RNA-dependent conversion of phosphoserine forms selenocysteine in eukaryotes and archaea

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The trace element selenium is found in proteins as selenocysteine (Sec), the 21st amino acid to participate in ribosome-mediated translation. The substrate for ribosomal protein synthesis is selenocysteinyl-tRNA^{Sec}. Its biosynthesis from seryl-tRNA^{Sec} has been established for bacteria, but the mechanism of conversion from Ser-tRNA^{Sec} remained unresolved for archaea and eukarya. Here, we provide evidence for a different route present in these domains of life that requires the tRNA^{Sec}-dependent conversion of *O*-phosphoserine (Sep) to Sec. In this two-step pathway, *O*-phosphoseryl-tRNA^{Sec} kinase (PSTK) converts Ser-tRNA^{Sec} to Sep-tRNA^{Sec}. This misacylated tRNA is the obligatory precursor for a Sep-tRNA:Sec-tRNA synthase (SepSecS); this protein was previously annotated as SLA/LP. The human and archaeal SepSecS genes complement *in vivo* an *Escherichia coli* Sec synthase (SelA) deletion strain. Furthermore, purified recombinant SepSecS converts Sep-tRNA^{Sec} into Sec-tRNA^{Sec} *in vitro* in the presence of sodium selenite and purified recombinant *E. coli* selenophosphate synthetase (SelD). Phylogenetic arguments suggest that Sec decoding was present in the last universal common ancestor. SepSecS and PSTK coevolved with the archaeal and eukaryotic lineages, but the history of PSTK is marked by several horizontal gene transfer events, including transfer to non-Sec-decoding Cyanobacteria and fungi.

aminoacyl-tRNA | evolution | formate dehydrogenase | pyridoxal phosphate

Amino acids enter protein synthesis as aminoacyl-tRNAs (aa-tRNAs). The identity of an amino acid inserted into the nascent polypeptide is determined by two factors: the interaction of the aa-tRNA anticodon with an appropriate mRNA codon, and the correct pairing of amino acid and tRNA anticodon in the aa-tRNA. Thus, faithful protein synthesis requires the presence in the cell of a full set of correctly aminoacylated tRNAs (1). Two routes to aa-tRNA synthesis exist (2): (*i*) direct acylation of the amino acid onto its cognate tRNA catalyzed by an aa-tRNA synthetase, and (*ii*) tRNA-dependent modification of a noncognate amino acid attached to tRNA. Although the majority of aa-tRNAs are made by direct aminoacylation, the indirect pathway is also widely used, e.g., for Gln-tRNA^{Gln} and Asn-tRNA^{Asn} formation (3).

The biosynthetic route of aa-tRNA formation is understood for all of the currently known cotranslationally inserted amino acids. The sole exception is the case of selenocysteine (Sec) in eukarya and archaea (e.g., refs. 4 and 5). The bacterial case was solved in the 1990s when genetic and biochemical studies with *Escherichia coli* revealed that bacterial Sec-tRNA^{Sec} was synthesized by an indirect tRNA-dependent amino acid transformation mechanism (6). Sec is cotranslationally inserted into proteins in response to the codon UGA; thus the tRNA^{Sec} species has the corresponding anticodon UCA (7). This tRNA is misacylated with serine by *E. coli* seryl-tRNA synthetase (SerRS) to form Ser-tRNA^{Sec} (8). Then a pyridoxal phosphate (PLP)-dependent Sec synthase (SelA) catalyzes the Ser-tRNA^{Sec} → Sec-tRNA^{Sec} conversion using selenophosphate as selenium donor (Fig. 1 *Upper*). For archaea and eukarya our understanding is still fragmentary. Serylation of tRNA^{Sec} by eukaryal (9–12) and archaeal (13–15) SerRS enzymes has been well established. However, the demonstration of an analogous reaction to the bacterial pathway converting Ser-tRNA^{Sec} → Sec-tRNA^{Sec} has remained elusive. In particular, the archaeal protein MJ0158, annotated as the ortholog to bacterial SelA, did not support *in vitro* Sec formation (15).

Recently, a pathway for cysteine formation was discovered in methanogens based on a tRNA-dependent conversion of *O*-phosphoserine (Sep) to cysteine (16). This route involves the attachment of Sep to tRNA^{Cys} by SepRS and the subsequent Sep-tRNA^{Cys} → Cys-tRNA^{Cys} transformation by Sep-tRNA:Cys-tRNA synthase, a PLP-dependent enzyme using a still unknown sulfur donor (Fig. 1 *Lower*). The implications of this pathway for Sec formation were obvious (16), especially because a phosphoseryl-tRNA^{Sec} kinase (PSTK) had just been identified (17). Its activity was characterized *in vitro* for the murine (17) and the *Methanocaldococcus jannaschii* (15) enzyme; this kinase phosphorylates specifically Ser-tRNA^{Sec} to Sep-tRNA^{Sec}. This finding would lend credence to early reports of the existence of mammalian Sep-tRNA formed from Ser-tRNA (18, 19). Because the initial search for a PLP-dependent SelA activity as manifested by *E. coli* SelA was unsuccessful (15), we considered other proteins. The most promising candidate was the human protein SLA/LP (soluble liver antigen/liver pancreas), which forms a ribonucleoprotein antigenic complex with tRNA^{Sec} in patients suffering from an autoimmune chronic hepatitis (20). This protein was classified computationally as a PLP-dependent serine hydroxymethyltransferase that might have SelA function (21). To lend further credibility, this protein (22, 23) has very well conserved archaeal orthologs but only in *Methanocaldococcus jannaschii* (MJ0610), *Methanococcus maripaludis* (MMP0595), *Methanopyrus kandleri* (MK0672), *Methanococcus voltae*, *Methanothermococcus thermolithotrophicus*, and *Methanococcus vannielii*; these are the known archaea that use Sec. Considering all of these data, it appeared probable that this protein was the missing Sep-tRNA:Sec-tRNA synthase (SepSecS) (Fig. 1 *Upper*).

Here, we report the identification of the human SLA/LP and *Methanococcus maripaludis* MMP0595 proteins as SepSecS en-

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Abbreviations: aa-tRNA, aminoacyl-tRNA; BV, benzyl viologen; FDH_H, formate dehydrogenase H; IPTG, isopropyl β-D-thiogalactoside; PLP, pyridoxal phosphate; PSTK, phosphoseryl-tRNA^{Sec} kinase; Sec, selenocysteine; SelA, selenocysteine synthase; SelB, elongation factor SelB; SelD, selenophosphate synthetase; Sep, *O*-phosphoserine; SepSecS, Sep-tRNA:Sec-tRNA synthase; SerRS, seryl-tRNA synthetase.

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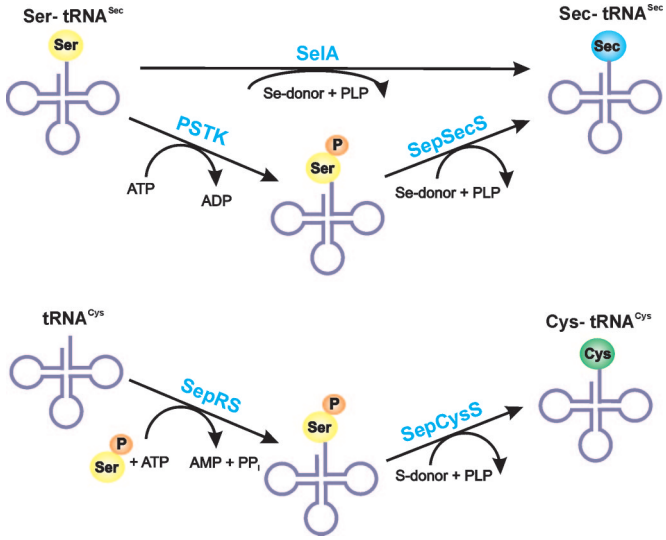

Fig. 1. tRNA-dependent amino acid transformations leading to Sec and cysteine. (*Upper*) The SelA route is the bacterial pathway (6). The PSTK/SepSecS is the archaeal/eukaryal route. (*Lower*) The SepRS/Sep-tRNA:Cys-tRNA synthase (SepCysS) pathway operates in methanogens to synthesize cysteine (16).

zymes (encoded by *spcS*) that convert Sep-tRNA^{Sec} → Sec-tRNA^{Sec}.

Results

SepSecS Rescues Selenoprotein Biosynthesis in an *E. coli selA* Deletion Strain. When grown anaerobically, *E. coli* produces the selenium-dependent formate dehydrogenase H (FDH_H). Its activity enables the cells to reduce benzyl viologen (BV) in the presence of formate, which is usually observed in agar overlay plates under anaerobic conditions (24). We therefore constructed an *E. coli selA* deletion strain (JS1) in which selenoprotein production is abolished. Complementation of the JS1 strain with archaeal SepSecS genes from *Methanococcus maripaludis* (MMP0595) and *Methanocaldococcus jannaschii* (MJ0610) and with the human homolog (Q9HD40) allowed us to test the ability of these genes to restore selenoprotein biosynthesis as detected by FDH_H activity. The *E. coli* cells were grown under anaerobic conditions for 24 h and overlaid with top agar containing BV and formate. Blue-colored cells indicated that BV was reduced and therefore FDH_H activity was present, whereas cells without FDH_H activity remained colorless. Neither the archaeal nor the human SepSecS genes were able to complement the *E. coli* JS1 strain (Fig. 2). However, complementation was achieved when the SepSecS genes were cotransformed with the gene encoding *Methanocaldococcus jannaschii* PSTK; the latter gene alone was not able to restore Sec synthesis in the *E. coli* JS1 strain. Confirmation of these results was with MacConkey nitrate agar plates where cells with inactive formate dehydrogenase N form dark red colonies (data not shown). These data imply that Sec formation was achieved by a two-step process: PSTK phosphorylates the endogenous Ser-tRNA^{Sec} to Sep-tRNA^{Sec}, and then this misacylated aa-tRNA species is converted to Sec-tRNA^{Sec} by SepSecS. Thus, the human and archaeal SepSecS enzymes are active in *E. coli*. The data also demonstrate that Ser-tRNA^{Sec}, present in *E. coli* JS1, cannot be converted to Sec-tRNA^{Sec}, and therefore is not a substrate for SepSecS.

The bacterial SelA is a PLP-dependent enzyme with a critical lysine residue engaged in PLP binding (25). As the archaeal and eukaryal SepSecS proteins also have PLP binding domains (21), we introduced Lys → Ala mutations to replace the critical lysine position in these enzymes (*Methanocaldococcus jannaschii* SepSecS K277A, *Methanococcus maripaludis* SepSecS K278A, and human SepSecS K284A). Even in the presence of PSTK, the mutant SepSecS genes were no longer able to complement the bacterial *selA* deletion (the result for the human SepSecS mutant is shown in Fig. 2). This finding strengthens the notion that the SepSecS proteins need PLP to carry out the Sep → Sec conversion.

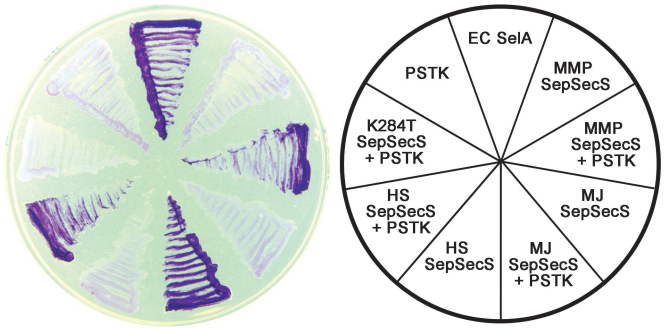

Fig. 2. SepSecS genes restore FDH_H activity in an *E. coli selA* deletion strain. The *E. coli* JS1 strains complemented with the indicated SepSecS genes were grown anaerobically on glucose minimal medium plates supplemented with 0.01 mM IPTG at 30°C for 2 days. FDH_H activity was observed by overlaying top agar containing formate and BV. Colonies with active FDH_H reduced BV to a blue color. The SepSecS genes were from *Methanocaldococcus jannaschii* (MJ), *Methanococcus maripaludis* (MMP), human (HS), and *E. coli selA* (EC). K284T denotes a mutant human SepSecS where the Lys residue critical to PLP binding is changed to Thr.

^{75}Selenium Incorporation into *E. coli* Proteins. To directly follow selenoprotein production in *E. coli*, we labeled with ^{75}Se the *E. coli* JS1 transformants (with SepSecS genes). These JS1 transformant strains were grown anaerobically in the presence of [^{75}Se]selenite and formate. Subsequently, the cell extracts were separated on a polyacrylamide gel and the radioactively labeled FDH_H was visualized by autoradiography. In agreement with the BV assay results, ^{75}Se-labeled FDH_H was detectable in the presence of archaeal SepSecS only in the strains also transformed with PSTK, whereas no labeling occurred when PSTK was absent (Fig. 3). The relatively lower labeling efficiency compared with the positive control, which was the transformant with *E. coli* WT *selA*, may be caused by the inefficient expression of the archaeal enzyme and the heterologous Sec biosynthesis

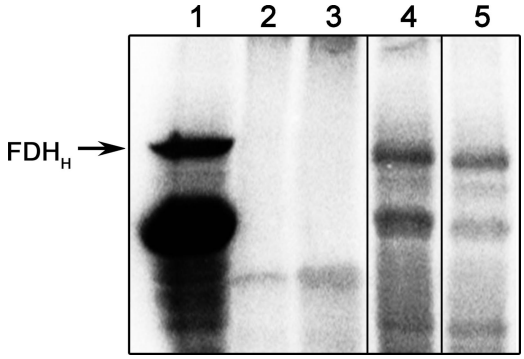

Fig. 3. ^{75}Se incorporation into the *E. coli* selenoprotein FDH_H. Cells were grown in the presence of [^{75}Se]selenite in TGYEP medium (see *Materials and Methods*) supplemented with 0.05 mM IPTG anaerobically at 37°C for 24 h. Cell extracts were separated by 10% SDS/PAGE, and the formation of ^{75}Se-containing FDH_H was followed by autoradiography. The *E. coli* strain JS1 was complemented with *E. coli* SelA (lane 1), empty plasmids (lane 2), *Methanocaldococcus jannaschii* SepSecS (lane 3), *Methanocaldococcus jannaschii* SepSecS with PSTK (lane 4), and *Methanocaldococcus jannaschii* SepSecS, PSTK with *Methanococcus maripaludis* tRNA^{Sec} (lane 5).

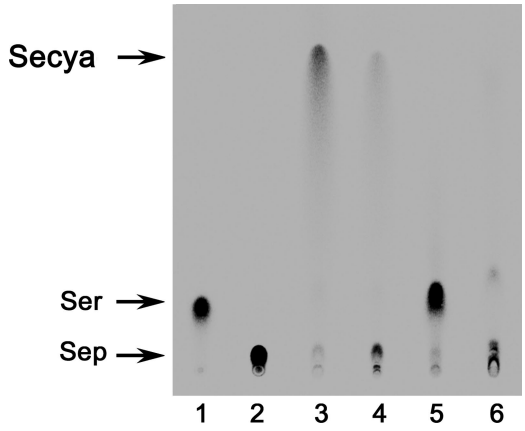

Fig. 4. Conversion of *in vitro*-synthesized Sep-tRNA^{Sec} to Sec-tRNA^{Sec}. Phosphorimages of TLC separation of [^{14}C]Sep and [^{14}C]Sec recovered from the aa-tRNAs of the SepSecS activity assay (see *Materials and Methods*). Sec was analyzed in its oxidized form as selenocysteic acid (Secya). Lane 1, Ser marker; lane 2, Sep marker; lane 3, Sep-tRNA^{Sec} with *Methanococcus maripaludis* SepSecS; lane 4, Sep-tRNA^{Sec} with human SepSecS; lane 5, Ser-tRNA^{Sec} with *Methanococcus maripaludis* SepSecS; lane 6, reaction of lane 3 with selenite omitted.

system. The second labeled band with lower molecular weight was likely caused by the degradation of FDH$_H$ considering the cells were harvested in stationary phase.

In a different experiment we investigated the effect of a homologous SepSecS:tRNA^{Sec} system on selenium incorporation by coexpressing archaeal tRNA^{Sec} in the *E. coli* JS1 strain. However, no significant increase in FDH$_H$ production was observed, which suggests that the endogenous *E.coli* tRNA^{Sec} is a good substrate for archaeal SepSecS. As the secondary structure of tRNA^{Sec} differs considerably among tRNA species from the different domains, SepSecS might display a relaxed recognition of the tRNA moiety of the Sep-tRNA substrate.

SepSecS Converts Sep-tRNA^{Sec} to Sec-tRNA^{Sec} *in Vitro*. To prove that SepSecS is a SelA that catalyzes a tRNA-dependent Sep → Sec synthesis we analyzed the conversion of [^{14}C]Sep-tRNA to [^{14}C]Sec-tRNA. To this aim *Methanococcus maripaludis* tRNA^{Sec} was acylated with [^{14}C]serine by using pure *Methanococcus maripaludis* SerRS (26). Phosphorylation was performed with pure *Methanocaldococcus jannaschii* PSTK. The isolated tRNA was then incubated under anaerobic conditions with purified recombinant SepSecS and *E. coli* selenophosphate synthetase (SelD) in the presence of selenite. After the reaction aa-tRNA was deacylated, and the amino acids were identified by TLC in two systems (one is shown in Fig. 4). The data show that SepSecS from both *Methanococcus maripaludis* (Fig. 4, lane 3) and human (Fig. 4, lane 4) were able to form Sec-tRNA^{Sec} from Sep-tRNA^{Sec}, but not from Ser-tRNA^{Sec} (Fig. 4, lane 5). Thus, Sep-tRNA is the crucial precursor for Sec-tRNA formation in archaea and eukarya.

Evolution of Two Sec Biosynthesis Pathways. Although genetic coding of Sec is limited in organismal distribution, selenoproteins are found in all domains of life. Phylogenetic evidence demonstrates that the core Sec-decoding genes (tRNA^{Sec}, SelB, and SelD), and thus translation of genetically encoded Sec, is an ancient process

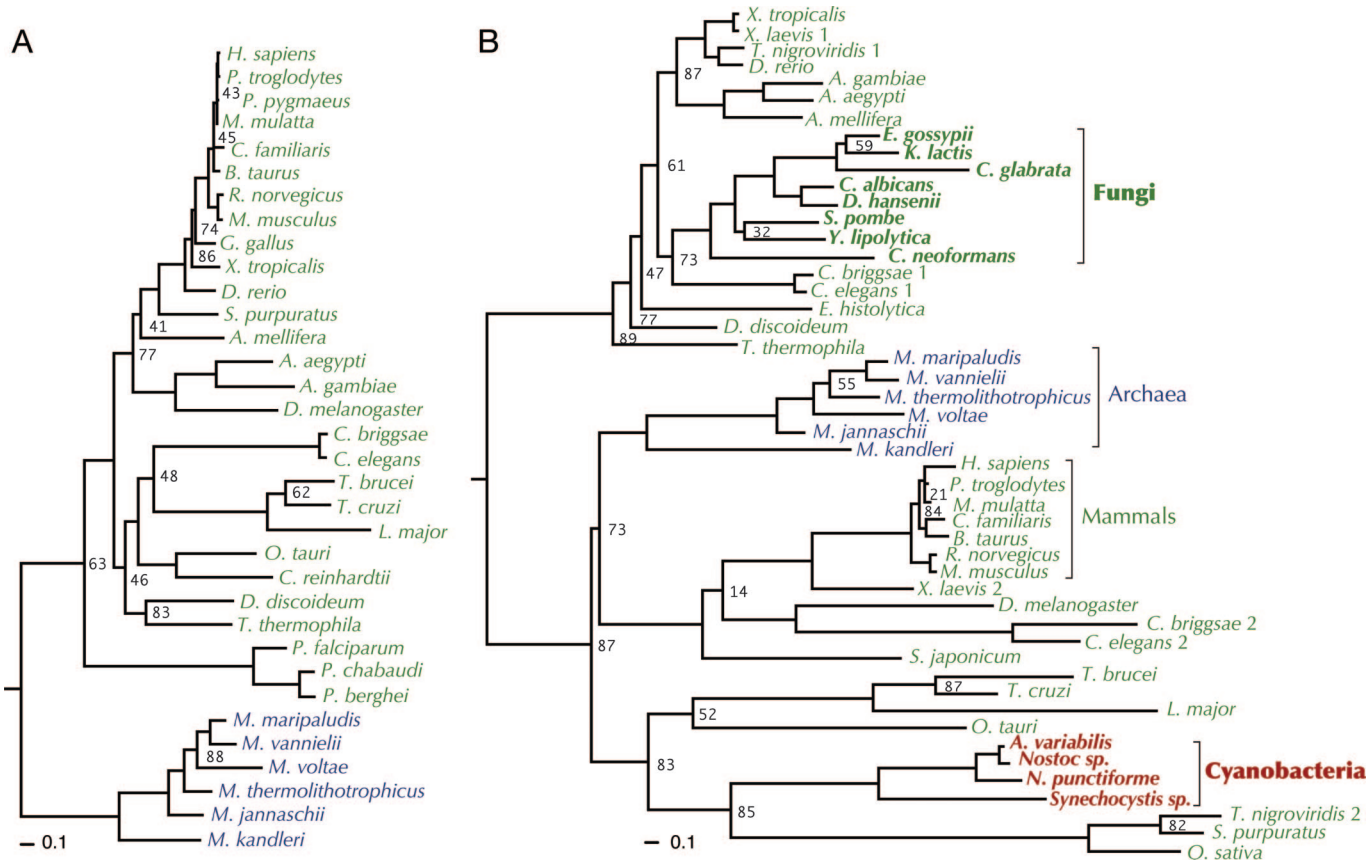

Fig. 5. Phylogenies of SepSecS (*A*) and PSTK (*B*). Organism names are color-coded according to the domain of life: Eukarya (green), Archaea (blue), and Bacteria (red). Non-Sec-decoding organisms are labeled in bold. Scale bar shows 0.1 changes per site. Only bootstrap values <90 are shown.

that was already in existence at the time of the last universal common ancestor (27–29). SelA is of bacterial origin and was vertically inherited in that domain (30). Phylogenetic analysis of SepSecS and PSTK reveals distinct archaeal and eukaryal versions of these enzymes. This pathway can be traced back (at least) to the evolutionary split between the archaeal and eukaryal sister lineages (Fig. 5). SepSecS is confined to Sec decoding archaea and eukarya. Its evolutionary history is consistent with vertical inheritance and gene loss in non-Sec-decoding lineages (Fig. 5*A*).

More interesting is the complex picture presented in the PSTK phylogeny (Fig. 5*B*). Like SepSecS, PSTK shows an initial deep divide between a strictly eukaryal group on the one hand and an archaeal group on the other. The archaeal PSTK appears to have been horizontally transferred to several of the Sec-decoding eukarya. The unexpected association between *Oryza sativa* and *Strongylocertrotus purpuratus* implies multiple horizontal gene transfer events, whereas a single transfer to mammals early in their evolution accounts for the clustering of these organisms within the archaeal genre. An additional horizontal gene transfer of the archaeal version to non-Sec-decoding Cyanobacteria is also suggested. The eukaryal PSTK is clearly discernable in a distinct group of non-Sec-decoding fungi that lack all other Sec biosynthesis components. These fungi form a coherent cluster in the eukaryal group, implying vertical inheritance. Retention of PSTK in this group suggests that it was recruited to perform a different function. Some eukarya (e.g., *Caenorhabditis* and *Xenopus*) retained both their native eukaryal PSTK and the acquired archaeal version, whereas in other lineages, including ancestors of mammals and trypanosomes, the archaeal version displaced its eukaryal counterpart. Most surprisingly, *Plasmodium falciparum* is Sec-decoding (30, 31), yet neither the PSTK nor SelA gene can be found, which contributes to the idea that a third pathway for Sec formation may exist in some organisms.

In summary, the last universal common ancestor genetically encoded Sec, and whether one or both pathways for converting Ser-tRNA^{Sec} → Sec-tRNA^{Sec} were present in the common ancestor remains to be seen.

Discussion

SepSecS has been suggested to be involved in Sec synthesis in eukarya since the early 1990s (20). In the current work, *in vivo* and *in vitro* results revealed that SepSecS is indeed a SelA analog able to catalyze the Sec-tRNA^{Sec} formation. However, in contrast to bacterial SelA that converts Ser-tRNA^{Sec} directly to Sec-tRNA^{Sec}, SepSecS acts on the phosphorylated intermediate, Sep-tRNA^{Sec} (Fig. 1). Therefore archaeal and eukaryal Sec biosynthesis requires three enzymes, SerRS, PSTK, and SepSecS, whereas bacteria accomplish the same task without PSTK. Both SelA and SepSecS are PLP-dependent enzymes. From a chemical standpoint a Sep → Sec conversion is desirable, as Sep would provide a better leaving group (phosphate) than Ser (water) for replacement with selenium. The detailed reaction mechanism of SepSecS should be subject to future structural and biochemical investigations. Sep-tRNA is reported to be more stable than Ser-tRNA (17), which is known to deacylate faster than any other aa-tRNA species (32). This fact would greatly improve the overall efficiency of the selenocysteinylation reaction and the rate of production of Sec-tRNA in all organisms that possess an extended selenoproteome. Most selenoprotein-containing bacteria only have one to three selenoproteins that are expressed under specific environmental conditions and a nonessential tRNA^{Sec} (33). In contrast, humans have an essential tRNA^{Sec} and an extended selenoproteome whose 25 members have been variously implicated in health and disease (4, 34).

Phylogenetic analyses showed that SelA is of bacterial origin, whereas SepSecS is confined to archaea and eukarya, and PSTK, principally an archaeal and eukaryal enzyme, has been horizontally transferred to the Cyanobacteria. tRNA^{Sec} has distinct secondary structure features: the long extra arm for recognition by SerRS, the elongated acceptor stem for SelB binding (35), and still unknown recognition sites for PSTK and SepSecS. Considering the number of proteins that tRNA^{Sec} interacts with, the clear division of bacterial and archaeal/eukaryal protein lineages and the discernable differences in tRNA^{Sec} structures in the three domains of life, it is interesting to see that *E. coli* tRNA^{Sec} can be recognized by archaeal and human SepSecS at least *in vivo* (35). Moreover, although the biological archaeal and mammalian selenium donors have not been characterized (6), our results indicate that selenophosphate provided by *E. coli* SelD can be used by SepSecS both *in vitro* and *in vivo*.

Are there other pathways? The absence of both PSTK and SelA genes in *P. falciparum* brings out interesting questions: Is there a third pathway or enzyme to make Sec? And why is Sec the only amino acid among the 22 naturally cotranslationally inserted protein building blocks that has not developed its own aa-tRNA synthetase?

Materials and Methods

General. Oligonucleotide synthesis and DNA sequencing was performed by the Keck Foundation Biotechnology Resource Laboratory at Yale University. [^{14}C]serine (163 mCi/mmol) was obtained from Amersham Pharmacia Biosciences (Piscataway, NJ). [^{75}Se]selenite was purchased from the University of Missouri Research Reactor Facility (Columbia, MO).

Bacterial Strains and Plasmids. The *selA* deletion strain JS1 was constructed by replacing the gene with kanamycin cassette in BW25113 *E. coli* strain as described (36). The T7 polymerase gene was inserted in the genome by P1 transduction. The *selC* deletion strain FM460 (37) was obtained from the Yale *E. coli* Stock Center. SepSecS genes from *Methanococcus maripaludis* (MMP0595) and *Methanocaldococcus jannaschii* (MJ0610) were amplified from genomic DNA, and the human SepSecS gene (GenBank accession no. BX648976) was amplified from an EST clone [clone ID: DKFZp686J1361, obtained from the RZPD German Resource Center for Genome Research, Berlin, Germany (38)] followed by cloning into the pET15b vector. *E. coli selD* was cloned into pET15b vector. *Methanococcus maripaludis* tRNA^{Sec} and *Methanocaldococcus jannaschii* PSTK gene were cloned into the pACYC vector individually or together. Human SepSecS K284T was generated by using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's directions.

Protein Expression and Purification. Human SepSecS, *Methanococcus maripaludis* SepSecS, and *E. coli* SelD were transformed into BL21(DE3) cd+ strain. Cells were grown to A_{600} = 1.0, and protein production was induced by the addition of 0.5 mM isopropyl β-D-thiogalactoside (IPTG). The cells were grown for 20 h at 15°C and then spun down and resuspended in 50 mM Hepes (pH 8.0), 300 mM NaCl, 10 mM imidazole, 3 mM DTT, 10 μM PLP, and protease inhibitor mix (Roche, Indianapolis, IN). After sonication, cell lysates were applied to Ni-NTA resin (Qiagen, Valencia, CA). The resin was washed, and the His_6-tagged proteins were eluted according to the manufacturer's manual. Proteins were then thoroughly dialyzed against 50 mM Hepes (pH 8.0), 300 mM NaCl, 10 mM DTT, 10 μM PLP, and 50% glycerol.

Complementation of an *E. coli* ΔselA Strain. *Methanocaldococcus jannaschii*, *Methanococcus maripaludis*, or human SepSecS were transformed into a ΔselA *E. coli* JS1 strain, with or without the *Methanocaldococcus jannaschii* PSTK gene. The deletion strain complemented with its own *selA* gene served as positive control. *E. coli* Δ*selA* strain plus PSTK, SepSecS, or both genes was plated on glucose-minimum medium agar plates with 0.01 mM IPTG and grown anaerobically for 2 days at 30°C. The plates then were

overlaid with agar containing 1 mg/ml BV, 0.25 M sodium formate, and 25 mM KH_2PO_4 adjusted to pH 7.0. The appearance of blue/purple color is the indication of active FDH_H (24). The same experiments were also carried out for SepSecS mutants. Formate dehydrogenase N activity was tested with MacConkey nitrate medium plates.

Metabolic Labeling with [^{75}Se]selenite. *E. coli* cells were grown aerobically overnight and diluted (1:50) under anaerobic conditions in 5 ml of TGYEP medium (0.5% glucose, 1% tryptone, 0.5% yeast extract, 1.2% K_2HPO_4, 0.3% KH_2PO_4, 0.1% formate, 1 μM Na_2MoO_4, pH adjusted to 6.5) (39) with 1 μCi [^{75}Se]selenite and 0.05 mM IPTG added. The cells were further grown anaerobically at 37°C for another 24 h and harvested. Cell lysates were prepared and subjected to SDS/PAGE, followed by autoradiography.

Preparation of tRNA Substrates. The *Methanococcus maripaludis* $tRNA^{Sec}$ gene was cloned into pUC18 with T7 promoter and terminator. The plasmid was transformed together with a pACYC vector encoding T7 RNA polymerase gene to the *E. coli selC* deletion strain (FM460). $tRNA^{Sec}$ was overexpressed and purified with biotinylated DNA oligonucleotides as described (40). Purified $tRNA^{Sec}$ (10 μM) was serylated in the presence of [^{14}C]Ser (200 μM) and *Methanococcus maripaludis* SerRS (6 μM) in reaction buffer (100 mM Hepes, pH 7.0/10 mM KCl/10 mM magnesium acetate/1 mM DTT/0.1 mg/ml BSA) at 37°C for 1 h. To prepare Sep-$tRNA^{Sec}$, 3 μM PSTK was added to the reaction. Aminoacylated tRNA products were purified by phenol extraction and ethanol precipitation followed by G-25 desalting column application.

***In Vitro* Sep → Sec Conversion.** Purified recombinant SepSecS was incubated in reaction buffer (100 mM Hepes, pH 7.0/300 mM KCl/10 mM $MgCl_2$) with Sep-$tRNA^{Sec}$ or Ser-$tRNA^{Sec}$ (10 μM), DTT (1 mM), Na_2SeO_3 (250 μM), and purified recombinant *E. coli* SelD (100 μM) at 37°C for 30 min. The oxygen in the buffer was removed, and the reaction was carried out in the anaerobic chamber. After incubation, proteins were removed by phenol extraction. The tRNAs were purified with G-25 column and ethanol precipitation to remove small molecules and salts. Purified tRNA products were deacylated in 20 mM NaOH at room temperature for 10 min. The released amino acids were oxidized with performic acid (16) and subjected to TLC analysis [system 1: 85% ethanol; system 2: butanol/acetic acid/water (4:1:1)].

Phylogenetic Analysis. Sequences, taken from the National Center for Biotechnology Information nonredundant database or the ERGO database, were aligned by using Multiseq in VMD 1.8.5 (41). The most parsimonious trees were generated with PAUP4b10 (42). Gaps were counted as missing data and a parsimony cost matrix, based on the Blosum45 substitution matrix (43), was used. Of the 1,000 most parsimonious trees, the topology with the maximum likelihood was chosen and branch lengths were optimized with PHYML v.2.4.4 (44). The JTT+Γ model with eight rate categories was used for amino acid substitution, and adjustable parameters were derived from maximum-likelihood estimates. Bootstrap values were computed by using the re-estimation of log likelihoods (RELL) method in PROTML from the Molphy 3.2 package (45).

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