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Sequence-dependent Cleavage Site Selection by RNase Z from the Cyanobacterium *Synechocystis* sp. PCC 6803*

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Biosynthesis of transfer RNA requires processing from longer precursors at the 5′- and 3′-ends. In eukaryotes, in archaea, and in those bacteria where the 3′-terminal CCA sequence is not encoded, 3′ processing is carried out by the endonuclease RNase Z, which cleaves after the discriminator nucleotide to generate a mature 3′-end ready for the addition of the CCA sequence. We have identified and cloned the gene coding for RNase Z in the cyanobacterium *Synechocystis* sp. PCC 6803. The gene has been expressed in *Escherichia coli*, and the recombinant protein was purified. The enzymatic activity of RNase Z from *Synechocystis* has been studied *in vitro* with a variety of substrates. The presence of C or CC after the discriminator nucleotide modifies the cleavage site of RNase Z so that it is displaced by one and two nucleotides to the 3′-side, respectively. The presence of the complete 3′-terminal CCA sequence in the precursor of the tRNA completely inhibits RNase Z activity. The inactive CCA-containing precursor binds to *Synechocystis* RNase Z with similar affinity than the mature tRNA. The properties of the enzyme described here could be related with the mechanism by which CCA is added in this organism, with the participation of two separate nucleotidyl transferases, one specific for the addition of C and another for the addition of A. This work is the first characterization of RNase Z from a cyanobacterium, and the first from an organism with two separate nucleotidyl transferases.

RNase Z is an endonuclease required for the processing of the 3′-end of tRNAs. RNase Z cleaves CCA-less pre-tRNAs just 3′ of the so-called discriminator nucleotide that protrudes from the aminoacyl stem. To the discriminator nucleotide the CCA sequence is added by nucleotidyl transferase. The gene coding for RNase Z has been identified (1), and the recombinant protein from several sources has been purified and characterized (1, 2). Recently, the crystal structures of RNase Z from *Bacillus subtilis* (3) and *Thermotoga maritima* (4) have been determined, and models for its interaction with the pre-tRNA substrate have been proposed. RNase Z belongs to a conserved group of proteins (ELAC1/ELAC2) within the metallo-β-lactamase superfamily and contains a phosphodiesterase domain. ELAC2 proteins are only present in eukaryotes, are longer, and are thought to have arisen by duplication of an ELAC1 family protein. In some cases zinc-dependent phosphodiesterase activity of RNase Z has been demonstrated (5).

The presence of the CCA sequence in the pre-tRNA is an antideterminant of RNase Z activity for eukaryotic (6) (7) and bacterial (2) RNase Z. RNase Z inhibition by the CCA sequence is thought to prevent a futile cycle of CCA addition by nucleotidyl transferase and removal by RNase Z. CCA-containing pre-tRNAs must be processed by alternative pathways. For instance in *Escherichia coli*, where all 86 tRNA genes encode CCA, 3′-end processing is carried out by exonucleases, after an initial cleavage of longer precursors by the endonuclease RNase E (8). Several exonucleases, functionally redundant, have been described that are able to generate the mature 3′-end (9). A similar exonucleolytic pathway is thought to be present in other bacteria for processing of CCA-containing pre-tRNAs like in *B. subtilis*, where RNase Z cannot process *in vivo* CCA-containing pre-tRNAs (2). *E. coli* encodes a functional RNase Z homologue, which has been identified as the previously described RNase BN (10); however, no obvious function can be ascribed to *E. coli* RNase Z, because all 86 tRNA genes encode CCA, and CCA-containing pre-tRNAs are not substrates for *E. coli* RNase Z (10, 11). An exception to the CCA inhibition of RNase Z is the bacterium *T. maritima* where RNase Z can cleave CCA-containing pre-tRNAs (11). In *T. maritima* CCA is encoded in all but one of the 46 tRNA genes, and RNase Z cleaves 3′ of the CCA sequence instead of 3′ of the discriminator in those CCA-containing pre-tRNAs.

Although RNase Z cleavage occurs mainly after the discriminator nucleotide, there is some heterogeneity in cleavage site selection *in vitro*. Furthermore, cleavage site can be displaced in a sequence-dependent manner. Variant sequences in the place of CCA can induce alterations in cleavage site (11). The purified plant enzyme does not recognize CCA as part of the tRNA and cleaves CCA-containing precursors after the discriminator, whereas crude extracts do not cleave CCA-containing substrates (6), suggesting that a cofactor is required for CCA recognition in this case.

Here we have studied the properties of RNase Z from the cyanobacterium *Synechocystis* sp. PCC6803, where CCA is not encoded in tRNA genes. We have identified between two possible candidates the gene that codes for RNase Z in *Synechocystis* and have purified the encoded protein. The effect of the sequence present at the CCA position in the precursor on activity and cleavage site selection has been studied. We discuss the observed effect of the sequence at the CCA site in relation with the fact that *Synechocystis* contains two separate nucleotidyl transferases for addition of CCA, one C-specific and another A-specific (12). *Synechocystis* is the first organism with two separate CCA-adding enzymes in which RNase Z is studied.

EXPERIMENTAL PROCEDURES

Construction of Synechocystis Mutant Strains—A genomic region of 2 kb, containing the slr0050 gene, was amplified with oligonucleotides 0050F2 and 0050R2. The PCR product was cloned into pGEM-T (Promega). A 0.7-kb HindIII fragment, partially overlapping the coding sequence of slr0050, was replaced by a chloramphenicol or a kanamycin resistance cassette (13). In a similar way, a genomic region of 1 kb, containing the sll1036 gene was amplified with oligonucleotides 1036F1 and 1036R1. The unique NcoI site within the coding sequence of sll1036

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was used to interrupt the coding sequence by inserting a chloramphenicol or kanamycin resistance cassette. The plasmids containing the interrupted genes were used to transform wild type *Synechocystis* cells. Cells were plated on chloramphenicol- or kanamycin-containing BG11 medium (14) and grown on the same medium for several segregation rounds. Segregation was checked by Southern blot and PCR.

Pre-tRNA Substrates—The template DNAs were obtained by PCR amplification based on the published complete genome sequence (15). In all cases the 5′-primer contains a BamHI site, the T7 RNA polymerase promoter and overlaps the 5′-end of the tRNA (for 5′ mature pre-tRNAs) or the 5′ of the leader sequence (for 5′ extended pre-tRNAs). The 3′-primer overlaps the 3′-end of the tRNA and contains variable extra nucleotides and a HindIII site. TABLE ONE shows the primers used for each pre-tRNA sequence. Primer N34GLUF1 and GLU(UUA)R1 were used to generate a template for $N_{34}tRNA^{Glu}UUAN_{17}$ that contains a 5′ leader sequence of 34 nucleotides and a 3′-trailer of 17 nucleotides after the genomic encoded UUA sequence at the CCA position. Primers GLUF1 and GLU(CCA)R1, GLU(CCU)R1, GLU(CUA)R1, GLU(UCA)R1, GLU(UUU)R1, or GLU(UUA)R1 were used to generate templates for $tRNA^{Glu}CCAN_{17}$, $tRNA^{Glu}CCUN_{17}$, $tRNA^{Glu}CUAN_{17}$, $tRNA^{Glu}UCAN_{17}$, $tRNA^{Glu}UUUN_{17}$, or $tRNA^{Glu}UUAN_{17}$, respectively. These pre-tRNAs contain a mature 5′-end and a 3′-trailer of 17 nucleotides after the different trinucleotides sequences indicated. Primers ILEF1 and ILER1 were used to generate a template for pre-$tRNA^{Ile(GAT)}$. Primers N10GLNF1 and GLN(UUU)R1 were used to generate a template for $N_{10}tRNA^{Gln}UUUN_{17}$. This pre-tRNA contains a 5′-leader sequence of 10 nucleotides and a 3′-trailer of 17 nucleotides after the genomic encoded UUU sequence at the CCA position. The PCR products were digested with BamHI and HindIII and cloned into pUC19. Templates for run-off transcription of the different pre-$tRNA^{Glu}$ or pre-$tRNA^{Gln}$ RNAs were prepared by digesting the resulting plasmids with HindIII, which generates a precursor with a 17-nucleotide tail after the CCA sequence or its variants as indicated above. Templates for run-off transcription of pre-$tRNA^{Ile(GAT)}$ with different 3′-tail length were prepared by digesting the resulting plasmid with HindIII (238 nucleotides tail), HincII (153 nucleotides tail), AvaII (86 nucleotides tail), and FokI (37 nucleotides tail). Other tRNA templates have been described (16). T7 RNA polymerase transcription was performed with the Ampliscribe kit (Epicenter).

RNA Labeling—RNAs were labeled at the 5′-end with [γ-^{32}P]ATP and T4 polynucleotide kinase after dephosphorylation with calf intestinal phosphatase as described (17). Labeling at the 3′-end was done with [5′-^{32}P]pCp by T4 RNA ligase as described (17).

Recombinant Protein Purification—The coding sequences of the *Synechocystis* proteins to be purified, deduced from the published sequence (15), were amplified by PCR and inserted in pET28a (Novagen) in-frame with the amino-terminal hexahistidine sequence as follows: slr0050 was amplified with oligonucleotides slr0050F3 and slr0050R3. The PCR fragment was digested with NdeI and SacI and cloned into pET28a digested with the same enzymes; sll1036 was amplified with oligonucleotides sll1036F1 and sll1036R1. The PCR fragment was digested with NheI and SacI and cloned in pET28a digested with the same enzymes; sll0825 (C-specific tRNA nucleotidyl transferase, Ntase(C))[3] (12) was amplified with oligonucleotides 0825F2 and 0825R2. The PCR product was cloned in pGEM-T. The resulting plasmid was digested with SacII, treated with the exonucleolytic activity of Klenow, and then digested with SacI. The fragment containing the sll0825 open reading frame was inserted in pET28a that had been digested with NdeI, filled in with Klenow, and finally digested with SacI. The expected proteins expressed from the recombinant plasmids would contain several extra amino acids at the amino end, including a $(His)_6$ tag. The recombinant plasmids were introduced in BL21(DE3)pLys (Novagen) by electroporation. Cells were grown at 37 °C in minimal M9 medium containing 50 μg/ml kanamycin. When A_{600} was around 0.5, 1 mM isopropyl 1-thio-β-D-galactopyranoside was added. Cells were harvested after 3-h incubation at 37 °C. The cell extract was prepared by sonication in 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 100 mM KCl, 1 mM phenylmethylsulfonyl fluoride (buffer A) and clarified by centrifugation at 30,000 × *g* for 30 min. His-tagged proteins were purified from the soluble protein fraction by nickel affinity chromatography on 1-ml His-trap columns using an Äkta fast-protein liquid chromatography system (Amersham Biosciences). The column was washed with 10 ml of 50 mM imidazole in buffer A before applying a 50–500 mM imidazole gradient in buffer A. His-tagged recombinant proteins eluted at 100–150 mM imidazole. The appropriate fractions were pooled, dialyzed against buffer A containing 50% glycerol, and frozen at −20 °C. The proteins purified this way were free of contaminating *E. coli* nuclease or phosphodiesterase activities (see below).

Phosphodiesterase Assay—Phosphodiesterase activity was measured as described (5) using the substrates bis(*p*-nitrophenyl)phosphate or thymidine-5′-monophosphate-*p*-nitrophenylester. Reaction mixes, containing 5 mM substrate and 5 μg of purified protein in 20 mM Tris-HCl, pH 7.5, were incubated at 22 °C. *p*-Nitrophenol production was followed spectrophotometrically at 405 nm.

RNase Z Assay—For single turnover experiments, 1000–5000 cpm (0.1 nM) of labeled pre-tRNAs were incubated in 40 mM Tris-HCl (pH 7.5), 2 mM dithiothreitol in the presence of 3 nM of the enzyme fraction at 37 °C. To terminate the reaction, one volume of loading dye containing 10 M urea and 25 mM EDTA was added, and the reaction products were separated in polyacrylamide/7 M urea gels, and the reaction products were detected with a Cyclone Phosphor System (Packard). The data were adjusted to a single exponential equation. For the determination of K_m and k_{cat} under steady-state conditions, assays were done in the same conditions with varying concentrations of substrate (0.05–5 μM). The data were fitted to the Michaelis-Menten equation. In competition assays, 5 nM RNase Z were incubated in the same reaction mix with 0.2 nM ^{32}P-labeled active pre-tRNA and variable amounts of unlabeled RNAs. As an unrelated control in inhibition assays, a 90-bp RNA (pBSK RNA), obtained by T7 RNA polymerase *in vitro* transcription from pBluescript SK− digested with XbaI, was used. The data were adjusted to a competitive inhibition equation as described previously (18).

Nucleotidyl Transferase Assay—10 μM pre-tRNA was incubated in buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 5 mM dithiothreitol, 0.5 mM ATP, and 0.05 mM [α-^{32}P]CTP (6.25 μCi/ml) at 37 °C for 30 min in the presence of 40 nM purified *Synechocystis* Ntase(C). In coupled assays, 50 nM purified RNase Z was added also to the reaction mix together with Ntase(C). Radioactively labeled RNAs were separated by electrophoresis on 8% polyacrylamide/7 M urea gels and detected with a Cyclone Phosphor System (Packard).

Primer Extension—A preparative RNase Z assay was set up with 125 ng of unlabeled substrate and enough enzyme to obtain almost complete processing in 60 min. The reaction products were phenol-extracted and precipitated with ethanol and used as templates in primer extension. Primer extensions were performed by standard procedures with oligonucleotides GLXPE and ILEPE (TABLE ONE) labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase.

RESULTS

Identification of slr0050 and sll1036—Blast searches of the *Synechocystis* genome identified two open reading frames (slr0050 and sll1036)

[3] The abbreviations used are: Ntase(C), C specific tRNA nucleotidyl transferase; pre-tRNA, precursor of tRNA; tRNA, transfer RNA.

TABLE ONE

Oligonucleotides used

Name	Sequence (5′→3′)
0050F1	AACGTCATATGGAAATTACTTTTCTTGGTACCAGCTCC
0050R1	CTTACGAGCTCTGTGGGGGAAATGGTGCCCTACG
0050F2	AAACCCAATTGCAAAACGGC
0050R2	GGTAGACCCCAATTTGTTGG
0825F1	ATTGCATGCTTTGTCCTGGCCACC
0825R1	TAAGGTTACCCCGGTTCAAGATTTTCCCAG
1036F1	AACGTGCTAGCACATTCAAGATCAAATTTTGGGG
1036R1	CTTACGAGCTCTTCGGCTATCTCCAACCAGTCC
N34GLUF1	CGACGGGATCCTAATACGACTCACTATAGTCTGAAATAACGAACTG
GLUF1	CGACGGGATCCTAATACGACTCACTATAGCCCCCATCGTCTAGAGG
GLU(CCA)R1	GTCCCAAGCTTGGATGGATCAATTGGTACCCCCAAGGG
GLU(CCU)R1	GTCCCAAGCTTGGATGGATCAATAGGTACCCCCAAGGG
GLU(CUA)R1	GTCCCAAGCTTGGATGGATCAATTAGTACCCCCAAGGG
GLU(UCA)R1	GTCCCAAGCTTGGATGGATCAATTGATACCCCCAAGGG
GLU(UUU)R1	GTCCCAAGCTTGGATGGATCAATAAATACCCCCAAGGG
GLU(UUA)R1	GTCCCAAGCTTGGATGGATCAATTAATACCCCCAAGGG
N10GLNF1	CGACGGGATCCTAATACGACTCACTATAGGTTAATCAATGGGGTG
GLN(UUU)R1	GTCCCAAGCTTGGATGGATCTTTAAACTGGGGTGCTAGG
ILER1	GTCCCAAGCTTCTTTTTGCTAACTAGG
ILEF1	CGACGGGATCCTAATACGACTCACTATAGGGCTATTAGCTCAGCTGG
GLXPE	GATTACGCCAAGCTTGGATGGATC
ILEPE	GGACTTACATCCAGC

with significant similarity to RNase Z from other organisms. Both proteins correspond to the short version (ElaC1) of RNase Z and contain the phosphodiesterase histidine motif (Fig. 1), although sll1036 lacks two of the conserved motifs present in the metallo-β-lactamase superfamily and that are found in known RNase Z (4). Similar proteins were identified in all the cyanobacterial sequenced genomes. They are grouped in two families (Fig. 2), one represented by *Synechocystis* slr0050 and the other represented by *Synechocystis* sll1036. All the cyanobacteria analyzed contain one single protein homologue in the slr0050 group. Of the thirteen cyanobacterial genomes analyzed, six (*Gloebacter violaceus* PCC7421, *Prochlorococcus marinus* MIT9313, *P. marinus* SS120, *P. marinus* MED4, *Synechococcus elongatus* PCC7942, and *Synechococcus* sp. WH8102) lack a representative in the sll1036 group. However, *Trichodesmium erythraeum* contains three, and *Anabaena variabilis* ATCC29413, *Nostoc punctiforme* PCC73102, and *Nostoc* sp. PCC7120 contain two proteins in this group.

Inactivation of slr0050 and sll1036—The coding sequence of slr0050 and sll1036 has been interrupted by the insertion of an antibiotic resistance cassette. The sll1036 insertion was readily fully segregated, indicating that the encoded protein is not essential for growth under normal culture conditions. No differences were observed in growth rate between the sll1036 null strain and the wild type strain under normal growth conditions. In contrast, it was not possible to obtain a strain with all the chromosomal copies of slr0050 interrupted even after many rounds of segregation at increasing antibiotic concentration, suggesting that slr0050 is an essential gene in *Synechocystis*.

In Vitro Activities of slr0050 and sll1036—To functionally characterize slr0050 and sll1036, the corresponding genes were cloned in pET28 with a histidine tag at the amino end and purified by nickel affinity chromatography. The purified proteins were assayed for RNase Z activity with radioactively labeled $tRNA^{Glu}UUAN_{17}$. Only the slr0050 protein showed significant RNase Z activity (Fig. 3). It has been shown that some RNase Z proteins have phosphodiesterase activity (5) with thymidine-5′-monophosphate-*p*-nitrophenylester or bis(*p*-nitrophenyl)-phosphate. Both slr0050 and sll1036 showed significant phosphodiesterase activity with bis(*p*-nitrophenyl)phosphate (Fig. 4), although the specific activity of slr0050 was about 4-fold higher. None had phosphodiesterase activity with thymidine-5′-monophosphate-*p*-nitrophenylester (not shown).

Effect of 5′ and 3′ Extensions on Synechocystis RNase Z Activity—The RNase Z activity of slr0050 was further characterized. We have previously shown that 5′ extended precursors are properly processed *in vivo* at the 3′-end (19) suggesting that RNase Z can act before RNase P in the pre-tRNA biosynthetic pathway. We have compared the RNase Z activity of slr0050 with a substrate containing an extension of 34 nucleotides at the 5′-end ($N_{34}tRNA^{Glu}UUAN_{17}$) and the 5′ mature substrate $tRNA^{Glu}UUAN_{17}$. No significant differences were observed (not shown). Similar result was obtained with $N_{10}tRNA^{Gln}UUUN_{17}$. The effect of the length of the 3′-tail was assayed with pre-$tRNA^{Ile(GAT)}$ with 3′-tails of different length (Fig. 5). $tRNA^{Ile(GAT)}$ is the only pre-tRNA in *Synechocystis* with CC encoded after the discriminator and is transcribed from the only duplicated tRNA gene in *Synechocystis*, within the duplicated ribosomal operons. There were some slight differences in cleavage efficiency that do not correlate in a simple way to tail length.

RNase Z Activity on Sequence Variants after the Discriminator Nucleotide—We have analyzed the effect on activity of the trinucleotide sequence after the discriminator in pre-tRNAs. For that purpose, we have compared the RNase Z activity of slr0050 with pre-$tRNA^{Glu}$ that contains different trinucleotides sequences at the CCA position followed by a constant tail of 17 nucleotides ($tRNA^{Glu}UUAN_{17}$, $tRNA^{Glu}CUAN_{17}$, $tRNA^{Glu}CCUN_{17}$, $tRNA^{Glu}UCAN_{17}$, $tRNA^{Glu}UUUN_{17}$, and $tRNA^{Glu}CCAN_{17}$). There was RNase Z activity with all substrates except with $tRNA^{Glu}CCAN_{17}$ (Fig. 6). Activity was compared under single turnover conditions (TABLE TWO). Maximum activity was observed with $tRNA^{Glu}UUAN_{17}$, which contains the genomic encoded UUA sequence. $tRNA^{Glu}CUAN_{17}$ and $tRNA^{Glu}CCUN_{17}$ were also active substrates, although with reduced reaction rates. Steady-state kinetics was also analyzed for $tRNA^{Glu}UUAN_{17}$, $tRNA^{Glu}CUAN_{17}$, and $tRNA^{Glu}CCUN_{17}$. The catalytic efficiency (k_{cat}/K_m) was reduced in

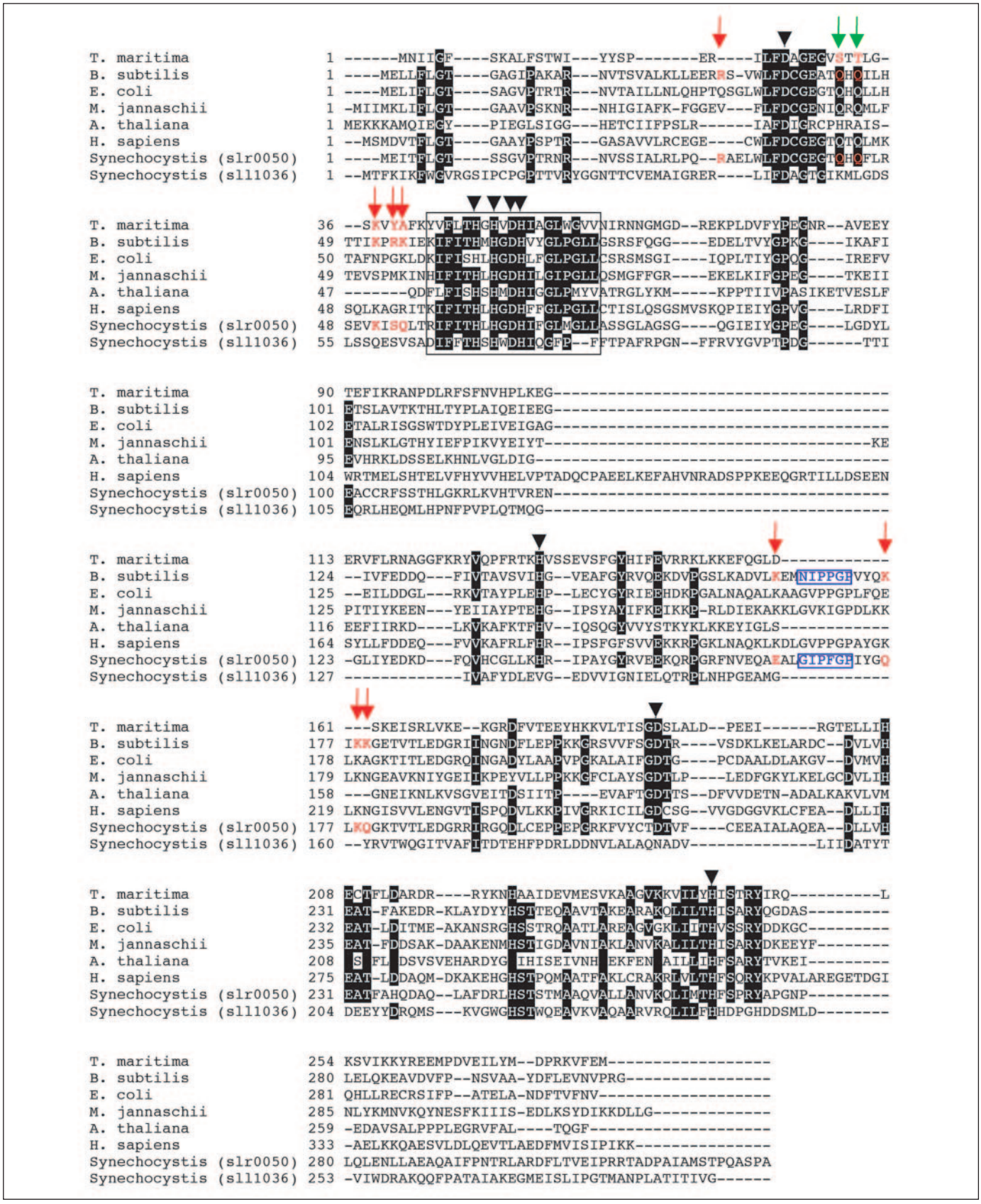

FIGURE 1. **Sequence alignment of RNase Z sequences.** The primary sequence of RNase Z from several organisms, as well as *Synechocystis* slr0050 and sll1036, were aligned with ClustalX (32). The sequences used were from *B. subtilis* (P54548), *E. coli* (Q47012), *Homo sapiens* (Q9H777), *M. jannaschii* (Q58897), *T. maritima* (NP_228673), and *A. thaliana* (NP_178532). Conserved residues are highlighted in *black*. The *square* indicates the phosphodiesterase histidine motif. The highly conserved amino acids within the five conserved motifs in the metallo-β-lactamase superfamily (33, 34) are also indicated (*black triangles*). *Green arrows* point to residues in *T. maritima* RNase Z that are important for cleavage site selection in this organism (11). *Red arrows* point to residues proposed to be involved in tRNA-enzyme interaction in *B. subtilis* (3). Residues highlighted in a *blue box* are the GP-motif within the exosite substrate binding module (30). See text for detailed discussion.

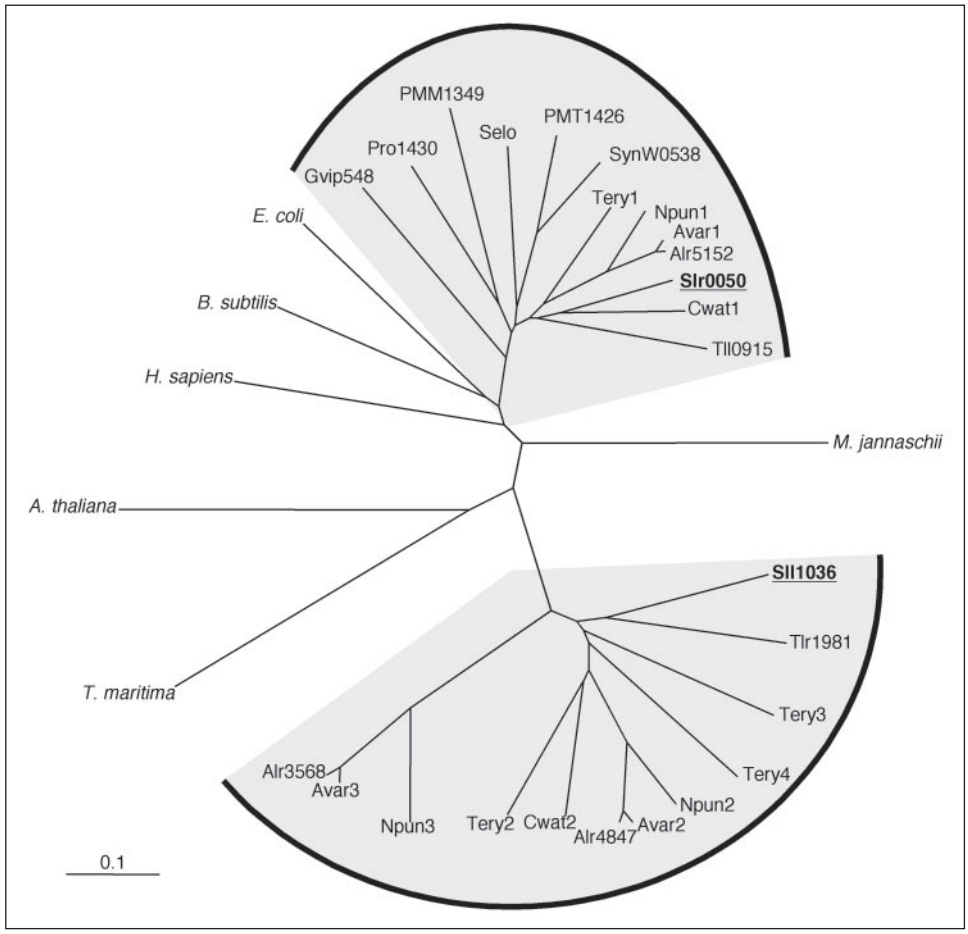

FIGURE 2. **Phylogenetic tree of RNase Z and related proteins from cyanobacteria.** Proteins deduced from the cyanobacterial sequenced genomes with significant similarity to RNase Z, as well as those studied from other organisms were aligned with ClustalX to generate a neighbor-joining tree. The *scale bar* represents the fraction of amino acids substitution. The accession number of the sequences are as follows: *A. variabilis* ATCC29413 (*Avar1*: ZP_00160370; *Avar2*: ZP_00160521; *Avar3*: ZP_00163033), *Crocosphera watsonii* WH8501 (*Cwat1*: ZP_00175784; *Cwat2*: ZP_00175736), *Gloebacter violaceus* PCC7421 (*Gvip548*: NP_927065), *Nostoc punctiforme* PCC73102 (*Npun1*: ZP_00112090; *Npun2*: ZP_00107194; *Npun3*: ZP_00112285), *Nostoc* sp. PCC7120 (*Alr5152*: NP_489192; *Alr3568*: NP_487608; *Alr4847*: NP_488887), *P. marinus* MIT9313 (*PMT1426*: NP_895253), *P. marinus* CCMP1375 (*Pro1430*: NP_87582), *P. marinus* CCMP1986 (*PMM1349*: NP_893466), *Synechococcus elongatus* PCC7942 (*Selo*: ZP_00165028), *Synechococcus* sp. WH8102 (*SynW0538*: NP_896633), *Thermosynechococcus elongatus* BP-1 (*Tll0915*: NP_681705; *Tlr1981*: NP_682771), and *T. erythraeum* IMS101 (*Tery1*: ZP_00326171; *Tery2*: ZP_00326754; *Tery3*: ZP_00325500; *Tery4*: ZP_00327925). Others are indicated in the legend of Fig. 1.

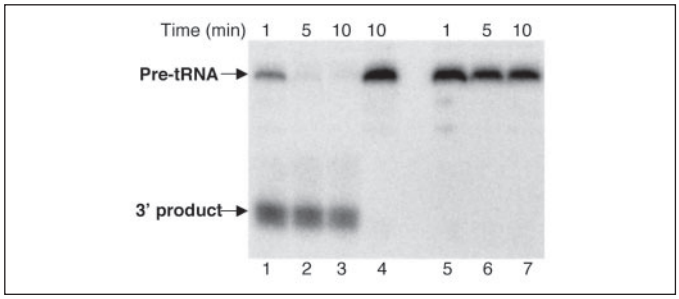

FIGURE 3. **RNase Z activity of slr0050 and sll1036.** RNase activity was assayed as described under "Experimental Procedures" with 3′-end labeled $tRNA^{Glu}UUAN_{17}$ and 25 nM His tag-purified slr0050 (*lanes 1–3*), sll1036 (*lanes 5–7*), or without protein (*lane 4*). After incubation for the indicated times, reaction products were separated on 20% polyacrylamide/7 M urea gels.

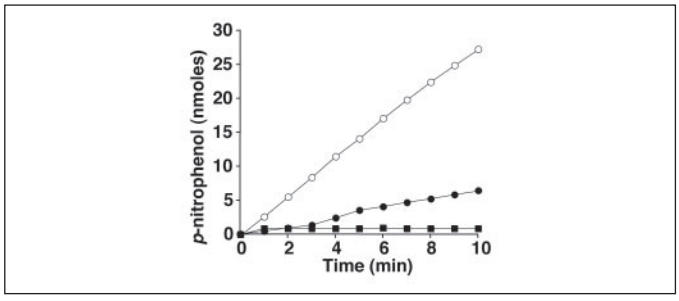

FIGURE 4. **Phosphodiesterase activity of slr0050 and sll1036.** Time course of the phosphodiesterase activity of slr0050 (*open circles*) and sll1036 (*black circles*) on bis(*p*-nitrophenyl)phosphate. The assay was performed as described under "Experimental Procedures" in a 1-ml reaction mix containing 120 nM purified protein. As a control of the possible presence of *E. coli* phosphodiesterase activity in the His-tagged purified proteins, His-tagged *Synechocystis* Ntase(C) (*squares*), which elutes at the same position than slr0050 and sll1036 in His-trap columns, was used.

$tRNA^{Glu}CUAN_{17}$, and $tRNA^{Glu}CCUN_{17}$ with respect to $tRNA^{Glu}UUAN_{17}$. This is due mainly to reduction of k_{cat}.

Identification of RNase Z Cleavage Site—The electrophoretic pattern shown in Fig. 6 suggests heterogeneity in the size of the 3′-fragment generated by RNase Z cleavage of pre-tRNAs. This could be due to heterogeneity in the 3′-end of the substrate by addition of non-template nucleotides by T7 RNA polymerase or to heterogeneity in cleavage site selection by RNase Z (see below). What is more, the size of the 3′-fragment generated by RNase Z seems to be slightly smaller in CUA and CCU-containing substrates than in the other three, which suggests a different cleavage site. The cleavage site of RNase Z was precisely determined by primer extension in three sequence variants of the pre-$tRNA^{Glu}$ substrate, those containing UUA, CUA, or CCU after the discriminator. The substrate with the native UUA sequence, $tRNA^{Glu}UUAN_{17}$, was cleaved at the correct expected site, but also a large amount of cleavage was detected before the discriminator (Fig. 7*A*). In contrast, the main cleavage site in $tRNA^{Glu}CUAN_{17}$ was 3′ of C. In $tRNA^{Glu}CCUN_{17}$ the main cleavage site was 3′ of CC. These results clearly indicate that cleavage site selection by *Synechocystis* RNase Z is dependent on the sequence around the cleavage site. The presence of C or CC 3′ of the discriminator nucleotide produces a displacement of one or two nucleotides of the cleavage site, respectively. These results were confirmed with two other pre-tRNAs. $tRNA^{Ile}CCUN_{86}$ was treated with RNase Z and subjected to primer extension with oligonucleotide ILEPE2, complementary to the 3′-tail. The result shown in Fig. 7*B* clearly indicates that RNase Z cleavage occurs mainly one and two nucleotides 3′ of the discriminator. The same result was obtained with

$tRNA^{Ile}CCUN_{37}$, $tRNA^{Ile}CCUN_{153}$, and $tRNA^{Ile}CCUN_{238}$ (not shown). A similar experiment with $N_{10}tRNA^{Gln}UUUN_{17}$ showed cleavage after the discriminator (Fig. 7*C*). The UUU sequence after the discriminator corresponds to the genomic encoded sequence. These results confirm that the sequence-dependent displacement of cleavage site is not a peculiarity of pre-$tRNA^{Glu}$, but is more general. It should be noted that in the pre-$tRNA^{Glu}$ and pre-$tRNA^{Gln}$ substrates that we have used, the sequence of the 3′-trailer is a synthetic sequence, whereas in pre-$tRNA^{Ile(GAT)}$ it corresponds to the genomic sequence encoded downstream the tRNA gene (*trnI*).

Nucleotidyl Transferase and RNase Z—To show that RNase Z generates a functional substrate for CCA addition, we have analyzed if the product of pre-tRNA cleavage by purified RNase Z activity can be labeled with [α-^{32}P]CTP in the presence of purified *Synechocystis* Ntase(C). CMP addition by Ntase(C) would indicate that the pre-tRNAs have been properly processed by RNase Z, because Ntase(C) would be able to add CMP only to tRNAs with mature 3′-ends. This is confirmed in the experiment shown in Fig. 8*A*, where only fully mature tRNA (*lane 3*) or 5′-extended but 3′-mature pre-tRNA (*lane 8*) are labeled by [α-^{32}P]CTP in the presence of Ntase(C). Upon incubation with RNase Z (Fig. 8*B*), $tRNA^{Glu}UUA$ (*lane 6*), and $tRNA^{Glu}UUAN_{17}$ (*lane 7*), which where not substrates of Ntase(C) in the absence of RNase Z, were labeled by [α-^{32}P]CTP, indicating their correct processing by RNase Z. Meanwhile $tRNA^{Glu}CCA$ (*lane 4*) or $tRNA^{Glu}CCAN_{17}$ (*lane 5*), after incubation with RNase Z, were not labeled by [α-^{32}P]CTP, as expected if they are not substrates of RNase Z. Incubation with purified sll1036 instead of RNase Z did not result in labeling by Ntase(C) of any of the pre-tRNAs tested (not shown).

RNase Z Inhibition by Inactive Pre-tRNA—To analyze if inactive CCA-containing pre-tRNA can bind to *Synechocystis* RNase Z, an inhibition assay was used (18). The inhibition assay (Fig. 9) suggests that the inactive $tRNA^{Glu}CCAN_{17}$ binds to the enzyme significantly stronger than an unrelated control RNA. The binding of the $tRNA^{Glu}CCAN_{17}$ is similar to the binding of a mature $tRNA^{Glu}$ that terminates at the discriminator nucleotide.

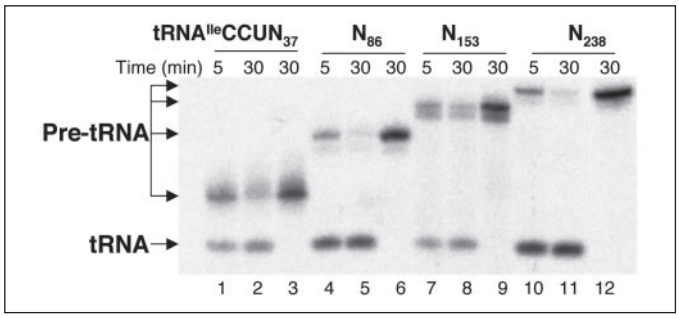

FIGURE 5. **Effect of 3′-tail length on RNase Z activity.** 5′-End-labeled $tRNA^{Ile}CCUN_{37}$ (*lanes 1–3*), $tRNA^{Ile}CCUN_{86}$ (*lanes 4–6*), $tRNA^{Ile}CCUN_{153}$ (*lanes 7–9*), and $tRNA^{Ile}CCUN_{238}$ (*lanes 10–12*) were assayed for RNase Z activity the indicated times as described under "Experimental Procedures" and separated in 8% acrylamide/7 M urea gels. *Lanes 3*, *6*, *9*, and *12*: control without enzyme.

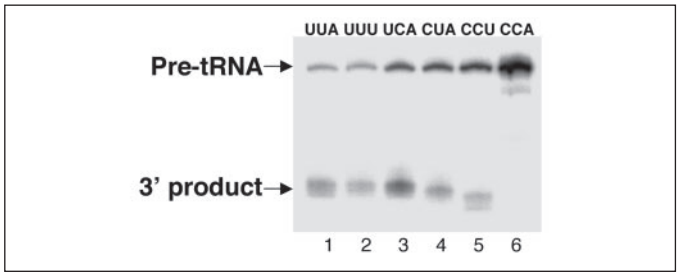

FIGURE 6. **Effect of nucleotide sequence at the cleavage site on RNase Z activity.** 3′-End-labeled $tRNA^{Glu}UUAN_{17}$ (*lane 1*), $tRNA^{Glu}UUUN_{17}$ (*lane 2*), $tRNA^{Glu}UCAN_{17}$ (*lane 3*), $tRNA^{Glu}CUAN_{17}$ (*lane 4*), $tRNA^{Glu}CCUN_{17}$ (*lane 5*), and $tRNA^{Glu}CCAN_{17}$ (*lane 6*), were assayed for RNase Z activity as indicated under "Experimental Procedures" and separated in 20% acrylamide/7 M urea gels.

DISCUSSION

In this work we have identified the functional RNase Z from the cyanobacterium *Synechocystis* among two candidate genes, slr0050 and sll1036. According to BLAST scores slr0050 is more similar to bacterial RNase Z, whereas sll1036 is more similar to plant RNase Z. Both proteins contain the phosphodiesterase histidine motif. However, under closer inspection of the aligned proteins (Fig. 1), only slr0050 contains all the conserved motifs of the metallo-β-lactamase superfamily, which are also conserved in the RNase Z family (Fig. 1). Our *in vitro* analysis has confirmed that sll1036 has no RNase Z activity and has lower phosphodiesterase activity than slr0050 (Figs. 3 and 4). tRNA genes in *Synechocystis* do not encode the 3′-CCA sequence, therefore it is expected that processing of pre-tRNAs requires RNase Z, as observed in *B. subtilis* for the processing of CCA lacking pre-tRNAs. We have concluded that slr0050 is essential, in agreement with its role as RNase Z. Furthermore, a close homologue to slr0050 is present in all cyanobacteria analyzed. sll1036 is not essential in *Synechocystis*, what is supported by its absence in several cyanobacteria, included the *Prochlorococcus* strains that have reduced genome size (20, 21). On the other side the nitrogen-fixing strains contain two or three homologues to sll1036, which points to a more relevant function of this protein family in the adaptation of nitrogen-fixing cyanobacteria. The function of this protein remains to be determined.

We have analyzed the effect of the presence of 5′ or 3′ extensions

TABLE TWO

Activity of purified slr0050 with $tRNA^{Glu}UUAN_{17}$ and its variants

	Single turnover[a]		Steady state[b]		
Substrate	K_{obs}	Relative activity	K_m	k_{cat}	k_{cat}/K_m
	min^{-1}	%	μM	min^{-1}	
$UUAN_{17}$	2.89 ± 0.25	100	0.96 ± 0.38	135.25 ± 18.28	140.9
$UUUN_{17}$	1.84 ± 0.29	64	ND[c]	ND	ND
$UCAN_{17}$	2.41 ± 0.09	84	ND	ND	ND
$CUAN_{17}$	1.13 ± 0.10	39	1.55 ± 0.31	17.83 ± 1.42	11.5
$CCUN_{17}$	0.46 ± 0.04	16	0.47 ± 0.10	8.21 ± 0.59	17.5
$CCAN_{17}$	0	0	ND	ND	ND

[a] Reaction rates were measured under single turnover conditions with 0.1 nM of 3′-labeled pre-tRNAs in the presence of 3 nM enzyme, and the data were adjusted to a single exponential equation.
[b] Reaction rates were measured under steady-state conditions with variable concentrations (0.1–5 μM) of 3′-labeled pre-tRNAs in the presence of 3 nM enzyme, and the data were adjusted to the Michaelis-Menten equation.
[c] ND, not determined.

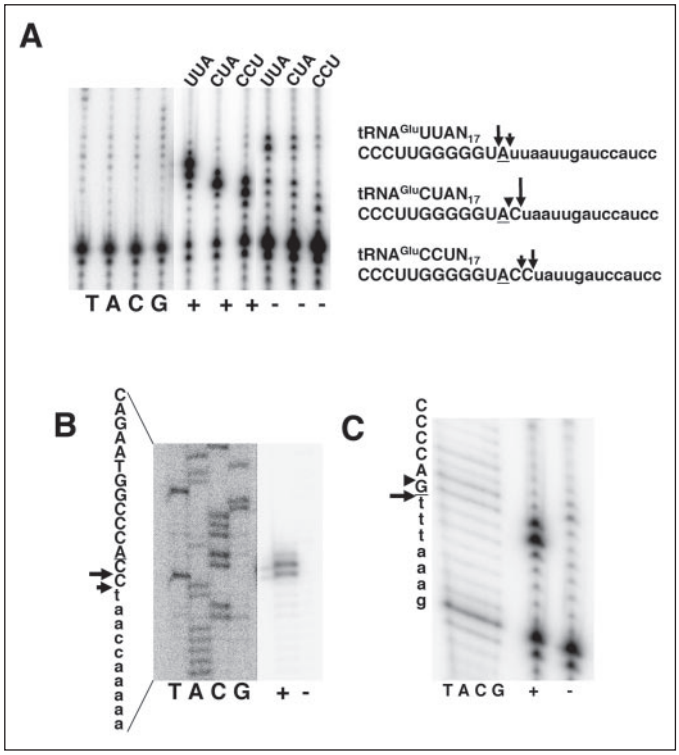

FIGURE 7. **Identification of RNase Z cleavage site.** 125 ng of the indicated pre-tRNAs were incubated with *Synechocystis* RNase Z in conditions that generated almost complete processing. The reaction products were annealed with ^{32}P-labeled oligonucleotide GLXPE for the three different pre-tRNA^{Glu} used and for pre-tRNA^{Gln}, or oligonucleotide ILEPE for pre-tRNA$^{Ile(GAT)}$, and subjected to primer extension as indicated under "Experimental Procedures." As a control of RNA-unspecific degradation or reverse transcription premature stops, samples not treated with RNase Z were subjected to the same procedure. Dideoxy sequencing reactions were performed on the corresponding plasmid templates with the same labeled oligonucleotides and used as size markers. The reaction products were separated on 6% (*B*) or 8% sequencing gels (*A* and *C*). *A*, primer extension results of RNase Z reaction products obtained with tRNA^{Glu}UUAN$_{17}$, tRNA^{Glu}CUAN$_{17}$, and tRNA^{Glu}CCUN$_{17}$. *B*, primer extension results of RNase Z reaction products obtained with tRNA^{Ile}CCUN$_{86}$. *C*, primer extension results of RNase Z reaction products obtained with N$_{10}$tRNA^{Gln}UUUN$_{17}$. *Arrows* on the pre-tRNA sequences indicate the deduced RNase Z cleavage sites. The *length of the arrows* is proportional to the relative radioactivity present in each band in the RNase Z-treated samples (*lanes* +), after subtracting the radioactivity present at the same positions in the control not treated with RNase Z (*lanes* −). The portion of the sequence corresponding to the mature tRNA is shown in *uppercase*. The discriminator nucleotide is *underlined*. A portion of the 3′-tail is shown in lowercase. In *A* and *C* the sequencing reactions cannot be read accurately, because they are too close to the primer, but they are useful as size markers.

in pre-tRNAs on RNase Z activity. We have used 5′-extended pre-tRNA^{Glu}, that contains a 34-nucleotide leader, and 5′-extended pre-tRNA^{Gln}, which contains a 10-nucleotide leader. The pre-tRNA^{Glu} leader length was based on the presence of a consensus promoter sequence. The pre-tRNA^{Gln} leader length has been determined *in vivo* (19). The presence of these 5′ extensions has no effect on 3′ processing by *Synechocystis* RNase Z *in vitro*, in agreement with the observation that *in vivo* depletion of RNase P activity does not affect 3′ processing (19). *Synechocystis* RNase Z seems to be more tolerant to 5′ extensions than other RNase Z enzymes. Pig liver RNase Z is inactive with pre-tRNAs with 5′ extensions >9 nucleotides (22). *B. subtilis* RNase Z has reduced activity with 5′ extensions of 33 nucleotides or more (2). To assay the effect of the length of 3′ extensions in pre-tRNA on RNase Z activity, we have used pre-tRNA$^{Ile(GAT)}$ with 3′-tails of variable length. tRNA$^{Ile(GAT)}$ gene is within the duplicated ribosomal RNA operon between rRNA 16S and 23S. The 3′-end of tRNA$^{Ile(GAT)}$ gene is 250 bp from the 5′-end of 23S rRNA. We have used pre-tRNA$^{Ile(GAT)}$ with up to 238 nucleotides out of the 250 nucleotides of the intergenic region. There were only minor differences in processing by RNase Z (Fig. 5), indicating that long 3′-trailer sequences do not affect *Synechocystis* RNase Z activity.

In contrast with the reduced effect of leader and trailer length on RNase Z activity, the sequence around the cleavage site has a significant effect on reaction rate. The presence of C or CC after the discriminator significantly reduced activity (TABLE TWO and Fig. 6). What is more, the cleavage site is displaced in a sequence-dependent manner (Figs. 6 and 7). When C or CC is already present after the discriminator nucleotide, they are not removed, but RNase Z cleavage proceeds at the 3′ side of these nucleotides. This behavior has not been described so far in any of the RNase Z studied. Several sequence variants (CCG, CUG, and UUG) after the discriminator nucleotide have been tested for cleavage by recombinant RNase Z from *Arabidopsis thaliana* and *Methanocaldococcus jannaschii*, and in all cases cleavage was after the discriminator (6). *B. subtilis* RNase Z cleavage site is not affected when the sequence after the discriminator is changed from UAA to UCA or CAA (2). In the case of *T. maritima*, sequence changes can alter cleavage site selection (11), although a consistent pattern cannot be discerned. For instance, pre-tRNA^{Phe} with the sequence CCAG or UCAG after the discriminator is cleaved after A, but with the sequence CUAG is cleaved after U, and with CCGG is not cleaved; pre-tRNA^{Arg} with the sequence CCGU is cleaved after the second C, but with GUGU is cleaved after the first U, with CCGG is not cleaved, and CCAU is cleaved after A. Cleavage site of sequence variants was analyzed by enzymatic sequencing of the 3′-end of the tRNA product; therefore, only the longest possible product was observed (11), and it is not known if there are additional cleavage sites. It should be remembered that *T. maritima* has the unique property that CCA is not an anti-determinant, but RNase Z cleaves after the CCA, that is present in most of their tRNA genes.

Our results highlight the importance of the 3′-trailer sequence in pre-tRNA processing. We have aligned the pre-tRNA sequence downstream of the discriminator for all tRNAs in *Synechocystis*. For comparative purpose we have done the same alignment in another fully sequenced cyanobacterium, the heterocyst-forming *Nostoc* 7120, and in *B. subtilis*. There is a strong bias in the nucleotide present after the discriminator, where there is never a purine, except an A in two tRNAs from *Nostoc*, and U is present in most cases. A similar but less strong bias is observed at the second position after the discriminator. Further downstream the sequence distribution is closer to random, with a slight preference for A or U. The presence of C or CC 3′ of the discriminator has been shown to be inhibitory in pig (7), *B. subtilis* (2), and *Synechocystis* (this work) RNase Z. In *B. subtilis* only 6 out of 26 pre-tRNAs that do not encode CCA contain C 3′ of the discriminator and none contains CC. In *Synechocystis*, only 6 out of 42 contain C, and only one (pre-tRNA$^{Ile(GAT)}$) contains CC, which corresponds to the only duplicated tRNA gene present in *Synechocystis* within the duplicated ribosomal RNA operon. In *Nostoc* 7120 there are several tRNA genes containing CC 3′ of the discriminator, and several that encode the complete CCA sequence. It would be interesting to analyze if in *Nostoc* 7120 RNase Z can process these CCA-containing pre-tRNAs, like in *T. maritima* (11), or if an *E. coli*-like exonucleolytic mechanism is used. In this respect, it is worth noting that *Nostoc* 7120 has putative genes coding for RNase D and RNase PH (23). However, *Synechocystis* 6803 lacks homologs to RNase T, RNase D, and RNase PH (24), three enzymes proposed to be involved in exonucleolytic 3′ processing of pre-tRNAs in *E. coli* (9), and polynucleotide phosphorylase from *Synechocystis* cannot generate a mature 3′-end of tRNA[4] *in vitro*.

Our results indicate that there is some degree of heterogeneity in cleavage site, as determined *in vitro*. If this heterogeneity is an *in vitro*

[4] M. Ceballos-Chávez and A. Vioque, unpublished data.

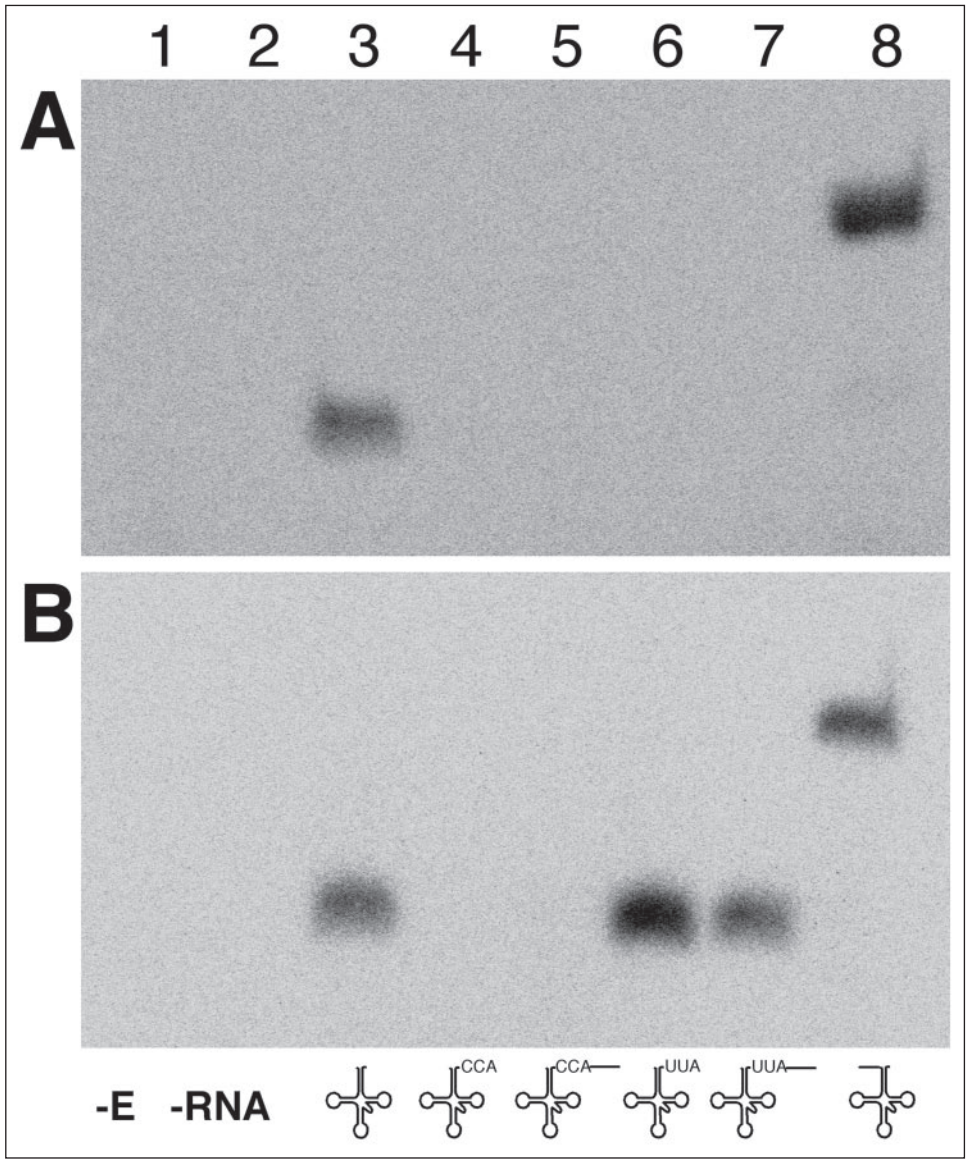

FIGURE 8. **CMP incorporation by purified Ntase(C) on diverse pre-tRNAs.** 10 μM $tRNA^{Glu}$ (*lane 3*), $tRNA^{Glu}CCA$ (*lane 4*), $tRNA^{Glu}CCAN_{17}$ (*lane 5*), $tRNA^{Glu}UUA$ (*lane 6*), $tRNA^{Glu}UUAN_{17}$ (*lane 7*), and $N_{34}tRNA^{Glu}$ (*lane 8*) were incubated with purified Ntase(C) (*A*), or a mixture of purified RNase Z and Ntase(C) (*B*), in the presence of [α-^{32}P]CTP as described under "Experimental Procedures." *Lane 1*, control without enzyme fractions; *lane 2*, control without RNA substrate.

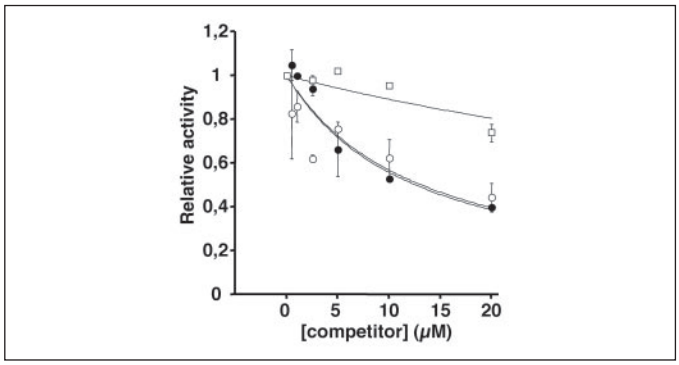

FIGURE 9. **Inhibition of RNase Z activity by different RNAs.** Inhibition assays were done as described under "Experimental Procedures." 3′-End-labeled $tRNA^{Glu}UUAN_{17}$ was used as substrate. The RNAs tested for inhibition were $tRNA^{Glu}CCAN_{17}$ (*black circles*), $tRNA^{Glu}$ (*open circles*), and pBSK RNA (*open squares*).

artifact or happens also *in vivo* remains to be determined. The same observation has been made in all cases where RNase Z cleavage site has been analyzed by primer extension (2, 6, 25–27) or size analysis (22).

Complete inhibition of activity by CCA seems to be a general feature of the RNase Z enzymes characterized so far, except in *Thermotoga*. We have analyzed if a CCA-containing pre-tRNA can bind to RNase Z by an inhibition assay (Fig. 9). This assay indicates that mature tRNA, the RNase Z reaction product, can bind to the enzyme with significantly higher affinity than an unrelated control RNA. A CCA-containing pre-tRNA has similar affinity than a mature CCA lacking tRNA, indicating that the 3′ extension in which the CCA sequence is imbibed does not contribute significantly in a positive or negative way to binding. This is congruent with the proposal that RNase Z interacts mainly with the mature tRNA part of the precursor (28, 29). However, this result is different from the effect observed with eukaryotic RNase Z, where there is a 10- to 30-fold increase in K_i between tRNA lacking or containing CCA (7).

It has been proposed that the inhibition by CCA of RNase Z processing avoids a futile cycle of CCA addition by nucleotidyl transferase and removal by RNase Z. The sequence-dependent cleavage site selection described here might represent a refinement of this idea, so that if C or CC is already present, they are not removed by RNase Z, saving cellular CTP. This could also be related to the fact that in *Synechocystis*, as well as in *Aquifex aeolicus* and *Deinococcus radiodurans*, CC addition and A addition are carried out by different enzymes (12). If there is no direct channeling of the tRNA substrate from the C adding enzyme (Ntase(C)) to the A adding enzyme, then the incomplete tRNA-CC could poten-

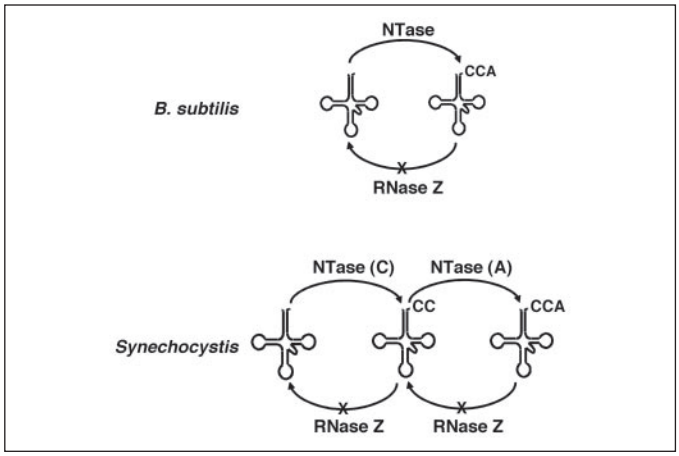

FIGURE 10. **Potential futile cycles in tRNA 3′ biosynthesis.** The inactivity of RNase Z on the indicated substrates is shown by the *X* on the *arrow* connecting them. Although in *B. subtilis* nucleotidyl transferase (*NTase*) generates directly the CCA sequence, in *Synechocystis*, C-specific NTase (*NTase(C)*) generates an intermediate containing CC before A-specific NTase (*NTase(A)*) adds the 3′-terminal A on the tRNA.

tially become available for futile RNase Z cleavage before A addition (Fig. 10). *Synechocystis* is the first bacteria with two separate tRNA nucleotidyl transferases where RNase Z is studied. It would be interesting to analyze RNase Z cleavage also in *A. aeolicus* and *D. radiodurans*.

What is the structural basis of the unique *Synechocystis* RNase Z cleavage site selection? Recently the crystal structures of RNase Z from *B. subtilis* (3) and *T. maritima* (4) have been determined, and models for their interaction with the substrate have been proposed. In addition, a substrate recognition module outside the active site has also been described (30). *Synechocystis* RNase Z seems to be more similar to RNase Z from *B. subtilis* than to RNase Z from *T. maritima*, both functionally and structurally. *Synechocystis* and *B. subtilis* share the complete inhibition of activity by CCA, whereas *T. maritima* RNase Z cleaves CCA-containing substrates after A. Both *Synechocystis* and *B. subtilis* contain the exosite module with a glycine- and proline-rich segment (GP-motif) (30). *T. maritima* RNase Z contains a shorter exosite and lacks the GP-motif (Fig. 1). At positions 42 and 44 (*Synechocystis* numbering) there are two conserved glutamines in *Synechocystis* and *B. subtilis*. In *T. maritima* RNase Z, they are replaced by serine and threonine, respectively (Fig. 1). These sites have been described as important for cleavage site selection in *T. maritima* (11). Differences in cleavage site and in the effect of CCA between RNase Z from *B. subtilis* and *T. maritima* can be rationalized as two different enzymes that interact in two different ways with their respective substrates. In the case of *Synechocystis*, where a single enzyme cleaves pre-tRNAs at different positions in a sequence-dependent manner, flexibility in enzyme-substrate interaction must be considered, so that a different scissile bond is placed at the active site of the enzyme. In the docking model of *B. subtilis* tRNA-enzyme interaction (3) the basic residues Arg-31, Lys-52, Arg-54, and Lys-55 (*B. subtilis* numbering) are involved in contacts with the acceptor arm of the tRNA. In *Synechocystis*, Arg-54 and Lys-55 are replaced by serine and glutamine (Ser-53 and Gln-54 in the *Synechocystis* sequence), respectively (Fig. 1). What is more, in *B. subtilis* Lys-164, Lys-176, Lys-178, and Lys-179 are present in the surface proposed to interact with the tRNA. From these basic residues only Lys-178 is conserved in *Synechocystis* (Fig. 1). We can only speculate if these potential differences in the tRNA-enzyme interaction between *T. maritima*, *Synechocystis* and *B. subtilis* RNase Z can account for the differences in cleavage site selection observed. The question could be addressed by site-directed mutagenesis of the relevant residues.

It might be useful in attempting to find a molecular mechanism for this flexibility to remind the nucleotidyl transferase mechanism, where the successive addition to the tRNA 3′-end of the nucleotides that compose the CCA sequence occurs at a single active site that becomes modified upon successive nucleotide addition without altering the enzyme-tRNA interaction (31). In this respect it is important to determine if the binding of C- or CC-containing pre-tRNAs to *Synechocystis* RNase Z is identical to the binding of pre-tRNAs with other sequences and if there is a sequence-dependent scrunching of nucleotides so that the appropriate scissile bond is place at the active site.

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