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Functional RNA production using the marine phototrophic bacterium *Rhodovulum sulfidophilum*

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Research aims

Functional RNAs, *e.g.*, aptamers, antisense oligonucleotides, and double-stranded RNA (dsRNA), hold promise for use as RNA drugs in the near future. However, the linear form of RNA without chemical modifications is rapidly degraded by endogenous nucleases. To address the problems outlined above, our research group has considered the possibility that functional RNAs can be expressed *in vivo* as circular RNAs for inexpensive RNA drug production because circular RNA molecules are resistant to exoribonucleases without any chemical modifications under cellular conditions[1,2].

Circular RNA can be produced by two methods. The first method makes use of ligase to ligate both ends of the linear form of RNA transcripts, while the second method uses a spontaneous group I intron self-splicing system, designated as the permuted intron-exon (PIE) method. The latter technique is the only methodology available for *in vivo* circular RNA production because it has no requirement for proteinaceous components, such as ligases. Therefore, the PIE method is a promising economical method for producing circular RNA drugs.

To increase the circular RNA expression in marine phototrophic bacteria *Rhodovulum sulfidofilum*, we developed the TOP (tandem one-way transcription of PIE) technique, which is a simple methodology for increasing the copy number of the PIE sequence in a single plasmid.

In this study, we constructed plasmids for circular RNA expression by the TOP method, evaluated the expression of the circular RNA from these TOP plasmids in the model bacterium *Escherichia coli*, and finally attempted to produce extracellular circular RNA using the marine phototrophic bacterium *R. sulfidophilum* as the host.

Methods

First, we amplified the transcriptional unit, which consists of the *lpp* promoter, the PIE sequence and the *rrn*C terminator in pM3-3E5, with both the 5′ flanking sequence containing *Kpn*I–*Xho*I sites and the 3′ flanking sequence containing a *Sal*I site. Next, we digested the amplified sequence with *Kpn*I and *Sal*I, and the resulting fragment was inserted into the M3 plasmid double-digested with *Kpn*I and *Xho*I. The digested *Xho*I site on the M3 plasmid and the *Sal*I site on the amplified fragment can hybridize with

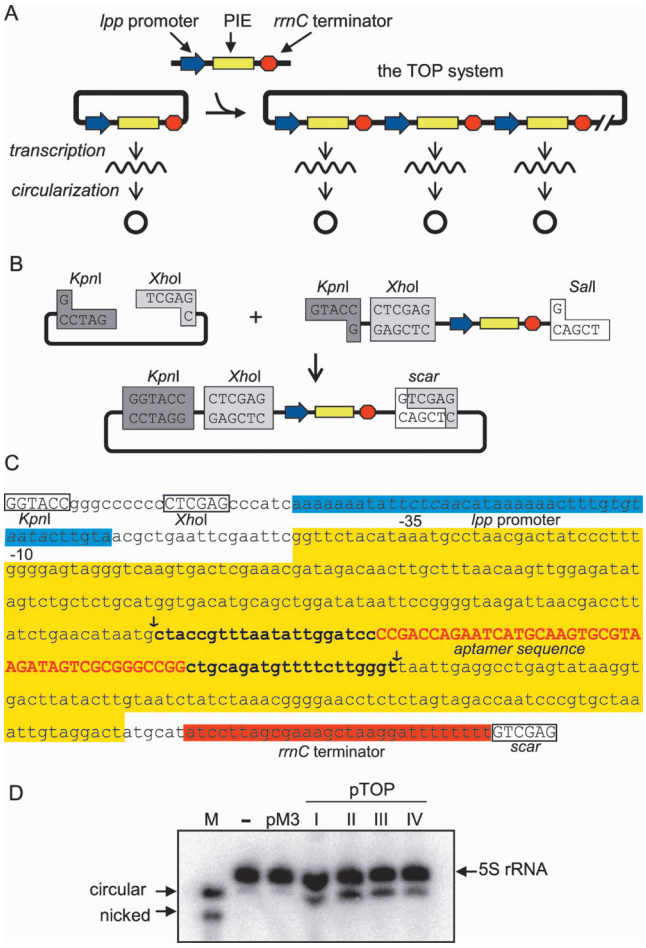

Fig. 1. Construction of the pTOP vectors, and the application of the TOP method for generating circular RNA in JM101Tr. (A) Outline of the TOP method. (B) Illustration of sequential insertion of the PIE sequence into the same plasmid. (C) Nucleotide sequence of one unit of the TOP system. Arrows represent splicing positions of this PIE sequence: yellow, the PIE sequence; blue box, *lpp* promoter sequence; italicized sequence in the blue box, −35 and −10 regions of the *lpp* promoter; red upper case letters, aptamer sequence and *rrn*C terminator sequence; lower case letters in the yellow region, intron sequence of the *td* gene; bold lower case letters, exon sequence of the *td* gene; bold, circularized sequence; boxed sequence, ligated sites. (D) Northern blotting analysis of the circular RNA expression by each member of the pTOP plasmid series. Arrows indicate the migration positions of the circular RNA (circular), nicked RNA (nicked) and 5S rRNA. Lane "M", the circular RNA control marker prepared by *in vitro* transcription (Umekage & Kikuchi, 2009a); lane "−", total RNA from JM101Tr; lane "pM3", negative control of the TOP system lacking the PIE sequence. Roman numerals I, II, III and IV represent the total RNA from JM101Tr harbouring pTOP(I), pTOP(II), pTOP(III) and pTOP(IV), respectively.

mutual 3′ protruding ends of the palindoromic TCGA sequence, and the resulting ligated fragment forms the sequence GTCGAG, which can be digested with neither *Xho*I nor *Sal*I (Fig. 1B). Therefore, the inserted sequence is as follows: 5′-*Kpn*I-*Xho*I-*lpp* promoter-PIE sequence-*rrn*C terminator sequence-GTCGAG site-3′ (Fig. 1C). Thus, the subsequent transcriptional unit can be inserted at the *Kpn*I–*Xho*I site. We constructed a series of four pTOP vectors from M3 [i.e., pTOP(I), pTOP(II), pTOP(III) and pTOP(IV), which were designated according to the number of inserted transcriptional units they contains]. These plasmids were transformed into the *E. coli* JM101Tr and the transformants obtained were cultivated for 18 hour at 30°C in the 2×YT broth. After the cultivation, total RNA was purified by the AGPC method and the circular RNAs in the total RNA fraction were detected by Northern analysis (Fig. 1D).

Next, the TOP plasmid was introduced into the *Rhodovulum sulfidophilum* DMS 1374^{T} with a heat shock procedure. The transformants were cultivated under light-anaerobic conditions. Total RNAs were recovered with the AGPC method and the expression of circular RNA was monitored by Northern analysis.

Results

We found that the circular RNA was expressed from all pTOP plasmids (pTOP(I), pTOP(II), pTOP(III) and pTOP(IV)) (Fig. 1D). As shown in the Fig. 1D, the expression level of circular RNA from the plasmid with two tandem insertions of the PIE [pTOP(II)] is the highest among the four plasmids constructed. These results indicated that the TOP system is a simple and potentially useful methodology for increasing the expression of circular RNAs in *E. coli*. The level of circular RNA expression with pTOP(II) was estimated to be about 9.7±1.0 ng per 1 μg of total RNA after 18 h of cultivation and this yield was approximately 2.7-fold higher than that obtained with the expression procedure using the pM3-3E5 system[2]. In addition, the amount of circular RNA produced in 1 L of culture medium was estimated to be approximately 0.19 mg, which is the highest yield of circular RNA in *E. coli* reported to date. In contrast, the expression level of the circular RNA was dramatically low with the *E. coli* host carrying pTOP(IV); the reason for this low expression is not yet clear. To address this problem, we recovered plasmid DNA from the *E. coli* strain JM101Tr carrying pTOP(IV) after 18 h of cultivation. The plasmid DNA was then digested with *Hin*dIII, which has a single cleavage site in the plasmid, and subjected to 1% agarose gel electrophoresis. Several fragments of pTOP(IV) with different size were observed (data not shown). These unexpected results suggested a marked instability of the pTOP(IV) in the *E. coli* host cells during cultivation.

Next we introduced the pTOP(II) plasmid into *R. sulfidophilum* with the heat pulse method and circular RNA expression was monitored by Northern analysis; however the expression of the circular RNA was not detected (data not shown). We currently investigate the possibility of using other promoters, such as the *rrnA* promoter, for the effective circular RNA expression by *R. sulfidophilum*.

Conclusion

The TOP system was effective when it was used with *E. coli*. However the system did not work well with *R. sulfidophilum*. Further optimization of the TOP system will be required to achieve effective expression of circular RNAs by *R. sulfidophilum*.

References

1) Umekage, S. & Kikuchi, Y., (2009). *In vitro* and *in vivo* production and purification of circular RNA aptamer. *J. Biotechnol*. **139**: 265–272.
2) Umekage, S. & Kikuchi, Y., (2009). *In vivo* circular RNA production using a constitutive promoter for high-level expression. *J. Biosci. Bioeng*. **108**: 354–356.