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Brief Communication

Aptazyme-Mediated Regulation of 16S Ribosomal RNA

Markus Wieland,[1] Barbara Berschneider,[1] Matthias D. Erlacher,[2] and Jörg S. Hartig[1,*]
[1]Department of Chemistry, Konstanz Research School Chemical Biology and the Zukunftskolleg, University of Konstanz, 78457 Konstanz, Germany
[2]Division of Genomics and RNomics, Innsbruck Biocenter, Innsbruck Medical University, 6020 Innsbruck, Austria
*Correspondence: joerg.hartig@uni-konstanz.de
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SUMMARY

Developing artificial genetic switches in order to control gene expression via an external stimulus is an important aim in chemical and synthetic biology. Here, we expand the application range of RNA switches to the regulation of 16S rRNA function in *Escherichia coli*. For this purpose, we incorporated hammerhead ribozymes at several positions into orthogonalized 16S rRNA. We observed that ribosomal function is remarkably tolerant toward the incorporation of large additional RNA fragments at certain sites of the 16S rRNA. However, ribozyme-mediated cleavage results in severe reduction of 16S rRNA stability. We carried out an in vivo screen for the identification of sequences acting as ligand-responsive RNA switches, enabling thiamine-dependent switching of 16S rRNA function. In addition to expanding the regulatory toolbox, the presented artificial riboswitches should prove valuable to study aspects of rRNA folding and stability in bacteria.

INTRODUCTION

Starting with the discovery of RNaseP (Guerrier-Takada and Altman, 1984) and self-splicing ribozymes (Bass and Cech, 1984), scientists have been revealing the fundamental roles of catalytic RNAs in nature. By solving crystal structures it became evident that even the ribosome is a ribozyme since the peptidyl-transferase reaction is catalyzed by RNA residues (Nissen et al., 2000). Moreover, ribozymes can also be part of regulatory elements such as the *glmS* mRNA-based riboswitch relying on a ligand-induced cleavage mechanism (Collins et al., 2007; Winkler et al., 2004). In addition to naturally occurring riboswitches, several artificial switches based on the hammerhead ribozyme (HHR) have been developed recently. The HHR is a small RNA motive catalyzing a phosphodiester cleavage reaction (Forster and Symons, 1987). It consists of a conserved catalytic core flanked by three helices, with helix I and II tertiary interactions locking the RNA in its catalytically active conformation (Canny et al., 2004; Khvorova et al., 2003; Martick and Scott, 2006). Inserted into the mRNA of a reporter gene in eukaryotic cells, the *Schistosoma mansonii* HHR allows for the control of gene expression upon addition of ribozyme inhibitors (Yen et al., 2006, 2004). Furthermore, aptamers have been attached to helices of the HHR in order to control ribozyme activity and thereby gene expression via addition of the respective ligand in yeast (Win and Smolke, 2007, 2008) as well as in bacteria (Wieland et al., 2009a, 2009b; Wieland and Hartig, 2008). The use of ligand-responsive ribozymes (aptazymes) should be more generally applicable than the incorporation of the ligand-binding aptamer sequence alone, since ligand recognition is transformed into an irreversible cleavage of the respective RNA. Hence, aptazymes could in principle be used for the regulation of other classes of RNA beyond mRNAs. Recently, it was demonstrated that aptazymes can also be used to control the activity of tRNAs (Berschneider et al., 2009) as well as miRNAs (Kumar et al., 2009). Here we extend the approach by engineering 16S rRNAs via inserting addressable ribozymes, enabling the regulation of translation by triggering the cleavage of the small ribosomal subunit RNA in *Escherichia coli*.

The 16S rRNA together with various proteins forms the small subunit (SSU) of the bacterial ribosome (Schluenzen et al., 2000; Wimberly et al., 2000). Among other tasks, it recruits mRNAs via the anti-ribosome binding sequence (A-RBS) located at its 3′ end and facilitates matching of mRNA and tRNA in the A site via stabilizing the codon/anticodon interaction (Shine and Dalgarno, 1974). Since interfering with the activity of any of the seven copies of the ribosomal RNA operons results in impaired growth ability (Condon et al., 1995; Hui et al., 1987), manipulations of the 16S rRNA stability are best carried out using an orthogonal translation machinery, which is not affecting gene expression levels of regular housekeeping genes. This can be achieved by introducing an orthogonal 16S rRNA/mRNA pair in *E. coli* (Hui and de Boer, 1987; Hui et al., 1987, 1988; Lee et al., 1996; Rackham and Chin, 2005). For our study, we transferred the orthogonal 16S rRNA/mRNA pair described by Rackham and Chin (2005) to our system to exclusively express an eGFP reporter gene. This setup allowed us to incorporate ligand-responsive ribozymes into the 16S rRNA, generating a system that enables external control of ribosomal function (Figure 1).

RESULTS

Implementing an Orthogonal Translation Machinery in *E. coli*

In order to manipulate the 16S rRNA without interfering with the cellular ribosomal activity, we first implemented an orthogonal 16S rRNA/mRNA pair in *E. coli* (Rackham and Chin, 2005). For

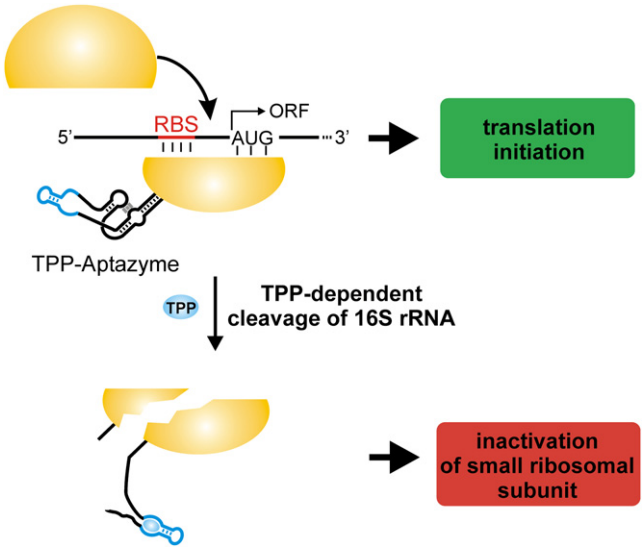

Figure 1. Simplified Scheme of Aptazyme-Mediated Inhibition of Gene Expression

An inactive TPP-dependent aptazyme incorporated into the 16S rRNA is not interfering with translation and the reporter gene is expressed. TPP-dependent activation of ribozyme activity, however, results in inhibition of the small ribosomal subunit and decreases reporter gene levels. ORF, open reading frame; yellow, ribosomal subunits.

this purpose, we changed both the ribosome binding sequence (RBS) of an eGFP reporter mRNA as well as the A-RBS of an additional ribosomal RNA operon [rrnB of the pT7-1 plasmid (Lewicki et al., 1993)]. The system was found to be truly orthogonal, since neither was any adverse effects of the orthogonal ribosome on natural mRNA gene expression (RBS•oA-RBS, terminology of the constructs as follows: RBS, natural ribosome binding sequence; oRBS, orthogonalized RBS; A-RBS, natural anti-ribosome binding sequence located in the 3′ region of the 16S rRNA; oA-RBS, orthogonalized A-RBS) observable nor was the orthogonal mRNA translated by the natural ribosome (oRBS•A-RBS) (see Figure S1 available online). In the case of co-transfection of the orthogonal pair oRBS•oA-RBS, eGFP was expressed by the artificial system at 10% of the natural (RBS•A-RBS) expression levels. The orthogonal system implemented in *E. coli* now allows the characterization of alterations of the 16S rRNA even if they impair ribosomal activity.

Insertion of a Hammerhead Ribozyme into 16S rRNA

After having established an orthogonal translation system, we surveyed the 16S rRNA for optimum sites for inserting an HHR. Such sites should fulfill two necessary requirements. First, insertion of the additional RNA motifs (of up to 120 nt in length) should not disturb the activity of the SSU in translation. Second, the activity should be inhibited upon ribozyme-mediated self-cleavage of the ribosomal RNA. To identify potential insertion sites, we considered three independent sets of information. First, the availability of huge phylogenetic data allowed for identifying sites that showed only little conservation, minimizing the danger of disrupting important features upon insertion (Van de Peer et al., 1996). Second, we examined crystal structures of the ribosome in order to prevent interference with protein, mRNA, or LSU binding (Schuwirth et al., 2005). Third, a recent study identified sites that tolerated insertion of a 31 nt sequence via a transposon-mediated strategy (Yokoyama and Suzuki, 2008). Taking these into consideration we chose seven sites within the 16S rRNA (Figure 2).

Next, we introduced HHR sequences as shown in Figure 2 into the respective sites of the 16S rRNA. We inserted the ribozymes together with connecting sequences of varying lengths in order to keep the original stem length more or less similar. For example, in helix 6 an already extended stem structure is present in the native 16S rRNA, hence we attached the HHR immediately to the end of the stem. In other places, we inserted additional connecting sequences to stabilize the catalytic core of the hammerhead. Importantly, in each site an inactive as well as an active ribozyme were inserted separately (the A to G point mutant indicated by a box in Figure 2B renders the ribozyme inactive). To measure whether insertion of the ribozyme was tolerated with respect to ribosomal activity we compared the 16S rRNAs containing the inactivated ribozymes to the orthogonal rRNA lacking the ribozyme (Figure 3A). Interestingly, high activity in translation comparable to the unmodified rRNA was observed if the inactivated HHR was inserted in helix 6. The other positions showed more or less pronounced decreases in activity. Subsequent activation via restoring the above mentioned mutation at the position in the active core of the HHR resulted in varying reduction of ribosomal activity in dependency of the insertion site. Except in helices 34 and 39, 5-fold or more inhibition was found upon self-cleavage of the ribozyme (Figure 3A). We also tested whether the length of the helix connecting the 16S rRNA and the ribozyme has an influence on ribosomal activity. When we varied the length of helix 6, similar results were obtained (Figure S2). In order to exclude that the different effects on translation in the various positions result from differential ribozyme cleavage activities, we carried out northern blots demonstrating that in all positions the active ribozymes cleaved efficiently (Figure 3B). We used a hybridization probe directed against the inserted HHR sequence to prevent the detection of the very abundant background of natural 16S rRNA. In case of inactivated HHRs, the orthogonal 16S rRNA is strongly expressed with only little degradation products visible on the blot. If activated, the full-length 16S rRNA is almost undetectable but the appearance of the probed 5′ cleavage product of the respective length demonstrates efficient and quantitative cleavage at all tested insertion sites (Figure 3B). Interestingly, all cleavage products are less abundant compared to the full-length 16S rRNA harboring the inactivated HHR. Although the ribozyme-containing 16S rRNA is cleaved and degraded efficiently at all insertion positions tested, at some positions the remaining full-length rRNA and/or the cleavage products still allow for significant translational activity (see, e.g., helices 34 and 39 in Figure 3).

Engineering Thiamine-Responsive 16S rRNA

Since insertion of the HHR in helix 6 of the 16S rRNA resulted in the highest absolute change of gene expression comparing the active and inactive ribozyme variants, we chose this position for the construction of a ligand-dependent ribosome. For this purpose, we inserted a thiamine pyrophosphate (TPP) aptamer

from the corresponding riboswitch (Serganov et al., 2006; Winkler et al., 2002) into stem III of the ribozyme (Figure 4A). We have previously shown that artificial riboswitches can be generated by incorporating TPP-dependent ribozymes (termed TPP-HHAz) into mRNAs (Wieland et al., 2009a). These ligand-dependent cleavage modules can be tuned by optimization of the connecting sequence between the aptamer and ribozyme to yield on as well as off switches of gene expression. We then randomized six nucleotides of the connection module and screened a library of *E. coli* clones for switchable variants of the ribosomal RNA upon addition of 500 μM thiamine to the growth medium. Thiamine is actively taken up and converted to TPP by *E. coli* (Settembre et al., 2003). By screening of about 4000 clones, we identified several clones that show a thiamine-dependent translation of the eGFP reporter gene. Upon addition of thiamine, gene expression levels of clone 3.1 are increased more than 5-fold, while clone 3.2 shows a more than 3-fold reduction (Figure 4B). Interestingly, the active and inactive variants of the HHR lacking the aptamer as shown in Figure 2B are not responsive to thiamine.

DISCUSSION

We describe the engineering of artificial ribosomes that can be specifically switched on or off via the external addition of a small molecule to the growth medium. We have realized these ligand-responsive ribosomes in the context of orthogonal 16S rRNA/mRNA pairs. Hence, the ribosomal RNA switch should allow for specifically switching a subset of mRNAs that carry altered RBSs. On the other hand, although the strategy of modulating rRNA function via triggered ribozyme cleavage should in principle work in eukaryotes as well, the need to use orthogonal mRNA/rRNA pairs limits the system in its present form to bacteria with defined mRNA/rRNA interactions. Within *E. coli*, the orthogonal ribosomes function sufficiently in order to implement the presented RNA switches, although reporter gene expression in the orthogonal system is reduced to 10% compared to the natural rRNA/mRNA pair. We are unable to compare the efficiency of the orthogonal system with the original report (Rackham and Chin, 2005) since Rackham and Chin used an antibiotic selection marker rather than reporter gene quantification as readout.

By attaching the inactivated HHR variants to different 16S rRNA helices we tested the tolerance of the 16S rRNA toward insertion of additional RNA sequences. Depending on the respective site, insertion of the HHR motif affected ribosome activity to varying degrees. For example, attachment to helix 17 resulted in substantial loss of gene expression (Figure 3A). In contrast, we identified helix 6 as very tolerant toward the additional ribozyme domain, showing almost no change in translation activity. In this respect, helix 6 seems to be generally suited to augment ribosomes with additional functional RNA sequences. To identify potential sites for aptazyme-mediated rRNA regulation, the results obtained with inactivated HHRs were then compared to cleavage-competent ribozymes. Interestingly, the cleavage reaction affected the ribosomal activity differently with respect to the insertion site. In general, more pronounced effects were observed with ribozymes inserted closer to the 5′ end of the rRNA (see ratios given in Figure 3A or helices 6, 10, 17, and 26). Contrastingly, cleavage close to the 3′ end did not result in pronounced inhibition compared to the inactive ribozymes. It is tempting to speculate that the folding and assembly of ribosomal RNA and proteins are already under way and hence a later cleavage of the rRNA affects ribosomal activity to a much lesser extent. In this respect it is noteworthy that many bacteria are known to contain fragmented ribosomal RNAs in their active ribosomes (Evguenieva-Hackenberg, 2005).

Subsequent northern blot analysis of the constructed HHR variants demonstrated that the active ribozymes cleaved very efficiently in vivo irrespective of the insertion site. This result proves that the HHR motif folds very robustly in vivo even in the context of long RNAs such as the 16S. The 5′-end product of the ribozyme cleavage reaction is detected by the hybridization probe. The inactivated variants show almost no degradation of 16S rRNA. Since the cleavage products of the active ribozymes are found to be much less abundant compared to the intact 16S rRNA, nuclease-mediated degradation of the cleaved 16S rRNA is likely to occur. It is known that incorrectly processed and assembled rRNAs are specifically degraded in bacteria (Deutscher, 2006, 2009). In this respect, our approach makes use of cellular mechanisms that sense and destroy apparently erroneous RNA species. The discrepancy between the residual in vivo translational activity of active ribozymes inserted at helices 34 and 39 (Figure 3A) on the one hand and the finding of almost complete ribozyme cleavage and partial degradation by northern blotting (Figure 3B) on the other could be due to ribozyme cleavage taking place during RNA isolation. Alternatively, the remaining cleavage products of helices 34 and 39 could be more active in translation compared to cleavage fragments generated at sites located closer to the 5′ end.

After identifying helix 6 as the best suited position for ribozyme-mediated control of 16S rRNA stability, we identified ligand-responsive variants using an in vivo screening procedure as we have already applied previously (Wieland et al., 2009a; Wieland and Hartig, 2008). We decided to use a naturally occurring TPP aptamer domain since, in contrast to theophylline, the most commonly used ligand for artificial RNA switches, even high concentrations of thiamine are tolerated well (Wieland et al., 2009a; Wieland and Hartig, 2008). Applying this in vivo screening, we identified two switches: clone 3.1 induces gene expression 5-fold whereas clone 3.2 exhibited a more than 3-fold inhibition of gene expression upon addition of thiamine. It is interesting to note that both on as well as off switches were identified in this context. Although the relative induction and inhibition rates of the respective clones are only moderate, the switches show satisfactory absolute changes of gene expression. Clone 3.1 spans more than 50% and clone 3.2 an even 70% of the absolute gene expression levels as defined by the maximum range of the active and inactive ribozyme constructs. Interestingly, clone 3.1 that was identified as an on switch in this work turned out to be identical to clone 2.5, identified previously in an mRNA screening context (Wieland et al., 2009a). In vitro cleavage assays carried out with the isolated TPP-dependent ribozyme displayed moderate inhibition of ribozyme activity (Wieland et al., 2009a). This not only highlights the efficacy and robustness of the applied in vivo screening method but demonstrates that the developed RNA switches are indeed transferable from one RNA class to another. In this respect, we

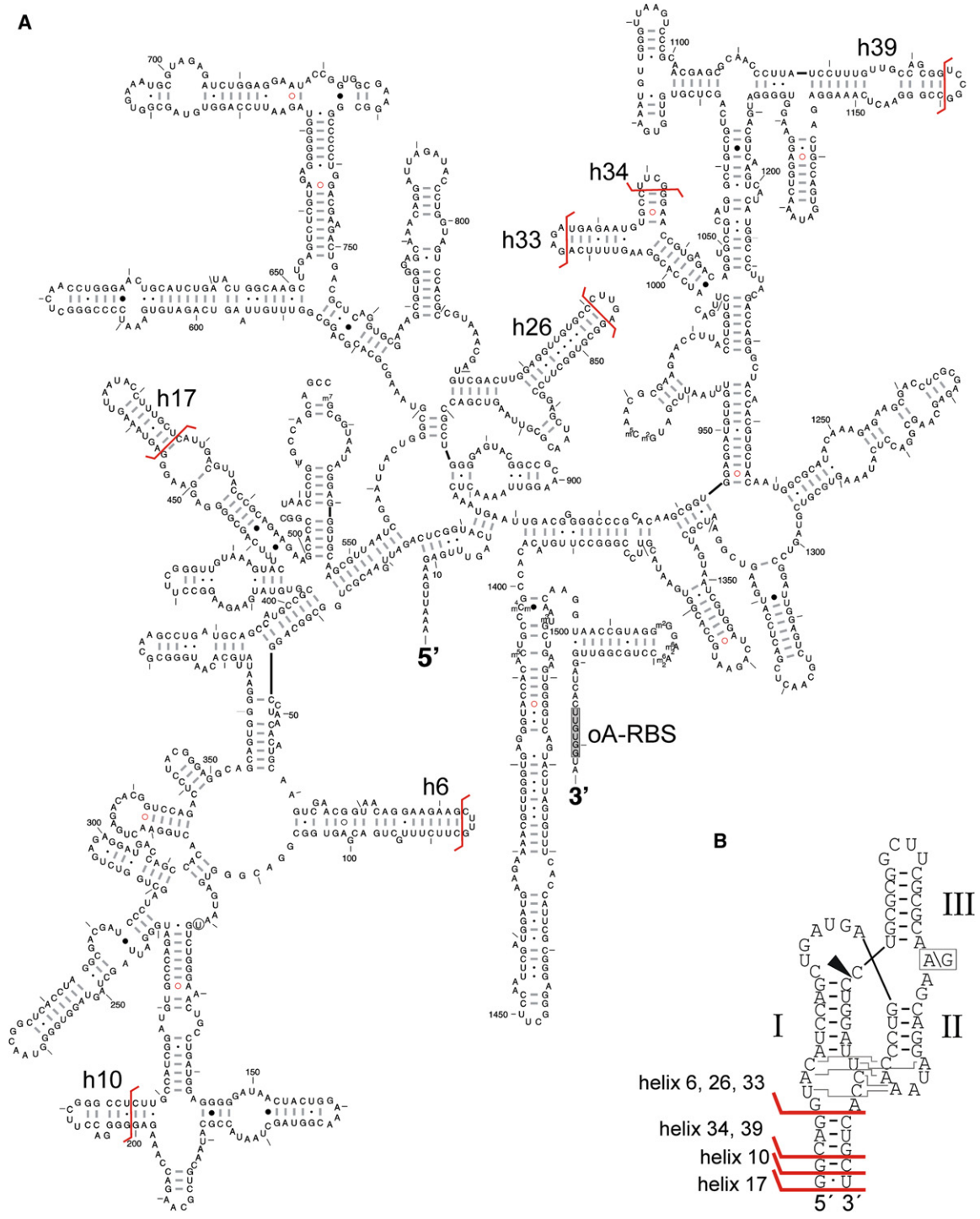

Figure 2. Insertion of the HHR into the 16S rRNA

(A) Secondary structure map [modified from Konings and Gutell (1995)] of the 16S rRNA containing the orthogonalized oA-RBS (boxed nucleotides) and the insertion sites (red lines).

(B) Nucleotide sequence and secondary structure of the inserted HHR. Red lines show the connectivity of the 16S rRNA at the respective sites. Inactivating A to G point mutation in the active core is highlighted by the boxed nucleotides.

have reported a similar transfer of a theophylline-dependent mRNA switch into the context of tRNA switching before (Wieland et al., 2009a; Wieland and Hartig, 2008). It is noteworthy that optimized communication modules such as our connection sequences have been generated by in vitro selection before. These modules allowed the exchange of different aptamer

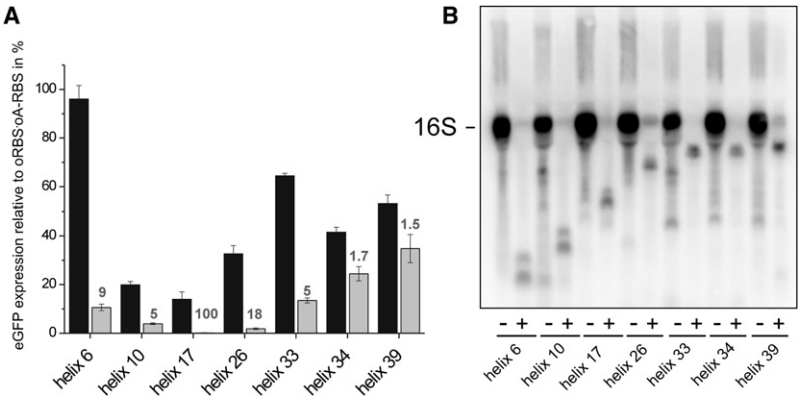

Figure 3. Effect of the HHR on Translation Efficacy and 16S rRNA Stability

(A) eGFP expression of the inserted inactive (black bars) and the active (gray bars) HHR variants. Numbers on top of the bars indicate the ratio of gene expression of inactive/active variants. Error bars represent the standard deviation of three independent experiments.

(B) Northern blot analysis of the inactive (–) and active (+) variants of the corresponding inserted HHRs using a labeled hybridization probe complementary to the 5′ region of the inserted HHR sequence.

sequences for controlling the HHR via stem II (Soukup and Breaker, 1999a, 1999b).

In conclusion, we demonstrated the successful construction of an orthogonal translational system that can be switched on as well as off via the external addition of a small molecule trigger. Surprisingly, the 16S rRNA tolerates insertion of large RNA sequences at some positions but its activity is dramatically affected upon site-specific cleavage of the 16S rRNA. If combined with other RNA-based switches, the presented strategy could be used to set up more complex information-processing devices. In addition to such synthetic biology applications the presented work should prove valuable for studying processes such as ribosome assembly and ribosomal RNA stability (Nierhaus, 1991). Since we have now proven the use of ribozymes for controlling mRNA, tRNA, and rRNA functions, ribozymes seem to be ideally suited to control a variety of RNA classes in cells.

SIGNIFICANCE

Artificial genetic switches for controlling gene expression via an external stimulus are important tools in chemical and synthetic biology. Here, we expand the application range of RNA switches to the regulation of 16S rRNA function in *Escherichia coli*. We incorporated HHRs at several positions into orthogonalized 16S rRNA. Ribosomal function is remarkably tolerant toward the incorporation of large additional RNA fragments. However, ligand-dependent ribozyme-mediated cleavage results in severe reduction of 16S rRNA stability. In addition to expanding the regulatory toolbox, the presented artificial riboswitches should prove valuable to study aspects of rRNA folding and stability in bacteria.

EXPERIMENTAL PROCEDURES

Construction of the 16S rRNA Variants

16S rRNA variants in plasmid pT7-1 and oRBS mutant in plasmid pHWB1 were constructed by performing a PCR with Phusion DNA Polymerase (Finzyme) using sequence-specific primers with the designed ribozyme sequences attached to the 5′ end of the primer (underlined) (see Supplemental Information for primer sequences). N represents an unbiased random position generated during solid phase DNA synthesis using a 1:1:1:1 mixture of nucleoside phosphoramidites. After PCR, the template plasmid was digested using the restriction enzyme *Dpn*I. The PCR products containing the ribozyme sequences were blunt end ligated (Quick Ligase; New England Biolabs) and afterwards transformed into *E. coli* BL21(DE3) gold (Stratagene) (Wieland and Hartig, 2009). Single colonies were picked and grown in LB medium supplemented with 100 μg ml^{-1} carbenecilline (Roth) or 25 μg ml^{-1} chloramphenicol (Roth). Successful cloning was verified by plasmid isolation and sequencing (Miniprep Kit; QIAGEN).

Bacterial Cultivation and eGFP Expression

For determining eGFP expression, pHWB1 oRBS and the corresponding pT7-1 plasmids were cotransformed into *E. coli* BL21(DE3) gold and grown in LB medium supplemented with 100 μg ml^{-1} carbenecilline and 25 μg ml^{-1} chloramphenicol. An overnight culture was used to inoculate a fresh culture at 25°C and 250 μM IPTG (isopropyl-beta-D-thiogalactopyranoside) was added to start transcription of the rrn operon when culture OD_{600} reached 0.5. Cultures were grown overnight (at least 12 hr) at 25°C and 300 rpm in 96 well plates. Subsequently, 100 μl of each culture was transferred into 96 well microplates and the fluorescence of the expressed eGFP was determined (excitation wavelength 488 nm and emission wavelength 535 nm; TECAN M200 plate reader). For background subtraction, an *E. coli* BL21(DE3) gold culture lacking eGFP was equally treated. The thiamine concentration-dependent eGFP measurements of the TPP-dependent clones were achieved equally except using M9 minimal medium instead of LB medium. Thiamine was added to the medium at indicated concentrations.

In Vivo Screening for TPP-Dependent Artificial Riboswitches

For the screening of TPP-dependent sequences, clones of the cotransformation of pHWB1 oRBS and pT7-1 h6 TPP-HHAz pool plasmids were picked into M9 minimal medium supplemented with 100 μg ml^{-1} carbenecilline and 25 μg ml^{-1} chloramphenicol. The library was replicated to 384 deep well plates and grown in the absence and presence of 500 μM thiamine at 25°C and 300 rpm. When culture OD_{600} reached 0.5, transcription of the additional rrn operon was induced by the addition of 250 μM IPTG. After at least 12 hr, 100 μl of each culture were transferred into 96 well plates and eGFP expression was determined via fluorescence. Finally, expression levels from each clone in the absence and presence of 500 μM thiamine were compared.

Northern Blot Analysis

Total RNA was isolated as follows: 10 ml cells were grown in LB medium supplemented with 100 μg ml^{-1} carbenecilline and 25 μg ml^{-1} chloramphenicol, induced with 250 μM IPTG when they reached E_{260} = 0.5, and followed by 3 hr transcription of the rrn operon at 25°C. After centrifugation, cells were resuspended in 10 ml of protoplasting buffer [15 mM Tris HCl (pH 8.0), 0.45 M sucrose, and 8 mM EDTA] and 80 μl of 50 mg/ml lysozyme was added and samples were incubated on ice for 15 min. The resulting protoplasts were resuspended after centrifugation in 500 μl of lysing buffer [10 mM Tris HCl (pH 8.0), 10 mM NaCl, 1 mM sodium citrate, and 1.5% (w/v) SDS] and 15 μl DEPC. Subsequent to incubation at 37°C, 250 μl of a saturated NaCl solution was added and the samples were kept on ice for 10 min and then centrifuged at high speed. The RNA in the supernatant was precipitated and purified by adding 1 ml of 100% ethanol. 20 μg of total RNA was loaded on a denaturing 0.8% agarose gel (2.2 M formaldehyde). Following standard capillary transfer

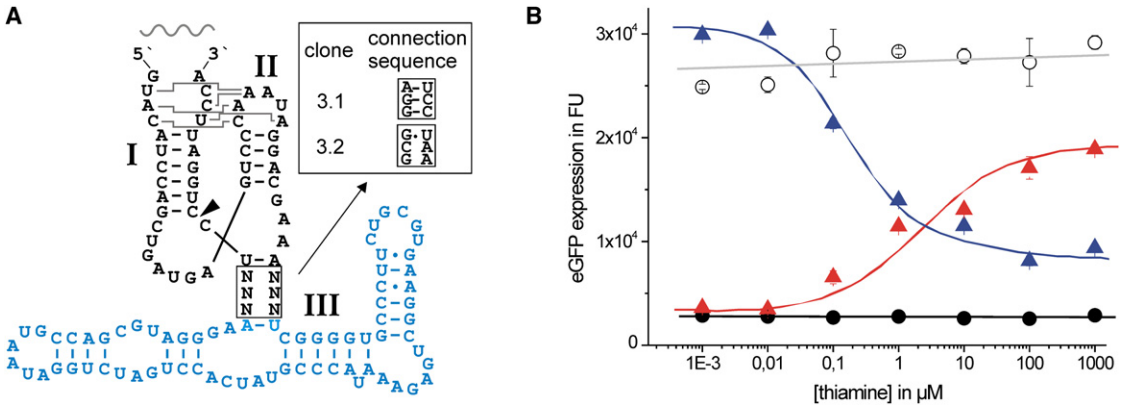

Figure 4. TPP-Mediated Regulation of Translation

(A) Nucleotide sequence of the TPP-dependent aptazymes (TPP-HHAz) inserted in helix 6 of the 16S rRNA. The TPP aptamer domain (Serganov et al., 2006; Winkler et al., 2002) inserted in stem III of the HHR is shown in blue; randomized nucleotides for in vivo screening are boxed. (Inset) Sequence of switches identified as on (3.1) and off switches (3.2).

(B) eGFP expression of the isolated clones 3.1 (red triangle) and 3.2 (blue triangle) is activated 5-fold and inhibited 3-fold, respectively, upon addition of thiamine. Gene expression levels of the inserted active (black circle) or inactivated (open circle) HHR clones lacking the aptamer are not affected by the addition of thiamine. Error bars represent the standard deviation of three independent experiments.

procedures, RNA was transferred in 20× SSC [175.3 g/l NaCl and 88.2 g/l citrate (pH 7.0)] to a positively charged nylon membrane (Nytran SPC). Subsequently, RNA was immobilized on the membrane by irradiation on a BioDocAnalyze (Biometra) for 2 min. After 60 min of prehybridization in ULTRAhyb-Oligo hybridization buffer (Ambion), 200 pmol of a 5′ ^{32}P-labeled probe (5′-CGCACCGAAGCGCGTCTCGTCCTATTTGGGACTCATCAGCTGG-3′, complementary to the inactivated HHR, and 5′-CGCACCGAAGCGCGTTTC GTCCTATTTGGGACTCATCAGCTGG-3′, complementary to the active HHR) was added and the blot was incubated over night at 42°C. Next, the blot was washed twice in buffer [2× SSC and 0.5% (w/v) SDS]. Visualization was performed using phosphorimaging.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.chembiol.2010.02.012.

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