs using epPCR (Supple-

mentary Materials) or other mutagenesis methods (34).

Frame-checking procedure

In initial studies, we observed that after TDEM, more than half of the clones contained inappropriate deletions or insertions. We speculated that this resulted from inaccurate digestion by the restriction enzymes and/or overdigestion by T4 DNA polymerase. Although the presence of inappropriate mutations is not a problem when an efficient screening system is used to positively select mutants, it results in a high background when isolating loss-of-function mutations. Therefore, the pFrameCheck vector (Figure 2 and Supplementary Figure 1) was constructed so that mutants containing a frameshift or stop codon would be eliminated. In frame-checking, the mutated gene is cloned between the gIII signal sequence of the coat protein of phage fd (35) and β -lactamase. Therefore, only when the gIII signal sequence and β -lactamase are linked in frame through the mutated gene, will the β -lactamase be transported into the periplasmic space and the clones survive in the presence of ampicillin or carbenicillin (Figure 2). Therefore, mutated target DNA

Figure 2. Schematic diagram of the frame-checking procedure. The mutant *ccdB* genes in pCompact-Kana were purified using *Bsa*I digestion and ligated into pFrameCheck-Kana. The ligation mixture was transformed into *E. coli* and grown on LB agar plates containing kanamycin and carbenicillin overnight. (A) The β -lactamase gene that is fused to mutant proteins without a frameshift or stop codon will be expressed in the periplasmic space and cells will survive in the presence of carbenicillin. (B) However, when a frameshift or stop codon is present, the β -lactamase gene fused to the mutant CcdB will not be expressed in the periplasmic space and the clones will be sensitive to carbenicillin resulting in cell death. Using this procedure, only mutants without a frameshift or stop codon are recovered.

with a frameshift or stop codon will be eliminated resulting in a mutant library in which most, if not all, of the clones contain the desired base substitution.

Mutagenesis of *lacZa* using TDEM

lacZa was cloned into pCompact-Kana (Supplementary Figure 1 and Supplementary Table 1). Use of this vector maximizes the possibility of introducing the transposon into the target gene. Plasmids with insertions into an essential part of the vector (origin and kanamycin resistance gene) will not grow. Transposition into *lacZa* (36,37) was carried out with Mu and Tn7 resulting in more than 20,000 colonies, and an IB sequence of GCC (GCC-MI) within the MI. Following transposition, transposon integration sites were analyzed as described in the “Materials and methods” section. In the gel, each band represents a site attacked by the transposon (Mu and Tn7) with the intensity of the band indicating the frequency of transposition into this site (Figure 3). Although not every base was attacked, when both transposons were used, a relatively even distribution was observed (Figure 3B). The result indicates that the sites mutated by TDEM are located throughout the *lacZa* gene.

lacZa mutants deficient in α -complementation after TDEM with GCC-MI were identified on LB plates containing X-gal. Eight white and 2 pale blue colonies were picked. The 2 pale blue clones contained amino acid substitutions at two different sites (L8A, P32A) (Figure 4 and Supplementary Table 2). Among the 8 white colonies, 2 had the same mutation (N19P) and showed no α -complementation activity (Figure 4 and Supplementary Table

2). Among the other 6 white colonies, 2 had one-base deletions and 4 had insertions of 1 or 2 bases, which resulted in frameshifts (Supplementary Table 2). In this study, TDEM was performed without frame-checking and yielded many clones containing unwanted base deletions or insertions. We used the N19P mutant as the target for the next round of mutagenesis.

Second round of mutation of *lacZa*

The N19P mutant was then used for a second round of TDEM with GCC-MI to generate revertants displaying α -complementation activity. Fifteen dark blue colonies were picked and sequenced. All revertants showing the dark blue phenotype had the same mutation, P19A. Two pale blue clones from the second TDEM showed different mutations, P19G and Q24G (Figure 4 and Supplementary Table 2).

In the initial reversion study, the spectrum of revertants recovered was limited because only one MI (GCC-MI) was used. In an attempt to broaden the spectrum of revertants, a mixture of MI containing 20 different IB (MI-mix) was used for TDEM. Subsequently, 20 dark blue clones were picked and sequenced. Although all dark blue revertants were still mutated at P19 (Figure 4 and Supplementary Table 2), these revertants contained a variety of one or two amino acid mutations. The pale blue colonies recovered contained the mutations P19G, Q24G, and T45K+D46N (Figure 4 and Supplementary Table 2). All mutants contained 3-base substitutions that were determined by the IB or IB-mix used, and there was an accumulation of mutations with repeated rounds of TDEM.

Figure 3. Analysis of target site preference of Mu and Tn7 transposons in *lacZa*. The sites attacked by the Mu and Tn7 transposons were analyzed as described in the “Materials and methods” section. The left lane represents a 10-bp DNA marker, M represents the sites attacked by the Mu transposase, and T represents the sites attacked by the Tn7 transposase. The size and the intensity of labeled fragments indicate the position and the frequency of transposition integration, respectively. (A) The target site preference was analyzed using the Mu and Tn7 transposition system separately. (B) The reaction mixtures following Mu and Tn7 transposition were mixed and analyzed.

Comparison with epPCR

To determine if a different spectrum of revertants could be obtained using a different mutagenesis method, the *lacZa* mutant N19P was subjected to mutagenesis using epPCR, cloned into pCompact-Kana, and screened on LB agar plates containing X-gal. Of the 856 colonies obtained, we observed only 5 dark blue colonies. Although the 5 clones carried multiple point mutations of 3–6 bases which changed 2 or 3 amino acids, all had a mutation at position 19 that substituted proline with histidine, arginine, leucine, serine, or alanine (Figure 4 and Supplementary Table 2). All of these amino acid substitutions, except for the leucine substitution, had been observed following TDEM with the MI-mix. Furthermore, TDEM performed with MI-mix resulted in a higher reversion frequency than epPCR (Supplementary Table 2).

Mutagenesis of *ccdB* using TDEM with or without frame-checking procedure

We also carried out TDEM of *ccdB* (38), which exerts a cytotoxic effect on *E. coli*.

Figure 4. *lacZα* mutagenesis using TDEM with GCC-MI or MI-mix compared with error-prone PCR (epPCR). Wild-type *lacZα* was mutated using TDEM with GCC-MI and mutant *lacZα* lacking α -complementation activity was selected on LB agar plates containing X-gal. Mutant *lacZα* N19P produces an inactive polypeptide (1). This mutant was subjected to a second round of mutation using various methods: (2) TDEM with GCC-MI, (3) TDEM with MI-mix and (4) epPCR. *lacZα* from the dark blue colonies or pale blue colonies recovered were sequenced. All revertants, irrespective of the mutagenesis method, showing the dark blue phenotype on X-gal plates had a mutation that replaced P19. However, pale blue colonies having mutations at a distant site were obtained: Q24G and T45K+D46N (3). The revertants recovered from epPCR contained several other point mutations as well as mutations at P19 complicating the interpretation (4). The nucleotide sequences substituted and the frequency of the mutations are shown in Supplementary Table 2.

In this experiment, we characterized the mutants that survived both with and without the frame-checking procedure. To

minimize the expression of the *ccdB* during TDEM, the *E. coli* lactose promoter (Plac) was not present in pCompact-Kana-CcdB

so that after TDEM mutant *ccdB* needs to be sub-cloned into pFinalScreen, which contains Plac promoter (Supplementary Figure 1). In these experiments, N-terminally deleted MuA and buffers optimized for increasing the transposition activity and diversity of the integration sites were used because commercial Tn7 and Mu transposase showed lot-to-lot variations in the activity (32). After transposition (see Methods) into *ccdB*, we obtained more than 20,000 colonies. When the integration sites were analyzed, they were found to be distributed throughout the *ccdB* gene, although the possibility of some site preference cannot be excluded (Supplementary Figure 4). This mixture was then analyzed both with and without frame-checking.

After performing TDEM with MI-mix without frame-checking, the mutated CcdB was cloned directly into pFinalScreen, transformed into Top10 cells, and screened. Of the 106 colonies picked and sequenced, 38 contained the intended 3-base substitution, 16 contained a 1-base deletion, 18 contained a 2-base deletion, 31 contained a deletion of >3 bases which disrupted the reading frame, and 2 contained a base insertion. Among the 38

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mutants containing a 3-base substitution, 6 contained a stop codon. The remaining 32 colonies contained amino acid substitutions. Thus, only 30% of clones after TDEM without frame-checking contained the intended 3-base substitution.

In contrast, when TDEM of *ccdB* was carried out with MI-mix and frame-checking, 44 of the 50 clones sequenced had a 3-base substitution (Table 1) while 5 had deletions that were multiples of 3 bases so that reading frame was maintained (data not shown). Only one clone had a 1-base deletion mutation. Thus, when the frame-checking step is used, the frequency of colonies containing the desired amino acid substitution is dramatically increased.

The amino acid substitution mutants isolated from TDEM without frame-checking can be divided into three groups based on the site of mutation: amino acids 2–3, 18–23, and 98 (Table 1). Although most mutations identified following TDEM with frame-checking were located at those 3 positions, novel amino acid substitutions, R31G and L83P+S84C, were also identified (Table 1).

Discussion

In the current study, we describe a novel mutagenesis method, TDEM, with properties that should facilitate both amino acid scanning mutagenesis and the directed evolution of proteins. TDEM uses Mu transposase's random integration property and following TDEM of *lacZa* and *ccdB*, all mutants that showed phenotypic changes had alterations only at a single site facilitating the interpretation of the effects of different mutants. Furthermore, we showed that repeated rounds of TDEM can be used to accumulate mutations in the target gene. In order to further assess the diversity of the resulting libraries, we performed TDEM without frame-checking on the HIV protease gene (300 bp) using MI-mix and randomly sequenced 170 clones from a library of 2×10^6 . All 170 clones had modified bases in the HIV protease gene and 73 clones (43%) showed the desired 3-base substitution. Among these 73 mutants there were 60 unique HIV protease variants (Supplementary Table 3). Based on these results, we conclude that a single round of TDEM generates libraries with diverse mutations at a single site, although there can be target site preferences depending on the base composition (39).

While we were preparing this manuscript, another group described a similar approach (TriNEx) using a variant Mu transposon (Mu-Del) containing *MlyI* sites to substitute three random

Figure 5. Structural analysis of *lacZa* (A) and *ccdB* (B) mutants. (A) Models of amino acid D16, N19 and V22 of wild-type and mutant *lacZa*. PDB entry of *E. coli* β -galactosidase was 1bpO. The wild-type and variants with an all-atom model were energy-minimized using MacroModel (Schrödinger, Portland, ME, USA) with the truncated-Newton conjugated gradient (TNCG) minimization algorithm in generalized born surface area (GBSA) implicit solvation model. The substructure was specified by residues 14–55, corresponding to the α -peptide (α -donor) and by residues of the α -acceptor within 5.0 Å of the α -peptide. The coordinates of the rest of the protein were fixed. The energy-minimized structures of (1) wild-type, (2) N19P, and (3) Q24G variant with N19P of β -galactosidase are shown with the magnesium ion drawn as a sphere and the main chain of α -peptide shown in stick representation. Distances (Å) measured between magnesium ion and main chain oxygen of the residues. (B) Change of secondary structure propensity in *ccdB* mutants; (1) wild-type, (2) R31A and (3) R31G. Three-state secondary structure propensity was calculated using the CSSP2 (<http://cssp2.sookmyung.ac.kr>) algorithm.

bases for three preexisting bases (40). Using this system, they efficiently isolated β -lactamase mutants showing increased activity. Because TriNEx requires only one *MlyI* digestion and religation for substituting the three bases, TriNEx provides a simple and straightforward approach for generating mutant libraries using transposition. However, this method is limited in that only a maximum of 3 bases can be substituted. Using TriNEx, undesired mutations containing a frameshift, a stop codon, or a changed start codon were seen in 6 out of 49 clones (12%).

In contrast to TriNEx, TDEM is not limited to single amino-acid substitution, but it can be used to substitute up to 11 bases within the target genes. Although a single amino acid substitution would be sufficient in many experiments, TDEM provides a valuable alternative for generating mutant libraries introducing more than one consecutive amino acid substitution. TDEM has two additional steps compared with TriNEx, and introduces significant numbers of frameshift mutations that are eliminated in the frame-checking step. Importantly, after the frame-checking step, only 2% of the library contained frameshifts or stop codons, which is less than the percentage of unwanted mutants found in the TriNEx library (however, without the frame-checking procedure, TDEM generates more frameshift mutations than does TriNEx).

Because carbenicillin and ampicillin are biostatic antibiotics and β -lactamase has robust activity toward these antibiotics, we assumed that *E. coli* containing only a few molecules of β -lactamase in the periplasm could survive and proliferate. However, we cannot exclude the possibility that the frame-checking step could introduce bias in the mutant library due to sequence preference for exporting the fusion protein. Future efforts will be directed at improving the frame-checking procedure with the goal of minimizing the production of frameshift mutants and simplifying the procedure.

We have used TDEM for the mutagenesis of two genes, *lacZa* and *ccdB*. For *lacZa*, we first isolated the inactive mutant, N19P. We sequenced only 10 white colonies because the purpose of this initial screen was to isolate a single mutant for generating revertants. We speculated that this N19 mutant did not represent a hot spot of Mu transposase based on the transposition site analysis (Figure 2) that showed even integration of the Mu transposon throughout *lacZa*. *lacZa* with N19P was then subjected to a second round of TDEM. All revertants showing full activity had a substitution of P19. Several revertants also showed partial activity. Among the mutants showing partial activity was a second site revertant, Q24G. Examination of the crystal structure of β -galactosidase provided some insights into why *lacZa*

(N19P) is inactive and why activity is regained in *lacZ* α (N19P+Q24G) (Figure 5A). Amino acids D16, N19, and V22 coordinate a Mg²⁺ ion required for enzyme activity (41,42). Comparison of the energy-minimized structure of wild-type and N19P showed that in N19P, the distance between the Mg²⁺ ion and the main chain oxygen atoms interacting with it increases. However, when the Q24G mutation is additionally introduced, there is a significant decrease in the Mg²⁺/D16 and Mg²⁺/V22 distances (Figure 5A). These decreased distances undoubtedly result in more efficient coordination of the Mg²⁺ and are probably responsible for the more favorable α -complementation seen with this second site revertant.

Overall, our results with *ccdB* mutagenesis were consistent with a previous study in which every residue of CcdB had been individually changed to either Ala, Asp, Glu, Lys, or Arg (43). However, we also identified the *ccdB* mutants V20Q, Q21W, Q21P, R31G, F98N, F98G, F98Q, and mutants containing 2 amino acid changes that had not been reported previously (Table 1). It was not expected that the mutant R31G would render *ccdB* inactive since in the previous study (43), the R31A mutant was active. R31 is located in the core β -sheet of the CcdB monomer structure and mutation of this residue may change the folding stability of the protein. Indeed, when the secondary structure propensity of this region of wild-type and the R31A and R31G mutants were compared (Figure 5B), it was observed that the R31G mutation significantly changed the β -sheet propensity in the region. In contrast, the R31A mutation does not show any significant change in the secondary structure propensity (Figure 5B). Thus this analysis suggests that the R31G mutation disrupts the formation of the core β -sheet that plays a critical role in folding and maintaining the global protein structure.

We have demonstrated that TDEM can be used to introduce alterations at a single position during each round of mutagenesis so that the number of mutations and the sequence to be introduced can be controlled. It is a versatile method in which up to eleven bases within the target can be substituted with any desired sequence. Frame-checking is especially useful for eliminating nonsense mutations when screening for loss-of-function variants. Therefore, TDEM should provide a useful tool for the directed evolution or affinity maturation of a protein as well as for scanning mutagenesis with alanine, cysteine or nonnatural amino acids (36,37,44,45). It should be partic-

ularly useful when proteins need to be mutated stepwise to gradually achieve the desired phenotype. TDEM with its versatility, flexibility and controllability facilitates efficient construction of diverse mutant libraries and provides a powerful mutagenesis system.

Acknowledgments

Y.C.K. conceived the study, designed the experiments and performed the mutagenesis and screening. S.L.M. conceived the study and supervised the work. H.S.L. and S.Y. performed the protein structure analysis of the mutants.

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The authors declare no competing interests.

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