Supporting Information

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SI Materials and Methods

Media and Culture Conditions for *Methanococcus maripaludis* Growth. *M. maripaludis* was grown at 37 °C in 28-mL aluminum seal tubes with 5 mL McC (rich medium) or McNA (minimal medium, 10 mM sodium acetate, 1 mM L-Ala) (1) reduced with 3 mM 2-mercaptoethanesulfonate. Tubes were pressurized with 275 kPa of H_2:CO_2 (4:1, vol/vol). The 1.5-L cultures were grown in 2-L bottles with 0.2 M sodium formate buffered with 0.2 M glycylglycine (pH 7.0). Puromycin (2.5 μg/mL) and neomycin (0.5 mg/mL in plates and 1 mg/mL in broth) were added when needed. When comparing the growth of the WT and mutants, antibiotics were omitted and the inoculum size was ~10^6 cells per 5 mL culture. Before inoculation, 3 mM sodium sulfide was added as the sulfur source.

Mutagenesis of *M. maripaludis*. The replacement of the *sepS* gene (*mmp0688*), encoding *O*-phosphoseryl-tRNA synthetase (SepRS), with a puromycin resistance (*pac*) cassette (2) was generated in the *M. maripaludis* Δ*hpt* strain Mm900 (3) by homologous recombination. Then the *pac* cassette was removed by transforming the Δ*sepS*::*pac* strain with the plasmid pFLPH, which encodes the Flp recombinase under the control of the *hmvA* promoter (4). Transformants were grown for 5–10 consecutive passages and screened by PCR for the removal of *pac* (4). The replacement of the *scsE* gene (*mmp1217*), encoding the SepRS/Sep-tRNA:Cys-tRNA synthase (SepCysS) pathway enhancer (SepCysE), with *pac* was generated in the markerless Δ*sepS* strain. The genotype of the Δ*sepS*/Δ*scsE*::*pac* strain (S761) was confirmed by Southern hybridization. The *scsE*$^-$ strain (S762) was obtained by transformation of S761 with pMEV2-*mmp0688* that was constructed for expression of N-terminal His_6-tagged SepRS under the control of the *hmvA* promoter (4). The *sepS*$^-$ strain (S763) was obtained by transformation of S761 with pMEV2-*mm1217* that was constructed for expression of N-terminal His_6-tagged SepCysE under the control of the *sla* promoter (5). The *sepS*$^+$*scsE*$^+$ strain (S764) was obtained by transformation of S761 with pMEV2-*mmp0688-mmp1217* that was constructed for expression of C-terminal His_6-tagged SepRS and SepCysE under the control of the *hmvA* and the *sla* promoter, respectively. The expression of His-tagged SepRS and SepCysE was confirmed by Western blot using an HRP-conjugated anti-His antibody (Thermo Fisher). The mutations of MMP1217—including the variants of K50A/Y54A/E58A/D60A (mut1), E58A/D60A/D61A/Y64A (mut2), and D61A/Y64A/K65A/K66A (mut3)—were constructed with the plasmid pMEV2-*mmp0688-mmp1217* using a QuikChange mutagenesis kit (Agilent Technologies) and then transformed into S761.

Pull-Down Assay and Protein Identification by Mass Spectrometry. The N-terminal His_6-tagged *pscS* (*mmp1240*), encoding SepCysS, was cloned into the vector pMEV2 (6) and transformed into the *M. maripaludis* WT strain S2 using the PEG method (7). The metal affinity purification was performed in an anaerobic chamber with an atmosphere of 95% (vol/vol) N_2 and 5% (vol/vol) H_2. Cells harvested from 1.5 L cultures were resuspended in 10 mL binding buffer [20 mM sodium 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (Hepes), 0.1 M NaCl, 5 mM $MgCl_2$, 20 mM imidazole, pH7.5], and then disrupted by twice freezing (−80 °C) and thawing. DNA and RNA were digested with 10 U of Benzonase Nuclease (Sigma). The cell lysate was centrifuged at 100,000 × *g* for 30 min at 4 °C, and the supernatant was applied to 1 mL TALON Metal Affinity Resin (Clontech). Proteins bound to the column were eluted with 10 mL elution buffer [20 mM sodium Hepes (pH 7.5), 0.1 M NaCl, 5 mM $MgCl_2$, 0.2 M imidazole]. The purified proteins were separated by SDS/PAGE and stained with silver. The protein bands were excised, in-gel digested with trypsin, and analyzed by liquid chromatography mass spectrometry at the Keck Mass Spectrometry and Proteomics Resource Laboratory (Yale University).

Purification of SepRS, SepCysS, and SepCysE for Gel Filtration and Biochemical Assays. The *Methanocaldococcus jannaschii sepS* (MJ1660) and *pscS* (MJ1678) genes with an N-terminal His_6-tag were cloned into the pRSF and pET15b (Novagen) vectors, respectively. The *M. jannaschii scsE* (MJ1481) gene with a C-terminal His_6-tag was cloned into the pDCH vector that is derived from the pCDF-Duet vector (Novagen).

For protein expression, the expression vectors were transformed into the *Escherichia coli* Rosetta 2(DE3) strain (Novagen). The transformants were grown in 1 L LB medium at 37 °C with shaking until they reached an absorbance at 600 nm of 0.6 to ~0.8. Then 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to induce overnight production at 25 °C. For anaerobic protein purification, the harvested *E. coli* cells were transferred into the anaerobic chamber [atmosphere of 95% (vol/vol) N_2 and 5% (vol/vol) H_2] and resuspended in 10 mL binding buffer [50 mM sodium Hepes (pH 7.5), 0.3 M NaCl, 5 mM $MgCl_2$, 20 mM imidazole]. Cells were disrupted by sonication in the presence of 0.5 mg/mL lysozyme and 0.1 mg/mL DNase I. *E. coli* host proteins were denatured at 70 °C for 30 min, and then the cell lysate was centrifuged at 100,000 × *g* for 30 min at 4 °C. All of the following processes were carried out at room temperature. The clarified supernatant was applied to 1 mL TALON Metal Affinity Resin (Clontech) equilibrated with the binding buffer. Proteins bound to the column were eluted with the elution buffer [50 mM sodium Hepes (pH 7.5), 0.3 M NaCl, 5 mM $MgCl_2$, 0.2 M imidazole] and dialyzed against the storage buffer [50 mM sodium Hepes (pH 7.5), 0.3 M NaCl, 5 mM $MgCl_2$, 20% (vol/vol) glycerol]. The purified proteins were stored at −80 °C until use.

Analytical Gel Filtration. The protein samples (10 μM of each protein subunit in 200 μL) were loaded onto a 24-mL Superdex 200 10/300 GL column (GE Healthcare) equilibrated with the running buffer [50 mM sodium Hepes (pH 7.5), 0.3 M NaCl, 5 mM $MgCl_2$]. The protein complexes were separated by fast protein liquid chromatography at a flow rate of 0.5 mL/min. Pooled fractions were concentrated by ultrafiltration before SDS/PAGE analysis.

Preparation of Labeled $tRNA^{Cys}$. The *M. jannaschii* $tRNA^{Cys}$ substrate was synthesized by in vitro T7-RNAP run-off transcription as described (8). Before use, the tRNA transcripts were folded by heating at 80 °C for 5 min, cooling down slowly to 45 °C, and further cooling on ice in the presence of 5 mM $MgCl_2$. Refolded transcript was ^{32}P-labeled on the 3′ terminus using the *E. coli* CCA-adding enzyme and [α-^{32}P]ATP as described (9).

Measurement of Protein Binding Affinity to $tRNA^{Cys}$. The affinity of proteins for $tRNA^{Cys}$ was measured by the filter-binding assay (10), in which 5 nM ^{32}P-labeled *M. jannaschii* $tRNA^{Cys}$ was incubated at room temperature for 30 min with the *M. jannaschii* proteins ranging from 0.5 nM to 10 μM in 25 μL binding buffer [50 mM sodium Hepes (pH 7.5), 0.3 M NaCl, 5 mM $MgCl_2$]. Then the reaction mixtures were filtered through a nitrocellulose membrane and an underlying Hybond-N^+ membrane (GE

Healthcare) with a 96-well vacuum manifold (Hybri-dot 96; Whatman Biometra). Aliquots of 7 μL from each binding reaction were spotted in triplicate and washed with 200 μL binding buffer at room temperature. The protein·tRNA complexes (bound to the nitrocellulose membrane) and the free $tRNA^{Cys}$ (bound to the Hybond membrane) were then exposed to imaging plates (Fuji Films), which were scanned on a Molecular Dynamics Storm 860 Phosphoimager and quantified using the ImageQuant software. The dissociation constants were calculated by fitting the data to the equation, [protein·tRNA complex] = ([total tRNA] × [protein])/(K_D + [protein]) using KaleidaGraph 4.0 (Synergy Software).

Measurement of Sep-$tRNA^{Cys}$ Formation. The activity of Sep-$tRNA^{Cys}$ formation was measured by Wolfson assay (11), in which 200 nM of the *M. jannaschii* enzymes (SepRS, SepRS+SepCysS, or SepRS·SepCysE·SepCysS) were incubated with 10 μM ^{32}P-labeled $tRNA^{Cys}$, 1 mM *O*-phosphoserine, 10 mM ATP, and 27 μg/mL pyrophosphatase (Roche) in 20 μL SepRS buffer [50 mM sodium Hepes (pH 7.5), 20 mM KCl, 10 mM $MgCl_2$] at 60 °C for 20 min. Time points were taken by removing 2-μL aliquots from the reaction followed by quenching with 3 μL of 0.66-mg/mL nuclease P1 (Sigma) in the 250 mM sodium citrate buffer (pH 4.5). Digestion was carried out for 30 min at room temperature. Then 1-μL aliquots were spotted on the polyethyleneimine-cellulose thin-layer chromatography (TLC) plates (EMD Chemicals) and developed in the running buffer containing 1 M ammonium acetate (pH 3.5) (12) for up to 120 min. The separated radioactive spots for AMP and Sep-AMP (derived from free $tRNA^{Cys}$ and Sep-$tRNA^{Cys}$, respectively) were then exposed to imaging plates (Fuji Films), which were scanned on a Molecular Dynamics Storm 860 Phosphoimager and quantified using the ImageQuant software. The relative positions of AMP and Sep-AMP were compared with those reported (12) under the same TLC separation condition. Kinetics were determined by varying tRNA concentrations from 0.5 to 20 μM in a 30-s reaction. Kinetic constants were derived from plotting the initial velocity versus [tRNA] and fitting to the Michaelis–Menten curve using KaleidaGraph 4.0 (Synergy Software).

Sep-$tRNA^{Cys}$ Hydrolysis Protection Assay. The ^{32}P-labeled $tRNA^{Cys}$ was acylated with Sep by the *M. jannaschii* SepRS·SepCysE·SepCysS complex and then purified by phenol/chloroform extraction. Hydrolysis of 10 μM Sep-$tRNA^{Cys}$ in the SepRS buffer [50 mM sodium Hepes (pH 7.5), 20 mM KCl, 10 mM $MgCl_2$] was monitored at 60 °C in the presence of 10 μM *M. jannaschii* SepRS, SepCysS, SepCysE, SepCysS·SepCysE, SepRS·SepCysE·SepCysS, or elongation factor 1A (EF1A). EF1A was activated as described (13) before addition to the protection assay solution. Time course and TLC procedures were the same as the Sep-$tRNA^{Cys}$ formation assay.

Measurement of Cys-$tRNA^{Cys}$ Formation. All steps of the Cys-$tRNA^{Cys}$ formation were performed in an anaerobic chamber with an atmosphere of 95% (vol/vol) N_2 and 5% (vol/vol) H_2. The *M. jannaschii* SepCysS (40 μM) was preincubated with 100 μM pyridoxal-5′-phosphate and 4 mM Na_2S in 5 μL SepRS buffer at 60 °C for 10 min. Then this mixture was supplemented with 10 μM ^{32}P-labeled $tRNA^{Cys}$, 10 μM SepRS or SepRS·SepCysE, 1 mM *O*-phosphoserine, 10 mM ATP, and 27 μg/mL pyrophosphatase (Roche) to a final volume of 20 μL in the SepRS buffer. In variation, 10 μM *M. jannaschii* EF1A was added to the reaction after activation as described (13). Reactions were carried out at 60 °C for 30 min. Time course and TLC procedures were the same as the Sep-$tRNA^{Cys}$ formation assay. The relative positions of AMP, Sep-AMP, and Cys-AMP (derived from free $tRNA^{Cys}$, Sep-$tRNA^{Cys}$, and Cys-$tRNA^{Cys}$, respectively) were compared with those reported (12) under the same TLC separation condition.

Preparation of the SepCysS·SepCysE Complex for Crystallization. The gene encoding the *M. jannaschii* SepCysE (MJ1481) was cloned into the pDCH vector derived from the pCDF-Duet vector (Novagen). The gene encoding the *M. jannaschii* SepCysS (MJ1678) with an N-terminal His_6-tag was cloned into the pET15b vector (Novagen). The *E. coli* strain B834 (DE3)-pRARE2 (Novagen) was cotransformed with these two plasmids. For protein expression, the transformants were grown in LB medium containing 50 μg/mL streptomycin, 100 μg/mL ampicillin, and 34 μg/mL chloramphenicol at 37 °C to an OD_{600} of 0.6. IPTG was then added to a final concentration of 0.5 mM to induce protein expression overnight at 25 °C. Then the cells were harvested by centrifugation at 4,000 × *g* for 15 min at 4 °C and disrupted by sonication in the binding buffer [50 mM sodium Hepes (pH 7.5), 300 mM NaCl, 5 mM $MgCl_2$] with 0.5 mg/mL lysozyme and 0.1 mg/mL DNase I. The homogenate was heat treated at 75 °C for 30 min to denature *E. coli* host proteins, and then clarified by centrifugation at 40,000 × *g* for 30 min at 4 °C. The following steps were carried out at room temperature. The clarified supernatant was loaded onto a HisTrap HP column (GE Healthcare) preequilibrated with the binding buffer. The column was washed with the binding buffer plus 20 mM imidazole, and proteins were eluted with a linear gradient of 20–250 mM imidazole. Pooled fractions were loaded onto a HiTrap Heparin column (GE Healthcare) and then eluted with a linear gradient from 300 mM to 1 M NaCl. The protein fractions were then loaded onto a HiLoad 26/60 Superdex 200 prep grade column (GE Healthcare) equilibrated with the buffer containing 20 mM sodium Hepes (pH 7.5) and 300 mM NaCl. Pooled fractions were concentrated by ultrafiltration to a final concentration of 10 mg/mL.

The vector for expression of the N-terminal domain of SepCysE (SepCysE_NTD, 1–103 aa) was constructed from the pDCH-SepCysE vector using a QuikChange mutagenesis kit (Agilent Technologies). The SepCysS·SepCysE_NTD complex was obtained with the same procedure as the SepCysS·SepCysE complex.

Crystallization and X-Ray Diffraction Data Collection. The crystals of both the SepCysS·SepCysE and the SepCysS·SepCysE_NTD complex were obtained by a sitting-drop vapor-diffusion method. The crystals of the SepCysS·SepCysE complex were obtained with a solution containing 180 mM ammonium citrate (pH 6.8) and 16% (wt/vol) PEG3350 at 20 °C. Then they were soaked into a reservoir solution containing 10% (vol/vol) glycerol. The diffraction dataset was collected on the beam line BL1A at the Photon Factory (Tsukuba, Japan) at −173 °C. The crystals of the SepCysS·SepCysE_NTD complex were obtained with a solution containing 100 mM succinic acid and 8% (wt/vol) PEG3350 at 20 °C. Then they were soaked into a reservoir solution containing 20% (vol/vol) glycerol. The diffraction dataset was collected on the beam line AR-NE3A at the Photon Factory (Tsukuba, Japan) at −173 °C. All data were processed using XDS (14). The crystal of SepCysS·SepCysE was merohedrally twinned with the twin operator (*h*, −*h*−*k*, −*l*), and the twin fraction was estimated to be 45% by Britton analysis using the program phenix.xtriage (15).

Structure Determination and Refinement. The SepCysS·SepCysE complex structure was solved by molecular replacement using Phaser (16) with the *Archaeoglobus fulgidus* SepCysS [Protein Data Bank (PDB) ID code 2E7J (17)] as a searching model. The SepCysS·SepCysE model was rebuilt using PHENIX AutoBuild (18) and LAFIRE (19) and modified manually using Coot (20). Structure refinement was performed using phenix.refine (21) and autoBUSTER (22). The twin operator (*h*, −*h*−*k*, −*l*) was applied during every round of refinement and the twin fraction was refined. The R_{work} and R_{free} factors were converged to

22.4% and 24.3%, respectively. The SepCysS·SepCysE_NTD structure was solved by molecular replacement using Phaser with the SepCysS·SepCysE structure as a searching model. After several cycles of refinement by phenix.refine and autoBUSTER and manual fitting by Coot, the R_{work} and R_{free} factors were converged to 18.9% and 24.0%, respectively. Data collection and refinement statistics are summarized in Table S1. All figures were prepared with PyMOL (The PyMOL Molecular Graphics System, Version 1.5.0.4).

Pull-Down Assays of Recombinant Proteins Expressed in *E. coli*. The expression vectors of SepCysE variants (with C-terminal His_6-tag), SepRS (tag-free), and SepCysS (tag-free) were constructed using a QuikChange mutagenesis kit (Agilent Technologies). The *E. coli* B834 (DE3)-pRARE2 strain was cotransformed with the vector pDCH-SepCysE together with pET15b-SepCysS or pET15b-SepRS. Cells were grown in 1 L LB medium and disrupted by sonication as described above. The homogenate was heat-treated at 70 °C for 30 min to denature *E. coli* host proteins, and then clarified by centrifugation at 40,000 × *g* for 30 min at 4 °C. The clarified supernatant was loaded onto 200 μL Ni-NTA resin (Qiagen) preequilibrated with the binding buffer plus 20 mM imidazole. After washing with 3 mL binding buffer plus 50 mM imidazole, the proteins were eluted with 500 mM imidazole and analyzed by SDS/PAGE.

Phylogeny and Bioinformatics. Protein homologs were identified using BLASTp searches (https://img.jgi.doe.gov/cgi-bin/w/main.cgi) against selected genomes. Sequence alignments were generated using the ClustalX 2.1 program (23). The sequence-based phylogenetic tree was constructed with MEGA5 (24) using the Minimum Evolution algorithm. Bootstrap analysis was performed with 1,000 replicates. For comparisons of ratios of evolutionary distances (RED) (25), the evolutionary distances (E_d) were calculated with MEGA5 using the PAM-t matrix with its default settings.

1. Whitman WB, Shieh J, Sohn S, Caras DS, Premachandran U (1986) Isolation and characterization of 22 mesophilic methanococci. *Syst Appl Microbiol* 7(2-3):235–240.
2. Gernhardt P, Possot O, Foglino M, Sibold L, Klein A (1990) Construction of an integration vector for use in the archaebacterium *Methanococcus voltae* and expression of a eubacterial resistance gene. *Mol Gen Genet* 221(2):273–279.
3. Moore BC, Leigh JA (2005) Markerless mutagenesis in *Methanococcus maripaludis* demonstrates roles for alanine dehydrogenase, alanine racemase, and alanine permease. *J Bacteriol* 187(3):972–979.
4. Hohn MJ, Palioura S, Su D, Yuan J, Söll D (2011) Genetic analysis of selenocysteine biosynthesis in the archaeon *Methanococcus maripaludis*. *Mol Microbiol* 81(1): 249–258.
5. Kansy JW, Carinato ME, Monteggia LM, Konisky J (1994) In vivo transcripts of the S-layer-encoding structural gene of the archaeon *Methanococcus voltae*. *Gene* 148(1):131–135.
6. Lin W, Whitman WB (2004) The importance of *porE* and *porF* in the anabolic pyruvate oxidoreductase of *Methanococcus maripaludis*. *Arch Microbiol* 181(1):68–73.
7. Tumbula DL, Makula RA, Whitman WB (1994) Transformation of *Methanococcus maripaludis* and identification of a PstI-like restriction system. *FEMS Microbiol Lett* 121(3):309–314.
8. Hohn MJ, Park HS, O'Donoghue P, Schnitzbauer M, Söll D (2006) Emergence of the universal genetic code imprinted in an RNA record. *Proc Natl Acad Sci USA* 103(48): 18095–18100.
9. Sherrer RL, O'Donoghue P, Söll D (2008) Characterization and evolutionary history of an archaeal kinase involved in selenocysteinyl-tRNA formation. *Nucleic Acids Res* 36(4):1247–1259.
10. Wong I, Lohman TM (1993) A double-filter method for nitrocellulose-filter binding: Application to protein-nucleic acid interactions. *Proc Natl Acad Sci USA* 90(12): 5428–5432.
11. Wolfson AD, Pleiss JA, Uhlenbeck OC (1998) A new assay for tRNA aminoacylation kinetics. *RNA* 4(8):1019–1023.
12. Hauenstein SI, Perona JJ (2008) Redundant synthesis of cysteinyl-tRNA^{Cys} in *Methanosarcina mazei*. *J Biol Chem* 283(32):22007–22017.
13. Park HS, et al. (2011) Expanding the genetic code of *Escherichia coli* with phosphoserine. *Science* 333(6046):1151–1154.
14. Kabsch W (2010) Xds. *Acta Crystallogr D Biol Crystallogr* 66(Pt 2):125–132.
15. Adams PD, et al. (2010) PHENIX: A comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr D Biol Crystallogr* 66(Pt 2): 213–221.
16. McCoy AJ, et al. (2007) Phaser crystallographic software. *J Appl Cryst* 40(Pt 4):658–674.
17. Fukunaga R, Yokoyama S (2007) Structural insights into the second step of RNA-dependent cysteine biosynthesis in archaea: Crystal structure of Sep-tRNA:Cys-tRNA synthase from *Archaeoglobus fulgidus*. *J Mol Biol* 370(1):128–141.
18. Terwilliger TC, et al. (2008) Iterative model building, structure refinement and density modification with the PHENIX AutoBuild wizard. *Acta Crystallogr D Biol Crystallogr* 64(Pt 1):61–69.
19. Yao M, Zhou Y, Tanaka I (2006) LAFIRE: Software for automating the refinement process of protein-structure analysis. *Acta Crystallogr D Biol Crystallogr* 62(Pt 2): 189–196.
20. Emsley P, Lohkamp B, Scott WG, Cowtan K (2010) Features and development of Coot. *Acta Crystallogr D Biol Crystallogr* 66(Pt 4):486–501.
21. Afonine PV, et al. (2012) Towards automated crystallographic structure refinement with phenix.refine. *Acta Crystallogr D Biol Crystallogr* 68(Pt 4):352–367.
22. Blanc E, et al. (2004) Refinement of severely incomplete structures with maximum likelihood in BUSTER-TNT. *Acta Crystallogr D Biol Crystallogr* 60(Pt 12 Pt 1):2210–2221.
23. Larkin MA, et al. (2007) Clustal W and Clustal X version 2.0. *Bioinformatics,* 23(21): 2947–2948.
24. Tamura K, et al. (2011) MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 28(10):2731–2739.
25. Farahi K, Whitman WB, Kraemer ET (2003) RED-T: Utilizing the Ratios of Evolutionary Distances for determination of alternative phylogenetic events. *Bioinformatics* 19(16): 2152–2154.

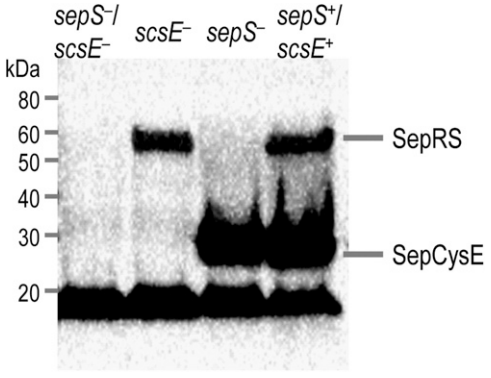

Fig. S1. The expression of His-tagged SepRS and SepCysE in *M. maripaludis* mutant strains detected by Western blot. Whole-cell lysates (from 5 mL culture) were separated by SDS/PAGE, transferred to a PVDF membrane, and incubated with the anti-His antibody conjugated to HRP.

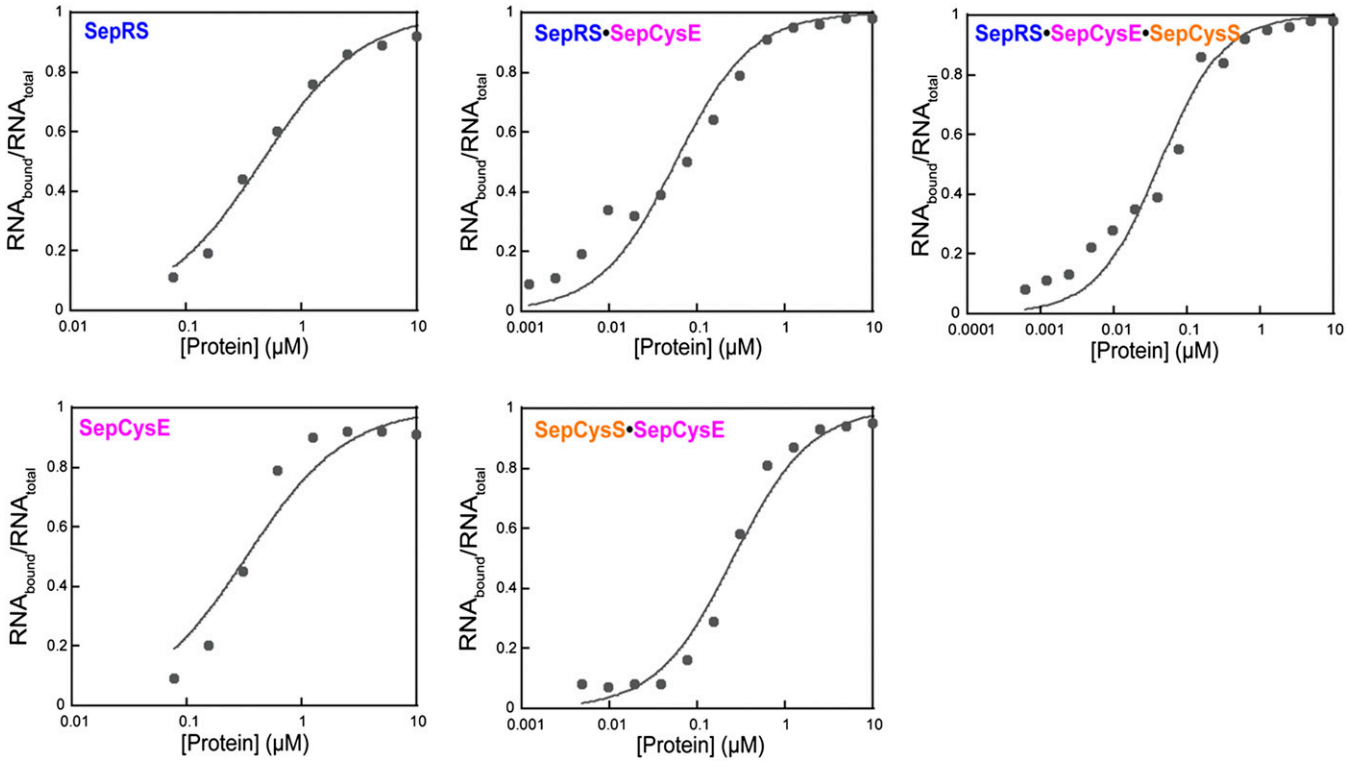

Fig. S2. Representative curves of the binding affinity of the *M. jannaschii* proteins for tRNA^{Cys} determined in a filter-binding assay.

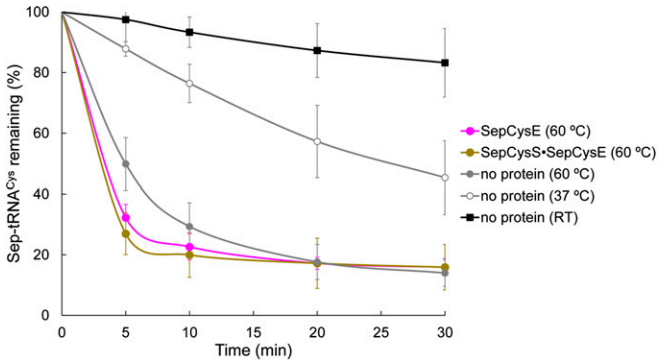

Fig. S3. Deacylation of Sep-tRNA^{Cys}. The ^{32}P-labeled Sep-tRNA^{Cys} (10 μM) was incubated at pH 7.5 at temperatures indicated. Each protein was present at 10 μM when included. Data are mean ± SDs (n = 3). RT, room temperature.

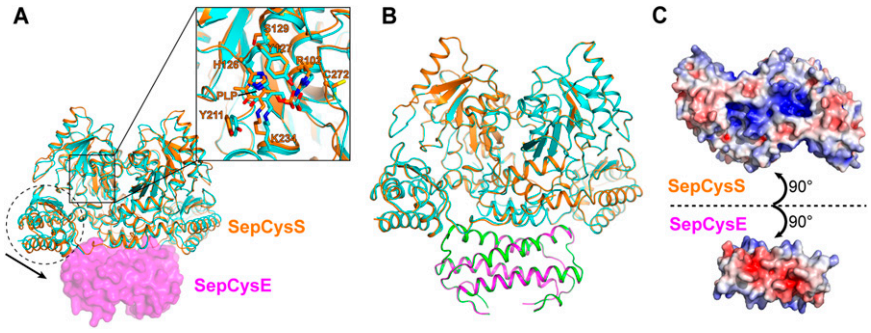

Fig. S4. Overlay of crystal structures and interactions between SepCysS and SepCysE. (*A*) The *Mj*-SepCysS dimer in the SepCysS·SepCysE complex (in orange; PDB ID code 3WKR) is superimposed on the *A. fulgidus* SepCysS dimer [in cyan; PDB ID code 2E7J (17)]. The active site of SepCysS is shown in the *Inset*. (*B*) The SepCysS dimer (orange) and the SepCysE dimer (magenta) in the SepCysS·SepCysE complex (PDB ID code 3WKR) are superimposed on the SepCysS dimer (cyan) and the SepCysE_NTD dimer (green) in the SepCysS·SepCysE_NTD complex (PDB ID code 3WKS). (*C*) Surface electrostatic potential of the SepCysS·SepCysE interface (red, −10 *kT*/e; blue, 10 *kT*/e).

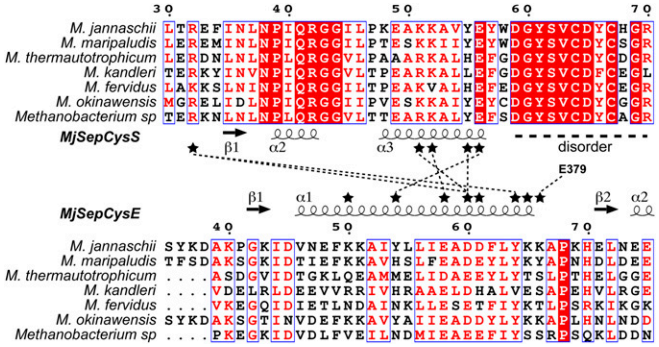

Fig. S5. Sequence alignment of SepCysS and SepCysE homologs. The hydrophilic residues located at the SepCysS·SepCysE interface are labeled with stars. Dashed lines indicate hydrogen bonds. The full species names are listed in Fig. S6.

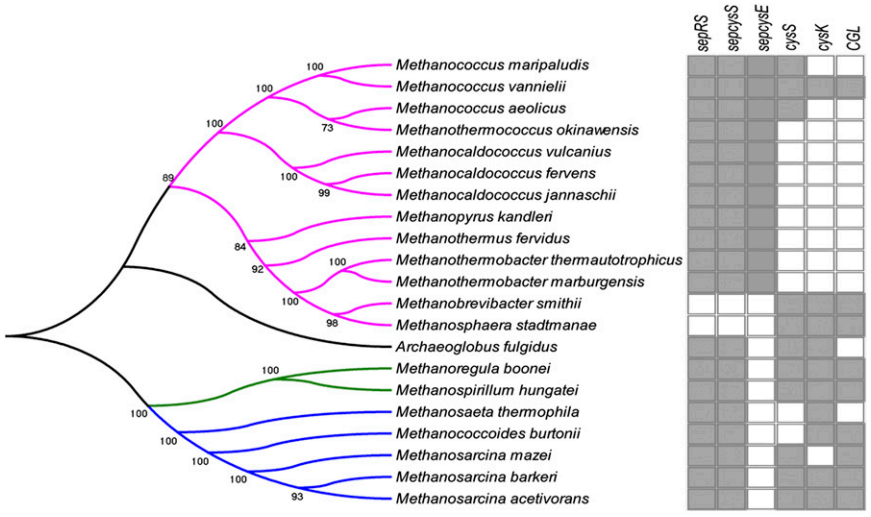

Fig. S6. Phylogenetic distribution of Cys biosynthesis genes in methanogenic archaea. The 16S rRNA phylogenetic tree was constructed with the Minimum Evolution algorithm using MEGA5 (1). Bootstrap analysis was performed with 1,000 replicates, and values >70% are labeled on the nodes. The magenta, green, and blue branches represent class I, II, and III methanogens, respectively. The gray and white boxes indicate the presence and absence of the Cys biosynthesis genes, respectively. *CGL*, cystathionine γ-lyase; *cysK*, *O*-acetylserine (thiol)-lyase-A; *cysS*, cysteinyl-tRNA synthetase.

1. Tamura K, et al. (2011) MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 28(10): 2731–2739.

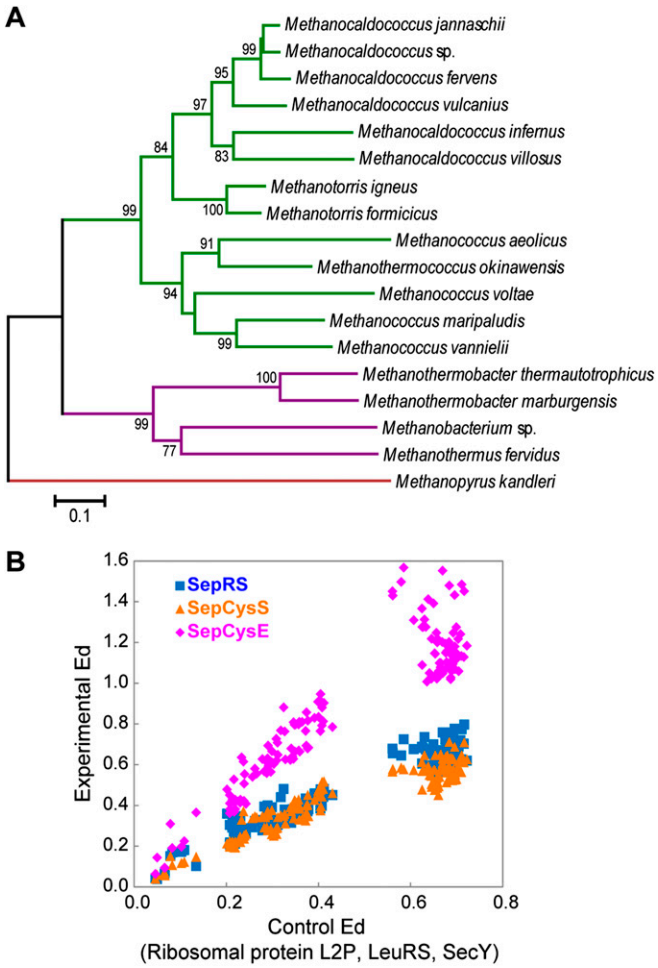

Fig. S7. Phylogeny of SepCysE. (*A*) The sequence-based phylogenetic tree of the SepCysE homologs from Methanococcales (green), Methanobacteriales (purple), and Methanopyrales (dark red) was constructed with the Minimum Evolution algorithm using MEGA5 (1). Bootstrap analysis was performed with 1,000 replicates, and values >70% are labeled on the nodes. (*B*) The RED plot of SepRS (blue), SepCysS (orange), and SepCysE (magenta) homologs. The control E_d are the mean E_d of the genes encoding the large ribosomal subunit protein 2 (L2P), leucyl-tRNA synthetase (LeuRS), and the protein translocase SecY.

1. Tamura K, et al. (2011) MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 28(10): 2731–2739.

Table S1. Data collection and refinement statistics

	SepCysS·SepCysE	SepCysS·SepCysE_NTD
PDB ID code	3WKR	3WKS
Data collection		
Space group	$P6_5$	$P2_12_12_1$
Cell dimensions, Å	a = 106.9, b = 106.9, c = 495.2	a = 95.1, b = 107.1, c = 110.8
Resolution, Å	47.30–2.80 (2.97–2.80)	49.20–3.03 (3.21–3.03)
R_{meas}, %*	12.3 (74.5)	19.2 (86.8)
I/σ	13.1 (1.5)	12.4 (2.9)
Completeness, %	95.0 (94.6)	99.7 (98.6)
Multiplicity	4.1 (3.2)	7.3 (7.3)
Refinement statistics		
No. of reflections	304,705	22,584
R_{work}/R_{free}, %	22.44/24.25	18.89/23.96
Twin fraction, %	44.9 (h, $-h-k$, $-l$)	—
$R_{twin,obs}$, $R_{twin,calc}$, %†	4.4, 29.7	—
No. of protein atoms	13,917	7,032
No. of water atoms	113	23
Average B factor of protein, Å^2	99.90	50.20
Average B factor of water, Å^2	52.80	32.70
rmsd of bond lengths, Å	0.002	0.002
rmsd of bond angles, °	0.53	0.65
Ramachandran favored, %	95.4	95.7
Ramachandran allowed, %	4.3	3.7
Ramachandran outliers, %	0.35	0.58

Each dataset was collected from a single crystal. Values in parentheses are for highest-resolution shell.

*R_{meas} is the redundancy-independent R_{sym}.

†$R_{twin} = \Sigma|I(h) - I(S_{twin}h)|/\Sigma(I(h) + I(S_{twin}h))$, where S_{twin} is the twin operator (1).

1. Lebedev AA, Vagin AA, Murshudov GN (2006) Intensity statistics in twinned crystals with examples from the PDB. *Acta Crystallogr D Biol Crystallogr* 62(Pt 1):83–95.