Kluyveromyces lactis γ-toxin, a ribonuclease that recognizes the anticodon stem loop of tRNA

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ABSTRACT

Kluyveromyces lactis γ-toxin is a tRNA endonuclease that cleaves *Saccharomyces cerevisiae* $tRNA^{Glu3}_{mcm^5s^2UUC}$, $tRNA^{Lys}_{mcm^5s^2UUU}$ and $tRNA^{Gln}_{mcm^5s^2UUG}$ between position 34 and position 35. All three substrate tRNAs carry a 5-methoxycarbonylmethyl-2-thiouridine (mcm^5s^2U) residue at position 34 (wobble position) of which the mcm^5 group is required for efficient cleavage. However, the different cleavage efficiencies of $mcm^5s^2U_{34}$-containing tRNAs suggest that additional features of these tRNAs affect cleavage. In the present study, we show that a stable anticodon stem and the anticodon loop are the minimal requirements for cleavage by γ-toxin. A synthetic minihelix RNA corresponding to the anticodon stem loop (ASL) of the natural substrate $tRNA^{Glu3}_{mcm^5s^2UUC}$ is cleaved at the same position as the natural substrate. In ASL^{Glu3}_{UUC}, the nucleotides $U_{34}U_{35}C_{36}A_{37}C_{38}$ are required for optimal γ-toxin cleavage, whereas a purine at position 32 or a G in position 33 dramatically reduces the cleavage of the ASL. Comparing modified and partially modified forms of *E. coli* and yeast $tRNA^{Glu}_{UUC}$ reinforced the strong stimulatory effects of the mcm^5 group, revealed a weak positive effect of the s^2 group and a negative effect of the bacterial 5-methylaminomethyl (mnm^5) group. The data underscore the high specificity of this yeast tRNA toxin.

INTRODUCTION

Killer strains of the dairy yeast *Kluyveromyces lactis* secrete a heterotrimeric toxin (zymocin), which causes an irreversible arrest of sensitive yeast cells, such as *Saccharomyces cerevisiae* in the G1 phase of the cell cycle (1–4). Zymocin consists of three subunits, α, β and γ, that are encoded by a linear plasmid (1,5). Upon secretion,

the α- and β-subunits dock the zymocin to the cell wall of susceptible yeasts and facilitate transfer of the γ-subunit into the cells (4). Cytotoxicity of zymocin resides within the γ-subunit (γ-toxin), since its intracellular expression in sensitive cells mimics the action of exogenous zymocin (6). Recently we showed that γ-toxin is a tRNA endonuclease that cleaves $tRNA^{Glu3}_{mcm^5s^2UUC}$, $tRNA^{Lys}_{mcm^5s^2UUU}$ and $tRNA^{Gln}_{mcm^5s^2UUG}$ between positions 34 and 35 (7). All substrate tRNAs have the modified nucleoside 5-methoxycarbonylmethyl-2-thiouridine (mcm^5s^2U) at position 34 (wobble position). The mcm^5 group present on the wobble uridine is important for tRNA cleavage, since tRNAs missing part of, or the entire mcm^5 side chain are less efficiently cleaved (7).

The γ-toxin and certain *Escherichia coli* proteins comprise a group of tRNA anticodon nucleases (ACNs) that cleave tRNA in the anticodon region. The PrrC protein is a nuclease found in prr^+ *E. coli* strains in a latent form. When *E. coli* is infected with phage T4, the nuclease is activated and cleaves $tRNA^{Lys}_{mnm^5s^2UUU}$ between positions 33 and 34 (8,9). Similar to γ-toxin, the efficiency of tRNA cleavage by PrrC is remarkably affected by the modified nucleosides present in the anticodon region (10,11). Colicin E5 and colicin D are produced by bacteria carrying the corresponding Col plasmids. When secreted, these colicins kill sensitive *E. coli* strains by inhibiting translation. Colicin E5 cleaves the queuosine (Q) containing tRNAs, $tRNA^{Tyr}_{QUA}$, $tRNA^{His}_{QUG}$, $tRNA^{Asn}_{QUU}$ and $tRNA^{Asp}_{QUC}$ (12) and colicin D targets all $tRNA^{Arg}$ isoacceptors (13). Cleavage by all the ACNs generates 5′-OH and 2′,3′-cyclic phosphate termini. The ACNs show no amino acid homology, however, the catalytic domains of colicin E5 and colicin D show similarities in their crystal structures (14,15). These similarities shared by colicin E5 and colicin D are also seen in other ribonucleases, such as ribonuclease T1 (16) and archaeal toxin aRelE (17).

Even though all three γ-toxin substrates, $tRNA^{Glu3}_{mcm^5s^2UUC}$, $tRNA^{Lys}_{mcm^5s^2UUU}$ and $tRNA^{Gln}_{mcm^5s^2UUG}$, contain mcm^5s^2U at the wobble position, the latter two tRNA species are cleaved with much lower efficiency (7).

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Table 1. Plasmids used in this study

Plasmids name	Description	Source
pABY1537	pUC18-T7-tRNA$_{UUC}^{Glu3}$ (G1-C72)	(22)
pABY1539	pUC18-T7-tRNA$_{CUC}^{Glu4}$ (G1-C72)	This study
pABY1542	pUC18-T7-tRNA$_{UUC}^{Glu4}$ (G1-C72, T34)	This study
pABY1714	pUC18-T7-tRNA$_{UUU}^{Lys}$ (G1-C72)	This study
pABY1738	pUC18-T7-tRNA$_{UUG}^{Gln}$ (T5-A68)	This study
pABY1746	pUC18-T7-tRNA$_{UUC}^{Glu}$ (G1-C72)-ASL$_{UUU}^{Lys}$	This study
pABY1747	pUC18-T7-tRNA$_{UUU}^{Lys}$ (G1-C72)-ASL$_{UUC}^{Glu3}$	This study
pABY1650	pETM-13-GST	(7)
pABY1644	pETM-13-γ-toxin-GST	(7)

This suggests that in addition to the wobble uridine modification, there are other features in tRNA$_{mcm^5s^2UUC}^{Glu3}$ that improve cleavage by γ-toxin. In this study, we have investigated the determinants in tRNA important for efficient cleavage by γ-toxin.

MATERIALS AND METHODS

Plasmid constructions

DNA manipulations, plasmid preparations and bacterial transformations were performed according to standard protocols. The plasmids used in this study are listed in Table 1. Plasmids containing T7 promoter-driven tRNA genes were constructed as previously described (18). Briefly, three pairs of oligonucleotides corresponding to the sequence of the T7 promoter and the various tRNA genes were ligated into the EcoRI/BamHI sites of pUC18 (Roche Applied Science). In the oligonucleotides used to construct genes of tRNA$_{UUC}^{Glu3}$, tRNA$_{UUU}^{Lys}$ and their derivatives, T1-A72 were changed to G1-C72 to facilitate *in vitro* transcription. In the plasmid carrying the T7 promoter-driven tRNA$_{UUG}^{Gln}$ gene, there is an MvaI site in the tRNA gene, which prevents the proper linearization of the plasmid for *in vitro* transcription. To circumvent this, the C5-G68 was changed to T5-A68 in the oligonucleotides used to construct the tRNA$_{UUG}^{Gln}$ gene. The C34 in the tRNA$_{CUC}^{Glu4}$ gene in plasmid pABY1539 was mutated to T using QuickChange® Site-Directed Mutagenesis kit (Stratagene), generating pABY1542.

RNA methods

For RNA preparation, *S. cerevisiae* cells were collected from mid-log phase cultures grown in YEPD medium at 30°C. *E. coli* cells were collected from mid-log phase cultures grown in LB medium at 37°C. Total RNA was prepared as described (19) followed by LiCl fractionation (20). The T7-transcribed radiolabeled tRNAs were prepared by using MvaI linearized vectors, 5′-[α-^{32}P] UTP (400 Ci/mmol, Amersham Biosciences), and the Riboprobe *in vitro* transcription system (Promega). The transcripts were purified as described (21). The RNA oligonucleotides used in this study are listed in Table 2. They were synthesized by Invitrogen and 5′-labeled using

Table 2. RNA oligonucleotides used in this study

Oligo name	Sequence[a]
Wt	5′-UCACGCUUUCACCGUGG-3′
No stem	5′-UCACGCUUUCAC<u>GCACU</u>-3′
GC stem	5′-<u>CCGC</u>CUUUCAC<u>GGCGG</u>-3′
AU stem	5′-<u>AUAAA</u>CUUUCAC<u>UUUAU</u>-3′
C39G	5′-<u>UCACG</u>CUUUCAC<u>G</u>GUGG-3′
C32A	5′-UCACG<u>A</u>UUUCACCGUGG-3′
C32G	5′-UCACG<u>G</u>UUUCACCGUGG-3′
C32U	5′-UCACG<u>U</u>UUUCACCGUGG-3′
U33A	5′-UCACGC<u>A</u>UUCACCGUGG-3′
U33C	5′-UCACGC<u>C</u>UUCACCGUGG-3′
U33G	5′-UCACGC<u>G</u>UUCACCGUGG-3′
U34A	5′-UCACGCU<u>A</u>UCACCGUGG-3′
U34C	5′-UCACGCU<u>C</u>UCACCGUGG-3′
U34G	5′-UCACGCU<u>G</u>UCACCGUGG-3′
U35A	5′-UCACGCUU<u>A</u>CACCGUGG-3′
U35C	5′-UCACGCUU<u>C</u>CACCGUGG-3′
U35G	5′-UCACGCUU<u>G</u>CACCGUGG-3′
C36A	5′-UCACGCUUU<u>A</u>ACCGUGG-3′
C36G	5′-UCACGCUUU<u>G</u>ACCGUGG-3′
C36U	5′-UCACGCUUU<u>U</u>ACCGUGG-3′
A37C	5′-UCACGCUUUC<u>C</u>CCGUGG-3′
A37G	5′-UCACGCUUUC<u>G</u>CCGUGG-3′
A37U	5′-UCACGCUUUC<u>U</u>CCGUGG-3′
C38A	5′-UCACGCUUUCA<u>A</u>CGUGG-3′
C38G	5′-UCACGCUUUCA<u>G</u>CGUGG-3′
C38U	5′-UCACGCUUUCA<u>U</u>CGUGG-3′

[a]Sequences different from the ASL$_{UUC}^{Glu3}$ are underlined.

adenosine [γ^{32}P]-triphosphate (5000 Ci/mmol, Amersham Biosciences) and polynucleotide kinase (Roche Applied Science).

Endonuclease activity assay

Recombinant γ-toxin-GST and GST were purified from an overnight culture of *E. coli* BL21(DE3) (Novagen) carrying corresponding expression plasmids by using Glutathione–Sepharose 4B (Amersham Biosciences) as described previously (7). Purified proteins were stored in 50% glycerol at −20°C. Under this condition, the proteins are stable for at least 12 months. To compare the cleavage of tRNA$_{mnm^5s^2UUC}^{Glu}$ from *E. coli* and tRNA$_{mcm^5s^2UUC}^{Glu3}$ from *S. cerevisiae*, 5 μg of total tRNA was mixed with purified γ-toxin-GST or GST proteins in buffer M (10 mM Tris–HCl, 10 mM MgCl$_2$, 50 mM NaCl, 1 mM dithiothreitol, pH 7.5) and incubated at 30°C for 10 min. The tRNAs were separated on 8% polyacrylamide, 8 M urea gels. The cleavage was analyzed by northern blot as previously described (7). The oligonucleotide used to detect *E. coli* tRNA$_{mnm^5s^2UUC}^{Glu}$ was 5′- CCTCTAGACGAAGG GGAC-3′. For detection of *S. cerevisiae* tRNA$_{mcm^5s^2UUC}^{Glu3}$ the oligonucleotide 5′-CTCCGCTACGGGGAGTCGA AC-3′ was used as a probe (7). The chemical trimethyla-mine N-oxide (TMAO) was reported to increase the cleavage of unmodified tRNA by a bacterial anticodon

nuclease, PrrC (11). We found that TMAO also enhanced the cleavage of unmodified tRNA and ASLs by γ-toxin-GST (data not shown). Therefore TMAO was included in the endonuclease assay when unmodified substrates were tested. To test cleavage of unmodified tRNAs, 2 nM of T7-transcribed radiolabeled tRNAs were mixed with purified γ-toxin-GST or GST proteins in buffer M containing 1.5 M TMAO and incubated at 30°C for 10 min. The tRNAs were separated on 8% polyacrylamide, 8 M urea gels. The gels were dried and cleavage monitored by autoradiography. The radioactivities of the full-length RNA and cleavage products were quantified. Extent of cleavage was calculated using the formula: extent of cleavage = cleavage products/(full-length tRNA + cleavage products). To compare the cleavage efficiencies of T7-transcribed $tRNA_{UUC}^{Glu3}$ and the RNA oligonucleotide corresponding to the anticodon stem loop of $tRNA_{UUC}^{Glu3}$ (ASL_{UUC}^{Glu3}), 5 μM of labeled tRNA or ASL_{UUC}^{Glu3} was incubated in buffer M containing 1.5 M TMAO and 2 nM purified γ-toxin-GST at 0°C. At intervals, 10 μl of the reaction was withdrawn, extracted with equal volume of phenol, and kept on ice. Samples were run on 8% (for tRNA transcript) or 10% (for RNA oligonucleotide) polyacrylamide, 8 M urea gels. The gels were dried and cleavage monitored by autoradiography. The extent of cleavage was calculated as described above. To compare ASL_{UUC}^{Glu3} and its derivatives, 5 μM of labeled ASLs were incubated in buffer M containing 1.5 M TMAO and 5 nM purified γ-toxin-GST at 0°C. The extent of cleavage at different time points was monitored as described above.

Strains

The *S. cerevisiae* strains are derivatives of W303-1A (MATa *ura3-1 leu2-3,112 trp1-1 his3-11,15 can1-100 ade2-1*). The *elp3* and *tuc1* mutant strains have been described previously (22,23). Bacterial strains used in this study, TH168 (F$^+$, *asnA, asnB, thi, lacIQ, relA, spoT, fadR3115::Tn10(Km), mnmA107*), TH169 (F$^+$, *asnA, asnB, thi, lacIQ, relA, spoT, fadR3115::Tn10(Km), val(R), mnmE*) and TH170 (F$^+$, *asnA, asnB, thi, lacIQ, relA, spoT, fadR3115::Tn10(Km), val(R)*) have been described elsewhere (24).

RESULTS

In vitro transcribed $tRNA_{UUC}^{Glu3}$, $tRNA_{UUG}^{Gln}$ and $tRNA_{UUU}^{Lys}$ are substrates for γ-toxin

Even though *in vitro* transcribed tRNAs lack modified nucleosides, they provide a useful tool to investigate the RNA sequence elements that are important for the interaction with proteins, e.g. PrrC and colicin E5 (11,12). To investigate whether unmodified tRNAs can be used to identify the primary sequence important for γ-toxin cleavage, *in vitro* transcribed ^{32}P-labeled $tRNA_{UUC}^{Glu3}$, $tRNA_{UUU}^{Lys}$ or $tRNA_{UUG}^{Gln}$ was incubated with different concentrations of purified γ-toxin protein. The extent of cleavage was determined by applying the samples to denaturing polyacrylamide gel followed by autoradiography. Interestingly, the unmodified $tRNA_{UUU}^{Lys}$ and $tRNA_{UUG}^{Gln}$ required ~100-fold higher concentration of γ-toxin to be cleaved

to a similar extent as $tRNA_{UUC}^{Glu3}$ (Figure 1B, data not shown). This difference in cleavage efficiencies was also found between native modified $tRNA_{mcm^5s^2UUU}^{Lys}$ and $tRNA_{mcm^5s^2UUG}^{Gln}$ when compared to $tRNA_{mcm^5s^2UUC}^{Glu3}$ (7), suggesting that it is caused by differences in the primary sequence. Thus, the unmodified tRNAs can be used to study the sequence identity elements for γ-toxin cleavage.

Anticodon stem loop in $tRNA_{UUC}^{Glu3}$ is important for cleavage by γ-toxin

To investigate what domain(s) of $tRNA_{UUC}^{Glu3}$ are important for γ-toxin cleavage, chimeric tRNA genes were constructed. Since γ-toxin cleaves the anticodon of substrate tRNAs, the anticodon stem loop (ASL) represents a candidate identity element of γ-toxin cleavage. A recombinant gene was created in which the ASL of $tRNA_{UUU}^{Lys}$ was replaced by the corresponding part of $tRNA_{UUC}^{Glu3}$ (designated $tRNA^{Lys}-ASL_{UUC}^{Glu3}$). In another construct, the ASL in $tRNA_{UUC}^{Glu3}$ was replaced with the ASL from $tRNA_{UUU}^{Lys}$, generating $tRNA^{Glu3}-ASL_{UUU}^{Lys}$. These chimeric tRNAs were *in vitro* transcribed and cleavage by γ-toxin was compared. The $tRNA^{Lys}-ASL_{UUC}^{Glu3}$ was cleaved with a similar efficiency as $tRNA_{UUC}^{Glu3}$, whereas the cleavage of $tRNA^{Glu}-ASL_{UUU}^{Lys}$ was comparable to $tRNA_{UUU}^{Lys}$ (Figure 1B).

Unmodified $tRNA_{UUC}^{Glu3}$ and $tRNA_{CUC}^{Glu4}$ share significant sequence homology: except for 3 nt in the acceptor stem and two in the anticodon loop, all the other nucleotides are identical (Figure 1A). In contrast to $tRNA_{UUC}^{Glu3}$, *in vitro* transcribed $tRNA_{CUC}^{Glu4}$ was resistant to γ-toxin cleavage (Figure 1B). Interestingly, a point mutation in the anticodon loop of $tRNA_{CUC}^{Glu4}$ (C_{34} to U) was enough to make the mutant $tRNA_{UUC}^{Glu4}$ as reactive as $tRNA_{UUC}^{Glu3}$ (Figure 1B). Taken together, these data show that the ASL in $tRNA_{UUC}^{Glu3}$ and position 34 are important for the cleavage by γ-toxin.

Unmodified ASL as γ-toxin substrate

The chimeric tRNAs data presented above showed that the ASL of $tRNA_{UUC}^{Glu3}$ carries the vital information for γ-toxin cleavage. We utilized an *in vitro* synthesized 17-mer RNA oligonucleotide with identical sequence to the ASL of $tRNA_{UUC}^{Glu3}$ (designated ASL_{UUC}^{Glu3}) to investigate if it acts as a substrate for γ-toxin. Incubation of the $5'-^{32}$P-labeled ASL_{UUC}^{Glu3} with γ-toxin generated a product of 8 nt in length (Figure 2A), showing that the ASL_{UUC}^{Glu3} was cleaved between nucleotides 8 and 9, which correspond to positions 34 and 35 in $tRNA_{mcm^5s^2UUC}^{Glu3}$. Thus, the ASL_{UUC}^{Glu3} is cleaved at the same site as $tRNA_{mcm^5s^2UUC}^{Glu3}$. The cleavage of 5'-labeled ASL_{UUC}^{Glu3} was compared with that of *in vitro*-transcribed radiolabeled $tRNA_{UUC}^{Glu3}$. As shown in Figure 2B, ASL_{UUC}^{Glu3} and $tRNA_{UUC}^{Glu3}$ displayed
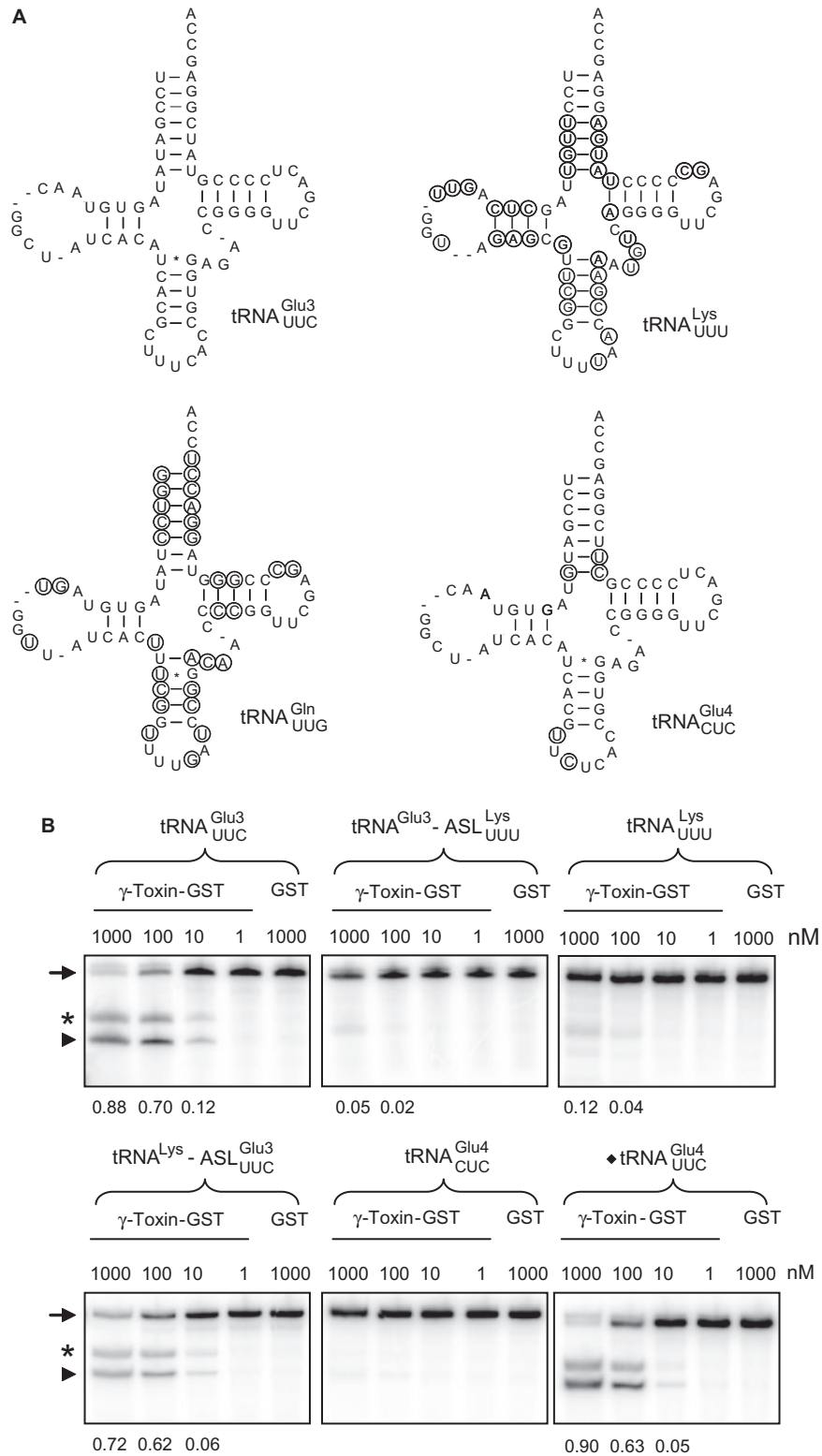
Figure 1. The determinants for γ-toxin cleavage reside within the anticodon stem loop. (**A**) Sequence of unmodified $tRNA_{UUC}^{Glu3}$, $tRNA_{UUU}^{Lys}$, $tRNA_{UUG}^{Gln}$ and $tRNA_{CUC}^{Glu4}$. Nucleotides circled in $tRNA_{UUU}^{Lys}$, $tRNA_{UUG}^{Gln}$ and $tRNA_{CUC}^{Glu4}$ identify differences compared to $tRNA_{UUC}^{Glu3}$. (**B**) *In vitro* cleavage of $tRNA_{UUC}^{Glu3}$, $tRNA^{Glu3}-ASL_{UUU}^{Lys}$, $tRNA_{UUU}^{Lys}$, $tRNA^{Lys}-ASL_{UUC}^{Glu3}$, $tRNA_{CUC}^{Glu4}$ and ◆$tRNA_{UUC}^{Glu4}$ by γ-toxin-GST. The tRNAs were T7 transcribed in the presence of $[\alpha\text{-}^{32}P]$ UTP and 2 nM of transcribed tRNAs were incubated with the indicated concentration of γ-toxin-GST or GST protein at 30°C for 10 min. Samples were analyzed on 8% polyacrylamide, 8 M urea gels and the cleavage products were quantified. Complete cleavage of substrate tRNA was set to 1.0 (for details see Materials and Methods section). Full-length, 5′-half and 3′-half of tRNAs are indicated with arrow, arrow head and asterisk, respectively. In ◆$tRNA_{UUC}^{Glu4}$, the anticodon was mutated from CUC to UUC.
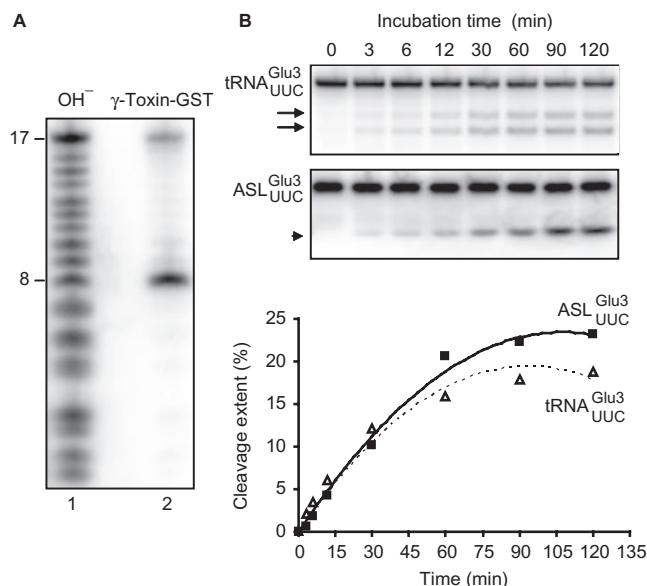
Figure 2. Cleavage of unmodified ASL$_{UUC}^{Glu3}$ and tRNA$_{UUC}^{Glu3}$ by γ-toxin. (**A**) The 5′-^{32}P-labeled ASL$_{UUC}^{Glu3}$ was incubated with γ-toxin-GST and the products (lane 2) were separated by 20% polyacrylamide, 8 M urea gel electrophoresis. As marker, a ladder (lane 1) was obtained by partial alkaline hydrolysis of the 5′-^{32}P-labeled ASL$_{UUC}^{Glu3}$. Numbers on the left indicate chain length in nucleotides. (**B**) The [α-^{32}P] UTP-labeled T7-transcribed tRNA$_{UUC}^{Glu3}$ (top panel) and the 5′-^{32}P-labeled anticodon stem loop (ASL$_{UUC}^{Glu3}$, middle panel), both at a concentration of 5 μM, were incubated with 2 nM γ-toxin-GST at 0°C. Aliquots of the reactions were taken at the indicated time points and analyzed on 8% or 10% polyacrylamide, 8 M urea gels, respectively. The cleavage products of tRNA$_{UUC}^{Glu3}$ and ASL$_{UUC}^{Glu3}$ are indicated by arrows and an arrow head, respectively. The extent of cleavage was quantitated for each time point and plotted (bottom panel).

similar cleavage kinetics upon γ-toxin treatment, supporting the notion that the ASL$_{UUC}^{Glu3}$ contains all the sequence information required for γ-toxin cleavage.

Influence of anticodon stem sequence on the cleavage of ASL

The observation that ASL$_{UUC}^{Glu3}$ constitutes a substrate equal to the full-length tRNA$_{UUC}^{Glu3}$ made it possible to use different variants of the ASL to pinpoint the sequence important for γ-toxin cleavage. In order to investigate the importance of the anticodon stem for cleavage by γ-toxin, variants of the ASL$_{UUC}^{Glu3}$ with different stem sequences were synthesized. The 5′-^{32}P-labeled ASLs were incubated with γ-toxin and the cleavage at different time points was analyzed by denaturing PAGE. In one of the ASLs, the sequence of the 3′ strand of the stem was changed to prevent base-pair formation in the stem, thus disrupting the stem structure (designated 'no stem'). Under the condition used in this study, this linear RNA oligonucleotide is not cleaved by γ-toxin (Figure 3B). A mutation, C$_{39}$ to G, that disrupts the lowest (31–39) base-pair in the stem expands the anticodon loop size (and at the same time, shortens the stem length) also abolished the cleavage of the ASL (Figure 3B). Changing the stem sequence of ASL$_{UUC}^{Glu3}$ to that of *E. coli* tRNA$_{mnm^5s^2UUC}^{Glu}$ (in total 5 G–C base pairs, Figure 4A) did not alter the cleavage, while a stem with a total of 5 A–U base pairs made the ASL 5-fold less sensitive

to γ-toxin (Figure 3B). Taken together, these data suggest that γ-toxin requires the canonical anticodon stem loop structure for efficient cleavage, and that ASL with a stable stem structure seems to be preferred by γ-toxin.

### Effects of mutations in the anticodon loop on the ASL cleavage by γ-toxin

In the anticodon loop, the three substrate tRNAs, i.e. tRNA$_{mcm^5s^2UUC}^{Glu3}$, tRNA$_{mcm^5s^2UUU}^{Lys}$ and tRNA$_{mcm^5s^2UUG}^{Gln}$, share identical nucleotides U$_{33}$U$_{34}$U$_{35}$ (25), which might be a sequence important for γ-toxin cleavage. However, ASLs carrying U$_{33}$U$_{34}$U$_{35}$, but with differences in other positions in the anticodon loop, still exhibit different cleavage efficiency (see below). This suggests that other nucleotides are also important for γ-toxin action.

To determine the nucleotides important in the ASL$_{UUC}^{Glu3}$ for γ-toxin cleavage, we designed a series of ASLs, each with one of the loop nucleotides changed. The ASLs were 5′-^{32}P-labeled and their cleavage efficiencies examined as described above. Changing C$_{32}$ to U in ASL$_{UUC}^{Glu3}$ caused a ∼30% drop in cleavage efficiency (Figure 3C), whereas replacement of C$_{32}$ by A or G abolished cleavage, suggesting that γ-toxin prefers a pyrimidine at position 32. At position 33 almost all tRNAs carry a U, which is required for the formation of U-turn, a signature of the canonical anticodon structure. Replacing U$_{33}$ by G caused a ∼85% reduction in cleavage, whereas a C$_{33}$ mutation displayed very similar kinetics as the ASL$_{UUC}^{Glu3}$. More surprisingly, ASL^{Glu3} carrying an A$_{33}$ mutation was cleaved even more efficiently than the ASL$_{UUC}^{Glu3}$ (Figure 3D). Any change of the nucleotides in the anticodon (position 34, 35 and 36) and position 37 abolished ASL cleavage (Figure 3E, F, G and H). Changing C$_{38}$ to U resulted in a ∼60% reduction in ASL cleavage, while a purine (A or G) at this position led to at least a ∼90% drop in cleavage efficiency (Figure 3I). Taken together, the nucleotides U$_{34}$U$_{35}$C$_{36}$A$_{37}$C$_{38}$ in the ASL$_{UUC}^{Glu3}$ are required for efficient cleavage by γ-toxin. A purine at position 32 or a G at position 33 dramatically reduced the ability of the ASL to act as a substrate.

The mnm^5 group in *E. coli* tRNA$_{UUC}^{Glu}$ is a negative element for γ-toxin cleavage

In contrast to the mcm^{5}s^{2}U-containing *S. cerevisiae* tRNA$_{mcm^5s^2UUC}^{Glu3}$, the *E. coli* tRNA$_{mnm^5s^2UUC}^{Glu}$ contains a 5-methylaminomethyl-2-thiouridine (mnm^{5}s^{2}U) residue at the wobble position (25). Moreover, the *E. coli* tRNA$_{mnm^5s^2UUC}^{Glu}$ has a 2-methyladenosine (m^{2}A) at position 37, whereas the *S. cerevisiae* tRNA$_{mcm^5s^2UUC}^{Glu3}$ has an unmodified A$_{37}$ (25). The other nucleotides in the anticodon loop are identical between the two tRNAs (Figure 4A). Total RNA isolated from a wild-type *E. coli* strain was treated with purified γ-toxin and tRNA cleavage was investigated by northern blot analysis. Compared with the *S. cerevisiae* tRNA$_{mcm^5s^2UUC}^{Glu3}$, at least a 1000-fold higher concentration of γ-toxin was required to observe the cleavage of *E. coli* tRNA$_{mnm^5s^2UUC}^{Glu}$ (Figure 4C, compare lane 25 with lane 1). The fact that unmodified *E. coli* and *S. cerevisiae* tRNA$_{UUC}^{Glu}$ share
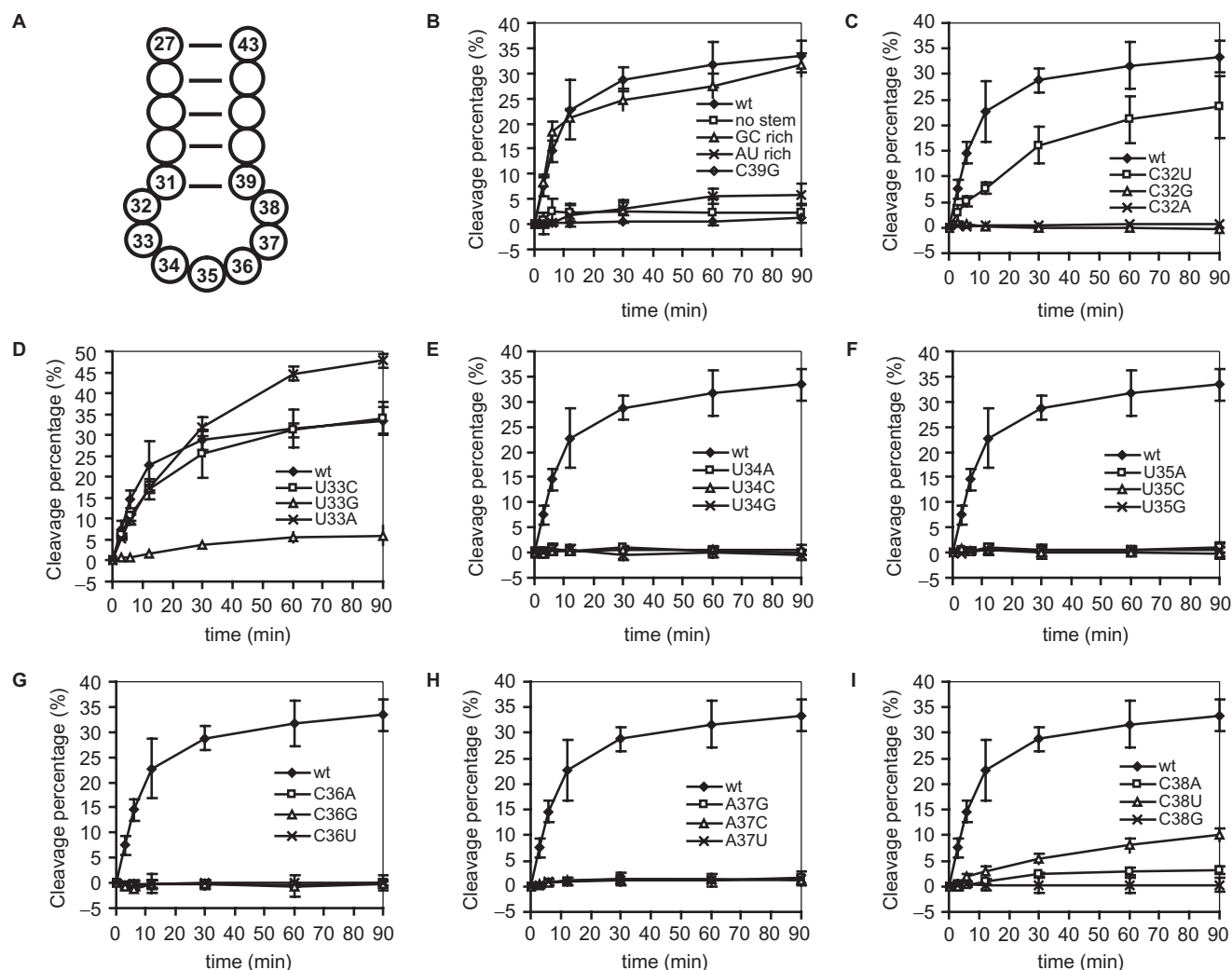
Figure 3. Cleavage of ASL_{UUC}^{Glu3} and its derivatives by γ-toxin. (**A**) Schematic drawing of the anticodon stem loop of tRNA. Positions of nucleotides are numbered according to conventional rules (25). (**B–I**) The $5'$-^{32}P-labeled wild-type and mutant forms of ASL_{UUC}^{Glu3}, all at a concentration of 5 μM, were incubated with 5 nM γ-toxin-GST at 0°C. Aliquots of the reactions were taken at the indicated time points and analyzed on 10% polyacrylamide, 8 M urea gels. The extent of cleavage of ASL_{UUC}^{Glu3} and its mutant derivatives were calculated for each time point and plotted. Each experiment was repeated at least three times, the standard deviations are shown as error bars.

identical anticodon loop sequence and their corresponding ASLs are cleaved by γ-toxin with the same efficiency (Figure 3B, compare wt and GC rich), makes it likely that $mnm^5s^2U_{34}$ and/or m^2A_{37} act negatively on γ-toxin cleavage of *E. coli* $tRNA_{mnm^5s^2UUC}^{Glu}$.

We individually investigated the effect of the mnm^5- or s^2-group on cleavage of *E. coli* $tRNA_{mnm^5s^2UUC}^{Glu}$. An *E. coli* *mnmE* mutant is defective in the formation of the mnm^5 but not the s^2 group (26). Similarly, a yeast *elp3* mutant lacks the mcm^5 side chain but contains the s^2 group in tRNA (22). Total tRNA isolated from these mutants and their corresponding wild-type strains were treated with serially diluted γ-toxin and the cleavage of $tRNA_{s^2UUC}^{Glu}$ was analyzed by northern blot analysis. Interestingly, $tRNA_{mnm^5s^2UUC}^{Glu}$ from the *mnmE* mutant (lacking the mnm^5 modification) was more sensitive to γ-toxin than the wild-type $tRNA_{mnm^5s^2UUC}^{Glu}$ (Figure 4C, compare lane 2 with lane 1, lane 5 with lane 4, respectively), indicating that the mnm^5 group has a negative effect on cleavage

of $tRNA_{mnm^5s^2UUC}^{Glu}$. Estimated by the amount of γ-toxin needed, *E. coli* $tRNA_{s^2UUC}^{Glu}$ lacking the mnm^5 modification served as an equally comparable substrate to yeast $tRNA_{s^2UUC}^{Glu3}$ lacking the mcm^5 modification (Figure 4C, compare lane 8 and lane 23, lane 5 and lane 20, respectively). This observation has two implications: First, despite sequence variations in other domains, the identical anticodon loop sequence in *E. coli* and yeast $tRNA_{s^2UUC}^{Glu}$ is enough to dictate their similar cleavage efficiencies, reinforcing the importance of previously identified sequence elements important for γ-toxin cleavage. Second, in this scenario, the presence of m^2A_{37} did not make *E. coli* $tRNA_{s^2UUC}^{Glu}$ a worse substrate than the yeast $tRNA_{s^2UUC}^{Glu3}$, suggesting that the m^2A_{37} is unlikely to be a negative element in *E. coli* $tRNA_{mnm^5s^2UUC}^{Glu}$ for γ-toxin cleavage.

The effect of the s^2 group was explored using an *E. coli* *mnmA* mutant and a yeast *tuc1* mutant, both of which lack the s^2 modification in tRNA (23,27). When tested with
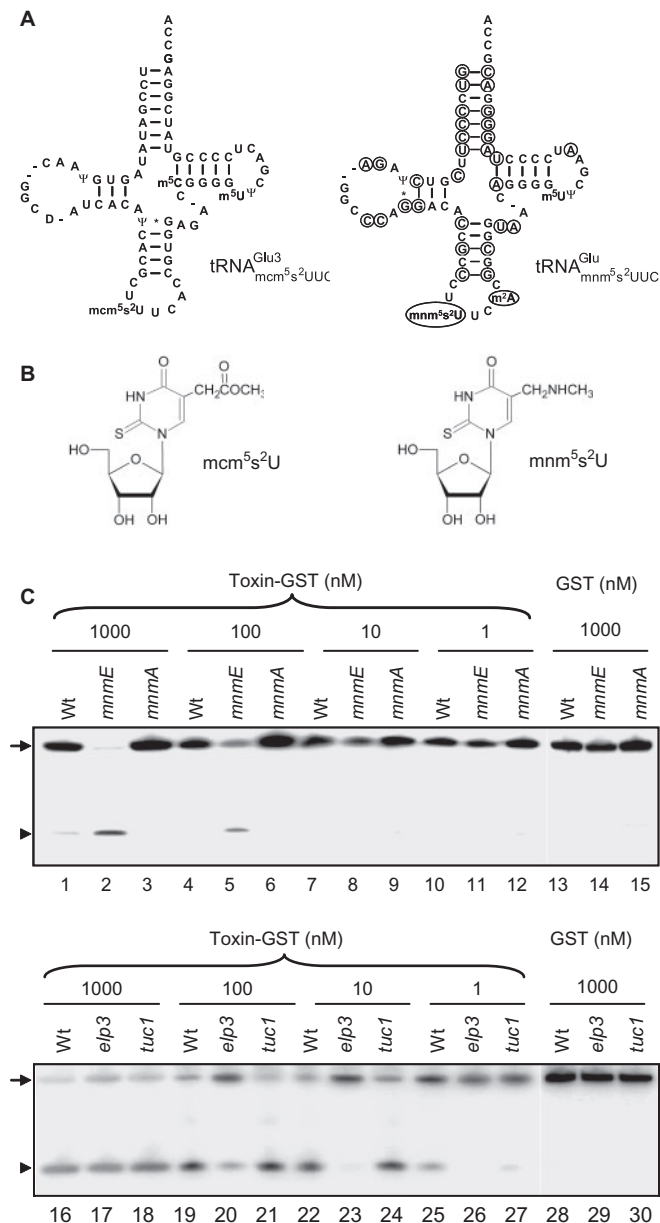
Figure 4. Effects of mnm[5], mcm[5] and s[2] groups on tRNA cleavage by γ-toxin. (**A**) Sequences of tRNA$^{Glu3}_{mcm^5s^2UUC}$ from *S. cerevisiae* (left) and tRNA$^{Glu}_{mnm^5s^2UUC}$ from *E. coli* (right). The nucleotides deviating from *S. cerevisiae* tRNA$^{Glu3}_{mcm^5s^2UUC}$ are circled. (**B**) Structure of mcm[5]s[2]U and mnm[5]s[2]U. (**C**) Northern blot analysis of total tRNA isolated from *E. coli* wild type, *mnmE*, *mnmA* and yeast wild type, *elp3*, or *tuc1* strains. In the reactions, 5 μg of total tRNA was incubated with the indicated concentrations of purified γ-toxin-GST or GST protein at 30°C for 10 min. Signals were detected using oligonucleotides specific for *E. coli* tRNA$^{Glu}_{mnm^5s^2UUC}$ or yeast tRNA$^{Glu3}_{mcm^5s^2UUC}$. Full-length and 5′-half of tRNAs are indicated with an arrow and arrow head, respectively.

γ-toxin at a concentration of 1000 nM, tRNA$^{Glu}_{mnm^5s^2UUC}$ from wild-type *E. coli* strain was cleaved with low efficiency but tRNA$^{Glu}_{mnm^5UUC}$ from the *mnmA* mutant was not detectably cleaved (Figure 4C, compare lane 1 with lane 3). The difference in cleavage between tRNA$^{Glu}_{mnm^5s^2UUC}$ and tRNA$^{Glu}_{mnm^5UUC}$ is not dramatic, but was detected repeatedly. A similar tendency was observed

using total tRNA isolated from a yeast wild-type strain and a *tuc1* mutant, but only at low γ-toxin concentration (Figure 4C, compare lane 25 and lane 27). Taken together, the mcm[5] group has a positive effect, while the mnm[5] group has a negative effect on cleavage of tRNA^{Glu}. In both yeast and *E. coli* tRNAs, the s[2] group has a weak stimulatory effect on cleavage by γ-toxin.

DISCUSSION

Sequence determinants for γ-toxin cleavage reside within the anticodon stem loop

The mcm[5] group on wobble uridines in tRNA was shown to be important for γ-toxin cleavage (7). However, the three γ-toxin substrate tRNAs are cleaved with different efficiency even though they all contain the same wobble nucleoside (7). This could be attributed to their sequence differences, or other tRNA modification differences, or a combination thereof. For example, tRNA$^{Lys}_{mcm^5s^2UUU}$ contains t[6]A$_{37}$ instead of the unmodified A$_{37}$ present in tRNA$^{Glu3}_{mcm^5s^2UUC}$. In addition, there are many sequence differences between these two tRNAs (25). Interestingly, the difference in cleavage was also observed for unmodified *in vitro* transcribed tRNAs, suggesting that the primary sequence accounts for the different cleavage efficiency of substrate tRNAs (Figure 1B).

Using unmodified tRNAs, we have shown that the anticodon stem loop (ASL) contains important sequence information for efficient γ-toxin cleavage (Figure 1B). In addition, an RNA oligonucleotide corresponding to the ASL$^{Glu3}_{UUC}$ is cleaved in a similar way as the full-length T7-transcribed tRNA$^{Glu3}_{UUC}$ (Figure 2). Assaying sequence variations of ASL$^{Glu3}_{UUC}$ indicated that the canonical stem loop structure is important for γ-toxin cleavage and that γ-toxin may prefer a stable stem structure in the ASL (Figure 3). Of the nucleotides in the ASL$^{Glu3}_{UUC}$, U$_{34}$U$_{35}$C$_{36}$A$_{37}$C$_{38}$ are required for optimal γ-toxin cleavage, whereas a purine at position 32 or a G in position 33 dramatically reduces the cleavage of the ASL (Figure 3). In contrast, a U$_{33}$ to A mutation stimulated cleavage of the ASL and a U$_{33}$ to C mutation had no effect. The presence of a U at position 33 is a conserved feature of tRNAs and contributes to a sharp turn of the phosphate backbone, the U-turn, which is crucial for the canonical anticodon loop structure (28). However, lack of U-turn has been reported for unmodified ASL^{Phe} (29) and unmodified ASL^{Lys} (30). The modifications normally present in the anticodon region of native tRNAs promote U-turn formation in these ASLs, either by disrupting the intra-loop Watson–Crick base pairs or stabilizing anticodon stacking (29,31,32). Even though the effect of mcm[5]s[2]U modification on the structure of ASL^{Glu} is not known, it is conceivable that the unmodified ASL^{Glu} might lack U-turn. Therefore, the different γ-toxin cleavage effects brought about by mutations in position 33 of ASL^{Glu} seem independent of U-turn formation.

The ASL also represents the minimal structure required for cleavage by a bacterial anticodon nuclease (ACN), PrrC. However, unlike γ-toxin, PrrC prefers a substrate with partially destabilized stem structure (10). On the

other hand, colicin E5, another bacterial ACN, seems to employ a different recognition mechanism. The dinucleotide GpUp corresponding to $G_{34}U_{35}$, a common moiety between the substrate tRNAs, was proposed to be the minimal recognition element for colicin E5 (33). Crystal structure of colicin E5 complexed with the substrate analog dGpdUp revealed a tight binding (34). However, it is noteworthy that the disruption of the stem structure in an RNA oligonucleotide corresponding to the ASL of substrate tRNA reduced the cleavage by colicin E5 (33). It remains to be seen if the dinucleotide GpUp and the ASL are cleaved with similar efficiency.

The effects of wobble uridine modifications on tRNA cleavage by the γ-toxin

Substrate tRNAs lacking part of, or the entire mcm^5 side chain are cleaved much less efficiently by γ-toxin, showing the importance of the mcm^5 side chain (7). In the present study, we have shown that the s^2 group at the wobble position has a positive effect on tRNA cleavage by γ-toxin, even though the effect is less significant than presence of the mcm^5 side chain (Figure 4C). On the contrary, the mnm^5 side chain that is present on the wobble uridine in *E. coli* $tRNA^{Glu}_{mnm^5s^2UUC}$ has a negative effect on cleavage. Both mcm^5 and mnm^5 side chains promote U-turn formation in the anticodon loop of $tRNA^{Lys}_{UUU}$ (32), and most likely also in $tRNA^{Glu}_{UUC}$. Therefore, the different effects of these two modifications on the tRNA cleavage by γ-toxin rather suggest that the wobble nucleotide might interact directly with γ-toxin. The positively charged amino group in the mnm^5 side chain of $tRNA^{Glu}_{mnm^5UUC}$ (32) might interfere with binding and/or catalysis by γ-toxin. A direct contact between tRNA endonuclease and wobble nucleotide has also been proposed for the interaction between PrrC and its substrate $tRNA^{Lys}_{mnm^5s^2UUU}$ (10,11). Interestingly, although all substrate tRNAs of colicin E5 contain the modified nucleotide queuosine (Q) at the wobble position, it seems that Q is not important for cleavage (12). Consequently, based on the crystal structure of colicin E5 in complex with the substrate analog, it was proposed that Q base does not interact with colicin E5 (34). Obtaining the crystal structure of -toxin complexed with substrate tRNA would provide the detailed picture of the interaction between tRNA and -toxin.

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