Supplementary Data

Supplemental Figure Legends

**Fig. S1.** *In vitro* synthesis of BstYI and the activity assay of the synthesized BstYI. (A) SDS-PAGE analysis of [³⁵S] methionine labeled BstYI synthesized in the reconstituted *E. coli* translation system (at 37°C, lane 1), the *T. thermophilus* cell extract (at 60°C, lane 2), and the reconstituted *T. thermophilus* system (at 60°C, without (lane 3) or with (lane 4) the energy regeneration enzymes, and at 37°C, with the energy regeneration enzymes (lane 5)). (B) agarose gel analysis of restriction digestion of lambda DNA (lane 1, before digestion) by aliquots of the translation reactions at 60°C from the reconstituted *T. thermophilus* system without (left panel) or with (right panel) BstYI mRNA. The digestion pattern of lambda DNA by purified recombinant BstYI is shown in lane 4 as a control. Lanes 2, 5 and 6: 0.1 µl aliquots; lanes 3, 7 and 8: 1 µl aliquots.

**Fig. S2.** Measuring the stability of the purified recombinant stGFP incubated at various temperatures (legends on the right). The stability of stGFP is indicated by the relative fluorescence of stGFP (%) compared to that of stGFP before the incubation (set as 100).

**Fig. S3.** The fluorescence of stGFP synthesized at 37°C in the reconstituted *T. thermophilus* system in the absence (open circle) or presence (black circle) of spermine.

**Fig. S4.** Testing the functional compatibility of individual initiation factors between *T. thermophilus* and *E. coli*. The translation reactions containing stGFP mRNA are conducted at 37°C for 4 hr in the reconstituted *T. thermophilus* system in which each of the *T. thermophilus* initiation factors is either removed (ΔIF1, ΔIF2, or ΔIF3) or replaced by its counterparts from *E. coli* (Ec IF1, Ec IF2, or Ec IF3, respectively). The relative synthesis yields (%) are based on the fluorescence of the reactions compared to that of the complete *T. thermophilus* system (Tt IFs) set as 100. The data are based on at least two independent experiments.
**Fig. S5.** Cladogram of the bacterial phylogeny highlighting nodes that represent the ancestral EF-Tu proteins assayed in the reconstituted *T. thermophilus* system (Fig. 5). Melting temperatures (Tm) for the ancestral EF-Tu proteins are shown in degrees (Celsius) (21). The bacterial lineages that contain the three modern EF-Tu proteins assayed in Fig. 5 are boxed (*E. coli* = gamma proteobacteria, *Thermus thermophilus* = Thermus/Deinococcus, *Thermotoga maritima* = Thermotogae).

**Fig. S6.** SDS-PAGE gels of 33 recombinant *T. thermophilus* proteins purified from over-expression strains of *E. coli* and used for the reconstitution of protein translation of *T. thermophilus*. Also shown is the SDS-PAGE gel of the *T. thermophilus* ribosomes purified from the cell extract of *T. thermophilus*. The molecular weight standards (MW, kDa) are shown on the left.

**Figure S7.** SDS-PAGE gel of purified resurrected ancient Tu, *Thermotoga* Tu and Tt Tu (indicated on the top). The molecular weight standards (MW, kDa) are shown on the left.

**Figure S8.** A poly(Phe) assay at 65°C showing the *in vitro* synthesis activity of the purified *T. thermophilus* ribosomes. Incorporation of [14C] phenylalanine into poly(phenylalanine) is determined at various time points in the presence of 2 mM (black circle) or 0.5 mM (open circle) spermine, and in the absence of spermine (open square) or ribosome (x). The data are based on three independent experiments.

**Figure S9.** Assaying the activities of 20 recombinant aminoacyl-tRNA synthetases of *T. thermophilus* (Tt aaRS). (A) The activity assays of Tt aaRS in the reconstituted *E. coli* translation system in which each *E. coli* aaRS is either removed (black bars, -aaRS) or replaced by its corresponding Tt aaRS (white bars, +Tt aaRS). The activity of the luciferase synthesized in the complete reconstituted *E. coli* system (grey bar, Complete) is set as 100. (B) The activity assays for the *T. thermophilus* AspRS, MetRS or TyrRS in the reconstituted *E. coli* system in which *E. coli* AspRS, MetRS or TyrRS, respectively,
is removed (black bars, -aaRS), replaced by its corresponding Tt aaRS (white bars +Tt aaRS) or replaced by its corresponding Tt aaRS with the addition of extra tRNAs from *T. thermophilus* (light grey bars, extra Tth tRNA) or *E. coli* (dark grey bars, extra Eco tRNA). The activity of the luciferase synthesized in the complete reconstituted *E. coli* system (not shown) is set as 100. (C) tRNA aminoacylation assays at 65°C for the *T. thermophilus* GluRS, ThrRS and TrpRS containing \[^3\text{H}\]Glu, \[^{14}\text{C}\]Thr and \[^{14}\text{C}\]Trp, respectively. Aliquots are taken at various time points from the reactions in the absence (open circle) or presence of 1 µM synthetases (grey circle). All data are based on two independent experiments.

**Fig. S10.** Testing the functional conservation of resurrected ancient elongation factors (indicated at the bottom) in the reconstituted *E. coli* system in which Ec Tu and Ec Ts is replaced by each of the ancient elongation factors (indicated at the bottom) (also see Fig. S5) and Tt Ts. The translation reactions containing stGFP mRNA are conducted at 37°C for 4 hr. The relative synthesis yields (%) are based on the fluorescence of the reactions compared to that of the original reconstituted system (Ec Tu, set as 100). All data are based on at least two independent experiments.
Table S1. List of key translation components in the reconstituted *T. thermophilus* system.

| *T. thermophilus* components | MW(kDa) including the tag | N- or C-terminal 6xhis tag in this study | Final conc. used in the reactions (µM) | *E. coli* homologs (gene name) | % identity to *E. coli* homologs | purification procedures reported in other studies* |
|-----------------------------|---------------------------|----------------------------------------|---------------------------------------|-----------------------------|-------------------------------|----------------------------------|
| **Initiation**              |                           |                                        |                                       |                             |                               |                                   |
| IF1                         | 9.1                       | C                                      | 2.0                                   | *infA*                      | 60                            | (48)                             |
| IF2                         | 64.3                      | C                                      | 0.4                                   | *infB*                      | 40                            | (9,49)                           |
| IF3                         | 17.9                      | C                                      | 0.7                                   | *infC*                      | 48                            | (48)                             |
| MTF                         | 34.4                      | C                                      | 0.6                                   | *fmt*                       | 40                            | (50) (*E. coli*)                 |
| **Elongation**              |                           |                                        |                                       |                             |                               |                                   |
| EF-Tu                       | 44.8                      | no tag                                 | 18                                    | *TufA*                      | 61                            | (8,51)                           |
| EF-Ts                       | 23.6                      | C                                      | 3.2                                   | *TsF*                       | 36                            | (8)                              |
| EF-G                        | 78.0                      | C                                      | 0.6                                   | *fusA*                      | 59                            | (8)                              |
| **Termination & recycling ‡|                           |                                        |                                       |                             |                               |                                   |
| RF1                         | 33.7                      | N                                      | 0.5                                   | *prfA*                      | 46                            | (10)                             |
| RRF (frr)                   | 23.2                      | N                                      | 2.8                                   | *frr*                       | 39                            | (52)                             |
| **aaRS**                    |                           |                                        |                                       |                             |                               |                                   |
| **Class I:**                |                           |                                        |                                       |                             |                               |                                   |
| ArgRS                       | 68.2                      | N                                      | 0.062                                 | *argS*                      | 24                            | (53)                             |
| CysRS                       | 92.6                      | C                                      | 0.048                                 | *cysS*                      | 38                            | (54) (*E. coli*)                 |
| IleRS                       | 121.6                     | N                                      | 0.36                                  | *ileS*                      | 28                            | (55)                             |
| LeuRS                       | 102.2                     | C                                      | 0.042                                 | *leuS*                      | 45                            | (56)                             |
| MetRS                       | 71.9                      | C                                      | 0.11                                  | *metG*                      | 25                            | (57)                             |
| ValRS                       | 96.7                      | C                                      | 0.034                                 | *valS*                      | 38                            | (58)                             |
| GluRS                       | 56.1                      | N                                      | 0.35                                  | *gltX*                      | 34                            | (59)                             |
| GlnRS                       | 64.4                      | C                                      | 0.060                                 | *glnS*                      | 51                            | (59) (*E. coli*)                 |
| TrpRS                       | 39.1                      | C                                      | 0.056                                 | *trpS*                      | 39                            | (60) (*E. coli*)                 |
| TyrRS                       | 49.8                      | C                                      | 0.076                                 | *tyrS*                      | 24                            | (61)                             |
| **Class II:**               |                           |                                        |                                       |                             |                               |                                   |
| HisRS                       | 49.0                      | N                                      | 0.086                                 | *hisS*                      | 42                            | (62) (*E. coli*)                 |
| ProRS                       | 56.4                      | N                                      | 0.25                                  | *proS*                      | 32                            | (63) (*M. jannaschii*)           |
| ThrRS                       | 77.7                      | N                                      | 0.084                                 | *thrS*                      | 39                            | (64)                             |
| SerRS                       | 48.9                      | C                                      | 0.078                                 | *serS*                      | 35                            | (65) (*E. coli*)                 |
| Protein      | % Conserved | AA  | % Conserved | AA  | % Conserved | AA  | % Conserved | AA  | % Conserved | AA  | % Conserved | AA  |
|--------------|-------------|-----|-------------|-----|-------------|-----|-------------|-----|-------------|-----|-------------|-----|
| LysRS        | 58.0        | C   | 0.11        | lysS| 46          |     | (66) (E. coli)|     |             |     |             |     |
| AsnRS        | 51.9        | C   | 0.42        | asnS| 39          |     | (67)        |     |             |     |             |     |
| AspS         | 67.2        | C   | 0.12        | aspS1| 48         |     | (68)        |     |             |     |             |     |
| AlaRS        | 99.7        | N   | 0.73        | alaS| 42          |     | (69), (70) (A. aeolicus)|     |             |     |             |     |
| GlyRS§       | 59.4        | C   | 0.13        | glyQ,glyS| n.a. |     | (71)        |     |             |     |             |     |
| PheRS a2β2   | 40.0        | N   | 0.20        | pheS pheT| 45 |     | (72)        |     |             |     |             |     |
|              | 87.4        | N   | 0.20        |     | n.a.        |     |             |     |             |     |             |     |

| Energy regeneration | PK     | NDK | ADK | PPA | PK   | NDK | ADK | PPA |
|---------------------|--------|-----|-----|-----|------|-----|-----|-----|
|                     | 52.3   | 15.7| 21.7| 20.3| 0.22 | 0.070| 0.26| 0.040|
| pykF                | 44     | 50  | 36  | 47  |      |      |      |      |
| ndk                 |        |     |     |     |      |      |      |      |
| adk                 |        |     |     |     |      |      |      |      |
| ppa                 |        |     |     |     |      |      |      |      |
| (73) (E. coli)      |        |     |     |     |      |      |      |      |
| (74) (E. coli)      |        |     |     |     |      |      |      |      |
| (75) (E. coli)      |        |     |     |     |      |      |      |      |
| (76) (A. aeolicus)  |        |     |     |     |      |      |      |      |

| T. th ribosomes | n.a. | n.a. | 2.4 | n.a. | n.a. | n.a. |
| T. th total tRNAs| n.a. | n.a. | 2 (mg/ml)| n.a. | n.a. | n.a. |

* References for the purification procedures of the components from organisms other than \( T. thermophilus \) are indicted by the names of the organisms in parentheses.

† RF2 was not used in this study since all mRNA in the translation reactions have UAA as the stop codon; there is no known release factor 3 (RF3) in \( T. thermophilus \), whereas in \( E. coli \), RF3 is encoded by the \( prfC \) gene.

§ The glycyl-tRNA synthetase (GlyRS) from \( T. thermophilus \) is a homodimer, with no significant identity with GlyRS of \( E. coli \), which is a alpha 2 beta 2 heterotetramer, encoded by two genes: \( glyQ \) and \( glyS \).

MTF: methionyl-tRNA formyltransferase; aaRS: aminoacyl-tRNA synthetases; PK: pyruvate kinase; NDK: nucleotide diphosphate kinase; ADK: adenylate kinase; PPA: inorganic pyrophosphatase. n.a. not available or applicable.
### Table S2. Protein identification using MS and MS/MS of the in vitro reaction without stGFP mRNA

| Group Num | Num Spectra | Num Peps Unique | Score Unique | Percent Coverage | Protein MW | Accession number | Entry_name |
|-----------|-------------|-----------------|--------------|------------------|------------|------------------|------------|
| 1         | 39          | 19              | 291.48       | 56               | 44782.4    | 46197258         | gb AAS81672.1 elongation factor Tu [Thermus thermophilus HB27] |
| 2         | 25          | 15              | 240.66       | 24               | 97480.4    | 46197409         | gb AAS81822.1 alanyl-tRNA synthetase [Thermus thermophilus HB27] |
| 3         | 16          | 14              | 222.49       | 52               | 51195.3    | 259450882        | gb AAS81953.1 pyruvate kinase [Thermus thermophilus HB27] |
| 4         | 18          | 14              | 205.19       | 41               | 59984.8    | 46197754         | gb AAS82166.1 SSU ribosomal protein S1P [Thermus thermophilus HB27] |
| 5         | 11          | 11              | 142.68       | 15               | 122311.9   | 46196634         | gb AAS81050.1 isoleucyl-tRNA synthetase [Thermus thermophilus HB27] |
| 6         | 11          | 7               | 109.7        | 28               | 40092.7    | 1785848          | dbj BAA13349.1 peptide chain release factor 1 [Thermus thermophilus HB8] |
| 7         | 10          | 7               | 107.49       | 22               | 63263.8    | 333966364        | gb AEG33129.1 translation initiation factor IF-2 [Thermus thermophilus SG0.5JP17-16] |
| 8         | 14          | 6               | 106.14       | 53               | 16411.3    | 149240892        | pdb 1VSA G Chain G, Crystal Structure Of A 70s Ribosome-Tma Complex Reveals Functional Interactions And Rearrangements. This File, 1vsa, Contains The 50s Ribosome Subunit. 30s Ribosome Subunit Is In The File 2ow8 |
| 9         | 6           | 6               | 89.04        | 39               | 13067.3    | 294979561        | pdb 3i8j I Chain I, Elongation Complex Of The 70s Ribosome With Three Tmas And Entry 3i8j Contains 50s Ribosomal Subunit. The 30s Ribosomal Can Be Found In Pdb Entry 3i8h. Molecule A In The Same Asym Unit Is Deposited As 3i8f (50s) And 3i8g (30s). |
| 10        | 6           | 6               | 87.28        | 41               | 29276.8    | 10835586         | pdb 1FJG B Chain B, Structure Of The Thermus Thermophilus 30s Ribosomal Subunit In Complex With The Antibiotics Streptomycin, Spectinomycin, And Paromomycin |
| ID | Label | Chain | Temperature | PDB/EMBL/GenBank Accession | Description |
|----|-------|-------|--------------|-----------------------------|-------------|
| 11 | 12    | 5     | 86.67        | 20994.1                     | 11513707 pdb 1EH1 A Chain A, Ribosome Recycling Factor From Thermus Thermophilus |
| 12 | 10    | 7     | 86.52        | 34                          | 206581907 pdb 3CM0 A Chain A, Crystal Structure Of Adenylate Kinase From Thermus Thermophilus Hb8 |
| 13 | 8     | 6     | 81.8         | 10                          | 383509358 gb AFH38790.1 valyl-tRNA synthetase [Thermus thermophilus JL-18] |
| 14 | 6     | 6     | 77.51        | 34                          | 1169484 sp P43895.1 EFTS_THET8 RecName: Full=Elongation factor Ts: Short=EF-Ts |
| 15 | 8     | 5     | 73.56        | 12                          | 67464648 pdb 2B11 A Chain A, Ribosomal Elongation Factor G (El-G) Fusidic Acid Resistant Mutant G16v |
| 16 | 12    | 4     | 64.41        | 58                          | 21901944 emb CAD42330.1 translation initiation factor 1 [Thermus thermophilus] |
| 17 | 5     | 4     | 60.93        | 21                          | 170292175 pdb 2EL7 A Chain A, Crystal Structure Of Tryptophanyl-Trna Synthetase From Thermus Thermophilus |
| 18 | 4     | 3     | 55.2         | 26                          | 134048675 sp Q5SKU2.2 IF3_THET8 RecName: Full=Translation initiation factor 1 IF-3 |
| 19 | 6     | 4     | 54.88        | 20                          | 1169713 sp P43523.1 FMT_THETH RecName: Full=Methionyl-IRNA formyltransferase |
| 20 | 7     | 4     | 52.43        | 13                          | 46197819 gb AAS82231.1 prolyl-tRNA synthetase [Thermus thermophilus HB27] |
| 21 | 3     | 3     | 48.51        | 31                          | 383508678 gb AFH38110.1 ribosomal protein L10 [Thermus thermophilus JL-18] |
| 22 | 3     | 3     | 45.72        | 9                           | 46196105 gb AAS80523.1 glycyl-IRNA synthetase [Thermus thermophilus HB27] |
| 23 | 3     | 3     | 45.08        | 25                          | 740019 prf 2004301A ribosomal protein L11 |
| 24 | 3     | 3     | 37.46        | 24                          | 347948331 pdb 3ZVP C Chain C, Crystal Structure Of The Hybrid State Of Ribosome In Complex With The Guanosine Triphosphatase Release Factor 3 |
| 25 | 3     | 3     | 34.43        | 7                           | 333965785 gb AEG32550.1 60 kDa chaperonin [Thermus thermophilus SG0.5J17-16] |
| 26 | 6 | 2 | 32.24 | 8 | 23218.7 | 14719583 | 14719583 pdb 1FEU A Chain A, Crystal Structure Of Ribosomal Protein T15, One Of The Ctc Family Proteins, Complexed With A Fragment Of 5s Rrna. |
|---|---|---|---|---|---|---|---|
| 27 | 2 | 2 | 31.71 | 10 | 50870.3 | 1004327 | 1004327 emb CAA62491.1 asparaginyl-tRNA synthetase [Thermus thermophilus HB8] |
| 28 | 5 | 2 | 30.73 | 5 | 39372.7 | 383510465 | 383510465 gb AFH39897.1 phenylalanyl-tRNA synthetase, alpha subunit [Thermus thermophilus JL-18] |
| 29 | 2 | 2 | 30.72 | 9 | 47813.1 | 46196451 | 46196451 gb AAS80868.1 seryl- tRNA synthetase [Thermus thermophilus HB27] |
| 30 | 2 | 2 | 30.55 | 6 | 54024.2 | 383510084 | 383510084 gb AFH39516.1 glutamyl-tRNA synthetase [Thermus thermophilus JL-18] |
| 31 | 3 | 2 | 29.76 | 17 | 16281.1 | 116668247 | 116668247 pdb 2J01 P Chain P, Structure Of The Thermus Thermophilus 70s Ribosome Complexed With Mrna, Trna And Paromomycin (Part 2 Of 4). This File Contains The 50s Subunit From Molecule I. |
| 32 | 3 | 2 | 27.02 | 25 | 14468.1 | 14278541 | 14278541 pdb 1194 L Chain L, Crystal Structures Of The Small Ribosomal Subunit With Tetracycline, Edeine And If3 |
| 33 | 2 | 2 | 24.57 | 30 | 15946.9 | 149240896 | 149240896 pdb 1VSA K Chain K, Crystal Structure Of A 70s Ribosome-Tma Complex Reveals Functional Interactions And Rearrangements. This File, 1vsA, Contains The 50s Ribosome Subunit. 30s Ribosome Subunit Is In The File 2ow8 |
| 34 | 3 | 2 | 24.51 | 4 | 75537.8 | 55773257 | 55773257 dbj BAD71698.1 threonyl-tRNA synthetase [Thermus thermophilus HB8] |
| 35 | 2 | 2 | 22.74 | 21 | 24431.7 | 46196415 | 46198792 ref YP_004459.1 transferase/hydrolase [Thermus thermophilus HB27] |
| 36 | 2 | 2 | 20.81 | 9 | 53921 | 55771823 | 55771823 dbj BAD70264.1 methyltransferase, HemK family [Thermus thermophilus HB8] |
| 37 | 1 | 1 | 20.68 | 5 | 44610.9 | 46197628 | 46197628 gb AAS82041.1 dihydrolipoamide succinyltransferase [Thermus thermophilus HB27] |
Table S3. Protein identification using MS and MS/MS of the in vitro reaction containing stGFP mRNA (the data of stGFP is highlighted in red).

| group Num | num Spectra | num Peps | score Unique | percent Coverage | protein_m w | accession_number | entry_name |
|-----------|-------------|----------|---------------|------------------|------------|-----------------|------------|
| 1         | 45          | 22       | 340.37        | 65               | 44782.4    | 46197258        | gb AAS81672.1 elongation factor Tu [Thermus thermophilus HB27] |
| 2         | 33          | 14       | 220.64        | 22               | 97480.4    | 46197409        | gb AAS81822.1 alanyl-tRNA synthetase [Thermus thermophilus HB27] |
| 3         | 21          | 13       | 192.56        | 39               | 60012.9    | 30141902        | emb CAD30282.1 3OS ribosomal protein S1 [Thermus thermophilus HB27] |
| 4         | 10          | 8        | 115.97        | 26               | 51195.3    | 259450882       | gb AAS81953.1 pyruvate kinase [Thermus thermophilus HB27] |
| 5         | 9           | 7        | 108.09        | 45               | 13067.3    | 294979561       | pdb 3I8I I Chain I, Elongation Complex Of The 70s Ribosome With Three Trnas And Entry 3I8I Contains 50s Ribosomal Subunit, The 30s Ribosomal Can Be Found In Pdb Entry 3I8H. Molecule A In The Same Asym Unit Is Deposited As 3I8f (50s) And 3I8g (30s). |
| 6         | 13          | 6        | 97.4          | 54               | 16411.3    | 149240892       | pdb 1VSA G Chain G, Crystal Structure Of A 70s Ribosome-Tma Complex Reveals Functional Interactions And Rearrangements. This File, 1vsa, Contains The 50s Ribosome Subunit. 30s Ribosome Subunit Is In The File 2ow8 |
| 7         | 8           | 7        | 95.05         | 22               | 63263.8    | 333966364       | gb AEG33129.1 translation initiation factor IF-2 [Thermus thermophilus SG0.5JP17-18] |
| 8         | 7           | 6        | 85.02         | 9                | 122311.9   | 46196634        | gb AAS81050.1 isoleucyl-tRNA synthetase [Thermus thermophilus HB27] |
| 9         | 8           | 5        | 82.25         | 39               | 20994.1    | 11513707        | pdb 1EH1 A Chain A, Ribosome Recycling Factor From Thermus Thermophilus |
| 10        | 9           | 5        | 69.97         | 38               | 29482.3    | stGFP           | stGFP |
| 11        | 5           | 4        | 65.19         | 17               | 40092.7    | 1785848         | dbj BAA13348.1 peptide chain release factor 1 [Thermus thermophilus HB8] |
12 5 5 60.47 28 29145.6 14278531 14278531 pdb 1194 B Chain B, Crystal Structures Of The Small Ribosomal Subunit With Tetracycline, Edeine And If3

13 7 4 56.76 58 8233.7 21901944 21901944 emb CAD42330.1 translation initiation factor 1 [Thermus thermophilus]

14 7 3 51.62 26 19866.4 134048675 134048675 sp Q5SKU2.2 IF3_THET8 RecName: Full=Translation initiation factor IF-3

15 4 3 50.96 16 26701.1 10835587 10835587 pdb 1FJG C Chain C, Structure Of The Thermus Thermophilus 30s Ribosomal Subunit In Complex With The Antibiotics Streptomycin, Spectinomycin, And Paromomycin

16 5 3 43.3 8 76921.7 67464648 67464648 pdb 2BM1 A Chain A, Ribosomal Elongation Factor G (EF-G) Fusidic Acid Resistant Mutant G16v

17 7 3 40.73 24 12056.6 116668256 116668256 pdb 2J01 Y Chain Y, Structure Of The Thermus Thermophilus 70s Ribosome Complexed With Mrna, Trna And Paromomycin (Part 2 Of 4). This File Contains The 50s Subunit From Molecule I.

18 4 3 39.42 22 22413.1 1169484 1169484 sp P43895.1 EFTS_THET8 RecName: Full=Elongation factor Ts: Short=EF-Ts

19 3 3 38.31 21 24843.8 347948831 347948831 pdb 3ZVP C Chain C, Crystal Structure Of The Hybrid State Of Ribosome In Complex With The Guanosine Triphosphatase Release Factor 3

20 3 2 34.96 7 54514.5 46197819 46197819 gb AAS82231.1 prolyl-tRNA synthetase [Thermus thermophilus HB27]

21 2 2 33.85 22 15962.9 116668248 116668248 pdb 2J01 Q Chain Q, Structure Of The Thermus Thermophilus 70s Ribosome Complexed With Mrna, Trna And Paromomycin (Part 2 Of 4). This File Contains The 50s Subunit From Molecule I.
| PDB Entry | Chain | Resolution | R-factor | Description |
|-----------|--------|------------|----------|-------------|
| 3CM0      | A      | 28.23      | 31       | Crystal Structure Of Adenylate Kinase From Thermus Thermophilus Hb8 |
| 2FJG      | L      | 24.96      | 16       | Structure Of The Thermus Thermophilus 30s Ribosomal Subunit In Complex With The Antibiotics Streptomycin, Spectinomycin, And Paromomycin |
| 3CMO      | A      | 26.83      | 11       | Crystal Structure Of Ribosomal Protein T15, One Of The Ctc Family Proteins, Complexed With A Fragment Of 5s Rrna |
| 4AFH      | D      | 32.81      | 15       | Structure Of The Thermus Thermophilus 70s Ribosome Complexed With Mrna, Trna And Paromomycin (Part 2 Of 4) |
| 2FJG      | L      | 29.54      | 22       | Structure Of Adenylate Kinase From Thermus Thermophilus Hb8 |
| 2FJG      | L      | 29.72      | 5        | Crystal Structure Of Ribosomal Protein T15, One Of The Ctc Family Proteins, Complexed With A Fragment Of 5s Rrna |
| 3CMO      | A      | 30.03      | 8        | Crystal Structure Of Ribosomal Protein T15, One Of The Ctc Family Proteins, Complexed With A Fragment Of 5s Rrna |
| 4AFH      | D      | 30.27      | 15       | Structure Of The Thermus Thermophilus 70s Ribosome Complexed With Mrna, Trna And Paromomycin (Part 2 Of 4) |
| 2FJG      | L      | 33.17      | 35       | Structural Basis For Translation Termination On The 70s Ribosome. This File Contains The 50s Subunit Of One 70s Ribosome. The Entire Crystal Structure Contains Two 70s Ribosomes As Described In Remark 400. |
| 2FJG      | L      | 33.17      | 35       | Structural Basis For Translation Termination On The 70s Ribosome. This File Contains The 50s Subunit Of One 70s Ribosome. The Entire Crystal Structure Contains Two 70s Ribosomes As Described In Remark 400. |
| 4AFH      | D      | 32.81      | 15       | Structure Of The Thermus Thermophilus 70s Ribosome Complexed With Mrna, Trna And Paromomycin (Part 2 Of 4) |
| 2FJG      | L      | 33.17      | 35       | Structural Basis For Translation Termination On The 70s Ribosome. This File Contains The 50s Subunit Of One 70s Ribosome. The Entire Crystal Structure Contains Two 70s Ribosomes As Described In Remark 400. |
| 4AFH      | D      | 32.81      | 15       | Structure Of The Thermus Thermophilus 70s Ribosome Complexed With Mrna, Trna And Paromomycin (Part 2 Of 4) |
| 2FJG      | L      | 33.17      | 35       | Structural Basis For Translation Termination On The 70s Ribosome. This File Contains The 50s Subunit Of One 70s Ribosome. The Entire Crystal Structure Contains Two 70s Ribosomes As Described In Remark 400. |
| 4AFH      | D      | 32.81      | 15       | Structure Of The Thermus Thermophilus 70s Ribosome Complexed With Mrna, Trna And Paromomycin (Part 2 Of 4) |
| 2FJG      | L      | 33.17      | 35       | Structural Basis For Translation Termination On The 70s Ribosome. This File Contains The 50s Subunit Of One 70s Ribosome. The Entire Crystal Structure Contains Two 70s Ribosomes As Described In Remark 400. |
Structure Analysis Of Ribosomal Decoding. This Entry Contains The 50s Ribosomal Subunit Of The First 70s Molecule In The Asymmetric Unit For The Cognate Trna-Leu Complex
Supplemental Methods

Cloning, expression and purification of recombinant protein factors of *T. thermophilus* and resurrected ancient elongation factors.

The genes encoding IF1, IF2, IF3, MTF, EF-Tu, EF-G, RF1, RRF, ADK, NDK, PK, PPA and 16 aminoacyl-tRNA synthetases of *T. thermophilus* HB27 and ancient elongation factors were synthesized by GeneScript (Piscataway, NJ) and DNA 2.0 (Menlo Park, CA) with codons optimized for expression in *E. coli*. The genes of EF-Ts and the other 4 aminoacyl-tRNA synthetases, ArgRS, HisRS, LysRS, and ProRS, were amplified by PCR from the genomic DNA of *T. thermophiles* HB8. EF-Ts was cloned into pET24b+ vector and transformed into *E. coli* BL21-CodonPlus (DE3)-RP competent cell (Agilent Technologies, Santa Clara, CA). The expression vectors for ancient elongation factors were constructed by Dr. M. Cole and J. Kratzer (School of Biology, Georgia Institute of Technology) and derived from pET24a vector to allow expression of proteins with a C-terminal his-tag. All other genes were cloned into the expression vector, pCOATexp, derived from pTYB1 vector (New England Biolabs), to allow expression of proteins with either N- or C-terminal his-tag. The expression vectors were transformed into *E. coli* strains ER3095 (New England Biolabs).

For each his-tagged protein, cells transformed with the expression vector were grown at 37°C to OD$_{600}$ of 0.6 in 2-6 L Luria-Bertani (LB) broth. Isopropyl-$\beta$-D-thiogalactoside (IPTG) was then added to a final concentration of 0.1 mM and the cells were grown for an additional 4-5 hr at 37°C. Cells were harvested by centrifugation and lysed by sonication in lysis buffer (50 mM Tris-HCl, pH7.5, 300 mM KCl, 10 mM MgCl$_2$, 20 mM imidazole, and 1 mM $\beta$-mercaptoethanol). Cell debris was removed by centrifugation at 16,000 g for 1 hr at 4°C and the supernatant was applied to a 10 ml HisTrap FF column (GE healthcare, Piscataway, NJ). After washing the column with 100 ml lysis buffer, the his-tagged protein was eluted with a linear gradient of 20 mM to 250 mM imidazole in lysis buffer. Fractions containing the his-tagged protein were combined and heated at 65°C for 30 min to denature contaminating *E. coli* proteins. After centrifugation to remove the denatured proteins, the purified protein was dialyzed against the storage buffer (25 mM Tris-HCl, pH7.5, 100 mM K-glutamate, 10 mM
Mg(OAc)$_2$, 30% glycerol, and 1 mM β-mercaptoethanol) and stored frozen in small aliquots at -80°C. In the case of his-tagged RF1, ArgRS, HisRS, LysRS, PheRS, ThrRS, and NDK, a high salt lysis buffer (50 mM Tris-HCl, pH7.5, 1M NH$_4$Cl, 10 mM MgCl$_2$, 20 mM imidazole, and 1 mM β-mercaptoethanol) and a high salt storage buffer (25 mM Tris-HCl, pH7.5, 1M NH$_4$Cl, 10 mM Mg(OAc)$_2$, 30% glycerol, and 1 mM β-mercaptoethanol) were used.

Almost all his-tagged recombinant proteins were purified to near homogeneity as indicated by the SDS-PAGE analyses (Figure S6 and S7). The purification of CysRS resulted in a significant amount of contaminants (Figure S6 CysRS). Since CysRS exhibited a high specific activity and only a small amount of the enzyme (0.4 % (w/w) of total protein factors) was used in the protein translation reaction, the effect of the contaminants was not expected to be significant. RF2 was not used in this study since all mRNA templates contained UAA as the stop codon.

EF-Tu from *T. thermophilus* (Tt Tu) was overexpressed without a tag in *E. coli* and purified to near homogeneity by several chromatographic steps (Figure S7, Tt Tu). In this case, cells were lysed in DEAE buffer A (50 mM Tris-HCl, pH8.0, 10 mM MgCl$_2$, and 1 mM β-mercaptoethanol). After centrifugation, the supernatant was applied to a Hi-Prep DEAE FF16/10 column (GE healthcare, Piscataway, NJ). After washing the column with 300 ml DEAE buffer, proteins were eluted with a linear gradient of 5 to 60% DEAE buffer B (50 mM Tris-HCl, pH8.0, 10 mM MgCl$_2$, 1M KCl, and 1 mM β-mercaptoethanol). The fractions containing Tt Tu were combined and heated at 65°C for 30 min to denature contaminating *E. coli* proteins. After centrifugation to remove the denatured proteins, the partially purified Tt Tu was dialyzed into SP buffer A (10 mM imidazole-HCl, pH6.0, 1 mM Mg(OAc)$_2$, and 1 mM β-mercaptoethanol) before loading onto HiTrap SP HP column (GE healthcare, Piscataway, NJ). The column was washed with 90 mL of SP buffer A and eluted with a linear gradient of 0-50% SP buffer B (10 mM imidazole-HCl, pH8.0, 1 mM Mg(OAc)$_2$, 100 mM NH$_4$Cl, and 1 mM β-mercaptoethanol). The final purified Tt Tu was dialyzed in the storage buffer and was frozen in small aliquots at -80°C.

**Determining the activities of the aminoacyl-tRNA synthetases of *T. thermophilus***
The activities of 20 purified aminoacyl-tRNA synthetases (aaRS) of *T. thermophilus* were first examined in a reconstituted *E. coli* system in which each *E. coli* aaRS was individually removed and replaced by its *T. thermophilus* counterpart in the same concentration. All other components in the reconstituted *E. coli* system remained essentially the same as those described in previously studies (14,80). The in vitro reaction mixtures containing the reconstituted *E. coli* system expressing the reporter gene for firefly luciferase (Fluc) were incubated at 37°C for 2-4 hr. Aliquots (5 µl) were taken and diluted 10-fold in 1x cell culture lysis reagent (Promega, Madison, WI) containing 1 mg/ml BSA. The activity of the synthesized Fluc was assayed using the Luciferase Assay System (Promega, Madison, WI) in a microplate luminometer (Centro LB 640, Berthold Technologies, Oak Ridge, TN). In the cases of AspRS, MetRS or TyrRS, lower Fluc activities were observed with the *T. thermophilus* aaRS in the reconstituted *E. coli* system (Fig. S9A). Addition of extra *T. thermophiles* tRNA (1.05 mg/ml) to the reactions significantly increased the Fluc activities (Fig. S9B). As controls, extra *E. coli* tRNA (1.59 mg/ml) was also added to the same set of reactions. The results in Fig. S9B suggest that the low activities observed for three *T. thermophilus* aaRS were mainly due to the preference of *T. thermophilus* AspRS and MetRS for *T. thermophilus* tRNA over *E. coli* tRNAs, and the requirement of *T. thermophilus* TyrRS for a higher concentration of tRNA.

In the absence of *E. coli* GluRS, PheRS, ThrRS or TrpRS, significant Fluc activities (>60%) still remained in the reconstituted *E. coli* system used in this study (Fig. S9A, black bars). Since addition of the corresponding *T. thermophilus* aaRS did not result in a significant increase in the Fluc activity (Fig. S9A, white bars), the activities of these *T. thermophilus* aaRS were verified by the poly(Phe) assay (PheRS, Fig. S8) or the tRNA aminoacylation assay (Gluc RS, ThrRS and TrpRS, Figure S9C) (81). The aminoacylation reactions were conducted at 65°C for 45 min in a 50 µl mixture containing 50 mM Heps-KOH, pH7.5, 50 mM KCl, 10 mM MgCl₂, 2 mM DTT, 2 mM ATP, 105 µM *T. thermophilus* tRNAs, one of the following radioactive amino acids: 4.03 µM[^3]H]Glu (49.6 Ci/mmol, PerkinElmer), 17.1 µM[^14]CThr (195 mCi/mmol, VWR), 2.6 µM[^14]C]Trp (53 mCi/mmol, PerkinElmer) and in the presence or absence of 2.6 µM of the purified synthetase. Aliquots were taken at different time points and deposited on 3
MM filter discs. After soaking in 5% TCA on ice for 5 min, the filter discs were washed twice with 5% TCA and once with cold ethanol for 10 min each step. The filter discs were dried and the radioactivity was measured in a scintillation counter.

Purification of *T. thermophilus* ribosome and total tRNA and preparation of *T. thermophilus* S30 extract

The ribosome of *T. thermophilus* was purified using established protocols with minor modification (82). All buffers and purification procedures were at 4°C, unless otherwise noted. Specifically, *T. thermophilus* strain HB8 was grown in small-scale fermentation (10 L) in NEB fermentation facility. The culture was cooled rapidly and the cells were harvested by centrifugation. Fresh cell paste (80 g) was washed twice in 200 ml of wash/lysis buffer (25 mM Tris-HCl, pH7.5, 100 mM NH₄Cl, 15 mM MgCl₂, 1 mM EDTA, 7 mM β-mercaptoethanol) and then resuspended in 150 ml of the same wash/lysis buffer. The cells were disrupted at 40 kpsi by a cell disruption system (Constant Systems, Low March, Daventry, Northants, United Kingdom) and cell debris was removed by centrifugation at 30,000 g for 1 hr. The supernatant was centrifuged again at 30,000 g for additional 30 min and saved as the *T. thermophilus* S30 extract. For ribosome purification, 25 ml of the S30 extract was overlayered onto 6 ml of a sucrose cushion (Cushion I: 25 mM Tris-HCl, pH7.5, 15 mM MgCl₂, 1 mM EDTA, 1.5 M Sucrose, 0.68 M CsCl, 7 mM β-mercaptoethanol) in each ultracentrifuge tube, and the ultracentrifugation was conducted in a Beckman SW28 rotor at 27,000 rpm for 21 hr. The top layer from each ultracentrifuge tube was saved for future purification of *T. thermophilus* total tRNA (see below) and the bottom layer (~4ml in each ultracentrifuge tube) was collected and mixed in a ratio of 3:7 (v/v) with the dilution buffer (25 mM Tris-HCl, pH7.5, 100 mM NH₄Cl, 15 mM MgCl₂, 7 mM β-mercaptoethanol). For the second ultracentrifugation step, 17 ml of the diluted fractions from the bottom layers of the previous step was overlayered onto 14 ml of a second cushion (Cushion II: 25 mM Tris-HCl, pH7.5, 15 mM MgCl₂, 1 mM EDTA, 1.8 M Sucrose, 0.86 M CsCl, 7 mM β-mercaptoethanol) in each tube. After ultracentrifugation at 28,000 rpm for 28 hr, the bottom layer (~10 ml) from each tube was collected and dialyzed overnight against 3 L
of the dialysis buffer (25 mM Tris-HCl, pH7.5, 150 mM NH₄Cl, 15 mM MgCl₂, 7 mM β-mercaptoethanol).

After the dialysis, 4M (NH₄)₂SO₄ was added in a ratio of 1:3 (v/v) to the ribosome solution. The ribosome was further purified on a HiTrap Butyl FF column on an AKTA system (GE healthcare, Piscataway, NJ), pre-equilibrated with the butyl buffer A (25 mM Tris-HCl, pH7.5, 400 mM NH₄Cl, 15 mM MgCl₂, 1M (NH₄)₂SO₄, 7 mM β-mercaptoethanol). After washing the column with the butyl buffer A, the ribosome was eluted with 0-100% linear gradient of the butyl buffer B (25 mM Tris-HCl, pH7.5, 400 mM NH₄Cl, 15 mM MgCl₂, 7 mM β-mercaptoethanol). The fractions containing the ribosome were dialyzed against the ribosome dialysis buffer (25 mM Tris-OAc, pH7.5, 200 mM KOAc, pH7.5, 75 mM NH₄OAc, 25 mM Mg(OAc)₂, 7 mM β-mercaptoethanol) overnight. The purified ribosome was concentrated to an appropriate concentration and stored frozen in aliquots at -80°C. The final concentration of the purified ribosome was measured at A260.

Total *T. thermophilus* tRNA was prepared according to the established protocol (83). Briefly, the top layer from the first ultracentrifugation step described above was mixed with an equal volume of the saturated phenol solution (pH 6.6) and incubated at 37°C for 30 min. After centrifugation at 13,000 g for 15 min, the upper layer was collected and subject to a second phenol extraction and centrifugation step. The nucleic acids (DNA and RNA) in the upper layer were then precipitated by adding 0.1 volume of 3M sodium acetate (pH5.3) and 2.5 volume of cold ethanol, and incubating overnight at -20°C.

Following centrifugation at 13,000 g for 30 min, the pellet was dried and then dissolved in 70 ml of 0.3 M sodium acetate (pH7.0). To precipitate DNA and high molecular weight RNA, isopropanol (38 ml) was added slowly in drops with a needle at 4°C. after centrifugation at 13,000 g for 30 min, the supernatant (~108 ml) was collected and mixed with 31 ml of isopropanol. Following centrifugation at 13,000 g for 30 min, pellet of the precipitated tRNA was dried and dissolved in H₂O. The final concentration of total tRNA was measured at A₂₆₀.

**Poly(Phe) assay**
The poly(Phe) assay (Fig. S8) was conducted in a reaction mixture (50 µl) containing 4 µM EF-Tu, 0.4 µM EF-Ts, 0.66 µM EF-G, 0.75 µM PheRS, 0.02 mg/ml pyruvate kinase, and 2 µM *T. thermophilus* tRNA<sub>Phe</sub>, 25 mM Tris-HCl, pH7.5, 100 mM NH₄OAc, 10 mM Mg(OAc)<sub>2</sub>, 1 mM DTT, 1.6 mM ATP, 1.6 mM GTP, 2 mM PEP, 0.21 mM Phe, 32.8 µM [¹⁴C]Phe (GE healthcare, Piscataway, NJ), and 0.5 or 2 mM spermine. After pre-incubation at 65°C for 30 min, 0.2 mg/ml poly(U) (Sigma, St. Louis, MO) and 25 pmol ribosome were added to the reaction mixture and the reaction continued at 65°C for 8 hr. As control reactions, no spermine or ribosome was added in the reaction mixture. Aliquots were taken at different time points and deposited on 3 MM filter discs. After soaking in 5% TCA on ice for 5 min, the filter discs were washed twice with 5% TCA at 90°C for 10 min. The filter discs were dried and the radioactivity was measured in a scintillation counter.

**Protein Identification using MS and MS/MS**

Protein samples were directly digested with Trypsin (New England Biolabs) and then analyzed by online nanoESI-MS/MS using an Agilent 6330 Ion Trap mass spectrometer with an integrated C18 Chip/nanoESI interface as described in detail by Swaim et al. (84). The in vitro translation reactions (with or without stGFP mRNA) (4 ml) were added to 25 ml of 1X Trypsin Buffer (New England Biolabs, Inc.) and digested with 500 ng (5 ml of 100 ng/ml stock) of Modified (TPCK-treated) Trypsin (New England Biolabs, Inc.). Injections of 4 ml of this digestion were subjected to nanoESI-MS/MS and data collected. The MS/MS data were analyzed using Spectrum Mill (Agilent Technologies). Peptides generated by a tryptic digest were searched against the sequences of stGFP and a metagenome constructed from all *Thermus thermophilus* entries in the NCBI database (26,403 entries on May 7, 2012) as described in Swaim et al. (84). The peptide identifications were validated using a reverse database search. Proteins scoring greater than 20 were considered valid identifications and were combined to generate the final lists of identified proteins (Tables S2 and S3).

**Testing the functional conservation of resurrected ancient elongation factors in the reconstituted *E. coli* system**
The functions of purified ancient elongation factors were also examined in the reconstituted *E. coli* system synthesizing stGFP (Fig. S10). In this case, Ec Tu was replaced by the equal amount of each ancient elongation factor. Since Tt Tu did not function well with Ec Ts (Fig. 4B) and we expected a similar case for the ancient Tu proteins, Ec Ts was replaced by the equal amount of Tt Ts. The reactions were conducted in a 384-well microplate (Corning, Lowell, MA) at 37°C for 8 hr in Spectramax M5 microplate reader (Molecular Devices, Sunnyvale, CA). The maximal fluorescence values were used to determine the relative protein synthesis yields.