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Short hairpin RNAs of designed sequences can be extracellularly produced by the marine bacterium *Rhodovulum sulfidophilum*

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Previously, we proposed a new method for production of RNA aptamers using the marine bacterium *Rhodovulum sulfidophilum*. A streptavidin RNA aptamer (an RNA which binds to streptavidin) was extracellularly produced by this bacterium containing engineered plasmid. The aptamer had full biological function. As a next step we attempted to produce another functional RNA, short hairpin RNAs (shRNAs) using this bacterial system. We have designed two types of shRNAs targeted to the luciferase gene. Here we report that shRNAs are successfully produced extracellularly by this system. Even if the shRNA has a long stem-loop structure which is thought to interfere with transcription in bacterial cells, the yield of the shRNA is almost the same as that of the streptavidin RNA aptamer. During the course of these experiments, we also found a new type of RNA processing for the double-stranded region of the shRNA.

Key words: extracellular RNA; marine Bacteria; *Rhodovulum sulfidophilum*; RNA medicine; short hairpin RNA

Introduction

It is well known that small RNAs have a wide variety of functional repertoires (reviewed by Nellen and Hamman, 2005). Ribozymes, small interfering RNAs (siRNAs), double-stranded RNAs and micro RNAs function as regulators of gene expression in cells. Especially after the discovery of RNA interference, these small RNAs have been considered to be one of the new approaches for therapy (Castanotto and Rossi, 2009). In this context, efficient methods for preparation of homogeneous RNA molecules are now required. In previous papers, we developed a new method for in vivo production of artificial RNAs using the marine phototrophic bacterium *Rhodovulum sulfidophilum* (Suzuki et al., 2010; 2011; Kikuchi, 2010; Kikuchi et al., 2010). This bacterium does not produce any detectable ribonucleases in culture medium but does produce extracellular nucleic acids in nature (Suzuki et al., 2009a, b, 2010). Using this bacterium and an engineered plasmid, we developed a method for extracellular production of an artificial RNA. As an artificial RNA model, we chose a streptavidin RNA aptamer (an RNA aptamer which binds to streptavidin). The streptavidin RNA aptamer was produced in the culture medium by this method (Suzuki et al., 2010). It has also been reported that the yield was improved by using a modified transcriptional promoter and a new plasmid (Suzuki et al., 2011).

To generalize this method, we tried extracellular production of short hairpin RNAs (shRNAs) using this bacterium. Since the synthetic shRNAs have been reported to be more potent inducers of gene silencing than siRNAs (Siolas et al., 2005), shRNAs are expected to be used for the treatment of disease in future. However, the shRNA contains a long stem and loop structure which is usually thought to cause transcriptional termination or pause for bacterial RNA polymerases (Wilson et al., 1995; Kyzer et al., 2007). Although this concerned us, we tried to design experiments for shRNA production.

Here we report that the shRNAs could be produced extracellularly by *R. sulfidophilum* containing appropriate engineered plasmids, although one shRNA was specifically cleaved by an unknown processing system of this bacterium. The yield was the same as in the case of the RNA aptamer previously published (Suzuki et al., 2011). It seemed that no transcriptional pause or termination specific to the shRNA structure occurs.

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Materials and Methods

Bacterial strains and growth conditions. The purple phototrophic marine alphaproteobacterium, *Rhodovulum sulfidophilum* DSM 1374 was used throughout this study. *R. sulfidophilum* was grown anaerobically at 30°C in a 1.5-ml tube filled with PYS medium (Nagashima et al., 1997) containing 2% (wt/vol) NaCl under incandescent illumination (about 3,000 lux). Aerobic growth of *R. sulfidophilum* was achieved by shaking a 10-ml culture in a 50-ml centrifuge tube at 150 rpm. Kanamycin was used at a concentration of 30 μg/ml for the strain harboring pBHSR3, pBHSR1RM-sh and pBHSR3-shb. Cell growth was evaluated by measuring the turbidity of the culture medium at 600 nm. Transformation of *R. sulfidophilum* was done by the method described previously (Suzuki et al., 2011).

Construction of shRNA-a expression plasmids. In the previous paper we constructed the plasmid pBHSR1-RM for production of streptavidin RNA aptamer (Suzuki et al., 2011). Using this plasmid, we tried to construct an shRNA expression plasmid. First, the shRNA-a coding fragment was prepared using synthetic oligodeoxynucleotides (oligoDNA) sh-L (5′-ATGAATTCGTTCAAGATCATCG ACTGAAATCCCTGGTAATCCGTTTGGGGCTCTG-3′) and sh-R (5′-ACAAGCTTCGAATTTTCCTGGTCATCGA CTAAAATCCCTGATAATCCATTTATCCCATAGCAGG GCAGAG-3′). The *Eco*RI and *Hin*dIII sites in the oligoDNAs are underlined. Because the 3′-terminal 5 nucleotides of sh-L and sh-R were complementary, two oligoDNAs were annealed, and the long recessed 3′ ends were filled in with dNTPs and Go *Taq* DNA polymerase (Promega) as described in the instructions of the enzyme supplier. The streptavidin RNA aptamer coding region of the plasmid pBHSR1-RM was replaced by this shRNA-a coding fragment using the *Eco*RI and *Hin*dIII sites to yield the new plasmid pBHSR1-sh. To obtain the in vitro transcripts, an shRNA-a coding DNA fragment was also inserted in appropriate sites of another vector, pGEM-3Z, to yield pGEM-sh.

Construction of shRNA-b expression plasmid. Production of another shRNA, shRNA-b, was performed. Before construction of the shRNA-b expression plasmid, we modified the RNA expression vector pBHSR1-RM. The streptavidin RNA aptamer expression plasmid pBHSR1-RM contains redundant sequences of more than 100 bases originating from the ribosomal RNA gene just after the mutated *rrn* promoter (Suzuki et al., 2011). To remove this sequence, first we prepared a linear plasmid sequence without this redundant sequence using a long PCR technique using a primer of inverted direction for the *rrn* promoter and another primer for the *puf* terminator. Using this prepared linear plasmid, a double-stranded oligoDNA containing *Eco*RI and *Bam*HI sites, and the In-fusion cloning technique (Zhu et al., 2007), we constructed a new RNA expression vector pBHSR3 which has no redundant sequence just after the *rrn* promoter. Using this vector, any sequence can be cloned between *Eco*RI and *Bam*HI sites. Since the *Eco*RI recognition site is directly attached to the transcription start adenine nucleotide, the sequence of the 5′-terminus of the products is expected to be pppAGAAUUC⋯.

The shRNA-b coding DNA fragment was prepared by similar technique to those described above. The oligoDNA shb-L (5′-CCGGAATTCGGTCGAAGTACTCAGCGTAA GTGATGTCCTTAAGGGTG-3′) and oligoDNA shb-R (5′-AAAAGGATCCCCAAAACACCCAACACAAACAA CACCCTTAAGGACATC-3′) were used. The *Eco*RI and *Bam*HI sites in the oligoDNAs are underlined. As described above, these oligoDNAs were annealed, and the long recessed 3′ ends were filled in with dNTPs and Go *Taq* DNA polymerase. This shRNA-b coding fragment was inserted into the *Eco*RI and *Bam*HI sites of pBHSR3 to yield the plasmid pBHSR3-shb. Similarly to the case of shRNA-a, to obtain in vitro transcripts, an -b coding DNA fragment was also inserted into appropriate sites of vector pGEM-3Z to yield pGEM-shb.

Preparation of intracellular and extracellular RNAs. Cultivated cells were collected by centrifugation, and the supernatant was transferred to a fresh tube. The intracellular RNAs were prepared by the acid guanidine phenol-chloroform (AGPC) method (Chomczynski and Sacchi, 1987). The nucleic acid fraction of the supernatant was precipitated with 2-propanol, and treated with DNase I (Promega, Madison, WI), and then the extracellular RNAs were collected by phenol-chloroform extraction and ethanol precipitation.

Northern blotting analysis. To determine the sizes and amounts of the shRNA products, Northern blotting analysis was conducted. Intracellular and extracellular RNAs were prepared from the cultures as described above. The RNAs were electrophoresed on 8 M urea-denaturing 10% polyacrylamide gels, and then the separated product bands were transferred onto Hybond-N+ membranes (GE Healthcare, Chalfont, St. Giles, UK). Hybridization was performed in PerfectHyb Hybridization Solution (Toyobo, Osaka, Japan) containing a 5′-^{32}P-labelled probe for 16 h. Probe-1 (5′-ACAAGCTTCGAATTTTCCTGGTCATCGACTAAAA TCCCTGATAATCCATTTATCCCATAGCAGGGCAGA G-3′) and probe-2 (5′-CCATTTATCCCATAGCAGGGCA G-3′) were used for shRNA-a, and probe-3 (5′-AAAA GGATCCCCAAAACACCCAACACAAACAACACCCTT AAGGACATC-3′) and probe-4 (5′-CCTTAAGGACATC ACTTACG-3′) were used for shRNA-b. Membranes were washed in low-stringency buffer (17.53 g/L sodium chloride, 8.82 g/L sodium citrate, 0.1% [wt/vol] SDS) twice at 50°C, and bands were detected by autoradiography using an Image Reader FLA-9000 (Fuji Film Co., Tokyo, Japan). The amount of shRNA-a was calculated from the band signal using Multi Gauge (Fuji Film Co.).

Results and Discussion

Design of shRNA-a production

To examine whether the shRNAs are also produced extracellularly by this bacterium, we first designed an shRNA sequence which has a microRNA-like secondary structure and is targeted to the firefly luciferase gene (Paddison et al., 2002; Fig. 1A). Additionally, this sequence contains a CCA sequence at the nucleotide number 83−85 (Fig. 1A) to imitate the 3′-end of transfer RNA. Since tRNA processing enzymes of *Escherichia coli* recognize these tRNA-like sequences of tRNA precursors and cleave them to produce the mature tRNAs (Ando et al., 2003), we expected this tRNA-like sequence in the shRNA sequence to be

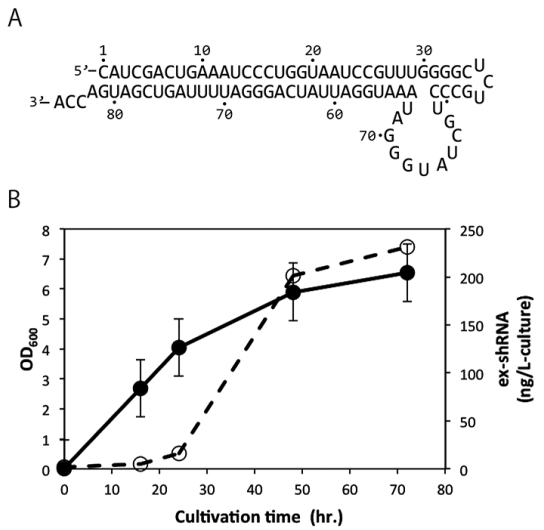

Fig. 1. Production of shRNA-a.

(A) Designed nucleotide sequence and secondary structure of shRNA-a. (B) The time course of extracellular production of shRNA-a. The amounts of shRNA-a were measured by Northern hybridization using probe-1(see Fig. 2A). Error bars indicate the respective standard deviations which were calculated from the results of three independent experiments. Open circles indicate cell growth (optical density at 600 nm). ex-shRNA, extracellular shRNA.

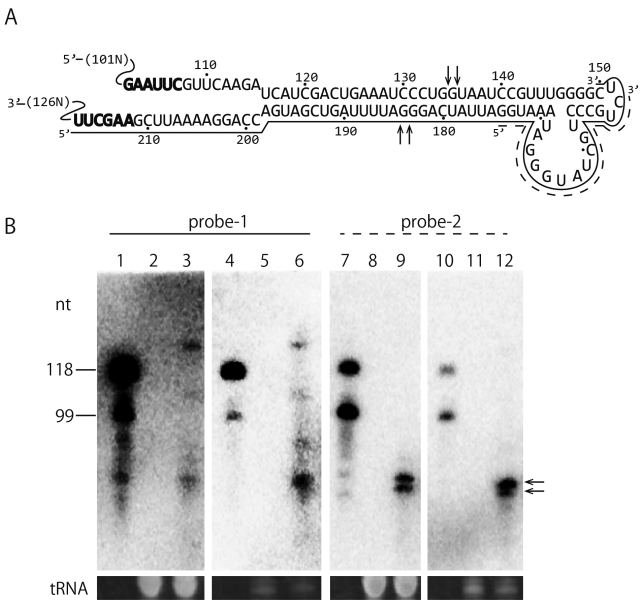

Fig. 2. Transcription and processing of shRNA-a.

(A) Predicted nucleotide sequence and secondary structure of the expressed shRNA-a. The shRNA-a with flanking sequences is shown. 101N and 126N indicate the predicted additional length expressed from the plasmid pBHSR1-sh. Bold letters (GAAUUC and AAGCUU) are the *Eco*RI and *Hin*dIII sites used for cloning (see MATERIALS AND METHODS). The solid line and dashed line along the sequence are hybridizable regions of probe-1 and probe-2, respectively. Arrows indicate cleavage sites by unknown enzyme(s) (see text). (B) Northern blotting analysis of the shRNA-a produced. Intracellular (lanes 2, 3, 8, and 9) and extracellular RNA samples (lanes 5, 6, 11, and 12) prepared were electrophoresed on 8 M urea-denaturing 10% polyacrylamide gels, and then transferred onto Hybond-N+ membranes. The shRNA-a sequence-containing bands were detected by probe-1 (lanes 1−6) and probe-2 (lanes 7−12) as indicated at the top of the panel. RNA samples from host cells only (no plasmid) were applied to lanes 2, 5, 8, and 11 as negative controls. Lanes 1, 4, 7, and 10, size markers (118 and 99 nucleotides long). These size markers were prepared by run-off in vitro transcription of pGEM-sh (see MATERIALS AND METHODS) and related plasmids. To compare relative amounts of the signals, tRNA bands stained with ethidium bromide on the gel are shown. Arrows at right side of the panel indicate the fragments containing loop regions generated by an unknown processing system (see text).

recognized and cleaved by tRNA processing enzymes of *R. sulfidophilum* to produce an appropriately trimmed shRNA (the sequence shown in Fig. 1A). However, the trimming occurred at other positions as described below.

Time course of the shRNA-a production

Time course and quantitative analysis of the shRNA-a production in the culture medium (extracellular production) were performed. *R. sulfidophilum* DSM 1374 was transformed with the plasmid pBHSR1-sh by the heat shock method as described (Suzuki et al., 2011). The transformed cells were cultivated under aerobic conditions. The amounts of products in the culture medium at various time points in the culture were determined by Northern hybridization using probe-1. Time course curves are shown in Fig. 1B. Probe-1 is shown in Fig. 2A. The maximum level of production (200 ng/L) was observed at 70 h of cultivation (stationary phase). This curve is very similar to the curve of the streptavidin RNA aptamer production which was published in our previous paper (Suzuki et al., 2011). The amount of the extracellular streptavidin RNA aptamer production was 195 ng/L after 70 h of cultivation (Suzuki et al., 2011). This indicates that the shRNA sequence, a relatively long stem and loop structure, has no serious inhibitory effect on the transcriptional elongation of this bacterium.

Detection of the shRNA-a and new type of processing

To examine whether the expected trimming of shRNA-a occurred, Northern hybridization using probe-1 and probe-2 was performed (Fig. 2). Figure 2A shows an expected primary transcript from the plasmid pBHSR1-sh. As shown in Fig. 2B, hybridized signals could be detected in the intracellular (lanes 3 and 9 of Fig. 2B) and extracellular (lanes 6 and 12 of Fig. 2B) RNA samples. This indicates that the shRNA sequences could be smoothly transcribed and that the shRNA products could be released to the culture medium by *R. sulfidophilum*. Using probe-1, which is complementary to the nucleotide 150−217 (see Fig. 2A), several bands were detected in both intra- and extracellular samples (lanes 3 and 6 of Fig. 2B). On the other hand, using probe-2, which is complementary to only the head loop region of shRNA-a (the nucleotide 150−174, see the dashed line of Fig. 2A), only the relatively clear double bands were detected (indicated by arrows in Fig. 2B). This indicates that the bands longer than 118 nt (in lanes 3 and 6 of Fig. 2B) contain only part of the 3′ terminal sequence of the shRNA-a and that all of the primary transcript from the plasmid pBHSR1-sh was cleaved and the head loop region converged to these double bands (arrows in Fig. 2B).

As described above, initially, we expected that the primary transcript of this shRNA-a could be processed by tRNA processing enzymes. If the expected processing occurred, the product should have been 85 nucleotides long (the nucleotide number 117−201 of Fig. 2A), but the size of the double bands seemed to be smaller than expected (lanes 9 and 12 of Fig. 2B). Therefore the cleavages by tRNA

processing enzymes did not occur, but other cleavages by unknown RNase(s) may have produced these double bands. From primer extension experiments (not shown), it was estimated that the longer of the double bands is a 50 nucleotide long fragment of nucleotide 135−184, and that the shorter one is a 48 nucleotide long fragment of nucleotide 136−183. Although we did not know at present what enzyme(s) cleaved the primary transcript of shRNA-a, it is possible that this RNA may be a good substrate for RNase III-like double-strand-specific RNase(s) of this bacterium. Since no bands longer than 50 nucleotides were observed in lane 9 or 12 of Fig. 2B, it may be concluded that almost all shRNA-a transcripts were processed completely by this unknown RNase(s). This efficient processing system may be used for the design of functional RNA production in future, although detailed elucidation of the specificity of the enzyme(s) is necessary.

Production of another model, shRNA-b

As described above, we found unexpected processing in the shRNA-a sequence, although a smooth transcription occurred. To generalize the shRNA production by *R. sulfidophilum* and to test whether the unexpected processing could be avoided, we designed another type of shRNA, shRNA-b (see Fig. 3A). For this production, we used a new vector, pBHSR3. As described in MATERIALS AND METHODS, this vector is derived from pBHSR1, but the redundant sequence of almost 100 bp, originating from the *rrn* promoter region just after the transcriptional start site, is removed. The synthetic DNA fragment of shRNA-b sequence was inserted into the *Eco*RI and *Bam*HI sites of pBHSR3 to yield pBHSR3-shb. Figure 3A shows an expected sequence of the in vivo transcript from this plasmid. Similarly to the case of shRNA-a, *R. sulfidophilum* DSM 1374 was transformed with the plasmid pBHSR3-shb. The transformant was cultivated and the RNA samples were prepared from the cells and the culture medium. These RNA samples were subjected to 10% PAGE/ 8 M urea and analyzed by Northern hybridization using probe-3 and probe-4 (Fig. 3B). As shown in Fig. 3B, clear bands longer than 165 nt (lanes 5 and 10) could be observed. Since no size markers longer than this band are in the marker lanes, the size of this band was extrapolated from the shorter size markers to be almost 200 nt. The size of the primary transcript was designed to be 199 nt (see Fig. 3A). Therefore this band seemed to be the primary transcript itself. This indicates that shRNA-b did not suffer any processing.

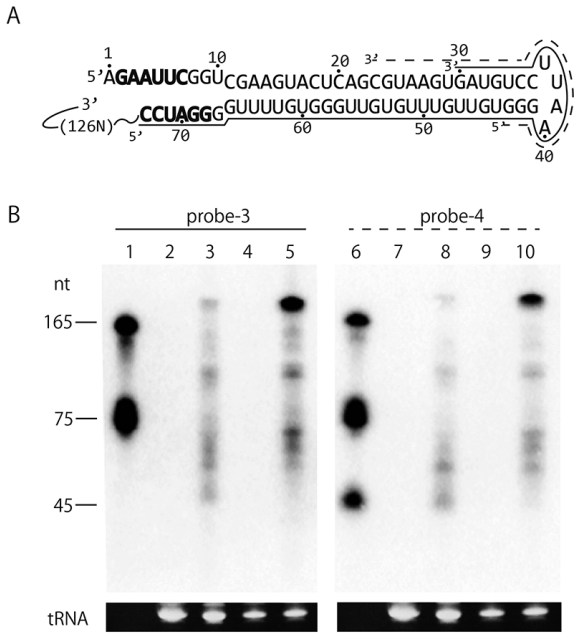

Fig. 3. Transcription of shRNA-b.

(A) Predicted nucleotide sequence and secondary structure of the expressed shRNA-b. The shRNA-b with flanking sequences is shown. 126N indicates the predicted additional length at the 3′ terminal region expressed from the plasmid pBHSR3-shb. Bold letters (GAAUUC and GGAUCC) are the *Eco*RI and *Bam*HI sites used for cloning (see MATERIALS AND METHODS). The solid line and dashed line along the sequence are hybridizable regions of probe-3 and probe-4, respectively. (B) Northern blotting analysis of the shRNA-b produced. Intracellular (lanes 2, 3, 7, and 8) and extracellular RNA samples (lanes 4, 5, 9, and 10) prepared were electrophoresed on 8 M urea-denaturing 10% polyacrylamide gels, then transferred onto Hybond-N+ membranes. The shRNA-b sequence-containing bands were detected by probe-3 (lanes 1−5) and probe-4 (lanes 6−10) as indicated at the top of the panel. RNA samples from host cells only (no plasmid) were applied to lanes 2, 4, 7, and 9 as negative controls. Lanes 1 and 6, size markers (165, 75 and 45 nucleotides long). These size markers were prepared by run-off in vitro transcription of pGEM-sh (see MATERIALS AND METHODS) and related plasmids. To compare relative amounts of the signals, tRNA bands stained with ethidium bromide on the gel are shown.

In conclusion, our extracellular production system for artificial functional RNAs using *R. sulfidophilum* can be applied for shRNA production. The shRNAs are widely used for gene knockdown. Our system may provide for the production of many useful shRNAs.

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