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Artificial RNA aptamer production by the marine bacterium *Rhodovulum sulfidophilum*: Improvement of the aptamer yield using a mutated transcriptional promoter

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Noncoding small RNAs and artificial RNA aptamers are now expected to be potential candidates for RNA therapeutic agents. We previously proposed a unique method for economical production of these RNAs using the marine phototrophic bacterium *Rhodovulum sulfidophilum*. This bacterium does not produce any ribonucleases but does produce extracellular nucleic acids in the culture medium in nature. Using this bacterium and an engineered plasmid containing the *rrn* promoter for the RNA expression, we developed a method for production of the streptavidin RNA aptamer in the culture medium. However, the yield of this RNA product in the culture medium by this method was not enough for practical use. In the present paper, we improved the yield of this product by modification of the −35 region of the *rrn* promoter so as to escape from the Fis protein control and the use of a new vector plasmid. Using this system, the extracellular RNA aptamer of approximately 200 ng and the total RNA aptamer (both extra- and intracellular form) of about 20 μg from 1 L culture were accomplished by constitutive expression of the gene.

[**Key words:** Functional RNA; Phototrophic bacterium; Promoter; RNA aptamer; Transcription]

RNAs have recently emerged as major players in current biology. Naturally occurring micro RNAs and artificial small interfering RNAs, double-stranded RNAs and ribozymes function as regulators of gene expression (1). In addition, an RNA aptamer, which is experimentally selected from randomized RNA pools, is a biologically functional molecule of specific binding ability to a target molecule (2). All these RNAs are important tools not only for RNA science but also potential RNA drug candidates for therapeutics (1–4). In this context, efficient methods for preparation of homogeneous RNA molecules are highly required. Currently, the RNAs are usually obtained by *in vitro* transcription or chemical synthesis (5,6). However, these methods are expensive and labor intensive, especially not suitable to produce large quantities. For RNA preparation on a large scale, the microbial production *in vivo* seems to be most suitable. In the previous paper (7), we developed a new method for *in vivo* production of a streptavidin RNA aptamer (an RNA aptamer which binds to streptavidin) (8) using the marine phototrophic bacterium *Rhodovulum sulfidophilum*. The streptavidin RNA aptamer was chosen as an RNA drug product model. This bacterium does not produce any detectable ribonucleases in culture medium (7). Using an engineered plasmid and this bacterium, we succeeded in producing the functional RNA aptamer *in vivo*. Especially it should be emphasized that the RNA aptamer was produced not only inside the cells but also in the culture medium by this method, because this organism produces extracellular nucleic acids in nature (7,9,10). This extracellular production system can be expected as an economically efficient method for large-scale production of functional RNAs. The yield of the RNA aptamer product by this method, however, was not promisingly high and this method in its form seemed to be not capable of industrial application. To obtain a high yield of the product, we previously suggested that improvement of the transcriptional promoter and increasing the copy number of the plasmid vector may be effective (7).

In the present paper, experimental results will be described on more favorable condition to obtain the RNA aptamer product in high yield using a mutated transcriptional promoter and a new plasmid vector of broad-host range.

MATERIALS AND METHODS

Bacterial strain and growth conditions The purple phototrophic marine alphaproteobacterium, *R. sulfidophilum* DSM 1374^T (11,12) was used throughout this study. Cultivation was performed essentially by the method of Hiraishi and Ueda (12). The strain was grown anaerobically at 25°C in screw-cap test tubes filled with PYS medium (13) including 2% (wt/vol) NaCl (PYS-M medium) under incandescent illumination (about 5000 lux). Streptomycin was used at a concentration of 25 μg/mL for the strain harboring pCF1010, pR2, pCF-rpWT, pCF-rpMT1, pCF-rpMT2, pCF-rpAM1 or pCF-rpAM2. Kanamycin was used at a concentration of 30 μg/mL for the strain harboring pBHSR1-RM. Cell growth was evaluated by measuring the turbidity of the culture medium at 600 nm.

Construction of plasmids First, we amplified the *rrn* promoter region from the genome of *R. sulfidophilum* DSM 1374^T by PCR using the primers rrn-2L

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5′-TCTGGCGCTTCGATTCCGGTCGAGTCGGGGCAGAGTCGCCACCTGACGCTGGGGCAATCTCAAGCTTCTGTGGGTA

AGTCCGGGGATAACTTTGGGCCCGTCAGTGTCGACCCAGGGGCGTCCGAAAAAAGCTGCGCCGGGCCCTTCATCAGCT

TGTGTGTTCTTTGGGCGTAAGCGCCAGATATTGGGTCGGTTTCCGGGTTGCTGCGAAAAATGTCATCGAACTGCAAAAAA

-35 -10

ACTGCTTGCGGTGTTCGAGTAGTATCCGTAGACAGCGCTTCACCGGCGGCGCTGAGGCGCTGACGGGGCGATGGCC

GGGTCGGTACGACACGGTGGTCGGACAAGCAGAGATGGTTGTGAGGCGGGCGGCGCCAAGTAGAAATGGCGCTTCTG

TCGAGTTTTTGTCTCTGATGCTCTTTGACATTGCTGGTATCTGAAGAGATATGCGGGCGGTTTGGTCGAGATCGACGGAA

CGAGCCTCGTATATCGGCTCTCTAGGGCTTCGGTCCGATGATGAGAGTGTCAGCTTCACTGTTTGATGTAGGTTCCGGTT

CCTGGTGAACTGGAGCACATTGAACAGAAGATGGACATTCGGGTTGTTGTCGCACTCTTGTAGTGGGGCGATTTCGCGA

ATGGACGATGTGCAGAGGTTCGAACGTCAAGAACTGCTAATGCAGTTTCAACCTGAGAGTTTGATCCTGGCTCAGaacgaa

cgctggcggcaggcctaaca – 3′

FIG. 1. The nucleotide sequence of *rrn* promoter of *R. sulfidophilum* (DDBJ accession no. AB513346). The bold types "A", "TAGACA" and "TTGCGG" indicate "transcription start point", "−10" and "−35" regions, respectively. The underlines indicate putative Fis binding sites (FBS). The mature 16S rRNA sequence of *R. sulfidophilum* is shown in lowercase letters.

(5′-TTGGTTGCGGCCGCTCTGGCGCTTCGATTCC-3′) and rrn-R (5′-TTAATGCATGAATTCATTTCTACTTGGCGCCG-3′) as described (7). The *Not*I and *Eco*T22I sites in the primers are underlined. The amplified region corresponds to almost the first five lines of Fig. 1. Using *Not*I and *Eco*T22I sites, this fragment was inserted to the upstream of the *lacZ* region of pCF1010 (14) to give pR2 (Fig. 2). To analyze whether putative FBS (see Results and discussion and Fig. 1) in the *rrn* promoter of *R. sulfidophilum* is involved in the promoter activity, we constructed plasmids containing FBS-deleted *rrn* promoter, pCF-rpWT (Fig. 2). For this deletion, PCR using the primers, rpWT-L (5′-TTGGTTGCGGCCGCGAACTGCAAAAAAACTG-3′) and rpWT-R (5′-TTAATGCATGAATTCGCCGGTGAAGCGCTGTCTAC-3′) and the genome DNA as a template was performed and the amplified fragment was inserted to the pCF1010 using *Not*I and *Eco*T22I sites to give pCF-rpWT. To obtain the mutated −35 and −10 regions, we used the primers, rpMT1-L (5′-TTGGTTGCGGCCGCGAACTGCAAAAAAACTGCttgacaTGTTCGAG-3′) and rpMT2-R (5′-TTAATGCATGAATTCGCCGGTGAAGCGCattataCGGA-3′), respectively. The mutated −35 and −10 regions were shown by lowercase letters. Using several combinations of these primers, we amplified several mutated promoter regions and constructed the plasmids, pCF-rpMT1, pCF-rpMT2, pCF-rpAM1 and pCF-rpAM2 (see Fig. 2).

For *in vivo* production of streptavidin RNA aptamer, we constructed the plasmid pBHSR1-RM, which was derived from the broad-host-range plasmid pBHR1 (purchased from MoBiTec) (15). First, for miniaturization of the plasmid, pBHR1 was digested by *Nar*I and *Eco*81I and self-ligated by DNA Blunting Kit (Takara Bio Inc.) to give pBBRKM. Then to the *Nae*I site of this plasmid, we inserted a fragment containing multiple cloning site which is a fragment from pGEM-3Z (Promega) cleaved by *Mva*I. Using this multiple cloning site, the structural gene of the RNA aptamer from pHSR1 (7) and the promoter region from pCF-rpAM1 (Fig. 2) were inserted appropriately to this plasmid to give pBHSR1-RM.

Transformation of *R. sulfidophilum* Transformation of *R. sulfidophilum* was done by the method of Fornari and Kaplan (16) with modification. Two milliliters of the bacterial culture of stationary phase was inoculated to 20 mL of new PYS-M medium. This was cultivated under aerobic-dark condition until $OD_{600} = 0.8$. The culture was centrifuged at 4°C. The cell pellet was washed in 10 mL of ice-cold 500 mM Tris–HCl (pH 7.2). The pellet was suspended in 1.2 mL of ice-cold 100 mM Tris–HCl (pH 7.2) containing 200 mM $CaCl_2$. Plasmid DNA was immediately added to 0.2 mL of competent cells, and an equal volume of cold 40% PEG-6000 in 100 mM Tris–HCl (pH 7.2) was slowly added. The suspension was gently shaken to mix the two phases and left on ice

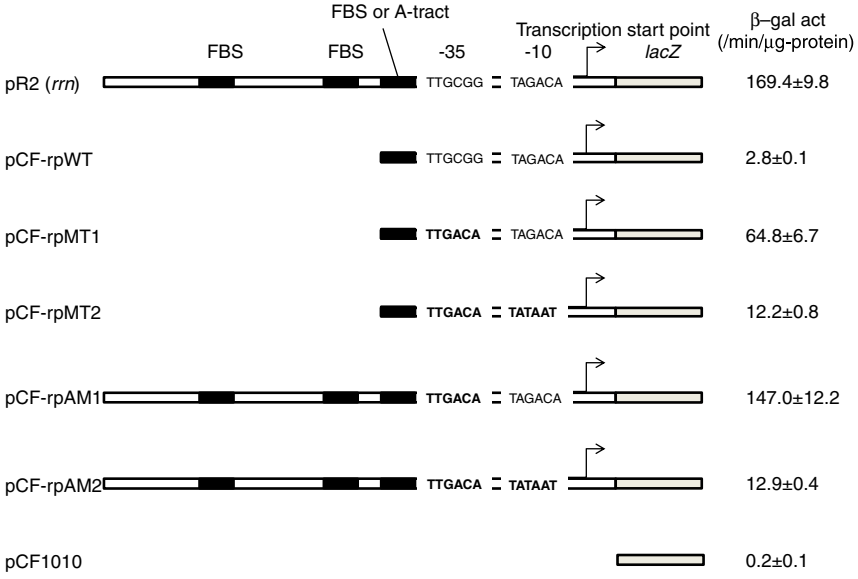

FIG. 2. The β-galactosidase activities under mutated *rrn* promoters. The closed boxes indicate putative Fis binding sites (FBS) or A-tract. The promoter elements (−10 and −35 regions) are shown as "TAGACA or TATAAT" and "TTGCGG or TTGACA". The transcription start point is indicated by arrows. The β-galactosidase activities obtained for constructs (see Materials and methods) are shown in the right. These are shown as units (A_{420}/min/μg protein). The data include standard deviations (±), which were derived from two independent experiments. The pR2 (*rrn*) is a plasmid containing the wild type *rrn* promoter. pCF1010 is shown as control.