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Disruption and complementation of the selenocysteine biosynthesis pathway reveals a hierarchy of selenoprotein gene expression in the archaeon *Methanococcus maripaludis*

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Summary

Proteins containing selenocysteine are found in members of all three domains of life, Bacteria, Eukarya and Archaea. A dedicated tRNA (tRNA^{sec}) serves as a scaffold for selenocysteine synthesis. However, sequence and secondary structures differ in tRNA^{sec} from the different domains. An *Escherichia coli* strain lacking the gene for tRNA^{sec} could only be complemented with the homologue from *Methanococcus maripaludis* when a single base in the anticodon loop was exchanged demonstrating that this base is a crucial determinant for archaeal tRNA^{sec} to function in *E. coli*. Complementation *in trans* of *M. maripaludis* JJ mutants lacking tRNA^{sec}, *O*-phosphoseryl-tRNA^{sec} kinase or *O*-phosphoseryl-tRNA^{sec}:selenocysteine synthase with the corresponding genes from *M. maripaludis* S2 restored the mutant's ability to synthesize selenoproteins. However, only partial restoration of the wild-type selenoproteome was observed as only selenocysteine-containing formate dehydrogenase was synthesized. Quantification of transcripts showed that disrupting the pathway of selenocysteine synthesis leads to downregulation of selenoprotein gene expression, concomitant with upregulation of a selenium-independent backup system, which is not re-adjusted upon complementation. This transcriptional arrest was independent of selenophosphate but depended on the 'history' of the mutants and was inheritable, which suggests that a stable genetic switch may cause the resulting hierarchy of selenoproteins synthesized.

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Introduction

Proteins containing the 21st proteinogenic amino acid selenocysteine are present in members of all three domains of life, Bacteria, Eukarya and Archaea. It is one of the few amino acids synthesized in a tRNA-dependent fashion. The selenocysteine-specific tRNA, tRNA^{sec}, is the largest known tRNA and the major identity determinant for selenocysteine biosynthesis and insertion appears to be the 13-bp-long extended acceptor arm (Commans and Böck, 1999). Initially, tRNA^{sec} is acylated with serine by seryl-tRNA synthetase before seryl-tRNA^{sec} is converted to selenocysteinyl-tRNA^{sec}. This conversion is catalysed in a single step by selenocysteine synthase in bacteria (Forchhammer and Böck, 1991), while eukaryotes and archaea employ a two-step mechanism. Both *in vitro* and *in vivo* analyses demonstrated that phosphoseryl-tRNA^{sec} kinase (PSTK) phosphorylates seryl-tRNA^{sec} to *O*-phosphoseryl-tRNA^{sec}, before phosphoseryl-tRNA^{sec}:selenocysteine synthase (SepSecS, also called SecS) converts this unusual intermediate into selenocysteinyl-tRNA^{sec} (Carlson *et al.*, 2004; Kaiser *et al.*, 2005; Yuan *et al.*, 2006; Xu *et al.*, 2007; Aeby *et al.*, 2009; Hohn *et al.*, 2011). Despite the occurrence of a phosphorylated aminoacyl-intermediate genetic analysis further demonstrated that selenophosphate is the *in vivo* selenium donor during selenocysteine synthesis in archaea (Stock *et al.*, 2010). Interestingly, the enzyme generating selenophosphate from ATP and a reduced selenium species, selenophosphate synthetase (SPS), is a selenoprotein itself in eukaryotes and in archaea (Guimaraes *et al.*, 1996; Stock *et al.*, 2010).

Translation of selenocysteine involves recoding of UGA, normally a termination codon, and is brought about by a dedicated translation factor (SelB in prokaryotes, eSelB or eEFsec in eukaryotes), which transfers selenocysteinyl-tRNA^{sec} to the ribosome, and by a secondary structure on the selenoprotein mRNA, the SECIS (selenocysteine insertion sequence) element, which in bacteria

is located directly adjacent to the UGA (Heider *et al*., 1992), while in eukaryotes and archaea it is found in the non-translated region (Berry *et al*., 1991; Rother *et al*., 2001).

Within the Archaea, only *Methanococcus, Methanocaldococcus* and *Methanopyrus* species were found to synthesize selenoproteins. These strictly anaerobic organisms grow by reducing CO_2 (with H_2 as the electron donor) or formate to methane, which is coupled to conservation of energy via a chemiosmotic mechanism (Thauer *et al*., 2008). Interestingly, eight of the 10 selenoproteins deduced from the genome sequence of *Methanococcus maripaludis* S2 (Hendrickson *et al*., 2004), namely two isoforms of subunits of formate dehydrogenase (FdhA1 and FdhA2), two isoforms of subunits of formyl-methanofuran dehydrogenase (FmdB and FwuB), a subunit of heterodisulphide reductase (HdrA), as well as of F_{420}-dependent (FruA) and F_{420}-independent hydrogenase (VhuD and VhuU), are directly involved in this essential energy conserving pathway. The genome also encodes distinct isoforms containing cysteine at the respective position of selenocysteine of all the selenoproteins involved in methanogenesis, except of formate dehydrogenase (Hendrickson *et al*., 2004). The consequential prediction that the organism's selenocysteine biosynthesis pathway is dispensable during growth with $H_2 + CO_2$ turned out to be true only if the stringent selenium-dependent (and probably also H_2-dependent) regulation of expression of the cysteine-encoding isogenes had been relieved (Hohn *et al*., 2011). A closely related strain to *M. maripaludis* S2, *M. maripaludis* JJ (Jones *et al*., 1983), for which no genome sequence is available, does apparently not require such measures as the selenocysteine biosynthesis pathway of this organism could be readily disrupted resulting in strains no longer synthesizing selenoproteins and no longer growing with formate as the sole energy source (Rother *et al*., 2003; Stock *et al*., 2010).

As bacterial and archaeal $tRNA^{sec}$ share most identity determinants for selenocysteine biosynthesis and insertion we addressed the functionality of archaeal $tRNA^{sec}$ in *Escherichia coli* by complementation analysis. The archaeal homologue did not support selenoprotein synthesis in *E. coli* unless a base in the anticodon loop was altered. Subsequent complementation of a *M. maripaludis* JJ mutant lacking $tRNA^{sec}$ showed that in this organism the respective base of $tRNA^{sec}$ is not crucial for function. Surprisingly, we also found that complementing mutants whose selenocysteine synthesis pathway had been previously disrupted no longer synthesized all of their selenoproteins, but only selenium-dependent formate dehydrogenase. Quantification of transcripts for selenoproteins and for their cysteine-containing isoforms suggests that this hierarchy of selenoproteins synthesized is caused by arrest of expression of the selenoprotein genes and of the cysteine-encoding isogenes in the mutant situation even though the ability to synthesize selenocysteine was restored. As this phenomenon depended on the strain's history and was inheritable a potential genetic switch established by a self-enforcing feedback loop is discussed.

Results

A G37→A variant of archaeal $tRNA^{sec}$ is functional in E. coli

It has been previously shown that a fraction of heterologously expressed $tRNA^{sec}$ of *Methanocaldococcus jannaschii* can be aminoacylated with selenocysteine *in vitro* when purified factors from *E. coli* were used in excess (Rother *et al*., 2000). To address heterologous as well as homologous functionality of archaeal $tRNA^{sec}$ *in vivo, M. maripaludis* strain JJ was used as model organism, because of its mesophilic lifestyle, the genetic methodology developed for this organism, and the fact that its selenoprotein synthesis machinery is not required when growing with $H_2 + CO_2$ (Rother *et al*., 2003; Stock *et al*., 2010). As no genome sequence is available for this strain the gene encoding $tRNA^{sec}$, henceforth designated *selC* in accordance with bacterial nomenclature (Leinfelder *et al*., 1988), was sequenced after its amplification by PCR and found to be identical to that of *M. maripaludis* strain S2 (Fig. 1) for which a genome sequence is available (Hendrickson *et al*., 2004). To test whether this $tRNA^{sec}$ is also a substrate for the *E. coli* selenoprotein synthesis machinery *in vivo,* the *E. coli* strain WL81460 (Table 1), which lacks $tRNA^{sec}$, was transformed with a plasmid containing the $tRNA^{sec}$ gene from *M. maripaludis*, or with a plasmid encoding a variant in which the guanine at position 37 (numbering according to Sprinzl *et al*., 1989) had been changed to adenine. Transformants were tested qualitatively for their ability to anaerobically produce gas from glucose (see *Experimental procedures*) which is an indirect assessment of their ability to synthesize selenoproteins: the selenoprotein formate dehydrogenase H is part of the formate:hydrogen lyase complex catalysing formation of H_2 and CO_2 from formate produced during mixed acid fermentation (Gest, 1954). Thus, glucose-dependent gas production is indicative of a functional system for selenoprotein synthesis. As previously shown, the parental strain of WL81460, FM433, produced substantial amounts of gas from glucose and the *selC* mutant WL81460 did not (Fig. 2, vials 1 and 2). While the *M. maripaludis* $tRNA^{sec}$ did not lead to gas formation (Fig. 2, vial 3) the $tRNA^{sec}$ G37→A variant was able to complement the phenotype of the *selC* lesion of *E. coli* (Fig. 2, vial 4), which shows that this base is crucial for $tRNA^{sec}$ function *in E. coli*.

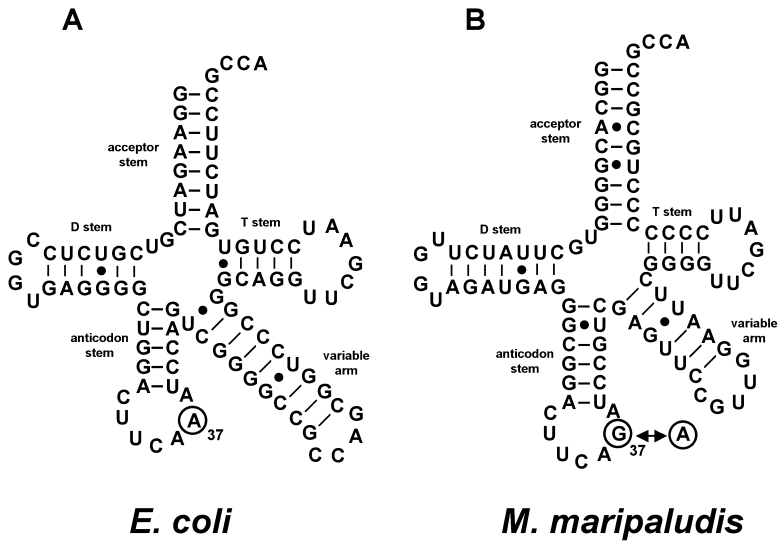

Fig. 1. Secondary cloverleaf structures of tRNA^{sec} from *E. coli* (A) and *M. maripaludis* (B); *E. coli* tRNA^{sec} adopts a 8/5 conformation in the 13-base-pair aminoacyl/T-stem helix (Baron *et al*., 1993) while a 9/4 conformation is proposed for tRNA^{sec} of *M. maripaludis*; bases at position 37 are circled; filled circles indicate non-Watson-Crick base pairing.

tRNA^{sec} is required for selenoprotein synthesis in M. maripaludis

As no known tRNA^{sec} from archaea contains an adenine at position 37 we wanted to see if the G37→A variant of *M. maripaludis* tRNA^{sec} is still functional in its natural host. To address this question, a mutant strain, JSC8, was constructed which carries the tRNA^{sec} gene disrupted by the *pacN* cassette (see *Experimental procedures*). Genotypic verification via Southern hybridization showed that the tRNA^{sec} encoding locus was altered in the mutant and that it carried a single chromosomal insertion of the *pacN* cassette (Fig. S1). Since JSC8 lacked only a 54 bp portion of the tRNA^{sec} gene, which is difficult to detect via Southern hybridization, absence of the gene product rather than absence of the encoding gene was addressed. To this end, crude tRNA was prepared from the wild type and JSC8, and subjected to Northern blot analysis using a digoxigenin labelled oligonucleotide complementary to the first 32 bases of tRNA^{sec} as the probe (see *Experimental procedures*). While *M. maripaludis* JJ contains a tRNA hybridizing with the probe (Fig. 3, lane 1) JSC8 contains no tRNA^{sec} transcript (Fig. 3, lane 2), which, together with the Southern analyses, confirms that this strain is a tRNA^{sec} mutant. JSC8 was unable to synthesize any selenoproteins, as evidenced by *in vivo* labelling of the strain with [^{75}Se]-selenite (Fig. 4A and B, lanes 2), which demonstrates that tRNA^{sec} is essential for selenoprotein synthesis in *M. maripaludis*. As expected from the phenotypic analysis of other *M. maripaludis* mutants lacking *trans*-active factors involved in selenoprotein synthesis (Rother *et al*., 2003; Stock *et al*., 2010), JSC8 was unable to grow with formate as the sole energy source due to the dependence of formate dehydrogenase of this organism on selenium (Fig. S4).

Restoring the path of selenoprotein synthesis only partially restores the selenoprotein pattern

Next, JSC8 was transformed with self-replicating plasmids containing either the *M. maripaludis* wild-type tRNA^{sec} gene (pWLJCwt) or that of the G37→A variant (pWLJCg37a). Both strains specifically incorporated radioactive selenium into a protein of *c.* 72 kDa (Fig. 4A, lanes 4 and 5; Fig. 4B, lane 3), which strongly suggests that the pathway of selenoprotein synthesis in these strains was restored. The molecular mass of the labelled selenoprotein corresponds to that deduced for FdhA1 and FdhA2 (MMP1298 and MMP138 in strain S2), the large subunit of formate dehydrogenase isoforms. The conclusion that the labelled selenoprotein is indeed FdhA is further supported by the ability of both strains to again grow with formate at a rate and to a final optical density very similar to that of the wild type (Fig. S4). Occurrence of selenocysteine-containing FdhA was irrespective of whether the wild-type tRNA^{sec} or its G37→A variant was expressed in JSC8 (lanes 4 and 5, Fig. 4A), which strongly suggests that both forms of tRNA^{sec} are equally functional in *M. maripaludis*. The empty vector did not restore the capacity of JSC8 to synthesize selenoproteins (Fig. 4A, lane 3).

Very much to our surprise, only selenium-containing FdhA but none of the other selenoproteins apparent in the wild type were synthesized after complementing JSC8 with its own tRNA^{sec} (or a variant of it). Northern analysis in complemented JSC8 cells showed significant increase of the level of tRNA^{sec} transcripts as compared with the wild type (compare lanes 4 and 5 with lane 1, Fig. 3), possibly due to the increased copy number of the gene provided in *trans*, or due to lack of *cis*-acting regulatory elements on the plasmid constructs. Such increased abundance of tRNA^{sec} could possibly lead to experimental

Table 1. Plasmids and strains used in this study.[a]

Plasmids/strains	Relevant genotype/description/construction[b]	Reference
Plasmids		
pUC19	Cloning vector, *p*lac promoter	Yanisch-Perron *et al.* (1985)
p19JJC	BamHI/HindIII PCR (primers B13.MM-T7-selC and oMjcT7-2) fragment encoding *M. maripaludis selC* cloned into pUC19	This study
p19JJCGA	p19JJC, product of inverse PCR (primers B33-MmselC and B32-MmselCG37-A) to generate G37→A variant of *M. maripaludis selC*	This study
pNPAC	Contains *pacN* cassette (*pac* gene from *Streptomyces alboniger* codon optimized for *M. voltae*)	Sun and Klein (2004)
pIJA03+neo	Contains *neo* cassette	Lie *et al.* (2007)
pCRPrtneo	Plasmid to create markerless deletions in *M. maripaludis*	Moore and Leigh (2005)
pdownSC	NheI/BamHI PCR fragment (primers odownSC/5′ and odownSC/3′) of downstream *selC* cloned into pNPAC	This study
pKoSC	SpeI/MluI PCR fragment (primers oupSC/5′ and oupSC/3′) of upstream *selC* cloned into pdownSC	This study
pMSec1	HindIII/AscI PCR fragment (primers oMP1490up/5′ and oMP1490up/3′) of upstream *pstK* and BamHI/AscI PCR fragment (primers oMP1490down/5′ and oMP1490down/3′) of downstream *pstK* cloned into pCRPrtneo	This study
pMSec2	KpnI/AscI PCR fragment (primers oMP0595up/5′ and oMP0595up/3′) of upstream *spcS* and BamHI/AscI PCR fragment (primers oMP0595down/5′ and oMP0595down/3′) of downstream *spcS* cloned into pCRPrtneo	This study
pupKoSec1	StuI/AscI fragment of pMSec1 blunted and ligated into NruI cut pNPAC	This study
pKoSec1	AscI/BamHI fragment of pMSec1 blunted and ligated into HincII cut pupKoSec1; for disruption of *pstK*	This study
pupKoSec2	KpnI/AscI fragment of pMSec2 blunted and ligated into NruI cut pNPAC	This study
pKoSec2	AscI/BamHI fragment of pMSec2 blunted and ligated into HincII cut pupKoSec1; for disruption of *spcS*	This study
pNMmselCWT	*M. maripaludis selC* (including 5′ and 3′ regions) cloned via assembly of osecsynt1-6 and ligation into SpeI/BglII-restricted pNPAC	This study
pNMmselCG37A	Like pNMmselCWT, only osecsynt2 and osecsynt5 replaced with MmselcG37A+ and MmselcG37A–	This study
pNpacpstK	NdeI/BglII PCR (primers oMmPSTK-For and oMmPSTK-rev) fragment encoding *M. maripaludis* S2 *pstK* cloned into pNPAC	This study
pNpacspcS	NdeI/BglII PCR (primers oMmSepSecS-For and oMmSepSecS-Rev) fragment encoding *M. maripaludis* S2 *pstK* cloned into pNPAC	This study
pWLG40NZ-R	Contains *neo* cassette; self-replicating in *M. maripaludis*	Lie and Leigh (2003)
pWLJCwt	Contains *M. maripaludis selC*; SpeI/BglII fragment of pNMmselCWT cloned into pWLG40NZ-R	This study
pWLJCg37a	Encodes G37→A variant of *M. maripaludis selC*; SpeI/BglII fragment of pNMmselCG37A cloned into pWLG40NZ-R	This study
pWLmmpstK	*P*sl–*pstK* fusion of pNpacpstK cloned via SpeI/BglII into pWLG40NZ-R	This study
pWLmmspcS	*P*sl–*spcS* fusion of pNpacspcS cloned via SpeI/BglII into pWLG40NZ-R	This study
pTSpstK	SpeI/BglII *p*sl–*pstK* fragment of pNpacpstK cloned into XbaI/BglII-restricted pIJA03+neo	This study
pTSpstK-selD	AflII/BamHI PCR (primers oRBSselDfor and oselD-Rev) fragment encoding *E. coli selD* and ribosome binding site from *p*sl cloned into pTSpstK	This study
pWLpstK-selD	XhoI/BamHI fragment of pTSpstK-selD cloned into XhoI/BglII-restricted pWLG40NZ-R	This study
pTS0904	For disruption of *sps* in *M. maripaludis*	Stock *et al.* (2010)
Strains		
E. coli		
DH10B	General cloning	Invitrogen
FM433	*rpsL*$^+$ *rpsE13* Δ(*srl-recA*)306::Tn*10*	Zinoni *et al.* (1990)
WL81460	FM433, Δ(*selC*)400::*Kan*	Zinoni *et al.* (1990)
M. maripaludis		
JJ	Wild type; type strain	Jones *et al.* (1983)
S2	Wild type	Whitman *et al.* (1986)
JSC8	JJ, Δ*selC::pacN* (pKoSC)	This study
JJ/pWLJCwt/KOC	Isogenic to JSC8 carrying pWLJCwt, only constructed in different order	This study
JA1	JJ, Δ*pstK::pacN* (pKoSec1)	This study
JJ/pWLmmpstK/KOK	Isogenic to JA1 carrying pWLmmpstK, only constructed in different order	This study
JA2	JJ, Δ*spcS::pacN* (pKoSec2)	This study
JJ/pWLmmspcS/KOS	Isogenic to JA2 carrying pWLmmspcS, only constructed in different order	This study
TS1	JJ, Δ*sps::pacN* (pTS0904)	Stock *et al.* (2010)
JJ/pWLpstK-selD/KOK	Isogenic to JA1 carrying pWLpstK-selD, only constructed in different order	This study

a. Primers used are listed in Table S1.
b. DNA sequences and maps of all plasmids are available upon request.

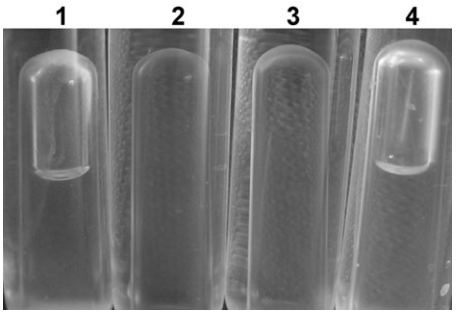

Fig. 2. Anaerobic gas production from glucose. *E. coli* strains (Table 1) were cultivated as described in *Experimental procedures* and gas accumulation in the Durham vials was qualitatively scored after 4 days. vial 1, FM433; vial 2, WL81460; vial 3, WL81460/pUC19JJC; vial 4, WL81460/pUC19JJCGA.

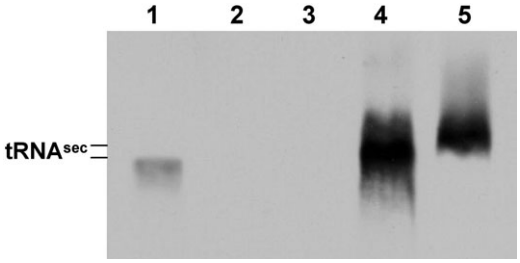

Fig. 3. Abundance of tRNA^{sec} in strains of *M. maripaludis*. For detection of tRNA^{sec} in crude tRNA preparations (see *Experimental procedures*) a DIG-labelled oligonucleotide complementary to the first 32 bases of *M. maripaludis* tRNA^{sec} was used as the probe; the migration position of tRNA^{sec} is indicated on the left; lane 1, *M. maripaludis* JJ; lane 2, JSC8; lane 3, JSC8 transformed with pWLG40NZ-R (vector control); lane 4, JSC8 transformed with pWLJCwt (wild-type tRNA^{sec}); lane 5, JSC8 transformed with pWLJCg37a (G37→A variant of tRNA^{sec}).

artefacts; in *E. coli*, the *in vivo* stoichiometry of SelB, tRNA^{sec} and SECIS is known to have a profound influence on the efficiency of co-translational selenocysteine insertion (Tormay *et al*., 1996). Still, other possibilities such as differences between the mechanism of selenocysteine translation for the *fdhA* transcript and the other selenoprotein mRNAs, or differentially regulated selenoprotein gene expression, could also cause the observed phenomenon.

To address this question, a strain isogenic to JSC8 complemented with pWLJCwt, only constructed in a different order of the genetic manipulations, was generated: *M. maripaludis* JJ was initially transformed with pWLJCwt, and subsequently the chromosomal copy of *selC* was disrupted. The resulting strain, JJ/pWLJCwt/KOC, which is genetically identical to JSC8 complemented with pWLJCwt (with respect to intended genetic alterations), synthesized selenoproteins like the wild type (Fig. 4B, lane 4). These data rule out experimental artefacts caused by the constructs used for the genetic manipulations, but instead show that the inability of *M. maripaludis* JSC8 complemented with pWLJCwt to synthesize most of its selenoprotein is a consequence of disrupting its pathway of selenocysteine synthesis. The pattern of selenoproteins did not change after six consecutive transfers (*c.* 25 generations) of the culture in to fresh medium containing various amounts of selenium (0.1–10 μM) prior to radioactive labelling (data not shown). Thus, the effect leading to only selenocysteine-containing FdhA being synthesized by

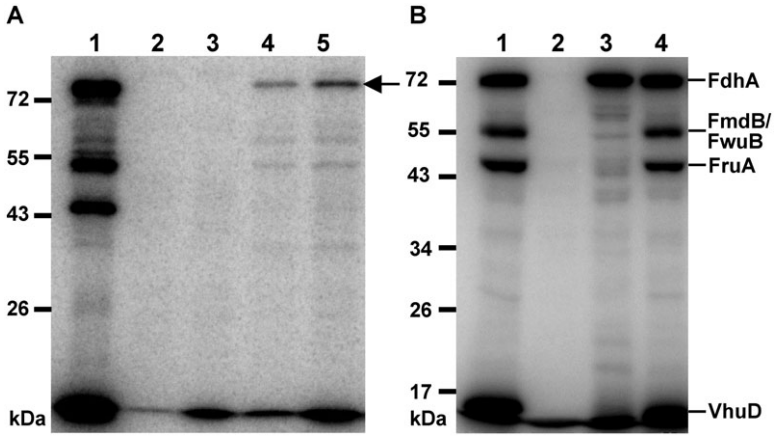

Fig. 4. tRNA^{sec}-dependent selenoprotein synthesis in *M. maripaludis*. Autoradiographs of sodium dodecyl sulphate polyacrylamide gels (SDS-PAGs) (Laemmli, 1970) after electrophoresis of ^{75}Se-labelled cell lysates, which had been treated with RNase A to remove selenium-containing RNA; the arrow depicts the migration position of selenocysteine-containing FdhA; the identity of the other main selenoproteins, according to Rother *et al.* (2001), is shown on the right; migration positions of standard proteins (in kDa) are indicated on the left.
A. Incorporation of ^{75}Se into proteins of *M. maripaludis* JJ (lane 1), JSC8 (lane 2), JSC8 transformed with pWLG40NZ-R (lane 3), JSC8 transformed with pWLJCwt (lane 4), JSC8 transformed with pWLJCg37a (lane 5); lysates were separated on a 10% SDS-PAG.
B. Order of genetic manipulation affects synthesis of ^{75}Se-labelled proteins in *M. maripaludis*. Lanes 1 and 2, same as (A); lane 3, JSC8 transformed with pWLJCwt; lane 4, JJ/pWLJCwt/KOC (see Table 1); lysates were separated on a 12.5% SDS-PAG.

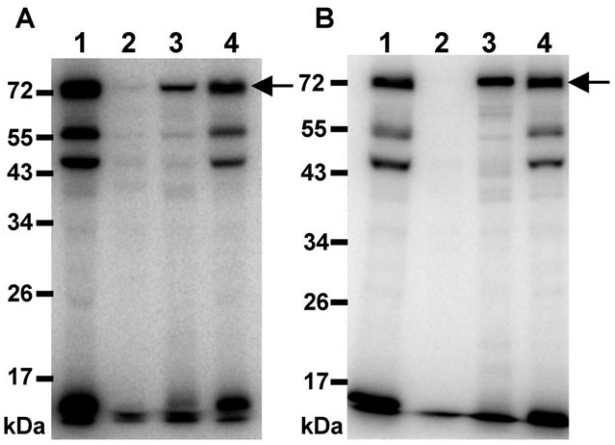

Fig. 5. PSTK, SepSecS and synthesis of selenoproteins in *M. maripaludis*. Autoradiographs of 12.5% SDS-PAGs after electrophoresis of ^{75}Se-labelled cell lysates, which had been treated with RNase A to remove selenium-containing RNA; the arrows depict the migration position of selenocysteine-containing FdhA; for the likely identity of other selenoproteins, see Fig. 4; migration positions of standard proteins (in kDa) are indicated on the left.
A. Lane 1, *M. maripaludis* JJ; lane 2, JA1; lane 3, JA1 transformed with pWLmmpstK; lane 4, JJ/pWLmmpstK/KOK (see Table 1).
B. Lane 1: *M. maripaludis* JJ; lane 2, JA2; lane 3, JA2 transformed with pWLmmspcS; lane 4, JJ/pWLmmspcS/KOS (see Table 1).

JSC8 complemented with pWLJCwt is stable, i.e. inheritable, and independent of the selenium supply.

To test if this hierarchy in selenoprotein synthesis, with selenium-dependent FdhA being the only selenoprotein synthesized, after disruption and complementation of the selenocysteine biosynthesis pathway, is a phenomenon only attributed to tRNA^{sec}, analogous deletion/complementation analyses were conducted with *pstK* (encoding PSTK, *O*-phosphoseryl-tRNA^{sec} kinase) and *spcS* (encoding SepSecS, *O*-phosphoseryl-tRNA^{sec}: selenocysteine synthase). Mutants carrying deletions in each of the two genes, respectively, were created (see *Experimental procedures*), and their genotype was verified by Southern hybridization (Figs S2 and S3). Like JSC8, *M. maripaludis* strains JA1 (*pstK* disrupted) and JA2 (*spcS* disrupted) were unable to synthesize selenoproteins (Fig. 5A and B, lanes 2), which shows that in *M. maripaludis* JJ, like in the *M. maripaludis* S2 derivative Mm900 (Hohn *et al*., 2011), PSTK and SepSecS are strictly required for selenoprotein synthesis. After transformation of each of the mutants with a plasmid containing the corresponding gene previously disrupted on the chromosome, each complemented strain synthesized of its selenoproteins again only selenocysteine-containing FdhA above background level (Fig. 5A and B, lanes 3). Consistent with the pattern of selenoprotein synthesis in the strains, JA1 and JA2 were unable to grow with formate as the sole energy source but regained this capacity after complementation with the plasmid containing the corresponding gene previously disrupted (Fig. S4).

The data presented clearly show that the phenomenon of only partial restoration of the selenoproteome upon disruption and complementation of the selenocysteine biosynthesis pathway is not restricted to tRNA^{sec} but also occurs when other *trans*-acting factors are affected, which further argues against experimental artefacts. This notion was again confirmed by analysing the selenoproteins of strains isogenic to the complemented mutants with only the order of the genetic alterations changed, i.e. transformation of *M. maripaludis* JJ with the complementing plasmid prior to disruption of the respective chromosomal gene. The strains JJ/pWLmmpstK/KOK (isogenic to JA1 complemented with pWLmmpstK with respect to intended genetic alterations) and JJ/pWLmmspcS/KOS (isogenic to JA2 complemented with pWLmmspcS with respect to intended genetic alterations) synthesized the full set of selenoproteins found in the wild type (Fig. 5A and B, lanes 4 respectively). To further minimize the possibility that genetic peculiarities of the individual clonal populations analysed (JSC8, JA1, JA2) were responsible for the observed phenomenon, other *selC, pstK* and *spcS* mutant clones (eight clones for each mutation) were complemented like JSC8, JA1 and JA2 (see above); in every case was the same phenotype of only partial restoration of the selenoprotein pattern observed (data not shown).

The hierarchy of selenoproteins synthesized in M. maripaludis *is not caused by selenophosphate depletion*

It was previously shown that selenophosphate synthetase of *M. maripaludis* is itself a selenoprotein (Stock *et al*., 2010). Therefore, disruption of the selenocysteine biosynthesis pathway may lead to the absence of this enzyme, and, thus, elimination of selenophosphate. We hypothesized that it may not be possible for the complemented *M. maripaludis* mutants to fully recover from selenophosphate deprivation resulting in the preferential synthesis of selenium-dependent FdhA. To address this question, a plasmid was constructed containing an artificial operon of *pstK* and *selD* (encoding selenophosphate synthetase from *E. coli*). In this construct, designated pWLpstK-selD, both genes are preceded by a ribosome binding site and transcribed from a *Methanococcus* promoter upstream of *pstK* (Fig. S5). The *M. maripaludis pstK* mutant JA1 was transformed with pWLpstK-selD and the pattern of selenoproteins was analysed by *in vivo* by labelling of the strain with radioactive selenium. As can be seen in Fig. 6, JA1/pWLpstK-selD only synthesized selenium-containing FdhA like JA1 carrying pWLmmpstK (compare lane 1, Fig. 6 and lane 3, Fig. 5A). Experimental artefacts caused by the plasmid were again ruled out by analysing a strain isogenic to JA1/pWLpstK-selD, for the generation of which the wild type was first transformed with pWLpstK-

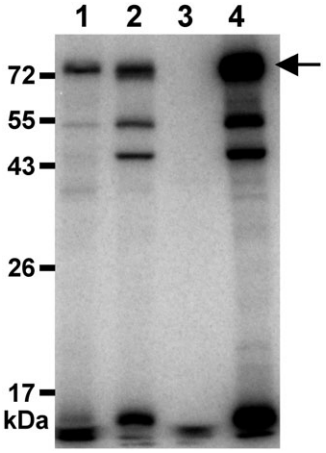

Fig. 6. Influence of selenophosphate availability on selenoprotein synthesis in *M. maripaludis*. Autoradiograph of a 12.5% SDS-PAG after electrophoresis of ^{75}Se-labelled cell lysates, which had been treated with RNase A to remove selenium-containing RNA; lane 1, JA1 transformed with pWLpstK-selD; lane 2, JJ/pWLpstK-selD/KOK; lane 3, TSI; lane 4, TSI transformed with pWLpstK-selD; the arrow depicts the migration position of selenocysteine-containing FdhA; for the likely identity of other selenoproteins, see Fig. 4; migration positions of standard proteins (in kDa) are indicated on the left.

selD before *pstK* was disrupted (Fig. 6, lane 2). As a further control, the *sps* mutant TS1 (Table 1) was transformed with pWLpstK-selD and shown to have wild-type capabilities to synthesize selenoproteins (Fig. 6, lane 4), which demonstrates that *selD* is functionally expressed from this artificial construct. These data strongly suggest that the observed hierarchy of selenoproteins synthesized by *M. maripaludis* upon disruption and complementation of the selenocysteine biosynthesis pathway is not caused by depletion of the cells for selenophosphate.

The hierarchy in selenoprotein synthesis is caused by differential transcription

The observed phenotype in the mutants, i.e. the hierarchy in selenoprotein synthesis upon disruption and complementation of the selenocysteine biosynthesis pathway, could be caused by differences in selenoprotein gene expression or by differences in selenocysteine translation. To address this question, quantitative reverse transcription PCR (qRT-PCR) was employed to determine the mRNA abundances of selected selenoprotein mRNAs and of those of the corresponding cysteine-encoding isoforms. We chose the genes for the large subunit of the selenium-containing F_{420}-dependent hydrogenase (encoded by *fruA*), of the corresponding selenium-free isoform (encoded by *frcA*), of the selenium-containing subunit of heterodisulphide reductase (encoded by $hdrA_U$), the corresponding selenium-free isoform (encoded by $hdrA_C$), and of selenium-containing FdhA. *M. maripaludis* S2 encodes two isoforms of FdhA (encoded by *fdhA*1 and *fdhA*2 respectively (Wood *et al.*, 2003). To test if the same is true for *M. maripaludis* JJ, its genome was probed by PCR and found to also encode two isoforms of FdhA with almost identical sequences to their counterparts in strain S2 (data not shown). The genes were therefore also designated *fdhA*1 and *fdhA*2 respectively. Abundances of the selected mRNAs were determined in the wild-type strain JJ, the *pstK* mutant JA1, JA1 complemented with pWLmmpstK, and its isogenic strain JJ/pWLmmpstK/KOK (Table 1). As a control, the *sps* mutant TS1 and the complemented TS1 strain carrying pWLSTselD (Table 1) was also analysed. From these strains total mRNA was isolated and subjected to qRT-PCR as described in *Experimental procedures*. The mRNA abundance data were normalized to that of *mcrB* (see *Experimental procedures*). The results of this analysis are summarized in Fig. 7.

While the *M. maripaludis* wild-type strain JJ (Fig. 7, black bars) contained *c.* 300 times more *fruA* transcript than *frcA* transcript, abundance of the former dropped by *c.* 40-fold and that of the latter increased by *c.* 200-fold upon *pstK* deletion resulting in *c.* 30-fold higher abundance of *frcA* than *fruA* in strain JA1 (Fig. 7, dark green bars). Similar results were obtained when analysing *hdrA* expression: $hdrA_U$ was *c.* 100-fold more abundant in the wild type than $hdrA_C$ and disruption of *pstK* led to a decrease of $hdrA_U$ by *c.* 30-fold, while $hdrA_C$ abundance increased *c.* 40-fold resulting in a $hdrA_C/hdrA_U$ ratio of *c.* 20. This apparent downregulation of selenoprotein gene expression concomitant to upregulation of the cysteine-encoding isogenes is fully consistent with a role of the selenium-independent system in substituting for the selenoproteins when the path of selenoprotein synthesis is disrupted or when selenium is limiting as proposed for this organism (Rother *et al.*, 2003) and for *Methanococcus voltae* (Sorgenfrei *et al.*, 1997). Strikingly, mRNA abundances neither of the selenoprotein genes nor of the cysteine-encoding isogenes changed much when JA1 was complemented with *pstK* (Fig. 7, light green bars), which suggest that the organism was unable to re-adjust its gene expression after its capacity to synthesize selenoproteins had been restored. The strain JJ/pWLmmpstK/KOK, isogenic to JA1 complemented with pWLmmpstK, contained levels of the mRNAs for the selenoproteins and the cysteine-containing isoforms very similar to those of the wild type (Fig. 7, hatched green bars).

Interestingly, abundance of *fdhA*1 mRNA, which was as least 100-fold higher than that of *fdhA*2 under any condition analysed, did not change when *pstK* was deleted or when JA1 was complemented with *pstK*. This finding strongly suggests that in *M. maripaludis* JJ *fdhA* expression is not regulated by the selenium supply but

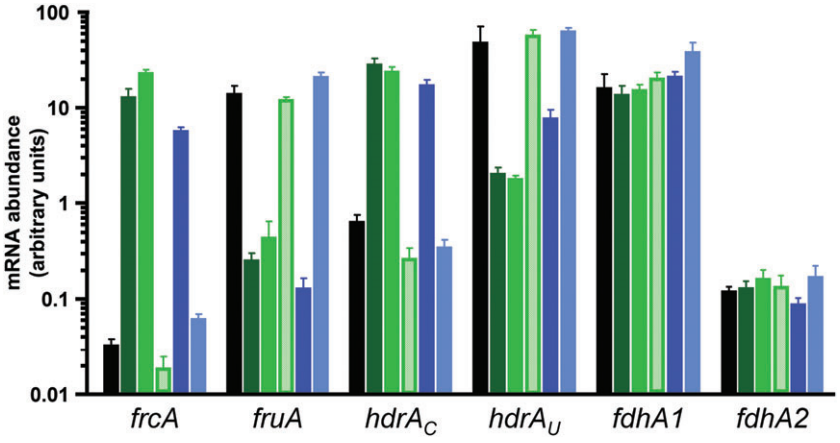

Fig. 7. Abundance of mRNAs encoding selenoproteins and their cysteine-containing isoforms. mRNA from *M. maripaludis* JJ (black bars), JA1 (dark green bars), JA1 transformed with pWLmmpstK (light green bars), JJ/pWLmmpstK/KOK (hatched green bars), TS1 (dark blue bars) and TSI transformed with pWLSTselD (light blue bars) was isolated and subjected to qRT-PCR as described in *Experimental procedures* to quantify the abundance of *frcA* (encoding a subunit of selenium-independent F_{420}-reducing hydrogenase), *fruA* (encoding a subunit of selenocysteine-containing F_{420}-reducing hydrogenase), *hdrA*$_C$ (encoding a subunit of selenium-independent heterodisulphide reductase), *hdrA*$_U$ (encoding a subunit of selenocysteine-containing heterodisulphide reductase), *fdhA1* (encoding a subunit of selenocysteine-containing formate dehydrogenase 1) and *fdhA2* (encoding a subunit of selenocysteine-containing formate dehydrogenase 2); mRNA abundance values were normalized to that of *mcrB* (encoding a subunit of methyl-coenzyme M reductase), which was set to 100 (arbitrary units); shown are mean values and standard deviations of three independent experiments.

constitutive. This conclusion is in full agreement with a previous immunological analysis addressing FdhA synthesis (Rother *et al.*, 2003). The differences in *fdhA*1 and *fdhA*2 expression levels further indicate that Fdh2 plays only a minor, if any, role in the energy metabolism of *M. maripaludis* JJ.

When the *sps* mutant TS1 was analysed, the reduction of selenoprotein gene expression and the increase in expression of the cysteine-encoding isogenes observed upon deletion of *sps* (Fig. 7, dark blue bars) reverted to the wild-type situation when the mutant was complemented with *selD* (Fig. 7, light blue bars), which shows that unlike JA1, TS1 is able to re-adjust expression of the selenoprotein genes and of their cysteine-encoding isoforms when the pathway of selenoprotein synthesis is restored.

Discussion

Synthesis of selenocysteine requires a number of dedicated *trans*-active factors and although the respective pathways are principally similar in members of the domains Bacteria, Archaea and Eukarya, peculiar differences remain, one of which regards the selenocysteine-specific tRNA. Comparison of the primary and the (predicted) secondary structure of bacterial and archaeal tRNA^{sec} (Fig. 1) indicated two major differences, an 8/5 (bacterial, Baron *et al.*, 1993) versus 9/4 (archaeal) conformation in the 13-base-pair aminoacyl/T-stem helix (Itoh *et al.*, 2009), and the presence (bacterial) or absence (archaeal) of the adenine base at position 37 (A37). Here, we show that archaeal tRNA^{sec} is functional in *E. coli* when the base following the anticodon was changed to adenine, which is invariant in bacterial elongator tRNAs (Dirheimer *et al.*, 1995). All other determinants of archaeal tRNA^{sec} evolved to ensure the stringency of selenocysteine insertion into growing polypeptides (Commans and Böck, 1999) appear to be sufficiently conserved for the archaeal species to function in bacteria. Notably, the proposed 9/4 aminoacyl/T-stem helix conformation experimentally validated for eukaryal, and proposed for archaeal tRNA^{sec} is no obstacle for its functionality in bacteria, a notion fully consistent with the observations made using tRNA^{sec} from *Xenopus laevis*, with A37 and a 9/4 aminoacyl/T-stem helix conformation (Baron *et al.*, 1994), and tRNA^{sec} from Gram-positive bacteria (Tormay *et al.*, 1994). In *E. coli*, A37 in tRNA^{sec} is modified to N^6-isopentenyl-adenosine (i^{6}A, Schön *et al.*, 1989). It is therefore very likely that this base is likewise modified in the heterologously synthesized molecule. tRNAs carrying i^{6}A 3′ of the anticodon (i^{6}A37) often recognize UNN codons and the modification both enhances the rather weak A36-U interaction (anticodon–codon) (Vacher *et al.*, 1984) and prevents non-cognate base recognition (Jukes, 1973). However, i^{6}A37 was so far not identified in Archaea (Björk, 1995). In fact, N^1-methyl-guanosine (m^{1}G) and N^6-threonylcarbamoyl-adenosine (t^{6}A) are the only modifications known to occur at position 37 in archaeal tRNAs (Phillips and de Crecy-Lagard, 2011). Unlike in *E. coli*, position 37 in archaeal tRNA^{sec} appears to play no significant role in the fidelity of codon/anticodon recognition during translation of UGA with selenocysteine. Whether the base at this position of tRNA^{sec} is modified

(e.g. with m^1G in the wild-type $tRNA^{sec}$ or t^6A in the $tRNA^{sec}$ variant) remains to be elucidated.

An unexpected outcome during the deletion/complementation analysis of the $tRNA^{sec}$-encoding gene, which we subsequently extended to *pstK* and *spcS*, was that in *M. maripaludis* JJ, complementation of genetic lesions affecting selenoprotein synthesis did not always restore the wild type's phenotype. Genetic analysis of methanogens is laborious both due to the organism's strict requirement to maintain anaerobic conditions and due to the still limited number of genetic tools available, as compared with classical genetic model organisms (Leigh *et al*., 2011). While complementation analysis is state of the art in *E. coli* genetics for over 30 years method development established this standard in *Methanococcus* only in the last few years (Sarmiento *et al*., 2011). The unexpected observations reported here illustrate the usefulness of such an analysis.

From the data presented it seems clear that the observed hierarchy in selenoprotein synthesis upon disruption and complementation of the selenocysteine synthesis pathway is based, at least mainly, on mRNA abundances, i.e. is a result of differential transcription of the selenoprotein genes. The expression pattern in the complemented *pstK* and *spcS* mutants seems to be 'arrested' in the mutant situation even when the selenocysteine biosynthesis pathway is restored. This transcriptional arrest is inheritable and depends on the strain's own history, i.e. the order in which the genetic manipulations occurred, which suggests that a bistable genetic switch may be responsible. However, the possibility that a secondary mutation causing arrest of expression of most of the selenoprotein genes occurred cannot be completely ruled out. Still, disrupting the pathway of selenocysteine synthesis does not create much of a selection pressure because the organism contains a full complement of cysteine-containing isoenzymes allowing growth on $H_2 + CO_2$ very similar to that of the wild type (Fig. S4). Further, such a mutation would have to have occurred independently thrice, while creating the mutants JSC8 (*selC* deleted), JA1 (*pstK* deleted) and JA2 (*spcS* deleted), and in each of the eight clones analysed for each mutation.

Although speculative, a plausible mechanism causing the inheritable transcriptional arrest observed here would be to obviate the ability of the complemented mutants to perceive that the capacity to synthesize selenoproteins was restored. If the amount or the activity of a sensor of the cellular selenium status required selenocysteine or one of its biosynthetic intermediates, disruption of the selenocysteine biosynthesis pathway would eliminate the sensor. If expression of the sensor-encoding gene(s) required presence of the sensor the situation would be locked-in, irrespective of whether the capacity to synthesize selenoproteins was restored. Regulation of the *lac* operon in *E. coli* is an example for such a positive feedback loop (Casadesus and D'Ari, 2002). Alternatively, a double-negative feedback loop involving two negative effectors is also feasible. For example, if *M. maripaludis* contained a repressor negatively effecting the sensor-encoding gene(s), they would remain repressed upon disruption and complementation of the selenocysteine biosynthesis pathway, if the sensor or its activity in turn negatively controlled said repressor. An example for such a switch was dissected in *Bacillus subtilis*, where the fate of cells, motile or sessile, is governed by a bistable genetic switch, realized through a double-negative feedback loop involving two repressor proteins; one, SinR, represses the gene for the other, *slrR*, and SlrR titrates SinR via direct binding, thus preventing SinR from repressing *slrR* (Chai *et al*., 2010). A similar mechanism could be operative downstream of a potential selenium sensor in such a signal transduction cascade, for example affecting a repressor of selenoprotein gene expression. However, no such regulator is known in *M. maripaludis*. Alternatively, disruption of the selenocysteine biosynthesis pathway could generate an RNA-based signal, e.g. a small regulatory RNA, which could directly or indirectly prevent transcription of most of the selenoprotein genes; small regulatory RNAs appear to be as abundant in archaea as in bacteria (Jäger *et al*., 2009; Soppa *et al*., 2009). Finally, aberrant epigenetic effects could also be responsible for the transcriptional arrest of most of the selenoprotein genes observed here. It is well established in mammals that aberrant gene regulation by epigenetic mechanisms, like DNA hypermethylation, can be the cause for cancer development. Interestingly, among numerous dietary compounds influencing DNA methylation in tumorigenesis, selenium deficiency was found to cause global methylation defects and in addition, promoter methylation of cancer-related genes (Li and Tollefsbol, 2010). However, no link of trace elements and epigenetic effects has thus far been unravelled for any archaeon. Consequentially, our current research is focused on exploring these possibilities.

Although we clearly show by coexpressing *selD* from *E. coli* that deprivation of selenophosphate is not the cue leading to the arrest of selenoprotein gene expression in the complemented *pstK* mutant, the endogenous SPS might still be key to this phenomenon. First, being itself a selenoprotein it is a suitable candidate to sense the cellular selenium status as proposed in eukaryotes (Guimaraes *et al*., 1996). Furthermore, it was recently shown that in the absence of selenium eukaryal selenium-dependent SPS synthesizes thiophosphate, which is incorporated as cysteine into selenoproteins via the selenoprotein synthesis machinery (Xu *et al*., 2011). While UGA codons on selenoprotein mRNAs of *M. maripaludis* are most

probably not translated with cysteine derived from this pathway due to the presence and the concerted expression of a whole set of cysteine-containing backup proteins, thiophosphate synthesized by the endogenous SPS could still be an effector leading to the transcriptional switch observed here and could also explain why the *selD*-complemented SPS mutant synthesized selenoproteins like the wild type (Stock *et al*., 2010). Clearly, further studies are warranted to clarify this issue.

The pathway for selenocysteine biosynthesis appears to be dispensable to different degrees among very closely related *M. maripaludis* strains. While it can be disrupted easily in strain JJ, we were previously not successful to do so in strain S2, where several attempt to disrupt the selenophosphate synthetase-encoding gene failed (Stock *et al*., 2010). In fact, we used a heterozygous *sps* mutant strain to show that the cellular ratio of wild-type and mutant *sps* alleles depends on the selection pressure and that the absence of selection leads to very fast equalization of genome copies in *M. maripaludis* S2, probably via a gene conversion mechanism (Hildenbrand *et al*., 2011). In a derivative of *M. maripaludis* S2, strain Mm900, deletion of *pstK* was possible only when either SPS or the transcriptional repressor HrsM, which suppresses expression of the selenium-free hydrogenases Frc and Vhc (Sun and Klein, 2004), were absent (Hohn *et al*., 2011). Interestingly, the amplitude of H_2-dependent regulation of methanogenesis genes, including that of most of the organism's selenoprotein genes and the genes for the selenium-independent isoforms is markedly reduced in strain Mm900 as compared with other *M. maripaludis* strains (Xia *et al*., 2009). Thus, stringency of gene control, obviously affected in Mm900, appears to be different between *M. maripaludis* JJ and S2 and induction of the selenium-free back-up system is more easily achieved in the former strain than in the latter strain.

The only selenoprotein genes apparently not affected by the transcriptional arrest found here are those encoding FdhA, which is constitutively synthesized. It is not clear why *M. maripaludis* JJ synthesizes formate dehydrogenase when growing on $H_2 + CO_2$, but the same was observed in *M. maripaludis* S2 (Wood *et al*., 2003). Still, from a physiological standpoint it would be beneficial to downregulate *fdhA* expression only if an alternative, selenium-independent, system was present to compensate. Such a system is apparently absent in *M. maripaludis* because mutants unable to synthesize selenoproteins do not grow on formate. Thus, downregulating expression of the other selenoprotein genes while constitutively expressing *fdhA* at a high level would be advantageous for the organism because it ensures preferential incorporation of selenium into formate dehydrogenase, even when this trace element is scarce.

In summary, the present analysis extends with $tRNA^{sec}$ the list of factors shown to be required *in vivo* for archaeal selenoprotein synthesis and suggests that a stable genetic switch in *M. maripaludis* JJ leading to selenium-independent transcriptional arrest may exist, which obviates synthesis of all but one of the selenoproteins involved in the organism's energy metabolism. Investigating the molecular basis of this switch, which causes a hierarchy in selenoprotein synthesis with formate dehydrogenase on top, will be a challenging task and promises novel insights into the biology of these unique and important prokaryotes.

Experimental procedures

Strains and growth conditions

For growth and transformation of *E. coli* strains (Table 1) standard conditions were used (Sambrook *et al*., 1989). Where appropriate, 100 μg ml^{-1} ampicillin was added to the medium for selection of strains carrying the *bla* gene. Anaerobic gas production from glucose, i.e. the activity of the formate-:hydrogen lyase, was qualitatively determined employing a previously described method (Stager *et al*., 1983) with modifications. *E. coli* was cultivated semi-anaerobically in 20 ml glass tubes with inverted Durham vials using 10 ml of Lysogeny Broth (Bertani, 2004) containing 0.8% glucose. Gas formation (i.e. gas accumulation in the Durhams) was scored after 4 days of non-agitated incubation at 37°C.

Methanococcus maripaludis JJ (DSMZ 2067; Jones *et al*., 1983) and its derivatives (Table 1) were cultivated at 37°C in McSe medium (Rother *et al*., 2003) containing casamino acids (Whitman *et al*., 1986) and 1 μM sodium selenite. A total of 2×10^5 Pa of H_2:CO_2 (80:20) served as the sole energy source. For growth on sodium formate (2%, w/v), 0.5×10^5 Pa of N_2:CO_2 (80:20) was applied and 80 mM morpholinepropanesulphonic acid, pH 6.8, added to keep the pH constant. Growth was monitored photometrically at 578 nm (OD_{578}). Transformation of *M. maripaludis,* plating on solid media, and selection for resistance towards puromycin and/or neomycin was conducted as described previously (Tumbula *et al*., 1994; Stock *et al*., 2010). *In vivo* labelling of *M. maripaludis* with [^{75}Se]-selenite and analysis of the selenoproteome was conducted as described (Stock *et al*., 2010).

Construction of plasmids and mutant generation/complementation

Standard molecular methods were used for manipulation of plasmid DNA from *E. coli* (Ausubel *et al*., 1997). Chromosomal DNA of *M. maripaludis* was prepared by a modified Cetyl trimethylammonium bromide/NaCl (CTAB) method (Pritchett *et al*., 2004). All DNA fragments derived from PCR, which were used for cloning, were sequenced by SRD (Bad Homburg) using the BigDye Terminator Cycle Sequencing protocol (Applied Biosystems). The plasmids and strains used in this study are listed in Table 1. Detailed description of plasmid construction, plasmid sequences and plasmid maps

are available upon request. As no genome sequence for *M. maripaludis* JJ is available, the DNA fragments used for gene disruptions in *M. maripaludis* JJ were amplified by PCR using genomic DNA from *M. maripaludis* S2 (Hendrickson *et al.*, 2004) as template. The oligonucleotides employed (Table S1) were also used to amplify the respective regions of the *M. maripaludis* JJ chromosome, which were subsequently sequenced to ensure sufficient similarity of the DNA of the two strains for homologous recombination. If the sequences from strain S2 compared with those of strain JJ were > 98% identical they were deemed suitable for use.

Generally, for disruption of a gene in *M. maripaludis*, the *pacN* cassette of pNPAC [encoding a codon-optimized variant of the *pac* gene, which confers resistance towards puromycin (Sun and Klein, 2004)] was flanked by *c.* 900 bp sequences comprising the up- and downstream regions of the target loci. The DNA fragments were generated by PCR thereby introducing suitable restriction sites for cloning. All resulting plasmids (Table 1) were linearized and transferred into *M. maripaludis* JJ, obtained from the DSMZ (Braunschweig, Germany) and stored as glycerol stock at –80°C, where target loci were replaced by the *pacN* cassette through a double recombination event. The genotype of mutants was analysed by Southern hybridization employing a previously described protocol (Pritchett *et al.*, 2004) using digoxigenin (DIG)-labelled DNA fragments as probes (Roche, Mannheim, Germany). For each target locus three probes were used, one specific for the *pacN* cassette, one specific for the target gene, and one specific for either the up- or the downstream region of the target gene. Comparison of the hybridization pattern of the wild type with that of a mutant verified the genotype of the latter.

For complementation of *M. maripaludis*, the structural genes *pstK* and *spcS* were amplified by PCR thereby introducing NdeI recognition sites at the 5′ ends overlapping with the annotated translation start codons and BglII sites at the 3′ ends. The fragments were cloned into pNPAC, thus creating fusions of the genes with the strong constitutive *p*sl promoter (Kansy *et al.*, 1994). The respective fusions were moved into the *E. coli*/*M. maripaludis* shuttle vector, pWLG40NZ-R (Lie and Leigh, 2003), likewise restricted, giving rise to pWLmmpstK and pWLmmspcS respectively (Table 1). To create an artificial operon of *M. maripaludis pstK* and *E. coli selD*, the *selD* gene was amplified by PCR, whereby the ribosome binding site of the *p*sl promoter (see above) was introduced 5′ of the translational start site, as well as suitable restriction sites at both ends of the fragment. This PCR fragment was placed downstream of *pstK*, which resulted in pTSpstK-selD (Table 1). The encoded artificial transcriptional unit was moved into pWLG40NZ-R, resulting plasmid in pWLpstK-selD (Fig. S5).

The gene for *M. maripaludis* $tRNA^{sec}$ (*selC*) and for the G37→A variant was synthesized via the assembly of six overlapping oligonucleotides (Table S1) introducing restriction sites 3′ and 5′ of the coding region (Meinnel *et al.*, 1988). Because expression signals for archaeal *selC* gene were unknown, *c.* 50 bp of its 5′ region (potentially containing a promoter) and *c.* 60 bp of its 3′ region (potentially containing a terminator and processing signals) were also included. The resulting fragments were cloned into pWLG40NZ-R, resulting in pWLJCwt and pWLJCg37a respectively (Table 1).

To complement *E. coli* with *M. maripaludis selC*, The gene was amplified by PCR, introducing a BamHI site at the 3′ end and a HindIII at the site and 5′ end of the fragment, and cloned into pUC19, resulting in p19JJC (Table 1). Inverse PCR using p19JJC as template was conducted to introduce the G37→A mutation and the BamHI/Hind III fragment of the PCR product was again cloned into pUC19 to generate p19JJCGA (Table 1).

Northern analysis of crude tRNA

For Northern blot analysis of crude tRNA from *M. maripaludis*, cells harvested from 250 ml of cultures were pressed five times through a syringe with a 0.8 mm gauge needle to enhance cell lysis. tRNAs were isolated as described (Chomczynski and Sacchi, 1987). The isolated tRNAs were separated on a 12% acid-urea polyacrylamide gel [acrylamide/bisacrylamid (29:1) containing 7 M urea], transferred onto a nylon membrane, and cross-linked (Köhrer and Rajbhandary, 2008). The membrane was probed using an oligonucleotide complementary to the first 32 bases of $tRNA^{sec}$ which was labelled with the DIG Oligonucleotide Tailing Kit 2nd Generation (Roche) following the manufacturer's instructions.

qRT-PCR with M. maripaludis *JJ RNA*

As no genome sequence for *M. maripaludis* JJ is available, portions of the respective genes were amplified by PCR using this strain's genomic DNA and primer oligonucleotides deduced from the known *M. maripaludis* S2 genome sequence (Hendrickson *et al.*, 2004). The sequence of the resulting PCR fragments was determined and used to deduce the oligonucleotides for the qRT-PCR analysis. One criterion for the oligonucleotides (Table S1) was to bind at portions of the respective mRNA most dissimilar to the respective portion of the corresponding isogene. The specificity of the oligonucleotides was ensured by extensive PCR and sequencing analysis.

Quantification of *M. maripaludis* RNA was conducted as described (Rother *et al.*, 2011). Briefly, cultures of *M. maripaludis* grown on $H_2 + CO_2$ to late exponential phase were harvested by centrifugation. Total RNA was isolated from the cells using the High Pure RNA isolation Kit (Roche, Mannheim, Germany) and following the manufacturer's instructions, except omitting the on-column DNase digestion step but treating the eluted RNA three times (for 2 h each) with RQ1 RNase-free DNase (Promega, Mannheim, Germany). The RNA preparation was treated with the Nucleospin RNA Clean-up Kit (Macherey-Nagel, Düren) to remove digested DNA and DNase and absence of DNA was confirmed via qPCR.

One gene-specific oligonucleotide was used for first-strand (cDNA) synthesis from each of the individual mRNAs to be analysed (Table S1). For cDNA synthesis from up to 12 mRNAs in one reaction, a 48 µl reaction mixture containing reaction buffer (provided with the enzyme), 2 µg of RNA, 10 pmol (of each) oligonucleotide, 0.5 mM dNTPs was combined. The sample was denatured at 70°C for 5 min and incubated at 55°C for 5 min for annealing of the

oligonucleotide(s). cDNA synthesis was started by addition of 2 µl of Moloney murine leukaemia virus (M-MLV) reverse transcriptase RNase H Minus, point mutant (200 U μl^{-1}, Promega). The reaction was allowed to proceed for 1 h at 55°C before the enzyme was inactivated by incubation at 80°C for 5 min. For every mRNA to be analysed two additional oligonucleotides were deduced (Table S1), both annealing within the cDNA for second-strand synthesis and amplification. This measure further increased the overall specificity of the analysis by reducing the amount of DNA amplified via unspecific priming. For the subsequent qPCR analysis, 1 µl of 1:10 diluted cDNA, 2 pmol of each oligonucleotide and 12.5 µl of Absolute QPCR SYBR Green Mastermix (Thermo Scientific, Dreieich, Germany) were combined in a 25 µl reaction. For the amplification a Rotor-Gene RG-3000 qPCR cycler (Corbett Research, Cambridge, UK) employing a 'hot start' protocol to activate the thermostable DNA polymerase contained in the Mastermix was used. The data were analysed using the $2^{-\Delta\Delta C}{}_T$ method (Livak and Schmittgen, 2001) and normalized to the expression of the *mcrB* gene (MMP1555 in the genome of strain S2) encoding the β-subunit of methyl-coenzyme M reductase, which is not transcriptionally regulated in *M. maripaludis* S2 (Xia *et al*., 2006; Hendrickson *et al*., 2007).

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