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Construction of a random circular permutation library using an engineered transposon

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ABSTRACT

Circular permutation is an important protein engineering tool used to create sequence diversity of a protein by changing its linear order of amino acid sequence. Circular permutation has proven to be effective in the evolution of proteins for desired properties while maintaining similar three-dimensional structures. Due to the lack of a robust design principle guiding the selection of new termini, construction of a combinatorial library is much preferred for comprehensive evaluation of circular permutation. Unfortunately, the conventional methods used to create random circular permutation libraries cause significant sequence modification at new termini of circular permutants. In addition, these methods impose additional limitations by requiring either relatively inefficient blunt-end ligation during library construction or redesign of transposons for tailored expression of circular permutants. In this study, we present the development of an engineered transposon for facile construction of random circular permutation libraries. We provide evidence that minimal modification at the new termini of the random circular permutants is possible with our engineered transposon. In addition, our method enables the use of sticky-end ligation during library construction and provides external tunability for expression of random circular permutants.

Circular permutation is an important protein engineering method that produces protein variants with linked backbone via the original N and C termini while cleaving elsewhere to introduce new termini [1,2]. For example, circular permutation improved proteolytic resistance and heterologous expression of a chaperonin thermosome from *Methanocaldococcus jannaschii* [3]. Circularly permuted *Candida antarctica* lipase B and TEM-1 β-lactamase (BLA)[2] displayed higher enzyme activity and altered substrate specificity [4,5]. In addition, circular permutation of a guest protein, BLA, which was inserted into a host protein, the *Escherichia coli* maltose binding protein, increased allosteric interactions between these two domains by altering their relative orientations [6]. The benefits of circular permutation are wide-ranging, where recently it brought about increased conformational stability of bacterial chemotaxis response regulator Y by facilitating the initial stages of folding toward the productive transition state ensemble [7]. Similarly, a circularly permuted avian interleukin-1β displayed greater stability against chemical and thermal denaturation compared with the wild-type protein [8]. Circular permutation has also been used to simplify production of native split *Nostoc punctiforme* DnaE intein for structural study [9]. Along with other protein engineering tools, such as random mutagenesis [10,11], DNA shuffling [12], and site-directed mutagenesis [13], circular permutation has proven to be effective for creating sequence diversity. When constructing circular permutants, a peptide linker is usually genetically incorporated between the original N and C termini [14]. Approximately 50% of single domain proteins have their N and C termini proximal [15], suggesting that circular permutation can be applied to a broad range of proteins. A backbone linkage between the two termini more distal can still be made with a relatively long linker, further increasing the applicability of circular permutation. Although one can produce circular permutants chemically [2], circular permutation based on DNA recombination is more straightforward.

Not all circular permutations lead to correctly folded variants [16]. In fact, the success of circular permutation heavily relies on the locations of new termini. Usually, loops distant from active sites or from other functionally critical regions of the protein are preferred as new termini [5,17]. Backbone flexibility of parental

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[2] *Abbreviations used:* BLA, β-lactamase; MuRCP, engineered Mu transposon for random circular permutation; Cm^R, chloramphenicol resistance; PCR, polymerase chain reaction; Cm, chloramphenicol; BCX, xylanase from *Bacillus circulans*; Amp, ampicillin; Amp^R, ampicillin resistance; ^{rcp}bcx, randomly circularly permuted *bcx* gene.

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proteins, which is well represented by B-factors, can also serve as an important guideline for the selection of new termini for circular permutants [3]. In this regard, rational circular permutation requires significant structural knowledge, which is not always available, and there is no robust strategy to ensure successful rational circular permutation. Instead, it is often necessary to thoroughly examine locations of potential new termini along the polypeptide backbone.

A more powerful approach to designing circularly permuted proteins is to use combinatorial methods to generate large libraries that survey a much larger number of potential termini [18]. Conventional construction of a random circular permutation library involves a few characteristic steps (see Fig. S1A in online Supplementary material). The 5′ and 3′ ends of a target gene are genetically attached with oligonucleotide sequences containing the same restriction enzyme site. Subsequently, digestion at the restriction enzyme site creates terminal sticky ends, which are used for circularization of the target gene. The terminal nucleotide modification also introduces sequences encoding a backbone peptide linker on DNA circularization. The circularized DNA is then subjected to treatment using a nonspecific endonuclease, such as a DNase, in order to introduce a single cut into the circular DNA construct. The random single cut produces linear permuted genes containing gaps and nicks that are later repaired by ligases and polymerases, creating blunt-ended DNA [19]. The resulting DNA construct is subsequently blunt-end ligated with a host plasmid. Unfortunately, this method of constructing random circular permutants has some limitations. For example, optimizing conditions to introduce single cuts using an endonuclease is technically difficult [20]. In addition, random DNA cuts using a nonspecific endonuclease frequently results in uncontrolled truncation and duplication of the target DNA sequence [20–22], which creates significant modifications at the new termini of the random circular permutants. Furthermore, blunt-end ligation is less efficient than sticky-end ligation, lowering library construction efficiency [23,24].

Recently, an alternative method of creating a random circular permutation library using a transposon was developed [25]. A transposon is a DNA element that can be randomly inserted into various host DNA sequences with high efficiency. Because of its low target site preference, the bacteriophage Mu transposon can readily be used for this purpose [26]. For the random transposition, the Mu transposon forms a transposome assembly with MuA transposase through recognition sequences [27,28], which are 22-bp symmetrical sequences located near both ends of the Mu transposon [27,29]. Then, the transposome assembly assists in self-cleavage of the Mu transposon at the cleavage site [30–32], resulting in a random 5-bp staggered cut in the host DNA sequence into which the Mu transposon is incorporated [26,27,31,33]. Although circular permutation using a current transposon-based method alleviates uncontrolled truncation and duplication, by virtue of the precise 5-bp duplication, it still imposes other significant limitations [25]. For example, construction of a random circular permutation library using this method leaves an additional 20 amino acids attached to the new termini (see Figs. S1B and C in Supplementary material) [25]. This amino acid attachment is unavoidable because the additional amino acids are encoded by necessary transposon elements, which need to be kept intact throughout library construction (Figs. S1B and C). Unfortunately, attachment of long amino acid sequences to the termini represents significant terminal modification, which frequently compromises important protein properties such as stability [34–37]. In this transposon-based method, an origin of replication, an antibiotic resistance gene, and a promoter are built in as part of the transposon (Fig. S1B) [25]. These elements remain intact during circular permutation and serve for expression of a random circular permutation library (Fig. S1C). Note that during library construction and screening, expression levels need to be carefully adjusted, which is difficult to carry out using this method due to inclusion of the built-in transposon elements. In other words, redesign of the transposon is necessary to adjust expression levels of a random circular permutation library—presenting significant obstacles.

In the current article, we describe the development of an engineered Mu transposon, MuRCP (an engineered Mu transposon for random circular permutation), for facile construction of a random circular permutation library using sticky-end ligation. We show that the engineered MuRCP transposon allowed construction of a random circular permutation library with minimal modification at the new termini of random circular permutants. Our engineered transposon does not require any permanent built-in elements for expression; instead, it is readily tunable externally by the selection of an appropriate cloning vector. These collective features should represent substantial improvements over the conventional methods and should significantly increase the applicability of random circular permutation.

Materials and methods

Materials

A wild-type Mu transposon containing the chloramphenicol resistance (Cm^R), MuA transposase, and bioassay dishes were purchased from Thermo Scientific (Rochester, NY, USA). High-fidelity platinum *Pfx* DNA polymerase used to carry out all polymerase chain reactions (PCRs) and *E. coli* DH5α cells were purchased from Life Technologies (Carlsbad, CA, USA). All DNA purification kits and columns were purchased from Zymo Research (Irvine, CA, USA). T4 DNA ligase and all other restriction enzymes were products of New England Biolabs (Ipswich, MA, USA).

Construction of an engineered transposon, MuRCP

The MuRCP transposon was created through PCR using the previously reported engineered Mu transposon as a template [38]. The template transposon was similar to the wild-type Mu transposon except for the presence of mutated sequences, which are the *Bcl*I site near the 5′ cleavage site, *Age*I + TCA at the 3′ cleavage site, and other PUC19-derived sequences outside the cleavage sites. Using PCR with forward primer (5′-TCAATTAGATCTTGCATATGCG CACGAAAAACGCGAAAGC-3′) and reverse primer (5′-ACGCATCT GTTGAACCGGT-3′), we attached AGATCTTG**CAT**ATG (where *Bgl*II and *Nde*I restriction sites are single- and double-underlined, respectively, and the complement of a start codon is shown in bold) at the 5′ end of the MuRCP transposon. Similarly, we carried out PCR with 5′-TCAATTAGATCTTGCATATGCGCACGAAAAACGC GAAAGC-3′ and 5′-GATAGTACTAGTTGAACCGGTGCACGAAAAAC GCGAAAGCGT-3′ as forward and reverse primers, respectively, to attach ACCGGTTCAACTAGT (where *Age*I and *Spe*I restriction sites are single- and double-underlined, respectively) at the 3′ end of the MuRCP transposon. The PCR products for the MuRCP transposon (~1.3 kbp) were separated by agarose gel electrophoresis and purified using the Zymo gel extraction kit. *Bgl*II and *Spe*I restriction sites were included in the MuRCP transposon for sticky-end ligation to a cloning vector, pDIMC8 [39], which was used for amplification of the MuRCP transposon.

Construction of pDIMC8–MuRCP plasmid

PCR was carried out on pDIMC8–MalE [39,40] to produce a linearized pDIMC8 with *Bgl*II and *Spe*I restriction sites attached at the 5′ and 3′ ends, respectively. The PCR product for the pDIMC8 vector with modified ends (~3.3 kbp) was isolated by agarose gel