Toxin-Antitoxin Systems on the Large Defense Plasmid pSYSA of Synechocystis sp. PCC 6803*

Received for publication, November 19, 2012. Published, JBC Papers in Press, January 15, 2013, DOI 10.1074/jbc.M112.434100

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Background: Cyanobacteria harbor a vast array of toxin-antitoxin modules, but their roles remain largely unknown.

Results: The sll7003/sll7004 system encodes a VapC-type RNA endonuclease targeting single-stranded regions with little specificity.

Conclusion: Seven different TA systems plus three free-standing components are located on plasmid pSYSA.

Significance: Some bacterial plasmids are safeguarded against post-segregational loss by multiple TA systems and an aggressive type of RNA-targeting toxicity.

Bacterial toxin-antitoxin (TA) systems are genetic elements, which are encoded by plasmid as well as chromosomal loci and mediate plasmid and genomic island maintenance through post-segregational killing mechanisms. TA systems exist in surprisingly high numbers in all prokaryotes, but cyanobacterial TA systems have been only very poorly experimentally characterized so far. Cyanobacteria are the only prokaryotes that perform oxygenic photosynthesis. As such, cyanobacteria are of high ecological importance and are considered promising for the production of biofuels. Here, we present the molecular characterization of the sll7003/sll7004 TA system encoded on plasmid pSYSA of the model cyanobacterium Synechocystis sp. PCC 6803 as involving a Mg2+-dependent RNA endonuclease activity targeting single-stranded RNA regions and demonstrate the functionality of four more TA systems encoded on this 100,749-bp plasmid. Furthermore, one additional type I, one additional type II, and three free-standing TA system components are predicted on pSYSA, all of which appear active judged by their expression. By harboring at least seven simultaneously active TA systems, pSYSA appears as the plasmid most strongly selected for among all plasmids studied in this respect thus far. These results point to a high biological relevance of pSYSA, whose coding capacity is 75% devoted to three distinct clustered regularly interspaced short palindromic repeats (CRISPR) systems mediating antiviral defense.

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* This work was supported by the Deutsche Forschungsgemeinschaft, Bonn, Grant HE 2544/6-1 (to W. R. H.).

** This article contains supplemental Table S1.

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2 The abbreviations used are: TA, toxin-antitoxin; asRNA, antisense RNA; RACE, rapid amplification of cDNA ends; TSS, transcriptional start sites(s); nt, nucleotide(s); CRISPR, clustered regularly interspaced short palindromic repeats; IPTG, isopropyl-β-D-thiogalactopyranoside; as, antisense.

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Gerdes (11) revealed that although most plasmids are likely to encode at least one TA system, the vast majority of TA systems are encoded at loci distributed throughout the chromosomal DNA. These findings and the observation that some TA systems are activated in response to environmental stress have led to the idea that TA systems not necessarily have functions only in maintaining certain genetic elements but may contribute to an enhanced stress resilience of the bacterial cell (12). In a more recent study, 750 complete genomes of archaea and bacteria were surveyed for the presence of type II TA systems, and an impressive number of 6797 TA pairs and a total of 51,319 putative toxin and antitoxin genes were identified (13). These include 175 individual toxin and antitoxin genes and 30 TA pairs in the model cyanobacterium *Synechocystis* sp. PCC 6803 (from here on “*Synechocystis*”). The most current update can be obtained in the Toxin-Antitoxin Database (TADB), where 10,753 type II TA gene pairs are listed within 1240 prokaryotic genomes (14), including 37 TA pairs in *Synechocystis*.

Because cyanobacteria are the only prokaryotes that directly convert solar energy into biomass using oxygenic photosynthesis, these are of tremendous relevance for primary carbon fixation and of rising biotechnological relevance. These organisms are functional in many ecosystems and have been experimentally characterized. RelE cleaves mRNAs in the ribosomal complex becomes permanently locked in this state, leading to DNA double-strand breaks (18). The cellular target of CcdB is the DNA gyrase subunit GyrA. Binding has two possible outcomes; besides an inhibition of DNA supercoiling, the more severe result is that the enzyme within the gyrase-DNA cleavage complex becomes permanently locked in this state, leading to DNA double-strand breaks (18).

However, mRNA-specific endonuclease activity appears as the most common activity of type II toxins. These toxins are also termed interferases (19, 20). HigB falls in this category (21), but interferases of the widespread RelE and MazF family are particularly well studied. RelE cleaves mRNAs in the ribosomal A site (22), whereas MazF cleaves single-stranded RNAs sequence specifically at ACA sites (23) or is even more selective, cleaving at UACAU, as in the case of the *Clostridium difficile* and *Staphylococcus equorum* MazF toxins (24, 25).

Among studied TA systems, the VapBC family constitutes the most frequent, diverse, and complex class. Toxins belonging to this family possess a PIN domain (homologues of the “piT N”-terminal domain). VapC-1 from *Haemophilus influenzae* shows an endoribonucleolytic activity (26), and *vapBC-LT2* and *vapBC-pMYSH* from *Salmonella* LT2 and *Shigella* plasmid pMYSH6000 inhibit translation due to the RNase activity encoded by the respective toxin gene (27). However, the biological function and molecular targets of the diverse *vapBC* loci abundant in various other bacteria are not yet known.

In addition to its single chromosome, seven plasmids have been identified in *Synechocystis*. The chromosomal reference sequence of *Synechocystis* was determined as early as 1996 (28), and the sequences of the large plasmids were reported in 2003 (29), whereas the three small plasmids had already been sequenced before (30–32). There are no genes on these seven plasmids known to have essential cellular functions. Nevertheless, the presence of all seven plasmids was verified during a recent resequencing study (33), suggesting the activity of efficient mechanisms for the maintenance of these plasmids. The plasmid pSYSA is a major defense plasmid because it encodes all components of three independent CRISPR systems, thought to be involved in the recognition and degradation of invading alien DNA molecules (for reviews, see Refs. 35–37). Due to two deletions in the repeat-spacer arrays of the CRISPR system in the *Synechocystis* strain PCC-M used here, the total length of plasmid pSYSA is only 100,749 bp (GenBank accession number CP003267 and Trautmann et al. (33)) as compared with 103,307 bp for the reference strain (29).

Among the 103 genes annotated on plasmid pSYSA are at least 12 genes coding for six different type II TA systems. Here we present a molecular characterization of the *ssl7003/ssl7004* TA system and demonstrate that at least four more TA systems encoded on plasmid pSYSA are functional.

**EXPERIMENTAL PROCEDURES**

**Growth Conditions**

Construction and propagation of plasmids pQE70::toxin, pBAD::antitoxin, and pET28::ssl7004 were conducted in host strain *E. coli* TOP10F (Invitrogen). Expression of recombinant toxin and antitoxin proteins was carried out in the expression strain *E. coli* M15 (pREP4) (Qiagen) and BL21 Rosetta™ (DE3) (Novagen). *E. coli* strains were grown in Luria broth (LB medium at 37 °C with shaking. Protein expression was induced with the addition of isopropyl-1-thiogalactopyranoside (IPTG) (final concentration 0.1 mM) and 1-arabinose (final concentration 0.2%). Antibiotics were added as needed at the following concentrations: 100 μg/ml ampicillin, 50 μg/ml kanamycin, 100 μg/ml chloramphenicol, 100 μg/ml streptomycin.

**Construction of Expression Plasmids pQE70::Toxin and pBAD::Antitoxin**

Each toxin was cloned into the expression vector pQE70 (Qiagen). For *ssl7003*, the ATG codon was shifted as no clones were obtained when using the pQE70 ATG codon and opti-
mized Shine-Dalgaro region, likely due to leaky expression. The ampicillin resistance gene of expression vector pBAD/Myc-His B (Invitrogen) was exchanged with the chloramphenicol or streptomycin resistance gene, respectively, resulting in plasmids pBAD_Cm and pBAD/Strep. Antitoxins were cloned into these vectors. Antitoxin gene ss17004 was also cloned in expression vector pET28a (Novagen). All oligonucleotides with engineered restriction sites and plasmids are given in supplemental Table S1.

**Growth Inhibition Assays**

*E. coli* M15 pQE70::toxin pBAD::antitoxin strains were inoculated and grown for several hours at 37 °C with shaking in LB medium containing appropriate antibiotics. These cultures were diluted to an 

\[ A_{600} = 0.1 \text{ in fresh LB medium also containing the appropriate antibiotics. Inducers IPTG and arabinose were added from the start or at an } A_{600} = 0.18. \]

Cultures expressing only toxin, only antitoxin, both proteins, and no protein were set up in duplicates and monitored for growth by measuring 

\[ A_{600} \text{ in 15-min intervals (VICTOR3™ multilabel plate reader, PerkinElmer Life Sciences).} \]

**Overexpression and Purification of Sll7003 and Ssl7004**

Overnight cultures of *E. coli* M15 pQE70::sll7003 and *E. coli* BL21(DE3) pET28::sll7004 were diluted in 1 liter of LB respectively and grown to an 

\[ A_{600} = 0.6–0.8 \text{. Overexpression of Sll7003(His)}_6 \text{ and Ssl7004(His)}_6 \text{ was induced by adding IPTG for 3 h at 37 °C with shaking. Protein purification was executed as described before (38) with slight modifications. For inhibition of proteases, phenylmethanesulfonyl fluoride and the Complete protease inhibitor mixture (Roche Diagnostics) were used. Wash buffer B composition was changed to 50 mM NaH_2PO_4, 300 mM NaCl, 40 mM imidazole (pH adjusted to 8.0 using NaOH).} \]

**In Vitro Synthesis of RNA**

*In vitro* transcription for 16 S rRNA was realized with the MEGAScript™ kit (Invitrogen), whereas for the smaller 127-nt RNA fragment, antisense to gene sll0406 and its complement the MEGAScript™ kit (Invitrogen) was employed. PCR products containing a T7 RNA polymerase promoter sequence were used as templates, and transcription was carried out according to the manufacturer’s instructions, including the optional DNase treatment and phenol/chloroform extraction.

**RNase Activity**

**Fluorescence Assay**—For the fluorescence assays, we employed the RNaseAlert™ (Integrated DNA Technologies) detection system, which uses a fluorescence-quenched oligonucleotide probe as substrate that emits a fluorescent signal after nuclease degradation. The substrate was incubated at 30 °C in 25 mM Tris-HCl (pH 7.5), 60 mM KCl, 100 mM NH_4Cl, 5 mM MgCl_2, and 0.1 mM DTT for 10–15 min before the addition of protein. Fluorescence was measured every minute with the Victor™ multilabel plate reader (PerkinElmer Life Sciences) with excitation at 480 nm and emission at 520 nm. To avoid evaporation during the measurement, samples were covered with 30 µl of RNase-free immersion oil. Magnesium dependence was tested with 8.5 pmol of substrate, 250 ng of Sll7003 toxin, and 60 mM EDTA. Antitoxin protection was tested with 5.8 pmol of substrate, 250 ng of Sll7003, and ~200 ng of Ssl7004. Each experiment was assayed in technical replicates.

**In Vitro Assay**—Purified Sll7003 and Ssl7004, 100–250 ng of each, were incubated with 400 ng of 16 S rRNA or 300 ng of sll0406, sll0406-as, or duplex sll0406 sense-antisense *in vitro* transcritps at 30 °C for 30–120 min in 25 mM Tris-HCl (pH 7.5), 60 mM KCl, 100 mM NH_4Cl, 5 mM MgCl_2, and 0.1 mM DTT. Reactions were stopped by adding 1 volume of RNA Loading buffer (New England Biolabs or Fermentas). Samples were heated for 5 min at 95 °C prior to electrophoretic separation on 6–10% 7M urea polyacrylamide gels (6 mA, 1.5–2.5 h). Duplex RNA formation was achieved as described in Stazic *et al.* (38) with the following modification. The reaction mixture was complemented with 25 mM Tris-HCl (pH 7.5), 60 mM KCl, 5 mM MgCl_2, 100 mM NH_4Cl, and 0.1 mM DTT.

**Determination of Cleavage Site Specificity**

*In vitro* transcribed full-length 16 S rRNA and a 127-nt-long sll0406-asRNA fragment were incubated with purified Sll7003 protein. Cleavage products were analyzed by 5’ and/or 3’ rapid amplification of cDNA ends (RACE).

3’-RACE—5’-RACE was performed following Steglich *et al.* (39) with certain modifications. The 16 S rRNA *in vitro* transcript was treated with DNase (2 units of Turbo DNase, Invitrogen) and tobacco acid pyrophosphylase (1 unit/1 mg of RNA; Epicenter). RNA was incubated with elution buffer and purified protein Sll7003 for 30 min at 37 °C followed by a phenol/chloroform extraction and ethanol precipitation at each time. A synthetic RNA oligonucleotide (3 pmol) (supplemental Table S1) was ligated to Sll7003-treated RNA (320 ng) and, as a control, to mock-treated RNA using T4 RNA ligase (Fermentas; 3 units/100 ng of RNA) for 1 h at 37 °C followed by phenol/chloroform extraction and ethanol precipitation. Linker reactions with no oligonucleotide were performed as controls. For reverse transcription, linked RNA and controls were preincubated with reverse transcription primer (1 µM final) at 65 °C for 5 min followed by 42°C for 10 min before incubation with Omniscript reverse transcriptase (Qiagen) and a solution of all four dNTPs (0.5 mM each) in the provided reaction. Incubation was carried out at 42°C for 2 h with a final inactivation step at 95°C for 5 min. cDNA was amplified by PCR using an RNA oligonucleotide-specific primer (0.2 µM) and several gene-specific primers (0.2 µM) binding to different positions within the 16 S rRNA. Primers are given in supplemental Table S1. Amplified PCR fragments that appeared specific for the Sll7003-treated sample were gel-excised and cloned into the pET plasmid (Fermentas). After transformation into *E. coli* Top10F™, plasmid inserts were amplified by colony PCR and sequenced.

3’-RACE—*In vitro* transcripts were treated with DNase (2 units of Turbo DNase, Invitrogen), purified with phenol/chloroform, and incubated with elution buffer and purified protein Sll7003 for 30 min at 37 °C. Samples were heated for 5 min at 95 °C prior to 6–10% 7 M urea PAGE separation. Bands of certain size ranges were cut out and gel-eluted overnight using RNA elution buffer (0.3 M NaAc; 1 mM EDTA, 0.1% SDS) fol-
lowed by ethanol precipitation. 5’ ends were blocked by dephosphorylation with FastAP (Fermentas) followed by phenol/chloroform extraction and ethanol precipitation. A 5’-monophosphorylated synthetic RNA oligonucleotide, with a 3’-dideoxynucleotide-blocked end, was ligated to elution buffer- and Sll7003-treated RNA as described under “5’-RACE.” Reverse transcription and PCR were executed as described above. Primers are given in supplemental Table S1. Amplicons that appeared specific for the Sll7003-treated sample were gel-excised and cloned into pGEM-T (Promega). After transformation into E. coli Top10F, plasmid inserts were amplified by colony PCR and sequenced.

RESULTS

TA Systems on Plasmid pSYSA of Synechocystis—We have recently characterized the main function of megaplasmid pSYSA of Synechocystis as harboring a sophisticated defense mechanism, indicated by the presence of three different types of CRISPR systems. However, CRISPR systems are not essential, and pSYSA also does not possess any other gene with a known essential function; therefore the presence of TA systems preventing loss of the megaplasmid appeared likely. Indeed, Makarova et al. (13) identified six putative TA pairs on plasmid pSYSA from a total of 30 type II TA pairs in Synechocystis. These TA systems are located at several distinct locations (Fig. 1), with five of the six putative toxins predicted as RNases, based on the presence of the respective domain. Three of these proteins possess PIN domains, and one protein each has a predicted RelE or MazF domain (Table 1). According to the criteria defined in a recent genome-wide mapping experiment of transcriptional start sites (TSS) (40), all these TA pairs appear to be transcribed, and distinct TSS could be identified (Table 1). It should be noted that the TSS of the ssl7039 toxin gene coincides with the start codon, pointing to a possible leader-less initiation of translation. For the ssl7007_ssl7006 module, we noticed with 207 nt a quite long 5’-UTR based on the existing annotation. Moreover, the annotated start of ssl7007 is an alternative start codon (GTG), and there is not a single stop codon within the 5’UTR. Comparing the possible translation product of a polypeptide 5’-extended by 60 amino acids revealed a very high degree of conserved residues (>85% positive and >65% identical positions) with predicted protein sequences from several other cyanobacteria, such as SYNPCC7002_F0004, N9414_16309, or CWATWH0003_5642 from Synechococcus sp. PCC 7002, Nodularia spumigena CCY9414, and Crocosphaera watsonii WH 0003. We conclude that the annotation of ssl7007 should be 5’-prolonged and that the start of translation likely occurs from an ATT alternative start codon at positions 3766–3768 on the reverse strand.

We also scanned for possible type I TA systems on pSYSA and identified with ssr7036 a gene coding for a short protein of high predicted pl (9.05) associated with very strong antisense transcription (Table 1), hence constituting candidacy for a type I TA system. Furthermore, pSYSA encodes at least three free-standing TA systems components, with ssl7046 and ssl7048 having close homologs on the plasmid pSYSG that are organized in the typical arrangement of a TA operon (genes sll8027 and ssl8028).

Expression of pSYSA TA Systems in E. coli Causes Growth Inhibition That Is Attenuated by the Associated Antitoxin—To test whether the pSYSA-borne potential TA pairs are bona fide
TA systems on plasmid pSYSA of Synechocystis sp. PCC 6803

TA systems, we heterologically expressed the toxin and antitoxin from different inducible promoters in E. coli. The respective toxins were cloned into the IPTG-inducible expression vector pQE70, resulting in the plasmid pQE70:toxin. The associated antitoxins were cloned into the arabinose-inducible expression vector pBAD, resulting in the plasmid pBAD:antitoxin. Five of the six predicted toxin-antitoxin systems (sll7003-sll7004; sll7007-sll7006; sll7033-sll7034; sll7039-sll7038; and slr7041-slr7040) were successfully cloned into the expression vectors. For the sixth TA system (sll7030-sll7031), no positive clones were obtained, whereby it was excluded from further analysis. The different E. coli strains containing both pQE70:toxin and pBAD:antitoxin were grown in LB broth plus the respective antibiotics until they reached a sufficient optical density. To test the effects separately for toxin and antitoxin expression, as well as for the toxin-antitoxin co-expression, we diluted the cultures 1:100 in fresh LB (with the respective antibiotics) plus the corresponding inducer and measured the optical density over time in intervals of 15 min. Each of the strains showed an inhibition in bacterial growth when the toxin was expressed alone as compared with strains with no expression or lone expression of the antitoxin (Fig. 2). Co-expression of toxin and antitoxin attenuated the growth inhibitory effect for all five systems tested (Fig. 2). We conclude that all five tested systems exhibit typical properties of a TA system in vivo. In the case of Slr7007, we observed toxicity despite the fact that the toxin was expressed according to the annotated reading frame and hence might have been missing about 60 residues at its N terminus.

Toxin Sll7003 Causes Growth Arrest and Decreases Colony-forming Units—We selected the toxic component Sll7003 to further test the toxicity of the protein by heterologously overexpressing it in E. coli strain pQE70::sll7003. The inducer IPTG was added at an appropriate optical density to ensure a sufficient amount of cell material for colony formation. Start of the Sll7003 expression arrested the bacterial growth for several hours (Fig. 3A). Toxicity was also seen by a significant decrease of colony-forming units over time (Fig. 3B).

The PIN Protein Sll7003 Is a Magnesium-dependent Ribonuclease—Ribonuclease activity of the purified PIN protein Sll7003 was tested with two different substrates. In the RNaseAlert™ nuclease detection system, a fluorescence-quenched oligonucleotide probe is used as substrate that emits a fluorescent signal after nuclease degradation. Upon the addition of the purified protein Sll7003, we observed a rapid increase of fluorescence over time, whereas there was no increase of fluorescence in the absence of Mg²⁺ (Fig. 4A). To verify the ribonuclease activity of the purified toxin Sll7003 further, in vitro synthesized Synechocystis 16 S rRNA was used as natural substrate. The substrate RNA was incubated with purified toxin and analyzed by PAGE separation. As shown in Fig. 4B, the 16 S rRNA substrate was cleaved into several fragments by the toxin, resulting in a distinct cleavage pattern.

Antitoxin Slr7004 Diminishes Toxin Sll7003 RNase Activity in Vitro—Co-expression of the respective toxin and antitoxin in vivo showed that the growth inhibition effect of the toxin could be attenuated by the antitoxin (Fig. 2). Therefore we tested whether the proposed toxic RNase activity of the purified toxin Sll7003 can be abolished by the purified antitoxin Slr7004 in vitro. As a first substrate, we again used the RNaseAlert™ nuclease detection system. RNase activity of Sll7003 can be seen by a rapid increase of fluorescence over time after the addition of the toxin as compared with an even level of fluorescence in the buffer and Slr7004 antitoxin control. Simultaneous addition of both toxin and antitoxin resulted in a significant lower increase of fluorescence (Fig. 5A). Furthermore, we investigated the diminishing effect of the antitoxin by incubating an in vitro synthesized RNA substrate with the purified toxin and antitoxin at the same time. Incubation with the toxin alone resulted in a distinct cleavage pattern, which could only be seen in a significantly extinguated form during synchronous incubation with toxin and antitoxin (Fig. 5B).

In Vitro Specificity of Sll7003 Ribonuclease—To determine the RNase specificity of the purified protein Sll7003, we applied 5′ and 3′ RACE on two different in vitro transscripts as substrates. We chose the 16 S rRNA and sll0406-as, a 127-nt RNA fragment antisense to the chromosomal gene sll0406, as substrates. The latter encodes another PIN domain-containing protein and is associated with a natural antisense RNA, but was selected here as an example for a short noncoding RNA. In total, we identified 34 cleavage sites, 31 in the 16 S rRNA and three in sll0406-as. Sequence analysis of these sites did not lead to the identification of a preferentially used recognition motif. However, the results indicated that cleavage is favored on the 3′ side of purine residues (~82% of the 34 sites).

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### TABLE 1

| Toxin   | Antitoxin | TSS position | TSS position PCC-M | Distance TSS to start | Type | Orientation |
|---------|-----------|--------------|---------------------|-----------------------|------|-------------|
| sll7003 | PIN       | sll7004 (RHH)| 2074 R             | 2074 R                | II   | AT-T        |
| sll7007 | PIN       | sll7006 (RHH)| 3798 R             | 3798 R                | II   | AT-T        |
| sll7030 | PIN       | sll7031 (RHH)| 29,663 R           | 27,264 R              | 24   | AT-T        |
| sll7033 | PIN       | sll7034 (COG2442)| 31,387 R | 28,988 R                  | 18   | AT-T        |
| ssl7039 | ReE (ribonuclease) | sll7038 (Xre)| 36,479 R          | 34,089 R              | 0    | T-AT        |
| slr7041 | Mzf (ribonuclease) | ssr7040 (AbIR)| 36,569 R          | 34,170 F              | 28   | AT-T        |
| ssr7036 |          | asRNA-ssr7036| 32,177 F/32,481 R | 29,778 F/30,082 R   | 112  | I           |
| ssl7046 | PIN (ribonuclease) | NK          | 40,539 R          | 38,140 R              | 25   |             |
| ssl7030-sll7031 |          |              |                     |                       |      |             |
Furthermore, analysis of the cleavage sites with regard to the known or inferred RNA secondary structure (16 S rRNA, Ref. 41; sll0406-as, RNAfold prediction) showed that single-strand RNA regions were preferred (Fig. 6). Therefore we tested the capability of Sll7003 to cleave double- versus single-stranded RNA molecules under defined conditions in vitro. The 127-nt sll0406-as fragment was annealed with the complementary sequence to create an RNA-RNA duplex. RNase activity was then tested against both ssRNA substrates and the dsRNA substrate. As shown in Fig. 7, Sll7003 was able to cleave the two single-stranded RNA substrates but not the double-stranded RNA target. We conclude that Sll7003 is a single-strand specific RNA endonuclease.

**DISCUSSION**

Here we have characterized the activity of the pSYSA-located Sll7003·Ssl7004 TA system. The toxic component Sll7003 is a Mg²⁺-dependent RNase, which is consistent with its annotation as a PIN domain-containing VapC-type protein (42–44).
FIGURE 4. Purified toxin Sll7003 is a magnesium-dependent ribonuclease. A, RNase Alert™ nuclease detection system. Fluorescence measurement is used as a function of time. The reagent is a fluorescence-quenched oligonucleotide probe that emits a fluorescent signal only after nuclease degradation. The addition of the toxin Sll7003 (indicated by the arrow) shows a significant increase in fluorescence (triangle line) as compared with the buffer control (diamond line) and toxin Sll7003 in the presence of the chelator EDTA, i.e., without MgCl₂ (asterisk line). The experiments were performed in triplicates, and standard deviations are indicated. RFU, relative fluorescent units. B, in vitro synthesized Synechocystis 16 S rRNA was incubated for 30 min with purified toxin Sll7003, resulting in a clear cleavage pattern (duplicate lanes Sll7003). Incubation in water (H₂O), elution (EB), and cleavage buffer (CB) served as controls. The substrate RNA band is labeled by an arrow. M, molecular size markers.
The activity of Sll7003 is inhibited in the presence of the protein Ssl7004, characterizing it as the corresponding antitoxin. Sll7003 activity was not unspecific but exhibited a wide range of accepted substrate configurations, with a clear preference for single-stranded RNA regions and a slight preference for cleavage on the 3' side of purine residues. However, the fact that it cleaved the 16 S rRNA more than 30 times and the fact that it still cleaved the shorter 127-nt-long artificial substrate sll0406-as at three sites underscores its activity as a fairly wide range RNA endoribonuclease, at least in vitro. In contrast, the analysis of VapC of the Shigella flexneri 2a plasmid and of Vap-CLT2 of Salmonella enterica serovar Typhimurium LT2 in vivo indicated that enterobacterial VapC toxins effectively act as tRNases inhibiting translation globally by the site-specific cleavage of the initiator tRNA tRNAfMet between the anticodon stem and loop (34).

We demonstrate that pSYSA harbors at least five functional type II TA systems. However, in the genome-wide analysis of 750 prokaryotic genomes, Makarova et al. (13) identified six likely type II TA pairs on the Synechocystis pSYSA plasmid. Additionally, there is evidence for a possible type I TA system on this plasmid and at least three freestanding TA components (Table 1). In the TADB database of type II TA systems (14), 150 of 337 analyzed prokaryotic plasmids encode only a single TA system. In contrast, only 18 plasmids (5.34%) are listed to possess six or more type II TA systems. Thus, pSYSA is in this top category regarding the number of TA systems. However, extending these bioinformatic predictions by inferring that these are actually transcribed, and for five TA systems, proving their actual toxicity/antitoxicity, we present to our knowledge the highest number of simultaneously active TA systems on any plasmid described thus far. These numbers indicate a high positive selection for possible plasmid maintenance systems on pSYSA, suggesting that it is a very relevant cyanobacterial plasmid. Although pSYSA does not contain any known gene in the classical sense, it has recently been characterized as a major defense plasmid, devoting 75% of its coding capacity to encode all components of three independent CRISPR systems.3
FIGURE 6. Identification of SII7003 ribonuclease specificity by RACE. In vitro synthesized Synechocystis 16 S rRNA was incubated for 30 min with purified toxin SII7003. Resulting RNA fragments were used to determine the cleavage sites by 5' and 3' RACE, indicated by the arrows. The RACE results indicate that toxin SII7003 has an in vitro preference for ssRNA.
The PIN protein SII7003 degrades ssRNA, but cannot degrade dsRNA. The 127-nt fragment of Synechocystis sII0406 (0406) and complement (0406-as) were synthesized in vitro. The RNA-RNA duplex between both molecules was annealed and gel-purified. Single- and double-stranded RNA species were incubated with purified toxin SII7003 for 2 h. Control incubations in elution buffer (EB) indicated the absence of any unspecific degradation. Toxin-inoculated ssRNA species (0406 and 0406-as) exhibited a clear cleavage pattern, whereas dsRNA (0406:0406-as Duplex) was not cut by SII7003. The positions of ssRNA and dsRNA substrates and of a selected marker band (M) are indicated.

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