Genetic Analysis of Selenocysteine Biosynthesis in the Archaeon *Methanococcus maripaludis*

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Summary

In Archaea selenocysteine (Sec) is synthesized in three steps. First seryl-tRNA synthetase acylates tRNA^{Sec} with serine to generate Ser-tRNA^{Sec}. Then phosphoseryl-tRNA^{Sec} kinase (PSTK) forms Sep-tRNA^{Sec}, which is converted to Sec-tRNA^{Sec} by Sep-tRNA:Sec-tRNA synthase (SepSecS) in the presence of selenophosphate produced by selenophosphate synthetase (SelD). A complete *in vivo* analysis of the archaeal Sec biosynthesis pathway is still unavailable, and the existence of a redundant pathway or of a rescue mechanism based on the conversion of Sep-tRNA^{Sec} to Cys-tRNA^{Sec} during selenium starvation, cannot be excluded.

Here we present a mutational analysis of Sec biosynthesis in *Methanococcus maripaludis* strain Mm900. Sec formation is abolished upon individually deleting the genes encoding SelD, PSTK, or SepSecS; the resulting mutant strains could no longer grow on formate while growth with H_2 + CO_2 remained unaffected. However, deletion of the PSTK and SepSecS genes was not possible unless the selenium-free [NiFe]-hydrogenases Frc and Vhc were expressed. This required the prior deletion of either the gene encoding SelD or that of HrsM, a LysR-type regulator suppressing transcription of the *frc* and *vhc* operons in the presence of selenium. These results show that *M. maripaludis* Mm900 is facultatively selenium-dependent with a single pathway of Sec-tRNA^{Sec} formation.

Keywords

Selenocysteine; Selenophosphate synthetase; *O*-phosphoseryl-tRNA^{Sec} kinase; Sep-tRNA:Sec-tRNA synthase

Introduction

Selenium is an important micronutrient for a subset of organisms from all three lines of descent (Bacteria, Archaea and Eukarya). Its most relevant biological form is the rare amino acid selenocysteine which is made in a tRNA-dependent manner. First tRNA^{Sec} is acylated with serine (Ser) by seryl-tRNA synthetase (SerRS), followed by conversion of Ser-tRNA^{Sec} to Sec-tRNA^{Sec}. Two pathways for Sec formation have been found in nature (Fig. 1). In bacteria Ser-tRNA^{Sec} to Sec-tRNA^{Sec} conversion occurs in a single step, catalyzed by the PLP-dependent enzyme selenocysteine synthase (SelA) (Forchhammer and Böck, 1991; Böck *et al.*, 2005). In eukaryotes and archaea, an additional step is required, in which Ser-tRNA^{Sec} is first converted to *O*-phosphoseryl-tRNA^{Sec} (Sep-tRNA^{Sec}) by *O*-phosphoseryl-

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tRNA kinase (PSTK) (Carlson *et al.*, 2004; Sherrer *et al.*, 2008a). The transformation from Sep-tRNA[Sec] to Sec-tRNA[Sec] is then catalyzed by a distant homolog of SelA, Sep-tRNA:Sec-tRNA synthase (SepSecS), in a PLP-dependent manner (Yuan *et al.*, 2006; Xu *et al.*, 2007). Both SelA and SepSecS use as selenium donor selenophosphate, which is produced by selenophosphate synthetase (SelD) from selenide and ATP (Leinfelder *et al.*, 1990). Besides its role in Sec formation, selenophosphate has also been described as a selenium donor for the synthesis of 2-selenouridine, a modified nucleoside found in certain tRNAs (Wittwer, 1983; Ching, 1984; Ching *et al.*, 1984; Ching *et al.*, 1985).

Sec is co-translationally inserted into proteins by tRNA[Sec] at certain UGA codons, that would otherwise signal stop (Flohe *et al.*, 1973; Rotruck *et al.*, 1973). Recoding of UGA into a Sec-codon requires two components. The first is the SECIS (Sec insertion sequence) element, a distinct stem-loop structure in the selenoprotein mRNA. The second is a Sec-specific elongation factor (SelB or EF Sec) that binds GTP and the SECIS element and delivers Sec-tRNA[Sec] to the ribosome (Forchhammer *et al.*, 1989; Berry *et al.*, 1991; Fagegaltier *et al.*, 2000).

Only a small number of archaea encode selenoproteins; this includes all representatives of the orders *Methanopyrales* and *Methanococcales* with known genome sequences (Rother et al., 2001; Kryukov and Gladyshev, 2004). Members of these orders are obligate anaerobes which gain energy exclusively by the reduction of CO_2 (with H_2) and/or formate to methane. *Methanococcus maripaludis* S2 encodes ten Sec containing proteins in its genome (Hendrickson *et al.*, 2004; Kryukov and Gladyshev, 2004), of which eight are involved in hydrogenotrophic energy metabolism. This includes subunits of F_{420}-dependent (FruA; MMP1382) and F_{420}-independent hydrogenase (VhuD; MMP1696 and VhuU; MMP1693), tungsten-containing formyl-methanofuran dehydrogenase (FwdB; MMP1691), molybdenum containing formylmethanofuran dehydrogenase (FmdB; MMP0511), heterodisulfide reductase (HdrA; MMP1697) and formate dehydrogenase (FdhA1; MMP1298 and FdhA2; MMP0138). In addition, a backup system consisting of selenium-free, cysteine-containing homologs for most of these selenoenzymes is encoded in the genome, with the exception of formate dehydrogenase. Consequently, under selenium limitation *M. maripaludis* should be able to grow with $H_2 + CO_2$ but not with formate. Indeed, this has been shown for *selB* and *selD* deletion strains of *M. maripaludis* JJ (Rother *et al.*, 2003; Stock *et al.*, 2010).

Apart from these genetic studies demonstrating the physiological functions of SelB (Rother *et al.*, 2003) and SelD (Stock *et al.*, 2010), most of our knowledge on Sec biosynthesis in archaea is based on *in vitro* analyses of PSTK from *Methanocaldococcus jannaschii* (Kaiser *et al.*, 2005; Sherrer *et al.*, 2008b) and SepSecS from *M. maripaludis* (Araiso *et al.*, 2008), as well as on *in vivo* complementation studies using *M. jannaschii* PSTK and SepSecS in a heterologous bacterial system (Yuan *et al.*, 2006; Araiso *et al.*, 2008; Sherrer *et al.*, 2008b). Although these experiments clearly indicate that Sec formation in archaea and eukaryotes is of a common origin, a number of questions remain unresolved. We recently demonstrated that Sep-tRNA:Cys-tRNA synthase (SepCysS), an enzyme catalyzing the conversion of Sep-tRNA[Cys] to Cys-tRNA[Cys] in a number of archaea, including *M. maripaludis* (Sauerwald *et al.*, 2005), can also recognize Sep-tRNA[Sec] as a substrate, and thus can replace SepSecS in the heterologous *Escherichia coli* system (Yuan *et al.*, 2010). This raises the intriguing possibility that SepCysS could rescue a SepSecS deletion in *M. maripaludis*. Moreover, it remains unknown why bacteria can convert Ser to Sec in a single step via the action of SelA, while archaea and eukaryotes require two enzymes, PSTK and SepSecS, which are phylogenetically remarkably distinct from SelA (Araiso *et al.*, 2008). Yet, it raises the question whether PSTK and/or SepSecS perform other physiological functions that are essential for these organisms.

In the present study, we took advantage of the genetic tractability of the *M. maripaludis* Mm900 strain (Moore and Leigh, 2005) to examine the archaeal mechanism of Sec biosynthesis *in vivo*, and to investigate the key roles of SelD, PSTK and SepSecS.

Results

Construction of marked and unmarked gene deletions in M. maripaludis

To analyze the Sec-tRNA^{Sec} formation pathway in *M. maripaludis* we constructed deletion mutants of the genes encoding SelD (MMP0904), PSTK (MMP1490; encoded by *pstK*) and SepSecS (MMP0595; encoded by *spcS*). We chose a "knock-in-knock-out" approach (Fig. 2) which allows generation of both marked and markerless gene deletions using the same suicide-plasmid. To construct marked gene deletions (Fig. 2, I–III), approximately 0.5 to 1.0 kb long DNA segments flanking the desired gene were first cloned into the suicide plasmid pJKM12, upstream and downstream of the puromycin resistance cassette (*pac*) which is flanked by two *flp* recombinase recognition sites (*FRT*). The resulting plasmid was then transformed into *M. maripaludis* Mm900 (*M. maripaludis* S2 Δ*hpt* mutant) with selection for puromycin resistance (Fig. 2, I). Since the plasmid cannot replicate in *Methanococcus*, recombinants arise by chromosomal integration of the vector into the flanking region of the target gene, mediated by a single homologous recombination event (Fig. 2, II). The resulting merodiploids were resistant to puromycin and were sensitive to the toxic purine analog 8-azahypoxanthine (zHyp) due to the presence of the vector-encoded *hpt*. Under continuous selection with puromycin, a second homologous recombination can take place, removing the wild-type allele together with the vector backbone from the chromosome, thus resolving the unstable merodiploid state. These recombinants were selected as puromycin and zHyp resistant clones (Fig. 2, III). To allow reuse of puromycin as a marker, the *pac*-cassette could be removed by intrachromosomal recombination (Fig. 2, IV) mediated by Flp-recombinase, which was expressed from plasmid pFLPH. By this method we readily generated marked deletion mutants of *selD*. Plating of ca. 10^3 cells that had undergone the second homologous recombination event gave rise to six puromycin and zHyp resistant colonies. Southern blot analysis revealed that in all six clones *selD* had been deleted (Fig S1 B). From one strain, designated FSD6, the *pac*-cassette was subsequently removed by Flp-recombinase mediated recombination to yield the markerless *selD* mutant FSD10 (Fig. S1). Surprisingly however, we were not able to delete the genes encoding PSTK and SepSecS from the *M. maripaludis* Mm900 chromosome, neither by using the "knock-in-knock-out" gene deletion strategy described above, nor by applying previously established methods (Stathopoulos *et al.*, 2001; Moore and Leigh, 2005).

Characterization of the SelD mutant

To determine how the deletion of SelD affects the growth phenotype compared to the wild-type, the knockout mutant FSD10 was subjected to cultivation experiments. Grown in the presence of $H_2 + CO_2$, the Δ*selD* strain had a slightly longer doubling time than the wild-type, and it was not able to grow on formate alone (Fig. 3). Given that both formate dehydrogenases in *M. maripaludis* are selenoenzymes (Wood *et al.*, 2003), this phenotype indicated that the Sec forming pathway has been impaired in this mutant. To directly follow selenium incorporation into protein and tRNAs, wild-type and deletion mutants were grown in the presence of [^{75}Se]selenite, followed by separation of cell extracts on a polyacrylamide gel and visualization by autoradiography. While in wild-type cell extract selenium incorporation occurred in both proteins and tRNAs (Fig. 4, lane 1), in cell extracts from the Δ*selD* mutant neither selenoproteins nor selenium-modified tRNAs were detectable (Fig. 4, lane 2). This confirms the previous finding that SelD is the predicted archaeal selenophosphate synthetase which provides the selenium donor for both Sec and selenouridine synthesis (Stock *et al.*, 2010). In an attempt to rescue the *selD* deletion,

FSD10 was transformed with plasmid pECSD from which *E. coli* SelD was expressed under the control of a constitutive *Methanococcus* promoter. *In vivo* labeling experiments with [^{75}Se]selenite show that both selenoprotein synthesis and tRNA modification were reestablished in this strain (Fig. 4, lane 3); thus, archaeal and bacterial SelD are interchangeable (Stock *et al.*, 2010). Consequently, the *E. coli selD* complemented strain is able to use formate as sole substrate, demonstrating the expression of a functional formate dehydrogenase (Fig. 3B).

Deletion of PSTK or SepSecS genes from the SelD mutant

Given that selenoprotein formation is not essential for the cultivation of *M. maripaludis* Mm900 on $H_2 + CO_2$, the finding that PSTK and SepSecS could not be deleted from this strain indicates that PSTK and/or SepSecS might have essential cellular functions in addition to Sec synthesis. If this were the case, the deletion of their corresponding genes should generally not be possible. Alternatively, an as-yet-unidentified regulatory process could make the presence of PSTK and/or SepSecS necessary as long as selenium is available to the organism. In that case, PSTK and SepSecS should become dispensable when *M. maripaludis* is grown on $H_2 + CO_2$ in a selenium-free medium or generally speaking, under conditions where no selenium donor for Sec formation is available. Due to the difficulty of preparing a medium free of any trace contamination with selenium, we decided to use a *selD* mutant (which is unable to form selenophosphate) as background to delete *pstK* and *spcS*. Transformation of FSD10 with suicide plasmids pJKM12ΔpstK or pJKM12ΔspcS yielded both knockouts of *pstK* (Fig. S2) and *spcS* (Fig. S3), respectively. Thus, neither PSTK nor SepSecS has further essential functions besides Sec formation.

SepSecS is required for Sec formation in M. maripaludis

In order to assess the phenotype of the SepSecS deletion, we complemented the *selD*/*spcS* double-deletion mutant (designated FSD103) with the *E. coli selD*-encoding plasmid. Cultivated on $H_2 + CO_2$, the resulting strain had a longer doubling time than the wild-type comparable to the *selD* deletion mutant, and growth on formate was still impaired (Fig. 5). Furthermore, no selenium containing proteins were detectable after *in vivo* labeling with [^{75}Se]selenite (Fig. 6, lane 3). The presence of active SelD, on the other hand, was evidenced by the formation of selenium-modified tRNAs. This result demonstrates that SepSecS is the only Sec synthase present in *M. maripaludis*. Also, the inability to grow on formate revealed that SepCysS cannot complement the deletion of SepSecS *in vivo*.

The archaeal and bacterial Sec formation pathways are compatible

In order to address the *in vivo* role of PSTK, we attempted to complement the *selD*/*pstK* double deletion mutant (designated FSD102) with *E. coli selD* in the same manner as described above for FSD103. The SelD expression plasmid, however, was unstable in this strain despite neomycin selection. In an alternative approach to construct a *pstK* deletion mutant, we transformed *M. maripaludis* Mm900 with plasmid pECSA, encoding *E. coli* Sec synthase (SelA), a neomycin marker, and the Hpt gene under control of constitutive archaeal promoters. From this transformant the PSTK gene could subsequently be deleted, resulting in a strain able to grow with $H_2 + CO_2$ as well as with formate (Fig 5). Selenoprotein formation was not impaired in this strain (Fig. 6, lane 4), demonstrating that the bacterial Sec formation pathway can replace its archaeal counterpart. Nevertheless, successive attempts to remove the *selA*-expression plasmid by counter selection with 8-azahypoxanthine failed. This observation is adding further support to our previous speculation that a regulatory mechanism may be present in *M. maripaludis* that renders the Sec biosynthesis pathway indispensable as long as selenium is available to the organism.

Deletion of PSTK requires expression of selenium-free [NiFe]-hydrogenases

Selenium-dependent gene regulatory mechanisms were observed previously in the closely related *Methanococcus voltae.* It has been shown that the two selenium-free [NiFe]-hydrogenases Frc and Vhc are only expressed under selenium starvation, whereas in the presence of selenium only the two Sec containing homologs Fru and Vhu are produced (Berghöfer *et al.*, 1994). In *M. voltae*, the LysR-family regulator HrsM was shown to repress the transcription of the Frc and Vhc operons when selenium is available (Sun and Klein, 2004). The presence of an HrsM homolog (MMP1712), together with a conserved organization for the Frc and Vhc operons, indicate that the same regulatory mechanism is likely to be present in *M. maripaludis* S2. To verify this, we constructed an *hrsM* deletion mutant (designated FHM1). RT-PCR experiments were used to evaluate transcription of the FrcA and VhcD encoding genes in *M. maripaludis* Mm900, FSD10 and FHM1 strains grown in selenite containing medium under an $H_2 + CO_2$ atmosphere. While transcription of the *frcA* and *vhcD* genes was repressed in the wild-type under these conditions, their expression was detected in the $\Delta hrsM$ and in the $\Delta selD$ strains (Fig. 7, lanes 1–6). On the other hand, transcription of the *vhuD* gene, encoding a subunit of the Sec-containing [NiFe]-hydrogenase Vhu, occurred at a significantly lower level in the $\Delta hrsM$ and in the $\Delta selD$ strains compared to wild-type (Fig. 7, lanes 7–9). Using suicide plasmid pJKM12NΔpstK, we were able to delete *pstK* in the $\Delta hrsM$ strain (resulting in strain FHM11; Fig. S4), indicating that the tight repression of the selenium-free [NiFe]-hydrogenases (Frc and Vhc) was affecting the dispensability of PSTK.

Sep-tRNA[Sec] is an essential intermediate in the archaeal Sec formation pathway

The *hrsM* deletion and *hrsM*/*pstK* double-deletion strains were characterized in cultivation experiments and by *in vivo* [^{75}Se] incorporation. The $\Delta hrsM$ mutant was able to synthesize [^{75}Se]-labeled proteins and seleno-modified tRNAs (Fig. 6, lane 5). Protein bands corresponding to FruA and VhuD, subunits of the selenium-containing hydrogenases, were not detectable. This did not significantly affect the growth rate of this strain on $H_2 + CO_2$ (Fig. 5), presumably due to the expression of Frc and Vhc. On formate medium, the $\Delta hrsM$ mutant exhibited a slightly slower growth rate compared to the wild-type (Fig. 5). This might be explained by a weaker expression of either one or both of the two formate dehydrogenases. In agreement with this, the labeled protein band corresponding to formate dehydrogenase subunits FdhA1 and FdhA2 appeared to be of significantly lesser intensity (Fig. 6, lane 5). On the other hand, the *hrsM*/*pstK* double-deletion mutant exhibited a slower growth rate on $H_2 + CO_2$ and completely lost the capacity to grow on formate (Fig. 5). Also, [^{75}Se] incorporation into both proteins and tRNAs was undetectable (Fig. 6, lane 6). The absence of [^{75}Se]-labeled tRNAs was anticipated, since *M. maripaludis* SelD, which provides the activated Se-donor for selenouridine formation, is a selenoenzyme itself (Moore and Leigh, 2005; Stock *et al.*, 2010). Our results confirm previous *in vitro* experiments showing that Sep-tRNA[Sec] formed by PSTK is a crucial intermediate in the archaeal Sec formation pathway (Yuan *et al.*, 2006).

DISCUSSION

A number of powerful genetic tools have been developed for *M. maripaludis*, making this organism an important model for molecular studies within the archaeal domain (Sandbeck and Leigh, 1991; Blank *et al.*, 1995; Argyle *et al.*, 1996; Whitman *et al.*, 1997; Moore and Leigh, 2005; Rother and Metcalf, 2005; Chaban *et al.*, 2007; VanDyke *et al.*, 2009). A recent report that *M. maripaludis* is polyploid and that single cells may contain more than 50 genome copies during exponential growth (Hildenbrand *et al.*, 2011) makes clear, however, that genetic manipulation of this organism is not trivial. Attempts to construct knockout mutants may result in strains carrying both the wild-type and mutant allele even when the

targeted gene is not essential, and careful evaluation of the resulting genotype is therefore crucial. A further limitation is the availability of only two antibiotic resistance markers (providing resistance against puromycin and neomycin) for selection in *Methanococcus.* Thus, it is challenging to construct strains carrying several mutations, an essential requirement for investigating interactions among various genes or to examine functional genetic redundancies. A previously reported mutagenesis method (Moore and Leigh, 2005) overcame this limitation by applying two consecutive allelic exchange reactions; they yield either a markerless deletion of the targeted gene, or restore the wild-type. Screening for the desired mutant is necessary, and is hampered when the mutant has a slower growth rate than the wild-type (Rother and Metcalf, 2005). A more straightforward strategy, the *Saccharomyces cerevisiae* Flp-*FRT* system (Sadowski, 1995), which allows *flp* recombinase-mediated removal of the antibiotic marker by an intra-chromosomal sequence-specific recombination, was successfully used in bacterial (Cherepanov and Wackernagel, 1995; Schweizer, 2003), archaeal (Welander and Metcalf, 2008) and eukaryotic (Dymecki, 1996; Lyznik *et al.*, 1996) systems. Our adaptation of the Flp-*FRT* recombination system for use in *M. maripaludis* to generate single and consecutive gene deletions was the basis for successfully obtaining deletions of the three genes involved in Sec biosynthesis and the demonstration that this biosynthetic role is their only cellular function.

Selenophosphate has previously been shown to serve as the activated selenium donor for both Sec biosynthesis and for the formation of selenium-modified tRNAs (Leinfelder *et al.*, 1990). In agreement with this, deletion of the selenophosphate synthetase encoding *selD* from the *M. maripaludis* JJ genome eradicated this organism's ability of selenium incorporation into proteins and tRNA (Stock *et al.*, 2010). However, attempts to delete *selD* (encoded by ORF MMP0904) in *M. maripaludis* S2 failed, leading the authors to conclude that viability of this strain is strictly selenium dependent (Stock *et al.*, 2010). In the present study MMP0904 was readily deleted from *M. maripaludis* S2 derived strain Mm900. Selenoprotein formation, and consequently the ability to grow on formate, was abolished in this mutant. Growth on $H_2 + CO_2$ remained unaffected demonstrating that, as in *M. maripaludis* JJ, the presence of SelD is not required for hydrogenotrophic growth. Complementation of the Δ*selD* strain FSD10 with *E. coli* SelD restored selenoprotein formation. The observed slower growth rate on formate for this complemented strain might be due to a lower efficiency of *E. coli* SelD in *M. maripaludis* or might result from a low expression level of the heterologous enzyme.

While earlier *in vitro* experiments and genetic studies with the heterologous *E. coli in vivo* system established an archaeal Sec biosynthesis pathway dependent on PSTK and SepSecS (Yuan *et al.*, 2006), the presence of an additional SepSecS-independent route could not be fully excluded. Indeed, *Methanocaldococcus* and *Methanococcus* species encode a bacterial SelA homolog; but the corresponding recombinant *M. jannaschii* enzyme (MJ0158) could not form Sec-tRNA[Sec] from either Ser-tRNA[Sec] or Sep-tRNA[Sec] *in vitro* (Kaiser *et al.*, 2005). While this led to the notion that this hypothetical SelA enzyme might require further proteins for activity, the experiments reported here reveal that in vivo *M. maripaludis* uses only the essential enzymes PSTK and SepSecS for Sec formation. Recent *in vivo* studies in *Trypanosoma brucei* indicate that this is the only pathway present in eukaryotes (Aeby *et al.*, 2009). Our experiments also showed that *E. coli* SelA complements the Δ*pstK* strain. This is was expected as *E. coli* Sec synthase converted archaeal Ser-tRNA[Sec] to Sec-tRNA[Sec] *in vitro* (Kaiser *et al.*, 2005). This leaves us with the intriguing question as to why during the eukaryotic and archaeal evolution these lineages maintained the two-step pathway for Sec-tRNA formation without any organism adopting the single-step bacterial mechanism via lateral gene transfer.

The deletion of *pstK* or *spcS* required the prior disruption of Sec-formation by deleting the gene encoding either SelD or the repressor HrsM. The deletion of *selD* or *hrsM* led to transcription of the genes encoding the selenium-free [NiFe]-hydrogenases Frc and Vhc, necessary for energy generation under selenium deprivation. LysR-type gene regulators (e.g., HrsM) usually require activation by an effector molecule. The effector for HrsM is unknown, but a likely candidate may be selenophosphate.

In this context, our finding that the selD/pstK double-mutant could be complemented with *E. coli selD* is surprising, as one would expect that the presence of selenophosphate (which is evidenced by the formation of selenium-modiefied tRNAs in this strain) would lead to repression of the Frc/Vhc operon. Possibly, the observed lower efficiency of *E. coli* SelD in the heterologous background results in cellular selenophosphate levels low enough to allow expression of the selenium-free hydrogenases.

Overall, our results demonstrate that in *M. maripaludis* Sec biosynthesis and the expression of certain selenoproteins (e.g., Fru and Vhu) are linked by a complex regulatory feedback mechanism.

The pathway catalyzed by PSTK and SepSecS is reminiscent of the indirect Cys-tRNA^{Cys} formation in methanogens where SepCysS converts Sep-tRNA^{Cys} to Cys-tRNA^{Cys} (Sauerwald *et al.*, 2005). We showed recently that an *E. coli* ΔselA strain expresses active formate dehydrogenase (normally a selenoenzyme in *E. coli*) when complemented with *M. jannaschii* PSTK and SepCysS (Yuan *et al.*, 2010). Since no selenoproteins were formed in this strain, SepCysS might convert Sep-tRNA^{Sec} to Cys-tRNA^{Sec}, probably leading to a Cys homolog of formate dehydrogenase H. A similar SepCysS induced read-through of Sec-codons might be imaginable in *M. maripaludis* mutants with a disrupted Sec biosynthesis pathway. However, neither the SelD, PSTK, nor SepSecS deletion mutant was able to grow with formate as the only carbon and energy source, indicating the absence of a sufficient amount of active formate dehydrogenase in these strains. The mutation of the Sec residue in the catalytic active site of *E. coli* formate dehydrogenase H to cysteine results in a 110-fold reduction of catalytic activity compared to the wild-type enzyme (Axley *et al.*, 1991). The effect of a Sec to Cys mutation in the *M. maripaludis* paralog formate dehydrogenase A has not been studied so far, but might render this enzyme inactive. Alternatively, control mechanisms might be present in *M. maripaludis* to prevent Cys-tRNA^{Sec} from entering protein biosynthesis. Further biochemical studies and genetic experiments using suitable reporter systems will be necessary to clarify these issues.

Experimental procedures

Microbial strains and growth conditions

M. maripaludis strains (Table S1) were grown at 37 °C on solid or liquid McCas medium (Moore and Leigh, 2005) under strictly anaerobic conditions with 276 kPa H_2/CO_2 gas (80:20 [vol/vol]) as described before (Balch *et al.*, 1979). Puromycin (2.5 μg/ml), neomycin (1.0 mg/ml), and 8-azahypoxanthine (0.25 mg/ml) were added to the medium as needed. For growth on formate, the medium contained 2% sodium formate and 80 mM morpholinepropanesulfonic acid, and cultures were pressurized with 100 kPa N_2/CO_2. *Escherichia coli* Dh5α, grown at 37 °C on Luria-Bertani medium supplemented with ampicillin (100 μg/ml) was used for plasmid cloning.

DNA methods, plasmid construction, and transformation

Standard methods were used for isolation of genomic DNA from *M. maripaludis* and of plasmid and genomic DNA from *E. coli*. All plasmids and primers used in this study are described in Tables S2 and S3. Southern blot analyses were performed using the DIG system

(Roche, Indianapolis, IN) according to manufacturer guidelines. *M. maripaludis* strains were transformed as described previously (Tumbula *et al.*, 1994).

Generation of M. maripaludis deletion strains

M. maripaludis gene deletions were generated in the Δ*hpt* strain Mm900 (Moore and Leigh, 2005) by homologous-recombination mediated gene replacement. Approximately 0.5 to 1.0 kb long DNA segments flanking the 5′ region and the 3′ region of the gene of interest were PCR amplified using *M. maripaludis* Mm900 genomic DNA as template. Each fragment was then cloned into plasmid pJKM12, upstream and downstream of the *pac*-cassette which is flanked by two *Frt* sites in the same orientation. 5 µg of the resulting plasmid were transformed into *M. maripaludis*, and transformants were incubated without antibiotic selection for 6 h. Cultures were then transferred into 5 ml McCas medium containing puromycin, grown to an $A_{600} \approx 0.8$ and, after adequate dilution, plated on McCas plates supplemented with puromycin and 8-azahypoxanthine. Single clones were PCR-screened, and replacement of the wild-type locus with the *Frt-pac-Frt* cassette was confirmed by Southern blotting.

When additional gene deletions were required, the puromycin marker was removed by transforming the knock-out strains with the replicative plasmid pFLPH, which encodes Flp recombinase under control of the *hmvA* promoter. Transformants were grown for 5 to 10 consecutive passages in McCas medium under neomycin selection, single colony purified, and screened by PCR for removal of the *pac* cassette. Markerless deletion of the wild-type locus was confirmed by Southern analysis. Strains were cured from the Flp recombinase encoding plasmid by 5 consecutive transfers on McCas medium without antibiotic selection. After plating, clones which had lost the plasmid were identified by PCR and by their sensitivity to neomycin.

Reverse transcriptase PCR

Total cellular RNA from 5 ml *M. maripaludis* cultures was purified using the RNeasy Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Two mg RNA in H_2O was treated with RNase-free DNase I (20 units mg^{-1} RNA) for 2 h at 37 °C. The DNase was inactivated by heating at 75 °C for 10 min. The RNA was then used as template for cDNA synthesis using random hexamer primers and the Superscript II RT system (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

Primer combinations FRCAF-FRCAR, VHCDF-VHCDR, and VHUF-VHUR (Table S3) were used to amplify fragments of *frcA*, *vhcD*, and *vhuD*, respectively.

Metabolic labeling with [^{75}Se]selenite

For labeling, *M. maripaludis* cultures grown to an A_{600} of 0.8 were diluted 1:50 in 5 ml McCas medium supplemented with 7.5 µCi [^{75}Se]selenite, and incubated at 37 °C for 48 h. Tubes were re-pressurized with H_2/CO_2 after 24 h. Cells were harvested by centrifugation, washed in McCas medium, and lysed by resuspending in H_2O_{bidest}. Cell lysates were subjected to SDS/PAGE, followed by autoradiography.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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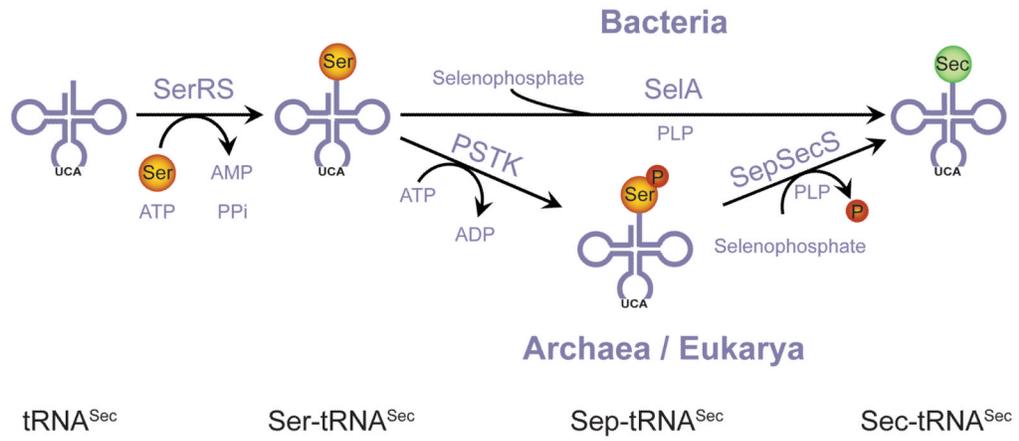
Fig. 1.
tRNA-dependent pathways leading to Sec-formation. In bacteria, Ser-tRNA^{Sec} to Sec-tRNA^{Sec} transformation is catalyzed by SelA. The archaeal/eukaryal route is dependent on PSTK and SepSecS.
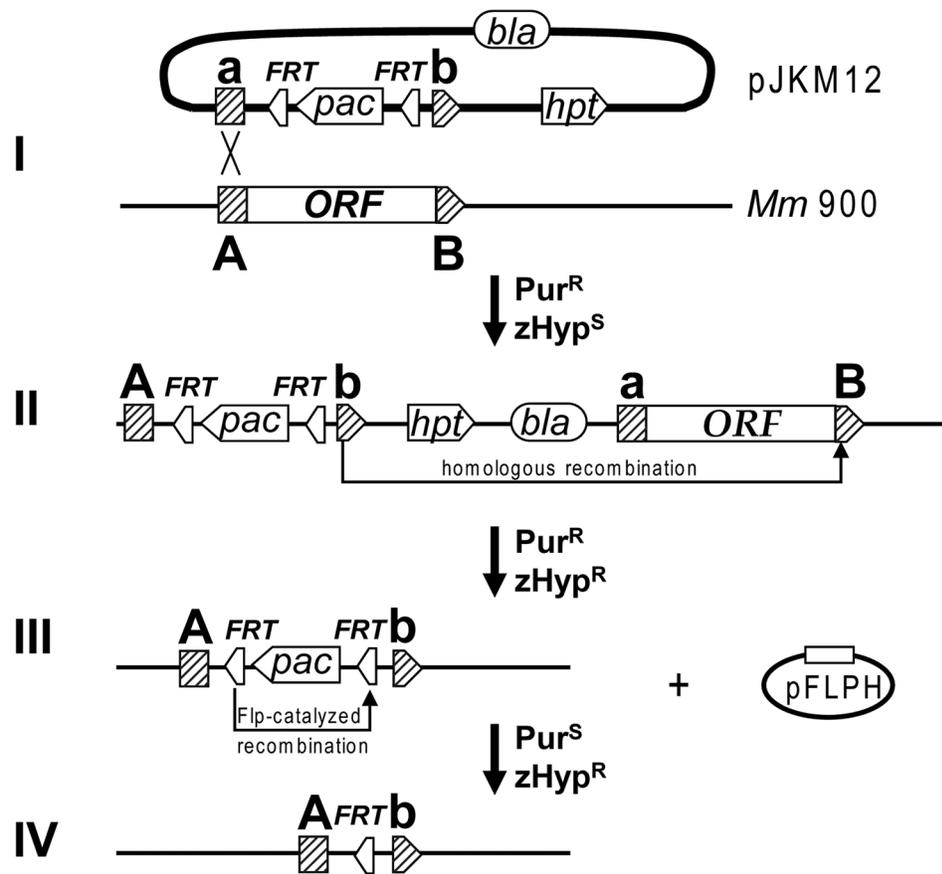
Fig. 2.
Generation of marked and markerless chromosomal deletions in *M. maripaludis*. (I) Plasmid pJKM12 which encodes *M. maripaludis hpt,* as well as a puromycin resistance cassette (*pac*) flanked by two *flp* recombinase recognition sites (*FRT*) and regions homologous to the target gene (a/A and b/B) is transformed into *M. maripaludis* Mm900 (Δ*hpt*). (II) Integration of pJKM12 into the chromosome creates an unstable merodiploid state; a second homologous recombination event removes the vector backbone together with the wild-type allele of the target gene from the chromosome. (III) The target gene is replaced by *pac* bracketed by two *FRT* sites; cells are resistant to puromycin and zHyp. (IV) Expression of Flp recombinase from plasmid pFLPH results in removal of the puromycin cassette.
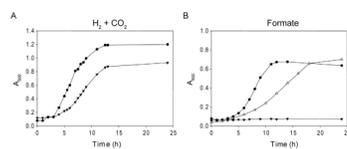
Fig. 3.
Effects of selenium on growth. (A) Growth of *M. maripaludis* Mm900 wild-type (solid circles) and of FSD10 (*selD* deletion mutant; solid triangles) cultivated on $H_2 + CO_2$. (B) Cultivation on formate. Same symbols as in A. Additionally: FSD10 was complemented with *E. coli selD* (open triangles).
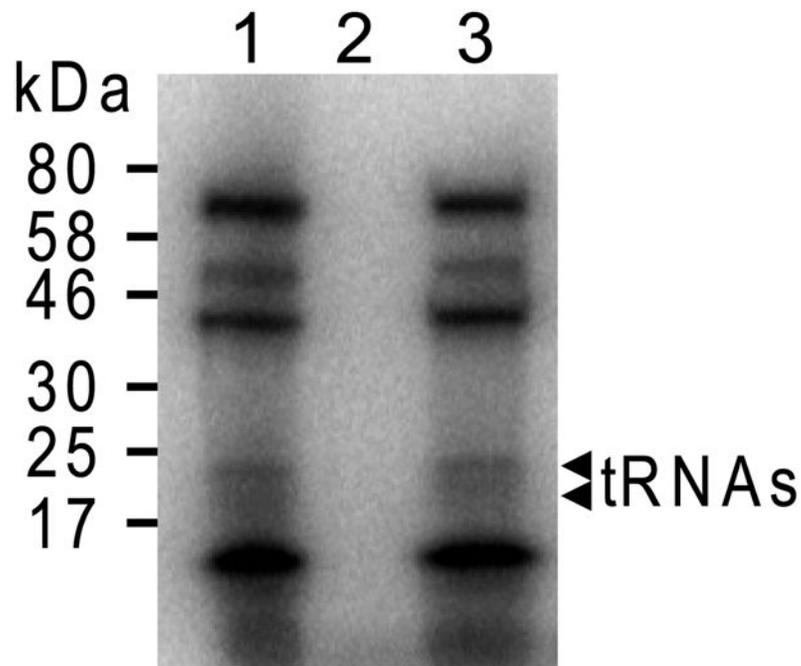
Fig. 4.
[75]Se incorporation into protein and tRNA. Cell extracts of wild-type Mm900 (*lane 1*), FSD10 (*lane 2*), and FSD10 containing the *E. coli selD* expression vector (*lane 3*), were prepared following growth on McCas medium in the presence of [^{75}Se]selenite. Extracts were separated on a SDS/PAGE gel (4–20%), and the insertion of ^{75}Se into macromolecules was followed by autoradiography. Migration position of ^{75}Se-labeled RNA is indicated on the right.
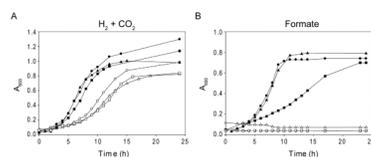
Fig. 5.
Effects of selenium on growth. (A) Growth of *M. maripaludis* wild-type Mm900 (solid circles), FSD10 (open circles), FSD103 (*selD spcS* double-deletion) containing the *E. coli selD* expression vector (open triangles), Mm900 *pstK* deletion mutant, pre-transformed with the *E. coli selA* expression vector (solid triangles), FHM1 (*hrsM* deletion; solid squares), and FHM11 (*hrsM pstK* double-deletion; open squares) cultivated on $H_2 + CO_2$. (B) Cultivation on formate. Same symbols as in A.
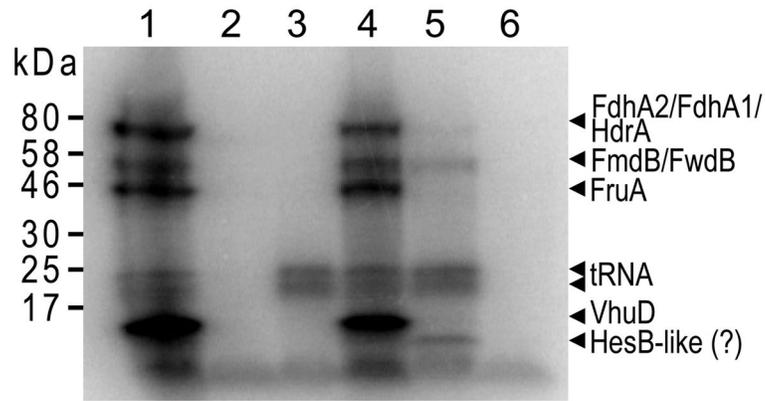
Fig. 6.
Selenoprotein synthesis by *M. maripaludis*. Autoradiograph of a SDS/PAGE gel after electrophoresis of cell lysates from ^{75}Se-labeled *M. maripaludis* Mm900 (*lane 1*), FSD103 (*selD spcS* double-deletion; *lane 2*), FSD103 containing the *E. coli selD* expression vector (*lane 3*), Mm900 Δ*pstK* mutant pre-transformed with the *E. coli selA* expression vector (*lane 4*), FHM1 (*hrsM* deletion; *lane 5*), and FHM11 (*hrsM pstK* double-deletion; *lane 6*), respectively. The migration positions of the standard proteins are indicated on the left; the positions predicted for the putative selenoproteins in *M. maripaludis* and of Se-containing tRNAs are indicated on the right side.
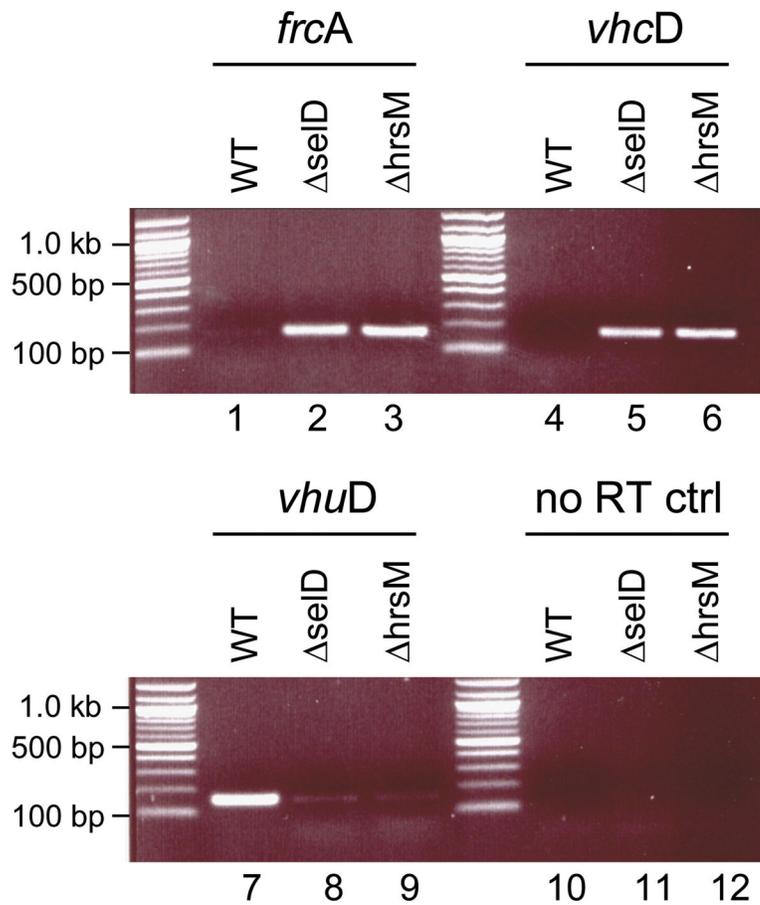
Fig. 7.
Transcription of genes encoding hydrogenase subunits in *M. maripaludis* Mm900, FHM1, and FSD10 strains, analyzed by RT-PCR. DNA-free total RNA from Mm900 wild-type (*lanes 1, 4, 7, 10*), or from deletion mutants carrying an insertion in the *hrsM* gene (*lanes 2, 5, 8, 11*) or the *selD* gene (*lanes 3, 6, 9, 12*) was subjected to RT-PCR. Primer pairs targeting fractions of the indicated genes encoding of the Se-free hydrogenases (*frcA* and *vhcA*), or on of the Se-containing hydrogenase (*vhuD*) were used. The primer pair directed against *vhuD* was also used in a control experiment where reverse-transcriptase has been omitted to exclude contamination with genomic DNA (*lanes 10–12*). The expected PCR product sizes were: *frcA*, 167 bp; *vhcD*, 165 bp; *vhuD*, 160 bp.