A small internal antisense RNA (*aftsH*) of *all3642* (*ftsH*) in *Anabaena* sp. PCC 7120

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Received September 12, 2011; accepted November 11, 2011

In bacteria, FtsH proteases are involved in quality maintenance of membrane proteins and cellular regulation at the level of protein stability. Among the 4 predicted *ftsH* genes in the cyanobacterium *Anabaena* sp. PCC 7120, namely *alr1262*, *all3642*, *all4776* and *all4936*, at least the first two showed reduced transcription in specialized N₂-fixing cells called heterocysts. In contrast, the transcription of a small internal antisense RNA (*aftsH*) of *all3642* was up-regulated in heterocysts. Overexpression of the antisense RNA suppressed the expression of *all3642*, *alr1262* and *all4776*. Control of promoter and modulation by antisense RNA may both contribute to gene regulation in heterocysts of filamentous cyanobacteria.

antisense RNA, *Anabaena* 7120, *ftsH* genes

Citation: Gong Y M, Xu X D. A small internal antisense RNA (*aftsH*) of *all3642* (*ftsH*) in *Anabaena* sp. PCC 7120. Chin Sci Bull, 2012, 57: 756–761, doi: 10.1007/s11434-011-4929-6

Cyanobacteria are a group of prokaryotes that perform oxygenic photosynthesis [1]. *Anabaena/Nostoc* sp. PCC 7120 (*Anabaena* 7120) can produce specialized N₂-fixing cells, called heterocysts, along their uniseriate filaments upon nitrogen stepdown. Unlike vegetative cells, heterocysts possess a secondary envelope consisting of the outside polysaccharide layer and the inner glycolipid layer, perform N₂-fixation and show significantly enhanced respiratory activity, but no longer perform oxygenic photosynthesis and cell division [2]. During the development process from vegetative cells to heterocysts, a large number of genes are up- or down-regulated [3].

FtsH is a transmembrane protein with a main cytosolic region consisting of AAA-ATPase and Zn²⁺-metalloprotease domains [4]. It is involved in cellular regulation at the level of protein stability and quality maintenance of membrane proteins. In the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 (*Synechocystis* 6803), one of the *ftsH* genes, *slr0228*, has been extensively studied. The *slr0228*-encoded FtsH was found to be required for quality control

of PSII [5], induction of inorganic carbon acquisition complex [6], and involved in osmoregulation by degrading GgpS [7]. In *Anabaena* 7120, no studies on FtsH-encoding genes have been reported.

Noncoding RNAs, especially antisense RNAs, have been found in different groups of cyanobacteria [8–13], and the regulatory effects of antisense RNAs have been shown in at least 2 species. In *Synechocystis* 6803, over-expression of an internal antisense RNA, *isrR*, significantly inhibited the expression of the target gene *isiA* [9]. In *Anabaena* 7120, the *furA* gene (*all1691*) is not only transcribed in its own direction, but also co-transcribed with *alr1690* in reverse direction, and interruption of *alr1690* increased the expression level of FurA protein [12]. Also, a non-coding RNA of about 60 nt specifically transcribed in heterocysts has been identified in *Anabaena* 7120 [14], but its role in gene regulation remains to be elucidated. More recently, some antisense RNAs of genes relating to heterocyst differentiation were identified by mRNA sequencing [10]. In this study, an internal antisense RNA of a gene encoding FtsH was identified in *Anabaena* 7120. The antisense RNA showed inhibitory effects on *ftsH* genes, two of which were down-regulated in heterocysts.

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1 Materials and methods

1.1 Strains and culture conditions

Anabaena sp. strain PCC 7120 and its derivatives were cultured in BG11 as previously described [15]. Conjugative transfer of plasmids into *Anabaena* 7120 was performed as described by Elhai and Wolk [16]. *Anabaena* strains are listed in Table 1.

1.2 Plasmid construction

Molecular manipulations were performed using standard methods. Tool enzymes were used according to the manufacturers' instructions. PCR fragments were cloned in the T-vector pMD18-T (TaKaRa, Japan) and confirmed by sequencing. The plasmids and primers used are listed in Table 1. The plasmids pHB1213 containing $P_{all3642}$ -*gfp*, pHB1295 containing $P_{alr1261}$ -*gfp* and pHB1296 containing $P_{all4776}$ -*gfp* were used for analyses of transcription from *ftsH* promoters. The plasmid pHB3143 carrying P_{rbcL} -*aftsH-omega* was used to overexpress *aftsH*, pHB3891 carrying P_{rbcL} -*omega* was used as the empty control, pHB3009 was used to produce the conserved portion of FtsH in *E. coli*.

1.3 Northern blot analyses

Northern blot hybridization was performed according to

Zhang et al. [18]. Primers used to generate the DIG-labeled probes are listed in Table 1.

1.4 Western blot analyses

Western blot analysis was performed as described by He and Xu [20]. FtsH proteins were detected with the rabbit antiserum raised against a conserved portion of FtsH.

1.5 Rapid amplification of cDNA ends

5' RACE was performed as described by Zhang et al. [18] using nested PCR. cDNA tailed with SMART II A Oligonucleotide was synthesized using a SMART RACE cDNA amplification kit (ClonTech, TaKaRa Bio) and random primers (Promega). The universal primer A mix provided with the kit and a gene-specific primer (aftsH5RACE-1) were used in the first-round PCR, and the nested universal primer A and the second gene-specific primer (aftsH5RACE-2) were used in the 2nd-round PCR.

3' RACE was performed as described by Argaman et al. [21] with minor modifications. Total RNA was ligated with 3' adapter (3' linker), reverse-transcribed with adapter-specific primer (3' linkerRTrev). The products of reverse transcription were used as the template for nested PCR using primer pairs *aftsH3RACE-1/3'* linkerPCRrev in the first-round PCR and *aftsH3RACE-2/3'* linkerPCRrev in the second-round PCR.

Table 1 *Anabaena* strains, plasmids and primers

Strain, plasmid or primer	Derivation, relevant characteristics or sequence	Source or reference
<i>Anabaena</i> strains		
<i>Anabaena</i> sp. PCC 7120	Wild type (WT)	FACHB ^{a)}
WT (pHB1835)	Sp ^f , pHB1835 carrying chromosomal DNA bp 4393690 to 4395128 upstream of <i>gfp</i> in pHB912 introduced into <i>Anabaena</i> 7120	[17]; this study
WT (pHB2918)	Sp ^f , pHB2918 carrying chromosomal DNA bp 4393694 to 4394934 upstream of <i>gfp</i> in pHB912 introduced into <i>Anabaena</i> 7120	This study
WT (pHB3143)	Nm ^f Sp ^f , pHB3143 carrying P_{rbcL} - <i>aftsH-omega</i> introduced into <i>Anabaena</i> 7120	This study
WT (pHB3891)	Nm ^f Sp ^f , pHB3891 carrying P_{rbcL} - <i>omega</i> (empty vector) introduced into <i>Anabaena</i> 7120	This study
Plasmids ^{b)}		
pDC8	Km ^r , pDU1-based CAT (chloramphenicol acetyl transferase)-reporting vector (NCBI GenBank accession number EU047752)	[18]
pET21b	Ap ^r , overexpression vector	Novagen
pHB1071	Ap ^r Sp ^f , <i>Bam</i> H I-cut pUC19, inserted into <i>Bgl</i> I II-cut pHB912, to provide <i>Sma</i> I- <i>Sse</i> 8387 I sites for cloning of DNA fragments upstream of <i>gfp</i>	[18]
pHB1192	Ap ^r , PCR fragment (<i>Anabaena</i> chromosomal bp 4395267 to 4396630) generated with primers <i>ftsH3642-1</i> and <i>ftsH3642-2</i> , cloned into pMD18-T	This study
pHB1193	Ap ^r , PCR fragment (<i>Anabaena</i> chromosomal bp 1493544 to 1495668) generated with primers <i>ftsH1261-1</i> and <i>ftsH1261-2</i> , cloned into pMD18-T	This study
pHB1245	Ap ^r , PCR fragment (<i>Anabaena</i> chromosomal bp 5691135 to 5691531) generated with primers <i>ftsH4776-1</i> and <i>ftsH4776-2</i> , cloned into pMD18-T	This study
pHB1213	Sp ^f , the DNA fragment excised from pHB1192 with <i>Sma</i> I and <i>Pst</i> I, cloned into <i>Sma</i> I- and <i>Sse</i> 8387 I-cut pHB1071, for expression of <i>gfp</i> from $P_{all3642}$ in <i>Anabaena</i> 7120	This study
pHB1295	Sp ^f , the DNA fragment excised from pHB1193 with <i>Sma</i> I and <i>Pst</i> I, cloned into <i>Sma</i> I- and <i>Sse</i> 8387 I-cut pHB1071, for expression of <i>gfp</i> from $P_{alr1261}$ in <i>Anabaena</i> 7120	This study
pHB1296	Sp ^f , the DNA fragment excised from pHB1245 with <i>Sma</i> I and <i>Pst</i> I, cloned into <i>Sma</i> I- and <i>Sse</i> 8387 I-cut pHB1071, for expression of <i>gfp</i> from $P_{all4776}$ in <i>Anabaena</i> 7120	This study

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Strain, plasmid or primer	Derivation, relevant characteristics or sequence	Source or reference
pHB1835	Sp ^f , <i>Anabaena</i> chromosomal bp 4393690 to 4395128 cloned upstream of <i>gfp</i> in pHB912, resulted from the random screening of <i>Sau</i> 3A I-cut genomic DNA fragments that drive the expression of GFP in <i>Anabaena</i> 7120	[17]; This study
pHB2900	Ap ^f , PCR fragment (<i>Anabaena</i> chromosomal bp 4393694 to 4394934) generated with primers PftsH-1 and PftsH-3, cloned into pMD18-T	This study
pHB2918	Sp ^f , the DNA fragment excised from pHB2900 with <i>Sma</i> I and <i>Pst</i> I, cloned upstream of <i>gfp</i> in pHB912 (<i>Sma</i> I- and <i>Sse</i> 8387 I-cut pHB1071), to detect the transcription activity of P _{aftsH} (trimmed) in <i>Anabaena</i> 7120	This study
pHB2937	Ap ^f , PCR fragment (<i>Anabaena</i> chromosomal bp 4393694 to 4395148) generated with primers PftsH-1 and PftsH-2, cloned into pMD18-T	This study
pHB2938	Sp ^f , the DNA fragment excised from pHB2937 with <i>Sma</i> I and <i>Pst</i> I, cloned upstream of <i>gfp</i> in pHB912 (<i>Sma</i> I- and <i>Sse</i> 8387 I-cut pHB1071), to detect the transcription activity of P _{aftsH} in <i>Anabaena</i> 7120	This study
pHB3008	Ap ^f , PCR fragment (<i>Anabaena</i> chromosomal bp 4394858 to 4394166) generated with primers ftsHex-1 and ftsHex-2, cloned into pMD18-T	This study
pHB3009	Ap ^f , the DNA fragment excised from pHB3008 with <i>Nhe</i> I and <i>Xho</i> I, cloned into <i>Nhe</i> I- and <i>Xho</i> I-cut pET21b, for overexpression of a portion of FtsH in <i>Escherichia coli</i>	This study
pHB3132	Ap ^f , PCR fragment containing P _{rbcl-aftsH} generated by two-step ligation PCR using primer pairs PrbcL-1/2 and aftsHOE-1/2, cloned into pMD18-T	This study
pHB3135	Ap ^f Sp ^f , <i>omega</i> cassette excised with <i>Dra</i> I from pRL57, cloned into <i>Sal</i> I-cut and T4 DNA polymerase-blunted pHB3132, downstream of the cloned P _{rbcl-aftsH}	This study
pHB3143	Km ^f Sp ^f , P _{rbcl-aftsH-omega} excised with <i>Bam</i> H I and <i>Sal</i> I from pHB3135, cloned into <i>Bam</i> H I- and <i>Sal</i> I-cut pDC8	This study
pHB3875	Ap ^f , PCR fragment (chromosomal bp 1784802 to 1785483) containing P _{rbcl} , generated with primers PrbcL-1 and PrbcL-2, cloned into pMD18-T	This study
pHB3889	Ap ^f Sp ^f , <i>omega</i> cassette excised with <i>Dra</i> I from pRL57, cloned into <i>Xba</i> I-cut and T4 DNA polymerase-blunted pHB3875	This study
pHB3891	Sp ^f Km ^f , P _{rbcl-omega} excised from pHB3889 with <i>Bam</i> H I and <i>Sal</i> I, cloned into <i>Bam</i> H I- and <i>Sal</i> I-cut pDC8	This study
pMD18-T	Ap ^f , pUC18-derived T-vector	TaKaRa
pRL57	Km ^f Sm ^f Sp ^f , cloning vector with the spectinomycin resistance cassette <i>omega</i>	[19]
Primers ^{c)}		
aftsH-N1	5'-CCAACAATCCCCAGATAGCG-3'	
aftsH-N2	5'-GCGTTGACTTATACGAAGGC-3'	
aftsHOE-1	5'-TAAGATCTATGGGGAACACTAGATTGCC-3'	
aftsHOE-2	5'-AGTTAACGGAATATGTAGATGCTGGACG-3'	
aftsH3RACE-1	5'-AGCGCCATCATTACGTGCTGGA-3'	
aftsH3RACE-2	5'-CCTGGGCTTCCACAATAGCTG-3'	
aftsH5RACE-1	5'-CTATTGTGGAAGCCCAGGA-3'	
aftsH5RACE-2	5'-CAGCACGTAATGATGGCGCT-3'	
all3642-N1	5'-CCAACAATCCCCAGATAGCG-3'	
all3642-N2	5'-GCGTTGACTTATACGAAGGC-3'	
alr1261-N1	5'-TGGAGAAATGCGGGGCTG-3'	
alr1261-N2	5'-TCCTTCATCGGTTTGTGGC-3'	
ftsH1261-1	5'-GGCAAGCAGACGTGACCA-3'	
ftsH1261-2	5'-ATAGCAGCGCGTACAGCC-3'	
ftsH3642-1	5'-ACCGACAGATGAACCACCA-3'	
ftsH3642-2	5'-AGCAAACGTCCCCTTGCCA-3'	
ftsH4776-1	5'-TCTAGGCGTTGCGCTGCT-3'	
ftsH4776-2	5'-TGCCATAGGCTGAGTGCTG-3'	
ftsHex-1	5'-AGCTAGCGAAGCCAAAAGTGGGGTGA-3'	
ftsHex-2	5'-ACTCGAGTCTGGCGGTAAGAATAGCGG-3'	
PaftsH-1	5'-TGGTCATCCAGTCACGAC-3'	
PaftsH-2	5'-CAGCTATTGTGGAAGCCCAGGA-3'	
PaftsH-3	5'-CCGTCGCTCTAATAATCTTCCG-3'	
PrbcL-1	5'-AAGATATCCGCCAAGAACCTACCCAG-3'	
PrbcL-2	5'-ACAGATCTCCACGTAATTTGTAACCTG-3'	
rnpB-N1	5'-TAGGGAGAGAGTAGGCGTTG-3'	
rnpB-N2	5'-GAGAGTTGGTGGTAAGCCG-3'	
3' linker	5'-AAGATGAATGCAACACTTCTGTACGACTAGAGCAC-3' (5' phosphorylated, 3' amino-modified)	
3' linkerPCRrev	5'-GTGCTCTAGTCGTACAGAAGTGTGCATTTCATC-3'	
3' linkerRTrev	5'-GTGCTCTAGTCGTACAGAA-3'	

a) FACHB, Freshwater Algal Culture Collection of the Institute of Hydrobiology, Chinese Academy of Sciences. b) Ap, ampicillin; Nm, neomycin; Km, kanamycin; Sm, streptomycin; Sp, spectinomycin; unless stated otherwise, the template for PCR reactions was *Anabaena* genomic DNA. c) N1 and N2 indicate primers used to generate probes for Northern blot hybridization; RT1 and RT2 indicate primers used in RT-PCR.

2 Results and discussion

2.1 A small internal antisense RNA (*aftsH*) of *all3642* (*ftsH*)

In a project using *gfp* (green-fluorescence protein) as the reporter gene to identify regulated promoters in heterocysts or developing proheterocysts in the filamentous cyanobacterium *Anabaena* 7120, we cloned many genomic DNA fragments that showed up- or down-regulated promoter activity in proheterocysts or heterocysts [17]. Some DNA fragments cloned upstream of the *gfp* reporter gene indicated that transcription started in an antisense direction within an ORF. One such fragment (*Anabaena* 7120 chromosomal 4393690–4395128 bp, pHB1835 in Table 1) was located within *all3642* (chromosomal 4395350–4393464 bp), a gene predicted to encode the FtsH protease. Transcription from this fragment or a PCR fragment located between chromosomal 4393694 and 4395148 bp (pHB2938 in Table 1) showed an up-regulation in heterocysts (P_{aftsH} in Figure 1 and data not shown), while trimming of chromosomal 4394935–4395148 bp from this fragment (pHB2918 in Table 1) greatly reduced the transcription activity in whole filaments and eliminated the up-regulation in heterocysts (P_{aftsH} trimmed in Figure 1). A Northern blot probed with an internal fragment, chromosomal 4394984–4395175 bp, showed a transcript of ~1.8 kb and a much smaller RNA of ~0.2 kb (Figure 2(c)). The larger transcript was the mRNA of *all3642*, whereas the smaller transcript was an internal anti-

sense RNA. 5'- and 3'-RACEs mapped the small RNA, denoted *aftsH*, between chromosomal 4394967 and 4395199 bp in opposite orientation to *all3642* (Figure 2(a); Figure 3).

2.2 Transcription of *ftsH* genes in heterocysts

In *Anabaena* 7120, 3 other genes, *alr1261*, *all4776* and *all4936* are predicted to encode FtsH proteases, in addition to *all3642*. At the nucleic acid level, *all3642* shows 79%, 86% and 51% identities with *alr1261*, *all4776* and *all4936*, respectively (Figure S1). At amino acid level, All3642 shares 63%, 74% and 48% identities with Alr1261, All4776 and All4936, respectively (Figure S2). All the 4 predicted FtsH proteases are composed of the N-terminal transmembrane segments and the C-terminal regions containing the protease and AAA⁺ domains, of which the AAA⁺ domain consists of conserved Walker A and Walker B motifs (Figure S2) [22].

We examined the expression of 4 *ftsH* genes using *gfp* as the reporter gene. Transcription from $P_{all3642}$ and $P_{alr1261}$ was down-regulated in heterocysts, while that from $P_{all4776}$ was not (Figure 1). No GFP fluorescence could be found in either vegetative cells or heterocysts of the strain carrying $P_{all4936}$ -*gfp* (data not shown). Consistently, no hybridization signal was detectable in Northern blot analysis of the expression of *all4936* (data not shown). The differential expression pattern of *ftsH* genes indicated that four *ftsH* genes in *Anabaena* 7120 might be involved in different cellular processes.

Figure 1 Bright field (I) and GFP fluorescence (II) images of *Anabaena* 7120 bearing P_{aftsH} -*gfp* (pHB1835), P_{aftsH} (trimmed)-*gfp* (pHB2918), $P_{all3642}$ -*gfp* (pHB1213), $P_{alr1261}$ -*gfp* (pHB1295) or $P_{all4776}$ -*gfp* (pHB1296) after nitrogen step-down for 24 h. Arrows point to heterocysts or proheterocysts.

Figure 2 Inhibitory effect of *aftSH* on the expression of *ftsH* genes in *Anabaena* 7120. (a) A schematic diagram showing *all3642* and the position/orientation of the *aftSH*-transcribed region. (b) A schematic diagram showing the clone for expressing *aftSH* from *P_{rbcL}* in *Anabaena*. The bent arrow indicates the start site of transcription; the stem-loop symbolizes the transcriptional terminator. (c) Northern blot analyses of *ftsH* transcripts and *aftSH* in *Anabaena* 7120 without (lane 1) or with *P_{rbcL}-aftSH* (lane 2). *rnpB* was used as the internal control. (d) Northern blot analyses of *ftsH* transcripts and *aftSH* in *Anabaena* 7120 without (lane 1) or with pHB3891 (carrying *P_{rbcL}* but lacking *aftSH*) as the empty vector control (lane 2). *rnpB* was used as the internal control. (e) Western blot detection of FtsH in *Anabaena* 7120 without (lane 1) or with *P_{rbcL}-aftSH* (lane 2).

2.3 Down-regulation of *ftsH* genes by overexpressed antisense RNA

Due to the down-regulation of the 2 *ftsH* genes in heterocysts, one may not see the effect of overexpression of the antisense RNA on their transcripts in heterocysts. Also, quantitative analysis of mRNA in heterocysts is hardly feasible. Extensive RNA degradation could have occurred before heterocysts are isolated from vegetative cells. Hence, we tested the effect of *aftSH* in vegetative cells. The antisense RNA was expressed from the promoter (chromosomal 1784801–1785483 bp) of *rbcL*, which encodes the large subunit of ribulose-1,5-bisphosphate carboxylase (WT (pHB3143) in Table 1; Figure 2(b)). *P_{rbcL}* is a strong promoter expressed in vegetative cells and down-regulated in heterocysts [23]. The DNA sequence of *aftSH* was fused to *P_{rbcL}* 19 bp downstream of its transcriptional start point. The overexpressed antisense RNA greatly reduced the abundance of *all3642*, *alr1261* and *all4776* transcripts (Figure 2(c) in *Anabaena* 7120). The empty vector with *P_{rbcL}* but lacking *aftSH* showed no effect on the expression of *alr1261* and *all3642* (WT (pHB3891) in Table 1; Figure 2(d)).

The regulatory effect of *aftSH* was also detected at the protein level. A 231-aa sequence, 73% to 96% identical to the 4 predicted FtsH proteins (Figure S2), was used to generate rabbit anti-FtsH antiserum. Western blot detection of FtsH showed a result consistent with the Northern blot detection (Figure 2(e)). The significantly reduced expression of *ftsH* genes in *Anabaena* 7120, however, affected the growth under neither nitrogen-fixing nor nitrogen-replete conditions, suggesting that the low level of FtsH was sufficient to support the cellular processes in the cyanobacterium.

All3642 shows 80% identity to Slr0228 and 49%–66% identity to the other 3 predicted FtsH proteins in *Synechocystis* 6803. Very probably, *all3642* is required for quality control of PSII in *Anabaena* 7120 as *slr0228* in *Synechocystis* 6803 [5]. Functions of the rest *ftsH* genes in *Synechocystis*

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CTTACGGTCGCTTTTGGGAATATGTAGATGCTGGACGTGTTACCAGCGTTGACTTATACGAAGGC
GAATGCCAGCGAAAAACCTTATACATCTACGACCTGCACAATGGTCGCAACTGAATATGCTTCCG
GGCAGAACAGCTATTGTGGAAGCCAGGATCAAGATATCGAAAACCGTGTCCAACGTTGGCGGGT
CCGTCTTGTGCGATAACACCTTCGGGTCTAGTTCTATAGCTTTTGGCACAGGTTGCAACCGCCCA
GGATTTGCCAGTTAGCGCTCCTGAGTTAATCAGCAAACCTTAAAGACAAGAAAGTAAGTTTGTATG
CCTAAACGGTCAATCGCGAGGACTCAATTAGTCGTTTGAATTTCTGTTCTTTCATTCAAAACTAC
CTCATCCAGCACGTAATGATGGCGCTATCTGGGGATTGTTGGGCAATCTAGTGTTCCCCATCTTA
GAGTAGGTCGTGCATTACTACCGGATAGACCCCTAACAACCGTTAGATCACAAGGGCTAGAAT
TSP

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Figure 3 The double-stranded sequence of the *aftSH* locus within *all3642* (partial). Transcriptional start point (TSP) and transcriptional termination point (TTP) are boxed. The shaded region is the *aftSH*-encoding sequence, and the underlined region is the sequence used as the probe for Northern blot analyses.

and *Anabaena*, however, are not identified yet. As a *cis*-encoded antisense RNA, *aftsH* may accelerate the down-regulation of *all3642* in developing heterocysts. Also, it may act in *trans* to down-regulate other *ftsH* genes. It is noteworthy that the corresponding regions of *alr1261* and *all4776* show only 43% and 30% identities to the *aftsH*-encoding sequence of *all3642* (Figure S1). Apparently, the regulation of *alr1261* and *all4776* by *aftsH* requires only a partial match between the antisense RNA and mRNAs, which is consistent with the view point that antisense RNAs can mediate inhibition by forming partial duplexes with target mRNAs [24].

The authors are indebted to Dr. Wang Yu for technical assistances. This work was supported by the National Natural Science Foundation of China (30825003, 30770049).

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Supporting Information

Figure S1 The alignment of sequences of *ftsH* genes in *Anabaena* 7120. Identical nucleotides are shaded in black. The *aftsH*-encoding sequence in *all3642* is indicated by a solid line.

Figure S2 The alignment of amino acid sequences of predicted FtsH proteins in *Anabaena* 7120. Identical amino acids are shaded in black and similar amino acids are shaded in gray. The region of *All3642* used for production of anti-FtsH antiserum is indicated by a solid line. The Walker A motif, Walker B motif and Zn²⁺-metalloprotease active site are denoted in green, yellow and red respectively.

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