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Colicin E5 Ribonuclease Domain Cleaves *Saccharomyces cerevisiae* tRNAs Leading to Impairment of the Cell Growth

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Colicin E5 is a ribonuclease that specifically cleaves $tRNA^{Tyr}$, $tRNA^{His}$, $tRNA^{Asn}$ and $tRNA^{Asp}$ of sensitive *Escherichia coli* cells by recognizing their anticodon sequences. Since all organisms possess universal anticodons of these tRNAs, colicin E5 was expected to potentially cleave eukaryotic tRNAs. Here, we expressed the active domain of colicin E5 (E5-CRD) in *Saccharomyces cerevisiae* and investigated its effects on growth. E5-CRD impaired growth of host cells by cleaving $tRNA^{Tyr}$, $tRNA^{His}$, $tRNA^{Asn}$ and $tRNA^{Asp}$ in *S. cerevisiae*, which is the same repertoire as that in *E. coli*. This activity of E5-CRD was inhibited by the co-expression of its cognate inhibitor (ImmE5). Notably, the growth impairment by E5-CRD was reversible; cells restored the colony-forming activity after suppression of the E5-CRD expression. This seems different from the sharp killing effect of E5-CRD on *E. coli*. These results may provide insights into the role and behaviour of cytosolic tRNAs on cell growth and proliferation.

Key words: transfer RNA, ribonuclease, *Saccharomyces cerevisisiae*, colicin, growth arrest.

Abbreviations: BPB, bromophenol blue; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; PCR, polymerase chain reaction; Q, queuine; SD, synthetic dextrose medium; SG, synthetic galactose medium; TBE, Tris–borate–EDTA buffer; TGT, tRNA-guanine transglycosylase; XC, xylene cyanol; YPD, yeast peptone dextrose medium; Ψ, pseudouridine; dGpdUp, deoxyguanylyl(3′-5′)deoxyuridine 3′-monophosphate.

Escherichia coli cells harbouring Col plasmids produce corresponding colicins that kill other cells lacking these plasmids (*1*). The N-terminal region to the central domain of colicin is necessary for receptor binding and translocation across the membrane, and the remaining C-terminal domain enters the cell to exert various toxic actions. Three types are known for those having nuclease activities in their C-terminal domains: a DNase-type (*2–4*) and two types of RNases that target 16S rRNA (*5–7*) and some tRNAs (*8*, *9*). An inhibitor protein, Imm, specific to each nuclease domain, is also produced by respective Col plasmid to protect the colicinogenic cells from lethality. In the tRNA-targeting type, colicin E5 cleaves $tRNA^{Tyr}$, $tRNA^{His}$, $tRNA^{Asn}$ and $tRNA^{Asp}$ (*8*), while colicin D cleaves four iso-accepter tRNAs for Arg in sensitive *E. coli* cells (*9*). Colicins are highly toxic to the sensitive strains, and only a single molecule is supposed to be sufficient to kill them in the mode of single-hit kinetics (*10*).

All colicin E5-sensitive tRNAs contain a modified base, queuine (Q) (*11*, *12*), at the wobble position of the anticodon and is cleaved between the first and second letters (*8*). The incorporation of Q into tRNA is catalysed by tRNA-guanine transglycosylase (TGT) (*13*, *14*). In *E. coli*, Q is found in tRNAs for Tyr, His, Asn and Asp. However, colicin E5 does not distinguish between Q and its precursor G since E5 can cleave tRNAs of TGT-deficient *E. coli in vivo* (*8*). Furthermore, we showed that colicin E5 is a sequence-specific RNase that recognizes YGUN (where Y is a pyrimidine and N is any nucleotide) sequence within single-stranded RNAs, and the diribonucleotide GpUp is the minimal substrate of colicin E5 (*15*, *16*).

Since all organisms possess tRNAs for Tyr, His, Asn and Asp with the common first and second letters of their anticodon, we expected that colicin E5 can cleave tRNAs of eukaryotic organisms. tRNAs are one of the most important factors in cellular reactions; however, with the increasing number of functions being ascribed to tRNAs (*17–19*), they appear to have yet more potentials to play some unknown roles in the cell But, to approach such new functions, tRNA genes are too abundant and dispersed in genomic DNA, so that it is difficult to genetically manipulate these genes. We expect that the C-terminal ribonuclease domain of colicin E5 (E5-CRD) can serve as a powerful tool to solve this issue.

In this study, we chose *Saccharomyces cerevisiae* as a model organism and investigated the effect of E5-CRD on *S. cerevisiae* tRNAs and the cells. E5-CRD exhibited almost the same effect on tRNAs as that observed in sensitive *E. coli*, and showed similar but distinct growth impairment effect on the cells.

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MATERIALS AND METHODS

Strain, Media and Growth Conditions—Yeast strains were grown in rich yeast peptone dextrose (YPD), synthetic dextrose (SD) or synthetic galactose (SG) media supplemented with appropriate amino acids at 30°C. *Saccharomyces cerevisiae* KFY1 strain (*MAT*a *ura3-52 leu2Δ1 trp1Δ63 hisΔ200 lysΔ202*) was used as the host strain. To suppress the E5-CRD expression, 2 mM L-methionine was added to the SD medium to inactivate the *MET3* promoter.

Plasmid Construction—The coding region of E5-CRD and ImmE5 was amplified from pColE5-099. The 3′ end of the *colE5* gene and the 5′ end of the *imm* gene are overlapped by 11 bp and possibly translationaly coupled in *E. coli* as in ColE3 (*20*). The *Sal*I–*Eco*RV fragment of pHAM8 (*21*) containing the *MET3* promoter and the *Xba*I–*Sac*I fragment of the PCR-amplified DNA encoding N terminal FLAG-tagged E5-CRD (FLAG-E5-CRD) were inserted into the *Sal*I–*Sma*I site and the *Xba*I–*Sac*I site, respectively, of both pRS316 (*22*), a low-copy plasmid containing *CEN6*/*ARSH4* and *URA3*, and pYO326, a multi-copy plasmid with 2 μm replication origin and *URA3*. The *MET3* promoter was induced to express FLAG-E5-CRD by methionine depletion from the medium.

We replaced the three catalytic residues of E5-CRD (Inoue *et al*., manuscript in preparation), namely, K25, R33 and K60, with Gln, and the resultant E5-CRD (K25Q), E5-CRD (R33Q), E5-CRD (K60Q) and E5-CRD (K25Q/K60Q) were expressed by the *MET3* promoter as a control. The killing activities of corresponding colicin mutants to sensitive *E. coli* cells were previously shown (*16*). The k_{cat}/K_m values of E5-CRD(K25Q), E5-CRD(R33Q), E5-CRD(K60Q) were all below 0.5% of that of E5-CRD, and the double mutant E5-CRD(K25Q/K60Q) completely abolished the activity (Inoue *et al*., manuscript in preparation). The *immE5* gene was PCR-amplified, and a *Bam*HI site and $(His)_6$-tag fragment were added at the 5′ end, followed by the addition of a *Kpn*I site at the 3′ end. This *Bam*HI–*Kpn*I fragment was inserted into the *Bam*HI–*Kpn*I site of pGMT10 containing *CEN6*/*ARSH4* and *TRP1* gene (*23*). The resultant N terminal $(His)_6$-tagged ImmE5 (HIS-ImmE5) was expressed under the *GAL1* promoter of pGMT10.

Expression of FLAG-E5-CRD and HIS-ImmE5 in S. cerevisiae—*Saccharomyces cerevisiae* KFY1 strain was transformed with two plasmids coding FLAG-E5-CRD and HIS-ImmE5 by the lithium acetate method. The transformant was seeded onto the SG medium supplemented with Met for the expression of HIS-ImmE5. After achieving an OD_{660} of 0.5, the cells were harvested, washed and suspended in the SG medium without Met for the induction of FLAG-E5-CRD. Two hours after the induction of FLAG-E5-CRD, the cells were harvested and lysed with Y-PER Yeast Protein Extraction Reagent (Pierce), and the proteins were resolved on a 14% gel. In the western blotting, anti-FLAG M2 monoclonal antibodies (Sigma) and HRP-conjugated anti-mouse IgG (Dako) were used for the detection of FLAG-E5-CRD, while Ni–NTA HRP conjugate (Qiagen) was used for HIS-ImmE5.

In Vitro Cleavage Assay of S. cerevisiae tRNA by E5-CRD—The methods of production of E5-CRD and ImmE5 in *E. coli* and their subsequent purification were described previously (*8*). Before the incubation of E5-CRD and total RNA from *S. cerevisiae*, E5-CRD was pre-incubated with ImmE5 or buffer (mock) for 15 min at 37°C to form a complex of E5-CRD/ImmE5. Total RNA (10 μg) was then added, and the reaction was initiated. The reaction buffer contained 20 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$ and 100 μg/ml BSA, and the reaction mixture was incubated for 15 min at 37°C. The reaction was terminated by adding an equal volume of the buffer containing 9 M urea, 0.02% xylene cyanol (XC) and 0.02% bromophenol blue (BPB). The samples were directly applied onto 10% acrylamide gel containing 7 M urea and 1× TBE buffer (89 mM Tris–borate and 2 mM EDTA).

Northern Blotting—Cells were treated with zymolyase 100T, and the total RNA was then extracted according to the method described previously (*24*). The probes used to detect tRNAs were as follows: 5′-AGTCGAACGCCCGATCTCAAGATTT-3′ for $tRNA^{Tyr}$, 5′-CTCCTAGAATCGAACCAGGGTTTCA-3′ for $tRNA^{His}$, 5′-GGGTTGAACTCACGATCTTGCGATT-3′ for $tRNA^{Asn}$, 5′-CGACGGGGAATTGAACCCCGATCTG-3′ for $tRNA^{Asp}$ and 5′-CCCCTAACCTTATGATTAAGAGTCA-3′ for $tRNA^{Lys}$. Northern blotting was performed as described previously (*9*), and hybridization was carried out at 42°C.

Cell Viability Test—The transformants possessing FLAG-E5-CRD- and HIS-ImmE5-expressing plasmids were cultured in the SD medium supplemented with Met. After reaching an OD_{660} of 0.3–0.45, cells were collected, washed and re-suspended into SD medium without Met at an OD_{660} of 0.2, and further incubated for the induction of FLAG-E5-CRD. Aliquots of 25 μl were withdrawn at intervals and kept on ice. After the completion of the incubation, samples were mixed with soft agar and plated onto the solid SD medium supplemented with Met to repress the expression of FLAG-E5-CRD. All plates were incubated at 30°C until the appearance of colonies.

RESULTS

E5-CRD Specifically Cleaves S. cerevisiae tRNA In Vitro—The anticodon loops of *S. cerevisiae* tRNAs for Tyr, His, Asn and Asp are highly homologous with corresponding *E. coli* tRNAs, which are the specific natural substrates of colicin E5 cleaving between Q and U at +1 and +2 positions of anticodons (Fig. 1A). The most obvious difference between yeast and *E. coli* tRNAs of this group is the presence of a G in yeast tRNAs instead of the Q in *E. coli* tRNAs. But, we recently showed that E5-CRD does not distinguish between Q and G, and that E5-CRD is ultimately a sequence-specific RNase recognizing a single-stranded YGUN, as seen at −1 to +3 of anticodons of tRNAs (*15*). We thus expected that yeast tRNAs for Tyr, His, Asn and Asp, matching the above criteria as the substrate, are cleaved by E5-CRD.

The total RNA was prepared from *S. cerevisiae* and incubated with E5-CRD for the analysis by northern blotting. *S. cerevisiae* $tRNA^{Tyr}$, $tRNA^{His}$, $tRNA^{Asn}$ and $tRNA^{Asp}$ were in fact cleaved by E5-CRD while $tRNA^{Lys}$

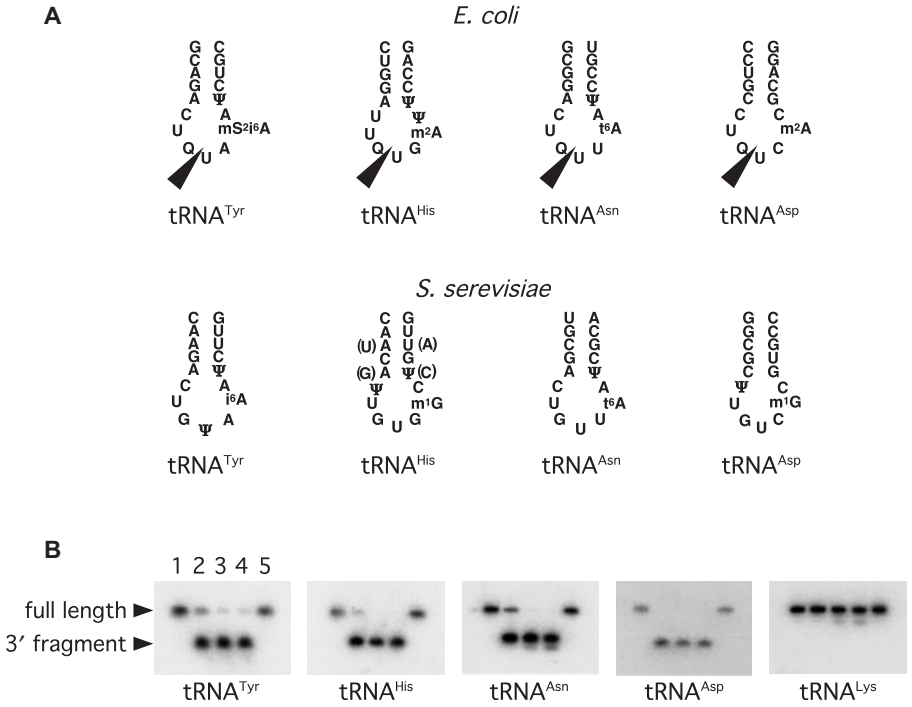

Fig. 1. ***In vitro* cleavage of *S. cerevisiae* tRNA by E5-CRD.** (A) Anticodon arms of *E. coli* tRNAs cleaved by E5-CRD both *in vitro* and *in vivo* and the corresponding ones of *S. cerevisiae* tRNAs are shown. The arrowhead shows the cleavage sites by E5-CRD. Nucleotides appearing in isoaccepting tRNAs are indicated in parentheses. (B) The susceptibility of *S. cerevisiae* tRNAs to E5-CRD was analysed by northern blotting. Lane 1, no addition (control); lanes 2–4, 12.5, 62.5 and 125 nM of E5-CRD, respectively, were incubated with the total RNA; lane 5; 125 nM of E5-CRD and 500 nM of ImmE5 were pre-incubated, and the total RNA was then added.

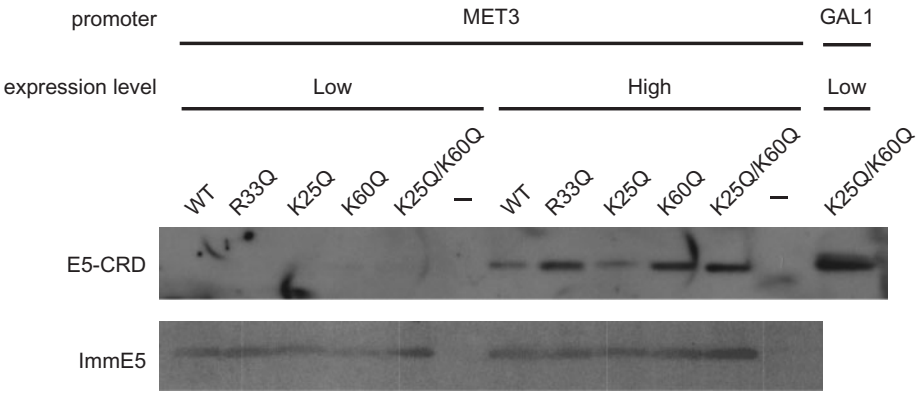

Fig. 2. **Expression of E5-CRD and ImmE5 in *S. cerevisiae*.** FLAG-E5-CRD and HIS-ImmE5 were expressed in *S. cerevisiae* and analysed by western blotting. R33Q, K25Q, K60Q and K25Q/K60Q indicate the expression of catalytic mutants whose active site residue(s) were replaced as indicated. '–' indicates transformants in which vector plasmids were introduced. Expression levels 'Low' and 'High' represent the expression from the low- and high-copy plasmids, respectively. Promoters 'MET3' and 'GAL1' indicate those on which the FLAG-E5-CRD expression was driven. In all cases, the expression of HIS-ImmE5 depended on the *GAL1* promoter.

was not, showing that the tRNA cleavage activity observed in yeast was specific one just like in *E. coli* (Fig. 1B). Notably, the second letter of the $tRNA^{Tyr}$ antidocon is pseudouridine (Ψ) instead of U in *S. cerevisiae*, implying that E5-CRD also cleaves the YGΨN sequence.

E5-CRD Impairs the Growth of S. cerevisiae by Cleaving Specific tRNAs—The effect of the FLAG-E5-CRD expression on the growth of *S. cerevisiae* cells was investigated. *Saccharomyces cerevisiae* KFY1 was transformed with both FLAG-E5-CRD- and HIS-ImmE5-encoding plasmids, and cells were grown in SG medium without Met to induce the expression. The total protein was prepared and FLAG-E5-CRD and HIS-ImmE5 were analysed by the western blotting. The expression of FLAG-E5-CRD by a high-copy plasmid was observed, but hardly detected by a low-copy plasmid (Fig. 2). The expression level of the *MET3* promoter was considerably

lower than that of the *GAL1* promoter as judged from the bands of FLAG-E5-CRD (K25Q/K60Q) (Fig. 2).

Colony formation was severely impaired on the expression of the wild-type FLAG-E5-CRD, but not affected at all on the expression of the mutant (Fig. 3). The impairment of colony formation was evident for the wild-type FLAG-E5-CRD produced even by the low-copy plasmid (Fig. 3, left upper panel), but the impairment seems complete for that produced by the high-copy plasmid (Fig. 3, left bottom panel). In addition, the growth impairment by FLAG-E5-CRD was completely suppressed by the co-expression of HIS-ImmE5, showing that HIS-ImmE5 was sufficiently operative even in *S. cerevisiae* (Fig. 3, right panels). The *MET3* promoter appears to be tightly regulated as no apparent impairment of the growth by FLAG-E5-CRD was observed in the presence of Met (Fig. 3, centre panels).

To analyse the inhibitory effect of FLAG-E5-CRD, the cell viability after the expression of FLAG-E5-CRD was followed (Fig. 4A). The wild-type and mutant FLAG-E5-CRD (K25Q/K60Q) were induced from low- or high-copy plasmids, and culture media were withdrawn at intervals of 2 h. Cell numbers at each time point were calculated from the colony-forming activity. The increase of the cell number stopped after 1 h from the induction of the wild-type FLAG-E5-CRD, regardless of the plasmid copy number. After 12 h of induction, the cells were harvested, and the total RNA was prepared, then the statuses of the potential E5-CRD substrates and control tRNA species were determined by the northern blot

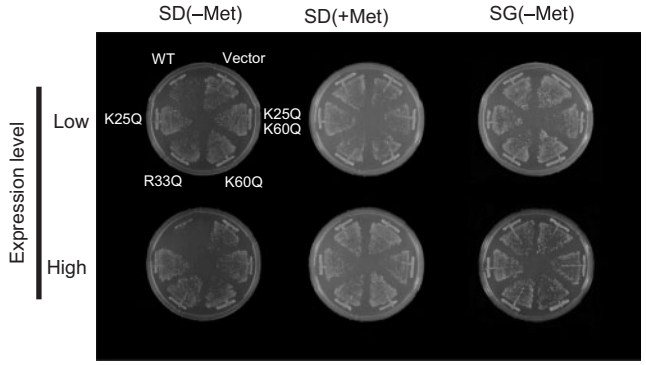

Fig. 3. **Colony formation of *S. cerevisiae* expressing FLAG-E5-CRD and HIS-ImmE5.** FLAG-E5-CRD (including the wild-type and mutants), HIS-ImmE5 or both FLAG-E5-CRD and HIS-ImmE5 were expressed in *S. cerevisiae*, and the colony formation was monitored. On the SD (+Met) plates, the expression of both FLAG-E5-CRD and HIS-ImmE5 was suppressed, while on the SD (–Met) plates, only FLAG-E5-CRD was expressed. On the SG (–Met) plates, both FLAG-E5-CRD and HIS-ImmE5 were expressed.

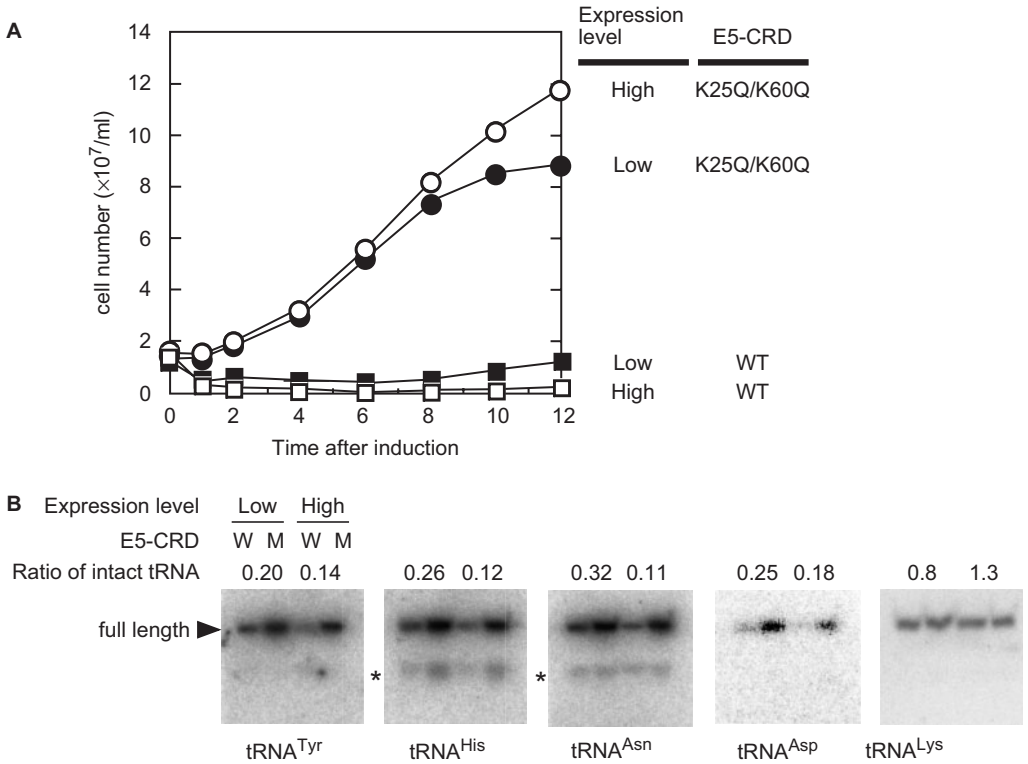

Fig. 4. **Cell viability and status of cytosolic tRNAs of transformants expressing FLAG-E5-CRD.** (A) Cell viabilities of the transformants expressing the wild-type and mutant FLAG-E5-CRD (K25Q/K60Q) were monitored for 12 h. Expression levels 'Low' and 'High' indicate those by a low-copy and high-copy plasmid, respectively, and 'cell number' indicates the colony-forming activity. (B) After 12 h of E5-CRD induction, cells were harvested, and the total RNA was prepared. tRNA cleavage was detected by northern blotting. Expression levels 'Low' and 'High' indicate those by a low-copy and high-copy plasmid, respectively. 'W' and 'M' mean the total RNAs prepared from the cells expressing the wild-type and an inactive mutant FLAG-E5-CRD (K25Q/K60Q), respectively. Ratios of intact tRNA bands are shown. Asterisks do not indicate specific tRNA cleavage fragments but possibly degradation products that appeared depending on the method for RNA preparation.

analysis (Fig. 4B). The amounts of intact $tRNA^{Tyr}$, $tRNA^{His}$, $tRNA^{Asn}$ and $tRNA^{Asp}$ were decreased after the expression of the wild-type FLAG-E5-CRD, and these extents were dependant on the expression level. Cleaved fragments were not visible, suggesting that these fragments were not so stable in cells.

DISCUSSION

In this study, we showed that E5-CRD, which is a natural toxin to *E. coli*, exhibited cell growth impairment activity in *S. cerevisiae* cells. Our recent results showed that transient expression of E5-CRD in HeLa cells also resulted in the impairment of cell growth (data not shown). From these results we concluded that E5-CRD is active in eukaryotic cells as well as in *E. coli*. E5-CRD cleaved *S. cerevisiae* $tRNA^{Tyr}$, $tRNA^{His}$, $tRNA^{Asn}$ and $tRNA^{Asp}$, which is the same tRNA repertoire as in *E. coli*. These four yeast tRNAs have GUN anticodons, without modification of G to Q, supporting our previous conclusion that GU is the minimum determinant of the substrate recognition of E5-CRD (*15*). E5-CRD cleaved the yeast $tRNA^{Tyr}$, which have a pseudouridine (Ψ) instead of U at the second letter of the anticodon. According to the crystal structure we determined of the E5-CRD complexed with a substrate analogue, dGpdUp, O4, N3 and O2 of dU formed hydrogen bonds with E5-CRD (*16*). Since these atoms and the statuses are conserved in Ψ, the same hydrogen bonds should be formed without any hindrance on the replacement U with Ψ.

ImmE5 interacted with E5-CRD to mask its activity in *S. cerevisiae* as in *E. coli* since the transformant co-expressing HIS-ImmE5 and FLAG-E5-CRD exhibited normal colony formation. When the wild-type FLAG-E5-CRD was expressed in *S. cerevisiae*, the host cell quickly terminated its multiplication but maintained the viable cell number until 12 h of induction. The cell numbers under the expression of the wild-type FLAG-E5-CRD at a low level and a high level were almost comparable, indicating that the amount of FLAG-E5-CRD expressed from the low-copy plasmid was sufficient to impair the growth.

It should be noted that the expression of E5-CRD exerted a static effect on the growth of *S. cerevisiae* and was not so lethal as observed in the *E. coli* cells challenged by colicin E5; the cells restored to grow when the expression of FLAG-E5-CRD was suppressed (Fig. 4A). This observation is not a result of the difference of susceptibilities between *S. cerevisiae* tRNAs and *E. coli* tRNAs, since both groups of tRNA are cleaved with almost the same sensitivity to E5-CRD *in vitro* (data not shown). The different effects of E5-CRD on *S. cerevisiae* and *E. coli* cells might possibly caused by its different relative stabilities as compared to different growth rates of the two organisms.

Recently, zymocin, a killer toxin produced by *Kluyveromyces lactis* that arrests the cell cycle of sensitive *S. cerevisiae* (*25*), was reported to specifically cleave anticodon loops of $tRNA^{Glu}_{mcm5s2}UUC$, $tRNA^{Lys}_{mcm5s2}UUU$ and $tRNA^{Gln}_{mcm5s2}UUG$ (*26*); this finding is consistent with our result that the tRNA cleavage leads to growth arrest in yeast. Taking our data into account, we suppose that not cell death but cell-cycle arrest of *S. cerevisiae* caused by reduction of the tRNA level is a physiologically possible situation. Moreover, tRNA cleavage in response to some stresses or cell differentiation has been widely observed in some prokaryote and eukaryote. Amino-acid-starved *Tetrahymena thermophila* accumulates tRNA half fragment (*27*), and oxidative stresses induce tRNA cleavage in *S. cerevisiae*, human cell lines and plants (*28*). tRNA halves are also observed in bacterium *Streptomyces coelicolor*, fungus *Aspergillus fumigatus* and protozoan *Giardia lamblia* in their cell differentiation (*29–31*). In all cases, the anticodon–stem/loop is the most susceptible to the cleavage, and an unidentified nuclease is supposed to be responsible for it. The significance of tRNA cleavage has not understood yet, but tRNA cleavage is expected to trigger the change of intra-celuller environment. If it is the case, E5-CRD should provide a novel and very specific tool to investigate such tRNA-mediated cell responses.

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CONFLICT OF INTEREST

None declared.

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