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Engineering of small sized DNAs by error-prone multiply-primed rolling circle amplification for introduction of random point mutations

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ABSTRACT

Small sized DNAs *per se* or their encoding peptides play various roles in biological systems and for biocatalyst development thus, engineering of those small sized DNAs/peptides is of great interest. By self-ligation of small sized DNAs, circular small sized DNA templates were prepared for error-prone rolling circle amplification using multiply-primed random hexamers to create tandem repeats of small sized DNAs and simultaneous introduction of random point mutations into those tandem repeats of small sized DNAs. We applied this method to randomize the signal peptide of a glucoamylase in recombinant *Saccharomyces cerevisiae*. Random point mutations were efficiently introduced into small sized DNA encoding the signal peptide of glucoamylase and the resulting recombinant *S. cerevisiae* with beneficial point mutations in its signal peptide was able to secrete ca. 30% more glucoamylase than that with native signal peptide.

1. Introduction

Small sized DNAs *per se* or their encoding peptides such as bacterial promoters [1], ribosomal binding sites [2], signal peptides, cofactor and substrates binding pockets of enzymes [3], and peptide hormones [4] play important roles in various biological systems. Engineering of those small sized DNAs or their encoding peptides for improved performance is of high interest. Some example are, the fine-tuning of expression level of target proteins/enzymes by modifying the strength of bacterial promoters, the engineering of signal peptides of proteins for high level of secretion, and change of the binding pockets of enzymes for a shift of substrate and cofactor specificity, etc.

Although various methods on biomolecular engineering have been developed [5–8], it is still challenging to engineer small sized DNA or its encoding peptide. Saturation mutagenesis is normally employed to randomize specific positions of DNAs or proteins for improved properties. By randomization using NNN for non-coding DNA or NNS for coding DNA at each position (where N denotes A, T, C, or G; S stands for C or G), a huge library will be created even for a small sized DNA template, which is challenging to screen [5,6]. At present, error-prone PCR (ep-PCR) is a commonly practiced method for randomization of target DNA sequences by taking advantage of the low fidelity of *Taq* DNA polymerase and the ability to efficiently introduce point mutations to DNA templates without any background information needed [7–9]. However, it requires the usage of a thermal cycler and optimizing the thermal cycling.

Recently, rolling circle amplification has emerged as an alternative method for biomolecular engineering due to its advantages over the conventional ep-PCR based evolution methods [10–12]. Rolling circle amplification (RCA) has been previously employed to amplify the whole genome of organisms and circular DNA templates [13,14] and recently explored to directly evolve enzymes (the encoding genes cloned in a plasmid) [10,11]. By taking advantage of the strand displacement DNA synthesis of Phi-29 DNA polymerase, RCA of circular DNA template with multiply-primed random hexamers will generate multiple replication forks. As RCA proceeds and product strands are rolled off the template as tandem copies of the circle. The advantages of Phi-29 DNA polymerase over normal DNA polymerase are its high capacity to perform strand displacement DNA synthesis for more than 70,000 nt without dissociating from the template and its stability for efficient DNA synthesis for many hours [13,15]. Besides that, no specific primers are required in RCA because random hexamers are universal primers for any DNA template. Most importantly, RCA is an isothermal amplification process and can perform at a constant temperature (30 °C), thus no optimization of thermal cycling conditions and thermal cycler are required [10,13].

In light of this, RCA was attempted in this research as an alternative to normal ep-PCR based directed evolution methods to introduce random point mutations to the small sized DNAs. To create tandem repeats of the small sized DNAs, the small sized DNAs

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themselves were initially circularized by self-ligation with T4 DNA ligase, followed by isothermal amplification using random primers and Phi-29 DNA polymerase. By including $MnCl_2$ during RCA, random point mutations were introduced into the tandem repeats of the small sized DNA units. To demonstrate that this strategy works, we applied RCA to the evolution of the signal peptide of glucoamylase from *Saccharomyces fibuligera* [16]. Signal peptides are known to control the entry of virtually all secretory proteins to the secretory pathways in both prokaryotes and eukaryotes [17–20] in which the NH_2-terminus of pre-protein is cleaved off while the mature protein is translocated through the membranes [18].

The pre-protein of mature glucoamylase from *S. fibuligera* which catalyzes a one-step depolymerization of starch to glucose is formed inside cells and contains a signal peptide of 27 amino acid residues encoded by 81 bp DNA. Our hypothesis is that the mutated signal peptide will significantly affect the interaction between the cellular machineries which in turn leads to the different secretion levels of the mature enzyme to the culture media. The glucoamylase gene containing the randomized signal peptide was cloned into *Saccharomyces cerevisiae*. The resulting recombinant *S. cerevisiae* containing glucoamylase gene with beneficial point mutations in its signal peptide secreted more mature glucoamylase into the culture. By standard starch-iodine colorimetric assay using 96-well plates, the beneficial point mutations in the signal peptide of glucoamylase were identified from recombinant *S. cerevisiae*.

2. Materials and methods

2.1. Microorganisms, plasmids and culture medium

Escherichia coli TOP10 (F-*mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G, Invitrogen) was used for plasmid maintenance, propagation and storage. *S. cerevisiae* INVSc1 strain (MATa *his3Δ1 leu2 ade2-1 trp1-289 ura3-52*) from Invitrogen (Carselberg, CA, USA) was employed as host for yeast transformation. Yeast plasmid pRS424 (New England Biolabs, Ipswich, MA) was used as backbone for cloning glucoamylase gene. *E. coli* cells were cultivated in LB broth and appropriate antibiotic was supplied where necessary. Yeast complex medium, YPAD, containing 1% yeast extract, 2% peptone, 0.01% adenine hemisulfate, and 2% dextrose was used to culture yeast cells. After transformation, yeast cells were plated onto proper synthetic complex dropout media or agar plates containing 0.7% Difco nitrogen base, 2% glucose and supplemented with appropriate 0.1% synthetic complete dropout mix without Trp from Sigma (St. Louis, MO).

2.2. Reagents

T4 polynucleotide kinase, T4 DNA ligase and Phi-29 DNA polymerase were from New England Biolabs (Ipswich, MA). The oligos were synthesized by IDT DNA (Coralville, IA) and the random hexamers, 5′-NpNpNpNp^{s}Np^{s}Np-3′, were synthesized by Sigma and thiophosphate-modified to prevent degradation during RCA due to the 3′-5′exonuclease activity of Phi-29 DNA polymerase.

2.3. Design of the synthetic oligos

The long oligo, 5′-aca taa aca aac aaa atg aaa ttc ggt gtt tta ttt tcc gtc ttt gct gct att gtt agt gct tta cct ttg caa gaa ggt cct ttg aac aaa aga gcc tat cct tct ttt g-3′ (underlined sequences are derived from pRS424-GLA1 for later subcloning by PCR into this vector by homologous recombination to create pRS424-sp-GLA1), were synthesized. Besides the flanking sequences underlined, the remaining sequence contains 81 nt encoding 27 amino acid residues of the signal peptide of glucoamylase.

2.4. Phosphorylation of 5′ end of oligos

Oligos were phosphorylated at 5′ ends with T4 polynucleotide kinase to facilitate subsequent self-ligation by following the protocol developed by Fire and Xu [21]. In brief, the phosphorylation reaction contained 10 μM oligos and 2 U polynucleotide kinase in 1× kinase buffer. The mixture was incubated at 37 °C for 2 h. The enzyme was inactivated at 70 °C for 15 min during the post incubation. The reaction was extracted by phenol–chloroform and the resulting oligos were ethanol-precipitated, air-dried and resuspended in 30 μl H_2O.

2.5. Circularization of the phosphorylated oligos

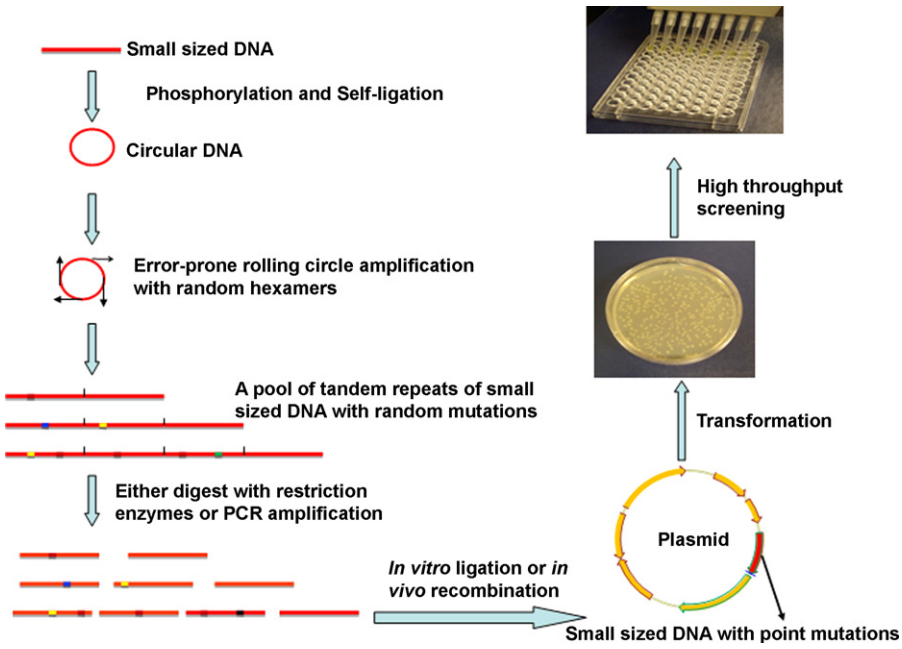
The phosphorylated oligos was circularized by self-ligation using T4 DNA ligase to create the circular templates for RCA. To facilitate self circularization of the phosphorylated oligos, a helper oligo (5′-ttt cat ttt gtt tag aag gat agg c-3′) and dilute annealing method described elsewhere [21] were used in this reaction. The reaction was incubated at 95 °C for 2 min to denature the secondary structure of the long oligos. The denatured oligos were slowly cooled down to 4 °C to facilitate the helper oligos to anneal to the denatured long oligos, followed by adding 2 U T4 DNA ligase and incubating at 16 °C overnight.

2.6. Ep-RCA with Phi-29 DNA polymerase

The ligation reaction was extracted with phenol-chloroform to remove enzymes followed by ethanol precipitation. The resulting circularized oligos were diluted and used as templates for rolling circle amplification using multiply-primed random hexamers. The 10 μl reaction was set up which containing 1 ng of circularized DNA templates, 2 μl of 10 μM random hexamers and 1 mM each dNTPs in 1× reaction buffer (New England Biolabs). The mixture was incubated at 95 °C for 3 min, then slowly cooled down to 4 °C. The reaction was initiated by adding 0.2 μl of 5 U/μl Phi-29 DNA polymerase and 1 μl of 15 mM $MnCl_2$ and incubated at 30 °C for 24 h. Post amplification, 2 μl of reaction was loaded onto 0.8% agarose gel and visualized under UV.

2.7. Subcloning of individual copies of small sized DNA units into yeast overexpression vector

A yeast overexpression vector, pRS424-GLA1, was firstly constructed by subcloning yeast TDH3 promoter and CYC1 terminator together with the GLA1 gene [16] which encodes mature glucoamylase without signal peptide sequence into pRS424 by known homologous recombination in yeast. To prepare cassette TDH3p-GLA1-CYC1t, the endogenous TDH3 promoter and CYC1 terminator were amplified from the genomic DNA of *S. cerevisiae* using oligos TDH3p-for (5′-acg act cac tat agg gcg aat tgg gta ccg gat act agc gtt gaa tgt tag-3′) & rev (5′-tga ata agc ttc aaa aga agg ata ggc gga tcc ttt gtt tgt tta tgt g-3′, *BamH* I underlined) and CYC1t-for (5′-ta agg tca agg ctt tgg ctt aaa cag gcc cct ttt cct ttg tcg-3′) & rev (5′-caa aag ctg gag ctc cac cgc ggt ggc ggc gca aat taa agc ctt cga-3′), respectively, then overlapped with GLA1 (without leading DNA sequence encoding the signal peptide) which was amplified using oligos GLA1-for (5′-aaa cac aca taa aca aac aaa gga tcc gcc tat cct tct ttt ga-3′, *BamH* I site underlined) and rev (5′-gac aaa gga aaa ggg gcc tgt tta agc caa agc ctt gac c-3′) by overlap extension PCR (OE-PCR). For subcloning the gene cassette into pRS424, we employed a method for rapid assembly of DNA fragments into yeast developed previously [22].



Scheme 1. Scheme for engineering of small sized DNAs by ep-RCA. Small sized DNAs are self-ligated to create circular template for error-prone rolling circle amplification using Phi-29 DNA polymerase and random hexamers. Random point mutations will be simultaneously introduced into those tandem repeats of small sized DNA units during RCA. Following either digestion with restriction enzymes or PCR amplification, the resulting individual small sized DNA units with random point mutations will be subcloned into an expression vector either by *in vitro* ligation or homologous recombination *in vivo*. The recombinant host with desired phenotypes will then be selected or screened by high throughput methods.

The cassette TDH3p-GLA1-CYC1t was co-transformed into *S. cerevisiae* INVSc1 with linear pRS424 (digested with *BamH* I and *Xho* I) by heat shock using the standard lithium acetate method [23]. After transformation, yeast transformants were plated on SC-Trp agar plates for selection of yeast cells with the right construct. Several single colonies were randomly picked up from selective agar plate and inoculated into SC-Trp liquid medium for yeast plasmid isolation using standard protocol with lyticase (Zymo Research, Orange, CA). The isolated yeast plasmids were resuspended into 50 μl H_2O, precipitated by butanol, air-dried and reconstructed in 5 μl Q-H_2O for re-transformation of *E. coli* TOP10 cells for plasmid propagation and maintenance. Yeast plasmids in *E. coli* were isolated using Qiagen's Miniprep Kit (Valencia, CA, USA) and confirmed by restriction digestion and DNA sequencing.

To further subclone the individual copies of the mutated small sized DNAs encoding signal peptides back to the 5′end of mature glucoamylase gene, the library was PCR-amplified with forward (5′-agt ttc gaa taa aca cac ata aac aaa caa aat ga-3′) and reverse (5′-ata gtt tga ata agc ttc aaa aga agg ata ggc-3′) primers for individual copies of small sized DNA units encoding the signal peptides. The PCR products with desired size were subcloned into pRS424-GLA1 and a library of *S. cerevisiae* containing various small sized DNAs in pRS424-sp-GLA1 (Fig. 2) by *in vivo* homologous recombination in *S. cerevisiae* was created for high throughput screening.

2.8. Library screening

After transformation, cells were plated on SC-Trp plates with starch as the sole carbon source. Colonies emerged after 6–8 days' incubation at 30 °C were randomly picked up from selective plates and inoculated into 96-well plates for high throughput screening for mutants with beneficial point mutations in their signal peptides.

Briefly, to normalize cell density, the transformants picked up from the selection plates were first inoculated into 400 μl of SC-Trp culture media with glucose as the sole carbon source in 96-well plates and grown for 3 days at 30 °C. The normalized cell cultures were resuspended and inoculated into the fresh SC-Trp/glucose in a new 96-well plate for overnight cell culture. At this stage, the cells with various signal peptides of glucoamylase secreted mature glucoamylase into culture media at different levels. Further analysis of glucoamylase concentration in the media was performed as follows. The cell cultures in 96-well plates were centrifuged at room temperature for 10 min using Eppendorf BenchTop Centrifuge. The supernatant containing glucoamylase was transferred into new 96-well plates with 400 μl of 50 mM starch in Tris buffer in each well, and incubated at 37 °C for 5 h without shaking. The 96-well plates were sealed with a sticker to prevent water evaporation. The starch in the reaction buffer was enzymatically depolymerized into glucose and the remaining starch in each well was quantified by colorimetric assay using the known starch-iodine method [24].

After 5 h incubation at 37 °C, the reaction was terminated by adding 30 μl of 1 M HCl to each well. The mixture, approximately 30 μl, was transferred into a new 96-well plate containing 240 μl of Q-H_2O in each well and 30 μl of 50 mM iodine solution was added to allow the color to develop. The OD value of each well was monitored at 560 nm using a microplate reader. Compared with the control – *S. cerevisiae* with gene encoding glucoamylase with native signal peptide on pRS424-GLA1 vector, wells with the lowest $OD_{560\,nm}$ values indicated that more starch was depolymerized to glucose. This suggested that more glucoamylase was secreted into the culture media and the gene encoding signal peptide in the parent strain was possibly mutated. The corresponding colonies were then picked up and spotted on a SC-Trp plate for short-term storage.

Once enough colonies with the lowest $OD_{560\,nm}$ values were identified after the first round of screening, the screened colonies were subjected to another two-round of screening using the same protocol as before to further screen and confirm the performance of the selected colonies. Compared with the control, the corresponding colonies which consistently had the lowest $OD_{560\,nm}$ value after the third round of screening were picked up for DNA sequencing to confirm mutations in the signal peptide.

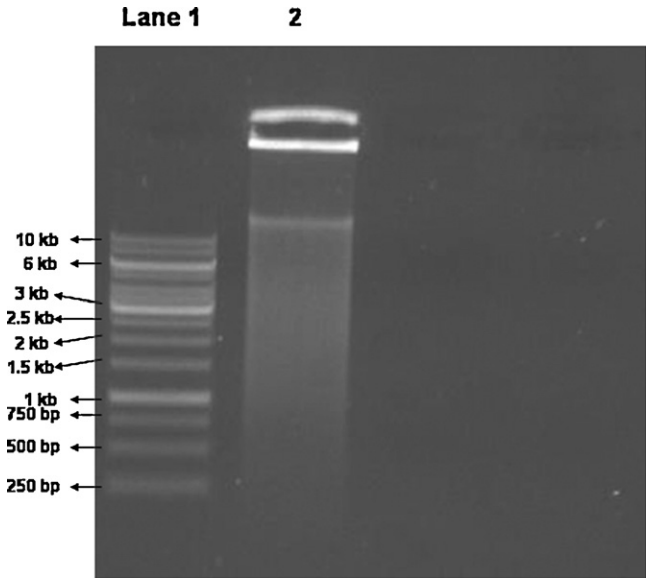


Fig. 1. A typical RCA products on 0.8% agarose gel. Lane 1, GeneRuler™ 1 kb DNA ladder from Fermentas (Cat# SM0311); lane 2, RCA products.

2.9. DNA sequencing

To isolate sufficient plasmid DNAs for DNA sequencing, 1-ml of recombinant *S. cerevisiae* overnight culture in selective medium was collected by centrifugation. Following the removal of cell wall by lyticase, the plasmids were isolated using Qiagen's Miniprep kit, and retransformed into *E. coli* TOP10 cells for propagation. The plasmids isolated from the retransformed *E. coli* were subjected to DNA sequencing for determination of the beneficial point mutations in the signal peptide.

3. Results

To apply RCA to the engineering of small sized non-coding or coding DNAs, circular templates of small sized DNAs must be created prior to RCA amplification using multiply-primed random hexamers. As illustrated in Scheme 1, besides flanking sequences, a long oligo which was identical to the sense strand of small sized DNA encoding signal peptide of glucoamylase was synthesized and phosphorylated at 5′-ends to facilitate self-ligation with T4 DNA ligase. The resulting circularized oligo templates were subjected to ep-RCA using Phi-29 DNA polymerase and random hexamers which led to a pool of tandemly repeated double-stranded small sized DNAs at various lengths. As indicated in Fig. 1, the RCA products were typically observed as a smear after electrophoresis on an agarose gel. In addition, the significant high molecular weight DNA molecules produced by RCA were also detected as a distinct band in the well of the agarose gel.

Normally, the tandem repeats of small sized DNAs were either digested by restriction enzymes or amplified by PCR to obtain individual copies of small sized DNA units. To facilitate subcloning of the individual copies of randomized small sized DNA back into pRS424-GLA1 by homologous recombination, two oligos which had ca. 30 bp homologous nt sequences at 5′ ends with the linearized pRS424-GLA1 backbones were employed to re-amplify RCA products to obtain individual copies of randomized small sized DNAs. As expected, a couple of bands of various sizes which correspond to certain repeats of signal peptide encoding DNA unit were observed (Fig. 2) due to the tandem repeats of small sized DNA templates created by RCA. The bands with expected size corresponding to a single copy of small sized DNA were excised from the agarose gel, purified, and used to transform *S. cerevisiae* together with the linearized pRS424-GLA1. The transformed *S. cerevisiae* cells were plated on SC-Trp plates supplemented with starch as the sole carbon and energy source for selection of yeast cells that were able to secrete glucoamylase and survive on the starch plate. Since the constitutive expression of glucoamylase gene was driven by TDH3 promoter, a strong yeast promoter, only recombinant *S. cerevisiae* which secreted glucoamylase was able to grow on the selection plates. Colonies emerged after 6–8 days' incubation at 30 °C during post transformation. A clear halo was observed around each colony which suggested the secretion of glucoamylase by recombinant *S. cerevisiae*.

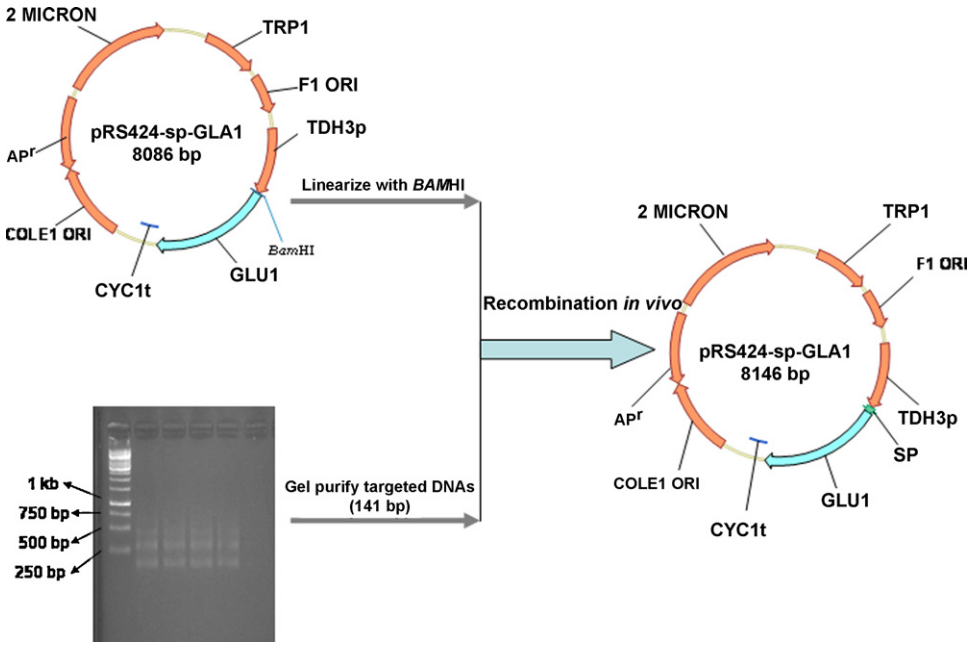


Fig. 2. Subcloning of the small sized DNAs into yeast expression vector. The amplified small sized DNAs (the lowest bands at ~141 bp, 81 bp DNA encoding the signal peptide plus flanking sequences) were further cloned at the 5′ end of gene encoding mature glucoamylase in pRS424-GLA1 vector for overexpression in *S. cerevisiae*.

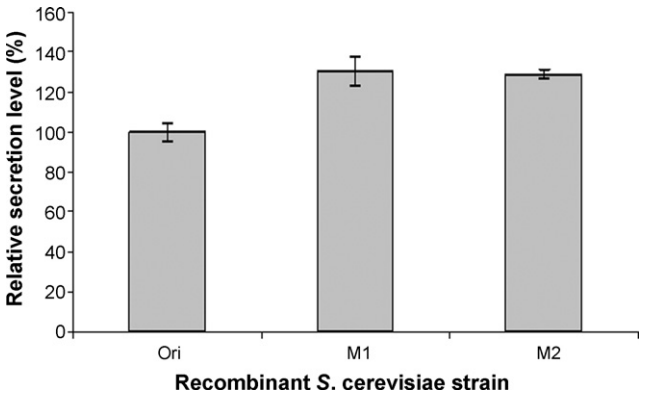


Fig. 3. A comparison of the secretion level of glucoamylase. M1 and M2 denote the screened single mutants and Ori represents the control with native signal peptide.

In this research, only signal peptide was randomized by ep-RCA while the structural gene encoding mature glucoamylase (without signal peptide sequence) was not mutated. In addition, prior to secretion of mature glucoamylase by recombinant *S. cerevisiae*, the signal peptide was cleaved off from the NH_2-terminal of pre-protein of glucomaylase before it was translocated through the membrane via certain protein secretory pathways. Therefore, the difference in glucoamylase activity in each 96-well plate only suggested the different secretion levels of glucoamylase in recombinant yeast which were caused by the perturbation of its signal peptide. Those colonies were randomly picked up from the selective plates and inoculated into 96-well plates for high throughput screening for mutants with beneficial point mutations in the signal peptide. To estimate the mutation rate, we randomly picked up 40 colonies from the library. Plasmid DNAs were isolated, and the small sized DNAs encoding signal peptides of glucoamylase were sequenced. In total, 3240 bp were sequenced and 12 point mutations were identified from 10 clones. The mutation frequency was, therefore, estimated to be 3.7 mutations/kb.

We screened ca. 2000 colonies in the first round and identified 70–80 colonies which indicated significant lower $OD_{560\,nm}$ values than the control recombinant yeast with un-mutated signal peptide. Another two rounds of screening were performed on these selected colonies from the first screening, and these finally led to the identification of 9 colonies which consistently indicated the lowest $OD_{560\,nm}$ value. Furthermore, we observed ca. 7.6% clones actually indicated lower glucoamylase activities compared with the control recombinant yeast with native signal peptide sequence. This suggested that unfavored mutations might have been introduced into their signal peptides. Even so, by applying error-prone multiply-primed RCA to randomize the signal peptide of glucoamylase, the secretion level of glucoamylase from one of the best mutants was increased by ca. 30% as indicated in Fig. 3.

Confirmation of mutations in the signal peptides were performed by DNA sequencing. As expected, random point mutations were identified in the signal peptide of glucoamylase in the screened recombinant yeast strains as shown in Fig. 4. Among the nine yeast colonies screened with best performance, five of them have the identical point mutation (A11T) in their signal peptides and the remaining four colonies share another point mutation (G22D). To further confirm their effects on the secretion level of glucoamylase, we retransformed *S. cerevisiae* using those plasmids with single point mutation. The resulting *S. cerevisiae* freshly transformed exhibited similar secretion level of glucoamylase with those previously screened from the library. To test whether the double mutant (A11T and G22D) had better performance over the single mutants in signal peptides, we created the double mutant by overlap extension PCR. However, no combined beneficial effect was observed. The double mutant had similar secretion level with the single mutants.

4. Discussion

By using rolling circle amplification, we introduced random point mutations to small sized DNAs for direct evolution of small sized DNAs *per se* or their encoding peptides. Although rolling circle amplification has previously been utilized for introduction of random mutations into genes in a plasmid, this work presents the first attempt for direct engineering of small sized DNA or its encoding peptide by ep-RCA.

Engineering of any DNA molecules including small sized DNAs can be theoretically performed by either rational design or random mutagenesis. Saturation mutagenesis, one of the rational design methods, is usually employed to explore the best mutations at specific positions of DNAs or proteins. It is therefore site-specific [6]. Without background information of DNAs or their encoding proteins, randomization of nucleotides at each position will

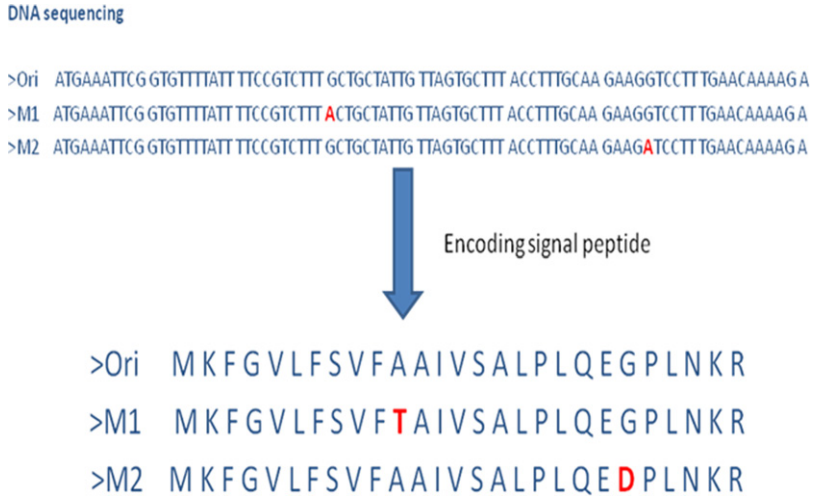


Fig. 4. DNA sequencing analysis of the small sized DNAs. Ori, M1, and M2 denote recombinant *S. cerevisiae* with original (un-mutated) and mutated signal peptides of glucoamylase, respectively. The point mutations identified were highlighted in red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

create a huge library. Taking an 81 bp non-coding small sized double stranded DNA as an example, saturation mutagenesis of nucleotides at each position will create a library size of 4^{81}, which is challenging to select or screen. Although normal ep-PCR based directed evolution methods are able to apply to small sized DNAs, RCA with multiply-primed random oligos presents an alternative to the conventional directed evolution methods for engineering of small sized biomolecules. In terms of simplicity, unlike the conventional directed evolution methods, ep-RCA performs isothermally at 30 °C using random hexamers. Therefore, thermal cycler, gene-specific oligos and optimization of thermal cycling conditions are not required. The step for *phosphorylation* of oligos in the procedure can be further simplified by directly purchasing the 5′ end phosphorylated oligos.

Using the $MnCl_2$ to decrease the fidelity of Phi-29 DNA polymerase, the mutation frequency of ep-RCA in this work was 3.7 mutations/kb, which is comparable to the conventional directed evolution methods and might possibly be promoted by the biased dNTPs. Furthermore, a mutant of Phi-29 DNA polymerase was reported to have a 16-fold increase of the mis-incorporation mutation frequency [25]. Thus, the mutation frequency of ep-RCA is potentially further promoted by using this mutant of Phi-29 DNA polymerase. Since it is not commercially available, we did not test in this work.

It is also known that protein secretion in both prokaryotic and eukaryotic cells is tightly regulated by protein secretion pathways [17,19,20]. Although the signal peptide plays an important role to control the entry of pre-protein to the secretory pathways [18,19], compared with other factors, such as correct folding foreign proteins, proper processing and glycosylation, in the complex protein secretion pathways, the perturbation of the signal peptide has a relatively minor effect on the secretion. In addition, the secretion is tightly regulated in yeast, and *S. cerevisiae* is known to secrete only 0.5% of its proteins [26]. This might explain the insignificant increase in secretion of glucoamylase in this research despite the introduction of beneficial point mutations into the signal peptides. However, it is clearly demonstrated in this work that ep-RCA using multiply-primed random primers is an alternative to the conventional methods for engineering of small sized DNAs *per se* or their encoding peptides. Besides engineering of the signal peptides, RCA might be further explored to engineer other biomolecules, such as peptide hormone drugs, interferon RNAs and micro RNAs (miRNAs) for high value-added products.

5. Conclusion

Ep-RCA using multiply-primed random hexamers is an alternative to the conventional directed evolution methods for introducing random point mutations to the small sized DNAs or their encoding peptides. Using ep-RCA, randomization of the signal peptide of glucoamylase led to ca. 30% more glucoamylase secretion in a recombinant *S. cerevisiae* than that with un-mutated signal peptide.

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