Characterization of a Chromosomal Type II Toxin–Antitoxin System mazEaFa in the Cyanobacterium Anabaena sp. PCC 7120

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Abstract

Cyanobacteria have evolved to survive stressful environmental changes by regulating growth, however, the underlying mechanism for this is obscure. The ability of chromosomal type II toxin-antitoxin (TA) systems to modulate growth or cell death has been documented in a variety of prokaryotes. A chromosomal mazEaFa locus of Anabaena sp. PCC 7120 has been predicted as a putative mazEF TA system. Here we demonstrate that mazEaFa form a bicistronic operon that is co-transcribed under normal growth conditions. Overproduction of MazFa induced Anabaena growth arrest which could be neutralized by co-expression of MazEa. MazFa also inhibited the growth of Escherichia coli cells, and this effect could be overcome by simultaneous or subsequent expression of MazEa via formation of the MazEa-MazFa complex in vivo, further confirming the nature of the mazEaFa locus as a type II TA system. Interestingly, like most TA systems, deletion of mazEaFa had no effect on the growth of Anabaena during the tested stresses. Our data suggest that mazEaFa, or together with other chromosomal type II TA systems, may promote cells to cope with particular stresses by inducing reversible growth arrest of Anabaena.

Introduction

Cyanobacteria are an ancient and diverse group of prokaryotes found in many different habitats [1], which reflects the considerable ability of cyanobacteria to adapt to variable or extreme environmental factors such as nutrient availability, light, temperature [2]. Indeed, in their long evolutionary history, cyanobacteria have developed highly refined response strategies including both those shared with other prokaryotes during various stresses and those specific for individual species as a result of a particular stress, such as heterocyst formation of Anabaena sp. during nitrogen limitation. One common successful survival strategy is the ability to undergo reversible growth arrest observed in almost all prokaryotes [3,4]. The roles of chromosomal toxin-antitoxin (TA) systems in regulation of bacterial growth is such a good example, which have been documented in many organisms, especially Escherichia coli, to cope with various stresses by reducing growth, inhibiting growth or killing a subpopulation of cells [5–8]. However, based on the authors’ best knowledge there is no similar report in cyanobacteria so far.

TA systems, also referred to as addiction or suicide modules, typically comprise an auto-regulated operon encoding a stable toxin and a labile antitoxin [9]. Based on the antitoxin nature and mode of action, TA systems are grouped into type I, II and III classes. Antitoxins of type I and III systems are small RNAs that inhibit toxin expression (type I) [10,11] or activity (type III) [12]. Antitoxins of type II systems are proteins that inactivate toxins by forming toxin-antitoxin (TA) complexes [9]. The identified TA toxins so far include several families with different biochemical activities [9,13,14]. The vast majority of type II toxins are mRNAs with specific endoribonucleases, also called mRNA interferases, such as MazF, RelE, HicA toxins [13]. Almost all described type II TA systems share mode of regulation: tight expression auto-regulation by a TA complex binding to the promoter region and toxin neutralization by formation of TA complex [9].

Type II TA systems were originally found in low copy number plasmids where they were first thought to be merely addiction modules to stabilize these systems. Recently, the discovery of their high prevalence on the chromosomes of free-living bacteria [15–17] led to the proposal that chromosomal type II TA systems are stress-response elements contributing to prokaryotes’ adaptation to stressful environments [5,9]. According to this hypothesis, under unfavorable conditions antitoxins would be degraded by stress-induced proteases, which causes relief of transcriptional repression of TA operons and release of toxins from TA complexes [9,13]. As a consequence, the free toxins would induce reversible growth arrest or cell kill by inhibiting an essential cellular process, such as protein synthesis or DNA replication [5,9]. This proposal has been supported by a series of recent experiment findings. For instance, in E. coli, activation of TA systems is triggered by various stresses [18–21], and ectopic expression of nearly all characterized chromosomal toxins could improve the ability to confer resistance to stress [22–24]. In addition, some TA knockout mutants have
apparent phenotype changes under stress conditions tested. For example, a mazEF-deletion mutant exhibited the phenotype resistant to a series of stressful inducements including nutrient starvation, addition of antibiotics, DNA damage, oxidative and heat-shock stresses [19,25,26]. Therefore, by inducing a reversible growth arrest, TA toxins could allow stressed cells to remain in a dormant or non-growing stress-tolerant state until more favorable environmental conditions return.

The sequenced cyanobacteria genomes were also found to encode a large number of putative TA systems [15,16]. However, it still remains to be investigated whether the homologs of the interesting TA systems of E. coli are as important in cyanobacteria. Anabaena sp. PCC 7120 (hereafter, Anabaena) is a filamentous heterocyst-forming cyanobacterium. This strain is amenable to genetic manipulation and has been used as a model to study multiple physiological processes in cyanobacteria. A comprehensive genome analysis revealed that the Anabaena chromosome possesses at least 38 putative TA loci belonging to various families of type II TA systems [16]. In this paper, we focus our study on characterization of a chromosomal gene pair mazEFa2 and mazEFa3 (hereafter referred to as mazEa and mazFa, respectively) of Anabaena, which were predicted to constitute a sequence homolog of the best-studied E. coli mazEF system [15,16]. This is the first functional characterization of TA loci in cyanobacteria.

Materials and Methods
Cyanobacterial strains, culture conditions and conjugations

Cells of E. coli strain DH5α or BL21(DE3) were grown in LB medium at 37°C with shaking at 200 rpm. When necessary, cultures were supplemented with ampicillin (Ap, 50 mg/l), spectinomycin (Sp, 100 mg/l) or kanamycin (Kn, 50 mg/l). Cells of Anabaena strains were statically cultured in BG11 (containing 240 μM K₂HPO₄ medium [27] in 200-mL transparent plastic bottles at 30°C with a photosynthetic photon flux density of ~30 μmol photons/m²s⁻¹, and shaken three times every day. As required, neomycin (50 mg/l) or spectinomycin (10 mg/l) was added. A series of modified BG11 media 1/10-P-BG11, 1/500-P-BG11, 1/1000-P-BG11 and P₀-BG11 was prepared according to Schreier [28] but simply modified here, which contain 24, 0.48, 0.24 and 0 μM K₂HPO₄, respectively. For the copper ion (Cu²⁺)-free cultures of Anabaena, deionized water with an electric conductivity of 18.2 μΩ was used for medium preparation, and cells of mid-logarithmic-phase cultures were harvested by centrifugation, washed three times with Cu²⁺-free BG11 (Cu₀-BG11). The washed cells were re-suspended and cultured in Cu₀-BG11 for at least 3-cycle passage to deplete intracellular Cu²⁺. Conjugations between E. coli and Anabaena were performed as previously described by Elhai [29].

Construction of plasmids

Tool enzymes (TaKaRa, Dalian) were used following the manufacturer instructions. The primer sequences used are listed in Table S1. To facilitate the sub-clone of PCR-amplified products, appropriate restriction sites were added in some primers. The template for PCRs was the genomic DNA of Anabaena unless stated otherwise. All clones of PCR products were verified by DNA sequencing for subsequent experiments.

To create the plasmid for mazEa/mazFa deletion, the left flank (LF) region of the mazEa/mazFa operon was amplified by PCR using the primers mazEa-1 and mazFa-2, and ligated to pMD18-T (TaKaRa, Dalian) in the clockwise direction generating pJS134. The right flank (RF) region was amplified using the primers mazFa-1 and mazFa-2, and digested with XhoI/SalI, and then cloned into the respective sites of pBJS34 producing pBS141. The C.K2 cassette containing the kanamycin-resistance gene (Kmr) was released from pRL446 [29] with XhoI, and ligated to the XhoI-digested pJS141 obtaining pJS153. The LF-C.K2-RF fragment was excised from pJS153 with BamHI/SalI and cloned in the BglII/SalI sites of pRL277 [30], obtaining pJS167.

For copper-inducible expression plasmids, the fragment containing the promoter region (P₉₀₋₂₅) along with the start codon (ATG) of the gene petE (all0258) was amplified with the primers PpetE-1 and PpetE-2 and ligated to pMD-18T in the clockwise direction yielding pJS314. The mazFa gene was amplified using primer mazFa-Xb and mazFa-K, digested with XhoI/KpnI and then cloned into the respective sites of pJS314 obtaining pJS334. The mazEaFa genes were amplified using the primers mazEa-Xb and mazFa-K, digested with XhoI/KpnI and then cloned into the same sites of pJS314 generating pJS335. These recombinant fragments were individually released with PdMI from pJS314, pJS334 and pJS335, and ligated to the PdMI/BglII, blunted with T4 DNA polymerase, sites of pHB912 [17], generating plasmids pJS350 (empty vector), pJS351 (containing P₉₀₋₂₅-mazEaFa fusion) and pJS352 (containing P₉₀₋₂₅-mazEaFa-mazFaFa fusion), respectively. These resulting shuttle plasmids can replicate in Anabaena cells.

To construct the plasmids for selective expression of MazEa and MazFa, the plasmid pJS298 was used, which contains P₉₀₋₂₅ and P₂₅₇ as well as the genes aatC and lacI encoding the regulator proteins of the respective promoters [31]. mazFa was amplified using the primers mazFa-N and mazFa-K, digested with NdeI/KpnI and cloned behind P₂₅₇ of pJS298 generating pJS301. The mazEa gene was amplified using the primers mazEa-S and mazEa-K, and digested with SacI/KpnI, and then placed under P₂₅₇ of pJS301 obtaining pJS302.

The mazEaFa genes were amplified with the primers mazEa-S and mazFa-X and cloned into pMD18-T obtaining pJS792. This insert was released with NdeI/XhoI, and placed under P₂₅₇ of PET30a (Novagen) resulting in pJS798, which allows the IPTG-inducible co-expression of MazEa together with MazFa tagged with 6 histidine (MazF-His6) at its C-terminus.

Extraction of RNA and RT-PCR reaction

Anabaena cells were collected from 200 ml culture with 0.5~1.0 optical density at 730 nm (OD₇₃₀) by centrifugation. The pellet of cells was used for RNA extraction as described previously [32]. Total RNA was converted to cDNA by reverse transcription using PrimeScript 1st strand cDNA synthesis kit (TaKaRa, Dalian) according to the manufacturer’s instructions. Using 1 μg of cDNA per reaction, the transcription abundance of mazEa and/or mazFa was determined by PCR amplification.

Analysis of cyanobacterial growth rate

To analyze growth rate of the Anabaena strains, cells were grown in 100 mL antibiotic-free medium in 500 ml flasks in triplicate, and OD₇₃₀ was measured at the selected time points. High-light exposure or phosphate limitation experiments were performed according to previous methods [33,34] but simply modified here. To analyze the effects of high-light intensities, Anabaena cultures with an initial OD₇₃₀ of 0.2 were grown under the light of 30, 100, 200 or 300 μmol photons/m²s⁻¹. For phosphate limitation experiments, Anabaena cells were collected from the mid-logarithmic-phase cultures (OD₇₃₀ of about 0.8), and washed twice with P₀-BG11 medium. The cells were resuspended in 1/10-P-BG11, 1/500-P-BG11 and 1/1000-P-BG11, respectively, and further incubated under normal growth conditions. The induction by nitrogen deprivation was performed as previously described [32],
and heterocyst formation of Anabaena cultures was investigated by microscopic analysis. To assess the effect of mazEaFa-encoded proteins, copper-free Anabaena cells harboring copper-inducible expression plasmids were cultured in BG11 containing 2 μM CuSO₄ (Cu-BG11) at an initial OD₇₃₀ of 0.2 under normal growth conditions.

Assays of toxicity, antitoxicity and growth recovery

E. coli cells containing respective selection-expression plasmids were incubated in 100 ml of LB medium with glucose to an OD₆₀₀ of about 0.6. For selective expression of the mazEaFa genes, 0.1 mM isopropyl β-D-thiogalactopyranoside (IPTG) or 0.2% (w/v) L-arabinose (Ara) was added for gene expression from the IPTG-inducible promoter Pᵣ₇₃ or the arabinose-inducible promoter Pᵣ₆₅₃. 0.2% (w/v) glucose (Glu) was added to suppress the background expression from Pᵣ₇₃ and Pᵣ₆₅₃. For assays of cell growth on LB agar plates with corresponding inducers, 1 ml of each culture were serially diluted in 10-fold steps, and 2 μl of each of diluted samples was dropped on the agar plates with glucose, glucose and IPTG, glucose and L-arabinose or glucose, L-arabinose and IPTG, and then incubated for 30 h. For assays of rescue of E. coli growth arrest, each culture with an OD₆₀₀ of 0.2 was divided into four equal parts, three of those were individually added with IPTG, L-arabinose or both, respectively. These cultures were further incubated as described above. Samples were taken every 30 min from each culture to measure OD₆₀₀, and then the cells were harvested and washed three times with PBS buffer. The washed cells were resuspended in an equal volume of liquid LB medium, and 100 μl of appropriately diluted samples was spread on the plates containing glucose or along with L-arabinose. The colony-forming unit (CFU) was counted after incubation at 37°C for 30 h.

Over-expression, purification and identification of MazEa and MazFa

Co-expression of MazEa with MazFa was induced by addition of 1 mM IPTG to the culture of E. coli BL21(DE3) containing pJS798 with an OD₆₀₀ of ~0.6, and growth was continued for 3 h. Co-purification of proteins was done by affinity chromatography using Ni²⁺-NTA His.Bind resins (Novagen, USA) under the native conditions, while purification of denatured proteins under the denaturing conditions according to the manufacturer's instructions. The purified products were separated with 15% SDS-PAGE. The densitometry of the bands purified under native conditions was then analyzed using Image J (http://rsb.info.nih.gov/nih-image/). The co-purified proteins were excised from gel, and determined by MALDI-TOF mass spectrometry (MS) analysis using a Shimadzu Biotech Axima TOF2 instrument (Shimadzu Biotech, Germany). The proteins embedded in gel slice was digested with trypsin and analyzed to determine the mass/charge (m/z) values according to the manufacturer instructions. The experimental peptide masses were compared to a theoretical cleavage with trypsin using the MS-DIGEST program (http://prospector.ucsf.edu).

Results

Genetic organization and transcriptional analysis of the mazEaFa operon

The mazEa (a3212) and mazFa (a3211) genes on the Anabaena chromosome (http://www.kazusa.or.jp/cyano/) encode MazEa and MazFa homologous to the E. coli MazE (31% identity) and MazF (39% identity), respectively. MazEa is predicted to be an 80-residue protein with a molecular weight of 8.9 kDa and an isoelectric point of 4.94, while MazFa is predicted to be a 146-residue protein with a molecular weight of 16.5 kDa and an isoelectric point of 8.1. A genetic organization analysis revealed that mazEa is located upstream of mazFa, and the two genes overlap by one base pair (Figure 1A), apparently organized in a bicistronic operon. The inferred −35 (TTGCG) and −10 (TATAA) sites and the putative ribosome-binding sites (RBS, GGAGA) upstream of mazEa suggest that mazEaFa comprises a transcriptional unit with a putative σ₇₀-family promoter (Figure 1A).

To characterize the coupling transcription between mazEa and mazFa, RT-PCR analysis was performed using primers that anneal to the 3’ end of mazEa and the 5’ end of mazFa. As seen in Figure 1B, the band with the expected size (564 bp) was observed after amplification of cDNA for mazEaFa, and confirmed by sequencing. The individual mazEa and mazFa genes were also amplified using the primers annealing to regions within the respective reading frames (Figure 1B). Possible DNA contamination can be excluded since no PCR products were evident in the negative controls (Figure 1B). These indicate that mazEa and mazFa are actively co-transcribed from a σ₇₀-family promoter under normal growth conditions. Taken together, on the basis of the genetic organization and sequence homology of its products, the mazEaFa locus may constitute a putative mazEF-family TA system, where mazFa encodes for a toxin with bacterial mRNA interferes activity, and mazEa for the cognate antitoxin.

Construction and characterization of mutant lacking mazEaFa

To investigate the physiological function of mazEaFa, the mazEaFa-deletion mutant was constructed by allelic exchange mutagenesis. The plasmid pJS167 was conjugally transferred into the Anabaena cells, the single-crossover and double-crossover recombinants were sequentially screened as previously described [30]. Twelve double-crossover recombinants were randomly selected and further cultured. As shown with PCR detection, mazEa and 5’ half of mazFa were replaced with C.K2 in 8 double recombinants, and the segregation was complete (Fig. 1C). Compared to their parental strain, the 8 double recombinants showed no any detectable difference in growth under the laboratory conditions (Figure 2), suggesting that the mazEaFa system is not essential for normal growth of Anabaena.

It has been documented that deletion of E. coli mazEF caused changes in response to a series of stresses, such as nutrient starvation, oxidative stress, antibiotic treatment, and DNA damage [19,25,26]. In nature, the adverse environmental factors cyanobacteria often encounter include high-light exposure and nutrient limitation, especially phosphorus or nitrogen starvation [2]. High-light exposure could reduce the photosynthetic electron transport components of cyanobacteria, resulting in excess production of reactive oxygen species (ROS) and eventually oxidative stress [35]. The nutrient limitation would inhibit growth of cyanobacteria [34,36]. We thus examined whether the mazEaFa deletion could affect the growth of Anabaena under these major environmental stresses [35]. In addition, Anabaena is able to differentiate heterocysts, specialized nitrogen-fixing cells, at semi-regular intervals after nitrogen deprivation [37], effect of mazEaFa deletion on heterocyst development was also determined by microscopy. All these experiments were performed with three mazEaFa-deletion mutant strains, and similar results were obtained. As seen in Figure 2, under high-light exposure or phosphate limitation, both mazEaFa-deletion and wild-type strains showed no difference in growth rate. Furthermore, the mutant strains could develop mature heterocyst as observed in the wild-type strain after nitrogen
starvation (Figure S1). These results demonstrate that, unlike the E. coli mazEF-deletion mutant, the mazEaF-deletion mutant exhibits no discernible phenotype under the tested conditions. We randomly selected one of the mazEaF-deletion strains for further experiments and designated it as DRJS167.

Ectopic production MazFa induces growth arrest of Anabaena

The crucial characteristic of TA systems is toxin-induced growth inhibition when in excess of their cognate antitoxins, we thus investigated the effect of ectopic expression of the mazEaF operon of Anabaena. For this, the tight copper-responsive promoter PpetE [38] was used to control expression of mazF from the plasmid pJS351 or co-expression of mazFa with mazFa from pJS352 (Figure S2) in Anabaena cells. To eliminate the possibility that the plasmid-encoded MazFa is interfered with the chromosome-encoded MazFa in the wild-type strain, pJS351 and pJS352 were conjugal transferred into the mutant DRJS167, generating strains DRJS167[pJS351] and DRJS167[pJS352], respectively. The strain DRJS167 [pJS350] (containing the empty vector) was used as a control. In the Cu-BG11 medium, neither DRJS167[pJS351] nor DRJS167[pJS352] showed any noticeable difference in the growth rate relative to the control strain (data not shown). However, in the Cu-BG11 medium, DRJS167[pJS351] exhibited the growth rate similar to the control strain and the wild-type strain of Anabaena, while DRJS167[pJS352] displayed significant growth inhibition (Figure 3A). These indicate that the Cu\(^{2+}\) -induced ectopic expression of mazFa alone led to growth inhibition of Anabaena, but the co-expression of mazEa abolished this growth-inhibition effect, thus validating that MazFa functions as a toxin while MazEa is an antitoxin against MazFa.

Previous studies of TA systems showed that growth-inhibition effect of some toxins could be abolished by stopping the toxin synthesis in the absence of the cognate antitoxins [22,39,40]. We thus determined whether the MazFa-induced growth inhibition of Anabaena would be overcome only by subsequent stop of MazFa production via removal of Cu\(^{2+}\). As seen in Figure 3B, the cells which had been incubated for 1 d in the Cu-BG11 medium began to recover growth after 4 d of incubation in the Cu\(^{0}\)-BG11 medium, while the cells incubated in Cu-BG11 for more than 2 d remained in an inhibited state under the same conditions. These suggest that growth inhibition of Anabaena by a prolonged induction (at least 2 d) with MazFa could not be relieved by stopping its production.

MazFa expression leads to growth inhibition of E. coli that can be rescued by subsequent expression of MazEa

In some characterized TA systems, the toxin toxicity could be counteracted only by subsequent production of antitoxin [41,42], we next tested whether later expression of MazEa may inhibit the growth-inhibition effect of MazFa. E. coli has been successfully used as a host for verification of heterogenic TA components due to its elaborate conditional expression systems established [43–48], we thus investigated rescue effect of MazEa using the selection-expression system constructed here. This expression system includes E. coli BL21(DE3) cells harboring the selection-expression
plasmids (Figure 4A). In these plasmids, the IPTG-inducible promoter $PT7$ and arabinose-inducible promoter $PBAD$ individually control the expression of $mazEaFa$ genes in trans. So the strain BL21(DE3)(pJS301) can express MazFa in the presence of IPTG but not express MazEa under all circumstances. However, BL21(DE3)(pJS302) can express MazFa and/or MazEa upon induction with IPTG and/or arabinose. We first tested the effect of MazFa and/or MazEa production on $E. coli$ growth on LB agar plates containing the respective inducers. As seen in Figure 4B, the presence of IPTG and/or arabinose has no effect on growth of BL21(DE3)(pJS301), but not in the presence of IPTG or along with L-arabinose. BL21(DE3)(pJS302) could grow on the plate containing arabinose or together with IPTG but not on the plate with IPTG alone. When these strains were cultured in liquid media containing IPTG, arabinose or both, the growth profiles of BL21(DE3)(pJS301) (data not shown) or BL21(DE3)(pJS302) (Figure 5A) were similar to those observed on the plates with the corresponding inducers. These data indicate that the expression of $mazFa$ alone led to growth inhibition of $E. coli$, and the co-expression of $mazEa$ in trans abolished this growth-inhibition effect.

We next investigated whether stop of MazFa production in the absence of MazEa can overcome MazFa-induced growth arrest of $E. coli$. For this, expression of the $mazEaFa$ components was selectively induced in BL21(DE3)(pJS302) with the respective inducers (Figure 5A), and subsequently switched off at the indicated time points by plating the cells on the plates without any inducer. As seen in Figure 5B, the earlier production of MazFa alone led to a reduction of about $10^4$ in CFU by 2 h of induction compared to the CFU at the time point of zero, whereas expression of MazEa or with MazEa showed no effect on CFU relative to the cells non-induced. Additionally, a subpopulation of cells ($2 \times 10^7$) exhibited colony-forming ability after the stop of MazFa synthesis, they were non-inheritable tolerant to MazFa because the similar reduction in CFU was observed when the cells from these colonies were re-plated on the plates with glucose (data not shown). This result, consistent with observation in $Anabaena$ cells (Figure 3B), indicates that MazFa production caused inability of most $E. coli$ cells to replicate even after the stop of MazFa synthesis in the absence of MazEa (Figure 5B). To examine whether subsequent expression of MazEa can reverse the MazFa-induced growth inhibition of $E. coli$, BL21(DE3)(pJS302) culture in the presence of IPTG alone, the CFU count was determined at time points of 0, 2, 4 and 6 h on the
plates with or without arabinose. As seen in Figure 5C, the subsequent induction of MazEa increased CFU of the cells previously expressing MazFa at least to a level at the zero time point, but the absence of MazEa did not. These results indicate that MazEa could rescue the growth of almost all cells within at least 6 h.

MazEa directly interacts with MazFa in vivo

To determine whether neutralization of the MazFa toxicity by MazEa is due to its interaction with MazFa to form the MazEa-MazFa complex in vivo, MazEa and MazFa-His6 were co-expressed in and co-purified from the strain *E. coli* BL21(DE3)(pJS798). As shown in Figure 6A, 9.9-kDa and 17.6-kDa proteins, consistent with the expected molecular masses of the recombinant MazEa and MazFa-His6 proteins, respectively, were co-expressed and successfully co-purified under native conditions (lane 3), but only the 17.6-kDa protein was purified under denaturing conditions (lane 4). MS analyses showed that 2 peaks (807.334 and 902.407 m/z) of the 8.9-kDa protein and three peaks (942.570, 1699.993 and 1811.912 m/z) of the 17.6-kDa protein were found in the theoretical peak list of MazEa and MazFa (Figure 6B and C), respectively, in MS-DIGEST program. These data indicate that MazEa directly interacts with MazFa to form the MazEa-MazFa complex in vivo. A densitometrical analysis using Image J revealed that the relative molar ratio of MazEa to MazFa was 1:2 (Figure 6A). Like many characterized TA complexes, the opposite pI values of MazEa (acidic) and MazFa (basic) may contribute to stabilization of the MazEa-MazFa complex [13].

Discussion

In the present study, we confirmed that the chromosomal genes *mazEa* and *mazFa* of *Anabaena* form a bicistronic operon that is co-transcribed from its putative σ70-family promoter. *mazFa* encodes a toxic protein MazFa due to its growth-inhibition effect demonstrated in both cells of *Anabaena* and *E. coli*. This growth-inhibition effect of MazFa could be neutralized by MazEa through formation of the MazEa-MazFa complex in vivo. Therefore, the *mazEaFa* locus has the properties required for a genuine TA system belonging to the *mazEF* family. By far, all of identified MazF homologues function as ribosome-independent endoribonucleases to effectively inhibit protein synthesis resulting in growth arrest, despite each with a different cleavage-site specificity among various species, such as ACA sequence for *E. coli* MazF [49] and GUUGC for *Myxococcus xanthus* MazF [50]. We thus reason that MazFa exerts its growth-inhibition effect via the mechanism of action similar to the identified MazF homologs.

Our studies also show that the MazFa-induced growth inhibition of *E. coli* could be completely abolished by subsequent production of MazEa at least within 6 h (Figure 5), suggesting bacteriostatic effect of MazFa. This result is in accordance with the previous demonstration that the overproduction of TA toxins caused reversible growth arrest [22,39,40,42]. Therefore, it is reasonably proposed that the chromosomal *mazEaFa* system of *Anabaena* might represent a growth modulator that induces a reversible dormancy state to enhance fitness and competitiveness under particular stress conditions. As a result, cyanobacteria can persist a long period of time under stress conditions and revive when suitable conditions arise.
Figure 5. Rescue of MazFa-induced E. coli growth arrest. (A) Growth curves of BL21(DE3)(pJS302) in the liquid LB media containing the indicated inducers. (B) CFU counts of BL21(DE3)(pJS302) after the stop of production of MazFa and/or MazEa. Cells of BL21(DE3)(pJS302) cultures as seen in (A) were transferred at the indicated time points to the LB plates containing only glucose, and CFUs were counted after incubation. (C) Effect of subsequent production of MazEa on viability of the cells previously induced with MazFa. The IPTG-induced cells of BL21(DE3)(pJS302) were transferred at the indicated time points to the LB plates containing only glucose or with arabinose, and CFU counts were measured after incubation. Error bars indicate the standard error of the means from three independent experiments with three replicate samples. doi:10.1371/journal.pone.0056035.g005

Figure 6. Interaction between MazEa and MazFa in vivo. (A) SDS-PAGE analysis of co-expression and purification of His6-MazFa and MazEa from BL21(DE3)(pJS798) cells. Lane 1, crude extract of un-induced cells; lane 2, crude extract of induced cells; lanes 3 and 4, products purified from induced cells under native and denaturing conditions, respectively; M, protein molecular weight standard with sizes as indicated in kDa. The bands indicated by arrows in lane 3 were subjected to mass spectrometer. The densitometry values and the relative molar ratio of MazEa to MazFa are shown below. (B) and (C) MS analysis of peptide mass fingerprinting of MazEa and MazFa-His6.
It is interesting to note that growth analysis of the mazEaFa-deleted mutant under the selected stress conditions could not allow us to make an intimate connection between stress adaptation of Anabaena and mazEaFa. Indeed, by far only several TA knockout mutants have deleterious phenotypes, many TA deletion mutants show no discernible phenotype [5]. The most recent work demonstrated that progressive deletions of all ten TA systems encoding mRNAase toxins in E. coli have cumulative effect on formation of persistor cells (a subpopulation of bacteria cells with low metabolic rates and antibiotic tolerance) [31], suggesting functional redundancy of TA systems. Comprehensive genome analyses also revealed the abundance and diversity of type II TA systems in cyanobacteria [15–17]. The unicellular cyanobacterium, Synechocystis sp. PCC 6803, contains as many as 34 putative TA systems [15–17], including our previously characterized neVe operon [31,52]. Characterization of this TA system carried out in E. coli demonstrated that the neVe operon constitutes a “hybrid” TA systems where the toxin gene neE belonging to the relE family is paired with the antitoxin gene relN with no sequence homology to characterized antitoxin genes [32]. Furthermore, the antitoxin protein RelN in E. coli could be degraded by both Lons and ClpP2s proteases from Synechocystis sp. PCC 6803, thus resulting in the activation of the toxin RelE from the RelN-RelEs complex [31]. Anabaena encodes at least 38 putative type II TA systems [15–17]. Perhaps because only one of these Anabaena TA genes was deleted here, the contribution of individual toxins might be marginal and detectable effects may require the cumulative action of multiple toxins in vivo.

Another reason for TA knockout mutants without deleterious phenotype may be due to our poor knowledge of the stressors activating TA systems of Anabaena cells. Various stresses have been documented to induce arrest of the cell cycle in cyanobacteria, as reported in other micro-organisms. For example, prolonged nitrogen starvation induced the cyanobacterium Synechococcus sp. PCC 7942 cells to enter a reversible growth arrest that represents a general acclimation process [36]. A long-term (at least 6 days) deprivation of phosphorus source also caused a reversible growth cessation of Anabaena sp. PCC 7120 [34]. Our data presented here show that the effect of mazEaFa-encoded products on growth of Anabaena or E. coli resemble the stress-induced reversible growth arrest. To determine a possible link of the TA system to a particular stress acclimation, we are investigating the conditions in which a particular toxin would be activated in the wild-type strain of Anabaena by promoter-reporter analysis.

Supposing their role in regulation of bacterial growth, chromosomal type II TA systems may promote cells to cope with various environment stresses. Therefore, the presence of a large number of type II TA systems in cyanobacteria may contribute to the impressive ability to acclimate variable environment conditions. For example, a bloom-forming cyanobacterium Microcystis aeruginosa NIES-843 contains as much as 97 putative TA systems, representing 1.5% with respect to the total number of predicted genes [17]. We thus speculate that such strikingly numerous TA systems might give the cyanobacteria cells a competitive advantage over other algae, resulting in cyanobacteria blooming under favorable environment conditions. Therefore, understanding of TA system-mediated regulation represents a promising avenue for clarifying the mechanism for environmental acclimation processes in cyanobacteria and developing novel and effective strategies to control blooms.

Supporting Information

Figure S1 Heterocyst formation of the mazEaFa-deletion mutant after starved of nitrogen. The heterocysts of the wild-type and mazEaFa-deletion mutant strains of Anabaena were detected under a microscope before (+N) or after 24 h of induction (−N) by nitrogen deficiency. Arrowheads point to mature heterocysts. (TIF)

Figure S2 Schematic diagram showing the plasmids for the copper-inducible expression of MazFa or with MazEa. The omega cassette is a spectinomycin (Sp+)/streptomycin/Sn+) resistance marker with stem loops at both ends that terminate background transcription. In the presence of copper, MazFa or together with MazEa was expressed from the copper-inducible promoter P0sit in Anabaena cells. (TIF)

Table S1 Primers used in this study. (DOCX)

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Author Contributions

Conceived and designed the experiments: DN ZL. Performed the experiments: DY JZ LZ. Analyzed the data: DN YJ ZL. Contributed reagents/materials/analysis tools: YJ ZL. Wrote the paper: DN.

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