RNase III-dependent down-regulation of \textit{ftsH} by an artificial internal sense RNA in \textit{Anabaena} sp. PCC 7120

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Received 22 March 2013; revised 16 April 2013; accepted 23 April 2013. Final version published online 13 May 2013.

DOI: 10.1111/1574-6968.12165

Editor: Olga Ozoline

Keywords
RNase III activity; \textit{aafsH} interference; \textit{Anabaena}.

Abstract
RNase III is a group of dsRNA-specific ribonucleases that play important roles in RNA processing and metabolism. Alr0280 and Al4107 in \textit{Anabaena} sp. PCC7120 are highly similar to RNase III enzymes. \textit{In vitro}, recombinant Alr0280 showed RNase III activity. In the same cyanobacterium, the expression of \textit{ftsH} (FtsH protease) could be suppressed by overexpression of an artificial sense RNA (\textit{aafsH}) that was complementary to \textit{afsH}, an internal antisense RNA. The \textit{aafsH} interference was abolished by inactivation of \textit{alr0280}, the RNase III-encoding gene, and restored by complementation of the mutant. A cyanobacterial homolog to \textit{hen1}, an RNA methyltransferase gene, may also be required for the \textit{aafsH} interference. This is the first report of RNase III-dependent sense RNA interference in cyanobacteria, and the underlying mechanism remains to be elucidated.

Introduction
RNase III is a group of double-stranded (ds) RNA-specific endonucleases found in both prokaryotes and eukaryotes (Conrad & Rauhut, 2002). They are phosphodiesterases that produce 5′-monophosphate and 3′-hydroxyl termini with a 2-nucleotide overhang at the 3′ end. A divergent metal activation is required for the catalytic activity (Dunn, 1982; Meng & Nicholson, 2008). RNase III can be divided into four classes based on the molecular weight and the polypeptide structure (Arraiano et al., 2010). Class I RNase III enzymes are composed of an endonuclease domain (endoND) and a dsRNA binding domain (dsRBD). Class 2 enzymes, called the Drosha family, contain two endoNDs and one dsRBD. Class 3 enzymes, called the Dicer family, include the largest RNase III that bear two endoNDs, one dsRBD, a DEAD-box helicase domain, and a PAZ domain. Class 4 only includes the mini-RNase III of \textit{Bacillus subtilis}, which has an endoND but no dsRBD.

RNase III enzymes function as homodimers, in which the two ribonuclease domains form a single processing center, each cleaving one strand of dsRNA (Zhang et al., 2004). The endoND contains a stretch of highly conserved amino acid residues, ERLEFLGD, known as the RNase III signature motif that contributes to an important portion of the catalytic center (Blaszczyk et al., 2004). For example, in \textit{Aquifex aeolicus} RNase III, this motif is located at amino acid (aa) residues 37–44. Of six aa residues that constitute the catalytic center, E37 is required for protein dimerization, E64 for substrate recognition and scissile-bond selection, E40, D44, D107, and E110 for hydrolysis of the scissile bond (Gan et al., 2006). In \textit{Escherichia coli} (\textit{E. coli}) RNase III, the 8 aa signature motif is located at residues 38–45, while the catalytic center is composed of E38, E65, E41, D45, D114, and E117 (Sun et al., 2004). Unlike endoND, dsRBD may not be essential for the activities of RNase III enzymes. A truncated form of \textit{E. coli} RNase III lacking dsRBD accurately cleaved small processing substrates (Sun et al., 2001). \textit{Giardia intestinalis} Dicer without the C-terminal dsRBD showed the catalytic activity \textit{in vivo} and \textit{in vitro} (Macrae et al., 2006).

RNase III enzymes play important roles in RNA processing, post-transcriptional gene regulation, and decay of RNAs (Nicholson, 1999). Especially, RNase III enzymes are involved in small interfering RNA (siRNA)-, microRNA (miRNA)-, or antisense RNA-mediated gene regulation (Ji, 2008). siRNAs- and miRNAs-mediated gene regulation, also called RNAi (RNA interference), is eukaryote specific, while antisense RNA-mediated gene regulation is found in both eukaryotes and prokaryotes (Brantl, 2002).
Cyanobacteria are a group of oxygenic photosynthetic prokaryotes. Certain N₂-fixing species of *Anabaena*/*Nostoc* has been proposed to be the ancestor of chloroplasts (Deusch et al., 2008). Antisense RNAs have been found in different groups of cyanobacteria (Csiszar et al., 1987; Dühring et al., 2006; Herrández et al., 2006, 2010; Steglich et al., 2008; Georg et al., 2009), and their regulatory effects have been shown in at least two species (Dühring et al., 2006; Herrández et al., 2006, 2010). In a previous report, we described a small internal antisense RNA (aftsH) of *all3642* (*ftsH*, encoding a transmembrane protein with cytosolic AAA-ATPase and Zn²⁺-metalloprotease domains) in *Anabaena* sp. PCC 7120 (*Anabaena* 7120) (Gong & Xu, 2012). When overexpressed, aftsH inhibited the expression of *ftsH* gene. Here, we report that overexpression of the sense RNA, which could form duplex RNA with the endogenous aftsH, also suppressed the expression of *ftsH*. Of two predicted RNase III proteins, the one showing activity in vitro was required for the down-regulation of *ftsH* by the artificial sense RNA. Consistently, a *Hen*1 homolog appeared to promote the RNase III- and sense RNA-mediated gene regulation.

**Materials and methods**

**Strains, culture conditions and transformation**

*Anabaena* 7120 was obtained from the Freshwater Algae Culture Collection of Institute of Hydrobiology, Chinese Academy of Sciences. The wild-type and its derivatives were cultured in BG11, as previously described (Ning & Xu, 2004). Conjugative transfer of plasmids into *Anabaena* 7120 was performed, as described by Elhai and Wolk (Elhai & Wolk, 1988). *Anabaena* mutant was generated by *sacB*-based positive selection of double-crossover homologous recombination (Cai & Wolk, 1990). Complete segregation of the mutant was confirmed by PCR.

**Plasmid construction**

Molecular manipulations were performed using standard methods or per manufacturers’ instructions. PCR fragments cloned into pMD18-T (Takara) were confirmed by sequencing. Plasmid construction processes are detailed in Supporting Information, Table S1 in supporting information but briefly described below.

1. Plasmids used to inactivate *alr0280* or *alr3730* in *Anabaena* 7120. DNA fragments carrying *alr0280*::Em' was generated using two T-vectors, pHB518 and pHB576, as described by Zhang et al. (2007). *alr3730*::Em' was generated by positioning a Cm'Em' cassette between the 5'-end and 3'-end fragments of *alr3730* in the T-vector pMD18-T (Takara, Japan) and deleting the Cm' marker. *alr0280*::Em' and *alr3730*::Em’ were cloned into a *sacB*-based positive-selection vector (Cai & Wolk, 1990), respectively, producing pHB3315 and pHB4057.

2. Plasmids used to produce recombinant Alr0280, Alr1158, or All4107 in *E. coli*. DNA fragments containing *alr0280*, alr1158, or *all4107* were cloned into pMD18-T, then excised and cloned into pET21b, producing pHB4046, pHB4556, or pHB4047.

3. Plasmids used to overexpress aftsH in *Anabaena*. *P_rbcL-aftsH* generated by overlap PCR (Horton et al., 1989) was cloned into pMD18-T, then the *omega* cassette (Prentki & Krish, 1984) was inserted downstream of *P_rbcL-aftsH*. *P_rbcL*aftsH-oom generated by PCR were cloned into pMD18-T, then P_rbcL was excised and cloned upstream of aftsH-oom. *P_rbcL-aftsH-omega* and *P_rbcL-aftsH-oom* were cloned into pDC8 (Zhang et al., 2007), respectively, producing pHB3144 and pHB3920.

4. Plasmids used to complement the mutants and overexpress aftsH. Addition of *alr0280* with upstream sequence to pHB3920 produced pHB4315 for complementing the *alr0280* mutation and overexpressing aftsH. Addition of *alr3730* with upstream sequence to pHB3920 produced pHB4327 for complementing the *alr3730* mutation and overexpressing aftsH.

5. Plasmids used to detect antisense transcription within *all3642*. DNA fragments generated by PCR were first cloned in pMD18-T, then excised and cloned upstream of gfp in pHB912 (Smal- and Sse8387I-cut pHB1071) (Wang & Xu, 2005), generating plasmids pHB2919 to pHB2923.

**Transcriptional analyses**

qRTPCR and Northern blot hybridization were performed, as previously described (Zhang et al., 2007; Gao & Xu, 2012) with modifications. Total RNA extracted from *Anabaena* 7120 with TRItol reagent was treated with RNase-free DNase I to eliminate chromosomal DNA. For qRT-PCR, total RNA was reverse-transcribed, and samples were tested in triplicates. Two independent experiments showed consistent results. *rnpB* (RNase P subunit B) (Vioque, 1992) was used as the internal control. Primers for *rnpB* and *all3642* were rnpB-RT-1/rnpB-RT-2 and 3642-RT-1/3642-RT-2, respectively. For Northern blot hybridization, DNA probes were prepared by PCR using primers rnpB-N1/rnpB-N2 or 3642-1/3642-N2 and labeled by incorporation of digoxigenin-dUTP.

**Production, purification, and activity assays of recombinant proteins**

His₆-tagged RNase III was overexpressed in *E. coli* BL21 (DE3) containing the plasmids pHB4046, pHB4556, or pHB4047, purified from total soluble proteins using
His-Bind Purification Kit (Novagen) and desalted using microcon YM-3 or YM-10 (MILLIPORE) per manufacturers’ instructions. Proteins were stored in 20 mM Tris-HCl (pH 8.0), 50 mM NaCl, 1 mM EDTA, 0.5 mM DTT, and 50% glycerol at −20 °C. Protein concentrations were determined by the Bradford method (Kruger, 2002).

Four micrograms of recombinant proteins were incubated with 0.5 μg of 30-bp biotin-labeled dsRNA or 30-nt ssRNA (Fig. S1) in reaction buffer containing 30 mM Tris-HCl (pH 8.0), 100 mM KCl, 0.1 mM EDTA, 0.1 mM dithiothreitol (DTT), and 10 mM MgCl₂ at 25 °C for different periods of time. The reaction mixture was then transferred to an ice bath and mixed with an equal volume of loading buffer (95% formamide, 0.025% SDS, 0.025% xylene cyanol, 0.025% bromophenol blue). After heating at 95 °C for 5 min, samples were separated by electrophoresis on a 15% polyacrylamide-urea gel and electro-blotted onto Immobilon-Ny⁺ membranes (Millipore). Detection of the biotin-labeled RNA was performed using North2South Chemiluminescent Detection Kits (Thermo).

Results

**arl0280 encodes RNase III in Anabaena 7120**

In the CyanoBase (http://genome.microbedb.jp/cyanobase), *arl0280* and *all4107* in *Anabaena 7120* were annotated as RNase III-encoding genes. A homolog search in NCBI GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) also identified *arl1158* as a candidate RNase III gene. *Ahr0280* and *All4107* both contain an N-terminal endoND and a C-terminal dsRBD, while *Alr1158* only possesses an endoND (Fig. S2A). Bacterial endoNDs possess a stretch of conserved residues, which is known as the RNase III signature motif (Blaszczyk et al., 2004). An alignment of partial sequences in predicted RNase III enzymes from *Anabaena* and other bacteria is shown in Fig. S2B. *Ahr0280* possesses the 8-aa motif, ERLEFLGD, *All4107* has two substitutions, while *Alr1158* does not have such a motif. In *E. coli* RNase III, E38, E41, D45, E65, D114, and E117 are required for the catalytic activity. Corresponding to D114, *Ahr0280* has a serine residue as the substitute; to E65, *All4107* has a valine residue as the substitute.

These three genes were cloned and expressed in *E. coli*. The recombinant *Ahr0280* was purified by Ni²⁺-affinity chromatography to homogeneity, while the recombinant *All4107* and *Alr1158* were only partially purified (Fig. S3). These proteins were assayed *in vitro* for RNase III activity. A 30-bp biotin-labeled dsRNA and a 30-nt biotin-labeled ssRNA were used as the substrates. As shown in Fig. 1, the recombinant *Ahr0280* cleaved dsRNA. After denaturation of the dsRNA products, a biotin-labeled RNA strand slightly larger than 20 nt was detected. Neither *Alr1158* nor *All4107* showed RNase III activity under the same conditions. Also, none of the three proteins showed activity to cleave the 30-nt ssRNA (Fig. S4). Of the three genes, at least *arl0280* encodes RNase III.

**arl0280 is required for aaftsH-induced down-regulation of ftsH**

RNase III enzymes play a role in antisense RNA-mediated gene regulation by cleaving the antisense RNA/mRNA duplex (Krinke & Wulf, 1987, 1990; Blomberg et al., 1990; Vogel et al., 2004; Huntzinger et al., 2005). Previously, we identified an antisense RNA called *aaftsH* internal to *all3642* (*ftsH*) in *Anabaena 7120*; Northern blot analysis showed that overexpression of *aaftsH* from P*rbcL* (the promoter of *rbcL* that encodes the large subunit of ribulose-1, 5-bisphosphate carboxylase) suppressed the expression of *ftsH* (Gong & Xu, 2012). However, when overexpressed from P*rbcL*, the sense RNA that was complementary to *aaftsH*, named *aftsH*, also suppressed the expression of *all3642* (Fig. 2).

To promote quantitative analysis of *ftsH* mRNA, we employed qRT-PCR to examine the regulation of mRNA
level by \( \text{aftsH} \) or \( \text{aafsH} \) using the primers indicated in Fig. 3. One primer was located within \( \text{aftsH} \), while the other one was located in a region upstream of \( \text{aftsH} \) with very low background antisense transcription. Using \( \text{gfp} \) as the reporter gene, we detected the antisense transcription of a series of fragments within \( \text{all3642} \). An additional antisense transcription was identified in a fragment located between chromosomal bp 4393694 and 4394264 (pHB2921), which was coincident with the antisense transcription from chromosomal bp 4394269 identified by Mitschke \textit{et al.} (2012) (Fig. 3). However, this antisense transcription apparently terminated before crossing the region bracketed by the primers (Fig. 3). Consistent with this result, directional deep RNA sequencing had identified no additional antisense transcription in this region (Flaherty \textit{et al.}, 2011). Therefore, this pair of primers can be used to evaluate the mRNA level of \( \text{all3642} \). qRT-PCR produced similar results with the Northern blot analysis, and the suppression of \( \text{ftsH} \) mRNA level by \( \text{aftsH} \) was even stronger than that by \( \text{aafsH} \) (Fig. 4).

We wondered whether \( \text{alr0280} \) was involved in the regulation by \( \text{aftsH} \) or \( \text{aafsH} \). An \( \text{alr0280} \) mutant of \textit{Anabaena} 7120 was generated by interrupting \( \text{alr0280} \) with an \( \text{Em}^r \) marker (DRHB3315, Table S1), and \( \text{aftsH} \) or \( \text{aafsH} \) was overexpressed in the mutant as in the wild-type strain. Inactivation of \( \text{alr0280} \) showed no influence on the regulation of \( \text{ftsH} \) mRNA level by \( \text{aftsH} \) (Fig. 4a) but almost abolished the regulation by \( \text{aafsH} \) (Fig. 4b). In order to complement the \( \text{alr0280} \) mutant, we cloned \( \text{alr0280} \) with upstream sequence into the plasmid carrying \( \text{P}_{\text{rbcL}}-\text{aftsH-oop} \) and introduced the resulting plasmid into the \( \text{alr0280} \) mutant. The addition of \( \text{alr0280} \) restored the strong suppression of \( \text{ftsH} \) mRNA level by \( \text{aafsH} \) in the \( \text{alr0280} \) mutant (Fig. 4b). The \( \text{aafsH} \) interference depended upon RNase III.

**RNase III-dependent \( \text{aafsH} \) interference may involve a Hen1 homolog**

In \textit{Anabaena variabilis}, there is a Hen1 homolog that is involved in RNA methylation and repair (Chan \textit{et al.}, 2009), and the Pnkp/Hen1 RNA repair system exhibits broad substrate specificity (Zhang \textit{et al.}, 2012). Because Hen1 in plants is required for accumulation of miRNA (Yu \textit{et al.}, 2005), we wondered if the \textit{Anabaena} Hen1 is related to the \( \text{aafsH} \) interference. In \textit{Anabaena} 7120, \( \text{alr3730} \) is the predicted Hen1-encoding gene. A \( \text{hen1} \) mutant was generated by interruption of the gene with an \( \text{Em}^r \) marker (DRHB4057, Table S1). For unknown reason, inactivation of \( \text{alr3730} \) decreased the level of \( \text{ftsH} \) mRNA (Fig. 4c). In contrast to that in the wild type, introduction of \( \text{P}_{\text{rbcL}}-\text{aafsH-oop} \) on plasmid to the mutant up-regulated the expression of \( \text{ftsH} \), and addition of \( \text{alr3730} \) with upstream sequence to the plasmid partially restored the \( \text{aafsH} \) interference (Fig. 4c). The Hen1 homolog appeared to play a role in the \( \text{aafsH} \) interference.

**Discussion**

RNase III enzymes are involved in generation of functional RNAs and gene regulation by noncoding RNAs therefore participate in many cellular activities (Nicholson, 1999; Blaszczyk \textit{et al.}, 2004). Even though many noncoding RNAs have been found in cyanobacteria, no study of cyanobacterial RNase III has been reported to date. In this study, we identified \( \text{alr0280} \) as the RNase III-encoding gene in \textit{Anabaena} 7120 and showed its role in down-regulation of \( \text{ftsH} \) by an artificial sense RNA.

In \textit{Anabaena} 7120, \( \text{Alr0280} \), \( \text{Alr1158} \), and \( \text{All4107} \), all contain a region similar to endoND. However, \( \text{Alr1158} \) lacks the 8-aa signature motif that is required for the formation of catalytic center (Blaszczyk \textit{et al.}, 2004), while \( \text{All4107} \) lacks E65 (numbered as in \textit{E. coli} RNase III) that is involved in substrate recognition and scissile-bond selection (Blaszczyk \textit{et al.}, 2001; Sun \textit{et al.}, 2004;
Fig. 3. Detection of antisense transcription within all3642 using gfp reporter gene. The upper part shows DNA fragments within all3642 cloned upstream of gfp in Anabaena-Escherichia coli shuttle plasmids pHB2938 and pHB2919-pHB2923. These fragments were generated by PCR using the same primer at the 5′ end but different primers at the 3′ end. Bent lines with arrowheads indicate transcription at different start sites. The solid leftward line stands for transcription of all3642, and the solid rightward line stands for transcription of aftsH. Dashed lines with arrowheads stand for other transcriptional start sites within all3642 detected by RNA sequencing (Mitschke et al., 2012). Multiple transcriptional start sites of all3642 are not indicated here. The two arrowheads opposite each other indicate primers (bracketing Anabaena chromosomal bp 4394859-4395029) for qRT-PCR (Table S1). all3642 is located at chromosomal bp 4395350-4393464, while aftsH is located at bp 4394967-4395199. The lower part shows bright-field (I) and GFP-fluorescence (II) microscopic images of Anabaena 7120 harboring these plasmids. Heterocysts were induced by nitrogen stepdown in BG11 minus nitrate.

Fig. 4. qRT-PCR analysis of ftsH mRNA showing the role of alr0280 and alr3730 in aftsH- or aaftsH-induced down-regulation of ftsH in Anabaena 7120. (a) Overexpression of aftsH in the wild type (wt) or alr0280 mutant. 1, wt; 2, wt with P\text{rbcL}-aftsH-omega; 3, alr0280::Em\textsuperscript{r}; 4, alr0280::Em\textsuperscript{r} with P\text{rbcL}-aftsH-omega. (b) Overexpression of aaftsH in the wild type or alr0280 mutant. 1, wt; 2, wt with P\text{rbcL}-aaftsH-oop; 3, alr0280::Em\textsuperscript{r}; 4, alr0280::Em\textsuperscript{r} with P\text{rbcL}-aaftsH-oop; 5, alr0280::Em\textsuperscript{r} with P\text{rbcL}-aaftsH-oop. (c) Overexpression of aaftsH in the wild type or alr3730 mutant. 1, wt; 2, wt with P\text{rbcL}-aaftsH-oop; 3, alr3730::Em\textsuperscript{r}; 4, alr3730::Em\textsuperscript{r} with P\text{rbcL}-aaftsH-oop; 5, alr3730::Em\textsuperscript{r} with P\text{rbcL}-aaftsH-oop.
Gan et al., 2006). Like All4107, *Thermotoga maritima* RNase III has a valine residue at the position corresponding to E65 but cleaves dsRNA \textit{in vitro}: the role of E65 in RNase III activity could have been replaced by the adjacent glutamic acid residue (Sun et al., 2004). In All4107, no such a glutamic acid residue can be found. In Alr0280, there is a substitution at the conserved site D114 (numbered as in *E. coli* RNase III), which is involved in hydrolysis of the scissile bond (Gan et al., 2006). As in *Helicobacter pylori* RNase III, it is substituted by serine. Due to a potential functional redundancy of E41 and D114 (Sun et al., 2004), the substitution at D114 may not necessarily eliminate the RNase III activity. Consistent with these analyses, Alr0280 showed RNase III activity \textit{in vitro}, while the other two did not. Even so, we cannot exclude the possibility that All4107 has RNase III activity \textit{in vivo} but with a different function.

Bacterial RNase III enzymes can play important roles in antisense RNA-mediated gene regulation (Krinke & Wulff, 1987, 1990; Blomberg et al., 1990; Vogel et al., 2004; Huntzinger et al., 2005). However, in *Anabaena* 7120, inactivation of alr0280 showed little effect on aftsH-induced down-regulation of *ftsH*. This could be due to the alternative mechanism that involves RNase E (Thomason & Storz, 2010). For example, in *Salmonella enterica*, AmgR-induced degradation of *mgtC* mRNA is RNase E dependent (Lee & Groisman, 2010). Usually, used as a control to show the effect of antisense RNA on target mRNA, overexpression of the complementary sense RNA should increase the mRNA level due to titration of the antisense RNA. In the unicellular cyanobacterium *Synechocystis* 6803, the sense RNA anti-istrR enhanced the expression of isiA by reducing istrR (Dühring et al., 2006). However, in *Anabaena* 7120, overexpression of aftsH greatly reduced the level of *ftsH* mRNA. To our knowledge, this is the first report of down-regulation of gene expression by anti-antisense RNA in bacteria. Because inactivation of alr0280 eliminated the aftsH interference and complementation of the mutant restored the interference, we conclude that the aftsH interference is dependent on RNase III.

The aftsH interference resembles RNA interference (RNAi) in some aspects: it is induced by a sense RNA that may form RNA duplex with an internal antisense RNA, dependent on RNase III activity and also dependent on a Hen1 homolog. However, no gene for argonaute protein (Hutvagner & Simard, 2008), the key player in RNAi, can be found in the genome of *Anabaena* 7120. Unlike the plant Hen1, the cyanobacterial homolog is in complex with Pnkp, and the Pnkp/Hen1 complex shows activity of ligase, in addition to methyltransferase, with colicin-cleaved tRNA (Chan et al., 2009) and probably many other small RNA as substrates (Zhang et al., 2012). Therefore, the aftsH interference is probably based on a different mechanism.

Both aftsH and aftsH are predicted to form extensive stem-loop structures (Fig. S5). Hypothetically, aftsH and aftsH form dsRNAs that are processed by RNase III into small dsRNAs. If some of the small dsRNAs are converted into ssRNAs, those partially matching up with each other (for example, those from the stem-loop regions) could form stem-open loop structures. According to Zhang et al. (2012), the two ends of open loops can be efficiently ligated by the Pnkp/Hen1 complex. Due to methylation at the ligation site, such small RNAs may be accumulated and cause inhibition of translation on *ftsH* mRNA, leading to RNase E-dependent degradation of the mRNA (Thomason & Storz, 2010).

In *Synechocystis* 6803, anti-istrR does not induce the degradation of isiA mRNA (Dühring et al., 2006). Therefore, sense RNA interference may be limited to *Anabaena* sp. It remains to be tested whether artificial internal sense RNA or dsRNA can induce the degradation of other mRNAs in *Anabaena* 7120. At least, our results showed a key role of RNase III in the aftsH interference. Also, for the first time, cyanobacterial Hen1 was related to the regulation of mRNA level.

**Acknowledgements**

This project was supported by the National Natural Science Foundation of China (Grant 31270132). The authors declare that there is no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Sequences of aftsH and synthetic double- or single-stranded RNAs.
Fig. S2. Bioinformatic analyses of candidate RNase III proteins in Anabaena 7120.
Fig. S3. SDS-PAGE analysis of purified recombinant proteins.
Fig. S4. Ribonuclease activity assay using 30-nt biotin-labeled ssRNA as substrate.
Fig. S5. Predicted secondary structures of aftsH (A, B) and aafsH (C, D).

Table S1. Anabaena strains, plasmids, and primers.