Supporting Information

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SI Text

Discussion. ***Cloning archaeal Thg1 homologs for expression in E. coli.*** Archaeal homologs of yeast Thg1 were identified by BLAST search (1) and cloned by PCR amplification from isolated genomic DNA using the indicated primers (Table S1). Genomic DNA from *M. thermoautotrophicus* str. delta H was kindly provided by Chuck Daniels, The Ohio State University; DNA from *M. kandleri* AV19 was kindly provided by John Reeve, The Ohio State University; and DNAs from *M. acetivorans* C2A and *M. barkeri* str. MS were kindly provided by Joe Krzycki, The Ohio State University. Primers contained appropriate sequence for ligation-independent cloning (LIC) into a pET vector for in-frame expression with an N-terminal His_6 tag (either BG1861 or AVA421, both were a gift from Elizabeth Grayhack, University of Rochester Medical Center), and the gel-purified PCR products for each archaeal Thg1 gene were inserted into each vector using standard LIC cloning protocols; clones were verified by sequencing.

The Thg1 homolog from M. acetivorans is unusual. BLAST search identifies two proteins with homology to Thg1 in *M. acetivorans* (Table S1). The first is a 141 amino acid hypothetical protein (GI:20089701) that aligns well to the N-terminal half of yeast Thg1, and the second is a 92 amino acid ORF (GI:20089700) that aligns well to the remaining C-terminal half of yeast Thg1; the two ORFs are separated by an in-frame amber (UAG) stop codon. Production of the full-length Thg1 homolog in this organism is likely to be the result of nonsense suppression of the UAG stop codon by the unusual amino acid pyrrolysine (Pyl), as is observed with other in-frame UAG-containing enzymes in methanobacteria (2, 3). To express full-length MaThg1 in *E. coli*, which does not synthesize Pyl, the TAG stop codon at position 142 in the Thg1 sequence derived by PCR from *M. acetivorans* genomic DNA was altered to TAT (encoding Y) or TGG (encoding W) by QuikChange mutagenesis (Stratagene) according to the manufacturer's instructions. Tyr and Trp were chosen to replace Pyl because both of these residues are found at the analogous position in full-length Thg1 sequences from other methanobacteria. The behavior of MaThg1 with either 142Y or 142W was indistinguishable in all assays indicating that Pyl is not required for function of this Thg1 homolog; all data reported here for MaThg1 were obtained with the 142Y enzyme.

Expression and purification of archaeal Thg1. Plasmids were transformed into *E. coli* strain Rosetta pLysS, and 1 L cultures were grown in LB media containing 100 μg/mL ampicillin at 37 °C, induced with isopropyl-β-thiogalactose upon reaching $OD_{600} = 0.4$, and shifted to 18 °C for approximately 20 h before harvest. Cells were harvested by centrifugation and lysed using French Pressure in buffer A containing 20 mM HEPES, 1 M NaCl, 4 mM $MgCl_2$, 0.5 mM β-mercaptoethanol (BME), 10% glycerol, pH 7.5, and protease inhibitors, followed by centrifugation to remove cellular debris. Despite varied levels of protein expression for each of the archaeal enzymes, when similar amounts of final purified protein were analyzed by SDS-PAGE, MaThg1, MbThg1, and MtThg1 were purified to similar levels of homogeneity. The peak of protein eluted from the MkThg1 preparation was significantly less pure than the others, but due to the absence of background 3′–5′ addition activity, the partially pure preparation could be used for in vitro activity assay.

Proteins were purified by immobilized metal-ion affinity chromatography using TALON resin (Clontech) (4). The cell-free crude extract from 0.5 L cells was added to 1 mL TALON resin in Buffer B (20 mM HEPES, 0.5 M NaCl, 4 mM $MgCl_2$, 0.5 mM BME, 10% glycerol, pH 7.5), and incubated with gentle shaking at 4 °C to allow binding of the His_6-tagged protein to the resin. The resin was pelleted by centrifugation at $2000 \times g$ for 2 m, and the supernatant containing unbound proteins was removed. The resin was washed by successive steps of mixing followed by centrifugation and removal of supernatant, first with a total of 40 column volumes of Buffer B, then with a total of 60 column volumes of Buffer B containing 10 mM imidazole. After the final wash in 10 mM imidazole buffer, the resin was poured into a disposable 5 mL column, and Thg1 was eluted from the column with 10 mL of Buffer B containing 250 mM imidazole. Fractions (1 mL each) were collected and the peak of protein was determined by BioRad protein assay (typically the first 2–3 fractions eluted from the column), pooled, and dialyzed against a buffer containing 20 mM tris-Cl, 0.5 M NaCl, 4 mM $MgCl_2$, 1 μM EDTA, 1 mM DTT, 50% glycerol, pH 7.5. The resulting purified protein concentrations were determined using BioRad protein assay and all proteins were stored at -20 °C. Yeast Thg1 was purified in the same way for comparison.

Preparation of labeled tRNA substrates. In vitro transcripts of wild-type (A_{73}-containing) and C_{73}-variant yeast $tRNA^{His}$ substrates were generated by standard methods, except that an additional step was added following NsiI linearization of the plasmid DNA template to enhance the yield of run-off transcription by T7 RNA polymerase. In this step, the 3′-overhang sequence generated by NsiI digestion was blunted by treatment with T4 DNA polymerase (Promega) in the presence of all four dNTPs, according to the manufacturer's instructions. The use of the blunt-ended DNA template significantly decreases the amount of high molecular weight end-initiated transcripts formed by T7 RNA polymerase during run-off transcription. To create 5′-^{32}P-labeled tRNA, each tRNA transcript was purified by polyacrylamide gel electrophoresis and 5′-end labeled using γ-[^{32}P]ATP (Perkin Elmer) and T4 polynucleotide kinase (New England Biolabs). For steady-state kinetic characterization, γ-^{32}P-labeled ppp-tRNA was created by in vitro transcription in the presence of γ-[^{32}P]GTP by standard techniques using T7 RNA polymerase.

Construction of archaeal Thg1 plasmids for yeast complementation assays. To test Thg1 function of the archaeal Thg1 homologs in yeast, each gene was cloned into a previously described single copy (*CEN*) shuttle plasmid (pAVA0042) containing a *LEU2* selectable marker and the *GAL1-GAL10* $_{UAS}$, such that each Thg1 gene is expressed under control of the *GAL1* promoter. Each of the homologs was amplified by PCR from the *E. coli* expression constructs described above, using primers that contain appropriate restriction site sequences for restriction cloning into the multiple cloning site of AVA0042. Mt and MbThg1 were inserted between BamHI and SalI restriction sites, MaThg1 was inserted between XbaI and HindIII sites and MkThg1 was inserted using BamHI and PstI sites; in each case the resulting plasmids were verified by sequencing before use.

1. Altschul SF, et al. (1997) Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res* 25(17):3389–3402.
2. 2.Hao B, et al. (2002) A new UAG-encoded residue in the structure of a methanogen methyltransferase. *Science* 296(5572):1462–1466.
3. Krzycki JA (2005) The direct genetic encoding of pyrrolysine. *Curr Opin Microbiol* 8 (6):706–712.
4. Gu W, Jackman JE, Lohan AJ, Gray MW, Phizicky EM (2003) tRNAHis maturation: an essential yeast protein catalyzes addition of a guanine nucleotide to the 5' end of tRNAHis. *Gene Dev* 17(23):2889–2901.

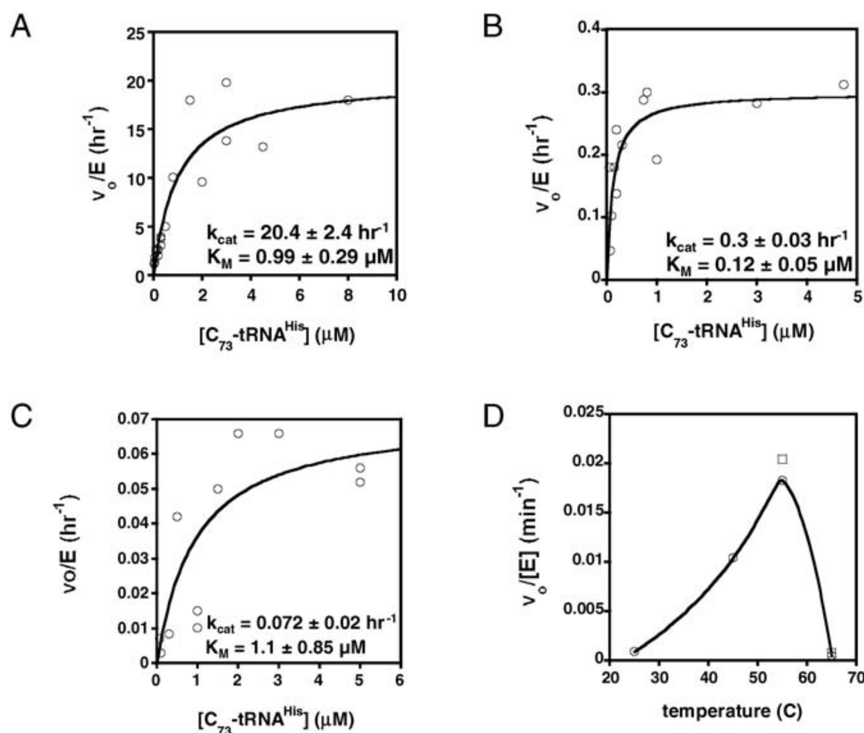

Fig. S1. Steady-state kinetic measurements of Thg1 activity with C_{73}-$tRNA^{His}$. (*A*)–(*C*) Initial rates of G_{-1} addition activity were determined at 25 °C for yeast Thg1 (*A*), MaThg1 (*B*), and MtThg1 (*C*). Initial rates were normalized to [Thg1] in each assay, and plotted as a function of [C_{73}-$tRNA^{His}$] (0.5–8 μM). Kinetic parameters shown on each plot were derived from fits of these data to the Michaelis-Menten equation. (*D*) Temperature dependence of activity of MtThg1 with C_{73}-$tRNA^{His}$. Initial rate (v_o/E) for MtThg1 was measured with 2 μM (circles) or 4 μM (squares) C_{73}-$tRNA^{His}$ at varied temperatures; at 55 °C, $v_o/E_{(2\ \mu M)} =$ 0.018 min^{-1} and $v_o/E_{(4\ \mu M)} = 0.020$ min^{-1}. Solid line shows an interpolated curve drawn to the data measured at 2 μM.

Table S1. Archaeal Thg1 cloning primers and accession numbers

Organism	GI accession number	Forward primer	Reverse primer
Methanothermobacter thermoautotrophicus	15678990	5′**ctcaccaccaccaccaccat**atggctggtcattctatg[a]	5′**atcctatcttactcac**ttatctcatgagaccc
Methanopyrus kandleri	19886374	5′**gggtcctggttcg**atgaagcccgtggaaccctgtgat	5′**cttgttcgtcgtgttta**ttactcttcaacctg atcgggggc
Methanosarcina acetivorans	20089700/20089701	5′**gggtcctggttcg**atgaaaacccgggaaa tatatgctgaa	5′**cttgttcgtgctgttta**ttagtttctattaa taagtttctctaaaaacg
Methanosarcina barkeri	72396415	5′**gggtcctggttcg**atgaaagaccgagaaattta tgctgag	5′**cttgttcgtgctgttta**tcaattcctat taataagtttctgtaaaaac

[a] Sequences in bold indicate LIC adapter sequences incorporated into the PCR product to facilitate cloning into vector BG1861 (*M. thermoautotrophicus*) and AVA421 (all other target genes).