

CysteinyI-tRNA^{Cys} Formation in *Methanocaldococcus jannaschii*: the Mechanism Is Still Unknown

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Most organisms form Cys-tRNA^{Cys}, an essential component for protein synthesis, through the action of cysteinyI-tRNA synthetase (CysRS). However, the genomes of *Methanocaldococcus jannaschii*, *Methanothermobacter thermautotrophicus*, and *Methanopyrus kandleri* do not contain a recognizable *cysS* gene encoding CysRS. It was reported that *M. jannaschii* prolyI-tRNA synthetase (C. Stathopoulos, T. Li, R. Longman, U. C. Voithknecht, H. D. Becker, M. Ibba, and D. Söll, *Science* 287:479-482, 2000; R. S. Lipman, K. R. Sowers, and Y. M. Hou, *Biochemistry* 39:7792-7798, 2000) or the *M. jannaschii* MJ1477 protein (C. Fabrega, M. A. Farrow, B. Mukhopadhyay, V. de Crécy-Lagard, A. R. Ortiz, and P. Schimmel, *Nature* 411:110-114, 2001) provides the “missing” CysRS activity for in vivo Cys-tRNA^{Cys} formation. These conclusions were supported by complementation of temperature-sensitive *Escherichia coli cysS*(Ts) strain UQ818 with archaeal *proS* genes (encoding prolyI-tRNA synthetase) or with the *Deinococcus radiodurans* DR0705 gene, the ortholog of the MJ1477 gene. Here we show that *E. coli* UQ818 harbors a mutation (V27E) in CysRS; the largest differences compared to the wild-type enzyme are a fourfold increase in the K_m for cysteine and a ninefold reduction in the k_{cat} for ATP. While transformants of *E. coli* UQ818 with archaeal and bacterial *cysS* genes grew at a nonpermissive temperature, growth was also supported by elevated intracellular cysteine levels, e.g., by transformation with an *E. coli cysE* allele (encoding serine acetyltransferase) or by the addition of cysteine to the culture medium. An *E. coli cysS* deletion strain permitted a stringent complementation test; growth could be supported only by archaeal or bacterial *cysS* genes and not by archaeal *proS* genes or the *D. radiodurans* DR0705 gene. Construction of a *D. radiodurans* DR0705 deletion strain showed this gene to be dispensable. However, attempts to delete *D. radiodurans cysS* failed, suggesting that this is an essential *Deinococcus* gene. These results imply that it is not established that *proS* or MJ1477 gene products catalyze Cys-tRNA^{Cys} synthesis in *M. jannaschii*. Thus, the mechanism of Cys-tRNA^{Cys} formation in *M. jannaschii* still remains to be discovered.

CysteinyI-tRNA synthetase (CysRS), a highly conserved essential enzyme, is a key component in protein biosynthesis. It is the smallest monomeric class I aminoacyl-tRNA synthetase (14, 20). A recent determination of the crystal structure of *Escherichia coli* CysRS (30) revealed that a zinc ion positioned at the active site is responsible for the precise binding of the substrate cysteine. This metal ion coordinates with the side chains of Cys28, Cys209, His234, and Glu238 (30). CysRS is well conserved in all three domains of life; phylogenetic studies suggest a transfer of *cysS* from bacteria to some archaea (24). Based on the knowledge of a large number of complete organism genomes, it is clear that only three methanogenic archaea, *Methanocaldococcus jannaschii* (8), *Methanothermobacter thermautotrophicus* (33), and *Methanopyrus kandleri* (32), do not

contain an open reading frame (ORF) resembling that for CysRS.

How is Cys-tRNA^{Cys} formed in these methanogenic archaea? One apparent answer came from reports that archaeal prolyI-tRNA synthetases (ProRSs) could form Cys-tRNA^{Cys} in addition to Pro-tRNA^{Pro} (26, 35). Apart from biochemical data, the conclusion was based on the persuasive in vivo result (35) that the *M. jannaschii*, *M. thermautotrophicus*, and *Methanococcus maripaludis proS* genes could restore the growth, albeit weakly, of temperature-sensitive *E. coli cysS*(Ts) strain UQ818 (5) at a nonpermissive temperature. Poor growth of the transformed strains was attributed to slow translation in *E. coli* of the large number of AGA codons present in the archaeal *proS* genes (35). A different route to archaeal Cys-tRNA^{Cys} formation was proposed on the basis of the existence of an unusual aminoacyl-tRNA synthetase that lacks the typical features of class I and class II synthetases and is encoded by the *M. jannaschii* MJ1477 ORF (15). The heterologously expressed MJ1477 protein was shown to cysteinylate in vitro both *M. jannaschii* total tRNA and purified *E. coli* tRNA^{Cys}, but the MJ1477 gene could not rescue the growth of *E. coli cysS*(Ts) strain UQ818 (15). Instead, DR0705, the *Deinococcus radiodurans* ortholog of MJ1477, was shown to complement strain UQ818. While these data suggested that MJ1477 provides Cys-

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TABLE 1. Strains and plasmids

Strain or plasmid	Genotype or relevant information	Reference or source ^a
Strains		
<i>E. coli</i>		
W3110	Wild type	
UQ818	<i>lacZ4 gyrA222 (nalR) aroE24 metB rpoB (rifR) cysS818</i>	5
BL21(DE3)		37
DY330	<i>lacU169 gal490 λcI⁸⁵⁷ Δ(cro-bioA)</i>	39
EC723	<i>lacU169 gal490 λcI⁸⁵⁷ Δ(cro-bioA) nad⁺ araCBAD::tolC</i>	J. A. DeVito, unpublished data
EC400	<i>lacU169 gal490 λcI⁸⁵⁷ Δ(cro-bioA) araCBAD::lox²-kan</i>	J. A. Mills, unpublished data
Δ <i>cysS</i>	EC723 <i>cysS::lox²-kan</i>	This work
<i>D. radiodurans</i> R1	ATCC 13939	ATCC; 17
Plasmids		
pET15b	Vector	Novagen
pET15b- <i>cysS</i>	<i>E. coli cysS</i>	This work
pET15b- <i>cysSV27E</i>	<i>E. coli cysS</i> (Ts)	This work
pET28a	Vector	Novagen
pBAD18-Cm	Vector	ATCC
pBADbr	Replace replication origin of pBAD18-Cm with that of pET28a	This work
pBADbr <i>cysS</i>	<i>E. coli cysS</i>	This work
pACYC184	Vector	NEB
pACYC-Tc-Mj-tRNA ^{Cys}	<i>M. jannaschii</i> tRNA ^{Cys}	This work
pCYB1	Vector	NEB
pCYB-MJ <i>proS</i>	<i>M. jannaschii proS</i>	This work
pCYB-MM <i>proS</i>	<i>M. maripaludis proS</i>	This work
pCYB-MM <i>cysS</i>	<i>M. maripaludis cysS</i>	This work
pCYB-DR0705	<i>D. radiodurans</i> DR0705	This work
pCYB-DR <i>cysS</i>	<i>D. radiodurans cysS</i>	This work
pCYB-DR <i>proS</i>	<i>D. radiodurans proS</i>	This work
pCYB- <i>cysE</i>	<i>E. coli cysE</i>	This work
pCYB-EC <i>cysS</i>	<i>E. coli cysS</i>	This work
pCYB- <i>cysEM256I</i>	<i>E. coli cysEM256I</i>	This work
pGEM-T	Vector	Promega
pTNK102	pGEM-T containing <i>PkatA-npt</i>	This work
pTNK301	DR0705 deletion cassette containing <i>PkatA-npt</i>	This work
pTNK302	DR1670 deletion cassette containing <i>PkatA-cat</i>	This work

^a ATCC, American Type Culture Collection; NEB, New England BioLabs.

tRNA^{Cys} in *M. jannaschii*, MJ1477 orthologs are not present in the genomes of *M. thermotrophicus* (33) or the viable *cysS* deletion strain (36) of *M. maripaludis* (J. Leigh, unpublished data).

In both of the above-mentioned studies (15, 35), the conclusions were based on the weak complementation of *E. coli* strain UQ818 by the archaeal *proS* genes or the *D. radiodurans* DR0705 gene. *E. coli* UQ818 has a thermolabile CysRS; cell extracts display little CysRS activity at 33°C (5). Strain UQ818 does not grow at the nonpermissive temperature of 41°C but does grow after complementation with *cysS* genes from *E. coli* (14, 20) or from *M. maripaludis* or *Methanosarcina barkeri* (24). A characterization of the *cysS* mutation in strain UQ818 has not been reported.

Although the finding that archaeal ProRSs form Cys-tRNA^{Cys} has been reported repeatedly (9, 26, 27, 34, 35), a biochemical reexamination of the amino acid recognition of ProRS enzymes showed that cysteine charging in vitro is a property of ProRS enzymes from all domains (1); however, the reaction product is the misacylated Cys-tRNA^{Pro} species (2). Therefore, we wanted to further examine the in vivo complementation of *E. coli cysS*(Ts) strain UQ818 and to analyze the complementation of an *E. coli cysS* deletion strain (Δ*cysS*). In

addition, we attempted to evaluate the physiological significance of DR0705 by using a gene deletion in *D. radiodurans* (3).

MATERIALS AND METHODS

General. [³⁵S]Cysteine (1,075 Ci/mmol) was purchased from Perkin-Elmer Life Sciences (Boston, Mass.). [¹⁴C]Serine (155 mCi/mmol) was obtained from Amersham Pharmacia Biotech (Little Chalfont, United Kingdom). *E. coli* total tRNA was obtained from Sigma (St. Louis, Mo.), and the TOPO-TA cloning kit was obtained from Invitrogen (Carlsbad, Calif.). Oligonucleotide synthesis and DNA sequencing were performed at the Keck Foundation Research Biotechnology Resource Laboratory at Yale University (New Haven, Conn.). Protein concentrations were determined by the method of Bradford (6) with bovine serum albumin as a standard.

Plasmids, strains, and culture medium. Table 1 lists the plasmids and strains used in this study. Primers were designed to PCR amplify each ORF and introduce the desired restriction sites. The forward primers contained a restriction site and 20 nucleotides identical to the start sequence of the 5' end; the reverse primers contained a restriction site and 20 nucleotides complementary to the sequence of the 3' end. Standard PCR procedures were used to generate the coding sequences of the *M. jannaschii proS*, *M. maripaludis proS* and *cysS*, *D. radiodurans* DR0705 and *cysS*, and *E. coli cysE* and *cysS* genes from their corresponding genomic DNAs. The amplified coding sequences were cloned into the pCR2.1-TOPO vector. After verification of the DNA sequences, the genes were subcloned into the desired vector. For in vivo complementation, the genes were subcloned into the pCYB1 vector between the *NdeI* and *BamHI* sites under

the control of an isopropyl- β -D-thiogalactopyranoside (IPTG)-inducible *tac* promoter. An *E. coli cysE* mutant allele, *cysEM256I* (10), was generated by PCR mutagenesis of the *cysE* gene (created above) with primers that contained the corresponding mutation.

A medium-copy-number vector, pBADbr, was constructed with the arabinose promoter from pBAD18-Cm and the replication origin from pET28a by using *Bgl*II and *Clal*I restriction sites. The *E. coli cysS* gene was cloned into its *Nhe*I and *Sac*I sites to generate pBADbr*cysS*.

The *M. jannaschii* tRNA^{Cys} gene behind the *lpp* promoter was subcloned from pTech-Mj-tRNA^{Cys} (35) into pACYC184 by using restriction enzymes *Ava*I and *Bst*Z171. This procedure removed the *cat* gene from pACYC184. The resulting plasmid, pACYC-Tc-Mj-tRNA^{Cys}, confers tetracycline resistance.

The following antibiotics at the concentrations indicated were used in Luria-Bertani (LB) medium (31): ampicillin (100 μ g/ml), chloramphenicol (34 μ g/ml), kanamycin (20 μ g/ml), and tetracycline (20 μ g/ml). The final concentrations of arabinose and isopropyl- β -D-thiogalactopyranoside (IPTG) in the culture medium were 0.02% and 1 mM, respectively.

Cloning of *E. coli cysS* genes and purification of CysRS enzymes. Genomic DNA of *E. coli* strain W3110 or UQ818 was used. PCR primers were designed to amplify the *cysS* ORF, and the resulting DNA fragment was cloned into the pET15b vector at the *Nde*I and *Bam*HI sites for expression of proteins as N-terminal His₆-tagged proteins. These pET15b-*cysS* clones were transformed into *E. coli* BL21(DE3) cells, and the transformants were grown in 500 ml of LB-ampicillin medium to a cell density (*A*₆₀₀) of 0.4. At this point, *cysS* expression was induced with 1 mM IPTG treatment for 2 h. Cells were harvested and lysed, and wild-type and mutant His₆-CysRS proteins were purified from cell extracts by Ni-nitrilotriacetic acid-agarose chromatography as described in the Qiagen (Valencia, Calif.) protein purification manual. Both proteins were judged to be >95% pure by sodium dodecyl sulfate-polyacrylamide gel electrophoresis after Coomassie brilliant blue staining. Active-site titration showed that the CysRS and CysRSV27E (CysRS with a V27E mutation) preparations contained 100 and 52% active enzymes, respectively. These values were used to determine enzyme concentrations for the calculation of *k*_{cat}. The enzymes were stored in 10% glycerol in 50 mM HEPES-KOH (pH 7.0)–50 mM KCl–15 mM MgCl₂–5 mM dithiothreitol at –20°C.

Assay for CysRS activity. Cys-tRNA formation was measured as acid-precipitable radioactivity as described previously (1). The standard reaction mixture contained 50 mM HEPES-KOH (pH 7.0), 50 mM KCl, 15 mM MgCl₂, 5 mM dithiothreitol, 10 mM ATP, 0.2 mM [³⁵S]cysteine, and 10 mg of unfractionated *E. coli* tRNA/ml (unless specified otherwise) in a final volume of 0.1 ml. The final enzyme concentrations were 0.37 nM for CysRS and 1.6 nM for CysRSV27E. Initial velocities were measured at 30°C. The substrate concentrations ranged from 0.01 to 20 times the *K_m* values, and the experiments were done in triplicate.

Active-site titration of CysRS enzymes. CysRS preparations were incubated at 30°C in 100 mM Tris-HCl (pH 7.5)–10 mM KCl–10 mM MgCl₂–4 mM ATP (pH 7)–200 μ M [³⁵S]cysteine (650 cpm/pmol)–5 mM dithiothreitol–1 U of inorganic pyrophosphatase in a total volume of 0.1 ml. Aliquots (25 μ l) were used at various times for active-site titration as described previously (1).

Growth of *E. coli* UQ818 at nonpermissive temperatures under various conditions. A cysteine gradient plate (12) was used to show the growth of UQ818 at nonpermissive temperatures in the presence of cysteine. UQ818 cells were grown overnight in LB medium. A 10^{–4} dilution of the overnight culture (0.1 ml) was spread on a λ plate (1% Bacto Tryptone, 0.5% sodium chloride, 1.5% agar). After the plate was dried, an aliquot (0.15 ml) of cysteine (0.4 M) was placed in a central well, and the plate was incubated for 2 days at 41°C.

In order to evaluate the growth restored by *cysEM256I*, UQ818 was transformed with pCYB1, pCYB-EC*cysS*, pCYB-*cysEM256I*, or pCYB-MM*cysS*. The transformants were cultured overnight in LB-ampicillin medium, and the liquid cultures (one loop) were streaked on LB-ampicillin agar plates containing 1 mM IPTG. The plates were grown at 30 or 41°C for 2 days.

In order to compare the growth restored by various genes, we measured at 42°C the growth curves for W3110 cells and UQ818 cells transformed with pCYB-EC*cysS*, pCYB-MM*cysS*, pCYB-DR0705, pCYB-MM*proS*, pCYB-MM*proS*, pCYB-EC*cysEM256I*, or pCYB1. UQ818 transformants with archaeal *proS* genes also contained an *M. jannaschii* tRNA^{Cys} gene (35). Each strain from an overnight culture was inoculated into LB medium with antibiotics and IPTG in triplicate and grown at 42°C for 8 h. Aliquots (1 ml) were taken every 0.5 h to measure the *A*₆₀₀.

Construction of an *E. coli cysS* chromosomal deletion strain. An *E. coli* chromosomal deletion strain was constructed by using the generalized recombination system of bacteriophage λ (39). The *cysS* gene was expressed from an arabinose regulon on pBADbr*cysS*, while the chromosomal *cysS* copy in *E. coli* strain EC723 was replaced with a *lox*²-*kan* cassette (18). The details of strain

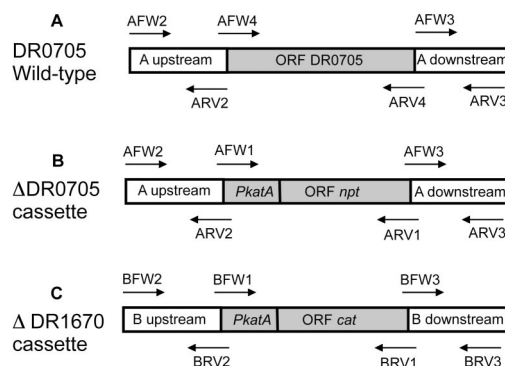


FIG. 1. Scheme of cassette constructs for *Deinococcus* gene deletions. (A) DR0705 in the *D. radiodurans* R1 chromosome. (B) DR0705 deletion cassette. (C) DR1670 deletion cassette. Primers indicated above or below the cassette were used for PCR. Sections of the diagrams are labeled as follows: A upstream, 971 bp immediately upstream of the initiation codon of DR0705; A downstream, 806 bp immediately downstream of the termination codon of DR0705; B upstream, 886 bp immediately upstream of the initiation codon of DR1670; B downstream, 957 bp immediately downstream of the termination codon of DR1670.

construction were adapted from the method of Yu et al. (39). The genomic DNA of *E. coli* strain EC400 served as the template for PCR amplification of the *lox*²-*kan* cassette (11), and the resulting cassette was flanked with 50-bp sequences identical to those found in the upstream and downstream regions of the *cysS* gene. The deletion cassette was transformed into heat-shocked (15 min) *E. coli* strain EC723 containing a rescue plasmid, pBADbr*cysS*. Transformants were selected on kanamycin plates, and strains containing a *cysS* chromosomal deletion were screened by PCR and nucleotide sequencing.

Plasmid exchange experiments with the *E. coli cysS* chromosomal deletion strain. The *cysS* chromosomal deletion strain was further transformed with pACYC-Tc-Mj-tRNA^{Cys}, which provides *M. jannaschii* tRNA^{Cys} for *M. jannaschii cysS* to function in *E. coli* (35). The resulting strain was transformed by electroporation (31) with pCYB derivative plasmids (50 ng) containing *cysS* or other genes at 30°C to prevent lysis of the host (39). Transformants were cultured in LB medium containing kanamycin and tetracycline for 60 min, and then a series of dilutions of the transformants were spread on LB agar plates containing kanamycin, tetracycline, ampicillin, L-arabinose, and IPTG. Cells were grown at 30°C for 1 day, and the numbers of ampicillin-resistant colonies were counted. Colonies then were replicated to LB agar plates containing either chloramphenicol (34 μ g/ml) or ampicillin (50 μ g/ml) and also containing kanamycin, tetracycline, L-arabinose, and IPTG. After 24 h of incubation, the growth of the replicants was checked.

Construction of plasmids for use in deleting DR0705 and *cysS* (DR1670) from the *D. radiodurans* R1 genome. The plasmid used for DR0705 deletion, pTNK301 (carrying the DR0705 deletion cassette) (Fig. 1B), was generated in a three-step process by splicing by overlap extension (13, 19). The Tn903 neomycin phosphotransferase gene (*npt*) was fused to a 120-bp sequence upstream of the initiation codon of the *D. radiodurans* R1 *katA* gene (*PkatA* in Fig. 1) (16). The primers shown in Fig. 1B were used for PCR to amplify the 806-bp sequence immediately downstream of the DR0705 termination codon and the 971-bp sequence upstream of the *PkatA-npt* cassette. The resulting cassette was cloned into pGEM-T to give pTNK301 for DR0705 deletion.

The plasmid used for *cysS* (DR1670) deletion, pTNK302 (carrying the DR1670 deletion cassette) (Fig. 1C), was generated in a manner similar to that used for constructing pTNK301. The chloramphenicol acetyltransferase (*cat*) gene from pBC (Stratagene, La Jolla, Calif.) was spliced to the *D. radiodurans katA* promoter. The resulting *PkatA-cat* fragment was fused to a 957-bp sequence downstream of DR1670 and to an 886-bp sequence immediately upstream of DR1670 by overlap extension, and the resulting product was cloned into pGEM-T to give pTNK302.

Deletion of DR0705 from *D. radiodurans* R1. DR0705 was disrupted by targeted mutagenesis as described previously (13). The DR0705 deletion cassette (Fig. 1B) was transformed into an exponential-phase *D. radiodurans* R1 culture, and the recombinants were selected on tryptone-glucose-yeast extract (TGY)

TABLE 2. Kinetic parameters of wild-type and mutant *E. coli* CysRS enzymes

Substrate	Enzyme	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu\text{M}^{-1} \text{s}^{-1}$)
tRNA ^{Cysa}	CysRS	0.64 ± 0.09	2.9 ± 0.17	4.5
	CysRSV27E	0.92 ± 0.08	0.68 ± 0.027	0.74
ATP	CysRS	338 ± 60	4.4 ± 0.34	0.013
	CysRSV27E	412 ± 54	0.48 ± 0.018	0.0012
Cysteine	CysRS	7.2 ± 1.0	4.8 ± 0.41	0.67
	CysRSV27E	28.2 ± 1.7	0.86 ± 0.015	0.030

^a The K_m for tRNA was measured with unfractionated *E. coli* tRNA containing 1% tRNA^{Cys} (determined by plateau charging with *E. coli* CysRS).

plates containing kanamycin (10 $\mu\text{g}/\text{ml}$). Since *D. radiodurans* is multigenomic, individual colonies had to be screened to determine whether they were homozygous for the disruption. Genomic DNAs of the individual recombinants were extracted and subjected to PCR analysis for the existence of wild-type DR0705 or the deletion cassette. Briefly, putative deletion mutants were subjected to PCR amplification for wild-type DR0705 with primers AFW4 and ARV4 (Fig. 1A). No PCR product should be obtained if DR0705 has been deleted. To verify the disruption, genomic DNAs from *D. radiodurans* R1 and the DR0705 deletion strain were PCR amplified to obtain DNA fragments of the deletion region with primers AFW2 and ARV3 (Fig. 1A and B), and the amplified DNA fragments (~2.7 kb) were further subjected to *Hind*III and *Xho*I digestions. The *npt* gene-containing fragments should be cut in half, because the *npt* gene, but not DR0705, contains single restriction sites for *Hind*III and *Xho*I.

Deletion of *cysS* from *D. radiodurans* R1. The *cysS* (DR1670) deletion cassette (Fig. 1C) was transformed into exponential-phase *D. radiodurans* R1 cells by the CaCl_2 method (13). The culture was spread on TGY plates containing chloramphenicol (3 $\mu\text{g}/\text{ml}$) and incubated at 30°C. Recombinants were observed on these plates within 6 days. Candidates were purified in three rounds of single-colony isolation. Then, chloramphenicol-resistant colonies were PCR screened for the loss of *cysS* by using genomic DNA isolated from each candidate.

RESULTS

The mutant CysRS in strain UQ818 is defective in cysteine binding. *E. coli* strain UQ818 was isolated as a spontaneous temperature-sensitive mutant and lacks CysRS activity in vitro at 42°C (5). In order to characterize the CysRS in this strain, we cloned and sequenced the *cysS*(Ts) gene. We found a single nucleotide change (T→A at position 80) leading to a V→E change at position 27 of the CysRS protein. Sequence alignment of 76 canonical CysRS proteins showed that valine is the most abundant amino acid at position 27 (in 51 out of 76 proteins), the other amino acids being leucine, threonine, cysteine, alanine, tyrosine, asparagine, and glycine.

To understand the effect of the V27E mutation on cysteinylolation, the N-terminal His₆-tagged CysRS and CysRSV27E enzymes were overexpressed and purified on an Ni-nitrilotriacetic acid matrix. Aminoacylation kinetics were determined at 30°C, a temperature at which the CysRSV27E enzyme retains activity. The results (Table 2) indicated a significant difference in the K_m values for cysteine (7.2 and 28.2 μM , respectively). The K_m values for the other substrates, tRNA and ATP, did not differ significantly for the mutant and wild-type enzymes. However, the k_{cat} values for both substrates were about four- to ninefold lower in the mutant, with a ninefold decrease in the value for ATP. Kinetic constants for other CysRS enzymes were determined earlier by ATP-PP_i exchange (23, 29); thus, they are not strictly comparable to our aminoacylation data.

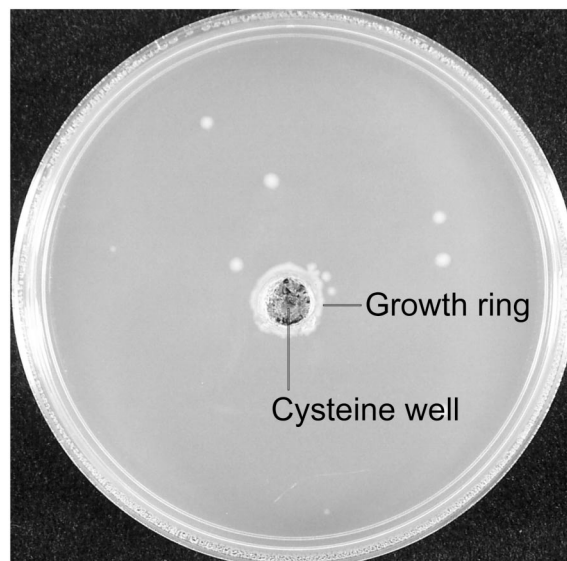


FIG. 2. Growth of *E. coli cysS*(Ts) strain UQ818 at 41°C on a cysteine gradient λ plate. The highest cysteine concentration is in the well (see Materials and Methods).

However, our values are in the range of values reported for CysRS enzymes from *E. coli* (0.4 μM for tRNA^{Cys}), mammals (0.8 μM for tRNA^{Cys}, 800 μM for ATP, and 11 μM for cysteine), and yeasts (0.54 μM for tRNA^{Cys}, 80 μM for ATP, and 8 μM for cysteine). The V27E mutation does not affect the discrimination of the enzyme for serine, which was not charged by either wild-type or mutant CysRS (data not shown).

Complementation of the temperature-sensitive phenotype of *E. coli cysS*(Ts) strain UQ818. Since the mutant CysRSV27E enzyme is impaired in cysteine binding, we tested whether elevated cysteine levels in *E. coli* UQ818 cells would restore their ability to grow at a nonpermissive temperature on a cysteine gradient plate (12). Only cells located close to the cysteine-containing well grew after 2 days of incubation at 41°C, apart from some revertant colonies (Fig. 2). Thus, elevated cysteine levels can restore the growth of the *cysS*(Ts) strain at a nonpermissive temperature.

To investigate the possibility that an elevation in cysteine biosynthetic capacity would also rescue growth, we transformed the *E. coli cysS*(Ts) strain with a *cysE* allele encoding serine acetyltransferase. This enzyme converts serine to *O*-acetylserine, the direct precursor of cysteine (10). Since serine acetyltransferase is feedback inhibited by cysteine, a feedback-resistant mutant enzyme, CysEM256I (10), was selected; an *E. coli cysEM256I* strain excretes up to 2.5 mM cysteine into the medium (10). Therefore, we transformed UQ818 cells with *cysEM256I* (cloned into pCYB) and used the *E. coli* and *M. maripaludis cysS* genes as controls. As expected, *cysEM256I* rescued the growth of strain UQ818 at 41°C, albeit not as well as did the *E. coli* and *M. maripaludis cysS* genes (Fig. 3). Taken together, these results suggest that increased cellular cysteine levels enable *E. coli* UQ818 to grow at a nonpermissive temperature.

Since it was previously reported that the *M. maripaludis proS* gene (35) or the *D. radiodurans* DR0705 gene (15) also rescues

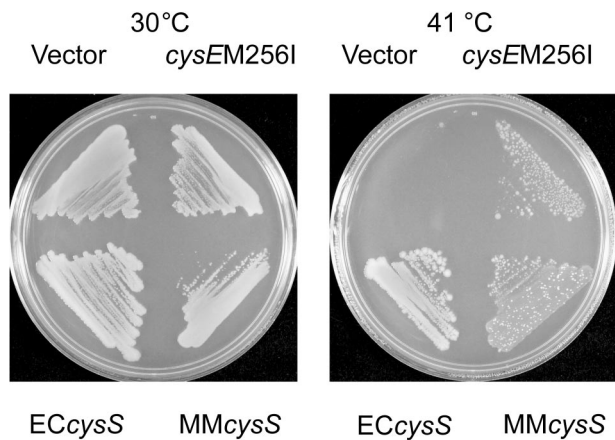


FIG. 3. Complementation of *E. coli* *cysS*(Ts) strain UQ818 with pCYB1, pCYB-EC*cysS*, pCYB-*cysEM256I*, or pCYB-MM*cysS*. LB agar containing ampicillin and IPTG was used for growth at 41°C (see Materials and Methods).

the high-temperature growth of *E. coli* strain UQ818, we wanted to compare the rates of growth of these transformants with those of transformants complemented with the *cysS* genes. *E. coli* strain UQ818 (carrying an *M. jannaschii* tRNA^{Cys} gene) does not grow at 42°C; however, strains complemented with the *cysS* genes grew at 42°C. The observed doubling times for W3110, UQ818 with *E. coli* *cysS*, and UQ818 with *M. maripaludis* *cysS* were 0.5, 1, and 2 h, respectively. However, no growth (defined as no appreciable change in the A_{600} during the experiment; after 8 h, the absorbance was <4% that of wild-type strain W3110) was observed in LB medium for strain UQ818 containing *D. radiodurans* DR0705, *M. jannaschii* *proS*, *M. maripaludis* *proS*, or *E. coli* *cysEM256I* (data not shown).

Only canonical *cysS* genes restore CysRS function to an *E. coli* *cysS* deletion strain. Because complementation of the *cysS*(Ts) gene in strain UQ818 is fraught with pitfalls, we constructed an *E. coli* *cysS* chromosomal deletion strain in order to perform an unambiguous test. Survival of a *cysS* deletion strain can be maintained with a rescue plasmid carrying *E. coli* *cysS*. This chloramphenicol-resistant plasmid, pBAD*bcysS*, can be replaced by transformation and selection for an incompatible plasmid carrying a functional *cysS* gene. We have constructed such incompatible plasmids (pCYB derivatives) that are ampicillin resistant. The pCYB plasmids contain various ORFs in order to test their ability to complement the *E. coli* *cysS* deletion strain. Upon transformation of the deletion strain, cells carrying only a pCYB plasmid (pBAD*bcysS* being lost due to plasmid incompatibility) would be chloramphenicol sensitive. Cells still containing both plasmids would be ampicillin and chloramphenicol resistant. Therefore, the CysRS activity encoded by genes cloned into pCYB can be assessed by their ability to replace the pBAD*bcysS* rescue plasmid in the *E. coli* *cysS* deletion strain.

pCYB transformants of the Δ *cysS* strain were selected on ampicillin and then screened for chloramphenicol sensitivity to assess the efficiency of plasmid exchange. When pCYB plasmids containing *cysS* genes from *E. coli* or *M. maripaludis* were tested, transformation efficiencies were increased and the efficiency of plasmid exchange was high (Table 3). Even though

TABLE 3. Plasmid exchange in the *E. coli* Δ *cysS* strain^a

Plasmid	No. of:		% Plasmid exchange ^c
	Amp ^r colonies	Amp ^r Cm ^r colonies ^b	
pCYB-EC <i>cysS</i>	240,000	47	94
pCYB-MM <i>cysS</i>	58,000	38	18
pCYB-MM <i>proS</i>	74	2	0.001
pCYB-DR0705	52	1	0.0004
pCYB empty vector	58	3	0.001

^a Because the rescue plasmid, pBAD*bcysS* (Cm^r), could be replaced by pCYB (Amp^r)-derived plasmids containing a functional *cysS* gene, the resulting transformants should be ampicillin resistant and chloramphenicol sensitive.

^b Fifty colonies from each strain were tested for ampicillin resistance and chloramphenicol sensitivity, a direct measure of the CysRS activity encoded by the test genes.

^c The percentage of cells containing the exchanged plasmids was calculated as follows: [(Amp^r Cm^r colonies/50) × (Amp^r colonies/240,000)] × 100. It was assumed that the same number of transformants (240,000/50 ng of plasmid) would be obtained with each plasmid tested and that the 50 tested colonies were representative of the entire pool of ampicillin-resistant colonies.

the same amount of plasmid DNA (50 ng) was used for transformation of the same batch of competent *E. coli* Δ *cysS* cells, the number of transformants was greatly reduced in the Δ *cysS* strain when pCYB plasmids without functional *cysS* genes were introduced (Table 3). Presumably, selection against the pBAD*bcysS* rescue plasmid did not allow enough *cysS* to be expressed for functional complementation of the *cysS* chromosomal deletion. When the transformants were tested for chloramphenicol resistance, >90% of the *proS* or DR0705 transformants retained the pBAD*bcysS* rescue plasmid (Table 3); this result indicates that *proS* or DR0705 does not provide *cysS* function. Thus, *proS* and DR0705 do not encode a CysRS that is functional in *E. coli*.

Deletion of DR0705 from *D. radiodurans* R1 generates a viable strain. DR0705 was deleted from the *D. radiodurans* R1 chromosome and replaced with a kanamycin resistance cassette under the control of a constitutively expressed *D. radiodurans* promoter. The results of experiments with the DR0705 deletion strain, designated TNK201, are shown in Fig. 4. Amplification of the 924-bp DR0705 ORF was not observed when genomic DNA of TNK201 was used as a template, although an ~900-bp fragment was obtained when *D. radiodurans* R1 genomic DNA was used as a template (Fig. 4, lanes 2 and 3). These results suggest that DR0705 had been replaced with the *PkatA-npt* cassette in TNK201. To verify the disruption, genomic DNAs from R1 and TNK201 were amplified with primers AFW2 and ARV3 (Fig. 1A and B), and DNA fragments of 2.7 kb were obtained (Fig. 4, lanes 4 and 5). Both purified DNA fragments were digested with *Hind*III and *Xho*I; *Hind*III cut the TNK201-derived PCR product into fragments of 1.2 and 1.5 kb, while R1-derived DNA remained intact (Fig. 4, lanes 6 and 7). Similarly, *Xho*I cut the TNK201-derived PCR product into 1- and 1.7-kb fragments but not the product amplified from R1 (Fig. 4, lanes 8 and 9). These results confirm that DR0705 had been deleted from strain TNK201 and replaced with the *PkatA-npt* cassette. The deletion strain (TNK201) grew with the same kinetics as its R1 parent (with a doubling time of ~1 h in TGY broth at 30°C). Thus, DR0705 is not essential for the growth of this *Deinococcus* strain.

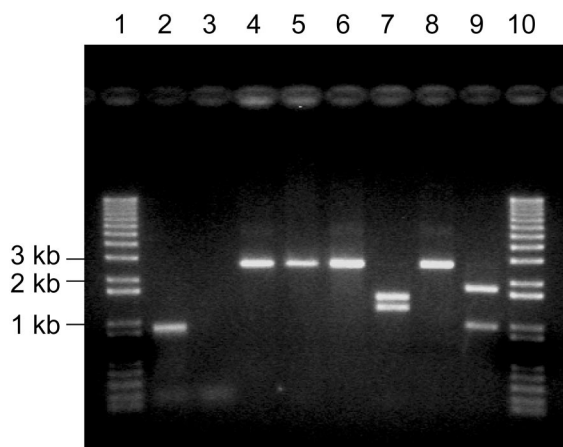


FIG. 4. Replacement of the *D. radiodurans* DR0705 gene with the *PkatA-npt* cassette. Lanes 1 and 10, DNA size markers; lanes 2, 4, 6, and 8, DNA from *D. radiodurans* R1; lanes 3, 5, 7, and 9, DNA from DR0705 deletion strain TNK201. Lanes: 2 and 3, PCR products of primers AFW4 and ARV4; 4 and 5, PCR products of primers AFW2 and ARV3; 6 and 7, *Hind*III digestion of DNA in lanes 4 and 5, respectively; 8 and 9, *Xho*I digestion of DNA in lanes 4 and 5, respectively. DR0705 has no restriction sites for *Hind*III and *Xho*I, while the *npt* gene is cut once by both enzymes.

Attempts to delete *cysS* from *D. radiodurans* R1 were unsuccessful. The same strategy as that used for DR0705 was pursued for replacing the *cysS* gene with a *cat* gene cassette under the control of the constitutively expressed *katA* promoter (Fig. 1C). However, all colonies screened were heterozygous for chloramphenicol resistance, containing both *cysS* and *cat* genes. This result suggests that *cysS* cannot be deleted from *D. radiodurans* R1.

DISCUSSION

The work presented here examined the earlier interpretation (9, 26, 27, 34, 35) of the data on Cys-tRNA^{Cys} formation in archaea. In vitro evidence has clearly shown that the ProRS enzymes from all three domains of life can charge cysteine to tRNA (1, 2). Even though the ProRS enzymes lack the amino acid landscape of the extremely conserved cysteine binding pocket of the canonical CysRS enzymes (30), the crystal structure of *M. thermautotrophicus* ProRS showed efficient binding of cysteine to the active site of the enzyme (22). However, the specificity for cognate tRNA prevails, and ProRS generates misacylated Cys-tRNA^{Pro}, which is not edited by the enzyme (1, 2, 4, 27).

Thus, we looked for an explanation for the successful in vivo rescue of the temperature-sensitive *cysS* phenotype by complementation with archaeal *proS* genes (9, 35). It had already been reported (7, 38) that rescue of a temperature-sensitive aminoacyl-tRNA synthetase phenotype can occur through factors other than the wild-type gene product. For instance, partial suppression of a temperature-sensitive *E. coli* valyl-tRNA synthetase strain by ribosomal protein mutations was reported (7, 38). Such mutations or metabolic changes slow down the growth rate suitably so that the mutant synthetase at the non-permissive temperature is able to provide sufficient aminoacyl-tRNA for adequate protein synthesis and cell survival. Stabi-

lization of the mutant CysRS in strain UQ818 through increased cysteine levels or through interactions with other proteins may yield the same result. Therefore, the inability of archaeal *proS* genes to restore viability to the *E. coli* Δ *cysS* strain indicates that archaeal *proS* genes do not generate Cys-tRNA^{Cys}, at least in *E. coli*. While all of the published data combined do not rule out the possibility that ProRS synthesizes some correctly charged Cys-tRNA, the conclusion (9, 35) that ProRS can form Cys-tRNA^{Cys} in vivo is not established. Therefore, we assume that archaeal ProRS does not supply Cys-tRNA^{Cys} in *M. jannaschii*. Whether this enzyme can provide this function with the help of an additional protein(s) is an open question (25).

The above findings may also explain the observation that the *D. radiodurans* DR0705 gene rescued the temperature-sensitive phenotype of strain UQ818 (15), even though this gene did not restore the growth of the *E. coli* Δ *cysS* strain. The fact that the DR0705 gene can be deleted from *D. radiodurans* without any effect on growth shows that its gene product is not required for the viability of the organism. However, attempts to delete the canonical *cysS* gene did not result in a viable strain, suggesting that CysRS is indeed a required enzyme in *D. radiodurans*. Unfortunately, genetic methods to establish unambiguously the essentiality of a gene do not yet exist for *D. radiodurans*.

Although the product of *M. jannaschii* MJ1477 ORF cysteinylates the homologous tRNA with cysteine in vitro (15), the nature of this protein as CysRS has been questioned by extensive computational analyses that instead predict a secreted polygalactosaminidase or a related polysaccharide hydrolase (21, 28). Moreover, no MJ1477 orthologs are present in the completed genome sequences of the methanogens *M. thermautotrophicus* (33) and *M. maripaludis* (Leigh, unpublished); the former organism contains no canonical *cysS* gene, while a viable *cysS* deletion strain of *M. maripaludis* exists (36). Therefore, MJ1477 cannot explain the lack of *cysS* in *M. thermautotrophicus*. Given the results of the different lines of experimentation, it cannot be convincingly deduced that the MJ1477 and DR0705 proteins function as CysRS enzymes.

All of these data compel us to conclude that the mechanism of Cys-tRNA^{Cys} formation in *M. jannaschii* is still unknown.

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