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An archael RNase P protein subunit is homologous to a eukaryotic nuclear protein

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ABSTRACT

A putative protein subunit of the RNase P holoenzyme from the methanogenic archaeon *Methanobacterium thermoautotrophicum* ΔH has been identified. A potential distant homolog to the *Saccharomyces cerevisiae* RNase P subunit Pop5p (MTH687) was identified by PSI-BLAST searching. The recombinant protein was produced in *E. coli*, highly purified and used to generate polyclonal antiserum. Specific antiserum against MTH687 immunoprecipitated RNase P activity and recognized a protein of the predicted size of MTH687 from partially a purified sample of *M. thermoautotrophicum* ΔH RNase P.

INTRODUCTION

Ribonuclease P is the enzyme responsible for the 5′ maturation of all precursor tRNAs (pre-tRNAs). RNase P is composed of a single RNA subunit and one or more proteins. All bacteria studied have a single protein, while yeast has at least nine proteins associated with the enzyme (1). The RNA alone from all known bacterial RNase P enzymes can cleave pre-tRNA *in vitro* given elevated salt conditions (2). A subset of methanogenic Archaea also have an RNase P RNA which is catalytic alone, but only under extreme ionic strength (3 M NaOAc, 300 mM $MgCl_2$, 3). No catalytic proficiency has yet been demonstrated for the RNA alone from any eukaryotic nucleus or organelle. Presumably, the protein component(s) of RNase P from Archaea and Eukarya have taken on a larger role in the enzyme compared to their bacterial counterparts. In *S. cerevisiae*, eight of the nine RNase P-associated proteins are also components of RNase MRP (1).

To date, no information is available for any archaeal RNase P protein subunit, despite the completion and annotation of four archaeal genomes (4, 5, 6, 7). More than 20 bacterial RNase P proteins are knowm, and none have obvious homology to any potential open reading frame from any archaeon. The RNase P RNA from the methanogenic archaeon *Methanobacterium thermoautotrophicum* ΔH is very similar in structure to bacterial RNase P RNAs, is catalytically active in high ionic conditions, and can be functionally reconstituted at low ionic strength with the *Bacillus subtilis* RNase P protein subunit (3). However, despite the availability of the genome sequence for this organism, no obvious homolog to any RNase P protein has been identified, and the buoyant density in cesium sulfate (1.42 g/ml, unpublished data) is not characteristic an RNA with a single small associated protein (eg, 1.55 g/ml for *E. coli*, 8).

We report here the identification of a probable RNase P protein subunit from *M. thermoautotrophicum* ΔH. This protein (conserved protein MTH687, accession no. AAB85192) was identified in the *M. thermoautotrophicum* ΔH genome as a weak homolog of the *S. cerevisiae* RNase P protein Pop5p. Polyclonal antiserum raised against recombinant his-tagged MTH687 has been used to immunoprecipitate enzyme activity from partially purified RNase P. Likewise, western blots have revealed a 14-15 kDA protein which co-purifies with a partial biochemical purification of RNase P and is specifically recognized by anti-MTH687 antiserum.

METHODS

Production of Polyclonal anti-MTH687 Antiserm: The MTH687 coding region was cloned by PCR into pET-16b

Figure 1. ClustalW (14) alignment of Pop5p, *M. thermoautotrophicum* ΔH MTH687 and five other clear archaeal homologs of MTH687. Other archaeal homologs were identified by NCBI BLAST searches (*M. junnaschii, P. horikoshii* and *A. fulgidus*), or from raw genome translations using BioEdit with NCBI BLAST tools (*P. abyssii* and *P. furiosus*).

(Novagen) to create an amino-His_{10}-tagged construct. The recombinant protein was expressed in *E. coli* BL21(DE3)-pLysS, purified over Ni-NTA resin (Qiagen), then highly purified by SDS-PAGE/electroelution. The purified protein (1-3 mg, in Titermax adjuvant, CytRx, Inc.) was injected into two rabbits (NC State Animal Facility). After one and two months, the rabbits were boosted with an additional 1-3 mg of protein. Antiserum was collected before each injection and one month after the last injection.

Partial Purification of M. thermoautotrophicum ΔH RNase P: Cleared lysates of *M. thermoautotrophicum* ΔH cell paste (12g in TMG [50 mM Tris-Cl, pH 8, 10 mM $MgCl_2$, 5% glycerol] with 60 mM NH_4Cl [TMGN-60]) were run through two Cs_2SO_4 gradients (1.39 g/ml starting density, 150,000 x g, 4°C, 42 hrs). RNase P-active fractions were combined, dialyzed against TMGN-20, and passed over DEAE trisacryl (Sigma).

Immunodepletion: 2.5 mg of protein A-sepharose beads (Sigma) were coated with 10 µl of serum, rocked at room temp. for 1 hr and washed 3X in TMGNT (TMGN-100 + 0.02% Tween-20). Beads were washed 2X with 200 µl TMGN-100. Partially purified RNase P (15 µl) was mixed with the beads and incubated 30 min, then drawn through a microcolumn prepared from a PCR filter-tip and collected into a fresh tube. 5 µl of column flow-throughs were assayed for RNase P activity.

Immunoprecipitation: The general protocol for immunodepletion was used for the first part of the experiment. Afterwards, the matrix was washed with 100 µl TMGNT followed by 100 µl TMGN-100. Columns were eluted 3X with 10 µl TMGN-100, pH 2.65. Elution volumes were combined immediately with neutralizing buffer and 5 µl of each elution were assayed for RNase P activity.

Western blot: Proteins from the active peak of DEAE-trisacryl fractionation as well as two fractions eluting before RNase P activity and two fractions eluting after were separated on SDS-PAGE (12%), electro-transferred to nitrocellulose and blocked overnight in 3% BSA. Anti-MTH687 and pre-immune sera (1:2000 dilution) were pre-absorbed with *E. coli* acetone precipitate (0.5%) for 30 min., then used to probe blots for 2 hrs at room temp. Blots were washed 3X, probed with anti-rabbit IgG-HRP (Sigma) 4 hrs, washed 4X, developed with Pierce SuperSignal (Pierce, Inc.), then exposed to X-ray film.

RNase P activity assays: Enzyme preparations were incubated for the indicated time with ^{32}P-labeled *in vitro* transcripts of *B. subtilis* $pre\text{-}tRNA^{Asp}$ at 60°C in 50 mM Tris, pH 8, 10 mM $MgCl_2$, 500 mM NH_4OAc. Assay products were separated on 12% denaturing acrylamide (8M urea) and analyzed by phosphorimaging.

RESULTS

***Methanobacterium thermoautotrophicum* ΔH has a potential distant homolog of yeast pop5p**: No ORF in the *M. thermoautotrophicum* ΔH genome has obvious homology to any bacterial RNase P protein. Several bacterial RNase P proteins and all recently identified yeast nuclear RNase P proteins (1) were used in queries for PSI-BLAST searches (9). Weak matches were found between yeast Pop5p and *M. thermoautotrophicum* ΔH MTH687 ($E=6x10^{-29}$, iteration 2, Fig. 1) and between yeast Rpp1 and *M. thermoautotrophicum* ΔH MTH688 ($E=3x10^{-38}$, iteration 4, not shown). MTH687 was cloned into a His-tagged expression vector (pET-16b), overexpressed in *E.coli*, highly purified, and used to raise polyclonal antiserum.

Anti-MTH687 serum immunoprecipitates enzyme activity from partially-purified *M. thermoautotrophicum* ΔH RNase P: Staphylococcal protein A-sepharose beads were coated with anti-MTH687 antiserum or with pre-immune serum and incubated with partially purified *M. thermoautotrophicum* ΔH RNase P. Enzyme activity was unaffected by beads alone or beads coated with pre-immune serum, but was virtually fully removed by the anti-MTH687 serum-coated beads (Fig 2A). Elution with buffer at pH 2.65 recovered RNase P activity from anti-MTH687 serum-coated beads, but not beads coated with pre-immune serum (Fig. 2B).

Anti-MTH687 serum specifically recognizes a protein in a partially-purified RNase P sample with the predicted molecular weight of MTH687: Partially purified RNase P was subjected to western blotting with either anti-MTH687 serum or pre-immune serum from the same rabbit. Anti-MTH687 serum specifically recognized a single band at 14-15 kDa in combined active fractions of a DEAE-trisacryl

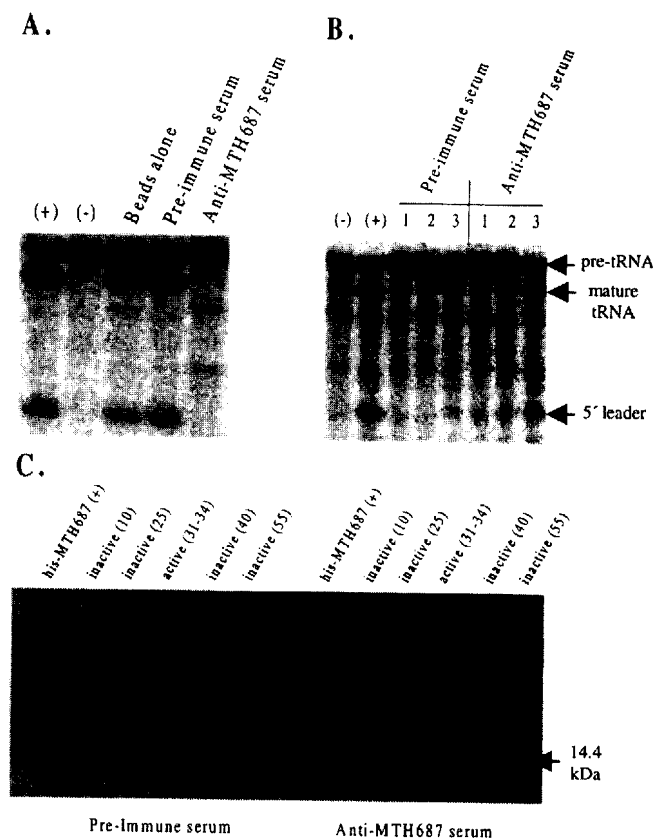

Figure 2. Immunodepletion (A) and immunoprecipitation (B) of RNase P activity from a partially purified sample, and a western blot showing a 14-15 kDa protein which co-purifies with RNase P activity and is specifically recognized by anti-MTH687 serum (C). In C, numbers next to fractions represent the DEAE-trisacryl fraction(s) from which the sample was taken.

seaparation but not in fractions flanking either side of the elution profile (Fig. 2C, right side). The predicted molecular weight of the non-tagged MTH687 protein is 14.56 kDa. Neither this band nor recombinant MTH687 were recognized by pre-immune serum (Fig 2C, left side).

DISCUSSION

The putative RNase P protein subunit MTH687 represents the first identification of a protein subunit of RNase P from an archaeon. It appears that at least one subunit of RNase P from *M. thermoautotrophicum* ΔH is evolutionarily related to a subunit from the eukaryotic nucleus, even though the RNA is clearly more similar to bacterial than eukaryotic RNase P RNAs. It is interesting that the protein subunit from the *Bacillus subtilis* RNase P, though it is not similar to any ORF in the *M. thermoautotrophicum* genome, can form a weakly functional complex with the *M. thermoautotrophicum* ΔH RNase P RNA *in vitro* (3). The same effect is seen with *M. formicicum* RNA and *B. subtilis* protein (3). At the time of this writing, we have not been able to reconstitute RNase P activity with the *M. thermoautotrophicum* ΔH RNase P RNA and recombinant his-tagged MTH687 (not shown).

The biochemical properties of the *M. thermoautotrophicum* ΔH RNase P holoenzyme also appear to be somewhat unique. Whereas RNase P holoenzymes typically have densities in Cs_2SO_4 close to that of pure RNA (*E. coli* = 1.55 g/ml, 8, *Haloferax volcanii* = 1.61 g/ml, 10), or pure protein (*Sulfolobus acidocaldarius* = 1.27 g/ml, 11), RNase P from *M. thermoautotrophicum* ΔH has a density of 1.42 g/ml in Cs_2SO_4 (unpublished data), suggesting an intermediate amount of protein relative to RNA content. The ionic optima (800 mM NH_4OAc, 5-10 mM $MgCl_2$, unpublished), also contrasts with the optima for *S. acidocalarius* (100 mM NH_4^+, 7.5 mM Mg^{2+}, 11) and *B. subtilis* (100 mM NH_4^+, 60 mM Mg^{2+}, 12). The temperature optimum for RNase P from *M. thermoautotrophicum* ΔH is at least 80°C (unpublished data). The Km for this enzyme is in the "normal" range, at ca. 40 nM (unpublished data).

Antibody generated against the MTH687 protein will be used in a large-scale immuno-purification attempt. The goal is to purify the RNase P holoenzyme to near homogeneity in order to identify all protein subunits. Characterization of the remaining subunits of the *M. thermoautotrophicum* ΔH RNase P will shed light on the evolutionary origins of this unique RNA enzyme. Although it has been clear for some time that the Archaea share distinct features with both the Eukarya and Bacteria (13), RNase P may represent a case where two subunits of the same enzyme have counterparts in both the Bacteria (the RNA) and the Eukarya (the protein).

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