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A new approach to directed gene evolution by recombined extension on truncated templates (RETT)

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Abstract

We describe a new approach to *in vitro* DNA recombination technique termed recombined extension on truncated templates (RETT). RETT generates random recombinant gene library by template-switching of unidirectionally growing polynucleotides from primers in the presence of unidirectional single-stranded DNA fragments used as templates. RETT was applied to the recombination of two homologous chitinase genes from *S. marcescens* ATCC 21074 and *S. liquefaciens* GM1403. When the shuffled genes were examined by restriction mapping and sequence analysis, it was found that chimeric genes were produced at a high frequency (more than 70%) between two chitinase genes with 83% of sequence identity. The number of crossovers within each chimeric gene ranged from one to four, and the recombination points were randomly distributed along entire DNA sequence. We also applied RETT to directed evolution of a chitinase variant for enhancing thermostability. Chimeric chitinases that were more thermostable than the parental enzyme were successfully obtained by RETT-based recombination.

Keywords: Directed evolution; *In vitro* recombination; Unidirectional single-stranded DNA fragments; Template-switching; Thermostability

1. Introduction

Genetic recombination is a key process for generating the genetic diversity, making organisms adapt and evolve in nature. Recently, *in vitro* recombination techniques such as DNA shuffling [1], staggered extension process (StEP) [2], random chimeragenesis on transient templates (RACHITT) [3], iterative truncation for the creation of hybrid enzymes (ITCHY) [4], and sequence homology-independent protein recombination (SHIPREC) [5], and so on have been developed to mimic and accelerate nature's recombination strategy. The directed gene evolution technology combining *in vitro* recombination techniques and high throughput screening system (HTS) is applicable to many industrial fields including protein pharmaceutical industry, agriculture, chemical industry, and biotechnology [6]. Especially, in the field of enzyme engineering, it is now widely applied to improve specific activity [7,8], enzyme stability [9–11], enzyme expression [12,13], and to alter substrate specificity [14–16].

In spite of the great importance of *in vitro* recombination techniques in directed gene evolution, the current techniques have still some drawbacks to be overcome for more efficient generation of gene library. DNA fragmentation process in DNA shuffling

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and RACHITT method may not be random because DNase I hydrolyzes DNA preferentially at sites adjacent to pyrimidine nucleotides, which introduces a sequence bias into recombination library [17]. A different *in vitro* recombination approach that relies only on PCR, StEP, was developed. In StEP method, PCR conditions have to be strictly controlled in order to achieve staggered extension of primers by shortening the polymerization time and lowering the reaction temperature. Failure to maintain the desirable range of temperature (e.g. too low temperature) during PCR process in StEP method may lead to non-specific annealing and further formation of undesirable recombinants. For the recombination of genes with low or no homology, ITCHY and SHIPREC were developed. However, these methods have limitations that only two parental genes can be recombined and the created hybrids are limited to one crossover.

In our way to develop new directed gene evolution methods, we devised a unique method for *in vitro* recombination, termed recombined extension on truncated templates (RETT). This method does not use DNA endonucleases for generation of shuffling blocks. Instead, it makes unidirectional single-stranded DNA (ssDNA) fragments by either DNA polymerase in the presence of random primers or serial deletion with exonuclease. These unidirectional ssDNA fragments only act as templates in PCR, not as primers. RETT generates random recombinant gene library by template-switching of unidirectionally growing polynucleotides from primers in the presence of unidirectional ssDNA fragments pool used as templates.

Here, we present the principle, advantages, and special applications of the RETT method. To test effectiveness of the RETT method, we applied RETT to the recombination of two homologous *Serratia* chitinase genes with 83% of sequence identity. We also describe the directed evolution of a chitinase variant by RETT-based recombination for enhancing thermostability.

2. Materials and method

2.1. Plasmid construction

Two chitinase genes, *m-chi* and *l-chi*, were amplified from *S. marcescens* ATCC 21074 and *S. liquefaciens* GM1403 by polymerase chain reaction (PCR) using forward primer (chiti-f: 5′-CCCAAGCTTC-CTCTCGGAATAAAGGAATCAG-3′) and reverse primer (chiti-r: 5′-GCTCTAGACCGGCAACGCAC-TGCAACCGATT-3′). Each 1.7-kb PCR product was purified from agarose gel using QIAquick gel extraction kit (Qiagen) and then digested with *Hin*dIII and *Xba*I. The digested 1.7-kb DNA fragments were inserted into the same restriction sites of pBluescript II KS (Stratagene) The resulting plasmids were designated as pBKS–*m-chi* and pBKS–*l-chi*, respectively, and used as starting polynucleotides for random recombination of two chitinase genes.

2.2. Preparation of unidirectional ssDNA fragments by reverse transcription

The plasmids, pBKS–*m-chi* and pBKS–*l-chi*, were linearized with *Ssp*I. An *in vitro* transcription was carried out using the linearized plasmids prepared above as template. The *in vitro* transcription reaction mixture contained 200 ng of the linearized plasmids, 40 mM Tris–HCl (pH 7.9), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM DTT, 0.5 mM each rNTP, 40 U of RNasin (Promega), and 17 U of T3 RNA polymerase (Promega) in a total volume of 20 μl, and incubated at 37 °C for 1 h. The RNA transcripts obtained by the above *in vitro* transcription were purified with RNAeasy column (Qiagen). Each RNA transcribed from the two chitinase genes was mixed. Reverse transcription was performed using the *in vitro* transcribed RNA as template. The reverse transcription mixture contained 200 μg of mixed RNA, 10 mM Tris–HCl (pH 8.3), 15 mM KCl, 0.6 mM $MgCl_2$, 0.2 mM DTT, 6 μg of random DNA hexamer, (Genotech Inc., Korea), 0.2 mM each dNTP, 40 U of RNasin and 50 U of M-MLV reverse transcriptase (Stratagene) in a total volume of 50 μl, and incubated at 37 °C for 1 h. After the reverse transcription, the RNA templates were removed by incubating the reaction mixture with 20 ng of RNase I at 37 °C for 1 h. The reverse transcription products were electrophoresed on agarose gel and the ssDNA fragments were purified using Geneclean kit (Bio 101).

The plasmids carrying thermostable chitinase variant genes were mixed and then linearized with *Ssp*I. The unidirectional ssDNA fragments of chitinase variant genes were prepared by *in vitro* transcription

and reverse transcription of *in vitro*-transcribed RNA by the same method as described above.

2.3. Preparation of unidirectional ssDNA fragments by serial deletion

Each 30 μg of pBKS–*m-chi* and pBKS–*l-chi* plasmid was mixed and linearized with *Not*I and *Sac*I. The resulting 25 μg of linearized plasmid was digested with 300 U of exonuclease III (Promega) at 30 °C. Each 2.5 μl of aliquot was then removed at intervals of thirty seconds, and mixed with 7.5 μl of solution containing 40 mM potassium acetate (pH 4.6), 304 mM NaCl, 2.28 mM $ZnSO_4$, 6.1% glycerol, and 25 U of S1 nuclease. The S1 nuclease reaction was placed at room temperature for 30 min and stopped by adding 1 μl of S1 stop solution (300 mM Tris base, 50 mM EDTA). The resulting serially deleted double-stranded DNA was dephosphorylated with calf intestinal phosphatase (Roche), and then digested with *Ssp*I. The double-stranded DNA fragments were purified from agarose gel using Geneclean kit and converted to unidirectional ssDNA fragments by further digestion with lambda exonuclease (New England Biolabs).

2.4. Recombinational synthesis

The 20 ng of unidirectional ssDNA fragments of chitinase genes prepared above served as templates to obtain full-length recombinant genes in PCR. First, a PCR reaction was carried out using 25 pmol of KS universal primer (5′-TCGAGGTCGACGGTATC-3′) under the condition of 30 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s. The PCR products were subsequently subjected to second PCR with reverse primer (5′-AATTCGAGCTCGGTACCCGGGGATCC-3′) under the condition of 30 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s. The resulting 1.8-kb PCR products were analyzed by 1% agarose gel electrophoresis. The recombined 1.8-kb DNA fragment was further amplified by PCR using chiti-f primer (5′-CCCAAGCTTCCTCTCGGAATAAAG-GAATCAG-3′) and chiti-r primer (5′-GCTCTAGAC-CGGCAACGCACTGCAACCGATT-3′). These PCR reactions were performed on an MJ Research thermal cycler (PTC-100) in a ExTaq polymerase buffer containing 0.2 mM each dNTP and 2 U of ExTaq polymerase (Takara) in a total volume of 50 μl.

2.5. Error-prone PCR

The pBKS-R24 carrying a chitinase gene variant was used as a template for error-prone PCR. Each 25 pmol of primers, chiti-f primer (5′-CCCAAGCT-TCCTCTCGGAATAAAGGAATCAG-3′) and chiti-r primer (5′-GCTCTAGACCGGCAACGCACTGCA-ACCGAT-3′) was used for an error-prone PCR reaction containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 4 mM $MgCl_2$, 0.2 mM dATP, 0.2 mM dGTP, 1 mM dCTP, 1 mM dTTP, 0.05 mM $MnCl_2$, 10 ng of template DNA and 5 U of ExTaq polymerase in a total volume of 50 μl. PCR was carried out on an MJ Research thermal cycler (PTC-100) under the conditions of 30 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min in a total volume of 50 μl.

2.6. Generation of chitinase mutant library

The full-length 1.8-kb PCR products of chitinase gene obtained by recombinational synthesis or error-prone PCR reaction were extracted from the agarose gel, digested with *Hin*dIII and *Xba*I, and then inserted to the same restriction sites of pBluescript II KS. The resulting recombinant plasmid was introduced to *E. coli* JM83 and transformants were selected on LB-agar plates supplemented with 100 μg/ml ampicillin.

2.7. Screening of chitinase mutants having improved chitin-resolving activity

To screen recombinant genes encoding chitinases which have specific activity higher than that of wild-type enzyme, the colonies from mutant library were picked to LB-agar plates containing 100 μg/ml ampicillin and 0.5% swollen chitin, and incubated at 37 °C overnight until clear halos were developed. Colonies showing clearer halo than that of wild type were selected for improved chitin-resolving activity.

2.8. Screening of chitinase mutants having improved thermostability

To screen the chitinase variant with improved thermostability, the colonies from mutant library were picked onto fresh plates and incubated at 37 °C for 20 h. The plates carrying the colonies were heated on a

water bath at an indicated temperature and then 50 mM sodium phosphate buffer solution (pH 6.8) containing 0.5% chitin and 1% agarose was poured onto the LB-agar plates. After the plates were incubated at 37 °C for further 24 h, colonies showing clear halos by hydrolyzing overlaid chitin were selected by staining with 0.2% Congo Red.

2.9. Enzyme purification

For purification of chitinases, each *E. coli* strain harboring recombinant wild-type or variant genes was grown at 30 °C in LB medium. After centrifugation of culture broth, supernatant was collected and concentrated with polyethylene glycol (PEG; MW 6000). The concentrated enzyme was dialyzed against 50 mM sodium phosphate buffer (pH 6.8) and adsorbed to swollen chitin at 4 °C. After obtaining enzyme–chitin precipitates by centrifugation, the adsorbed chitin were removed by incubating the enzyme–chitin complexes at 37 °C. The purified chitinases were analyzed by using SDS–polyacrylamide gel electrophoresis.

2.10. Enzyme assay

Chitinase activity was determined by measuring reducing sugars produced from chitin hydrolysis. Reducing sugars in the reaction were measured by DNS method [18] with *N*-acetyl-D-glucosamine as a calibration standard.

2.11. Thermostability test

Heat treatment of the purified enzymes was carried out on a temperature controller. Enzymes were incubated at different temperature and cooled immediately in an ice bath. The residual enzyme activity was measured.

3. Results and discussion

3.1. Description of the RETT method

The RETT method is based on two key processes: preparation of unidirectional ssDNA fragments of target genes to be recombined and recombinational synthesis of full-length genes in the presence of the ssDNA fragments which serve as templates for PCR (Fig. 1(a) and (b)). Random recombinations of target genes occur by template-switching of unidirectionally growing specific primers during PCR.

The ssDNA fragments used as templates in the RETT method are characterized by unidirectionality. The unidirectional ssDNA fragments can be generated by (1) reverse transcription using *in vitro*-transcribed RNA as template in the presence of random primers or (2) unidirectional serial deletion with exonuclease III. The unidirectionality of ssDNA fragments does not allow cross-priming and extension among them during PCR. Therefore, these unidirectional ssDNA fragments only act as templates in PCR, not as primers.

After mixing the unidirectional ssDNA fragments and specific primers that recognizes the 3′-end of target genes, the recombinational synthesis reaction is conducted by PCR. During one cycle of PCR, the specific primer is annealed to a ssDNA fragment which contains 3′-end of target genes and extended. During the next cycles of PCR, short fragments extended from the specific primer are annealed to other unidirectional ssDNA fragments and extended. In this process, elongating fragments can anneal to different templates based on sequence complementarity. Usually, the higher the nucleotide sequence similarity of parental genes to be recombined, the higher probability of choosing different template in template-switching process. Finally, full-length recombinant genes should be produced by annealing of DNA fragments extended from the specific primer to unidirectional ssDNA fragments containing 5′-end of target genes and further extension. In summary, the specific primer that recognizes the 3′-end of target genes grows unidirectionally by using unidirectional ssDNA fragments as template. Namely, RETT-derived full-length recombinant genes are totally synthesized from the specific primer and crossovers occur by template-switching of the growing primers during PCR. We termed this process as a “recombinational synthesis”. The RETT method produces full-length recombinant genes not by a reassembly process of shuffling blocks but by a recombinational synthesis process during PCR.

RETT has several advantages over the other *in vitro* recombination techniques. Compared with DNA shuffling and RACHITT which use enzymatic cleavage (most commonly DNase I digestion) to generate shuffling blocks, RETT may increase randomness

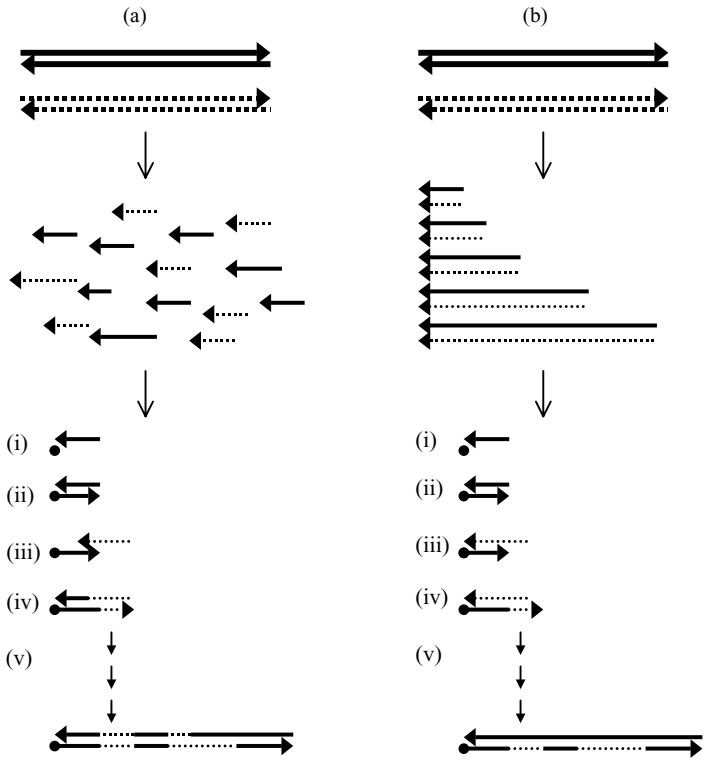

Fig. 1. General concept of RETT. According to truncation pattern of unidirectional ssDNA fragments, two methods are described separately. (a) Two homologous genes are presented to simplify the model. Unidirectional ssDNA fragments are prepared by reverse transcription using *in vitro*-transcribed target RNA as template in the presence of random primers. Recombinational synthesis reaction is conducted; (i) Specific primer is annealed to ssDNA fragment. (ii) Specific primer is extended during one cycle of PCR. (iii, iv) Short fragments extended from primer are annealed to other ssDNA fragment by template-switching and extended during another cycle of PCR. (v) Steps are repeated until full-length ssDNA genes are generated. (b) Unidirectional ssDNA fragments are prepared by serial deletion with exonuclease III. Recombinational synthesis reaction is conducted by steps (i–v).

in recombination process because random-priming synthesis and exonuclease III digestion don't cause sequence bias in generating unidirectional ssDNA fragments. In RETT process, the recombinational synthesis and template-switching of growing primers are carried out under normal PCR conditions of annealing and DNA elongation unlike StEP which uses extremely abbreviated annealing and DNA elongation conditions. RETT may also generate more random library than StEP because it is not influenced by sequence-specific pause sites met by DNA polymerase like StEP process.

The RETT technique using reverse transcription can generate a broad genetic diversity by point mutations introduced by reverse transcriptase (error rate, one per about 500 nucleotides) [19]. This may be advantageous in some cases of directed gene evolution of single gene because random recombinant library can be generated by single step of RETT without using error-prone PCR or any other mutational methods.

3.2. Random recombination of two homologous chitinase genes by RETT

To test the effectiveness of RETT method, we applied the RETT technique to a random recombination of two homologous genes encoding *S. marcescens* chitinase and *S. liquefaciens* chitinase, respectively. Fig. 2 shows the nucleotide sequence alignment of two chitinase genes. These two genes have 83% of nucleotide sequence identity.

```
l-chi AAGCTTCCTCTCGGAATAAAGGAATCAGTTATGCGCAAATTTAATAAACCGCTGTTGGCGTTGCTGATCGGCAGCACGCTGTGCTCTGCGGCGCAGGCCG 100
m-chi AAGCTTCCTCTCGGAATAAAGGAATCAGTTATGCGCAAATTTAATAAACCGCTGTTGGCGCTGTTGATCGGCAGCACGCTGTGTTCCGCGGCGCAGGCCG 100
      ************************************************************ ** ******************** ** **************

l-chi CTGCACCGGGCAAACCTACGTTGGCCTGGGGCAATACCAAATTCGCCATTGTCGAAGTCGATCAAGCGGCGACGGCTTATAATAATCTGGTGAAAGTAAA 200
m-chi CCGCGCCGGGCAAGCCGACCATCGCCTGGGGCAACACCAAGTTCGCCATCGTTGAAGTTGACCAGGCGGCTACCGCTTATAATAATTTGGTGAAGGTAAA 200
      * ** ******** ** ** ************ ***** ******** ** ***** ** ** ***** ** ***************** ******* *****

l-chi AAGTGCCGCCGACGTTTCTGTTTCATGGAATTTATGGAATGGCGATACCGGTACCACGGCAAAAGTATTATTAAATGGCAAAGAAGTTTGGAGTGGTGCC 300
m-chi AAATGCCGCCGATGTTTCCGTCTCCTGGAATTTATGGAATGGCGACGCGGGCACGACGGCCAAGATTTTATTAAATGGTAAAGAGGCGTGGAGTGGTCCT 300
      ** ********* ***** ** ** ********************  * ** ** ***** **  * ************ ***** *  ************

l-chi TCAACCGGTAGTTCGGGAACCGCAAACTTTAAGGTGAATAAAGGCGGCCGTTATCAAATGCAGGTGGCGTTATGCAACGCCGACGGCTGTACCGCCAGCG 400
m-chi TCAACCGGATCTTCCGGTACGGCGAATTTTAAAGTGAATAAAGGCGGCCGTTATCAAATGCAGGTGGCATTGTGCAATGCCGACGGCTGCACCGCCAGTG 400
      ********   ******* ** ** ** ***** ************************************ ** ***** *********** ******** *

l-chi ATGCAACCGAAATTGTGGTGGCAGATACCGACGGTAGCCATTTGGCACCTTTAAAAGAACCTTTGTTGGAAAAGAATAAGCCTTATAAACAAGACTCCGG 500
m-chi ACGCCACCGAAATTGTGGTGGCCGACACCGACGGCAGCCATTTGGCGCCGTTGAAAGAGCCGCTGCTGGAAAAGAATAAACCGTATAAACAGAACTCCGG 500
      * ** ****************** ** ******** *********** ** ** ***** **  ** ************** ** ********  *******

l-chi CAAAGTGGTTGGCTCTTATTTCGTTGAATGGGGCGTTTACGGCCGTAATTTCACCGTCGATAAACTTCCGGCTCAGAACCTGACGCACCTGCTGTACGGC 600
m-chi CAAAGTGGTCGGTTCTTATTTCGTCGAGTGGGGCGTTTACGGGCGCAATTTCACCGTCGACAAGATCCCGGCGCAAAACCTGACCCACCTGCTGTACGGC 600
      *********  ** *********** ** *************** ** *************** **  * ***** ** ******** ***************

l-chi TTTATCCCTATCTGTGGCGGTGACGGCATCAACGACAGCCTGAAAGAGATCGAAGGCAGCTTCCAGGCGTTACAGCGTTCCTGTCAGGGGCGTGAAGACT 700
m-chi TTTATCCCGATCTGCGGCGGCAATGGCATCAACGACAGCCTGAAAGAGATTGAAGGCAGCTTCCAGGCGTTACAGCGCTCCTGCCAGGGCCGCGAGGACT 700
      ******** ***** *****  * ************************************************************ ***** *****  * ** ****

l-chi TTAAGGTATCGATCCACGATCCGTTCGCTGCGCTGCAGAAAGGTCAGAAGGGCGTGACCGCCTGGGACGACCCCTACAAAGGCAACTTCGGCCAGTTGAT 800
m-chi TCAAAGTCTCGGTCCACGATCCGTTCGCCGCGCTGCAAAAAGCGCAGAAGGGCGTGACCGCCTGGGATGACCCCTACAAGGGCAACTTCGGCCAGCTGAT 800
      * ** ** *** ***************** ******** ****   ********************** ********** *************** ****

l-chi GGCGTTGAAACAGGCGCGCCCGGACCTGAAAATCCTGCCGTCGATCGGTGGCTGGACGTTATCCGATCCGTTCTTCTTTATGGGCGATAAGGTGAAGCGC 900
m-chi GGCGCTGAAGCAGGCGCATCCTGACCTGAAAATCCTGCCGTCGATCGGCGGCTGGACGCTGTCCGACCCGTTCTTCTTCATGGGCGACAAGGTGAAGCGC 900
      **** **** *******  ** ************************** ********* * ***** ********** ******** ************

l-chi GATCGCTTCGTCGGCTCGGTGAAGGAGTTCCTGCAAACCTGGAAGTTCTTTGATGGCGTAGATATCGACTGGGAATTCCCGGGCGGGCAGGGTGCTAACC 1000
m-chi GATCGCTTCGTCGGTTCGGTGAAAGAGTTCCTGCAGACCTGGAAGTTCTTCGACGGCGTGGATATCGACTGGGAGTTCCCGGGCGGCAAAGGCGCCAACC 1000
      ************** ******** *********** ***************** ***** ************** ***********  * ** ** ****

l-chi CGAAACTGGGCAGTACGCAGGATGGGGCAACCTATGTGCAGCTGATGAAAGAGCTGCGCGCCATGCTGGATCAGCTTTCGGCGGAAACGGGCCGTAAGTA 1100
m-chi CTAACCTGGGCAGCCCGCAAGACGGGGAAACCTATGTGTTGCTGATGAAGGAGCTGCGGGCGATGCTGGATCAGCTGTCGGCGGAAACCGGCCGCAAGTA 1100
      * ** ********  **** ** **** **********  ********* ******** ** ************** *********** ***** *****

l-chi TGAACTGACCTCTGCGATCAGCGCCGGCAAGGATAAAATCGATAAGGTGGATTACAACACCGCACAAAACTCGATGGATCACATTTTCCTGATGAGTTAC 1200
m-chi TGAGCTGACCTCCGCCATCAGCGCCGGTAAGGACAAGATCGACAAGGTGGCTTACAACGTTGCGCAGAACTCGATGGATCACATCTTCCTGATGAGCTAC 1200
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l-chi GACTTCTATGGGGCATTCGATCTGAAAAATCTGGGCCACCAGACTGCGCTGAAAGCGCCGGCCTGGAAACCGGATACGGCGTATACCACGGTGAATGGCG 1300
m-chi GACTTCTATGGCGCCTTCGATCTGAAGAACCTGGGGCATCAGACCGCGCTGAATGCGCCGGCCTGGAAGCCGGACACCGCTTACACCACGGTGAACGGCG 1300
      *********** ** *********** ** ***** ** ***** ******** ************** ***** ** ** ** *********** ****

l-chi TTAATGCACTGCTCACGCAGGGCGTGAAGCCGGGCAAAATCGTGGTGGGCACCGCCATGTACGGTCGCGGTTGGACCGGGGTGAACGGTTACCAGAACAA 1400
m-chi GCGCTGCTGGCGCAGTCAATGGCGTCAAGCCGGGCAAGATCGTGGTCGGCACCGCCATGTATGGCCGCGGCTGGACCGGGGTGAACGGCTACCAGAACAA 1400
         ***           * ****************** ******** *************** ** ***** ****************** ***********

l-chi CATTCCGTTTACCGGCACCGCCACTGGCCCGGTGAAAGGCACCTGGGAAAATGGCATCGTGGATTACCGCCAGATCGCCAATGAGTTTATGAGCGGCGAA 1500
m-chi CATTCCGTTCACCGGTACCGCCACTGGGCCGGTTAAAGGCACCTGGGAGAACGGCATCGTGGACTACCGCCAAATCGCCGGCCAGTTCATGAGCGGCGAG 1500
      ********* ***** ********** ***** *************** ** ********** ******** ******     **** ***********

l-chi TGGCAGTACAGCTACGATGCTACCGCTGAAGCACCCTATGTCTTCAAACCTTCCACTGGCGATCTGATCACCTTCGACGATGCGCGCTCGGTGCAGGCGA 1600
m-chi TGGCAGTATACCTACGACGCCACGGCGGAAGCGCCTTACGTGTTCAAACCTTCCACCGGCGATCTGATCACCTTCGACGATGCCCGCTCGGTGCAGGCCA 1600
      ******** * ****** ** ** ***** ** ** ** ************** ************************* ***************

l-chi AGGGCAAATATGTGCTGGATAAGCAGCTGGGCGGGTTGTTCTCATGGGAAATTGACGCCGACAACGGCGATATTCTGAATAACATGAACAGCAGCCTGGG 1700
m-chi AAGGCAAGTACGTGCTGGATAAGCAGCTGGGCGGCCTGTTCTCCTGGGAGATCGACGCGGATAACGGCGATATTCTCAACAGCATGAACGCCAGCCTGGG 1700
      * ***** ** ************************* ******* ***** ** ***** ** ************** ** * ********  *********

l-chi CAACAGCGTCGGTACGCCTTAAATCGGTTGTAGTGCGTTGCCGGTCTAGA 1750
m-chi CAACAGCGCCGGCGTTCAATAA-TCGGTTGCAGTGCGTTGCCGGTCTAGA 1749
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Fig. 2. Nucleotide sequence alignment of *S. marcescens* ATCC 21074 chitinase (*m-chi*) and *S. liquefaciens* GM1403 chitinase gene (*l-chi*), respectively. Asterisks indicate the same nucleotide identity. The starting ATG and stop codons are indicated by boldface.

RETT-based random recombination using unidirectional ssDNA fragments prepared by reverse transcription with random primers was conducted as described in Section 2. Restriction enzyme mapping analysis of randomly selected 14 recombinant plasmids from library revealed that restriction patterns of 10 plasmids were different from those of parent plasmids (data not shown). This result indicated that recombination occurred at a high frequency above 70%. Further nucleotide sequence analysis of the 10

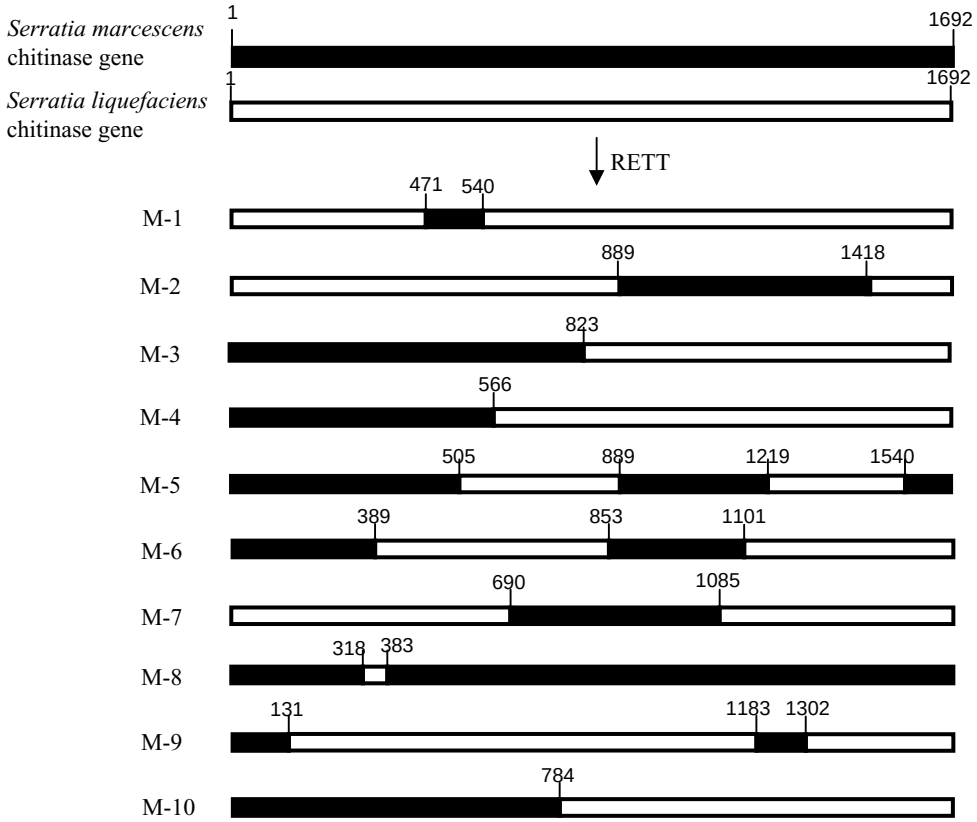

Fig. 3. Chimeric structures of RETT-based recombinant chitinase genes. Recombinant chitinases were generated by RETT method using unidirectional ssDNA fragments generated by reverse transcription of *in vitro*-transcribed RNA with random primers. The crossover sites are indicated by vertical lines. The numbers in RETT-derived recombinant chitinase genes indicate the position of crossovers. Sequences derived from *S. marcescens* chitinase gene and *S. liquefaciens* chitinase gene are shown by black and white boxes, respectively.

recombinant chitinase genes showed that the number of apparent recombination sites within each recombinant gene ranged from one to four, and the recombination points were randomly distributed along entire DNA sequence (Fig. 3).

When the screening of mutants having improved chitinolytic activity was performed against randomly selected 800 clones, one positive clone showed increased chitinase activity on chitin–agar plates (Fig. 4(a)). The recombinant chitinase gene derived from the positive clone, R-24, has four apparent recombination sites (Fig. 4(b)). The purified R-24 chitinase has 1.5 times higher specific activity than that of *S. marcescens* chitinase and 1.3 times higher than that of *S. liquefaciens* chitinase.

RETT-based random recombination using unidirectional ssDNA fragments prepared by serial deletion with exonuclease III was also conducted as described in Section 2. Restriction enzyme mapping of the recombinant plasmids prepared from randomly selected 10 colonies revealed that restriction patterns of seven plasmids were different to those of parent plasmids (data not shown). Further nucleotide sequence analysis of the seven recombinant chitinase genes showed that the number of apparent recombination sites within each recombinant gene ranged from one to four, and the recombination points were randomly distributed along entire DNA sequence (Fig. 5).

When the screening of mutants having improved chitinolytic activity was performed against randomly

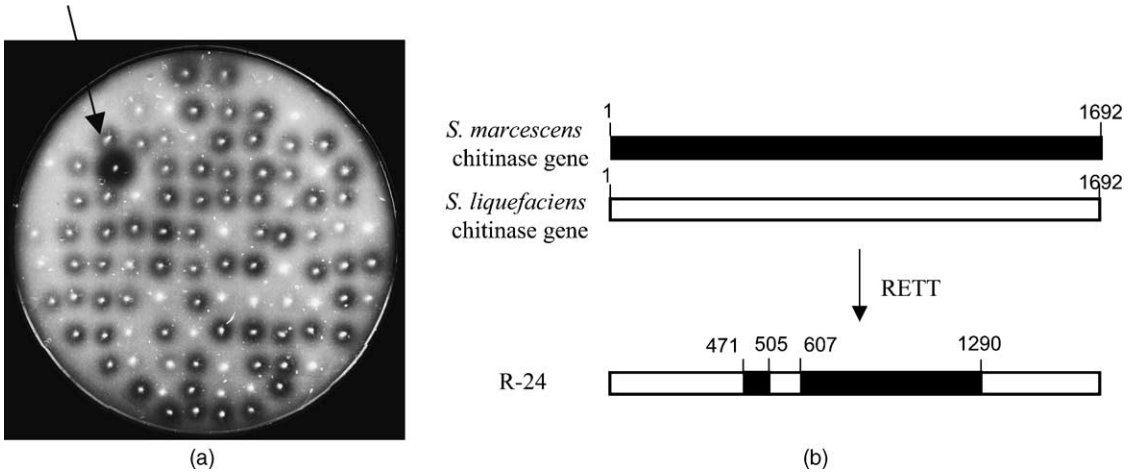

Fig. 4. (a) Screening of recombinant chitinase library on chitin–agar plate for improved chitin-resolving activity. Recombinant library were generated by RETT method using unidirectional ssDNA fragments generated by reverse transcription of *in vitro*-transcribed RNA with random primers. The R-24 clone showing increased chitinase activity on chitin–agar plates is indicated by an arrow. (b) Chimeric structure of R-24 chitinase gene. The numbers in R-24 chitinase gene indicate the position of crossovers. Sequences derived from *S. marcescens* chitinase gene and *S. liquefaciens* chitinase gene are shown by black and white boxes, respectively.

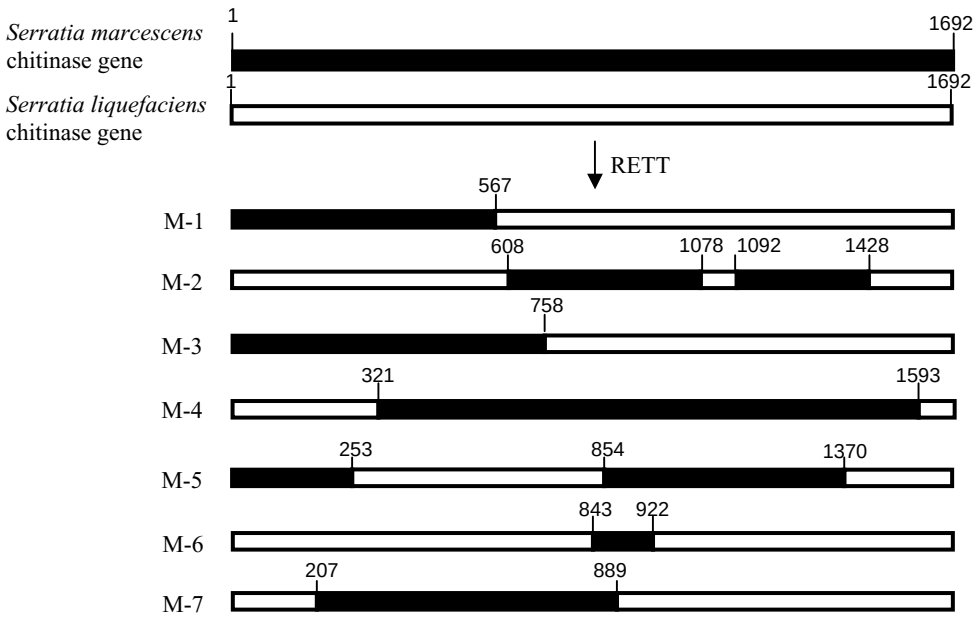

Fig. 5. Chimeric structures of RETT-based recombinant chitinase genes. Recombinant chitinases were generated by RETT method using unidirectional ssDNA fragments generated by serial deletion with exonuclease III. The sites of crossovers are indicated by vertical lines. The numbers in RETT-derived recombinant chitinase genes indicate the position of crossovers. Sequences derived from *S. marcescens* chitinase gene and *S. liquefaciens* chitinase gene are shown by black and white boxes, respectively.

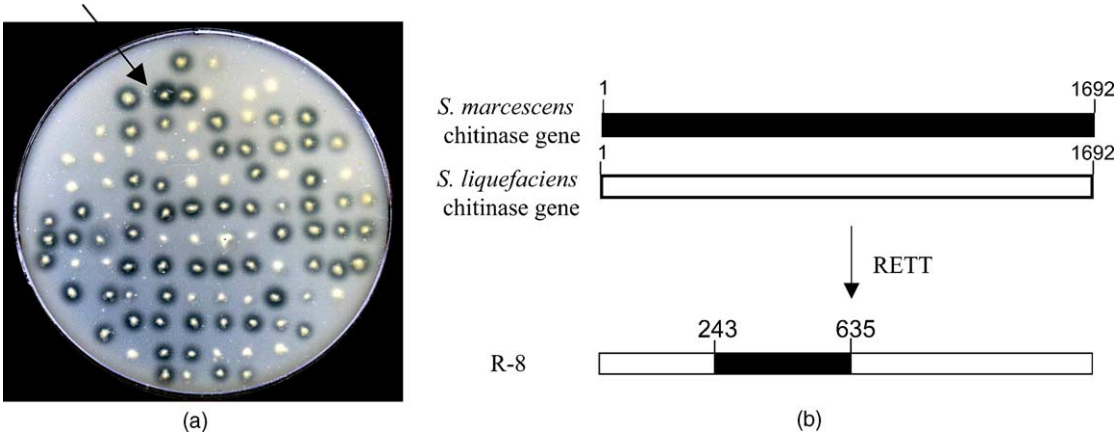

Fig. 6. (a) Screening of recombinant chitinase library on chitin–agar plate for improved chitin-resolving activity. Recombinant library were generated by RETT method using unidirectional ssDNA fragments generated by serial deletion with exonuclease III. The R-8 clone showing increased chitinase activity on chitin–agar plates is indicated by an arrow. (b) Chimeric structure of R-8 chitinase gene. The numbers in R-8 chitinase gene indicate the position of crossovers. Sequences derived from *S. marcescens* chitinase gene and *S. liquefaciens* chitinase gene are shown by black and white boxes, respectively.

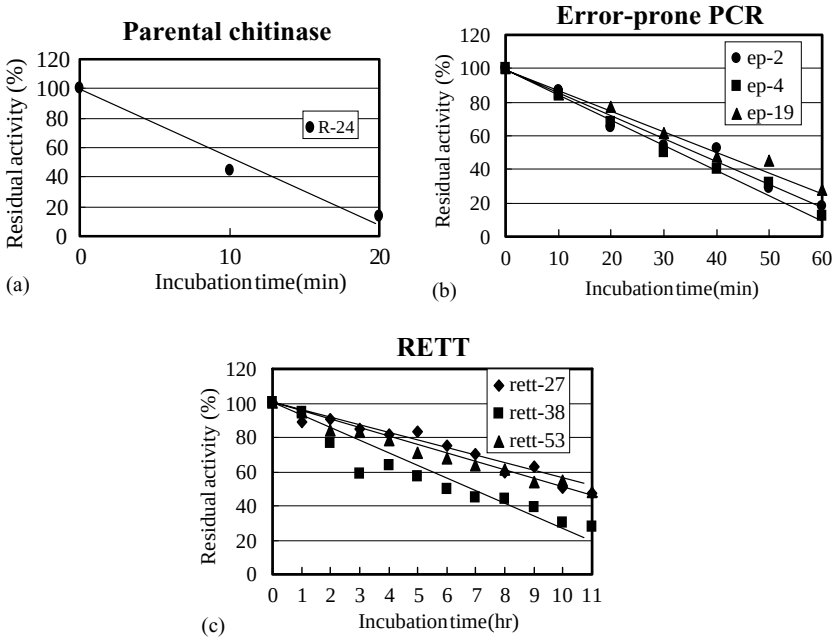

Fig. 7. Thermostability of parental chitinase (a), error-prone PCR-derived variants (b), and RETT-derived recombinant variants (c). Each purified enzyme was treated at 60 °C for indicated times. The residual activity was expressed as percentage of the original activity. The half-lives of R-24, ep-2, ep-4, ep-19, rett-27, rett-38, and rett-53 chitinase at 60 °C were 11 min, 37 min, 34 min, 41 min, 11.2 h, 6.7 h, and 10.1 h, respectively.

selected 2000 colonies, one positive clone showed increased chitinase activity on chitin–agar plates (Fig. 6(a)). The recombinant chitinase gene, R-8, derived from the positive clone has two apparent recombination sites (Fig. 6(b)). The purified R-8 chitinase has 2.1 times higher specific activity than that of *S. marcescens* chitinase and 1.6 times higher than that of *S. liquefaciens* chitinase.

3.3. Application of RETT to directed evolution of chitinase for improved thermostability

Plasmid pBKS-R24 carrying chitinase R-24 gene was used as a template in error-prone PCR to introduce random point mutations. Mutant libraries were created under conditions that average one to two amino acid substitutions per gene. When approx. 10,000 colonies were screened for improved thermostability, three chitinase variants were obtained. They have approximately three to four times longer half-lives at 60 °C than that of parental chitinase R-24 (Fig. 7(a) and (b)). Plasmids encoding each mutant chitinase were isolated and pooled with equal amounts. Genes for these chitinase variants were subjected to random recombination by RETT method. From ∼15,000 recombinant chitinase library, three recombinant chitinases were obtained for improved thermostability. The purified recombinant chitinases, rett-27, rett-38, and rett-53, have 61, 37, and 55 times longer half-lives at 60 °C than that of parental chitinase R-24, respectively (Fig. 7(a) and (c)).

Nucleotide sequence analyses of chitinase variants were carried out and the deduced amino acid substitutions are shown in Fig. 8. Chitinase variants, ep-2, ep-4, and ep-19, derived from error-prone PCR have one or two amino acid substitutions at different sites in amino acid sequence. RETT-derived chitinase variants have two common amino acid substitutions, N474D and Q404H, which were originated from ep-4 and ep-19, respectively. These two amino acid substitutions in chitinase R-24 endowed a very high thermostability, about 60 times increase in rett-27. N70S substitution of ep-2 gave a slight increase in thermostability of chitinase. However, N425S substitution in rett-38, generated by a point mutation during RETT-based random recombination gave a deteriorative effect on thermostability of chitinase. This substitution can be replaced with original amino acid by back-crossing with parental chitinase, R-24.

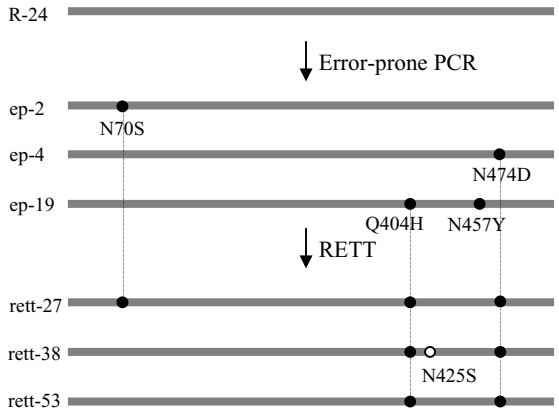

Fig. 8. The deduced amino acid changes are indicated in each chitinase variant. The filled circles indicate amino acid substitutions derived by error-prone PCR. The empty circle indicates an amino acid substitution generated during RETT process.

4. Conclusions

We have developed a new method for *in vitro* DNA recombination technique termed recombined extension on truncated templates (RETT). RETT generates random recombinant gene library by template-switching of unidirectionally growing polynucleotides from primers in the presence of unidirectional ssDNA fragments used as templates. Since RETT method does not use DNA endonucleases for generation of shuffling blocks, RETT may increase randomness in recombination process compared with DNA shuffling and RACHITT. In RETT process, the recombinational synthesis and template-switching of growing primers are carried out under normal PCR conditions of annealing and DNA elongation unlike StEP, which uses extremely abbreviated annealing and DNA elongation conditions. RETT may generate more random library than StEP because it is not influenced by sequence-specific pause sites met by DNA polymerase-like StEP process. We verified the effectiveness of RETT method by applying it to the recombination of two homologous chitinase genes from *S. marcescens* and *S. liquefaciens*. RETT was also applied to directed evolution of a chitinase for enhancing

thermostability. Chimeric chitinases that were more thermostable than the parental enzyme were successfully obtained by RETT-based recombination.

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