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RNA polyadenylation and degradation in different Archaea; roles of the exosome and RNase R

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ABSTRACT

Polyadenylation is a process common to almost all organisms. In eukaryotes, stable poly(A)-tails, important for mRNA stability and translation initiation, are added to the 3′ ends of most mRNAs. Contrarily, polyadenylation can stimulate RNA degradation, a phenomenon witnessed in prokaryotes, organelles and recently, for nucleus-encoded RNA as well. Polyadenylation takes place in hyperthermophilic archaea and is mediated by the archaeal exosome, but no RNA polyadenylation was detected in halophiles. Here, we analyzed polyadenylation in the third archaea group, the methanogens, in which some members contain genes encoding the exosome but others lack these genes. Polyadenylation was found in the methanogen, *Methanopyrus kandleri*, containing the exosome genes, but not in members which lack these genes. To explore how RNA is degraded in the absence of the exosome and without polyadenylation, we searched for the exoribonuclease that is involved in this process. No homologous proteins for any other known exoribonuclease were detected in this group. However, the halophilic archaea contain a gene homologous to the exoribonuclease RNase R. This ribonuclease is not able to degrade structured RNA better than PNPase. RNase R, which appears to be the only exoribonucleases in *Haloferax volcanii*, was found to be essential for viability.

INTRODUCTION

Polyadenylation is an important post-transcriptional modification of prokaryotic, eukaryotic and organellar RNA. In bacteria and organelles, such as plant mitochondria and chloroplasts, polyadenylation is transient and occurs mainly on fragmented molecules as part of the RNA decay pathway (1–3). Non-abundant, polyadenylated RNA fragments are also present in human and trypanosome mitochondria (4,5). In general, this decay process consists sequentially of endonucleolytic cleavage, addition of degradation-stimulating poly(A) or poly(A)-rich sequences to the proximal cleavage products, and exonucleolytic degradation. In contrast to this form of degradation-stimulating polyadenylation, stable poly(A)-tails are added to the mature 3′ ends of most nuclear encoded mRNAs and are important for proper translation initiation, mRNA stability and, at least in some cases, nuclear export (6–9). In animal and trypanosome mitochondria, in addition to the presence of transient polyadenylation mentioned above, RNA molecules are characterized with stable poly(A)-tails that are post-transcriptional added to their mature 3′ ends. This is not the only example of stable and degradation-stimulating poly(A) coexistence as recently, a quality control mechanism, including transient polyadenylation which targets nucleus-encoded yeast RNA for degradation by the exosome complex, was described (10–13). In addition, non-abundant and truncated polyadenylated nucleus-encoded RNA molecules were detected in human cells (14,15). These accumulated observations suggest that polyadenylation-stimulated RNA degradation is a common process which occurs in most of the life kingdoms including bacteria, chloroplasts, plant and animal mitochondria and nuclear encoded transcripts (3).

In search of an organism in which RNA polyadenylation does not occur, we analyzed the halophilic archaea, as none of the known prokaryotic polyadenylating enzyme homologues were identified in their sequenced genomes. Indeed, uniquely, neither stable nor transient polyadenylation were found in *Haloferax volcanii*, as well as several other halophilic archaea. No other organism is known to lack any and all forms of polyadenylation (16). However, polyadenylation was found in the hyperthermophiles of the genus *Sulfolobus* and was due to the archaeal exosome (16). Polyadenylation is believed to assist the exoribonucleases in overcoming stem–loops and other complicated secondary RNA structures during digestion. Therefore, it was somewhat surprising to observe that halophilic archaea that lives in high salt environment, encouraging the formation of RNA structures, do not polyadenylated RNA while hyperthermophiles, living at very high temperatures, do polyadenylate RNA. In order to gain more insight to RNA polyadenylation in Archaea, we examined polyadenylation in the third group of Archaea, the methanogens. This group is comprised of members who have an exosome and others lacking this complex.

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In this work, we searched for the presence of RNA polyadenylation in various methanogenic archaea and asked whether polyadenylation, if it indeed occurs in methanogens, is produced, as in hyperthermophiles, by the archaeal exosome. Our results show that heteropolymeric polyadenylation indeed occurs in methanogens, but only in those containing an exosome. Additionally, in order to explore the mechanism of RNA degradation in the absence of polyadenylation, we studied the RNase R homologue of the halophilic archaea, *H.volcanii*. We found that this exoribonuclease is expressed in archaea cells, essential for viability and most likely plays a pivotal role in the process of RNA degradation without polyadenylation.

METHODS

Organism

H.volcanii was grown at 42°C in a medium containing 3.4 M NaCl (16,17) (http://www.microbiol.unimelb.edu.au/staff/mds/HaloHandbook/). *Sulfolobus solfataricus* (strain P2) was grown as described (18). The methanogens *Methanopyrus kandleri*, *Methanococcus maripaludis* and *Methanococcus jannaschii* were grown at the University of Regensburg, Germany. Purification of DNA was performed as described (17), and RNA was isolated using the 'hot phenol' method (16,19). A soluble protein extract of *H.volcanii* was prepared by breaking logarithmically growing cells resuspended in a buffer E containing 3 M KCl (20) in a French Pressure cell at 20 000 psi. The extract was cleared by centrifugation, desalted, and stored in aliquots at −80°C.

Determination of poly(A)-tails

First, 20 μg of total RNA from the methanogenic archaea, *M.jannaschii* and *Escherichia coli* were 3′ end-labelled with [^{32}P]pCp and T4 RNA ligase for 24 h at 4°C. Then, the RNA was digested with 25 μg of RNase A and 300 U of RNase T1 for 1 h at 37°C (16,21). Poly(A)-tails were resolved in 14% polyacrylamide sequencing gels containing 7 M urea and detected by autoradiography.

Analysis of the poly(A)-tails by oligo(dT)-primed reverse transcription PCR was performed as described (16,21). The gene specific PCR primers that were used are listed in the supplementary information.

Expression of the *H.volcanii* RNase R homologue and the *Synechocystis* PNPase in *E.coli*

The *rnr* gene was PCR-amplified from genomic DNA using oligonucleotides GGATCCCTAGTCGGGGCTATGTCAGAC and CTGCAGGCGACCGCCGGGTTATTCCTG and cloned into the *E.coli* pQE30 expression vector using the BamHI and PstI restriction endonuclease sites (underlined). Expression and purification on NTA-agarose column were performed as described previously (20) with an additional purification step using an anion exchange monoQ column (19). The column was developed with a KCl gradient in buffer E (20). The recombinant RNase R was eluted from this column at the relatively high salt concentration of 0.4 M KCl, probably due to its evolutionary adaptation to the high salt concentration living environment. The *Synechocystis* PNPase was expressed in *E.coli* and purified as previously described (19).

In vitro RNA degradation activity assay

In vitro RNA degradation assays using recombinant RNase R homolog were performed as previously described (20). Several substrates, all [^{32}P] uniformly labeled, were applied in this work. An RNA 267 nt corresponding to the 3′ end of the spinach chloroplast *petD* transcript (20). This RNA contained a stable stem–loop structure located 58 nt from the 3′ end. A 190 nt RNA corresponding to the tRNA^{ala} of *H.volcanii* (plasmid for *in vitro* transcription kindly obtained from Anita Marchfelder) (22), and the 110 nt *E.coli* RNA I (23).

The reaction products were resolved in a denaturing polyacrylamide gel and analyzed by autoradiography. Alternatively, the reaction products were analyzed by thin layer chromatography as described (20).

Production of antibodies

Recombinant RNase R produced and purified in *E.coli* cells was injected into chickens, and antibodies were produced as previously described (19). Protein was quantified using the Bio-Rad assay.

The construction of the *rnr* knock-out mutant of *H.volcanii*

The pop in/pop out system developed by Dr Mevarech (Tel Aviv University) was used (24). The 3′ and 5′ flanking ends of the *rnr* gene were PCR amplified using genomic DNA and the primers GAATTCCCGGAATCGTGTCGTGTGCAT and GGATCCAGACGGGGCAGGGAGAAATGA for the 5′ flanking end (indicated by a grey box in Figure 6) and GGATCCCCGTTCGACCACTTGTTTTTG and GGTACCGGCTGGACGACGCTGGACGGT for the 3′ end (indicated by a black box in Figure 6). Sites for the corresponding restriction enzymes are underlined. The two PCR fragments were cloned in tandem with the *pyrE* gene using the EcoRI and KpnI restriction sites of the plasmid pGB70 (24). Transformation of *H.volcanii* cells was performed as described following selection on plates lacking uracil in the growth medium (24). Colonies were analyzed by PCR or DNA blot analysis using the [^{32}P] labeled 3′ end fragment (indicated by a black box in Figure 6) as a probe. For the pop out step, cells containing the 476 bp fragment (indicating the success of the pop-in event), were grown on plates containing 5-fluoroorotic acid (5-FOA) (24). Colonies obtained were subjected to PCR and DNA blot analysis to resolve the presence or absence of the *rnr* gene. Since all the examined colonies contained the *rnr* gene, the pop-in/pop-out procedure was repeated with cells in which the RNase R was expressed from a plasmid. To this end the *rnr* gene, PCR amplified from genomic DNA using the primers CCATGGGCCATCACCATCACCATCACCTAGTCGGGGCTATGTCAGAC and GGTACCGCGACCGCCGGGTTATTCCTG, was cloned into the *H.volcanii* expression plasmid pWL-Nov using the *NcoI* and *Acc65I* restriction sites with the addition of a sequence for His_6 tag at the N-terminus end (25). Transformation and induction of the expression of the protein were performed as described (25).

Table 1. Polyadenylation takes place only in exosome containing archaea

Archaea	Exosome subunits	RNase R	Gene (Gene ID number)	# Of sequenced clones (# of analyzed sites)	# Of identified poly(A)-tail
M.kandleri (Meth.)	+	–	16S rRNA (3161737)	69 (2)	5
			exosome complex exonuclease 2 (1477683)	15 (1)	4
M.maripaludis (Meth.)	–	–	16S rRNA (2762295)	52 (3)	—
M.jannaschii (Meth.)	–	–	S1-layer structural protein (1451705)	8 (1)	—
			SSU ribosomal protein (1451330)	33 (2)	—
			LSU ribosomal protein (1451370)	7 (2)	—
			Ferredoxin (1451048)	2 (1)	—
			M reductase I (1451734)	15 (3)	—
			16S rRNA (1451003)	145 (3)	—
H.volcanii (Hal.)	–	+	16S rRNA	40 (2)	—
			RNase R	25 (2)	—
S.solfataricus (Hyper.)	+	–	16S rRNA (2974190)	53 (3)	50
			NADH dehydrogenase (1454848)	16 (1)	3

The results of the oligo(dT)-primed RT–PCR analysis of RNA purified from several archaea are presented as well as the present in the genome of putative genes encoding subunits of the archaeal exosome and RNase R. In *M.maripaludis* and *M.jannaschii*, no homology gene for a known exoribonuclease was detected. The gene annotation is indicated as well as its Gene ID number in parenthesis. The number of RT–PCR clones that were sequenced for each gene and the number of sites that were analyzed for each gene are indicated.

RESULTS

RNA polyadenylation occurs only in Archaea containing an exosome

We have previously characterized halophilic archaea as the first described organism to metabolize RNA with no polyadenylation (16). However, polyadenylation was observed in the hyperthermophiles of the genus *Sulfolobus* and was mediated by the archaea exosome complex (16). The third archaea group, the methanogens, contains members with genes encoding the exosome proteins, and therefore believed to contain an active exosome, while other methanogens lack these genes. In order to verify the correlation between the presence of the archaeal exosome and RNA polyadenylation, several methanogenic archaea were analyzed for the presence or absence of poly(A)-tails. RNA was purified from *M.kandleri,* containing the exosome proteins, as well as from *M.maripaludis* and *M.jannaschii* cells, lacking the exosome proteins genes, and subjected to analysis. Because of the low abundance and rapid digestion of polyadenylated intermediate degradation products, the highly abundant ribosomal RNA was chosen for this analysis, as well as several mRNAs. The results disclosed truncated RNA harboring polyadenylated tails from the exosome-containing archaea, *M.kandleri* (Table 1). However, out of 262 analyzed clones created by oligo(dT)-RT–PCR applied to *M.maripaludis* and *M.jannaschii* RNA, none contained inserts originating from polyadenylated RNA. As mentioned, these two methanogens do not contain genes for exosome proteins (Table 1). Furthermore, as previously described (16), no tails were obtained from the halophilic archaea *H.volcanii* while poly(A)-tails were observed in the hyperthermophilic archaea *S.solfataricus* (Table 1).

In order to verify that indeed no polyadenylation occurs in exosome-lacking archaea, RNA purified from *M.jannaschii* was analyzed by the 3′ end labeling and ribonucleases A and T1 digestion assay (Figure 1). While RNA from *E.coli* displayed short poly(A)-tails, as described before, (16,26) no poly(A)-tails were detected in RNA purified from *M.jannacshi*.

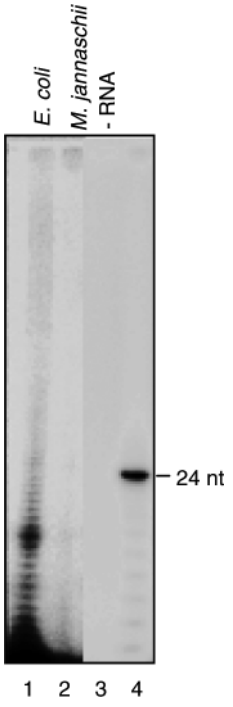

Figure 1. No polyadenylation is detected in the methanogenic archaea *M.jannaschii*, lacking the exosome. RNA was purified from *E.coli* and the methanogenic archaea *M.jannaschii*. The RNA was labelled with [^{32}P]pCp at the 3′ end and digested to completion with RNase T1 and RNase A. The remaining poly(A)-tails were then fractionated on 14% denaturing PAGE alongside a [^{32}P]-labelled 24 nt oligonucleotide size marker and detected by autoradiography. The lane marked –RNA (number 3) indicates a control sample where no RNA was added to the reaction mixture.

Interestingly, in addition to the lack of genes encoding the exosome proteins, genes homologues to any ribonuclease of the RNR family could not be identified in these methanogens as well (Table 1). Genes encoding these hydrolytic exoribonucleases were found in almost all the bacterial genomes analyzed so far including the small genome of the parasitic bacteria Mycoplasma (27). In addition, an RNase

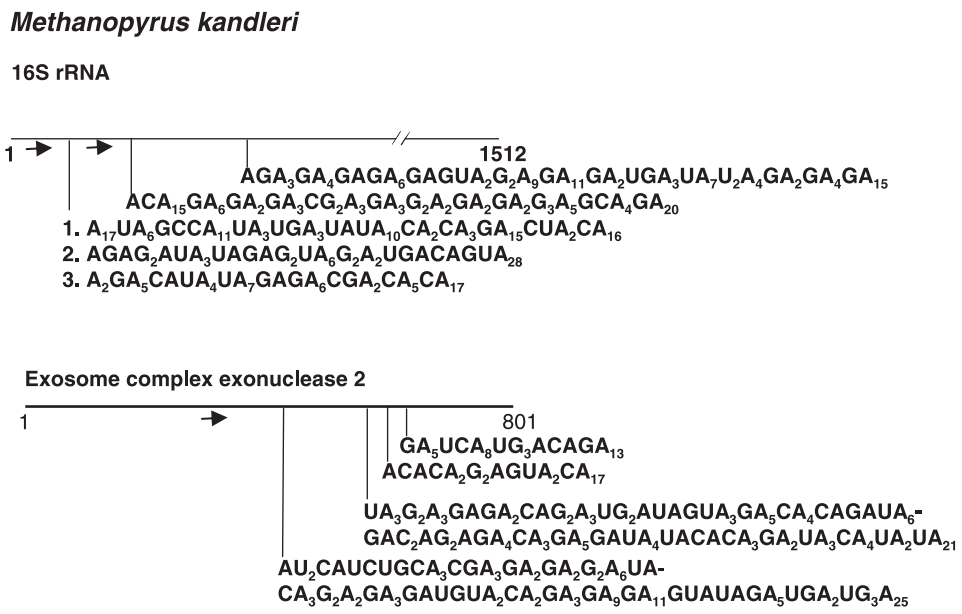

Figure 2. Post-transcriptional added heteropolymeric tails in the exosome containing archaea, *M.kandleri*. RNA prepared from the methanogenic archaeon *M.kandleri* was analyzed for polyadenylated tails using oligo(dT)-primed RT–PCR. The gene specific oligonucleotides used for the PCR amplification are indicated by arrows. The relative locations of the tails are indicated by vertical lines, and the heteropolymeric sequence of the tail is presented. The gene specific PCR primers are listed in the supplementary information.

R homologue is encoded in the genome of halophilic archaea lacking the exosome complex (16,27). Therefore, the lack of both exosome and RNase R encoding genes in *M.maripaludis* and *M.jannaschii* is the first known case in which both exoribonucleases homologues are absent. Therefore, the protein responsible for the exonucleolytic degradation of RNA is yet to be identified.

Together, the results of the poly(A)-tail analysis disclosed a correlation between the presence of the exosome complex and that of polyadenylation in methanogens.

Heteropolymeric poly(A)-rich tails in *M.kandleri*

Since it was previously found that tails produced by the archaeal exosome are heteropolymeric, the nature of the poly(A)-tails in the methanogenic archaea, *M.kandleri* was analyzed next.

Tails produced by the enzyme PNPase that is present in bacteria and organelles and by the *S.solfataricus* exosome were shown to be heteropolymeric, containing the other 3 nt in addition to adenosines (16). We therefore examined the nature of the poly(A)-tails in the methanogen *M.kandleri*. Oligo(dT)-primed RT–PCR analysis of RNA purified from these Archaea using forward primers specific to the 16S rRNA and the mRNA encoding the exosome complex exonuclease 2 protein disclosed heteropolymeric 3′ extensions containing all 4 nt (Figure 2). The poly(A)-rich tails resembled those produced by the exosome in the hyperthermophilic archaea *S.solfataricus*, suggesting that they too are produced by the exosome in *M.kandleri* (16).

In the absence of an exosome, RNase R is the major exoribonuclease in *H.volcanii*

In order to analyze the mechanism of RNA degradation in the absence of the exosome and polyadenylation, while lacking any exoribonuclease candidates in the sequenced methanogen genomes (Table 1), we decided to characterize the putative RNase R homologue, present in the halophilic archaea genomes. These Archaea contain only one gene for a putative RNA-degrading exoribonuclease with significant homology to the RNR family (Table 1) (TIGR Microbial Database:. http://www.tigr.org/tbd/mdbinprogress.html) (27,28). We compared the amino-acid sequence of the *H.volcanii* RNR homolog to the homologues *E.coli* proteins, revealing 34 and 26% identity to RNase R and RNase II, respectively. Therefore, we considered the *H.volcanii* protein to be an RNase R homologue of the *E.coli* enzyme.

The *H.volcanii* RNase R protein was expressed in *E.coli* and purified to near homogeneity (Figure 3A). The corresponding gene is expressed in *H.volcanii* cells, as observed by the reaction of specific antibodies with a polypeptide of slightly smaller size, found in a protein extract (Figure 3B). The difference in sizes between the recombinant protein and the one detected in the *H.volcanii* extract is attributed to the addition of the His_6 tag in the case of the recombinant protein. Incubation of the recombinant *H.volcanii* RNase R with RNA resulted in digestion of the RNA to nucleoside monophosphates, in accordance with the expected activity of a hydrolytic enzyme (Figure 3C). These results demonstrated that the protein encoded by the *rnr* gene in *H.volcanii* is a hydrolytic exoribonuclease.

Since the *H.volcanii* lives in the Dead Sea at a salt concentration nearing saturation and the internal potassium ion concentration is ~4 M (29,30), we next asked whether its RNase R would display evolutionary adaptation for optimal activity at high salt concentration. The recombinant protein was incubated with [^{32}P]-RNA in a buffer containing high salt concentrations. Following the incubation, the RNA was purified, fractionated on denaturing gel and analyzed by autoradiography. Optimal RNA degradation activity was obtained at 25°C

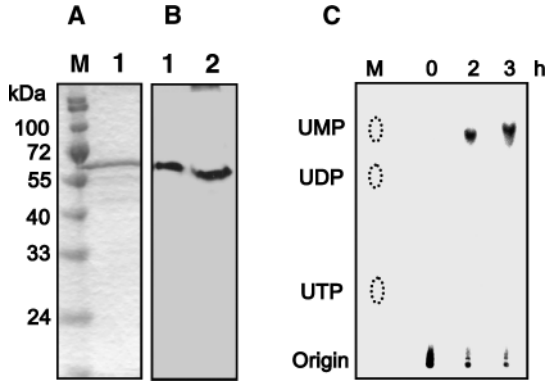

Figure 3. Characterization of *H.volcanii* RNase R. (**A**) The protein product of the *H.volcanii rnr* gene was expressed in *E.coli* and purified (lane 1). M, molecular weight markers. (**B**) RNase R homologue antibodies, produced against the *E.coli*-expressed protein, detect the protein in a soluble protein extract of *H.volcanii* (lane 2) in comparison with the purified *E.coli* expressed protein (lane 1), using immunoblot analysis. The size of RNase R in the protein extract is slightly smaller than the recombinant protein due to the addition of the His_6 tag. (**C**) Ribonuclease activity of the RNase R homologue. The purified *E.coli*-produced protein was incubated with [^{32}P]RNA corresponding to *H.volcanii* $tRNA^{ala}$. RNA was purified at time points indicated and analyzed by thin-layer chromatography and autoradiography. The migration patterns of the markers analyzed on the same plate and visualized by fluorescence quenching are indicated with circles (lane M).

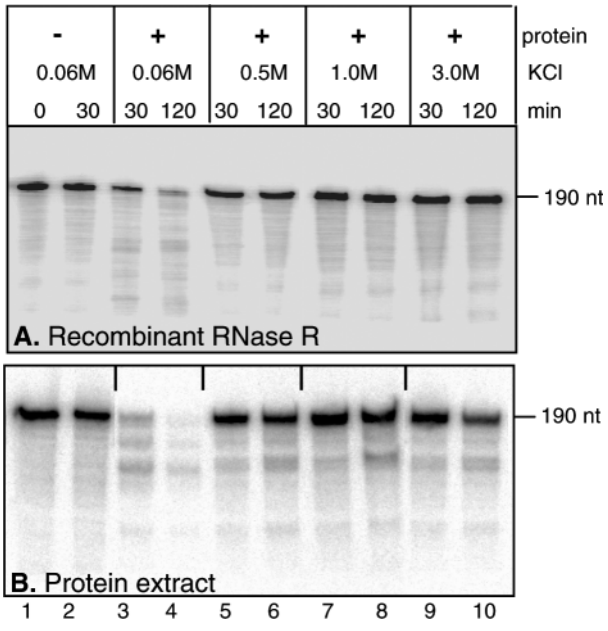

Figure 4. *H.volcanii* RNase R activity is optimal at low salt concentration. A 190 nt long [^{32}P]RNA, corresponding to the *H.volcanii* $tRNA^{ala}$ precursor, was incubated without (lanes 1 and 2) or with (lanes 3–10) the addition of purified recombinant *H.volcanii* RNase R (**A**) or soluble protein extract of *H.volcanii* (**B**) in a buffer containing KCl at the concentrations as indicated in the figure. RNA was purified at the time points indicated and analyzed by denaturing polyacrylamide gels and autoradiography.

at a salt concentration of 60 mM KCl, with very low activity at salt concentrations higher than 0.5 M (Figure 4A). Similar results of optimal activity at low salt concentration were obtained previously for *H.volcanii* RNase Z (22).

To verify that this result was not obtained because the recombinant protein was expressed in *E.coli* at low salt concentration, soluble protein extracts of *H.volcanii* cells, that were grown at 3.4 M NaCl, were analyzed for RNA degradation activity. The result disclosed, also in this case, an optimal activity at low salt concentration with very little activity at salt concentrations higher than 0.5 M KCl (Figure 4B). These results suggested that the halophilic RNase R did not undergo evolutionary adaptation for optimal activity at internal high salt concentration of the archaea. Therefore, it seems that its residual activity at high salt concentration is sufficient for the RNA degradation activity required for the archaeal life at its natural living place (29,30). It is also possible that the activity of RNase R in the intact archaeal cell is more efficient than detected in *in vitro* assays, due to inhibitory factors in the soluble protein extract.

The *H.volcanii* RNase R does not degrade RNA containing secondary structures more efficiently than PNPase

The proposed function of RNA polyadenylation during the degradation process is to enable the processive exoribonucleases, PNPase, RNase II, RNase R or the archaeal exosome to bind their substrates. The poly(A)-tail produces a single-stranded platform to which the exoribonucleases, mostly displaying high binding affinity to poly(A) (31,32), can bind and possessively digest the structured RNA molecule (1,33). In addition, the *E.coli* RNase R was reported to more efficiently digest RNA characterized with extensive secondary structures as compared to RNase II and PNPase (33). Therefore, we next compared the activity of the *H.volcanii* RNase R to that of the PNPase of the cyanobacteria *Synechocystis*.

Both enzymes were incubated with [^{32}P]-RNA corresponding to molecules known to harbour extensive secondary structures, archaeal tRNA and the *E.coli* RNAI (22,23). The results disclosed that under the experimental conditions, the RNase R activity paused at secondary structures and was less efficient than that of PNPase (Figure 5). Similar results were obtained when other RNA substrates were analyzed (data not shown). Therefore, these results suggest that the lack of polyadenylation in *H.volcanii* is not compensated by improved activity of the RNase R on structured RNA molecules.

The *rnr* gene is required for viability in *H.volcanii*

In the absence of PNPase and an exosome, an important or even essential role for RNase R in RNA degradation in *H.volcanii* could be predicted. Thus, the deletion of the corresponding gene should result in poor growth or lethality. Methods for the deletion of a specific gene from the genome of *H.volcanii* and for expression of a protein from a plasmid have been described (24,25), and were used here to delete the *rnr* gene (Figure 6). In the first step, defined as 'pop-in,' the *pyrE* selective marker is introduced into the *H.volcanii* genomic DNA in the vicinity of the *rnr* gene by homologous recombination. Two transformants were obtained from the homologous recombination during the pop-in process and were identified by PCR and DNA-blot analysis (A1 and A2 in Figure 6A). The second step, defined as 'pop-out,' includes selection on 5-FOA resulting with two possible recombination events leading to deletion or restoration of the *rnr* gene

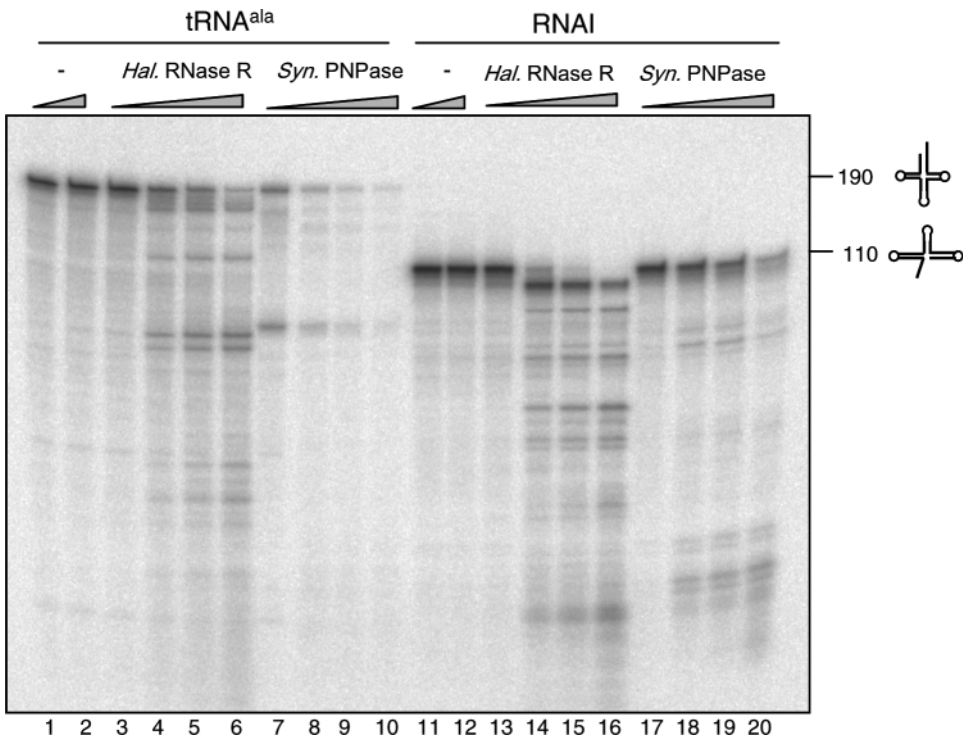

Figure 5. Activity of the RNase R on structured RNA. A 190 nt long [^{32}P]RNA corresponding to the tRNA^{ala} of the halophilic archaea *H.volcanii* and a 110 nt long [^{32}P]RNA corresponding to the *E.coli* RNAI were incubated at 25°C with purified *H.volcanii* RNase R (*Hal*. RNase R) or *Synechocystis* PNPase (*Syn*. PNPase) (4 μM of each), both produced in *E.coli*. 10 mM Pi was added to the reactions analyzing the PNPase activity. RNA was purified at time points indicated and analyzed by denaturing polyacrylamide gels and autoradiography. A schematic presentation of the corresponding RNA molecules is shown on the right. Lanes 1, 2, 11 and 12 showed control reactions in which the corresponding RNA molecules were incubated without protein for 0 min (lanes 1 and 11) and 120 min (lanes 2 and 12). In each serial of lanes 3–6, 7–10, 13–16 and 17–20, the time points were 0, 30, 60 and 120 min, respectively.

from the genomic DNA (Figure 6B). Therefore, if the gene product is not essential for viability, both possibilities are expected. Out of many colonies obtained, 30 were analyzed and were all found to contain the *rnr* gene. To validate that the deletion of the *rnr* gene and not the homologous recombination events was the cause of lethality, this experiment was repeated using *H.volcanii* cells that were first transformed with an expression plasmid harboring the *rnr* gene. This time, out of 9 colonies analyzed, 3 were obtained in which the *rnr* gene was depleted from the genomic DNA (Figure 6B). Together, these results strongly suggest that that the *rnr* gene is required for the viability of the *H.volcanii* cells, as would be expected of an enzyme with an essential role in exonucleolytic digestion of RNA, in the absence of the archaeal exosome.

DISCUSSION

Polyadenylation-stimulated RNA degradation is broadly dispersed throughout the domains of life, excluding the halophilic and several methanogenic archaea lacking the exosome

Following its initial discovery in *E.coli*, the polyadenylation-stimulated RNA degradation pathway was described in other Bacteria, as well as in Archaea, chloroplasts and mitochondria (2,3,16,19,21,34–38). Interestingly, polyadenylation of mitochondrial RNA has a varying function or doesn't occur at all, depending on the organism (3,39). While the polyadenylation stimulated degradation pathway exists in plant mitochondria, it is not present in yeast mitochondria (39–41). However, in animal and trypanosome mitochondria, the coexistence of stable poly(A)-tails and transient tails which stimulate degradation has been described (4,5). In both systems the molecular mechanism that differentiates between the stable poly(A)-tails and those that play a role in the RNA degradation process is yet unknown.

In addition, a polyadenylation-stimulated degradation pathway was recently described for nuclear encoded transcripts in yeast, revealing yet an additional system that includes both types of polyadenylation (10–12). Last, the presence of non-abundant poly(A)-tails for nuclear encoded transcripts in human cells were recently described, suggesting that the polyadenylation stimulated degradation pathway accounts for these transcripts as well (14,15). Therefore, this mechanism is broadly dispersed throughout the life domains and most likely evolved before the onset of the division between archaea and bacteria.

The exosome as the polyadenylation enzyme in archaea

While searching for an organism in which polyadenylation does not exist, the domain of Archaea was chosen. There are no genes for PNPase, described to be the polyadenylating enzyme in spinach chloroplast, cyano- and several gram positive bacteria and *E.coli* under certain conditions. In addition, most archaea contain only one gene for nucleotidyltransferase and no homologue for the known eukaryotic

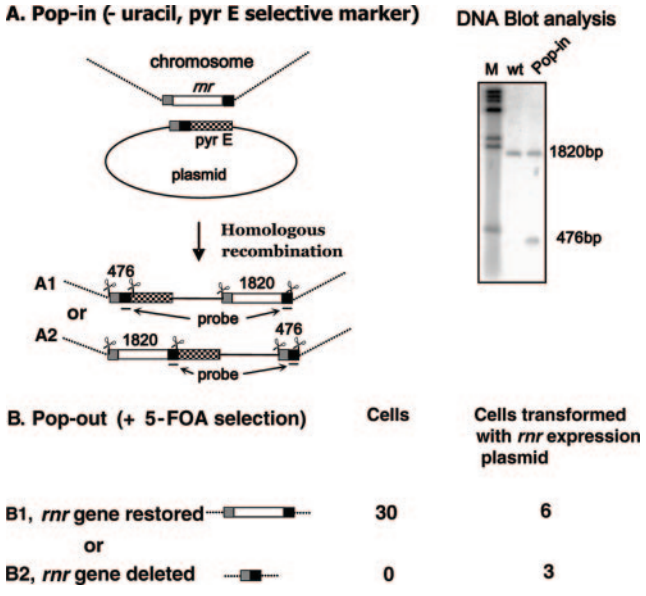

Figure 6. RNase R is required for viability of *H.volcanii* cells. The *rnr* gene encoding the RNase R protein was deleted from the *H.volcanii* genome using the pop in/pop out system (24) as schematically described in the figure. (**A**) In the first step (pop in) the selection marker, *pyrE* gene, is introduced into the genome by homologous recombination using two flanking sequences of the *rnr* gene (indicated as gray and black boxes). The two possible results of the homologous recombination step are expected to be produced (A1 and A2) and were confirmed by DNA blot analysis. The probe was a DNA fragment representing the flanking sequence displayed by the black box. Scissors and numbers indicate the restriction enzyme sites and the number of nucleotides, respectively. In the lane marked M of the DNA blot, lambda DNA digested with HindIII and [^{32}P] labeled was ran on the same gel as molecular weight marker. It should be noted that the results of the DNA blot analysis showed that the recombination occurred but did not allow discrimination between the A1 and A2 intermediate. (**B**) The two possible results of the second step (pop out) are schematically shown (B1 and B2) indicating that in half of the colonies the *rnr* gene would be deleted (the B2 option). However, if this protein is required for viability all of the colonies obtained should contain the *rnr* gene in the genome (the B1 option). Many colonies were obtained and out of 30 colonies analyzed, all contained the *rnr* gene, indicating that this protein is essential for viability. To validate that the lethality was indeed due to deletion of the *rnr* gene, *H.volcanii* cells were first transformed with an expression plasmid for the RNase R and then were subjected to the pop in/pop out procedure. Under these conditions, in which the RNase R was expressed from a plasmid, the deletion of the genomic *rnr* gene was possible, indicating that it is required for viability.

poly(A)-polymerases. The poly(A)-tails produced by PNPase were found to be heteropolymeric, containing the other nucleotides in addition to adenosines (4). However, the hyperthermophiles and several methanogens contain genes encoding the archaeal exosome. While the eukaryotic exosome is composed of 10–11 proteins, that of Archaea contains only four that share homologies to the first and second phosphorylase core domains of the PNPase as well as the S1 RNA binding domain. In eukaryotes this complex is assigned to the 3′–5′ exoribonucleolytic activity and the crystallographic structure of the archaea exosome is very similar, a ring-like shape, to the bacterial and organelles trimeric PNPase (42–44). Indeed, when polyadenylation was analyzed in the hyperthermophilic archaea, *Sulfolobus sulfataricus*, it was found that the exosome is the polyadenylating enzyme and like the PNPase in bacteria and organelles, the tails produced were heteropolymeric (16).

Since the third archaea group, the methanogens, includes members which contain the exosome subunits genes and those lacking these genes, we decided to determine whether also in this group a correlation between the presence of the exosome and polyadenylation takes place. Indeed, we found that in methanogenic archaea containing the genes for the exosome subunits RNA is polyadenylated and the tails are heteropolymeric, similar to the hyperthermophiles of the genus *Sulfolobus* and tails produced by PNPase in bacteria and organelles (3). In addition, similar to the situation previously described in halophilic archaea, in which no exosome is present, no polyadenylation was detected in the methanogens that do not contain this complex, further supporting the correlation between polyadenylation and the presence of PNPase or the archaeal exosome. In addition to the cases of various archaea described above, no polyadenylation is present in yeast mitochondria and accordingly, no PNPase is present in this organelle (39–41). Another organism that lacks PNPase is the small genome bacteria of the genus *Mycoplasma*, although experimental data concerning polyadenylation is still lacking. An exception, however, to the correlation between the presence of PNPase or the archaeal exosome and polyadenylation is the mitochondria of trypanosomes. Both stable and unstable poly(A)-tails were detected there but no PNPase could be identified (3,5).

RNA degradation without polyadenylation

Polyadenylation is believed to stimulate exonucleolytic degradation since the bacterial exoribonucleases: RNase II, RNase R, PNPase as well as the yeast exosome all display high binding affinities to poly(A). In addition, the poly(A)-tail provides a single-stranded platform which allows the enzyme to bind and then progress and overcome secondary structures. In the absence of PNPase and the exosome, it could be hypothesized that the hydrolytic enzyme RNase II/R would be responsible for the exoribonucleolytic activity. Indeed, members of the *rnr* family could be detected in most genomes of the prokaryotes and eukaryotes (27,28). However, while organisms of these domains usually contain both RNase II/R and phosphorylases homologues, most of the Archaea genomes sequenced so far contain either the archaeal exosome or an RNase R homologue (28). Surprisingly, no homologue for RNase II/R gene could be identified in the methanogens that also lacked the archaeal exosome (Table 1). Since it is believed that exonucleolytic degradation takes place during RNA turnover in these cells, we hypothesized that either an exoribonuclease that is not related to the *rnr* and the exosome families is responsible for this process, or that the homology of the hydrolytic enzyme to the *rnr* family is so low that it could not be detected bioinformatically. This question awaits more intensive bioinformatic search and biochemical detection such as analysis of the exoribonucleolytic activity in extracts of these cells.

However, a clearly observed RNase R homologue was detected in the halophilic archaea genome. Therefore, in order to analyze exoribonucleolytic degradation in the absence of polyadenylation, we studied the activity of this protein. Indeed, this protein is expressed in archaea and is essential for viability. However, unlike the *E.coli* RNase R (33), that of *H.volcanii* displayed no enhanced activity

degrading structured RNA as compared to the PNPase of cyanobacteria. It is still elusive why during evolution the present situation where there is no polyadenylation in halophilic archaea living at relatively low temperatures in high salt medium, while there is polyadenylation in hyperthermophiles growing at very high temperatures, where the formation of RNA structures is limited, has evolved. The methanogenic archaea, living at both very high and intermediate temperatures and in an anaerobic atmosphere, are divided between those harboring an exosome and polyadenylation and those lacking these two characters. Clearly more research of the RNA metabolism and polyadenylation processes in additional archaea is required in order to answer this question.

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