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Effective Family Shuffling Method Using Complementary DNA Fragments Produced by S1 Nuclease

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Abstract An efficient method for the *in vitro* reassembly of homologous DNA sequences is presented. The proposed method involves obtaining single strands of homologous genes and hybridizing them to obtain partially hybridized heteroduplex DNA; cleaving the single-stranded regions of the heteroduplex DNA using S1 nuclease to generate double-strand DNA fragments; denaturing the double-strand DNA fragments to generate single-strand DNA fragments; conducting a series of polymerase chain reactions (PCR) using the single-strand DNA fragments as internal primers and a mixture of homologous DNA as templates to obtain elongated reassembled DNA; and finally, amplifying the reassembled DNA by a PCR using terminal primers. As a result, DNA reassembly could be achieved between homologous genes with a sequence similarity as low as 78%.

Key words: Family shuffling, S1 nuclease

A lot of effort has been made to improve enzyme properties, such as their heat or acid stability, enzyme activity, and substrate specificity, for industrial application [6, 7, 9, 12], and directed evolution employing mutant library construction by *in vitro* DNA reassembly as well as *in vitro* or *in vivo* screening has emerged as one of the most effective approaches to achieve this goal [9]. Several different methods have been developed for efficient library construction, including DNA shuffling [11], a staggered extension process (StEP) [15], random-priming recombination (RPR) [10], incremental truncation for the creation of hybrid enzymes (ITCHY) [8], and degenerate oligonucleotide gene shuffling (DOGS) [2]. Nonetheless, family shuffling among homologous genes recovered from nature is known to be much more effective than reassembly among randomly introduced mutants to obtain enzymes with expected properties [1].

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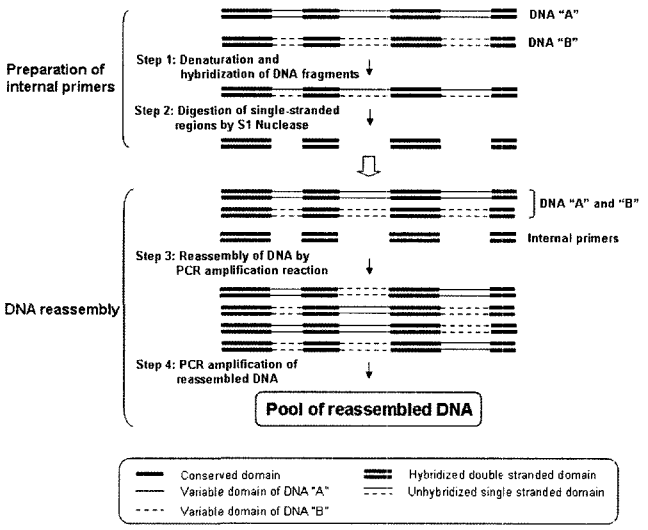

Fig. 1. General scheme of DNA reassembly.

Accordingly, to increase the *in vitro* DNA reassembly efficiency among DNA fragments with a low sequence similarity, this study reports on a new method of constructing mutant libraries from homologous genes, using complementary DNA fragments that have conserved sequences between the genes. The overall scheme, action mechanism, and examples using partial DNA fragments of bacterial chitinase genes are presented.

The DNA reassembly method introduced in this study consists of 4 steps, as illustrated in Fig. 1: 1) hybridization between homologous genes; 2) digestion of an unhybridized DNA region of heteroduplex using S1 nuclease; 3) reassembly of genes by repeated PCR amplification reaction using original genes as templates and DNA fragments produced in steps 1) and 2) as internal primers; and 4) amplification of reassembled DNA. The first and second steps involve the preparation of internal primers that have complementary DNA

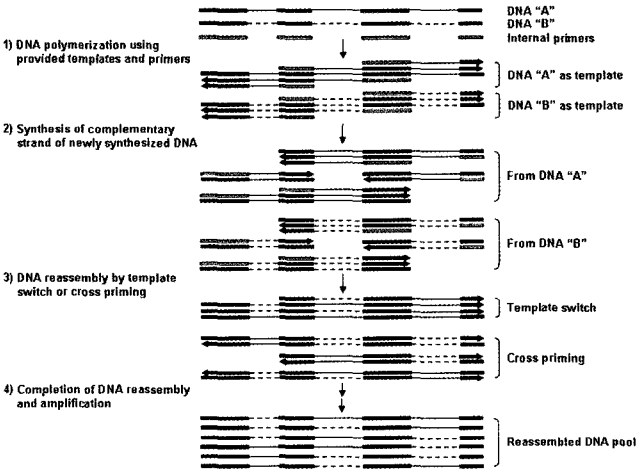

Fig. 2. Mechanism of DNA reassembly.
The internal primers and DNA fragments supplied or synthesized in previous steps are represented as pink and black lines, respectively, and newly synthesized DNA regions are represented as blue lines.

sequences that are conserved in both of the homologous genes. Annealing between homologous genes leads to partially hybridized heteroduplex DNA composed of double-stranded regions with conserved sequences and single-stranded regions with variable sequences. Thereafter, since double-stranded regions are resistant to the activity of S1 nuclease, whereas single-stranded regions are digested by S1 nuclease, treating the partially hybridized heteroduplex between homologous genes with S1 nuclease produces double-stranded DNA fragments with complementary sequences. These DNA fragments are then used as internal primers in the next step to reassemble homologous genes. The third step, involving the reassembly of DNA, is conducted using a PCR amplification reaction in the presence of template DNA, the internal primers produced in the previous steps, and with or without a small amount of external primers, as illustrated in Fig. 2. In the first round of DNA polymerization, the internal primers anneal to the conserved domains and the polymerization proceeds to the end of the template DNA. Then, in the second round of DNA polymerization, complementary strands are synthesized by annealing the end primers or internal primers to the newly synthesized DNA strands. However, since the synthesis of a complementary strand cannot be completed to the full size of the original template DNA, the synthesis of the complementary strands is completed using a new template DNA, which can be the original template DNA or partial DNA fragments in the opposite direction synthesized in the previous steps. When the new template is a homologous gene with a different sequence in the variable domain, DNA reassembly occurs by template switching or cross-priming. The annealing efficiency of primers or short DNA fragments to the template DNA, or cross-priming between two partial DNA fragments, is greatly influenced by the sequence conservation at the 3' end of the primer or short DNA fragments. Thus, when DNA reassembly between genes with a low sequence similarity is attempted, the DNA fragments anneal to the template DNA with the same sequence. Therefore, the unique feature that distinguishes the proposed method from previous methods, such as DNA shuffling [11], PRP [10], or StEP [15], is that all the DNA fragments participating in the annealing process have conserved sequences in the terminal regions. This is achieved by using DNA fragments from conserved domains as the primers in all the DNA polymerization reactions. As a result, it is expected that the annealing bias to the self template and DNA reassembly efficiency will be improved.

Therefore, to test the above rationale and examine the reassembly efficiency, the chitinase genes from *Aeromonas hydrophila* (KCTC 2358), *Aeromonas punctata* (KCTC 2944), and *Pantoea agglomerans* (KCTC 2578) were used. The partial gene fragments were amplified using the forward primer Chi600f (GGCATCAACGACAGCNTN-AAAG) and reverse primer Chi1200r (GTCNTAGCTCA-TCAGGAAGATG). The PCR products were then gel purified and cloned in the pGEM T-vector (Promega, Madison, WI, U.S.A.). The sequences of the cloned genes were determined using the primers pGTf (TACGACTCA-CTATAGGGCGA) and pGTr (ACTCAAGCTATGCATC-CAAC), and BigDye terminator cycle sequencing kits (Applied Biosystems, Foster City, CA, U.S.A.). The template DNA for the DNA reassembly was prepared by restriction digestion of the cloned genes using *Sac*II and *Spe*I, which cut multiple cloning sites of the vector, and gel purification.

To prepare the internal primers, chitinase gene fragments of *P. agglomerans* and *A. hydrophila* or *A. punctata* were mixed (1 μg each) and the final volume adjusted to 38 μl with water. The double-stranded DNA fragments were then denatured by heating at 94°C for 10 min and rapid cooling on ice. The salt concentration was increased by adding 5 μl of a 10× S1 nuclease buffer and 5 μl of 3 M NaCl, followed by incubation at 65°C for 2 h. Thereafter, the single-stranded regions of the DNA fragments were digested by adding 2 μl of diluted S1 nuclease (35 units/μl, Invitrogen, Carlsbad, CA, U.S.A.), followed by incubation at 45°C for 1 h. The digested DNA fragments were then purified by phenol:chloroform extraction and ethanol precipitation, and the resulting DNA pellets washed with 70% ethanol, dried in a vacuum, and dissolved in 15 μl of distilled water. The reassembly of the DNA fragments was achieved by PCR amplification in the presence of the same template DNA as used in the production of the internal primers (0.1 ng), the two end primers Chi600f and Chi1200r (0.1 pmol each), 5 μl of the internal primers produced in the previous step, 5 μl of a 10× *Taq* reaction buffer [100 mM Tris-HCl, 400 mM KCl, 15 mM $MgCl_2$, pH 9.0], 2 μl of 25 mM $MgCl_2$, 4 μl of 2.5 mM dNTPs, and 1 unit of *Taq* polymerase (Bioneer, Daejeon, Korea). The final

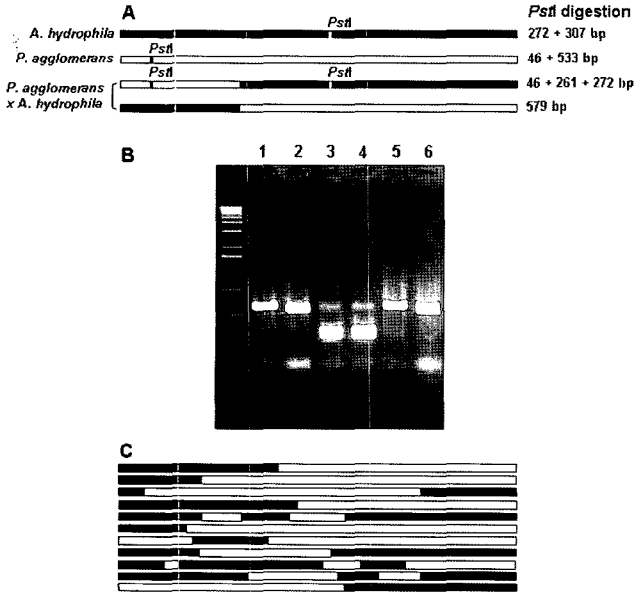

Fig. 3. DNA reassembly between *A. hydrophila* and *P. agglomerans*.
A. PstI restriction map of chitinase gene clones and expected restriction sites in reassembled DNA fragments. **B**. Restriction digestion with PstI of six randomly chosen clones. **C**. Map of reassembled DNA determined by sequence analysis.

volume was adjusted to 50 μl with deionized water, and the PCR amplification conducted using the following conditions: initial denaturation at 94°C for 3 min, 45 amplification cycles at 94°C for 30 s, 45°C for 30 s, and 72°C for 30 s, plus a final extension phase at 72°C for 30 min. The reassembled genes were then amplified by a PCR amplification reaction with Chi600f and Chi1200r and cloned into the pGEM-T vector (Promega, Madison, WI, U.S.A.). The frequency of recombination was examined by restriction digestion and the sequencing of randomly selected clones.

The sequence similarity of the cloned chitinase genes between *A. hydrophila* and *P. agglomerans*, *A. hydrophila* and *A. punctata*, and *A. punctata* and *P. agglomerans* was 95%, 79%, and 78%, respectively. The chitinase gene clone of *A. hydrophila* had a *Pst*I restriction site at nucleotide position 307, and when it was digested with *Pst*I, 272-bp and 307-bp DNA fragments were produced (Fig. 3A). The chitinase gene clone of *P. agglomerans* had a *Pst*I restriction site at nucleotide position 46, and when it was digested with *Pst*I, 46-bp and 533-bp DNA fragments were produced. Thus, when reassembling the two genes between nucleotide positions 46 and 307, the resulting DNA fragments would be expected to have no restriction sites or two restriction sites at nucleotide positions 46 and 307. In addition the clones without a *Pst*I site should maintain an intact DNA fragment of 579 bp, whereas the clones with two *Pst*I sites should be digested by PstI to produce three DNA fragments of 46,

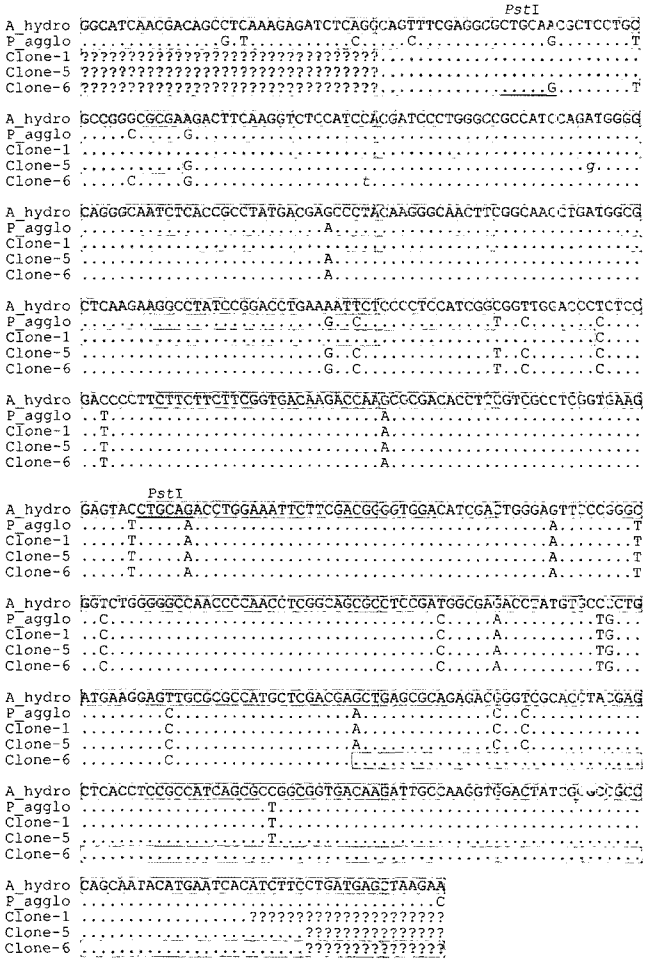

Fig. 4. Sequence alignment of chitinase genes from *A. hydrophila*, *P. agglomerans*, and reassembled clones 1, 5, and 6.
The sequence regions with homology to *A. hydrophila* are shaded. The two nucleotide positions with a point mutation during the PCR reaction are represented by small italicized letters.

261, and 272 bp. Based on these predictions, the reassembled DNA clones were screened by restriction digestion with PstI among randomly selected clones (Fig. 3B). Among 6 clones, clone 1 and clone 5 were not digested by PstI. The DNA reassembly was also examined by sequencing the clones, which revealed that three of the six selected clones were reassembled (Figs. 3C and 4). Clones 1 and 5 correspond to the first and second diagram in Fig. 3C, respectively, where clone 1 maintained the same sequence as *A. hydrophila* to nucleotide position 235, and then the homology of the sequence was switched to *P. agglomerans* from nucleotide position 236. Meanwhile, clone 5 maintained the same sequence as *A. hydrophila* to nucleotide position 71, and then the homology of the sequence was switched to *P. agglomerans* from nucleotide position 72. Although clone 6 had the same restriction pattern as the sequence of *P. agglomerans*, the sequence analysis showed that it was a reassembled clone, as represented in the third diagram in

Fig. 3C and Fig. 4, since it maintained the same sequence as *A. hydrophila* to nucleotide position 50, at which point the sequence was replaced with that for *P. agglomerans,* creating a PstI site and continuing to nucleotide position 450; then, the homology of the sequence switched again to *A. hydrophila* at nucleotide position 451. In repeated experiments, the frequency of reassembled gene clones ranged from 50% to 70%, and reassembly events occurred 1–5 times per reassembled clone. The reassembly of the chitinase genes of *A. punctata* and *P. agglomerans* was carried out under the same conditions as for the reassembly of the chitinase gene clones of *A. hydrophila* and *P. punctata*. Among 6 clones examined, one clone exhibited a reassembly event (data not shown).

In conclusion, this paper presented a new scheme of *in vitro* DNA reassembly, devised to improve the reassembly efficiency between homologous genes with a low sequence similarity. In the DNA reassembly of bacterial chitinase genes, up to 70% of the clones were reassembled, with 1–5 reassembly events per clone, when homologous genes with a 95% similarity were used. When homologous genes with a 78% sequence similarity were used, 1 clone was reassembled out of 6 randomly selected clones. The relatively low reassembly efficiency between genes with a low sequence similarity may have been caused by a low efficiency of the heteroduplex formation during the preparation of the internal primers, or low annealing efficiency between the DNA fragments originating from different genes during the PCR reaction for DNA reassembly. Thus, when the annealing conditions are optimized by changing the annealing temperature or salt concentration, or by introducing restriction digestion steps [3], the proposed method will be a promising *in vitro* DNA reassembly method that can be conducted without knowledge of the template DNA sequences.

Directed evolution by *in vitro* DNA reassembly together with screening *in vitro* or *in vivo* is one of the most efficient methods among currently used protein engineering techniques. Accordingly, this paper presented a new family shuffling method for efficient DNA reassembly between homologous genes with a low sequence similarity. Thus, coupling the proposed method with gene mining from genomic and metagenomic approaches [4, 5, 13,] will have a significant impact on the development of enzymes for the food, pharmaceutical, clothing, and leather industries. In addition, the proposed method can also be applied to basic science to understand the adaptation of proteins in extreme environments by comparing the structures and functions of proteins created by DNA reassembly.

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