

# ***In vitro* selection of restriction endonucleases by *in vitro* compartmentalization**

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## **ABSTRACT**

**Restriction endonucleases are widely used in laboratory applications from recombinant DNA technology to diagnostics, but engineering of restriction enzymes by structure-guided design and *in vivo* directed evolution is at an early stage. Here, we report the use of an *in vitro* compartmentalization system for completely *in vitro* selection of restriction enzymes. Compartmentalization of a single gene in a rabbit reticulocyte *in vitro* transcription/translation system serves to isolate individually synthesized enzymes from each other. In each compartment, an active enzyme cleaves only its own encoding gene, whereas genes encoding inactive enzymes remain intact. Affinity selection of the cleaved DNA encoding active restriction endonucleases was accomplished by the use of streptavidin-immobilized beads and dUTP-biotin, which was efficiently incorporated into the cohesive end of the cleaved DNA using a DNA polymerase. We confirmed that genes encoding active restriction endonuclease FokI could be selected from a randomized library. This method overcomes the limitations of current *in vivo* technologies and should prove useful for rapid screening and evolution of novel restriction enzymes from diverse mutant libraries, as well as for studies of catalytic and evolutionary mechanisms of restriction enzymes.**

## **INTRODUCTION**

Restriction endonucleases that occur naturally in bacteria as host defense systems have been widely used in laboratory applications from recombinant DNA technology to polymorphism detection for diagnostics. Although more than 3500 restriction enzymes, including 240 distinct specificities, have been isolated from various bacteria, many sequence specificities have not yet been discovered (1). Creation of artificial restriction enzymes that recognize a desired or long DNA sequence is expected to be useful for various applications, including genome engineering. However, studies on design

and directed evolution of restriction endonucleases have been limited so far (2–6).

Numerous other enzymes have been improved by directed evolution, which usually involves iterations of random mutagenesis followed by selection using living cells (7,8). However, the scope of this *in vivo* approach is rather limited because of the restriction of library sizes based on transformation efficiencies of cells and the bias of selected clones with toxicity toward the cells (9,10). As an alternative approach, Tawfik and Griffiths (11) developed an *in vitro* compartmentalization (IVC) system that allows completely *in vitro* evolution of enzymes. In this system, artificial cell-like compartments provide the linkage of genotype (DNA) and phenotype (protein) for directed protein evolution (8,10,11). So far, IVC has been used for the selection of enzymes such as methyltransferases (12,13), a bacterial phosphotriesterase (14), and *Taq* DNA polymerase (15). In this study, we have applied the IVC approach for *in vitro* selection of restriction endonucleases, using FokI as a model enzyme.

FokI is a type IIS restriction endonuclease isolated from *Flavobacterium okeanokoites* and recognizes the nonpalindromic nucleotide sequence 5'-GGATG(N)<sub>9/13</sub>-3' (16). FokI comprises two separate domains: a DNA recognition domain that binds the recognition sequence specifically and a catalytic domain that cleaves DNA nonspecifically in the presence of Mg<sup>2+</sup> (17). It has been demonstrated that the FokI catalytic domain can be fused to zinc finger DNA-binding proteins to yield hybrid restriction enzymes with novel recognition specificities (18–20). One of the important goals of the study of *in vitro* protein evolution is the improvement of such artificial enzymes so that they are efficient enough for practical use as laboratory reagents, such as naturally occurring restriction enzymes. Here, we report the use of IVC for completely *in vitro* selection of restriction enzymes. We confirmed that the method is useful for selecting active enzymes from a randomized mutant FokI library.

## **MATERIALS AND METHODS**

### **DNA construction**

The FokI gene (1740 bp) was amplified from the genomic DNA of *F. okeanokoites* (obtained from IFO; Institute for Fermentation, Osaka, Japan) by PCR using the primers FokIF

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**Table 1.** Oligonucleotide sequences

Oligo	Sequence
CATmutF	GCTTATAGCGGAGGTTATAATCTGCC
CATmut3R	GGCAGATTATAACCTCCGCTATAAGCENNAG- TNNNCACGATCACACCATAGTCGATCGGAG- ATCCGACAGTATAAATTGCTCCNCCGGTT- TCCTTGATCCACCCAAAT
DDRmutF	CGGTGTGATCGTGGATACGAGAGCTTATAGC- GGAGG
DDRmutR	CCTCCGCTATAAGCTCTCGTATCCACGATCAC- ACCG
DEKmutF	GATTACGGTGTGATCGTCGAGACTAAAGCTTA- TAGCGGAGG
DEKmutR	CCTCCGCTATAAGCTTTAGTCTCGACGATCACACCG- TAATC
EDKmutF	GGATCAAGGAAACCGGAGGGTGCAATTTATAC- TGCGG
EDKmutR	CCGACAGTATAAATTGCACCCTCCGGTTTCCTTG- ATCC
FokI112F	CGTTGGGCACATGCTTTAGGATTTATT
FokI112R	AATAAATCCTAAAGCATGTGCCCAACG
FokI459F	CCTATTGATTACGGTGTGATCGTG
FokI459R	CACGATCACACCGTAATCAATAGG
FokIF	CGCGGATCCATGGTTTCTAAAATAA- GAACCTTCGGTTGG
FokIR	CAACTGCAGTAAAAAGTTTATCTCGC- CGTTATTAATTTCCG
FokmutF	GTTGGACTTGCTTACTCTAAATAAGG- TCGACGGCAGCCATTG
FokmutR	CAATGGCGCTGCCGTCGACCTTATTTAGAGTAAGC- AAGTCCAAC
ORIR2	CAGCAGATTACGCGCAGAAAAAAGG
T7F	CGGCATATGATCCCGCAAATTAATACG
T7kumaF	AATTCATAGCCGTCTCAGCAGCCAAC
T7kumaR	GTTGGTGCTGAGACGGCTATGAAATT

N = A, C, G or T.

and FokIR (all primer sequences used in this study are listed in Table 1). The PCR product was digested with BamHI and PstI, and then cloned into a pT7K vector comprising a T7 promoter, T7-tag sequence (21), and kanamycin resistance gene. The resulting plasmid pT7K-FokI was used for the construction of mutant FokI genes as follows. A plasmid pT7K-FokIΔ containing an inactive FokI mutant gene (containing a mutation of the S140 codon TCA to a stop codon TAA) was constructed from pT7K-FokI by use of a QuickChange XL site-directed mutagenesis kit (Stratagene) using primers FokmutF and FokmutR. Similarly, plasmids containing a D450E, D467E or K469R mutant gene were constructed using primers EDKmutF and EDKmutR, DEKmutF and DEKmutR or DDRmutF and DDRmutR, respectively. Template DNAs for *in vitro* transcription/translation of the wild-type and the mutant FokI were amplified from the above plasmids by PCR using primers T7F and ORIR2.

### Library preparation

A library of mutated FokI with randomized amino acids at residues 450, 467 and 469 was constructed as follows. The N- and C-terminal fragments of the FokI gene were amplified by PCR using two sets of primers T7F-FokI459R and FokI459F-ORIR2, respectively, and then cloned with a TOPO TA cloning kit (Invitrogen). From the resulting plasmids, two DNA fragments were re-amplified by PCR using two sets of primers T7F-CATmut3R and CATmutF-ORIR2.

The PCR products were purified using a QIAquick PCR purification kit (Qiagen) and then reacted with DpnI (New England Biolabs) for digesting the template plasmids. The reaction products were further purified using a QIAquick gel extraction kit (Qiagen) after 1% agarose gel electrophoresis. The purified DNA fragments were mixed and the library of full-length mutated FokI genes was amplified by overlap-extension PCR with KOD-plus DNA polymerase (Toyobo) using primers T7F and ORIR2. The PCR program was 25 cycles of denaturation at 94°C for 15 s, annealing at 65°C for 30 s and extension at 68°C for 320 s.

### *In vitro* compartmentalization

*In vitro* transcription/translation reactions in water-in-oil emulsions were performed essentially as described elsewhere (22) using the following modifications. The TNT quick-coupled transcription/translation system (Promega) based on a rabbit reticulocyte lysate was used as the water phase, and mineral oil (Nacalai tesque) containing 4% (v/v) Span85 (Nacalai tesque) and 1% (v/v) Tween-80 (Nacalai tesque) was used as the oil phase. The DNA concentration was 50 pM. The emulsions were incubated for 2 h at 30°C. To recover the water phase, the emulsions were spun at 2000g and 200 μl of supernatant was mixed with 100 μl of quenching buffer (phosphate buffered saline with 100 mM EDTA) and 900 μl of water-saturated diethyl ether. The mixture was vortex-mixed, then centrifuged, and the ether phase was removed. The water phase was washed with ether, dried and further purified using a QIAquick PCR purification kit.

### Biotinylation and affinity selection of cleaved DNA

Incorporation of dUTP-16-biotin (Roche) into cohesive ends of digested DNA fragments in the purified water phase was done with ΔTth DNA polymerase (Toyobo) (23,24) for 10 min at 75°C. The reactions were purified using a QIAquick PCR purification kit and mixed with streptavidin magnisphere paramagnetic particles (Promega) and buffer B (10 mM Tris-HCl, pH 7.9, 1 M NaCl, 1 mM EDTA) on a rotator for 90 min at 4°C. The streptavidin beads were washed twice with buffer B, twice with buffer W (25 mM Tris-HCl, pH 7.9, 1 M NaCl, 1 mM EDTA, 2.25 M guanidinium chloride), with buffer B and with PCR buffer, and then resuspended in 15 μl of PCR buffer. The DNA captured on the beads was amplified by PCR with KOD-plus DNA polymerase using primers T7F and T7kumaR (30 cycles at 94°C for 15 s, at 60°C for 30 s and at 68°C for 140 s). The PCR products containing promoter and open reading frame were re-assembled with 3'-untranslated region (3'-UTR) DNA (amplified from pT7K using primers T7kumaF and ORIR2) by overlap-extension PCR using primers T7F and ORIR2. The products were electrophoresed onto 1% agarose gel, purified with a QIAquick gel extraction kit and used as template DNAs for the next round of selection.

### Cloning and characterization of selected mutants

The PCR products amplified from beads in each round of selection were re-amplified by PCR with KOD-plus DNA polymerase using primers FokI112F and T7kumaR (25 cycles at 94°C for 15 s, at 60°C for 30 s and at 68°C for 90 s). The PCR fragments containing the randomized portion of the FokI gene were cloned using a TOPO TA cloning kit and

sequenced using an ABI PRISM 3100 genetic analyzer (Applied Biosystems).

For the activity assay of the selected clones, full-length FokI mutant genes were reconstructed from the partial fragment of each clone as follows. Two common fragments were amplified from pT7K-FokI using primers T7F and FokI112R and from pT7K using primers T7kumaF and ORIR2. The mutated region of each clone was amplified from each plasmid using primers FokI112F and T7kumaR. The three fragments were purified and then joined by overlap-extension PCR using primers T7F (labeled with fluorescein) and ORIR2. The resulting DNAs (2 nM final) were used as the template for the rabbit reticulocyte *in vitro* transcription/translation system. As the negative control, 10% heparin was added to the reaction mixture. Digestion of the fluorescence-labeled DNA with the translated enzymes was analyzed by SDS-PAGE using a Molecular Imager FX (Bio-Rad Laboratories).

## RESULTS AND DISCUSSION

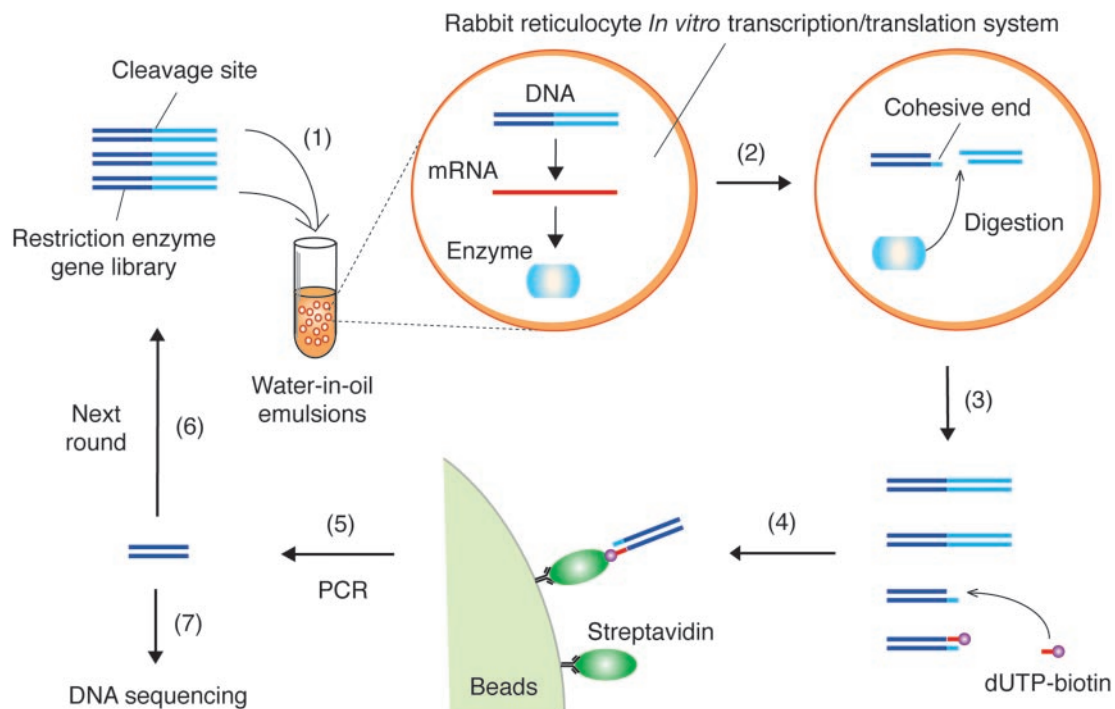
### Strategy for *in vitro* selection of restriction enzymes

The scheme for *in vitro* selection of restriction endonucleases by IVC is shown in Figure 1. IVC utilizing water-in-oil emulsions was developed by Tawfik and Griffiths to cage a single gene per micelle for linking the gene (genotype) and the gene product (phenotype), and was first applied for *in vitro* selection of methyltransferase M.HaeIII (11). In the M.HaeIII selection

procedure, a DNA encoding an active enzyme in a biotinylated DNA library was methylated in a compartment and rendered resistant to cleavage by restriction endonuclease HaeIII; thus, the biotin-label at the end of the DNA remained and could be used for affinity selection of uncleaved DNA encoding active methyltransferase with streptavidin beads (11). In this study, on the other hand, affinity selection of cleaved DNA encoding active restriction endonucleases is required. Thus, we examined methods of introducing biotin into cleaved DNA in a library recovered from emulsions. Our early attempts at ligating a biotinylated DNA adaptor with the cleaved DNA were unsuccessful because of the low efficiency of the ligation reaction (data not shown). As described below, success was achieved by using dUTP-biotin, which was efficiently incorporated into the cohesive end of the cleaved DNA with a DNA polymerase (Figure 1).

Although we have previously used wheat germ extract (22,25) as an *in vitro* transcription/translation system compartmentalized in emulsions, here we used a rabbit reticulocyte lysate system, because the ligation and polymerization reactions of the FokI-cleaved DNA recovered from the wheat germ reaction mixture were inhibited, probably due to endogenous factors such as exonucleases that eliminate the cohesive end of DNA (data not shown). DNA from the rabbit reticulocyte reaction mixture accepted the incorporation of dUTP-biotin and thus had retained its cohesive end.

Addition of the rabbit reticulocyte *in vitro* transcription/translation reaction mixture to stirred mineral oil gave a



**Figure 1.** Schematic representation of the *in vitro* selection of restriction enzymes. (1) A DNA library encoding mutant restriction enzymes is introduced into a rabbit reticulocyte *in vitro* transcription/translation system. (2) A single DNA molecule compartmentalized in a reversed micelle of water-in-oil emulsions is transcribed and translated *in vitro*. In a compartment, a translated enzyme with high activity cleaves its encoding DNA. In other compartments, genes that do not express active enzymes remain intact. (3) The mixture of cleaved and intact DNA is recovered from the emulsions. A biotinylated derivative of deoxyribonucleoside 5'-triphosphate (e.g. dUTP-biotin) is specifically incorporated into the cohesive end of the cleaved DNA by DNA polymerase. (4) The biotinylated DNAs encoding active restriction enzymes are affinity-selected with streptavidin-immobilized beads. (5) The selected DNA is amplified by PCR and used for (6) the next round of enrichment or (7) cloning and sequencing.

water-in-oil emulsion with a mean droplet diameter (by vol) of 3.5  $\mu\text{m}$  as measured by laser diffraction (data not shown). Accordingly, an emulsion made from 50  $\mu\text{l}$  of reaction mixture dispersed in 1 ml of oil contains  $2 \times 10^9$  compartments, each of which contains a single gene when the DNA concentration is  $<70$  pM. In this study, all IVC experiments were thus performed at 50 pM DNA.

### Enrichment of FokI genes

First, we confirmed that genes encoding active FokI restriction endonuclease could be selected from at least a  $10^4$ -fold excess of genes encoding inactive mutants in a model experiment.

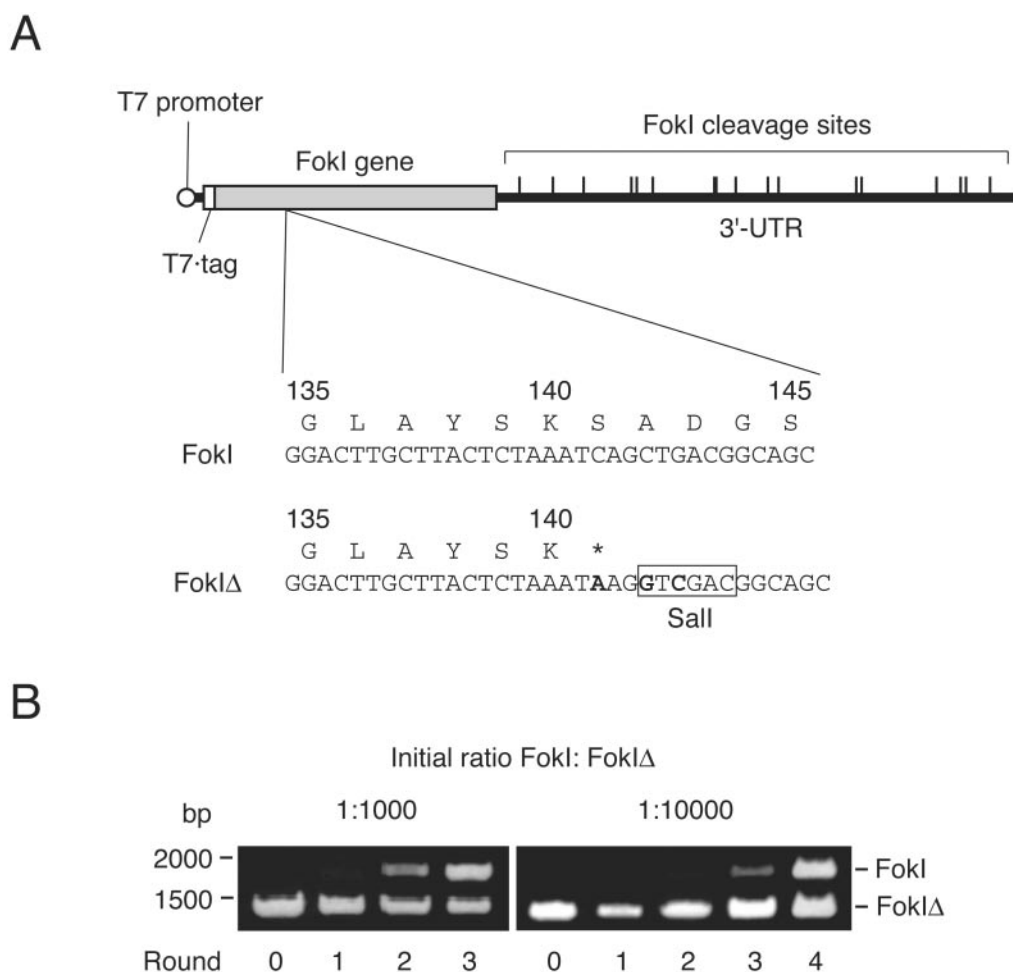
Figure 2A shows the DNA construct encoding the active FokI or an inactive FokI $\Delta$ . The N-terminal T7-tag (MASMTGGQMQGRGS) was used as an epitope tag in western blotting analysis. The 5'-UTR contains a T7 promoter. The FokI recognition and cleavage sites were present only within the 3'-UTR. As the length of the 3'-UTR of mRNA is known to affect the protein yield in a cell-free translation system (26), we investigated the yield of FokI in the rabbit reticulocyte transcription/translation system supplemented

with DNA template using various lengths of 3'-UTR (~1000, 2000 and 3000 bp). The DNA with 3000 bp of 3'-UTR (Figure 2A) gave the highest yield of FokI (data not shown), and thus this DNA construct was used in further experiments.

The wild-type FokI and the mutant FokI $\Delta$  genes were mixed in a ratio of 1:1000 or 1:10 000, and then subjected to several rounds of IVC selection procedure. As shown in Figure 2B, three rounds of selection of the 1:1000 ratio of FokI:FokI $\Delta$  genes and four rounds of selection of the 1:10 000 ratio each resulted in a roughly 1:1 final gene ratio, indicating an enrichment factor of ~10-fold per round. The efficiency is not so high, but IVC selection can be performed at the rate of one round per day and thus our system using dUTP-biotin should be useful for rapid selection of active restriction enzymes from a mutant library.

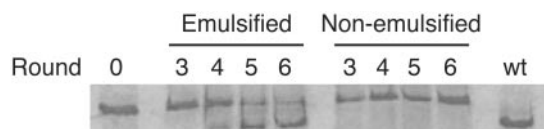
### *In vitro* selection of a randomized FokI library

Next, we applied the dUTP-biotin method for the selection a mutant FokI library. In the library, codons corresponding to three residues D450, D467 and K469 in the catalytic domain



**Figure 2.** Selections for restriction endonuclease FokI by using IVC. (A) DNA templates for *in vitro* expression of FokI. The 1740 bp FokI gene is fused with the N-terminal T7-tag coding sequence (42 bp). The 108 bp 5'-UTR fragment contains T7 promoter. The 3045 bp 3'-UTR fragment contains 17 FokI cleavage sites. A mutant FokI $\Delta$  gene contains three nucleotide mutations (boldface), yielding a stop codon (\*) and a Sall cleavage site (box). (B) Enrichment of the active FokI gene in multiple rounds of selection. Reaction mixtures containing 1:1000 or 1:10 000 molar ratio of FokI:FokI $\Delta$  genes were emulsified. The DNA after each round of selection was digested with Sall and analyzed by agarose gel electrophoresis.





**Figure 4.** DNA digestion activity at each round of emulsified or non-emulsified selection. Fluorescently labeled DNA after each round of selection was transcribed and translated *en masse* and analyzed by SDS-PAGE using an imaging analyzer. A result for the wild-type DNA (wt) is also shown.

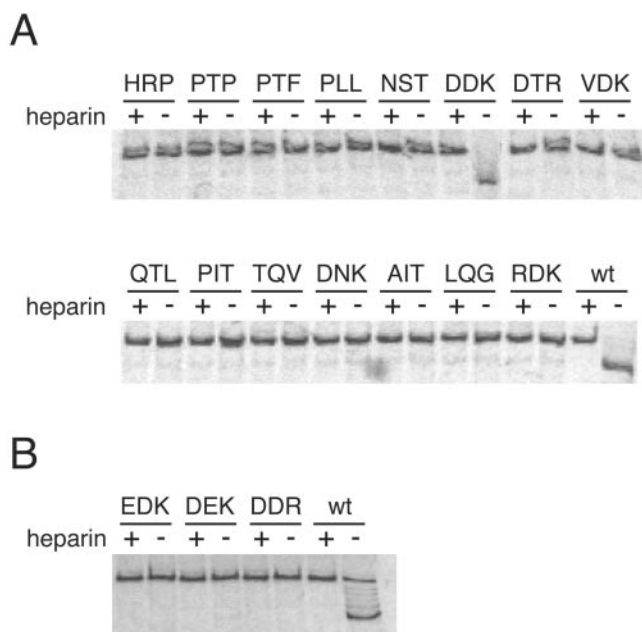
**Table 2.** Amino acid sequences of selected clones

Round	Number of clones	Amino acid residues			Activity
6	21	450	467	469	+
	4	D	D	K	
	4	Q	T	L	
	4	T	Q	V	
	2	H	R	P	
	2	P	T	F	
	2	P	L	L	
	2	P	I	T	
	2	A	I	T	
	2	L	Q	G	
	2	R	D	K	
	1	P	T	P	
	1	D	N	K	
1	25 other sequences				
4	2	D	D	K	+
	2	N	S	T	
	2	D	T	R	
	2	P	L	L	
	1	V	D	K	
	1	H	R	P	
	1	20 other sequences			

The amino acid residues at positions 450, 467 and 469 are shown for cloned mutants isolated from the library after four or six rounds of selection. The number of clones containing each sequence is indicated. Five clones at round 4 and one clone at round 6 contain stop codons. The activity of each mutant is also shown in Figure 5A.

### Cloning and characterization of the selected FokI mutants

The library DNA from the 4th and 6th rounds of emulsified selection was cloned and randomly chosen clones were analyzed by DNA sequencing. As listed in Table 2, 21 of 76 clones (6th) and 2 of 36 clones (4th) had Asp, Asp and Lys at positions 450, 467 and 469, respectively, as in wild-type FokI. The DNA sequences of all clones were distinguished from that of the original wild-type DNA by pre-designed silent mutations (Figure 3B), indicating that the selected wild-type sequence was not a contaminant, but was derived from the library. In the sequences of 55 (6th) and 34 (4th) non-wild-type clones, the frequencies of the 20 natural amino acids at the three positions seemed to be random and did not show any obvious bias, such as acidic or basic amino acids, as in wild-type FokI. Further, these clones did not reveal distinct activity (Figure 5A). These results suggest that the genes encoding FokI mutants other than the wild-type were randomly selected from the library.



**Figure 5.** DNA digestion activity of FokI mutants. (A) Activity of selected mutants listed in Table 2. The sequence of three mutated amino acids at residues 450, 467 and 469 in each mutant is shown at the top of each lane. The DDK protein has the same amino acids as in native FokI, though the DNA sequence is different from that of the wild-type FokI (wt). Fluorescently labeled DNA encoding each mutant gene was translated and analyzed by SDS-PAGE with an imaging analyzer. Heparin was added to inhibit translation. (B) Activity of site-directed mutants D450E, D467E and K469R.

The proposed active site motif of the restriction enzyme (27) permits Glu as well as Asp at position 467. Further, mutations between amino acids with similar properties are often tolerated without complete loss of activity. However, FokI mutants D450E, D467E and K469R were not selected in this study. To test whether these mutants were functional or not, we constructed the mutants D450E, D467E and K469R by site-directed mutagenesis and analyzed their activity. As shown in Figure 5B, no DNA digestion with these mutants was observed in this assay. Because the reaction conditions were not optimized for the FokI activity (e.g. the  $Mg^{2+}$  concentration in the rabbit reticulocyte reaction is 0.5 mM, whereas the optimum for FokI is 10 mM), the mutants analyzed in this study may possess weak activity, but no mutant having activity comparable with that of wild-type FokI was present in the 8000 mutants.

### Applications of *in vitro* selection of restriction enzymes

In this study, completely *in vitro* selection of a mutant restriction enzyme library was accomplished by using IVC and dUTP-biotin for the first time. This method allows screening of a large library of  $10^9$ – $10^{10}$  molecules within one day per selection round, and overcomes the limitations of current technologies of *in vivo* directed evolution (the library sizes of  $10^4$ – $10^6$  and the speed of several days per round) (2–6,30). Thus, the *in vitro* system should prove useful for screening and evolution of novel restriction enzymes from diverse mutant libraries.

Although restriction enzymes that can be selected by the use of dUTP-biotin are limited to those that create cohesive ends containing one or more nucleotide dAs, other biotinylated nucleotides can also be incorporated by  $\Delta$ Tth DNA polymerase (23). Biotinylated dUTP was also used in directed evolution studies of novel DNA polymerases by means of phage display (31,32). If an improved DNA polymerase with a higher incorporation efficiency of dUTP-biotin becomes available, it may lead to a higher enrichment factor in our *in vitro* selection system.

One application of this system would be the fine-tuning of hybrid restriction enzymes based on the linkage of a zinc finger DNA-binding domain to the FokI DNA-cleavage domain (18–20). In principle, designed zinc finger proteins capable of recognizing desired DNA sequences can be produced (33,34). Thus, hybrid restriction enzymes with desired specificity can also be obtained, though further improvement of the activity would be required (19). Such enzymes that recognize a sequence long enough to specify a unique site in a genome would be useful for gene mapping and gene targeting (35) on the genome.

The *in vitro* selection system may also be utilized to study mechanisms of natural evolution of restriction enzymes by testing evolutionary scenarios in the laboratories. For example, *in vitro* co-evolution of restriction endonucleases and methyltransferases can make it possible to clarify the evolutionary mechanism of restriction-modification systems.

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