Maturation of the 5S rRNA 5' end is catalyzed in vitro by the endonuclease tRNase Z in the archaeon *H. volcanii*

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ABSTRACT

Ribosomal RNA molecules are synthesized as precursors that have to undergo several processing steps to generate the functional rRNA. The 5S rRNA in the archaeon *Haloferax volcanii* is transcribed as part of a multicistronic transcript containing both large rRNAs and one or two tRNAs. Release of the 5S rRNA from the precursor requires two endonucleolytic cleavages by enzymes as yet not identified. Here we report the first identification of an archaeal 5S rRNA processing endonuclease. The enzyme tRNase Z, which was initially identified as tRNA processing enzyme, generates not only tRNA 3' ends but also mature 5S rRNA 5' ends in vitro. Interestingly, the sequence upstream of the 5S rRNA can be folded into a mini-tRNA, which might explain the processing of this RNA by tRNase Z. The endonuclease is active only at low salt concentrations in vitro, which is in contrast to the 2–4 M KCl concentration present inside the cell in vivo. Electron microscopy studies show that there are no compartments inside the *Haloferax* cell that could provide lower salt environments. Processing of the 5S rRNA 5' end is not restricted to the haloarchaeal tRNase Z since tRNase Z enzymes from a thermophilic archaeon, a lower and a higher eukaryote, are as well able to cleave the tRNA-like structure 5' of the 5S rRNA. Knock out of the tRNase Z gene in *Haloferax volcanii* is lethal, showing that the protein is essential for the cell.

Keywords: tRNase Z; RNase Z; tRNA processing; rRNA processing; archaea; *Haloferax volcanii*

INTRODUCTION

In archaea, ribosomal RNAs are encoded in operons and are transcribed as multicistronic precursor molecules containing the 16S rRNA, the 23S rRNA, the 5S rRNA, and one or two tRNAs (Klug et al. 2007). The number of operons encoding these RNAs varies from one (extreme thermophiles) to four (*Methanococcus vannielii*) operons per genome (Garrett et al. 1991). In Euryarchaeota, such as *Haloferax volcanii*, the rRNA operon contains the 16S rRNA gene, a tRNA gene in the internal transcribed spacer (ITS), the 23S rRNA gene, the 5S rRNA gene, and one distal tRNA gene (Fig. 1A; Dennis et al. 1998). Bacterial rRNA operons have a similar organization with the 16S rRNA gene preceding the ITS with one or more tRNA genes, the

23S rRNA gene, the 5S rRNA gene, and one or more distal tRNA genes (Gegenheimer and Apirion 1981). In eukaryotes, the 18S rRNA, 5.8S rRNA, and 25S rRNA are transcribed into a single long 35S rRNA precursor (Venema and Tollervey 1999). The 5S rRNA is encoded separately and transcribed by a different polymerase, RNA polymerase III, instead of polymerase I (Venema and Tollervey 1999).

The bacterial and archaeal pre-rRNA transcripts, in general, contain inverted repeats surrounding the 16S and 23S rRNA sequences, which may form extended helical structures and contain the sites for initial endonucleolytic cleavage and excision of pre-16S and pre-23S from the primary transcript (Fig. 1A). In *Escherichia coli*, the endonuclease responsible for pre-rRNA excision is the helix-specific RNase III (Gegenheimer and Apirion 1981), but in archaea, no RNase III-like enzyme has been identified (Condon and Putzer 2002). The inverted repeats surrounding the large rRNAs in archaea contain a bulge-helix-bulge (BHB) motif that might be recognized and cleaved by the splicing endonuclease (Chant and Dennis 1986). To define the intermediates generated during processing of the primary rRNA

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FIGURE 1. (A) Structure of the multicistronic rRNA transcript. The *H. volcanii* genome contains two copies of the rRNA operon, which differ in the 3' part. Both operons contain the genes for 16S rRNA, tRNA^{Ala}, 23S rRNA, and the 5S rRNA, but only one operon encodes in addition the distal tRNA^{Cys}. The primary transcript is processed by several endonucleolytic cuts (indicated by black arrows). The tRNAs are removed by RNase P and tRNase Z, and the 16S rRNA and the 23S rRNA are removed from the primary transcript by the splicing endonuclease (SE), which cleaves at the bulge-helix-bulge motifs. Both large rRNAs still contain additional 5' and 3' sequences, which have to be removed by as yet unidentified endonucleases or exonucleases. The 5S rRNA is also excised as pre-5S rRNA by as yet unidentified endonucleases. Potentially remaining nucleotides have to be removed by exonucleases. (B) A protein extract from *H. volcanii* processes the 5S rRNA precursor. Two different 5S rRNA precursors were incubated with a soluble protein extract from *H. volcanii*. The longer precursor contains the 5S rRNA and 5' as well as 3' additional sequences (lanes: long). The short precursor contains only the 5S rRNA and the 5' extension (lanes: short). (Lanes p) Incubation with protein extract; (lanes c) incubation without proteins. (Lane m) DNA size marker, sizes are given at the left. Precursor and products from the long precursor are shown schematically at the right; precursor and products from the short precursor are shown at the left.

transcript, primer extension and nuclease protection assays were used in studies with *Halobacterium salinarum* and *Haloarcula marismortui* (Chant and Denni 1986; Dennis et al. 1998). Several endonucleolytic processing sites were identified in these studies (Fig. 1A), but only few of the enzymes cleaving at these sites are known: the tRNA processing enzymes RNase P (Frank and Pace 1998) and tRNase Z (Vogel et al. 2005), as well as the splicing endonuclease (Kleman-Leyer et al. 1997). According to these studies, the 5S rRNA is excised as a precursor containing several nucleotides 5' and 3', which are subsequently trimmed, but the endonucleases responsible for these cleavages were not identified. In *E. coli*, the 5S rRNA is released in a similar fashion from the long primary transcript (Gegenheimer and Apirion 1981). RNase E cleaves three nucleotides upstream of and downstream from the 5S rRNA, generating a pre-5S rRNA (Szeberenyi et al. 1983). The remaining nucleotides at the 3' end are subsequently removed by the exonuclease RNase T (Szeberenyi et al. 1983; Li and Deutscher 1995); maturation of the 5' end is still a mystery. In yeast, the 5S rRNA is transcribed by RNA polymerase III, which generates a 5S precursor with a mature 5' end but with additional nucleotides at the 3' end (Venema and Tollervey 1999). Removal of these nucleotides requires in yeast the RNA82 gene product, although the 3' unprocessed 5S rRNA does not detectably reduce

ribosomal function in strains where this 3' processing is impaired (Venema and Tollervey 1999).

H. volcanii used in this study is a halophilic archaeon, which lives in an environment with high concentrations of salt (2–4 M) (Mullakhanbhai and Larsen 1975). Halophilic archaea adapt to these salt concentrations by raising their intracellular concentrations to equal amounts (2–4 M salt), conditions at which many conventional proteins are known to denature and precipitate (Lanyi 1974). The mechanisms by which enzymes are active at these salt concentrations inside the cell are still not fully clear.

Here we report the first identification of a 5' processing activity for the 5S rRNA in archaea. We show that the mature 5' end of the 5S rRNA in *H. volcanii* can be generated by the endonuclease tRNase Z.

RESULTS

A 5S rRNA substrate is processed by a soluble protein extract from *H. volcanii*

To identify endonucleases responsible for generation of the mature 5S rRNA in archaea, we isolated a soluble cell extract (S100) from the halophilic archaeon *H. volcanii* (Schierling et al. 2002) and incubated it with 5S rRNA precursor molecules.

We generated two different precursors of the *H. volcanii* 5S rRNA, one containing 5' and 3' extensions and the other one containing only a 5' extension and a mature 5S rRNA 3' end. Both substrates were incubated with a soluble cell extract (S100) from *H. volcanii* cells (Fig. 1B). After 30 min of incubation, processing products were detectable that correspond in length to the 5' leader and either the 5S rRNA with 3' trailer or the mature 5S rRNA, respectively, depending on the substrate used (Fig. 1B). To identify the enzyme responsible for the endonucleolytic cut, we initiated purification of the protein. The soluble S100 extract was fractionated using polyethylene glycol. 5S rRNA processing activity fractionated with the 3.6%–5.2% PEG fraction, which was subsequently further purified on an anion exchange column (Source30 Q), from which it eluted with 0.3 M KCl. Since the purification pattern resembled the one for the tRNA 3' processing enzyme tRNase Z (Schierling et al. 2002; Schiffer et al. 2002; Rösch 2004), we investigated whether tRNase Z is able to process the 5S rRNA precursor.

The tRNA endonuclease tRNase Z catalyzes the 5' processing reaction

tRNase Z is the endonuclease that generates the mature tRNA 3' end (3.1.26.11) (Vogel et al. 2005). The tRNase Z activity from *H. volcanii* has been characterized earlier using partially purified protein fractions from *H. volcanii* cells (Schierling et al. 2002). Only after cloning of the first tRNase Z gene in plants was the identification of the tRNase Z genes in other organisms (Schiffer et al. 2002) like *Haloferax* possible (Späth et al. 2008). The gene for the *Haloferax* tRNase Z protein was cloned and expressed in *E. coli*, yielding a pure recombinant protein that is as efficient in tRNA 3' processing as the tRNase Z proteins from other organisms (Fig. 2; Späth et al. 2008). The recombinant tRNase Z from *H. volcanii* (HvoTrz) was subsequently incubated with the long 5S rRNA precursor containing 5' and 3' extensions (Fig. 3A). The precursor was cleaved by the recombinant tRNA endonuclease, giving rise to the same products as seen with the S100 extract. To further characterize the processing products of the recombinant tRNase Z, we incubated HvoTrz with a 3' end labeled substrate (Fig. 3B). Processing of this substrate yielded only a single product of ~125 nucleotides (nt) in length, which corresponds to the size of the 5S rRNA. Thus HvoTrz, which was initially identified as tRNA 3' processing enzyme, also catalyzes the 5' end cleavage of the 5S rRNA.

The 5' leader can be folded into a mini-tRNA structure

To analyze whether tRNase Z recognizes a structure in the 5' leader sequence and not the 5S rRNA, we used RNAfold

FIGURE 2. Expression of the *H. volcanii* tRNase Z gene. The gene for the *Haloferax* tRNase Z was cloned into expression vector pET29a (Novagen), expressed in *E. coli*, and purified using the S-tag (Novagen). Purification yields a pure recombinant tRNase Z as shown by the silver-stained SDS PAGE. (Lane m) Protein size marker; sizes are given in kDa at the left. (Lane p) Recombinant tRNase Z, indicated by the arrow.

of the Vienna RNA package (Hofacker et al. 1994) to fold the 40 nt upstream of the 5S rRNA (Fig. 4A). Interestingly, the sequence can be folded into a mini-tRNA with a 6-base-pair (bp) acceptor stem (instead of the usual 7-bp acceptor stem), a D replacement loop (instead of a D arm and an anti-codon arm), and a 6-bp T-stem (instead of the usual 5-bp T stem) and T-loop. The canonical secondary structure of a tRNA is shown in comparison in Figure 4B. Some of the conserved tRNA nucleotides are also present in that structure, two guanosines in the D replacement loop, and the sequence GUUCNANNC in the T arm. Further three-dimensional (3D) modeling of the structure showed that even the tertiary interaction typical for a tRNA is sterically possible: interaction of the D replacement loop and the T loop (Fig. 4C,D). According to this modeling, tRNase Z could recognize the mini-tRNA structure in the 5' leader and cleave it at the 3' end (Fig. 4A).

tRNase Z cleaves pre-tRNAs more efficiently than the pre-5S rRNA

To determine whether the mini-tRNA structure in the 5S rRNA substrate is an equally efficient substrate for tRNase Z as the pre-tRNA, we compared the processing efficiency of both substrates. The pre-tRNA substrate was processed more efficiently than the 5S rRNA substrate. When setting pre-tRNA processing to 100%, 5S rRNA processing was only 66% as efficient.

Determination of the cleavage site

To identify the exact cleavage site we isolated the processed 5' leader, used circularized RNA-RT-PCR (CR-RT-PCR) to reverse transcribe the RNA, and amplified and sequenced

FIGURE 3. The *H. volcanii* tRNase Z catalyzes the 5S rRNA 5' end processing reaction. (A) Uniformly labeled 5S rRNA substrate. The long 5S precursor was incubated with the *H. volcanii* tRNase Z. (Lane c) Incubation without proteins; (lane p) incubation with tRNase Z. (Lane m) DNA size marker; sizes are shown at the left. Precursor and products are shown schematically at the right. (B) 3' end labeled 5S rRNA substrate. The short 5S precursor was labeled at the 3' end and incubated with the recombinant *H. volcanii* tRNase Z. (Lane c) Incubation without proteins; (lane p) incubation with tRNase Z. (Lane m) DNA size marker; sizes are shown at the right. Precursor and products are shown schematically at the left.

the resulting cDNA. Analysis of the cDNA clones showed that the majority of the sequences represented RNAs, which were cut between nucleotides -1 and $+1$ (Fig. 5). Thus the tRNase Z directly generates the mature 5S rRNA 5' end.

Characterization of the processing products

tRNase Z generates upon pre-tRNA cleavage a tRNA with a 3' hydroxyl group and a 3' trailer with a 5' phosphate group (Kunzmann et al. 1998). To identify the end groups of the pre-5S rRNA processing products, we isolated the 5' leader molecule and incubated it with RNA ligase and ^{32}P -pCp (data not shown). A single product of the 5' leader size is detectable on an 8% PAA gel. The pCp is successfully ligated to the 5' leader RNA, which indicates an accessible 3' terminal hydroxyl group essential for this ligation. The end groups generated upon pre-5S rRNA cleavage by tRNase Z are thus the same as upon pre-tRNA cleavage, a 3' hydroxyl group and a 5' phosphoryl group.

The activity is inhibited by high salt concentrations

To determine the optimal reaction conditions for the 5S rRNA processing reaction, we analyzed the processing efficiency in a range of different conditions. The optimal

conditions for 5' end processing of the 5S rRNA are summarized in Table 1. Interestingly, the pre-5S rRNA is most efficiently processed at KCl concentrations of 10 mM KCl, and KCl concentrations higher than 100 mM inhibit the reaction in vitro. Generally proteins isolated from halophilic archaea require high salt concentrations for activity in vitro, but several proteins have been identified that are similarly inhibited by high salt concentrations in vitro, e.g., the fatty acid synthetase, RNA-dependent RNA polymerase, and amylase (Lanyi 1974). Since halophilic archaea counterbalance the high salt concentration in the medium by high intracellular salt concentrations, pre-5S rRNA processing inside the cell must be possible at salt concentrations of 2–4 M KCl. We therefore investigated whether there might be salt-free compartments in *Haloferax* cells, which would allow some enzymes to act in an environment with lower salt concentrations.

There are no visible compartments in the Haloferax cell

In an attempt to identify compartments inside *H. volcanii* cells, we analyzed high-pressure-frozen and freeze-substituted *H. volcanii* cells with electron microscopy (Fig. 6). Using this technique, no compartments are visible inside the cell.

Do other tRNase Z enzymes cleave the 5S precursor?

To analyze whether tRNase Z proteins from other organisms would also be capable of processing this archaeal 5S rRNA substrate, we tested recombinant tRNase Z enzymes from a thermophilic archaeon (*Pyrococcus furiosus*), yeast (*Saccharomyces cerevisiae*), and a higher eukaryote (*Arabidopsis thaliana*, tRNase Z^{S1}) (Fig. 7). These tRNase Z enzymes were also able to process the 5' end of the haloarchaeal 5S rRNA. All three enzymes generated the 5S rRNA and the 5' leader.

Biological functions of the tRNase Z protein

tRNase Z was initially purified and identified as tRNA 3' processing endonuclease (Schiffer et al. 2002). The halophilic tRNase Z has been isolated and shows in vitro the same tRNA 3' processing activity as the previously characterized tRNase Z proteins (Späth et al. 2008). Here we show that this enzyme is also capable of processing pre-5S rRNAs. To identify other biological functions of the tRNase Z enzyme, we attempted to generate a knock out strain of *H. volcanii* using the pop-in/pop-out system (Fig. 8A; Bitan-Banin et al. 2003; Allers et al. 2004). However we were not able to isolate a strain homozygous for the knock out (Fig. 8B). *Haloferax* cells contain several copies of the chromosomal DNA (up to 20 copies) (Breuer et al. 2006), and after the pop-out step, cells did contain the marker replacement gene but also still the wild-type gene, suggesting

FIGURE 4. Structure of the 5S rRNA leader sequence. (A) Using the program RNAfold of the Vienna RNA package (<http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi>), a potential two-dimensional structure of the 5' leader sequence was calculated. (B) The canonical tRNA secondary structure is shown. Conserved nucleotides are indicated. (C) A 3D model structure was generated based on the similarity of the 5S rRNA leader sequence and predicted secondary structure to the tRNA acceptor stem, T-stem, and T-loop regions (see Materials and Methods). Bases that correspond to acceptor stem, T-stem, and T-loop regions are indicated as blue, red, and green stick representation, respectively (nucleic acid backbone in gray). For clarity, only the backbone trace of the D-replacement loop is shown except for two guanine bases (atom color code) that allow for similar D-loop/T-loop contacts as seen in tRNA structures. (D) Structure of yeast tRNA^{Phe} with the same view and color coding as in B (only the backbone trace is shown for the whole D-arm and anti-codon-arm region).

that the knock out is lethal (Fig. 8B). Further proof for the essentiality of the tRNase Z gene was achieved by transforming the tRNase Z wild-type gene on a plasmid (pTA409-zwt) into the heterozygous pop-out strain. Propagation of this strain resulted in a homozygous genomic background in which the wild-type gene was completely lost (Fig. 8C), but critically, the plasmid-borne copy of the gene was retained. Cells that were forced to lose the plasmid died.

DISCUSSION

To elucidate rRNA maturation in archaea, we investigated processing of two different 5S rRNA precursor molecules

with a protein extract from the halophilic archaeon *H. volcanii*. In vitro generated precursor molecules are processed efficiently, showing that nucleotide modifications of the 5S rRNA are not essential for 5S processing. In addition, the short substrate containing the 5S rRNA and additional 5' sequences is sufficient for recognition by the processing enzyme.

Interestingly, we identify here the tRNA endonuclease tRNase Z as the enzyme responsible for 5S rRNA 5' processing in vitro. Several studies had suggested that the tRNase Z enzymes might have additional functions besides tRNA processing, but none has been clearly identified so far (for review, see Späth et al. 2007). A screen for ribonucleases in yeast revealed a role for the yeast tRNase Z in 35S rRNA processing, but its exact function could not be determined (Peng et al. 2003). Yeast strains with mutations in the tRNase Z gene can be rescued by complementation with *REX2* (Chen et al. 2005). Rex2p is an RNA exonuclease involved in U4 snRNA processing, 5.8S rRNA maturation, U5 snRNA maturation, and RNase P RNA processing. In *E. coli*, Perwez and Kushner (2006) showed that tRNase Z is involved in mRNA decay of specific mRNAs.

Hitherto the main substrate identified for tRNase Z was the tRNA precursor. In vitro studies showed that the D arm and the anti-codon arm of the tRNA are dispensable for tRNase Z processing (Nashimoto et al. 1999; Mayer et al. 2000; Schiffer et al. 2001). A mini-pre-tRNA that consists of the acceptor stem, the T arm, and the 3'

trailer and that can fold into a long stem-loop was identified as minimal substrate for processing (Nashimoto et al. 1999). Thus substrates do not have to contain the complete tRNA structure to be processed by tRNase Z. Structure modeling of the 5' leader sequence of the 5S rRNA substrate used in this study showed that it is sterically possible to adopt a mini-tRNA structure. Therefore tRNase Z may recognize the tRNA-like structure in the 5' leader and process it at its 3' end, thereby releasing a mature 5S rRNA 5' end. Comparison of processing efficiency of pre-tRNA and pre-5S rRNA showed that the pre-tRNA substrate is cleaved slightly more efficient in vitro.

FIGURE 5. Determination of the cleavage site. 5' leader products were analyzed in respect to the cleavage site of tRNase Z. The majority of clones obtained showed that cleavage occurs between nucleotides –1 and +1 of the 5S rRNA precursor.

tRNase Z enzymes from a thermophilic archaeon, as well as from a lower and a higher eukaryote, are likewise capable of cleaving the archaeal 5S rRNA precursor. This cleavage is thus not a specific feature of the halophilic enzyme but seems to be a general tRNase Z characteristic, confirming the hypothesis that a tRNA-like structure is recognized in the substrate and cleaved.

Unfortunately tRNase Z is essential for *Haloferax*, and we could not analyze in vivo whether this endonuclease has additional RNA substrates besides the now identified tRNA and rRNA precursors. At this point, further substrates for the halophilic tRNase Z will have to be identified by in vitro approaches, and in silico calculations will show whether more tRNA-like elements are present in the genome, allowing recognition and subsequent processing by tRNase Z.

The 5' end of the 5S rRNA generated by the recombinant tRNase Z is the same as the 5' end determined for the *H. volcanii* 5S rRNA in vivo (Daniels et al. 1985). The end groups of the processing products are the same as those generated upon cleavage of pre-tRNAs, suggesting that cleavage of both types of RNA might occur by the same mechanism.

Optimal reaction conditions for processing of the rRNA substrate by the halophilic tRNase Z are similar to those for tRNA processing (Table 1; Schierling et al. 2002; Rösch 2004). For both reactions, low KCl concentrations are optimal, which is in contrast to the high KCl concentration inside the cell. Halophilic archaea do not use compatible solutes to cope with the high extracellular salt concentrations but raise the intracellular salt concentrations to isotonic levels with the surrounding medium (Oren 1999). Generally halophilic enzymes require high salt concentrations in vitro, but several exceptions to this rule behave similarly to the tRNase Z, like the RNA-dependent RNA polymerase of *Halobacterium cutirubrum* (Lanyi 1974). According to the electron microscopy images, no compart-

ments are visible that could provide an environment with lower ionic concentrations inside the cell for these low-salt preferring enzymes. Therefore the tRNase Z enzyme must be stabilized inside the cell by other proteins or cofactors.

Processing of 5S rRNA in archaea is similar to the process observed in bacteria; in both domains, the 5S rRNA is excised by an endonuclease from the precursor molecule. While in bacteria the excised 5S rRNA still contains residual nucleotides at the 5' and 3' ends, it seems that in *Haloferax* at least the 5' end is generated in one step by the endonuclease, which cleaves directly between nucleotides –1 and +1. 5S rRNA processing in eukaryotes differs, since in yeast the 5S rRNA is transcribed by RNA polymerase III, yielding a pre-5S rRNA that starts directly at the 5' end of the 5S rRNA; the 10 nucleotides of the 3' trailer are removed by an exonuclease (Venema and Tollervey 1999).

MATERIALS AND METHODS

Strains and culture conditions

H. volcanii strains H53 (*DpyrE2*, *DtrpA*) and H119 (*DpyrE2*, *DtrpA*, *DleuB*) (Allers et al. 2004) were grown aerobically at 45°C in Hv-YPC or Hv-Min medium (Allers et al. 2004).

Isolation of the 5S rRNA gene and the tRNase Z gene from *H. volcanii*

Chromosomal DNA from *H. volcanii* was isolated by using the alternative rapid chromosomal isolation method as published in the Halohandbook v6.06 (http://www.microbiol.unimelb.edu.au/people/dyallsmith/resources/halohandbook/HaloHandbook_v6_06.pdf). The gene for the 5S rRNA was amplified from *H. volcanii* DNA by PCR using primers Hvo5S1T7 and HvoS2 (primer sequences and PCR programs are available upon request) according to the sequence published in TIGR (<http://archaea.ucsc.edu>). The resulting PCR product contains the 5S rRNA gene and additional 5' and 3' sequences (66 bp and 88 bp, respectively). The PCR product was subcloned into pUC18 to yield clone pUC18-5SI. The gene for tRNase Z was amplified with primers Hvvz1 and Hvvz2 and cloned into pET29a (Novagen), yielding pET29a-hvoz.

TABLE 1. Optimal reaction conditions of tRNase Z

Parameter	5S	tRNA
T	40°C	37°C
pH	5.5	5.5
Mg ₂ Cl	5 mM	5 mM
KCl	10 mM	10 mM

The optimal reaction conditions for processing of pre-tRNAs (data taken from Rösch 2004; Späth et al. 2008) and pre-5S rRNA (this study) are shown.

FIGURE 6. Electron microscopy of *H. volcanii* cells. *H. volcanii* cells were high-pressure frozen and freeze-substituted for electron microscopic analysis.

Substrate preparation

Templates for 5' extended 5S rRNA and 5' and 3' extended 5S rRNA from *H. volcanii* were synthesized from clone pUC18-5SI using PCR. The resulting template pHv5S-5 contains the T7 promoter, the 5S rRNA gene (129 bp), and the 5' leader (66 bp); template pHv5S-5 + 3 additionally contains the 3' trailer (88 bp). In vitro transcription and purification of transcripts were performed as described (Marchfelder et al. 1990).

Purification of the processing activity

H. volcanii cells (H53) were grown to an OD₆₀₀ of 0.6–0.8 in 1 L medium. Cells were collected by centrifugation at 7,500g for 15 min at 4°C. After washing of the cell pellet with buffer C (50 mM Tris-HCl at pH 7.5, 2 M KCl, 5 mM MgCl₂), cells were disrupted by sonification, and a high-speed supernatant (S100) was obtained by ultracentrifugation at 100,000g for 60 min at 4°C. The S100 fraction was dialyzed against buffer E (50 mM Tris-HCl at pH 7.5, 5 mM MgCl₂).

PEG precipitation

A 3.6%–5.2% PEG-fraction was prepared as follows: a 40% PEG stock solution (40% PEG 6000 in buffer E) (w/w) was slowly added to the S100 fraction until the final PEG concentration was 3.6% (w/w). The solution was stirred for 45 min, and the precipitate was pelleted for 30 min at 30,000g. To the resulting supernatant, a 40% PEG stock solution was added to a final concentration of 5.2%. After centrifugation, the resulting pellet was dissolved overnight in 1 mL buffer E containing 10% glycerol (v/v), yielding the fraction 3.6%–5.2% PEG.

Resource Q column

The 3.6%–5.2% PEG fraction was loaded onto a 1 mL Resource 30Q column (GE Healthcare). The column was washed with buffer A (50 mM MES at pH 5.5, 5 mM MgCl₂), and bound proteins were eluted with a KCl step gradient (0.1, 0.2, 0.3, 0.5 M KCl in buffer A). The processing activity eluted with 0.3 M KCl. This fraction was dialyzed against buffer A using centriplus filtration units (Millipore). For in vitro processing assays, 10 µg of the protein fractions was used.

Expression of HvoTrz in *E. coli*

PET29a-hvoz were transformed into the strain Rosetta (DE3) pLys (Novagen) and expressed and purified according to the manufacturer's protocol using S-protein agarose (Novagen).

Optimization of processing assays

The initial processing assay was performed with 100 ng HvoTrz in buffer hvoz (50 mM MES at pH 5.5, 10 mM KCl, 5 mM MgCl₂, 2 mM DTT) in a volume of 100 µL for 30 min at 37°C. Pre-5S rRNA was incubated in different buffers depending on the parameter examined. These were the optimal reaction conditions determined earlier for tRNA processing by HvoTrz (Rösch 2004). For pH determination, the following buffers were used: MES for pH 5.5–6.5, Tris for pH 7.0–8.0. Processing reactions were terminated by phenol and chloroform extractions. Nucleic acids were precipitated, and reaction products were analyzed on 8% polyacrylamide gels. According to the optimal reaction conditions determined, the initial conditions were the optimal reaction conditions, thus all processing reactions were carried out with 100 ng protein in 100 µL buffer hvoz (40 mM MES at pH 5.5, 5 mM MgCl₂, 5 mM KCl) for 30 min at 37°C.

Comparison of reaction efficiencies

To determine the cleavage efficiencies, in vitro processing products of internally labeled precursors were separated by PAGE, which were subsequently dried. Gels were analyzed using a Fuji BAS 1000 instrument (FujiFilm); processing products were quantified using the software MacBAS (FujiFilm). All experiments were carried out in triplicates, and the resulting data were

FIGURE 7. The 5S rRNA precursor is also processed by tRNase Z enzymes from another archaeon and eukaryotes. tRNase Z enzymes from *Saccharomyces cerevisiae* (lane Sce), *Arabidopsis thaliana* (tRNase Z^{S1}; lane Ath), and *Pyrococcus furiosus* (lane Pfu) were incubated with the short 5S rRNA substrate. (Lane M) DNA size marker; sizes are given at the left. (Lane c) Control reaction without the addition of proteins. Precursor and products are shown schematically at the right.

FIGURE 8. Knock out of the tRNase Z gene is lethal. The gene for tRNase Z in the *Haloferax volcanii* genome was replaced by the *trpA* gene by the pop-in/pop-out transformation procedure. (A) Schematic presentation of the knock out strategy. SalI sites important for Southern hybridizations are indicated (S). Strain H119 containing the wild-type *trpA* gene (shown in yellow and labeled *hvtorz*) was transformed with the integrating plasmid pTA131-Z1/4-trpA. Successful integrants (pop-in events) were selected on medium without tryptophan and uracil. The plasmid was forced out by plating the cells on 5-FOA, which yielded a heterozygous pop-out (B). This pop-out was transformed with plasmid pTA409-zwt, which contains the wild-type *trpA* gene. After several cycles of cultivation, a homozygous strain was obtained (C). (B) The deletion of the chromosomal *trpA* gene was investigated by Southern analysis. As a probe, the upstream sequence of the *trpA* gene was used (probe Z1/2). Replacement of the *trpA* gene by the *trpA* gene results in a 864-bp SalI fragment, whereas the wild-type gene gives rise to a 1754-bp fragment. Both fragments are visible, indicating that cells still contain wild-type copies of the gene in some of the chromosomal DNA copies present in the *Haloferax* cells. (Lanes a–d) Heterozygous pop-out strains; (lane e) wild-type strain. (C) To generate a homozygous chromosomal knock out strain, the heterozygous cells were transformed with plasmid pTA409-zwt, which contains the tRNase Z gene. Southern analysis of this strain shows that it is homozygous for the pop-out. Only the 864-bp SalI fragment of the pop-out is visible and the plasmid borne *trpA* gene, which is 6223 bp. (Lane a) Wild-type strain; (lane b) heterozygous strain without the plasmid (as in B); (lane c) homozygous pop-out strain. A DNA size marker is given at the right in base pairs.

averaged. The cleavage efficiency of the tRNA precursors was set to 100%.

Characterization of end groups of the processing products

In vitro processing assays were performed with unlabeled 5S rRNA precursor. Processing products were separated on 8% polyacrylamide gels, and 5' leader products were identified by the migration of processing products of a parallel assay with radio-labeled substrates, excised and eluted in buffer C (0.5 M NH₄OAc at pH5.0/ 0.1 mM EDTA/ 0.1% SDS), precipitated, and dissolved in 29 μ L H₂O. Eluted unlabeled 5' leader products were incubated with ³²P-pCp (10 μ Ci) and T4 RNA ligase (20 units) in a final volume of 40 μ L with pCp-buffer; (50 mM Tris-HCl at pH 7.5/10 mM DTT/ BSA [0.5 mg/ mL]), 4 μ L dimethyl sulfoxide, and 1 unit of RNasin for 16 h at 4°C. Products were analyzed on 8% PAA gels.

Determination of cleavage site

The 5' leader product was isolated as described in "characterization of processing products." After elution, the 5' leader was dephosphorylated and subsequently phosphorylated. The resulting RNA was circularized by addition of RNA ligase and reverse transcribed using primer LeadRT5S. The cDNA was amplified by PCR with primers LeadRT5S and Lead5S2. PCR products were cloned into pUC18 and sequenced.

Electron microscopy

For electron microscopy, the samples were prepared by high-pressure freezing and freeze substitution. For this purpose, the cells were absorbed in small cellulose capillary tubes (inner diameter, 200 μ m) as described (Hohenberg et al. 1994). High-pressure freezing was performed with the HPF Compact 01 high-pressure freezing apparatus (Engineering Office M. Wohlwend GmbH). Freeze substitution was done in acetone containing 1.6% (w/v) osmium tetroxide, 0.1% (w/v) uranyl acetate, and 5% (v/v) water (Walther and Ziegler 2002) by slowly warming the samples over a period of 18 h from –90°C to 0°C. The samples were then kept at 0°C and at room temperature for 30 min, washed with acetone, and embedded in a two-step Epon series (Fluka) of 50% Epon in acetone for 1 h and 100% Epon for 6 h. The Epon was polymerized for 3 d at 60°C. Thin sections (~60–80 nm) were imaged with a Philips 400 transmission electron microscope at an acceleration voltage of 80 kV.

Generation of a *trpA* knock out strain

The *trpA* gene was replaced in the *H. volcanii* strain H119 by the *trpA* marker gene using the pop-in/pop-out method (Bitan-Banin et al. 2003; Allers et al. 2004). The upstream and downstream regions of the *trpA* gene were amplified by PCR using chromosomal DNA from *H. volcanii* and primers KOZ1 and KOZ2 and KOZ3 and KOZ4, respectively, yielding fragments Z1/2 and Z3/4, both of

~1 kb in length. The *trz* gene sequence was taken from TIGR (http://tigrblast.tigr.org/ufmg/index.cgi?database=h_volcanii seq). PCR primers contained different restriction sites XhoI (KOZ1), EcoRV (KOZ2 and KOZ3), and BamHI (KOZ4). The *trpA* gene was amplified by using plasmid pTA132 (Allers et al. 2004) as template and oligos TRP1 and TRP2, which both contain EcoRV sites. All PCR fragments were first cloned into pBlue-scriptII (Stratagene), yielding plasmids pblue-Z1/2, pblue-Z3/4, and pblue-TrpA, and were subsequently subcloned into the integrative vector pTA131 containing the *pyrE2* marker (Allers et al. 2004), yielding pTA131-Z1/4-trpA. This plasmid was integrated into the chromosomal DNA of *H. volcanii* (strain H119, pop-in) (Fig. 6A). The plasmid containing the *pyrE2* marker was forced out by plating the cells on 5-fluoroorotic acid (5-FOA; pop-out). Southern blot analysis was carried out as described in method of Sambrook and Russell (2001) with the following modifications. Chromosomal DNA was isolated from wild-type and knock out strains (Allers et al. 2004) and digested using SalI. Ten micrograms of digested DNA were separated on an 0.8% agarose gel and transferred to a nylon membrane (Hybond-N, GE-Healthcare). Hybridization probe Z1/2 was generated by PCR using primers TZ1 and KOZ2 on template pblue-Z1/2, yielding a 500-bp fragment, which was subsequently radioactively labeled using the random prime kit Readiprime II (GE Healthcare).

Construction of shuttle vector pTA409

A synthetic operon, consisting of *H. volcanii pyrE2* and *hdrB* genes under control of the *H. salinarum* ferredoxin promoter, was constructed. A 685-bp PCR product, comprising the *hdrB* coding sequence (Ortenberg et al. 2000), was inserted at the XbaI–HindIII sites directly downstream of the *pyrE2* gene in pGB70 (Bitan-Banin et al. 2003). To generate pTA409, the 1253-bp *pyrE2::hdrB* operon was inserted at the PstI site in pBluescript II, and a 948-bp BmgBI–AclI fragment containing the *ori-pHV1/4* DNA replication origin of *H. volcanii* plasmids pHV1 and pHV4 (Norais et al. 2007) was inserted at the PciI site.

Cloning of the pTA409-zwt construct

The tRNase Z gene, including 70 bp upstream and downstream sequences each, was amplified by using primers ZWT3 and ZWT4, which contain the restriction sites for ApaI (ZWT3) and BclI (ZWT4). The resulting PCR product was digested with ApaI and BclI and ligated into pTA409 digested with the same restriction enzymes yielding pTA409-zwt. Plasmid pTA409-zwt was transformed into the heterozygous pop-out strain. After several cultivation cycles in Hv-Min, chromosomal DNA was isolated and investigated by Southern analyses.

Structural modeling of the 5S leader region

A modified version (Zacharias 2001) of the Jumna (Junction Minimization of Nucleic Acids) program (Lavery et al. 1995) was used for all molecular modeling calculations. The Jumna program uses a combination of helicoidal and internal coordinates to describe nucleotide placements and flexibility of a nucleic acid molecule. The helicoidal description allows easy nucleotide addition and substitution in a given structural motif. Structural modeling was performed in two steps: Based on the similarity of

the 5S leader sequence and secondary structure to tRNA acceptor stem, T-stem, and T-loop regions, an initial model was generated by using corresponding regions of the high-resolution yeast tRNA^{Phe} X-ray structure (Protein Data Bank [PDB] entry 1EHZ) as a template. In a second step, the structure was extensively energy minimized to optimize the bonded geometry and to remove any sterical overlap.

SUPPLEMENTAL DATA

Supplemental material can be found at <http://www.rnajournal.org>.

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