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Substrate recognition ability differs among various prokaryotic tRNase Zs

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Abstract

There exists a significant difference in pre-tRNA preference among prokaryotic tRNase Zs. This is an enigma, because pre-tRNAs should form the common L-shaped structure and tRNase Zs should form the common structure based on the αβ/βα-fold. To address this issue, we examined six different eubacterial and archaeal tRNase Zs including two newly isolated tRNase Zs for cleavage of 18 different pre-tRNA substrates. Two *Thermotoga maritima*, one *Thermus thermophilus*, one *Bacillus subtilis*, one *Thermoplasma acidophilum*, and one *Pyrobaculum aerophilum* enzymes were tested. To our surprise, the newly isolated proteins *T. maritima* TM0207 and *T. thermophilus* YP_145327 showed the weak tRNase Z activity, even though their primary amino acid sequences are, on the whole, quite different from those of the typical tRNase Zs. We confirmed that substrate recognition ability is quite different among those tRNase Zs. In addition, we found that the optimal conditions as a whole differ significantly among the enzymes. From these results, we provided several clues to solve the enigma by showing the potential importance of the 74th–76th nucleotide sequence of pre-tRNA, the flexible arm length of tRNase Z, the divalent metal ion species, and the histidine corresponding His222 in *T. maritima* tRNase Z.

Keywords: CCA sequence; Pre-tRNA; *Pyrobaculum aerophilum*; *Thermoplasma acidophilum*; *Thermotoga maritima*; *Thermus thermophilus*; tRNA 3′ processing; tRNase Z

Every single tRNA molecule ends with the sequence CCA [1], which is essential for tRNA aminoacylation [2] and translation on the ribosome [3] in all organisms. The tRNAs are transcribed as larger precursor molecules, which subsequently undergo various processing steps such as removal of 5′ and 3′ extra sequences to generate mature tRNAs [4]. tRNA 3′ processing endoribonuclease (tRNase Z) is one of the tRNA-maturing enzymes, which removes a 3′ trailer from pre-tRNA [5–10]. Most tRNase Zs cleave pre-tRNAs immediately downstream of a discriminator nucleotide, onto which the CCA residues are added to produce mature tRNA. *Thermotoga maritima* tRNase Z is an exception, which cleaves pre-tRNAs containing the $_{74}CCA_{76}$ sequence precisely after the A_{76} residue to create the mature 3′-termini [9].

tRNase Zs can be divided into two groups: a long form (tRNase Z^L) that contains 800–900 amino acids and a short form (tRNase Z^S) that consists of 300–400 amino acids [11]. Eukaryotic genomes encode either only tRNase Z^L or both forms, and eubacterial and archaeal genomes contain a tRNase Z^S gene only. The C-terminal half region of tRNase Z^L has high similarity to the whole region of tRNase Z^S, and these regions contain a well-conserved histidine motif, which has been shown to be essential for the tRNase Z activity in the *T. maritima* and *Drosophila melanogaster* enzymes [9,12].

Sequence analysis has suggested that tRNase Zs belong to the metallo-β-lactamase superfamily, and their crystal

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structures [13,14] have revealed a four-layer αβ/βα-sandwich fold that is typically found in the metallo-β-lactamase superfamily. From the structural and functional studies of the superfamily enzymes, the well-conserved six histidine and two aspartate residues together with metal ions are assumed to form the tRNase Z catalytic center. Models for pre-tRNA docking are proposed, in which one or two pre-tRNA molecules bind a positively charged surface of the enzyme dimmer [13,14]. The flexible arm of *B. subtilis* tRNase Z (or the exosite module of *Escherichia coli* tRNase Z) [14,15], which corresponds to the long disordered region between β9 and β10 in the *T. maritima* tRNase Z structure [13], appears to be found typically in tRNase Zs and may play a role in pre-tRNA binding.

Almost all eukaryotic tRNA genes do not encode the $_{74}$CCA$_{76}$ sequence, and 3′ trailers are believed to be removed by tRNase Z [4]. On the other hand, percentages of the CCA-coding tRNA genes in eubacteria and archaea vary with species from 0% to 100%, and the mechanism to remove 3′ trailers appears to change accordingly [9]. The CCA sequences of all *E. coli* tRNAs are encoded in its genome, and the five exoribonucleases RNase II, polynucleotide phosphorylase (PNPase), RNase PH, RNase D, and RNase T in addition to tRNase Z (or RNase BN) are involved in the removal of 3′ trailers to generate the CCA termini [16]. The tRNase Z gene, however, appears to be dispensable under normal growth conditions [17]. In *Bacillus subtilis*, where ~73% of the total tRNA genes encode the $_{74}$CCA$_{76}$ sequence, tRNase Z cleaves only CCA-less pre-tRNAs and RNase PH plays a major role in CCA-containing pre-tRNA processing [8,18]. All 46 *T. maritima* pre-tRNAs contain $_{74}$CCA$_{76}$ with only one exception and would be cleaved after the A$_{76}$ residue by tRNase Z in vivo [9].

We have shown that pre-tRNA preference appears to differ depending on tRNase Z species [9]. The *T. acidophilum* enzyme cleaves human pre-tRNA^{Arg}(GUG) and *T. maritima* pre-tRNA^{Arg}(GUG) and the *P. aerophilum* enzyme cleaves *T. maritima* pre-tRNA^{Arg}(CCA), while the *T. maritima* tRNase Z cleaves all these three substrates. Because each tRNase Z should basically form the common structure based on the four-layer αβ/βα-fold containing the histidine motif (Fig. 1) and each pre-tRNA should form the common L-shaped structure (Fig. 2), we wondered what would make this difference. In this paper, to address this issue, we examined various eubacterial and archaeal tRNase Zs including two newly isolated tRNase Zs for cleavage of various pre-tRNA species.

Materials and methods

cDNA cloning for T. maritima tRNase Z2 and T. thermophilus tRNase Z. The full-length coding regions of *T. maritima* tRNase Z2 (624 bp) and *T. thermophilus* tRNase Z (825 bp) were PCR-amplified from their genomes. The following primer pairs 5′Tm2/3′Tm2 and 5′Tt/3′Tt were used for the amplification of *T. maritima* and *T. thermophilus* genes, respectively. 5′Tm2, 5′-CGGGATCCATGAAGATCACCTGGTTT-3′; 3′Tm2, 5′-ACGCGTCGACTCACTGCACCTCCATGAC-3′; 5′Tt, 5′-ACATGC ATGCATGAACGGGGGCGGCTTTTA-3′; 3′Tt, 5′-CCCAAGCTTCT AAAACTCAAAGAAGGCCG-3′. The amplified genes were cloned between the *Bam*HI and *Sal*I sites and between the *Sph*I and *Hin*dIII sites, respectively, of pQE-80L (Qiagen). We confirmed that the insert regions of pQE/Tm2 and pQE/Tt are the same as the sequences previously published (GenBank Accession Nos. TM0207 and YP_145327, respectively).

Expression and purification of various tRNase Zs. Histidine-tagged recombinant tRNase Zs from *T. maritima*, *T. thermophilus*, *B. subtilis*, *T. acidophilum*, and *P. aerophilum* were produced in *E. coli* cells and purified using nickel–agarose beads as described previously [9].

Pre-tRNA synthesis. The pre-tRNAs were synthesized in vitro with T7 RNA polymerase (Takara Shuzo) from the synthetic pre-tDNAs containing its promoter. The transcription reactions were carried out under the conditions recommended by the manufacturer (Takara Shuzo), and the transcribed pre-tRNAs were gel-purified. The pre-tRNAs were subsequently labeled with fluorescein according to the manufacturer's protocol (Amersham-Pharmacia Biotech). Briefly, after the removal of the 5′-phosphates of the transcripts with bacterial alkaline phosphatase

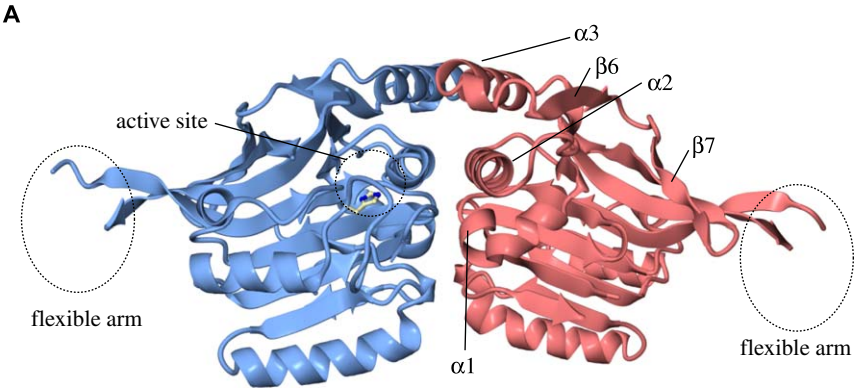

Fig. 1. Sequence alignment of tRNase Zs. (A) Dimer structure of *T. maritima* tRNase Z. The two subunits are colored cyan and pink, respectively. The non-crystallographic symmetry 2-fold axis is perpendicular to the paper. The side chain of His222 is represented by ball-and-stick model. (B) The amino acid sequences of *T. maritima* (*Tma*), *B. subtilis* (*Bsu*), *T. acidophilum* (*Tac*), and *P. aerophilum* (*Pae*) tRNase Zs are aligned using ClustalW [26] and represented using ESPript [27]. (C) The amino acid sequence of *T. maritima* (*Tma*) tRNase Z is aligned with that of *T. maritima* (*Tma2*) tRNase Z2 based on their structures. The *T. maritima* tRNase Z and tRNase Z2 secondary structures for α-helices, β-strands, and 3_{10} helices are indicated by α, β, and η, respectively. The red box and the blue rectangle denote the conserved and moderately conserved residues, respectively. The regions of the histidine motif and the flexible arm are shown. The red triangle in (C) indicates the histidine that may be used as a proton donor. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

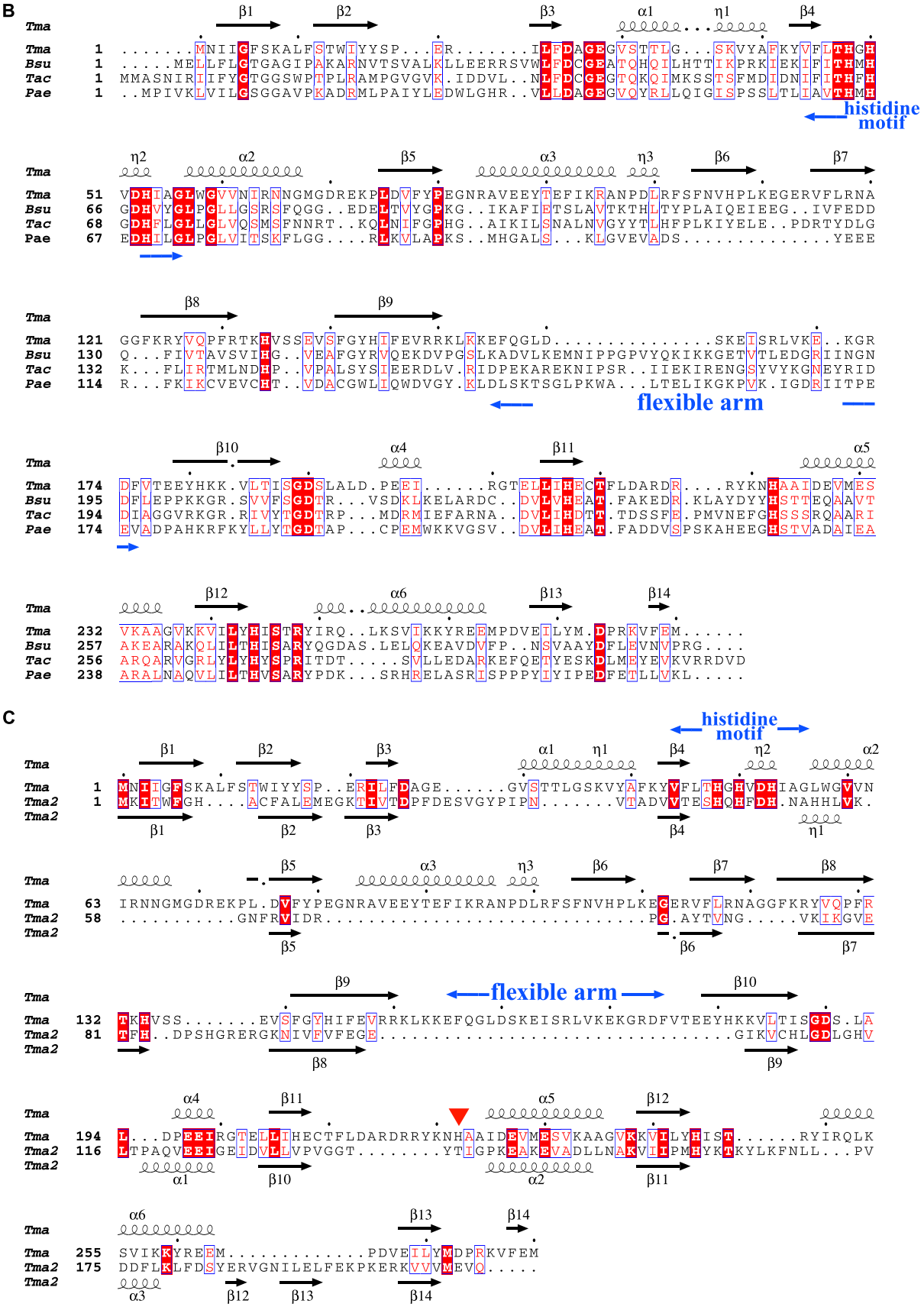

Fig. 1 (*continued*)

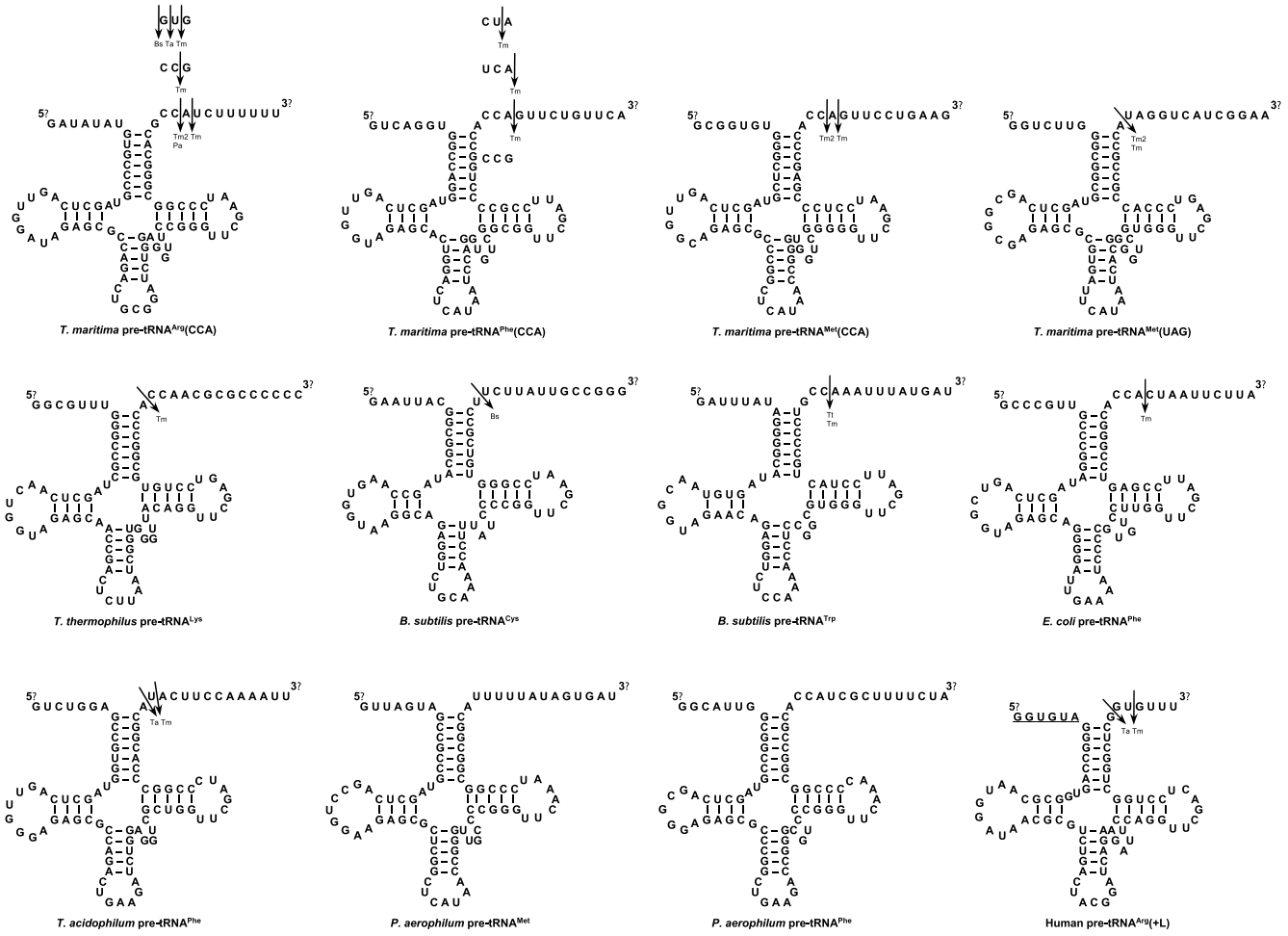

Fig. 2. Various pre-tRNAs from *T. maritima*, *T. thermophilus*, *E. coli*, *B. subtilis*, *T. acidophilum*, *P. aerophilum*, and human. *T. maritima* pre-tRNA^{Arg}(CCG) and pre-tRNA^{Arg}(GUG) were derived from *T. maritima* pre-tRNA^{Arg}(CCA) by replacing $_{74}$CCA$_{76}$ with CCG and GUG, respectively. In the same way, *T. maritima* pre-tRNA^{Phe}(CCG), pre-tRNA^{Phe}(UCA), and pre-tRNA^{Phe}(CUA) were derived from *T. maritima* pre-tRNA^{Phe}(CCA). Only replaced nucleotides are shown. Human pre-tRNA^{Arg}(+L) without the underscored sequence is pre-tRNA^{Arg}(−L). The primary cleavage site by each enzyme is indicated by an arrow on the pre-tRNA secondary structure.

(Takara Shuzo), the transcripts were phosphorylated with ATPγS using T4 polynucleotide kinase (Takara Shuzo). Then a single fluorescein moiety was appended onto the 5′-phosphorothioate site. The resulting pre-tRNAs with fluorescein were gel-purified before assays.

In vitro tRNA 3′ processing assay. The standard 3′ processing reactions for fluorescein-labeled pre-tRNAs (0.1 pmol) were performed with tRNase Zs (10 pmol) of various origins in a mixture (6 μl) containing 10 mM Tris–HCl (pH 7.5), 1.5 mM dithiothreitol, 25 mM NaCl, and 10 mM $MgCl_2$. The reaction mixture was incubated at 35 °C (for *T. thermophilus* tRNase Z), 50 °C (for *B. subtilis* and *T. acidophilum* tRNase Zs), or 60 °C (for *T. maritima*, *T. maritima* 2nd, and *P. aerophilum* tRNase Zs) for 10 min. After resolution of the reaction products on a 10% polyacrylamide-8 M urea gel, the gel was analyzed with a Typhoon 9210 (Amersham-Pharmacia Biotech).

Gel-shift assays. To determine the dissociation constant of a tRNase Z/pre-tRNA complex, fluorescein-labeled pre-tRNA (0.01–0.1 μM) was incubated at 25 °C for 20 min with various amounts of *T. maritima* tRNase Z (0.01–0.72 μM) or tRNase Z2 (14.3–57.4 μM) in a buffer (6 μl) containing 10 mM Tris–HCl (pH 7.5), 1.5 mM dithiothreitol, and 10 mM $MgCl_2$. After the incubation, the sample was mixed with the same volume of a loading buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA, and 50% glycerol), and electrophoresed on a 5% non-denaturing polyacrylamide gel with TBE buffer (90 mM Tris-base, 90 mM boric acid, and 1.5 mM EDTA, pH 8.3). After electrophoresis, the labeled RNAs were quantitated with the Typhoon 9210.

Results and discussion

Another T. maritima tRNase Z has a weak tRNA processing activity

A structure search using DALI [19] for known three-dimensional protein structures similar to *T. maritima* tRNase Z revealed that the overall architecture of *T. maritima* tRNase Z shares a common fold with those of the metallo-β-lactamases [13]. Despite the modest Z score and the low level of structure-based sequence identity (less than 10%) (Fig. 1C), *T. maritima* tRNase Z was structurally aligned with the *T. maritima* putative zinc-dependent hydrolase of metallo-β-lactamase superfamily (TM0207, PDB ID 1VJN) with an r.m.s.d. of 2.5 Å for 155 Cα atoms [13].

To see if TM0207 has tRNase Z activity, we isolated the TM0207 gene and generated its product in *E. coli* (Fig. 3A). The purified histidine-tagged product was tested for the activity using 12 different pre-tRNAs from *T. maritima*, *E. coli*, and human (Fig. 2 and Table 1). *T. maritima* pre-tRNA^{Met}(CCA) and pre-tRNA^{Met}(UAG) were cleaved albeit very inefficiently (Fig. 3B). The cleavage sites were identified by comparing with cleavage products by *T. maritima* tRNase Z on a sequencing gel. Pre-tRNA^{Met}(CCA) and pre-tRNA^{Met}(UAG) were cleaved after C_{75} and after the discriminator, respectively. *T. maritima* pre-tRNA^{Arg}(CCA) and pre-tRNA^{Arg}(GUG) were also cleaved very inefficiently (data not shown). The cleavage site of pre-tRNA^{Arg}(CCA) was identified after C_{75}, but that of pre-tRNA^{Arg}(GUG) could not due to its very inefficient cleavage (data not shown). From these observations, we propose to call TM0207 tRNase Z2.

To understand what causes these very inefficient cleavages by tRNase Z2, we compared the strength of enzyme/substrate interactions by measuring the

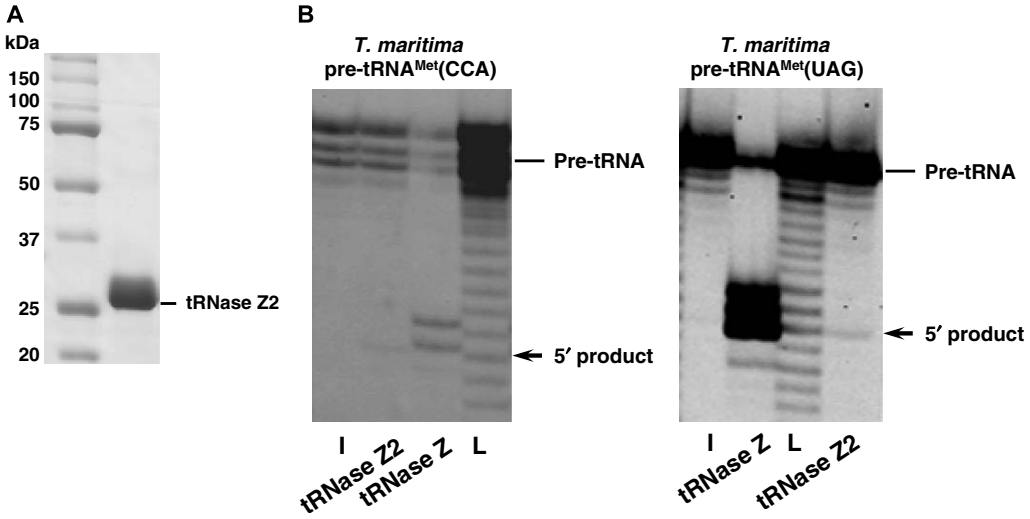

Fig. 3. The tRNA 3′ processing activity of tRNase Z2 from *T. maritima*. (A) A protein profile of histidine-tagged tRNase Z2 from *T. maritima*. The purified protein (5 μg) was analyzed on an SDS-10% polyacrylamide gel, and visualized by staining the gel with Coomassie brilliant blue R-250. (B) The 3′ processing assays for *T. maritima* tRNase Z and tRNase Z2 (10 pmol) using the two fluorescein-labeled pre-tRNAs (0.1 pmol). The reaction mixtures were incubated at 60 °C for 10 min and the products were subsequently analyzed on a denaturing sequencing gel. The 5′-cleavage products are indicated by arrows. The 5′-cleavage products by *T. maritima* tRNase Z are used as size standards. I, input RNA; L, alkaline ladder.

Table 1
Pre-tRNA processing efficiencies of prokaryotic tRNase Zs

Substrate	*T. maritima*	*T. maritima* 2nd	*T. thermophilus*	*B. subtilis*	*T. acidophilum*	*P. aerophilum*
T. maritima pre-tRNA^{Arg}(CCA)	+++[c]	++	+[b]	–	–[c]	++[c]
T. maritima pre-tRNA^{Arg}(GUG)	+++[c]	+[b]	–	+++	++[c]	–[c]
T. maritima pre-tRNA^{Arg}(CCG)	+[c]	–	ND	ND	ND	ND
T. maritima pre-tRNA^{Phe}(CCA)	++[c]	–	ND	ND	ND	ND
T. maritima pre-tRNA^{Phe}(UCA)	+++[c]	–	ND	ND	ND	ND
T. maritima pre-tRNA^{Phe}(CUA)	+++[c]	–	ND	ND	ND	ND
T. maritima pre-tRNA^{Phe}(CCG)	–[c]	–	ND	ND	ND	ND
T. maritima pre-tRNA^{Met}(CCA)	+++[c]	++	ND	ND	ND	ND
T. maritima pre-tRNA^{Met}(UAG)	+++[c]	++	–	ND	ND	ND
T. thermophilus pre-tRNA^{Lys}	++	ND	–	–	ND	ND
B. subtilis pre-tRNA^{Cys}	–	ND	–	+++[a]	ND	ND
B. subtilis pre-tRNA^{Trp}	+++	ND	++	–	ND	ND
E. coli pre-tRNA^{Phe}	+++	–	+[b]	ND	–	–
T. acidophilum pre-tRNA^{Phe}	++	ND	ND	ND	++	–
P. aerophilum pre-tRNA^{Met}	+++[b]	ND	–	ND	ND	–
P. aerophilum pre-tRNA^{Phe}	+++[b]	ND	–	ND	ND	–
Human pre-tRNA^{Arg}(+L)	+++	–	–	ND	ND	ND
Human pre-tRNA^{Arg}(−L)	+++[c]	–	–	+++[b]	+++[c]	–[c]

The cleavage sites are shown in Fig. 2. +++, efficient cleavage; ++, weak cleavage; +, cleavage barely detected; –, cleavage not detected; and ND, not determined.

[a] The cleavage occurred only in the presence of 0.2 mM Mn^{2+}.

[b] The cleavage site was not determined.

[c] The data are from the previous report [9].

Table 2
Dissociation constants for pre-tRNA/tRNase Z complexes

Substrate	*T. maritima* (μM)	*T. maritima* 2nd (μM)
T. maritima pre-tRNA^{Arg}(CCA)	0.05 ± 0.012	26 ± 1.8
T. maritima pre-tRNA^{Arg}(GUG)	0.17 ± 0.035	37 ± 2.1
T. maritima pre-tRNA^{Arg}(CCG)	0.06 ± 0.004	11 ± 2.5
T. maritima pre-tRNA^{Met}(UAG)	0.24 ± 0.041	270 ± 5.1
T. maritima pre-tRNA^{Phe}(UCA)	0.08 ± 0.005	17 ± 2.4
T. maritima pre-tRNA^{Phe}(CUA)	0.04 ± 0.002	30 ± 5.3
T. maritima pre-tRNA^{Phe}(CCG)	0.13 ± 0.012	130 ± 3.2
Human pre-tRNA^{Arg}(+L)	0.08 ± 0.039	39 ± 3.6

Data are the means ± SD of three independent experiments.

dissociation constant K_d. Seven *T. maritima* and one human pre-tRNAs were examined for the affinity to tRNase Z or tRNase Z2. The K_d values for tRNase Z ranged from 0.04 to 0.24 μM, while those for tRNase Z2 was between 11 and 270 μM (Table 2). The very high K_d values for tRNase Z2 may be due to inefficiency of dimer formation, which appears to be essential for pre-tRNA binding [13,14]. Gel filtration analysis showed that indeed, only ~20% of tRNase Z2 molecules formed dimers (data not shown), while tRNase Z molecules existed as dimers [13]. This inefficient tRNase Z2 dimer formation may be attributed to the lack of α1, α2, and α3 that are important for dimerization of tRNase Z (Fig. 1) [13,14].

The ~50 to 7000-fold lower affinity to tRNase Z2 than tRNase Z suggests that the very low cleavage efficiencies may be because of the very week interaction of pre-tRNA with tRNase Z2. However, *T. maritima* pre-tRNA^{Met}(UAG) was cleaved by tRNase Z2 in spite of the highest K_d value, while cleavages of four other *T. maritima* and one human pre-tRNAs with smaller K_d values were not detected (Tables 1 and 2). This observation argues against this rationalization and implies additional factors that lower the cleavage activity.

One of the potential factors may be nucleotide species near the scissile bond as shown with respect to *T. maritima* pre-tRNA^{Phe}(CCG), which binds to tRNase Z with K_d of 0.13 μM but is not cleavable probably due to the presence of G_{76}. Another factor may be the lack of the histidine (His222 in tRNase Z) that may be used as a proton donor in *T. maritima* tRNase Z (unpublished observation). In the case of tRNase Z2, the corresponding amino acid that may act as a proton donor is Tyr138 (pK_a 10.1) or Thr139 (pK_a 13.6) (Fig. 1C), the pK_a values of which are much higher than that (pK_a 6.0) of His.

Thermus thermophilus tRNase Z

We found that the *T. thermophilus* protein YP_145327 annotated as Zn-dependent hydrolase contains a highly similar region to the histidine motif although the similarity to tRNase Zs along the whole region is not high (data not shown). Thus, we cloned its cDNA from the *T. thermophilus* genome and expressed it in *E. coli* cells (Fig. 4A). The purified histidine-tagged product was assayed for the

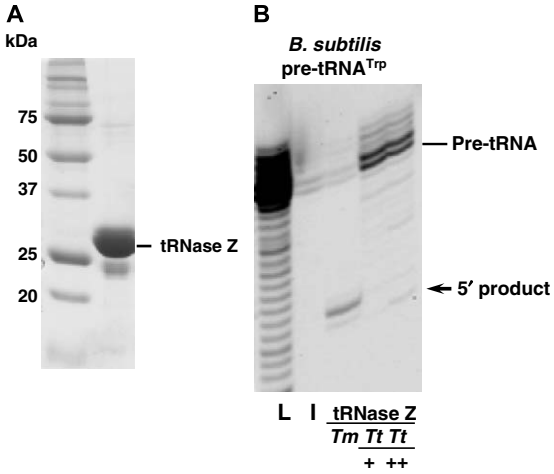

Fig. 4. The tRNA 3′ processing by *T. thermophilus* tRNase Z. (A) A protein profile of histidine-tagged *T. thermophilus* tRNase Z. The purified protein (5 μg) was analyzed on an SDS–10% polyacrylamide gel, and stained with Coomassie brilliant blue R-250. (B) The 3′ processing assays. The fluorescein-labeled *B. subtilis* pre-tRNA^{Trp} (0.1 pmol) was incubated for 10 min with *T. thermophilus* (*Tt*) tRNase Z (5 or 10 pmol) at 35 °C or with *T. maritima* (*Tm*) tRNase Z (10 pmol) at 60 °C, and the products were subsequently analyzed on a denaturing sequencing gel. The 5′-cleavage product by *T. thermophilus* tRNase Z is indicated by an arrow. The 5′-cleavage product by *T. maritima* tRNase Z is used as a size standard. I, input RNA; L, alkaline ladder.

tRNase Z activity using several pre-tRNAs and this product cleaved three pre-tRNAs albeit very inefficiently. *B. subtilis* pre-tRNA^{Trp} was cleaved after C_{75} relatively well (Fig. 4B), and cleavages of *T. maritima* pre-tRNA^{Arg}(CCA) and *E. coli* pre-tRNA^{Phe} were barely detected (Fig. 2 and Table 1). Cleavage of other eight pre-tRNA substrates tested including *T. thermophilus* authentic pre-tRNA^{Lys} was not detected at all. From these data, we believe that the *T. thermophilus* protein YP_145327 possesses the tRNase Z activity.

Comparison of optimal conditions among tRNase Z

To determine optimal pre-tRNA processing conditions for tRNase Zs, we assayed each enzyme activity under the standard conditions by varying one of the parameters pH, temperature, concentrations of NaCl, $MgCl_2$, and $MnCl_2$. One of the cleavable pre-tRNAs was used for each enzyme: *T. maritima* pre-tRNA^{Arg}(CCA), *T. maritima*; *T. maritima* pre-tRNA^{Met}(UAG), *T. maritima* 2nd; *B. subtilis* pre-tRNA^{Trp}, *T. thermophilus*; *B. subtilis* pre-tRNA^{Cys}, *B. subtilis*; *T. acidophilum* pre-tRNA^{Phe}, *T. acidophilum*; *T. maritima* pre-tRNA^{Arg}(CCA), and *P. aerophilum*. Optimal pH and temperature ranged from 7 to 10 and from 35 to 70 °C, respectively, and optimal concentrations of NaCl, $MgCl_2$, and $MnCl_2$ were 25–200, 1–10, and 0.1–1 mM, respectively (Fig. 5). Cleavages by *T. thermophilus*, *T. acidophilum*, and *P. aerophilum* tRNase Zs were not detected in the presence of $MnCl_2$.

The optimal pH for tRNase Zs was 7–8.2 with the exception of *T. maritima* tRNase Z2, which showed the

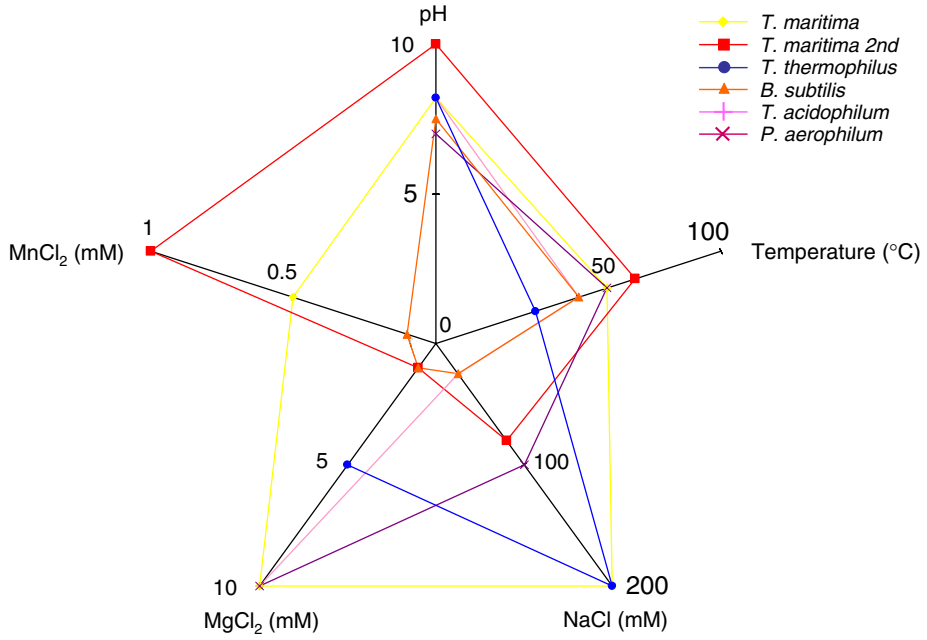

Fig. 5. Optimal conditions for tRNase Zs. Optimal pH, optimal temperature, and optimal concentrations of NaCl, $MgCl_2$, and $MnCl_2$ were shown for *T. maritima*, *T. maritima* 2nd, *T. thermophilus*, *B. subtilis*, *T. acidophilum*, and *P. aerophilum* tRNase Zs. Cleavages by *T. thermophilus*, *T. acidophilum*, and *P. aerophilum* enzymes were not detected in the presence of $MnCl_2$.

unusually high optimal pH (~10). This observation is consistent with the above supposition that Tyr138 (pK_a 10.1) or Thr139 (pK_a 13.6) may be used as a proton donor in *T. maritima* tRNase Z2. The optimal temperature for *T. thermophilus* tRNase Z was 35 °C, which was unexpectedly low compared with those for the other tRNase Zs, even though *T. thermophilus* grows best at 75 °C. In the cells living at a high temperature, some protein modification and/or an auxiliary factor might be needed for its maximum activity, or some pre-tRNA modification might be essential for the proper interaction. The difference in the optimal Mg^{2+} or Mn^{2+} concentration among the tRNase Z species may reflect a difference in the Mg^{2+} or Mn^{2+} affinity to the catalytic center activated by pre-tRNA binding.

It is astonishing that the optimal conditions as a whole differ significantly among the enzymes. These tRNase Zs would have evolved quite differently depending on the change in their intracellular milieu and/or in pre-tRNA sequences.

Substrate recognition ability differs among tRNase Zs

We examined six different tRNase Zs from four eubacterial and two archaeal species for the endoribonuclease activity using 18 different pre-tRNA substrates from seven species. As below, we found that substrate recognition ability is quite different among tRNase Zs. It should be noted that all the tested pre-tRNAs except *T. maritima* pre-$tRNA^{Phe}$(CCG) were cleaved at least one of the tRNase Zs, indicating that these cleavable pre-tRNAs are correctly folded.

As listed in Table 1 and Fig. 2, *T. maritima* tRNase Z was the most versatile enzyme, and processed most of the pre-tRNAs from any species very efficiently. Interestingly, *T. thermophilus* pre-$tRNA^{Lys}$ was cleaved after the discriminator in spite of the presence of the $_{74}CCA_{76}$ sequence. This would be because of the presence of a $_{71}CCA_{73}$ sequence that may be recognized by the enzyme as the $_{74}CCA_{76}$ sequence though a tRNA conformational change like the case of *T. maritima* pre-$tRNA^{Met}$(UAG) [9]. Cleavage of *T. maritima* pre-$tRNA^{Phe}$(CCG) and *B. subtilis* pre-$tRNA^{Cys}$ by this enzyme was not detected and cleavage of *T. maritima* pre-$tRNA^{Arg}$(CCG) was barely detected. In contrast, *T. maritima* tRNase Z2 was very unrobust and processed only four *T. maritima* pre-tRNAs among 12 tested species (Table 1 and Fig. 2). As mentioned above, *T. thermophilus* tRNase Z was also a very weak enzyme, and cleaved only three pre-tRNA species among assayed 11 (Table 1 and Fig. 2).

Bacillus subtilis tRNase Z has been well characterized by Condon's group [8]. Generally, the *B. subtilis* enzyme can process only CCA-less pre-tRNAs. We tested three CCA-less pre-tRNAs and three CCA-containing pre-tRNAs for cleavage by this enzyme. As expected, this tRNase Z cleaved only CCA-less pre-tRNAs from *T. maritima*, *B. subtilis*, and human (Table 1 and Fig. 2). Interestingly, *B. subtilis* pre-$tRNA^{Cys}$ was cleaved in the presence of Mn^{2+} but not in the presence of Mg^{2+}.

Two archaeal tRNase Zs from *T. acidophilum* and *P. aerophilum* were examined for cleavage of several pre-tRNAs. The *T. acidophilum* enzyme cleaved three CCA-less pre-tRNAs from *T. maritima*, *T. acidophilum*, and human fairly well, but did not cleave two pre-tRNAs containing $_{74}CCA_{76}$ from *T. maritima* and *E. coli* (Table 1 and Fig. 2). With respect to *P. aerophilum* tRNase Z, two *T. maritima*, one *E. coli*, one *T. acidophilum*, two

P. aerophilum, and one human pre-tRNAs were tested for cleavage. Only cleavage of *T. maritima* pre-tRNA^{Arg}(CCA) was detected, and even two *P. aerophilum* authentic pre-tRNAs were not cleaved at all (Table 1 and Fig. 2).

What makes the difference in substrate recognition ability?

With respect to mammalian tRNase Z^{L}s, we have identified several pre-tRNA elements that affect the cleavage reaction. The efficiency of pre-tRNA cleavage by mammalian tRNase Z^{L} is affected by the length of a 5′ leader and a 3′ trailer, the complementarity degree between a 5′ leader and a 3′ trailer, and the 74th–76th nucleotide sequence [5,20]. The cleavage site selection depends on the total length of the T-stem and the acceptor-stem [21,22]. We have also shown that the La protein reduces the pre-tRNA cleavage efficiency through binding the 3′-terminal uridine stretch of pre-tRNA [23]. In this paper, to elucidate what makes the difference in substrate recognition ability among prokaryotic tRNase Zs, we tried to identify elements in pre-tRNA that are discriminated by each enzyme and domains in tRNase Z that are involved in the difference in substrate preference. As discussed below, the present results provided several clues to complete understanding of the substrate preference issue.

The most versatile *T. maritima* tRNase Z can cleave almost any pre-tRNAs very efficiently, but has some difficulty in cleaving pre-tRNAs containing $_{74}$CCG$_{76}$ or $_{74}$UCU$_{76}$. In general, the cleavages of CCA-containing pre-tRNAs occur primarily after C$_{76}$, while the cleavages of CCA-less pre-tRNAs take place primarily after the 75th nucleotide. In some cases, the cleavage sites fluctuate by one nt. In the case of *T. maritima* pre-tRNA^{Met}(UAG) and *T. thermophilus* pre-tRNA^{Lys}, which contain the sequence $_{71}$CCA$_{73}$, the cleavage occurred after the discriminator A$_{73}$. The versatility of this enzyme may be attributed to the shortness of the flexible arm region [14,15] between β9 and β10 of the enzyme (Fig. 1B), in the crystal structure of which this region was disordered [13].

Thermotoga maritima tRNase Z2 can process only a subset of pre-tRNAs regardless of the presence of $_{74}$CCA$_{76}$, and the cleavage site appears to be selected in the similar fashion to *T. maritima* tRNase Z. The lack of amino acid sequences corresponding to α1, α2, α3, β6, and the flexible arm region between β9 and β10 of *T. maritima* tRNase Z may be responsible for the limited number of cleavable pre-tRNAs (Fig. 1C).

Thermus thermophilus tRNase Z preferentially cleaved CCA-containing pre-tRNAs, but two of them including authentic *T. thermophilus* pre-tRNA^{Lys} were not its substrates. The substrate preference rule for these tRNase Zs does not seem to be straightforward. Currently we know neither pre-tRNA elements nor their interacting tRNase Z domains are responsible for the discrimination.

The rule to govern the preference of the *B. subtilis* enzyme seems to be simple. This enzyme cleaves CCA-less pre-tRNAs after the discriminator but not CCA-containing pre-tRNAs. Usually, Mg^{2+} or Mn^{2+} ions are required for the activity, but *B. subtilis* pre-tRNA^{Cys} was cleaved only in the presence of Mn^{2+}, suggesting that Mn^{2+} ions may need to be coordinated to form the activated catalytic center with some pre-tRNAs that may have specific Mn^{2+}-requiring nucleotides near the scissile bond.

The substrate preference and the cleavage site selection of *T. acidophilum* tRNase Z are basically the same as those of the *B. subtilis* enzyme, although the cleavage site of *T. maritima* pre-tRNA^{Arg}(GUG) was fluctuated one nt downstream of the discriminator. The rule how *P. aerophilum* tRNase Z discriminates substrates remains unknown. This enzyme cleaved only one substrate, *T. maritima* pre-tRNA^{Arg}(CCA), after C$_{75}$ among seven tested substrates, which are three CCA-containing pre-tRNAs and four CCA-less pre-tRNAs. Although we could not find any elements in pre-tRNAs that may distinguish the cleavable substrate from the others, the lack of the amino acid sequence corresponding to β6 and/or β7 of *T. maritima* tRNase Z might be related to this discrimination.

Conclusion and outlook

The significant difference in tastes for pre-tRNAs among tRNase Zs is an enigma, because pre-tRNAs should form the common L-shaped structure and tRNase Zs should form the common structure based on the αβ/βα-fold. Here, we provided several clues to solve this enigma by showing the potential importance of the 74th–76th nucleotide sequence, the flexible arm length, the divalent metal ion species, the histidine corresponding His222 in *T. maritima* tRNase Z, and the amino acid sequence corresponding to β6 and/or β7 of *T. maritima* tRNase Z (Fig. 1).

It was surprising that *T. maritima* TM0207 and *T. thermophilus* YP_145327 showed the weak tRNase Z activity, because their primary amino acid sequences are, on the whole, quite different from those of the typical tRNase Zs although they share the common αβ/βα-structure containing the histidine motif. The weakness of the activity implies that these enzymes may need additional cellular factors for the full activity or may play other roles in the cells. In any case, this observation would provide an insight into the evolution of the metallo-β-lactamase superfamily, in which the αβ/βα-fold is typically found.

This superfamily contains various enzymes such as β-lactamase, glyoxalase II, and tRNase Z [24], which would have evolved from one common ancestral protein. The proteinous enzyme tRNase Z that was essential for tRNA generation might have emerged earliest, and might have evolved to the other enzymes by losing the flexible arm domain and/or gaining their functional domains. Alternatively, if the RNA world really existed, the removal of pre-tRNA 3′ trailers would have been executed by a ribozyme [25]. tRNase Z, however, contains no reminiscent RNA component unlike the tRNA 5′ processing endoribonuclease RNase P, suggesting that one of the superfamily proteins may have happened to obtain the tRNA 3′

processing endoribonuclease activity by gaining the flexible arm domain late in evolution and may have taken over the ribozyme's job.

Further analyses for various prokaryotic tRNase Zs together with eukaryotic tRNase Z^Ss and tRNase Z^Ls using various pre-tRNAs are currently under way. In addition to these, the experiments using tRNase Z chimeras and deletion or addition variants and the crystal structures of pre-tRNA/tRNase Z complexes would help to elucidate what makes the substrate preference and to understand the evolution of tRNase Z.

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