Supplementary data

Multisite-specific archaeosine tRNA-guanine transglycosylase (ArcTGT) from *Thermoplasma acidophilum*, a thermo-acidophilic archaeon

Takuya Kawamura¹, Akira Hirata¹, Satoshi Ohno², Yuichiro Nomura², Tomoko Nagano¹, Nobukazu Nameki³, Takashi Yokogawa², and Hiroyuki Hori¹

¹Department of Materials Science and Biotechnology, Graduate School of Science and Engineering, Ehime University, Bunkyo 3, Matsuyama, Ehime 790-8577, Japan
²Department of Biomolecular Science, Faculty of Engineering, Gifu University, Yanagido 1-1, Gifu, Gifu 501-1193, Japan
³Department of Applied Chemistry and Biochemistry, Faculty of Engineering, Gunma University, Tenjin 1-5-1, Kiryu, Gunma 376-8515, Japan
Outline of genetic manipulations using *Thermococcus kodakarensis*

*Thermococcus kodakarensis* is a hyperthermophilic euryarchaeon and can grow at 60-100°C under anaerobic conditions (1, 2). The medium for *T. kodakarensis* requires supplementation with starch, chitin, or peptides as a carbon source and S\(^0\) or H\(^+\) for anaerobic respiration (ref. 2 and see Supplementary Materials and Methods section).

*Thermococcus kodakarensis* is naturally competent for DNA uptake and incorporates donor DNA into its genome by homologous recombination (3, 4). The unique nature of *T. kodakarensis* enabled the development of various selective markers and genetic techniques (5, 6). In this study, we developed a new *T. kodakarensis* strain KUWA (SFig. 1), and this strain was used for the construction of the *arcTGT* gene disruption (∆*arcTGT*) strain. The KUWA strain was derived from *T. kodakarensis* strain KUW1 (2, 3, and STable 1). Because the KUWA strain is auxotrophic for uracil, tryptophan, and agmatine, multiple gene selection is possible (STable 1). In this study, we used the auxotrophy for uracil to construct the ∆*arcTGT* and Ta1493 complimentary (KTA1493) strains, and the auxotrophy for agmatine to supply tRNA\(^{Leu}\) genes using plasmid vectors.

The agmatine auxotrophy-selection system was previously devised by using the *pdaD* gene (Tk0149: arginine decarboxylase) as a selection marker (7). The *pdaD* gene is essential for cell viability (8) and agmatine is not included in the rich and synthetic media (MA-YT and ASW-AA media). We disrupted the *pdaD* gene in the genome of strain KUW1 by a two-step method. In the first step, we performed the so-called “single crossover insertion” (9), which introduces the entire plasmid with the *pyrF* marker (Tk2276: orotidine 5'-phosphate decarboxylase) into the genome (SFig. 1). The candidate clones were selected in the absence
of uracil, which is synthesized by PyrF. In the second step, the plasmid region (Tk0147-Tk0148-pdaD-Tk0150-pyrF) in the genome was removed by the so-called “pop-out recombination” (9) process in the presence of 5-fluoroorotic acid (SFig. 1). Because 5-fluorouracil synthesized from 5-fluoroorotic acid by PyrF is toxic, the colonies without the pyrF gene can survive and the pdaD gene in the resultant strain is disrupted (SFig. 1). We named the constructed strain (ΔpyrF, ΔtrpE, ΔpdaD) as KUWA (STable 1).

Thermococcus kodakarenis arcTGT (Tk0760) gene disruption (ΔarcTGT) strain was constructed from the strain KUWA by the same method (SFig. 2). A plasmid vector, pUDΔarcTGT (SFig. 2), was used in this construction instead of pUDpdaD (SFig. 1).

The KTA1493 strain was constructed by homologous recombination (SFig. 3). The pyrF and Ta1493 genes were introduced into the Tk1765 (chiA) region. Tk1765 gene encodes chitinase. The deletion of Tk1765 gene does not cause any growth defect unless chitin is used as a carbon source (10).

The variants of T. acidophilum tRNALeu were constitutively expressed using plasmid vectors as described in the main text. The plasmid vectors for supplying the tRNALeu gene were constructed from the pTK02 vector (7).
MATERIALS AND METHODS

The strains and plasmids used in this study are listed in Supplementary Table 1. The primers used for genetic manipulations in *T. kodakaren*sis are listed in Supplementary Table 2.

Strain and growth media

*Thermococcus kodakaren*sis strain KUW1 was a kind gift from Dr. Tamotsu Kanai (Kyoto University). *Thermococcus kodakaren*sis strains were cultured under anaerobic conditions at 60°C or 85°C in a nutrient-rich medium (MA-YT) or a synthetic medium (ASW-AA). MA-YT medium (1 L) contains 0.8 × Marine Art SF1 reagent (Osaka Yakken Co. Ltd., Osaka, Japan), 5 g yeast extract (Y), and 5 g tryptophan (T). Sodium pyruvate (5 g) or elemental sulfur (2 g) was supplied into 1 L MA-YT medium prior to culture. ASW-AA medium is a synthetic medium that contained a mixture of vitamins, modified Wolfe’s trace minerals and the 20 canonical amino acids dissolved in 0.8 × artificial seawater (2, 3). Elemental sulfur (2 g) was added into 1 L ASW-AA medium prior to culture. For colony isolation, solid ASW-YT and MA-YT media containing 1 g of gelrite and 0.4 g of polysulfide per 0.1 L were used.

Construction of *T. kodakaren*sis strain KUWA

To construct the plasmid vector, pUDPdaD (SFig. 1, SFig. 4 and STable 1), for the Tk0149 (*pdaD*) gene disruption in *T. kodakaren*sis strain KUW1, the *pdaD* gene with the 5′- and 3′-flanking regions (1,000 base pairs each) was amplified from the *T. kodakaren*sis genomic DNA
by polymerase chain reaction (PCR) using a primer set (AgF/AgR) (SFig. 4 and STable 2). The amplified DNA was inserted downstream of the pyrF gene in a plasmid vector, pUD3 (11). Next, reverse PCR was performed with a primer set (delAgF/delAgR) to remove the pdaD gene. The amplified fragment was then self-ligated to generate the plasmid, pUDpdaD (SFig. 1 and SFig. 4). pUDpdaD was used to transform T. kodakarensis strain KUW1, and transformants were cultured in a uracil-free ASW-AA liquid medium containing agmatine at 85°C for 24 h. The cultures were then spread onto an ASW-AA solid medium supplemented with 5-fluoroorotic acid. Single colonies were picked up and then cultured in MA-YT medium containing agmatine at 85°C for 12h. The cells were harvested and then suspended in distilled water. Genomic DNA was extracted from the cells by phenol-chloroform treatment. DNA sequencing of the recombination region was performed.

Construction of the ΔarcTGT strain

To construct the plasmid vector, pUDΔarcTGT (SFig. 2, SFig. 5 and STable 1), for the Tk0760 (arcTGT) gene disruption in T. kodakarensis strain KUW1, the arcTGT gene with the 5´- and 3´- flanking regions (1,000 base pairs each) was amplified from the T. kodakarensis genomic DNA by PCR using a primer set (arcTGTF/arcTGTR) (SFig. 5 and STable 2). The amplified DNA was inserted downstream of the pyrF gene in a plasmid vector, pUD3 (11). Next, reverse PCR was performed with a primer set (delarcTGTF/delTGTR) to remove the arcTGT gene. The amplified fragments were then self-ligated to generate the plasmid, pUDarcTGT (SFig. 5). The pUDΔarcTGT was used to transform T. kodakarensis strain KUWA. Transformants were cultured in a uracil-free ASW-AA liquid medium containing agmatine at 85°C for 24 h. The
cultures were then spread onto an ASW-AA solid medium supplemented with 5-fluoroorotic acid. Single colonies were picked up and then cultured in MA-YT medium containing agmatine at 85°C for 12h. The cells were harvested and suspended in distilled water. Genomic DNA was extracted from the cells by phenol-chloroform treatment. DNA sequencing of the recombination region was performed.

**Construction of the KTA1493 strain**

The conditional expression system in *T. kodakarensis* was previously developed by Hirata *et al* (10). The *trpE*-fructose 1, 6-bisphosphatase (FBPase) promoter-based selection-expression cassette in pCTF (STable 1) was modified as follows: the *trpE* selection marker in pCTF was replaced with the *pyrF* gene and the resultant plasmid was designated as pCTP (SFig. 6 and STable 1). To express the Ta1493 gene product in the *ΔarcTGT* strain, we constructed the Ta1493 gene expression cassette by using the pCTP. The Ta1493 gene was amplified by PCR from *T. acidophilum* genomic DNA with a primer set TAIF/TAIR (STable 2). The amplified fragment was inserted downstream of the FBPase promoter in the pCTP by the in-fusion reaction (SFig. 7). The resultant plasmid is designated as pCTTA (SFig. 3, SFig. 7 and STable 1). The pCTTA was used to transform the *ΔarcTGT* strain, and the transformants were selected at 85°C on ASW-AA medium plates, which contains agmatine but not uracil. The introduction of Ta1493 gene into the genome of *ΔarcTGT* strain was confirmed by PCR and DNA sequencing.

**Expression of the *T. acidophilum* wild-type and mutant tRNA\textsuperscript{Leu} species in the KTA strain**
The replicable plasmid in *T. kodakarensis*, pTK02, was a gift from S. Fujiwara, Kwansei Gakuin University in Japan (7). DNA fragments that carried the *T. acidophilum* tRNA^{Leu} gene (TA_RS03210) with artificial 5′- and 3′- flanking regions (15 base pairs each), were amplified by PCR from *T. acidophilum* genomic DNA with a primer set (LeuWF/LeuWR) (SFig. 8 and STable 2). The LeuWF and LeuWR primers include the promoter sequence of the glutamate dehydrogenase (Tk1431) gene and the terminator sequence of chitinase (Tk1765) gene, respectively. The amplified fragment was inserted between the Ndel and NotI sites in pTK02 and the resultant plasmid was named wild-type pTK02-tRNA^{Leu} (SFig. 8 and STable 1). The plasmid vectors for the expression of *T. acidophilum* tRNA^{Leu} variants (A13G15, G13A15 and A13A15) were constructed by the same method using primer sets Leu13F/LueWR, Leu15F/LeuWR, and Leu13/15F/LeuWR, respectively. The resultant plasmids were designated pTK02-tRNA^{Leu} (A13G15), pTK02-tRNA^{Leu} (G13A15), and pTK02-tRNA^{Leu} (A13A15), respectively (SFig. 8 and STable 1). These plasmids were used to transform the KUWA or KTA1493 cells and the transformants were selected at 85°C on MA-YT medium plates in the absence of agmatine.
Supplementary Figure 1. Construction of *T. kodakarensis* strain KUWA (ΔpyrF, Δ*trpE* and ΔpdaD). *Thermococcus kodakarensis* strain KUW1 (ΔpyrF and Δ*trpE*) was used as the host strain and the *pdaD* gene in the genome was disrupted in two steps. In the first step, the entire region of the plasmid vector, pUD*pdaD* was introduced into the genome by homologous recombination (single crossover insertion). In the second step, the plasmid region, which contains Tk0147 to *pyrF* gene, was deleted by culture in the presence of 5-fluoroorotic acid (pop-out recombination). As a result, the *pdaD* gene (Tk0149) was deleted from the genome of the strain KUW1.
Supplementary Figure 2. Construction of the \( \Delta \text{arcTGT} \) strain. The \( \text{arcTGT} \) gene (Tk0760) in the genome of the \( T. \ kodakarensis \) strain KUWA was disrupted in two steps. In the first step, the entire region of the plasmid vector, \( \text{pUD}\Delta \text{arcTGT} \) was introduced into the genome by homologous recombination (single crossover insertion). In the second step, the Tk0759-\( \text{pyrF} \) gene region derived from the plasmid was deleted by culture in the presence of 5-fluoroorotic acid (pop-out recombination). As a result, the \( \text{arcTGT} \) gene (Tk0760) was deleted from the genome of the strain KUWA.
Supplementary Figure 3. Construction of the KTA1493 strain. The \( \Delta arcTGT \) strain was used as the host strain and homologous recombination with a plasmid vector, pCTTA, in which the Ta1493 gene was inserted between a part of the TK1765 gene with the \( pyrF \) gene and the Tk1766 gene. The vector, pCTTA, is not maintained as a plasmid in \( T. kodakarensis \) cells due to the lack of a replication origin. Given that the Tk1765 (\( chiA \)) gene encodes chitinase, the deletion of Tk1765 gene does not cause any growth defect unless chitin is used as a carbon source. In this system, the Ta1493 gene product was constitutively expressed under the fructose-1, 6-bisphosphatase (FBPase) promoter.
**Supplementary Figure 4.** Construction of pUDpdaD. The *pdaD* gene with the 5’- and 3’-flanking regions (1,000 base pairs each) was inserted in a plasmid vector, pUD3. Reverse PCR was performed using a primer set (delAgF/delAgR) to remove the *pdaD* gene and the amplified fragments were self-ligated to generate a plasmid, pUDpdaD.
Supplementary Figure 5. Construction of pUDΔarcTGT. The arcTGT (Tk0760) gene with the 5´- and 3´- flanking regions (1,000 base pairs each) was inserted in a plasmid vector, pUD3. Reverse PCR was performed using a primer set (delarcTGF/delTGR) to remove the arcTGT gene and the amplified fragment was self-ligated to generate a plasmid, pUDΔarcTGT.
**Supplementary Figure 6.** Construction of pCTP. The *pyrF* gene with its promoter was amplified from the *T. kodakarensis* genomic DNA by PCR. The *trpE* region in pCTF was replaced by the amplified DNA. The resultant plasmid was designated as pCTP.
**Supplementary Figure 7.** Construction of pCTTA used for complementation with the Ta1493 gene product. The Ta1493 gene was inserted at the downstream of the FBPase promoter in the pCTP by the in-fusion reaction. The resultant plasmid was named pCTTA.
Supplementary Figure 8. Construction of tRNA^{Leu} expression vectors. DNA fragments that carried the *T. acidophilum* tRNA^{Leu} gene (TA_RS03210) with artificial 5′- and 3′- flanking regions was inserted between the NdeI and NotI sites in pTK02 and the resultant plasmid was named wild-type pTK02-tRNA^{Leu}. The plasmid vectors for the expression of *T. acidophilum* tRNA^{Leu} variants (A13G15, G13A15 and A13A15) were constructed by the same method.
Supplementary Figure 9. (A) Cloverleaf structure of *T. kodakarensis* tRNA<sup>Leu</sup>. The hybridization region with DNA probe is illustrated. (B) The total RNA from the KTA1493 strain and purified tRNA<sup>Leu</sup> fraction were analyzed by 10% PAGE (7 M urea). (C) The tRNA<sup>Leu</sup> fraction was digested with RNase A and then its fragment was analyzed by MS spectrometry. (D) The sequence of fragment (m/z = 1343.2) was determined as GAGCp by MS/MS analysis.
Supplementary references

1. Morikawa, M., Izawa, Y., Rashid, N., Hoaki, T. and Imanaka, T. (1994) Purification and characterization of a thermostable thiol protease from a newly isolated hyperthermophilic Pyrococcus sp. *Appl. Environ. Microbiol.* **60**, 4559-4566.

2. Atomi, H., Fukui, T., Kanai, T., Morikawa, M. and Imanaka, T. (2004) Description of *Thermococcus kodakaraensis* sp. nov., a well studied hyperthermophilic archaeon previously reported as *Pyrococcus* sp. KOD1. *Archaea* **1**, 263-267.

3. Sato, T., Fukui, T., Atomi, H. and Imanaka, T. (2003) Targeted gene disruption by homologous recombination in the hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1. *J. Bacteriol.* **185**, 210-220.

4. Sato, T., Fukui, T., Atomi, H. and Imanaka, T. (2005) Improved and versatile transformation system allowing multiple genetic manipulations of the hyperthermophilic archaeon *Thermococcus kodakaraensis*. *Appl. Environ. Microbiol.* **71**, 3889-3899.

5. Atomi, H., Sato, T. and Kanai, T. (2011) Application of hyperthermophiles and their enzymes. *Curr. Opin. Biotechnol.* **22**, 618-626.

6. Hileman, T.H. and Santangelo, T.J. (2012) Genetics Techniques for *Thermococcus kodakarensis*. *Front. Microbiol.* **3**, 195.

7. Nagaoka, E., Hidese, R., Imanaka, T. and Fujiwara, S. (2013) Importance and determinants of induction of cold-induced DEAD RNA helicase in the hyperthermophilic archaeon *Thermococcus kodakarensis*. *J. Bacteriol.* **195**, 3442-3450.

8. Fukuda, W., Morimoto, N., Imanaka, T. and Fujiwara, S. (2008) Agmatine is essential for the cell growth of *Thermococcus kodakarensis*. *FEMS Microbiol. Lett.* **287**, 113-120.

9. Atomi, H., Imanaka, T. and Fukui, T. (2012) Overview of the genetic tools in the Archaea. *Front. Microbiol.* **3**, 337.

10. Hirata, A., Kanai, T., Santangelo, T.J., Tajiri, M., Manabe, K., Reeve, J.N., Imanaka, T. and Murakami, K.S. (2008) Archaeal RNA polymerase subunits E and F are not required for transcription in vitro, but a *Thermococcus kodakarensis* mutant lacking subunit F is temperature-sensitive. *Mol. Microbiol.* **70**, 623-633.

11. Yokooji, Y., Tomita, H., Atomi, H. and Imanaka, T. (2009) Pantoate kinase and phosphopantothenate synthetase, two novel enzymes necessary for CoA biosynthesis in the Archaea. *J. Biol. Chem.* **284**, 28137-28145.
Supplementary Table 1. Strains and plasmids used in this study

| Strain and plasmid | Relevant characteristics | Source or reference |
|--------------------|--------------------------|---------------------|
| **Strains**        |                          |                     |
| *T. kodakarensis*  |                          |                     |
| KUW1               | ΔpyrF, ΔtrpE             | (3)                 |
| KUWA               | ΔpyrF, ΔtrpE, ΔpdaD      | This study          |
| ΔarcTGT            | KUWA ΔTk0760            | This study          |
| KTA1493            | KUWA ΔTk0760, ΔchiA::pyrF-P_{tk2164-Ta1493} | This study |
| **Plasmids**       |                          |                     |
| pUD3               | pUC118 derivative, pyrF marker cassette (P_{pyrF-pyrF}) | (9)                 |
| pUDP_{pdaD}        | pUD3 derivative, P_{pyrF-pyrF-Tk0148-Tk0150} | This study          |
| pUDP_{ΔarcTGT}     | pUD3 derivative, P_{pyrF-pyrF-Tk0759-Tk0761} | This study          |
| pCTF               | Tk1765::trpE-P_{tk2164-rpoF-Tk1766} in pUC18 | (7)                 |
| pCTP               | Tk1765::pyrF-P_{tk2164-rpoF-Tk1766} in pUC18 | This study          |
| pCTTA              | Tk1765::pyrF-P_{tk2164-Ta1493-Tk1766} in pUC18 | This study          |
| pTK02              | pdaD cloned in pTK01    | (10)                |
| pTK02-tRNA\text{Leu} (Wild-type) | pTK02 derivative, *T. acidophilum* tRNA\text{Leu} (Wild-type) | This study          |
| pTK02-tRNA\text{Leu} (A13G15) | pTK02 derivative, *T. acidophilum* tRNA\text{Leu} mutant (A13G15) | This study          |
| pTK02-tRNA\text{Leu} (G13A15) | pTK02 derivative, *T. acidophilum* tRNA\text{Leu} mutant (G13A15) | This study          |
| pTK02-tRNA\text{Leu} (A13A15) | pTK02 derivative, *T. acidophilum* tRNA\text{Leu} mutant (A13A15) | This study          |
### Supplementary Table 2. Primers used in this study

| Primer name      | Primer sequence                                      |
|------------------|------------------------------------------------------|
| **Construction of plasmids for gene disruption strains in *T. kodakarensis*** |
| agF              | 5'-CCAGTGCCAAGCTGCGCACCACAACAGATAACATCCAAATCC-3'     |
| agR              | 5'-ATGATTACGAATCCGAGCGTGCGATTATCAGCT-3'              |
| delagF           | 5'-CTTCTTTCTTTTCTACCCAAAGTGATCCCTTTCCC-3'           |
| delagR           | 5'-CTCAACCTCTCCTTTTCTCTAGGATACC-3'                  |
| arcTGTF          | 5'- CCAGTGCCAAGCTGCGTACATGAAACTATAGACACAATA -3'     |
| arcTGTR          | 5'- ATGATTACGAATCCGACCTCAGGAATATGCGG-3'             |
| delarcTGTF       | 5'- AAAAAATATAAAACCCTCTCTTTTTC -3'                  |
| delarcTGTR       | 5'- TTCTCACCTACCTGGAATGGAA -3'                      |
| **Construction of plasmid for complementation with Ta1493 gene** |
| TAIF             | 5'-CCGGTGGTTATCATGAAAGATAGGAAAGGGACGG -3'           |
| TAIR             | 5'-AGAAGAGAGGGTCACTATTTCCTGATCTGC -3'               |
| **Construction of plasmids for expression of tRNA^{Leu} (Wild-type, A13G15, G13A15 and A13A15)** |
| LeuWF            | 5'- AACCACCTCCATATGGTTATCGAAAAAGGTTATATATGCAAACGCTAAGTGAATCTCAGGAAATATGACAAACGGAGTTGTCGAGACTCG -3' |
| LeuWR            | 5'- TGCTGACGGCCTGCTGTCTCTCAATGACCAAAAGAGGAGAGGAGGAGGGTCATTACATAAAATGCTGAGGAGGG -3' |
| Leu13F           | 5'- AACCACCTCCATATGGTTATCGAAAAAGGTTATATATGCAAACGCTAAGTGAATCTCAGGAAATATGACAAACGGAGTTGTCGAGACTG -3' |
| Leu15F           | 5'- AACCACCTCCATATGGTTATCGAAAAAGGTTATATATGCAAACGCTAAGTGAATCTCAGGAAATATGACAAACGGAGTTGTCGAGACTG -3' |
| Leu13/15F        | 5'- AACCACCTCCATATGGTTATCGAAAAAGGTTATATATGCAAACGCTAAGTGAATCTCAGGAAATATGACAAACGGAGTTGTCGAGACTG -3' |

Underlined sequences show the regions used for the connection of DNA fragments by the in-fusion reaction.