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In Vivo Circular RNA Expression by the Permuted Intron-Exon Method

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1. Introduction

Functional RNAs, *e.g.*, aptamers (Lee *et al*., 2005; Que-Gewirth & Sullenger, 2007), ribozymes (Malhbacher *et al*., 2010), antisense oligonucleotides (Hnik *et al*., 2009), and double-stranded RNA (dsRNA) (Watts & Corey, 2010), hold promise for use as RNA drugs in the near future. However, the linear form of RNA without chemical modifications is rapidly degraded in both human serum and cell extracts due to endogenous nucleases. Therefore, it will likely be necessary to chemically modify these RNA drugs (Pestourie *et al*., 2005; Watts *et al*., 2008) to protect them from nuclease-dependent degradation. In fact, the recently developed aptamer drug pegaptanib sodium (Macugen®; Pfizer) for use against macular degradation consists of 2'-F- or 2'-OCH_3-substituted nucleotides, thus preventing its rapid degradation in the ocular environment. Although at present it is the only commercially available RNA drug, we infer from the selling price of Macugen® that similar novel chemically modified RNA drugs are likely to be expensive because production of a chemically modified RNA molecule and scaling up the production yield of the RNA are expensive in principle. Therefore, the development of not only inexpensive but also durable RNA drugs will facilitate the widespread use of easily administered RNA drugs. To address the problems outlined above, our research group has considered *in vivo* circular RNA expression as a model for inexpensive RNA drug production because circular RNA molecules are resistant to exoribonucleases without any chemical modifications under cellular conditions. Therefore, the circular form of RNA would be a promising RNA drug candidate without requiring chemical modification.

Circular RNA can be produced both *in vitro* and *in vivo* using two methodologies. The first makes use of ligase to ligate both ends of the linear form of RNA transcripts (Chen & Sarnow, 1998; Beaudry & Perreault, 1995), while the second uses a spontaneous group I intron self-splicing system, designated as the permuted intron-exon (PIE) method (Puttaraju & Been, 1992). 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 methodology for producing circular RNA drugs. In this chapter, we describe our circular streptavidin RNA aptamer expression by the PIE method as a model for RNA drug production (Umekage & Kikuchi, 2006, 2007, 2009a, 2009b). Then, we discuss our recent improvements in the circular RNA expression technique, *i.e.*, the **t**andem **o**ne-way transcription of **P**IE (TOP) method, to achieve higher yields of *in vivo*

circular RNA expression. In this system, we achieved production of approximately 0.19 mg of circular RNA from a 1-L culture of the *Escherichia coli* strain JM101Tr. To our knowledge, this is the highest circular RNA expression yield reported to date. Finally, we will discuss *in vivo* circular RNA expression by the marine phototrophic bacterium *Rhodovulum sulfidophilum*. This bacterium produces RNA both within the cell and in the culture medium in nature and produces no RNases in the culture medium (Suzuki *et al*., 2010), whereas strong RNase activity is observed in the culture medium of a conventional *E. coli* strain that can be used for RNA production. Therefore, we speculated that *Rdv. sulfidophilum* would be a suitable strain for RNA production in the culture medium bypassing the total RNA extraction procedure to break the cell membrane, such as the acid guanidinium thiocyanate phenol chloroform (AGPC) method (Chomczynski & Sacchi, 1987).

2. Group I intron self-splicing and the permuted intron-exon (PIE) method

Group I intron self-splicing RNA from the ciliate *Tetrahymena* was the first discovered ribozyme (Cech *et al*., 1981). The group I intron sequence has been widely detected in eukaryotes (Cech *et al*., 1981), prokaryotes (Xu *et al*., 1990) and some bacteriophages (Ehrenman *et al*., 1986). This self-splicing does not require any proteinaceous components but does require the presence of Mg^{2+} and guanosine nucleotides (Cech *et al*., 1981). After self-splicing, the concomitant ligation of the two exons takes place (Fig. 1). This self-splicing mechanism consists of a well-defined two-step transesterification mechanism, and the sequential self-splicing steps take place after formation of the higher-order intron architecture. In the first step, a guanosine nucleotide attacks the phosphate at the 5' splicing site and scission occurs between the upstream exon and the intron, and the guanosine nucleotide is then ligated to the 5' side of the intron. Next, the hydroxyl group of the 3' end of the upstream exon shows nucleophilic attack of the downstream splicing site of the phosphorus, and intron circularisation and exon ligation occur. Therefore, it is assumed that both the 5' end of the 5' half exon and 3' end of the 3' half exon are somehow ligated before self-splicing occurs, and the resulting spliced exon product has a circular conformation. Several biochemical (Galloway-Salbo *et al*., 1990) and structural investigations of group I intron self-splicing (Stahley & Atrobel, 2006) indicated that the peripheral region of the intron architecture and internal open reading frame (ORF) sequence does not participate in formation of the intron architecture and the self-splicing event mentioned above. Theses investigations allowed us to permute the order of the intron and exon sequence without distorting the tertiary structure of the permuted intron architecture. Puttaraju & Been (1992) first reported that circular permutation of the group I intron from both the *Anabena* pre-tRNA intron and the *Tetrahymena* intron generated a circular RNA exon *in vitro*. Another PIE from the T4 phage group I intron was later shown to be applicable for generating the circular exon (Ford & Ares, 1994). As the exon sequence does not participate in the self-splicing reaction, the exon sequence in the PIE sequence is replaced with another foreign sequence. Based on this concept, several circular RNAs have been developed by the PIE method, *i.e.*, the tat-activated response (TAR) RNA (Puttaraju & Been, 1995; Bohjanen *et al*., 1996; Bohjanen *et al*., 1997), rev responsive element (Puttaraju & Been, 1995), HDV ribozyme (Puttaraju *et al*., 1993; Puttaraju & Been, 1996), tRNA (Puttaraju & Been, 1992), *Bacillus subtilis* PRNA (Puttaraju & Been; 1996), mRNA encoding GFP (Perriman & Ares, 1998), yeast actin exon (Ford & Ares, 1994), hammerhead ribozyme (Ochi *et al*., 2009), and streptavidin RNA aptamer (Umekage & Kikuchi, 2006, 2007, 2009a, 2009b) (Table 1).

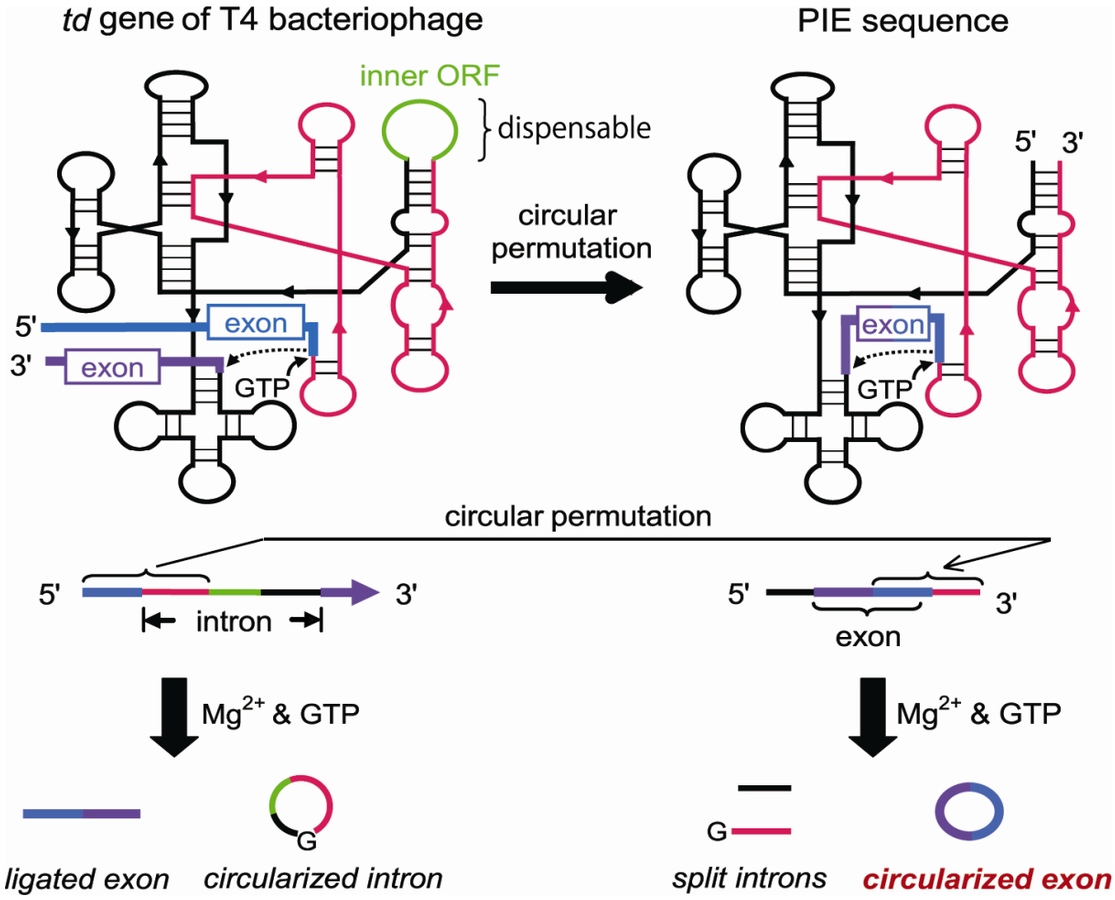

Fig. 1. Group I intron self-splicing RNA and the permuted intron-exon (PIE). Predicted secondary structure of the *td* group I intron sequence (upper left side) and the PIE sequence (upper right side). The pink and black lines show the intron sequence and the green line indicates the internal ORF sequence. Coloured horizontal lines shown in the middle of this figure illustrate the circular permutation of the *td* intron. In the normal *td* intron (left side), after transesterification, the exon sequence is ligated. In the permuted *td* intron (right side), the exon sequence is circularised and a split intron sequence appears. Circularisation of the exon sequence by the PIE method also requires magnesium ions and guanosine nucleotides.

These results confirmed the availability of the PIE method to yield a wide variety of circular RNAs. As circularisation is driven only by magnesium ions and guanosine nucleotides, the circularisation of RNA in *E. coli* (Puttaraju & Been, 1996; Perriman & Ares, 1998; Umekage & Kikuchi, 2007, 2009a, 2009b) and *Saccharomyces cerevisiae* (Ford & Ares, 1994; Puttaraju & Been, 1996) have been demonstrated, and the *in vivo* expressed circular RNAs reported above are functionary active. Our group also showed that the circular streptavidin RNA aptamer produced both *in vitro* and in *E. coli* (Umekage & Kikuchi, 2006, 20007, 2009a, 2009b), and the expressed circular streptavidin RNA aptamer was purified from the total RNA fraction by the solid-phase DNA probe method (Suzuki *et al.*, 2002). This is the first evidence that both *in vitro* and *in vivo* circularisation of an RNA aptamer and the *in vivo* circularised RNA generated by the PIE method can be purified (Umekage & Kikuchi, 2009a).

Category of circularised RNA	Intron	Expression	Reference
tRNA exon from *Anabaena* PCC7120	Anabaena & Tetrahymena	*in vitro*	Puttaraju & Been, 1992
HDV ribozyme	Anabaena	*in vitro*	Puttaraju *et al*., 1993; Puttaraju & Been, 1996
td exon from T4 phage	T4 phage	*in vitro* & *E. coli* DH5α & *S. cerevisiae* IH1097	Ford & Ares, 1994
actin ORF from yeast	T4 phage	*in vitro* & *E. coli* DH5α& *S. cerevisiae* IH1097	Ford & Ares, 1994
Rev-responsive element RNA	Anabaena	*in vitro*	Puttaraju & Been, 1995
tat-activated response RNA	Anabaena	*in vitro*	Puttaraju & Been, 1995 Bohjanen *et al*., 1996; Bohjanen *et al*., 1997
B. subtilis PRNA	Anabaena	*in vitro* & *E. coli* BL21(DE3)	Puttaraju & Been, 1996
GFP ORF	T4 phage	*in vitro* & *E. coli* BL21(DE3)	Perriman & Ares, 1998
streptavidin aptamer	T4 phage	*in vitro* & *E. coli* JM109(DE3) & *E. coli* JM101Tr	Umekage & Kikuchi, 2006, 2007, 2009a, 2009b
hammerhead ribozyme	T4 phage	*in vitro*	Ochi *et al*., 2009
streptavidin aptamer	T4 phage	*Rdv. sulfidophilum* DSM 1374^{T}	this study (see 2.4)

Table 1. Summary of circular RNA production. "Category of circularised RNA": Source of the circularised exon sequence. "Intron": Source of the intron sequence used for constructing the PIE sequence. "Expression": Circular RNA production *in vitro* or *in vivo* (the expression host strain is listed).

On the other hand, we found that this circularisation affected the original activity of the linear form of functional RNAs. The dissociation constant (K_d) of the circular streptavidin RNA aptamer increased (Umekage & Kikuchi, 2009a) and ribozyme activity of the hammerhead ribozyme decreased (Ochi *et al*., 2009). These observations suggest that structural constraints were induced by circularisation. Although it is also important to take into consideration the circular RNA structure before constructing the PIE sequence, it is difficult to predict the tertiary structure of the circularised RNA molecule.

Therefore, optimisation of the circularised sequence would be required involving randomising the spacer sequence, inserting the poly(A) sequence, *etc*. We succeeded in recovering the functional activity of the circular hammerhead ribozyme by adding a poly(A) spacer between the ribozyme sequence and the indispensable linkage sequence derived from the exon sequence for circularisation by the PIE method (Ochi *et al*., 2009), and the recovered ribozyme activity of the circular hammerhead ribozyme was dependent on the length of the poly(A) spacer (Ochi *et al*., 2009).

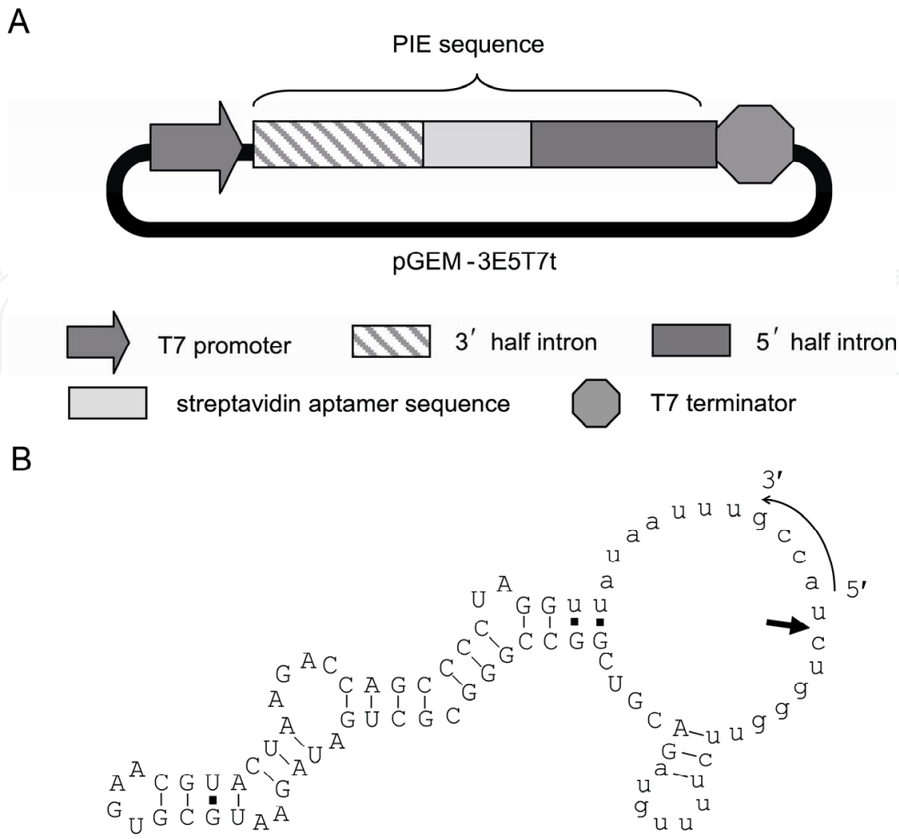

Fig. 2. Schematic representation of the plasmid pGEM-3E5T7t, and the predicted secondary structure of the circular streptavidin RNA aptamer produced from the plasmid. (A) The figure shows pGEM-3E5T7t. The PIE sequence is located between the T7 promoter sequence and the T7 terminator sequence. The PIE sequence consists of the 3' half intron, streptavidin aptamer sequence and 5' half intron sequence. The intron sequence is derived from the *td* intron of bacteriophage T4. (B) Predicted secondary structure of the circular streptavidin RNA aptamer. Upper and lower case letters indicate the aptamer sequence and exon sequence derived from the original exon sequence of the *td* gene, respectively. Streptavidin RNA is derived from the S1 aptamer reported by Srisawat & Engelke (2001). The thick arrow represents the self-ligated junction. The thin arrow (anticlockwise) indicates the orientation of the circular RNA from 5' to 3'. The thin line and black dot represent Watson–Crick base pairing and G-U Wobble base pairing, respectively.

2.1 *In vivo* circular streptavidin RNA aptamer expression by the PIE method

To demonstrate *in vivo* circular RNA expression, our group designed the circular streptavidin RNA aptamer as a model RNA dug and the PIE sequence for production of the circular streptavidin RNA aptamer. The PIE sequence consists of the 3' half intron, aptamer sequence and 5' half intron sequence in this order (Fig. 2A), with omission of the internal ORF sequence (Fig. 1, shown as the green-coloured line) in the *td* intron for this PIE construction that does not participate in the self-splicing reaction of the intron. We constructed a circular streptavidin RNA aptamer expression vector, pGEM-3E5T7t, which consists of three parts: the T7 RNA promoter, the PIE sequence and the T7 terminator

sequence in the multicloning site in the standard cloning vector, pGEM-3Z (Promega) (Fig. 2A). The streptavidin RNA aptamer sequence in the PIE sequence was derived from the S1 aptamer sequence reported by Srisawat & Engelke (2001), and the intron sequence derived from the *td* intron of bacteriophage T4. Both ends of the resulting circular streptavidin RNA aptamer sequence were ligated by an indispensable linker sequence derived from a partial exon sequence of the *td* gene of bacteriophage T4 (Fig. 2b, lower case).

To express the circular streptavidin RNA aptamer *in vivo*, pGEM-3E5T7t was transformed into JM109(DE3) (*endA*1 *recA*1, *gyr*A96, *thi*-1, *hsdR*17 (r_K^-, m_K^+), *relA*1, *supE*44, Δ(*lac-proAB*), [F', *traD*36, *proAB*, *lacI*^{q}Z, ΔM15], λ(DE3)) (Promega), which encodes an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible T7 RNA polymerase on its genomic sequence. The full-growth culture of JM109(DE3) harbouring pGEM-3E5T7t was transferred into 1 L of fresh LB broth and cultured until the optical density at 600 nm (OD_{600}) reached 0.7 at 30°C. Then, IPTG was added to a final concentration of 0.4 mM and circular RNA expression was induced by cultivation for 2 h at 30°C with vigorous shaking. After extraction of the total RNA, which included the circular RNA, using the AGPC method to break the cell membrane (Chomczynski & Sacchi, 1987), the circular RNA expression in the total RNA fraction was monitored by ethidium bromide staining and Northern blotting analysis.

We performed two-dimensional (2D) denaturing polyacrylamide gel electrophoresis (2D PAGE) (Schumacher, 1983; Feldstein, 2007) to monitor whether the circular RNA was present in the recovered total RNA fraction. This 2D-electrophoresis is based on the differences in migration behaviour between linear and circular RNA under denaturing gel conditions. Unlike linear RNA, migration of a circular RNA molecule of the same length varies with the acrylamide and/or bis-acrylamide concentration on denaturing polyacrylamide electrophoresis (PAGE). Therefore, after 2D denaturing gel-electrophoresis, the linear RNA fraction has migrated in the diagonal direction, whereas the circular RNA appears beside the diagonal line of the linear molecule. Both ethidium bromide staining and Northern blotting analysis showed a single spot beside the diagonal migration line, indicating that the circular RNA was present in the total RNA fraction. This spot was eluted and subjected to partial alkaline digestion. The partially digested nicked circular RNA migrated faster than the intact circular molecule on denaturing 10% PAGE, confirming that the eluted RNA was a circular form. The degradation products of circular RNA were not detected by Northern blotting analysis. This clearly showed that the circular RNA expressed in *E. coli* cells was protected against exonuclease-induced degradation, such as that induced by ribonuclease II (RNase II) (Frazão *et al.*, 2006). The expression level of the circular RNA aptamer was determined to be 2.5 ± 0.46 ng per 1 µg of total RNA by Northern blotting analysis. The yield of the circular RNA aptamer in that total RNA was estimated to be approximately 24 µg.

Next, we developed a circular RNA purification method for future inexpensive and economical RNA production and purification. We showed that the circular streptavidin RNA aptamer was successfully purified with the solid-phase DNA probe technique (Suzuki *et al.*, 2002). To purify the circular RNA produced by the PIE method, we designed a 5' biotinylated DNA probe that can hybridise with the circular RNA and the circular RNA-DNA hybrid can be easily trapped using a streptavidin-coated column. The trapped circular RNA-DNA hybrid is also denatured with a high concentration of urea (7 M) solution and the circular RNA is eluted. We can also choose the elution buffer such that the DNA probe

still binds to the solid-phase and it can be reused for another round of RNA purification (data not shown). Using a streptavidin-coated column (GE Healthcare), the circular streptavidin RNA aptamer was eluted under denaturing conditions and yielded 21 μg of the circular RNA (about 88% recovery) from 1 L of *E. coli* cell culture. Electrophoretic mobility shift assay (EMSA) also showed that the purified circular streptavidin RNA aptamer from JM109(DE3) retained its binding properties toward streptavidin.

To verify the suitability of the circular RNA for future RNA therapeutic uses, we measured the half-life of the purified circular RNA aptamer in HeLa cell extracts as a model of intracellular conditions. The estimated half-life of the purified circular streptavidin RNA aptamer was at least 1,386 min, while that of the S1 aptamer, which is the linear form of the streptavidin RNA aptamer, was 43 min. These observations suggested that the circular RNA escapes exoribonuclease-dependent RNA degradation under intracellular conditions. However, the circular RNA degraded completely within 15 s in 25% human serum. This is reasonable because human serum contains the RNaseA family ribonucleases (Haupenthal *et al*., 2006; Haupenthal *et al*., 2007; Turner *et al*., 2007). These findings indicated that the circular RNA would be useful under cellular conditions only when delivered into the cell in a precise manner, *e.g*., by using cationic liposomes (Sioud & Sorensen, 2003; Sorensen *et al*., 2003) or virus vector systems (Mi *et al*., 2006), to prevent RNaseA family ribonuclease-dependent degradation.

2.2 Constitutive *in vivo* circular streptavidin RNA aptamer expression by the PIE method

We then considered the constitutive circular RNA expression, as the previous expression procedure requires monitoring of the optical density for optimal IPTG induction (see 2.1). For constitutive expression of the RNA sequence in *E. coli*, we followed the procedure of Ponchon & Dardel (2004). They reported that the M3 vector containing the strong constitutively active lipoprotein (*lpp*) promoter, which is one of the strongest promoters in *E. coli* (Movva *et al*., 1978; Inoue *et al*., 1985), is applicable for *in vivo* RNA expression in the *E. coli* strain JM101Tr (Δ(*lac pro*), *supE*, *thi*, *recA56*, *srl-300*.:Tn*10*, (*F'*, *traD36*, *proAB*, *lacIq*, *lacZ*, Δ*M15*)). In addition, total RNA expression in JM101Tr is higher than that of JM109(DE3) (our unpublished observation).

Before constructing the constitutive PIE expression plasmid, we replaced the original $tRNA^{Met}$ sequence between the *lpp* promoter and *rrn*C terminator sequence in the M3 vector with the PIE sequence from pGEM-3E5T7t. The resulting expression vector is designated as pM3-3E5. The PIE sequence was amplified from the PIE sequence in pGEM-3E5. After transformation of pM3-3E5 into the JM101Tr strain, cell density (OD_{600}) was measured at several time points during cultivation and 1-mL aliquots were collected from 200 mL of 2×YT medium. Total RNA was recovered by ISOGEN (Nippon Gene) and Northern blotting analysis was performed. At various time points in culture from early logarithmic phase to stationary phase, circular RNA was visible in each lane on electrophoretic analysis even with ethidium bromide staining. The presence of circular RNA, but not the nicked form, was clearly detected on Northern blotting analysis and the amount of circular RNA increased with cell growth. These results suggested that the *lpp* promoter was active and drove expression of the PIE sequence without any induction. The stain JM101Tr is positive for ribonucleases, such as ribonuclease II (Frazão *et al*., 2006). Therefore, these observations

indicated that the circular RNA also accumulated in the *E. coli* JM101Tr strain, escaping degradation by exonucleases as seen in the previous expression system described in Section 2.1. The resulting yield of circular RNA after 18 h of cultivation at 30°C was estimated to be 3.6 ± 0.15 ng per 1 µg of total RNA, which was approximately 1.5-fold higher than that of the previous method (Umekage & Kikuchi, 2009a) (see 2.1). These observations indicated effective constitutive circular RNA expression in this system.

2.3 Improving circular RNA expression with the tandem one-way transcription of PIE (TOP) technique

To augment the circular RNA expression in *E. coli*, we developed the TOP (**t**andem **o**ne-way transcription of **P**IE) technique, which is a simple methodology for increasing the copy number of the PIE sequence in a single plasmid. The TOP technique is shown schematically in Fig. 3A. With this technique, it is easy to amplify the copy number by sequential insertion of the transcriptional unit in a single plasmid (Fig. 3B). First, we amplified the transcriptional unit, which consists of the *lpp* promoter, PIE sequence and *rrn*C terminator in pM3-3E5 (see Section 2.1) 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 hybridise with mutual 3' protruding ends of the palindromic TCGA sequence, and the resulting ligated fragment forms the sequence GTCGAG, which can be digested with neither *Xho*I nor *Sal*I (Fig. 3B). Therefore, the inserted sequence is as follows: 5'-*Kpn*I-*Xho*I-*lpp* promoter-PIE sequence-*rrn*C terminator sequence-GTCGAG site-3' (Fig. 3C). Thus, the subsequent transcriptional unit can be inserted at the *Kpn*I–*Xho*I site. We constructed four series of pTOP vectors using M3 designated as pTOP(I), pTOP(II), pTOP(III) and pTOP(IV) in parallel with the number of inserted transcriptional units.

This pTOP plasmid has a constitutive *lpp* promoter and therefore the constitutive expression of the PIE sequence in JM101Tr is expected, similar to that using the constitutive expression plasmid pM3-3E5 described in Section 2.2. To demonstrate the availability of the TOP technique, we then analysed the circular streptavidin RNA aptamer expression in *E. coli* by Northern blotting analysis and we detected that the circular RNA expression was expressed in all pTOP vectors (pTOP(I), (II), (III) and (IV)) (Fig. 3D).

As shown in the Fig. 3D., the circular RNA expression increased until two tandem insertions of the PIE, and the expression yields were almost the same using pTOP(II) and pTOP(III) (Table 2). These results indicated that the TOP system is a potentially useful and simple methodology for increasing circular RNA expression in *E. coli*. The circular RNA expression using 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 of the expression procedure using the pM3-3E5 system as described in Section 2.2. In addition, the circular RNA expression in 1 L of culture medium was estimated to be approximately 0.19 mg, which is the highest yield of circular RNA expression in *E. coli* reported to date. In contrast, expression of the circular RNA dropped dramatically when using pTOP(IV); the reason for this drop in expression level is not yet clear. To address this problem, we collected pTOP(IV) after 18 h of cultivation in JM101Tr and the plasmid was single-digested with *Hin*dIII and then subjected to 1% agarose gel electrophoresis. A few single-digested pTOP(IV) fragments

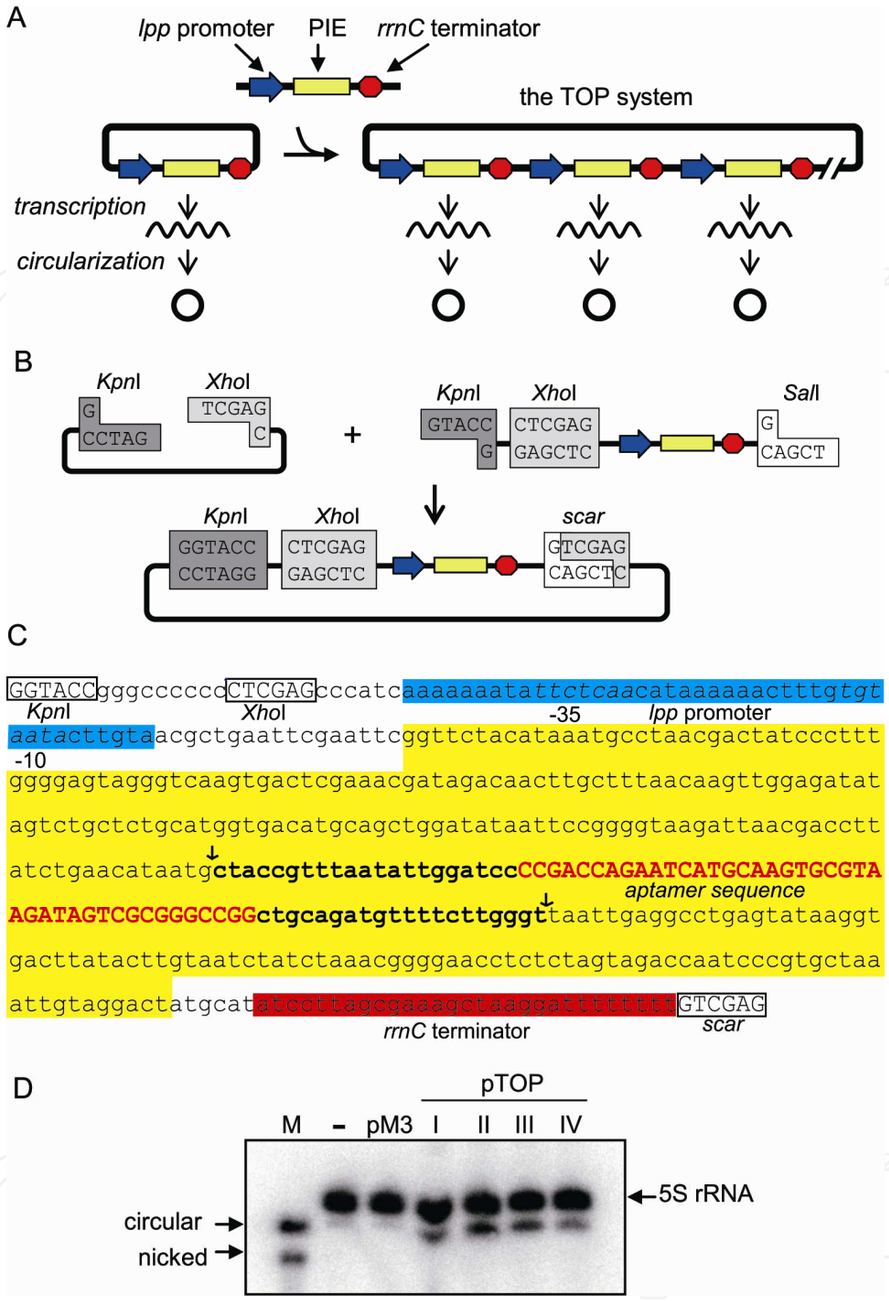

Fig. 3. Construction of the pTOP vectors, and the availability of the TOP method for generating circular RNA in JM101Tr. (A) Outline of the TOP method. (B) Illustration of sequential insertion of the PIE sequence into the same plasmid. First, *Kpn*I and *Xho*I double digested plasmid and *Kpn*I and *Sal*I double digested insertion sequence were prepared. Both the *Kpn*I site from the plasmid and the insertion sequence are ligated and the *Xho*I-digested site in the plasmid and the *Sal*I-digested site in the insertion sequence are ligated, resulting in the sequence GTCGAG at the 3' side of the inserted site. (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; italicised 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, circularised sequence; boxed sequence, ligated sites. (D) Northern blotting analysis of the circular RNA expression by each pTOP series. Total RNA derived from JM101Tr containing the *in vivo* expressed circular streptavidin RNA aptamer was fractionated by 10% denaturing PAGE. In addition, the circular RNA expression monitored using the ^{32}P-labelled complementary oligo-DNA probe of the aptamer sequence (5'-CCAATATTAAACGGTAGACCCAAGAAAACATC-3'). 5S rRNA was monitored as an internal control using the ^{32}P-labelled complementary oligo-DNA probe sequence (5'- GCGCTACGGCGTTCACTTC-3'). Arrows indicate the migration positions of the circular RNA (circular), nicked RNA (nicked) and 5S rRNA. Circular RNA control marker (M) was prepared by *in vitro* transcription (Umekage & Kikuchi, 2009a). "-", Total RNA from JM101Tr; "M3", 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.

showed unexpected migration behaviour (data not shown), suggesting that it was difficult for pTOP(IV) to undergo replication in JM101Tr during 18 h of cultivation. Although the expressional host strain JM101tr has the *recA56* mutant, which results in defects in recombination, this genetic mutation is not sufficient to confer stability on pTOP(IV). This instability of pTOP(IV) in JM101Tr indicates the necessity for optimisation of the TOP technique for further augmentation of circular RNA expression; *e.g.*, optimisation of the intervening sequence between the two transcriptional units, considering the direction of transcription, changing the expressional host to a strain lacking another gene that results in defective recombination, such as *sbcB*, *C* or another *rec* gene (Palmer *et al*., 1995), and optimising the copy number of PIE sequences in the single transcriptional unit to avoid accumulation of *lpp* promoter in the single plasmid.

2.4 Circular RNA expression by the marine phototrophic bacterium *Rhodovulum sulfidophilum*

Finally, we would like to discuss our new project to develop an economical and efficient method for RNA production using the marine phototrophic bacterium *Rdv. sulfidophilum* (Fig. 4), taking advantage of its unique characteristics in that nucleic acids are produced extracellularly (Suzuki *et al*., 2010). In addition this bacterium produces no RNases in the culture medium (Suzuki *et al*., 2010). Although the mechanism of extracellular RNA production by this bacterium has not been fully characterised, this extracellular RNA expression system represents an economical and efficient methodology for RNA production as it is only necessary to collect the culture medium containing extracellularly produced RNA and purify the RNA of interest with a column bypassing the need for a cell extraction procedure using phenol or various other extraction reagents to rupture the cell membrane.

We began by constructing the engineered circular RNA expression plasmid, pRCSA, based on the broad-host range plasmid pCF1010 (Lee & Kaplan, 1995). The PIE sequence was amplified from pGEM-3E5T7t, and the *rrn*A promoter and *puf* terminator sequence were amplified from the genomic DNA of *Rdv. sulfidophilum* DSM 1374^T (Hansen & Veldkamp, 1973; Hiraishi & Ueda, 1994). The resulting amplified DNA fragments were inserted into pCF1010 to give pRCSA, which was then transformed into *Rdv. sulfidophilum* DSM 1374^T by

conjugation using the mobilising *E. coli* strain S-17 as a plasmid donor (Simon *et al*., 1983). The heat shock transformation method can also be used (unpublished observation) (Fornari & Kaplan, 1982). The transformed *Rdv. sulfidophilum* DSM 1374^T was cultured under anaerobic conditions under incandescent illumination (about 5,000 lx) for 12 – 16 h at 25°C in PYS-M medium (Nagashima *et al*., 1997, Suzuki *et al*., 2010). Cultured cells were harvested and the total intracellular RNA was extracted with the AGPC method. The estimated yield of the intracellular circular RNA was approximately 1.3 ng per 1 L of culture medium by Northern blotting analysis. On the other hand, the circular RNA expression in the culture medium was barely detected by Northern blotting analysis; however, RT-PCR analysis demonstrated the existence of circular RNA in the cultured medium (data not shown). At present, neither intracellular nor extracellular expression of the circular RNA aptamer can be achieved at practical levels for economic and efficient circular RNA expression, and the overall improvement of RNA expression using this bacterium is strongly promoted.

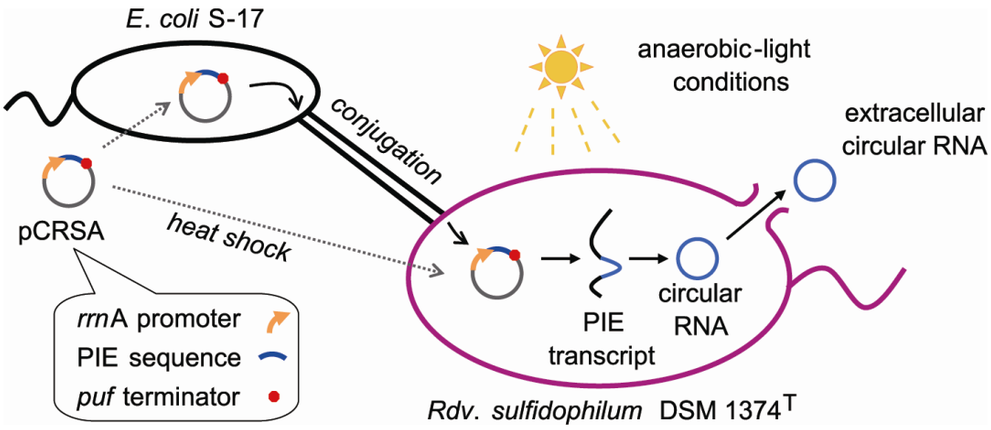

Fig. 4. Overview for circular RNA expression using *Rdv. sulfidophilum* DSM 1374^T. Circular RNA expression plasmid, pRCSA, was transformed into *Rdv. sulfidophilum* DSM 1374^T by conjugation using the mobilising *E. coli* strain S-17 (Simon *et al*., 1983) or by direct transformation using the heat shock method (Fornari & Kaplan, 1982). The transformed *Rdv. sulfidophilum* was grown under anaerobic-light conditions. The PIE sequence in pRCSA was transcribed with the endogenous RNA polymerase and circular RNA was generated from the PIE sequence. The circular RNA produced inside the cell was released extracellularly into the culture medium.

3. Conclusions

Our circular streptavidin RNA aptamer expression system described in Sections 2.1, 2.2 and 2.3 is summarised in Table 2. To our knowledge, the TOP method is the most effective means of circular RNA expression, and the *in vivo* constitutive RNA expression is suitable for circular RNA expression, as the spontaneously expressed circular RNA can exist stably within the cell avoiding endogenous exoribonuclease-dependent degradation. By using the circular streptavidin RNA aptamer expression plasmid pTOP(II) and *E. coli* JM101Tr as a host stain, the expression yield of the circular RNA was estimated to be approximately 0.19 mg per 1 L of culture. Although the TOP method requires further improvement to augment circular RNA expression, it is notable that this method easily increased the level of circular RNA expression by simple multiplying the copy number of transcription units in the single

plasmid. Therefore, we assumed that the TOP strategy will be more effective especially using a low copy number plasmid, because increasing the plasmid copy number by genetic engineering is not easy. We also presented the solid-phase DNA probe method as a simple purification procedure for *in vivo* expressed circular RNA, because this technique does not require electrophoresis for purifying the circular RNA (Umekage & Kikuchi, 2009a).

The most remarkable advantage of circularising functional RNAs is protection from exoribonuclease-induced degradation without the need for chemical modifications, such as use of 2'-protected nucleotides (*e.g.*, 2'-fluoro, 2'-*O*-methyl, LNA) (Schmidt *et al*., 2004; Burmeister *et al*., 2005; Di Primo *et al*., 2007; Pieken *et al*., 1991) or phosphorothioate linkages (Kang *et al*., 2007). Although chemical synthesis of RNA molecules is currently the main methodology used for synthetic RNA production, the *in vivo* circular RNA production technique described in this chapter is a promising method for future RNA drug production because it is both economical and the product can be purified simply. In addition, circular RNA without any chemical modification would be safer than chemically modified RNA for therapeutic human use.

This PIE method can be applied in any species because it requires only magnesium ions and guanosine nucleotides. However, the expression of circular RNA inside human cells or other mammalian cells in culture has not been examined. Therefore, we are currently examining circular RNA expression in human cells based on this method for future development of gene therapy methodologies. We assume that PIE transcription and concomitant RNA circularisation take place in the nucleus, and therefore the circular functional RNA (including aptamers, ribozymes, dsRNA *etc.*) expression within the nucleus will represent a novel gene regulation method targeting nuclear events, such as transcription (Battaglia *et al*., 2010), RNA splicing (van Alphen *et al*., 2009), telomere repairing (Folini *et al*., 2009) and chromatin modification (Tsai *et al*., 2011).

Plasmid	Host strain	Expression	Yield (ng/μg)	Reference
pGEM-3E5T7t	JM109(DE3)	IPTG	2.5 ± 0.46	Umekage & Kikuchi, 2009a
pM3-3E5	JM101Tr	constitutive	3.6 ± 0.15	Umekage & Kikuchi, 2009b
pTOP(I)	JM101Tr	constitutive	5.0 ± 1.5	this study
pTOP(II)	JM101Tr	constitutive	9.7 ± 1.0	this study
pTOP(III)	JM101Tr	constitutive	9.0 ± 1.8	this study
pTOP(IV)	JM101Tr	constitutive	1.8 ± 0.70	this study

Table 2. Summary of circular RNA expression. "IPTG" and "constitutive" indicate that the circular RNA expression was induced by the addition of IPTG and constitutive expression of the circular RNA by the constitutive *lpp* promoter, respectively. "Yield" represents the circular RNA expression yield (ng) per 1 μg of total RNA recovered from the harvested cells. The data include standard deviations (±), which were derived from three independent experiments (n = 3).

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