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In vivo requirement of selenophosphate for selenoprotein synthesis in archaea

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Summary

Biosynthesis of selenocysteine, the 21st proteinogenic amino acid, occurs bound to a dedicated tRNA in all three domains of life, Bacteria, Eukarya and Archaea, but differences exist between the mechanism employed by bacteria and eukaryotes/archaea. The role of selenophosphate and the enzyme providing it, selenophosphate synthetase, in archaeal selenoprotein synthesis was addressed by mutational analysis. Surprisingly, MMP0904, encoding a homologue of eukaryal selenophosphate synthetase in *Methanococcus maripaludis* S2, could not be deleted unless *selD*, encoding selenophosphate synthetase of *Escherichia coli*, was present in *trans*, demonstrating that the factor is essential for the organism. In contrast, the homologous gene of *M. maripaludis* JJ could be readily deleted, obviating the strain's ability to synthesize selenoproteins. Complementing with *selD* restored selenoprotein synthesis, demonstrating that the deleted gene encodes selenophosphate synthetase and that selenophosphate is the *in vivo* selenium donor for selenoprotein synthesis of this organism. We also showed that this enzyme is a selenoprotein itself and that *M. maripaludis* contains another, HesB-like selenoprotein previously only predicted from genome analyses. The data highlight the use of genetic methods in archaea for a causal analysis of their physiology and, by comparing two closely related strains of the same species, illustrate the evolution of the selenium-utilizing trait.

Introduction

Proteins containing the unusual amino acid selenocysteine are present in all three domains of life, Bacteria, Eukarya and Archaea (Lu and Holmgren, 2009; Stock and Rother, 2009). Biosynthesis of selenocysteine proceeds bound to a dedicated tRNA, $tRNA^{sec}$, which is initially mischarged with serine (Hatfield *et al.*, 1982; Leinfelder *et al.*, 1988; Kaiser *et al.*, 2005). In bacteria, conversion of seryl-$tRNA^{sec}$ (ser-$tRNA^{sec}$) to selenocysteinyl-$tRNA^{sec}$ (sec-$tRNA^{sec}$) is catalysed in one step by selenocysteine synthase (the *selA* product in *Escherichia coli*), which uses (mono)selenophosphate as the *in vivo* selenium donor (Leinfelder *et al.*, 1990). It is generated by selenophosphate synthetase (the *selD* product) from a reduced selenium species and ATP (Veres *et al.*, 1994). In contrast, Archaea and Eukarya employ a two-step mechanism for sec-$tRNA^{sec}$ synthesis. Phosphoseryl-$tRNA^{sec}$ kinase (PSTK) (Carlson *et al.*, 2004; Kaiser *et al.*, 2005) phosphorylates ser-$tRNA^{sec}$ to *O*-phosphoseryl-$tRNA^{sec}$ (sep-$tRNA^{sec}$), which is subsequently converted into sec-$tRNA^{sec}$ by phosphoseryl-$tRNA^{sec}$: selenocysteine synthase (SepSecS, also called SecS; Xu *et al.*, 2006; Yuan *et al.*, 2006). Whether selenophosphate is the physiological selenium donor in this reaction has not been unequivocally demonstrated. The physiological function of sep-$tRNA^{sec}$ is also not clear, although it was argued that phosphoserine would provide a better leaving group (phosphate) than serine (water) for replacement with selenium (Yuan *et al.*, 2006). Therefore, the aminoacyl-mojety could be sufficiently activated to make phosphorylation of selenium obsolete. The selenocysteine-specific translation factor (SelB in prokaryotes, eSelB or eEFsec in eukaryotes) binds sec-$tRNA^{sec}$ (Forchhammer *et al.*, 1989; Fagegaltier *et al.*, 2000; Rother *et al.*, 2000; Tujebajeva *et al.*, 2000) and transfers it to the ribosome where it is inserted into a nascent polypeptide at the position corresponding to a dedicated UGA codon on a selenoprotein mRNA. This recoding from a stop codon to a selenocysteine codon is mediated by the selenocysteine insertion sequence (SECIS) element. In bacteria, it is located directly downstream of the UGA selenocysteine codon on the mRNA, whereas in archaea and eukaryotes it was moved out of the reading frame into the non-translated region (Zinoni *et al.*, 1990; Berry *et al.*, 1991; Rother *et al.*, 2001). Selenoproteins and, thus, the selenoprotein synthesis machinery is essential in higher eukaryotes as evidenced by the embryonic lethality of a $tRNA^{sec}$ mutation (Bosl *et al.*, 1997) and the fact that

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selenophosphate synthetase 2 cannot be deleted but only reduced by silencing the encoding mRNA (Xu *et al.*, 2007). To date, only in trypanosomes and archaea is unambiguous mutational analysis of the *in vivo* requirement of factors potentially involved possible (Rother *et al.*, 2003; Aeby *et al.*, 2009).

Within the Archaea, selenoproteins have so far only been found in *Methanococcus*, *Methanocaldococcus* and *Methanopyrus* species, strictly anaerobic organisms conserving energy for growth only via the hydrogenotrophic pathway of methanogenesis. There, CO_2 (with H_2 as the electron donor) or formate is reduced to methane in a stepwise fashion involving coenzyme-bound C1 intermediates (Thauer, 1998). In *Methanococcus maripaludis* S2, most of the selenoproteins deduced from the genome sequence (Hendrickson *et al.*, 2004), namely subunits of formate dehydrogenase (FdhA), tungsten-containing formyl-methanofuran dehydrogenase (FwuB), heterodisulfide reductase (HdrA), as well as F_{420}-dependent (FruA) and F_{420}-independent hydrogenase (VhuD and VhuU), are directly involved in this essential pathway. However, 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). Consequently, loss of selenoproteins should not affect the organism's ability to grow with $H_2 + CO_2$ but obviate formate-dependent growth. This prediction was proved through mutational analysis of a closely related strain, *M. maripaludis* JJ (Jones *et al.*, 1983), for which no genome sequence is available; a mutant in which the *selB* gene had been disrupted could no longer synthesize selenoproteins and no longer grew with formate as the sole energy source, which was attributed to the strict selenium dependence of formate dehydrogenase (Rother *et al.*, 2003). Beside two seleno-modified RNAs six selenoproteins were detected in this strain (Rother *et al.*, 2001), which probably correspond to those deduced from the *M. maripaludis* S2 genome. Based on the analysis of the *Methanocaldococcus jannaschii* genome (Kryukov and Gladyshev, 2004), two additional selenoproteins can be predicted for *M. maripaludis* S2. One, HesB-like protein, is a *c.* 11 kDa protein of unknown function distantly related to IscA, which is possibly involved in iron-sulphur cluster assembly (Huang *et al.*, 1999; Cupp-Vickery *et al.*, 2004); the other is a homologue of eukaryal SPS2, selenocysteine-containing selenophosphate synthetase (Guimaraes *et al.*, 1996). However, neither in *M. jannaschii* nor *M. maripaludis* could the latter selenoprotein be detected via metabolic labelling with [^{75}Se]-selenite, which was explained by its presumed low abundance (Wilting *et al.*, 1997; Rother *et al.*, 2001).

In the present study, we addressed the role of archaeal selenophosphate synthetase in selenoprotein synthesis and whether it is a selenoprotein, by mutational analysis. Surprisingly, we found the encoding gene to be essential in *M. maripaludis* S2 but not in *M. maripaludis* JJ. Heterologous expression of the *selD* gene from *E. coli* in conjunction with *in vivo* labelling of complemented mutants unambiguously shows that selenophosphate synthetase is essential for selenoprotein synthesis in *M. maripaludis* JJ, that selenophosphate is the *in vivo* selenium donor in archaeal selenoprotein synthesis and that selenophosphate synthetase is a selenoprotein itself. Our findings illustrate the apparent difference in selenoprotein requirement among very closely related strains of *M. maripaludis* species, the basis of which was further investigated by analysing expression in *M. maripaludis* S2 of the genes coding for the cysteine-containing isoforms of the selenoproteins.

Results and discussion

MMP0904 is essential in M. maripaludis *S2*

As selenocysteine biosynthesis in archaea involves a phosphorylated aminoacyl intermediate, *O*-phosphoseryl-tRNA^{sec}, which could be sufficiently activated to make phosphorylation of selenium obsolete, the role of selenomonophosphate as the selenium donor in this pathway was at issue. *M. maripaludis* strain JJ, for which no genome sequence is available, synthesizes at least six selenocysteine-containing proteins and two seleno-modified RNAs. Furthermore, its machinery for biosynthesis and incorporation of selenocysteine is dispensable due to the presence of a set of selenium-independent isoforms of its selenoproteins involved in hydrogenotrophic methanogenesis (Rother *et al.*, 2003). Based on inspection of its genome sequence, the situation was predicted to be identical for *M. maripaludis* strain S2, which was therefore chosen as the model.

To investigate selenium metabolism in *M. maripaludis* S2, attempts were made to disrupt MMP0904, the only open reading frame (ORF) that could, based on its homology to eukaryal SPS2 (the amino acid sequence of MMP0904 is 39–43% similar and 25–28% identical to mammalian SPS2 homologues), be a selenophosphate synthetase and, at the same time, a selenoprotein. For this purpose, plasmid pTS0904 was constructed in which the *pacN* cassette is flanked by 913 and 834 bp portions, respectively, of the genomic region surrounding MMP0904 (Fig. 1). The plasmid was linearized by restriction and the linear DNA fragment containing the knock-out construct was transferred into *M. maripaludis* S2. To acquire puromycin resistance, two homologous recombination events have to take place, thereby replacing the wild-type allele by the disrupted allele (Fig. 1). More than 40 puromycin-resistant transformants were screened by PCR and shown to contain the disrupted allele (see *Experimental proce-*

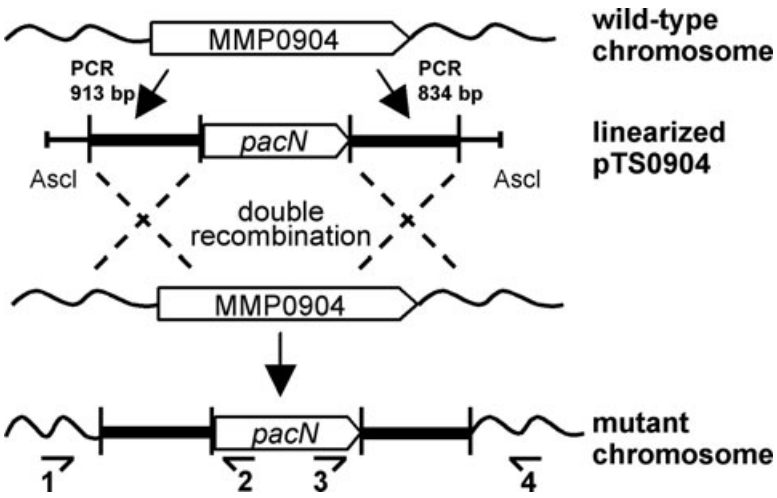

Fig. 1. Scheme for the disruption of MMP0904 in *M. maripaludis*. pTS0904 was constructed by flanking the *pacN* resistance cassette with flanking sequences (black bars) overlapping with the ORF. *M. maripaludis* was transformed with linear DNA (*pacN*-containing AscI fragment of pTS0904) to obtain puromycin-resistant transformants through double recombination events (dashed lines); screening for deletion/disruption of the ORF was conducted using the primer combinations (arrows) 1 + 2, 3 + 4 and 1 + 4 (1, oselDintegFor; 2, oNpacinteg/rev; 3, oNpacinteg/for; 4, oselDintegRev; see Table S1).

dures); however, all of them also contained the wild-type ORF as well (data not shown). The presence of the wild-type allele was confirmed by hybridizing genomic DNA of a randomly selected clone, which was designated SKoD4, with a probe corresponding to an internal portion of MMP0904 (Fig. 2A, lane 5). Using a probe hybridizing with the *pacN* cassette (Fig. 2B) revealed a single insertion of the resistance cassette into the genome; further, the size of the fragment (*c.* 5.6 kb, Fig. 2B, lane 5), corresponds in size to the one, resulting from *in silico* prediction, generated by the expected double homologous recombination event. Strikingly, a probe hybridizing to the upstream region of MMP0904 resulted in two fragments, one corresponding to the wild-type signal (compare lanes 4 and 5, Fig. 2C) and one corresponding in size to the allele disrupted by the *pacN* cassette. Integration of the circular plasmid containing the knock-out construct via a single recombination event as well as its tandem duplication resulting from selective pressure, as reported for *M. maripaludis* and *Methanococcus voltae* (Sandbeck and Leigh, 1991; Pfeiffer *et al.*, 1998) would have resulted in a different pattern (not shown). Experiments with a culture derived from re-streaking a single colony three times gave identical results (data not shown), which rules out that the observed heterogeneities in the DNA resulted from an impurity of the culture. Instead, these data strongly suggest that SKoD4 is heterozygous and contains both alleles located at the same respective position although on distinct chromosomes, some carrying the wild-type allele while others carry the mutated allele. This implies that the organism contains multiple copies of its genome. Genome polyploidy has been observed in archaea (Majernik *et al.*, 2005; Breuert *et al.*, 2006) and also in bacteria (Maldonado *et al.*, 1992). In *M. jannaschii*, distribution of chromosomes appears to follow a random mechanism (Malandrin *et al.*, 1999), which raises the question how the type of polyploidy

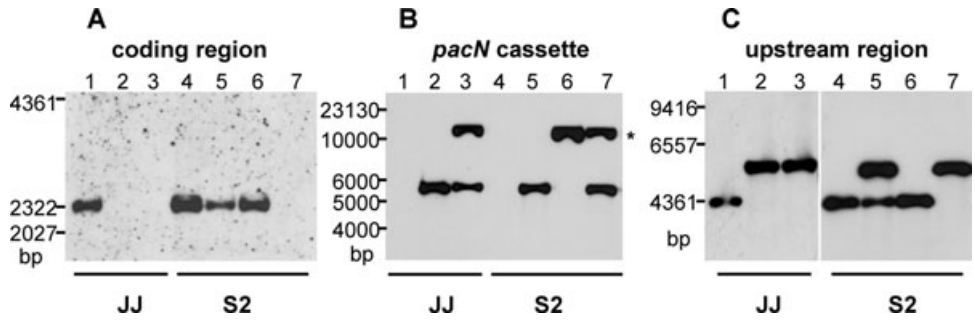

Fig. 2. Verification of the genotype of selenophosphate synthetase mutants via Southern hybridization. Genomic DNA of the strains JJ (lanes 1), TS1 (lanes 2), TS1 transformed with pWLSTselD (lanes 3), S2 (lanes 4), SkoD4 (lanes 5), S2 transformed with pWLSTselD (lanes 6) and TS904 (the MMP0904 disruption mutant pre-transformed with pWLSTselD, lanes 7) was restricted with EcoRI (A) or BglII (B and C) and probed with DIG-labelled DNA fragments.
A. Analysis of the structural gene (coding region) using a DIG-labelled probe hybridizing to an internal 304 bp portion of MMP0904.
B. Analysis of genomic integration of the resistance cassette (*pacN* cassette) was performed with a DIG-labelled probe hybridizing to an internal 570 bp portion of the *pacN* cassette; the signal of < 10 kb (asterisk) stems from pWLSTselD, which is a derivative of pWLG30 containing portions of the *pacN* cassette (Gardner and Whitman, 1999).
C. Analysis of the genomic region surrounding the structural gene (upstream region) was performed with a DIG-labelled probe hybridizing to 902 bp of the region upstream of the structural gene.

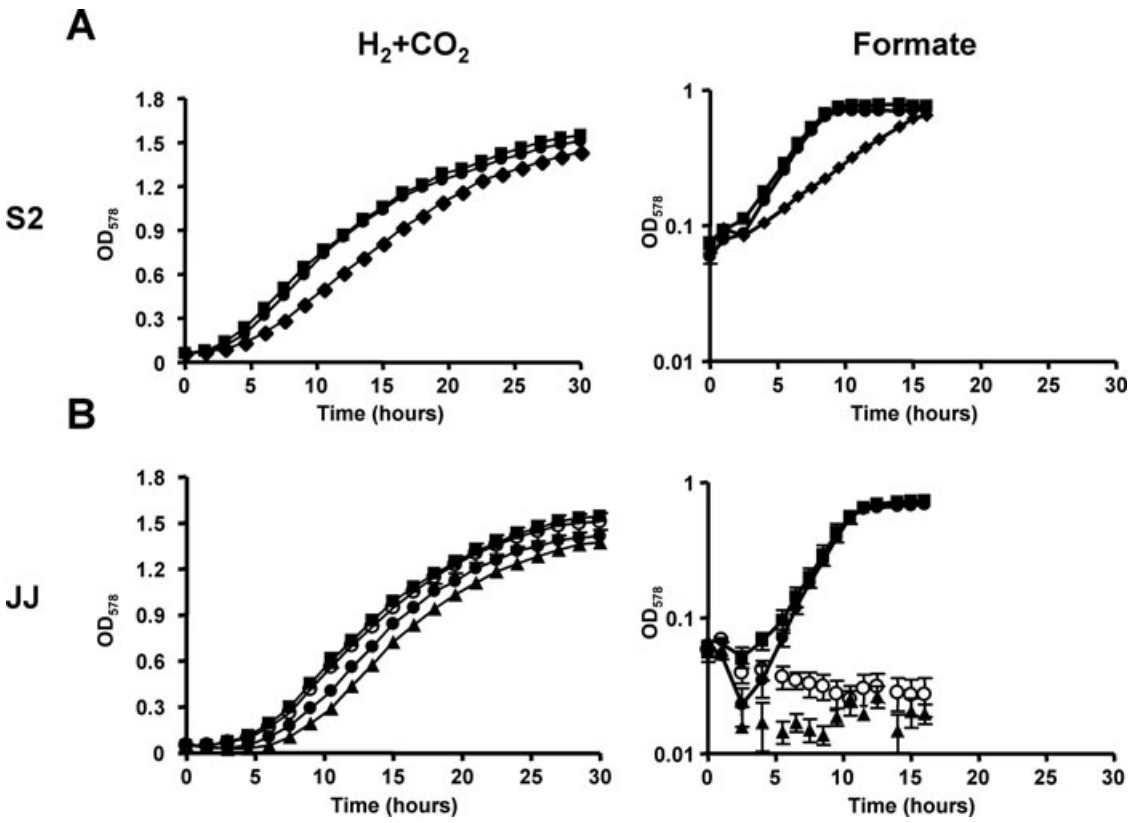

Fig. 3. Selenophosphate synthetase-dependent growth of *M. maripaludis*. Cultures of derivatives of strain S2 (A) and JJ (B) were pre-grown on $H_2 + CO_2$, transferred to fresh medium containing $H_2 : CO_2$ (left panels; cultures were re-pressurized every 3 h) or formate (right panels), and growth was monitored at 578 nm; shown are average values and standard deviations of triplicates of at least two independent experiments; note that growth of *M. maripaludis* on $H_2 + CO_2$ is mostly non-exponential due to limited gas/liquid mass transfer; see *Experimental procedures* for details.
A. Solid square, S2 (wild type); solid diamond, SKoD4 (heterozygous *sps* mutant); filled circle, TS904 (homozygous MMP0904 disruption mutant pre-transformed with pWLSTselD).
B. Solid square, JJ (wild type); open circle, TS1 (homozygous *sps* mutant); filled triangle, TS1 transformed with pWLG40NZ-R (vector control); filled circle, TS1 transformed with pWLSTselD (complementation with *selD*).

observed here is maintained, i.e. how the different 'kinds' of chromosomes are segregated and the distinct alleles on different chromosomes equalized during cell division (Rosenshine and Mevarech, 1991). Efforts to elucidate the molecular basis of this phenomenon, which is also observed in the archaeon *Haloferax volcanii,* are under way (C. Lange and J. Soppa, pers. comm.).

The fact that SKoD4 stably maintained (an) undisrupted (copy) copies of MMP0904 suggested that the function encoded might be essential for the organism. To test this notion, the wild type was transformed with pWLSTselD, carrying *selD* (encoding selenophosphate synthetase from *E. coli*) under the control of a strong constitutive methanococcal promoter (see *Experimental procedures*). Using this strain as parent, a homozygous MMP0904 disruption mutant, could be readily isolated, as evidenced by Southern blot analysis (lanes 6 and 7, Fig. 2A–C). Compared with the S2 wild-type strain, SKoD4 showed a slight growth defect with $H_2 + CO_2$ (Fig. 3A, left panel), and a more pronounced growth defect with formate (growth rate 0.17 $\pm$ 0.02 h^{-1} versus 0.31 $\pm$ 0.01 h^{-1} in S2, Fig. 3A, right panel), as the energy source; the homozygous MMP0904 disruption mutant carrying *selD* was designated TS904 and grew indistinguishably from the wild type on both substrates, which might indicate that the reduced copy number of MMP0904 in SKoD4 (see above) caused its growth defect (Fig. 3A).

The ability to disrupt a gene only in the presence of a complementing copy is compelling evidence for its essentiality (Rother *et al*., 2005; Thomas and Bolhuis, 2006): Therefore, our findings show that MMP0904 is essential in *M. maripaludis* S2 and that this essentiality can be relieved by *selD* complementing its function. pWLSTselD was stably maintained in the MMP0904 mutant without antibiotic selection for more than 100 generations (Fig. S1). This finding suggests that a strong selective pressure exists in this strain for maintaining *selD* adding further support to the conclusion regarding essentiality of

MMP0904. A shuttle plasmid containing the identical backbone as pWLSTselD is rapidly lost in *M. maripaludis* under non-selective conditions (Gardner and Whitman, 1999).

As the selenocysteine-specific translation factor SelB and, thus, the system for selenocysteine insertion into nascent polypeptides is not essential in *M. maripaludis* JJ (Rother *et al*., 2003), we wanted to determine if this also applies for selenocysteine biosynthesis, i.e. the putative selenophosphate synthetase of this strain. To check whether pTS0904 could be used in *M. maripaludis* JJ, the genomic region encompassing the MMP0904 homologous ORF was amplified from this strain and sequenced (see *Experimental procedures*). Both the flanking regions and the coding region of the S2 and the JJ sequence were >98% identical (data not shown) making pTS0904 suitable for use in strain JJ. It was transformed with the linearized vector, and one puromycin-resistant clone obtained, which was designated TS1, was examined further. Analysis of the genotype by Southern hybridization revealed that TS1 is a homozygous mutant, in which the MMP0904-homologous ORF is disrupted by the *pacN* cassette (lanes 1 and 2, Fig. 2A–C). This is in contrast to *M. maripaludis* S2 and either due to the presence of a gene complementing the gene loss in strain JJ, which is absent in strain S2, or to the dispensability of the function encoded.

TS1 grew indistinguishably from the wild type with $H_2 + CO_2$ (Fig. 3B, left panel) but was unable to grow with formate as the sole energy source (Fig. 3B, right panel), which is consistent with the phenotype of the *selB* deletion strain (Rother *et al*., 2003) and might indicate a required function of the deleted protein in selenium metabolism of TS1. The formate-dependent growth defect was fully compensated in the presence of pWLSTselD (Fig. 3B, right panel), indicating that lack of selenophosphate synthetase activity caused the growth defect in TS1. To determine the *in vivo* function of the ORF deleted in *M. maripaludis* JJ more rigorously, it was subjected to metabolic labelling analysis.

Selenophosphate is the in vivo *selenium donor in* M. maripaludis *JJ*

M. maripaludis JJ, TS1 and TS1 carrying pWLSTselD were metabolically labelled with [^{75}Se]-selenite (see *Experimental procedures*). Crude extracts were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and the selenium-containing macromolecules were visualized by phosphoimaging. As shown in Fig. 4. *M. maripaludis* TS1 is unable to synthesize selenoproteins (Fig. 4A, lane 3) present in the wild type (Fig. 4A, lane 1). This finding demonstrates that the ORF deleted in *M. maripaludis* JJ is not crucial for growth but for selenoprotein biosynthesis. When TS1 was complemented with *selD* from *E. coli* its ability to synthesize selenoproteins was restored (Fig. 4A, lane 4). These data, together with the known activity of SelD, provide compelling evidence for the notion that the MMP0904-homologous ORF in *M. maripaludis* JJ encodes selenophosphate synthetase and that selenophosphate is a required intermediate for archaeal selenoprotein synthesis *in vivo*. These findings also rule out that the basis for evolving a two-step mechanism for conversion of ser-tRNA^{sec} to selc-tRNA^{sec} found in archaea, rather than a one-step mechanism found in bacteria, lies in activating the aminoacyl-moiety of ser-tRNA^{sec} rather than activating selenium. This conclusion is fully consistent with *in vitro* analyses of archaeal and eukaryal systems in which synthesis of selenocysteinyl-tRNA^{sec} required selenophosphate (Xu *et al*., 2006; Yuan *et al*., 2006). In contrast to the *selB* deletion strain (Rother *et al*., 2003), TS1 has lost its ability to generate the [^{75}Se]-labelled RNA species (compare lanes 1 and 3, Fig. 4A), which demonstrates that in *M. maripaludis* JJ the selenium donor for biosynthesis of both the seleno-modified RNAs and the selenoproteins is selenophosphate, as was shown for bacteria (Leinfelder *et al*., 1990; Veres *et al*., 1992).

Selenophosphate synthetase of M. maripaludis *is a selenoprotein*

Metabolic labelling of *M. maripaludis* S2 with [^{75}Se]-selenite revealed the same pattern of selenoproteins as in *M. maripaludis* JJ (Rother *et al*., 2001; data not shown). All of the six readily visible selenoproteins correspond in their apparent masses with those of the selenoproteins deduced from the genome sequence (Hendrickson *et al*., 2004; Stock and Rother, 2009). When the amount of protein subjected to SDS-PAGE was increased (*c.* 3-fold) and the exposure time during autoradiography extended (*c.* 5-fold), another selenium-labelled protein with a mass of *c.* 36 kDa became visible (Fig. 4B, arrow), which, however, is absent in TS904 (the MMP0904 mutant carrying pWLSTselD), and thus, complemented with SelD (compare lanes 3 and 6, Fig. 4B). This finding strongly argues that MMP0904, replaced in the complemented mutant by SelD from *E. coli*, which is no selenoprotein (Ehrenreich *et al*., 1992), is a selenoprotein itself. The same was found for *M. maripaludis* JJ. While the wild type synthesizes a *c.* 36 kDa selenoprotein of low-abundance compared with the other selenoproteins visible (Fig. 4A, arrow) and the selenophosphate synthetase mutant synthesizes no selenoproteins at all (Fig. 4A, lane 3), complementing with SelD fails to confer the ability to the mutant to synthesize this low-abundant selenoprotein (Fig. 4A, lanes 4, 5), because the enzyme deleted is the respective selenoprotein itself. Analysis of the genome sequence of *M. jannaschii* (Bult *et al*., 1996), a close relative of

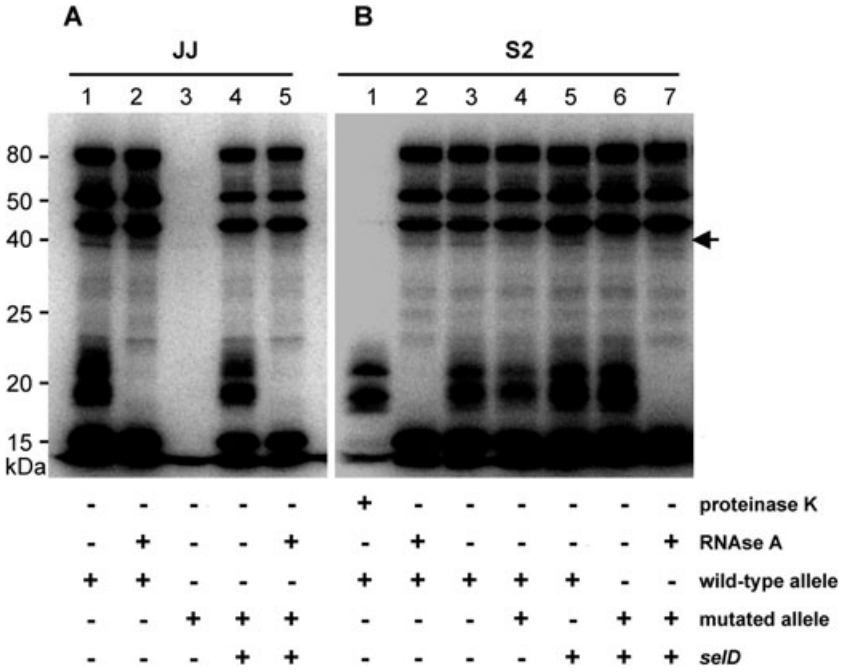

Fig. 4. Selenophosphate synthetase and synthesis of selenoproteins in *M. maripaludis*. Autoradiographs of 12.5% SDS-PAGs (Laemmli, 1970) after electrophoresis of cell lysates from ^{75}Se-labelled *M. maripaludis*. The presence of the wild-type, mutated, or heterologous (*E. coli selD*) allele encoding selenophosphate synthetase is indicated at the bottom; the arrow depicts the migration position of selenocysteine-containing selenophosphate synthetase; migration positions of standard proteins (in kDa) are indicated on the left.
A. Incorporation of ^{75}Se into proteins and RNAs of *M. maripaludis* JJ (lanes 1 and 2), TS1 (lane 3) and TS1 transformed with pWLSTselD (lanes 4 and 5); to identify labelled RNA, extracts were treated with RNAse A (lanes 2 and 5), see *Experimental procedures*.
B. Incorporation of ^{75}Se into proteins and RNAs of *M. maripaludis* S2 (lanes 1–3), SkoD4 (lane 4), S2 transformed with pWLSTselD (lane 5) and TS904, the MMP0904 disruption mutant pre-transformed with pWLSTselD (lanes 6 and 7); to distinguish between labelled protein and RNA, extracts were treated with proteinase K (lane 1) or RNAse A (lanes 2 and 7), see *Experimental procedures*.

M. maripaludis, led to the proposal that this organism might encode a selenocysteine-containing selenophosphate synthetase (MJ1591); however, experimental evidence for this proposal could not be gathered by metabolic labelling and was explained by the presumed low abundance of this protein, thereby evading detection (Wilting *et al*., 1997). Our data support this conclusion by showing that compared with the other selenoproteins of *M. maripaludis*, selenophosphate synthetase is synthesized at a much lower level. MMP0904 is still annotated as a *c.* 32 kDa non-selenoprotein (Fig. 5; http://cmr.jcvi.org; http://genome.ornl.gov/microbial/mmar/). However, our data are consistent with a N-terminally extended protein of 335 amino acids (calculated mass 36.1 kDa) containing a selenocysteine residue at position 7 (Fig. 5A). Additionally, the 3′ non-translated regions of the deduced mRNAs in both strains are apt to fold into 'classical', identical archaeal SECIS structures (Fig. 5B) containing all consensus elements (Rother *et al*., 2001; Kryukov and Gladyshev, 2004). *M. voltae*, *Methanococcus vannielii* and *Methanopyrus kandleri* apparently also encode such a selenocysteine-containing selenophosphate synthetase, which raises the mechanistically interesting question how selenocysteine synthesis can be initiated employing an enzyme that itself is a selenoprotein. *Haemophilus influenzae* apparently solves this problem by initially operating selenophosphate synthetase that is either devoid of selenocysteine, or by forming sec-tRNA^{sec} in a selenophosphate-independent fashion (Wilting *et al*., 1998).

M. maripaludis *synthesizes a single HesB-like selenoprotein*

The fact that selenophosphate synthetase is essential in *M. maripaludis* S2 but not in *M. maripaludis* JJ raised the question about the physiological basis for this striking difference in the two strains. One possibility is that one (or more) of the selenoproteins, and thus the system for its synthesis, is essential in the former strain, as is the case in higher eukaryotes (Bosl *et al*., 1997; Xu *et al*., 2007). Analysis of the genome sequence of *M. maripaludis* S2 indicated the presence of a previously unrecognized gene encoding a small selenoprotein similar to HesB (MMP0252) but of no isogene encoding cysteine at the position of the selenocysteine (Kryukov and Gladyshev, 2004), which, if essential, would render the selenoprotein biosynthesis pathway essential as well. To test whether

Fig. 5. mRNA of selenophosphate synthetase in *M. maripaludis*.
A. Alignment of deduced mRNA nucleotide sequences of MMP0904 from *M. maripaludis* S2 (top) and the homologous gene from *M. maripaludis* JJ (bottom). The translational start codons (bold) annotated in the databases (Start2) and the ones deduced from this study (Start1) are indicated by round arrows; the deduced masses (in kDa) corresponding to the use of the two respective start codons are depicted on top of the sequence; the UGA selenocysteine codons at position seven (sec) are italicized; the translational termination codons are underlined; intervening sequences including their length (in nt) are indicated by '…'; alignments were generated by hand.
B. Secondary structure model of the putative SECIS element from the selenophosphate synthetase mRNA of *M. maripaludis*. The mRNA structure is located in the 3′ non-translated region of MMP0904 immediately downstream of the coding region; the termination codon of the coding region preceding the putative SECIS element is underlined.

the *hesB*-like gene might be absent or duplicated in *M. maripaludis* JJ, chromosomal DNA of both strains was probed with a labelled DNA fragment hybridizing with the coding region of the *hesB*-like gene of *M. maripaludis* S2. As can be seen in Fig. 6A, both strains contain a single fragment encompassing the gene (the *hesB*-like gene of *M. maripaludis* S2 contains a BstYI restriction site; Fig. 6A, lane 6) but no homologous sequences giving rise to additional signals, which indicates that both strains encode a single HesB-like protein. To address the requirement of this protein directly the encoding gene was disrupted in both strains (see *Experimental procedures*, Fig. S2). Both mutants deleted for the *hesB*-like gene (JKHes2 derived from strain JJ and SKHes7 derived from strain S2) grew with formate as sole energy source indistinguishably from their respective parent, which indicated that selenium metabolism in the mutants is not adversely affected (data not shown). Metabolic labelling with [^{75}Se]-selenite revealed that both *M. maripaludis* JJ and S2 synthesize a small *c.* 10–11 kDa selenoprotein of low abundance, which is absent in the mutants (Fig. 6B, arrow). This finding provides compelling evidence for the notion that HesB-like protein of both strains is a selenoprotein. However, as it is not required in *M. maripaludis* S2, it cannot be the basis for the requirement for selenophosphate synthetase in this strain.

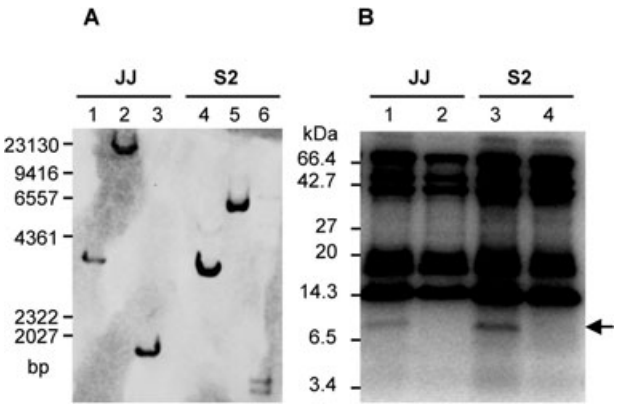

Fig. 6. Analysis of the HesB-like protein of *M. maripaludis*.
A. Genomic DNA of the strains JJ (lanes 1–3) and S2 (lanes 4–6) was restricted with HindIII (lanes 1 and 4), BglII (lanes 2 and 5) and BstYI (lanes 3 and 6) and probed with DIG-labelled DNA fragments hybridizing to a 260 bp internal portion of MMP0252.
B. Autoradiograph of a 16% SDS-PAG (Schägger and von Jagow, 1987) after electrophoresis of cell lysates from ^{75}Se-labelled *M. maripaludis* JJ (lane 1), JKHes2 (lane 2), S2 (lane 3) and SKHes7 (lane 4); the arrow depicts the migration position of selenocysteine-containing HesB-like protein; migration positions of standard proteins (in kDa) are indicated on the left.

M. maripaludis *S2 expresses the genes for the selenoproteins and their cysteine-containing isoforms*

For all of the selenoproteins (with the exception of selenophosphate synthetase, the HesB-like selenoprotein and the subunit of formate dehydrogenase) selenium-independent isoforms are encoded in *M. maripaludis* S2 (Hendrickson *et al*., 2004). To test whether lack of expression of any of the cysteine-encoding isoforms of the selenoproteins could cause the requirement for the selenoprotein biosynthesis machinery during growth of *M. maripaludis* S2 on $H_2 + CO_2$, transcription of the genes was qualitatively assessed by reverse transcription PCR. The primers were chosen to specifically target the respective TGA- (for the selenoprotein genes) or TGT/TGC- (for the non-selenoprotein genes) containing sequences, which were confirmed by sequencing the PCR products (data not shown). As can be seen in Fig. 7, all cysteine-

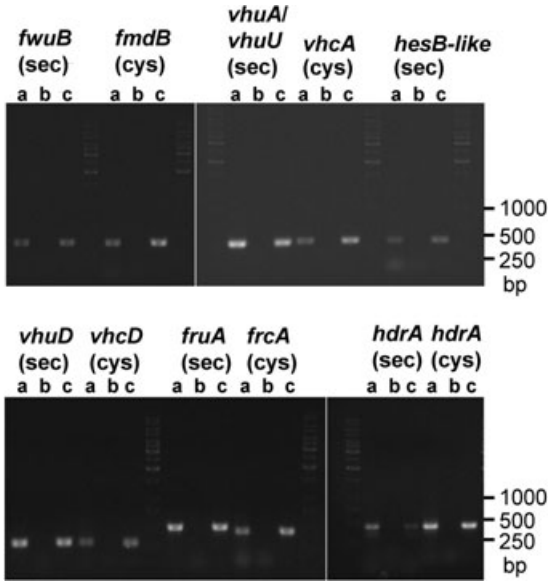

Fig. 7. Expression of selenoprotein genes and their isogenes in *M. maripaludis* S2. DNA fragments amplified by PCR using primer pairs specific for the selenoprotein genes (sec) or their respective cysteine-encoding isogenes (cys) were separated by agarose electrophoresis and stained with ethidium bromide; templates used for the PCR, prepared as described in *Experimental procedures*, were: cDNA obtained by reverse transcription (lanes a), total RNA (lanes b), chromosomal DNA (lanes c); migration positions of standard DNA fragments (in bp) are indicated on the right; note that the cysteine-containing isoform of VhuU is part of VhcA (Sorgenfrei *et al.*, 1997).

encoding isogenes of the selenoprotein genes the products of which are involved in methanogenesis from $H_2 + CO_2$ are transcribed under selenium-limited conditions in *M. maripaludis* S2. These findings are broadly consistent with the results of a global proteomics study assessing the cellular response of *M. maripaludis* S2 to different nutritional conditions in which most cysteine-containing isoforms could be detected; however, MMP0820, the cysteine-containing FrcA subunit of F_{420}-dependent hydrogenase (i.e. the selenium-independent homologue of FruA) could not be detected (Xia *et al.*, 2006), which might support speculating that the encoding mRNA is generated but not translated. If so, this would lead to a strict selenium dependence of hydrogenotrophic growth of *M. maripaludis* S2. Furthermore, neither this study nor our data allows inferring that any of the back-up proteins for the selenoproteins are synthesized in a functional or sufficiently active form, or are sufficiently abundant, to support growth with $H_2 + CO_2$ in the absence of selenoproteins. For example, *M. voltae* expresses, like *M. maripaludis*, a set of genes for selenocysteine-containing and selenium-independent hydrogenases (Halboth and Klein, 1992; Berghöfer *et al.*, 1994), but the gene for a small, selenocysteine-containing subunit of its F_{420}-independent hydrogenase (*vhuU*) could not be deleted (Pfeiffer *et al.*, 1998). Efforts addressing the question whether all of the selenoproteins of *M. maripaludis* S2 involved in hydrogenotrophic methanogenesis can be replaced by their corresponding selenium-independent isoforms are under way in our laboratory, the results of which will be reported elsewhere.

Last, it is noteworthy that selenium utilization is an ancient but very asymmetrically distributed trait among modern archaea (Romero *et al.*, 2005; Yuan *et al.*, 2006), which indicates that it was, and is being, lost in this group (Stock and Rother, 2009). The two *M. maripaludis* strains compared here might represent two 'moments' in this ongoing evolution, where strain S2 has not (yet) functionalized its selenium-independent back-up system sufficiently. Its growth on $H_2 + CO_2$ is therefore still depending on selenoproteins, and thus, the machinery to synthesize them while strain JJ can dispense with it under the same condition. This view is also supported by the presence of selenocysteine/cysteine forms of formate dehydrogenase among methanogens. *M. jannaschii* and *M. maripaludis* solely contain the selenocysteine form, *M. vannielii* synthesize both forms (Jones and Stadtman, 1981) while most other methanogens only generate the cysteine form (Stock and Rother, 2009). Determining the genome sequence of *M. maripaludis* JJ would be useful, as its comparison with those of the other four *M. maripaludis* strains in the databases could reveal molecular details underlying this evolutionary process.

Experimental procedures

Strains and growth conditions

Standard conditions were used for growth and transformation of *E. coli* (Sambrook *et al.*, 1989). Where appropriate, 100 μg ml^{-1} ampicillin was added to the medium for selection of strains carrying the *bla* gene.

Methanococcus maripaludis strains S2 (DSMZ 14266; Whitman *et al.*, 1986) and JJ (DSMZ 2067; Jones *et al.*, 1983) were cultivated at 37°C in McSe medium (Rother *et al.*, 2003) containing casamino acids (Whitman *et al.*, 1986) and 1 μM sodium selenite. Cultures were pressurized with 2×10^5 Pa of $H_2 : CO_2$ (80:20), which 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 by following the optical density at 578 nm (OD_{578}) using a spectronic 20 photometer (Thermo, Dreieich, Germany). For growth on solid medium 1% (w/vol) Bacto Agar (Difco) was added to the medium. Where appropriate, the medium was supplemented with 2.5 μg ml^{-1} puromycin and/or neomycin (0.5 mg ml^{-1} in agar plates; 1 mg ml^{-1} in broth) to select for presence of the codon-optimized *pac* (*pacN*, encoding puromycin acetyltransferase; Gernhardt *et al.*, 1990; Sun and Klein, 2004) or the APH3′ gene (encoding aminoglycoside 3′-phosphotransferase; Argyle *et al.*, 1996) respectively. Transformation of

M. maripaludis was conducted as described previously (Tumbula *et al*., 1994).

In vivo *labelling of* M. maripaludis *with [^{75}Se]-selenite*

Methanococcus maripaludis was pre-grown in the absence of added selenium and in the presence of 1 mM DL-Methionine to increase specific incorporation of radioactive selenium. The cultures were diluted into fresh medium supplemented with Na-[^{75}Se]-selenite (Hartmann Analytik, Braunschweig) to a final concentration of 0.5 μM (specific activity 16 Ci mmol^{-1}) and incubated at 37°C for 24 h. Cells were harvested by centrifugation, washed once with medium and lysed in HMK buffer (20 mM HEPES, 5 mM $MgCl_2$, 1 mM KCl, 1 μg ml^{-1} DNaseI; pH 7.0) to a volume corresponding to an OD_{578} of 10 of the culture. If appropriate the cell lysate was treated with proteinase K (1.5 mg ml^{-1}) or RNase A (0.5 mg ml^{-1}) to differentiate between labelled RNAs and labelled proteins. Lysates (5–30 μl) were separated by SDS-PAGE according to Laemmli (1970) or Schägger and von Jagow (1987). Gels were stained with Coomassie, dried and subjected to autoradiography by Phosphoimaging using a phosphor screen and a Typhoon 9400 (GE Healthcare, Freiburg).

Construction and complementation of M. maripaludis *mutants*

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 method (Pritchett *et al*., 2004). Primers used in this study are listed in Table S1. Plasmids constructed were verified by extensive restriction analysis and 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). For disruption of the selenophosphate synthetase encoding gene, a fragment of 913 bp comprising the upstream region of the gene MMP0904 (encoding the SPS2 homologue of *M. maripaludis* S2) was amplified by PCR with primers oup0904F and oup0904R using chromosomal DNA as template. The fragment was cloned via SpeI and HindIII into pNPAC (Sun and Klein, 2004) resulting in plasmid pTSup0904. A fragment of 834 bp comprising the downstream region of MMP0904 was generated in an analogous fashion using primers odo0904F and odo0904R and cloned via NheI and BamHI into pTSup0904. The resulting plasmid was named pTS0904. The vector was linearized with AscI prior to transformation.

To complement loss of selenophosphate synthetase in *M. maripaludis* with SelD from *E. coli*, the encoding gene was amplified by PCR using chromosomal DNA from *E. coli* as template and the primers oselD/EcoFor and oselD/EcoRev; the product was cloned via NdeI and BamHI, together with the HindIII/NdeI fragment of pNPAC (comprising the strong constitutive *p*sl promoter; Kansy *et al*., 1994), into HindIII/BamHI-linearized pSU2719 (Martinez *et al*., 1988). The SpeI/BglII fragment of the resulting plasmid pSUSTselD comprising the *p*sl–*selD* fusion was subsequently ligated into an *E. coli*/*M. maripaludis* shuttle vector conferring neomycin resistance, pWLG40NZ-R (Lie and Leigh, 2003), likewise restricted, giving rise to pWLSTselD. Stability of pWLSTselD in *M. maripaludis* was assessed by consecutively streaking single colonies onto agar plates lacking neomycin. Single colonies could be used to inoculate neomycin-containing medium at any time during the procedure without any delay in growth. After 10 rounds of streaking, a single colony was transferred to liquid broth (lacking neomycin); the DNA of the culture was isolated and subjected to Southern hybridization analysis (see below).

For disruption of the gene encoding the HesB-like protein, a fragment of 833 bp, comprising the upstream region of MMP0252 was amplified by PCR with primers oHesBupFor and oHesBupRev using chromosomal DNA of *M. maripaludis* S2 as template; this fragment was cloned via SpeI and HindIII into pNPAC resulting in pNpacHesBup. With primers oHesBdownFor and oHesBdownRev a fragment of 679 bp comprising the downstream region of the ORF was amplified and cloned via NheI and BamHI into pNpacHesBup, resulting in pKoHesB. The vector was linearized with AscI prior to transformation.

Genotypic verification of M. maripaludis *mutants*

For screening potential MMP0904-deficient mutants of *M. maripaludis* S2, primers oselDintegFor and oselDintegRev targeting the whole genomic region of 2408 bp including the MMP0904 structural gene were used in PCR reactions with chromosomal DNA as template, which allows detection of both the wild-type allele and the one disrupted by the *pacN* cassette (Fig. 1). The same primer pair was used to amplify the respective region from *M. maripaludis* JJ, which was subsequently sequenced (GenBank Accession Number: GQ884168). To exclusively detect the disrupted allele, the primer combinations oselDintegFor/oNpacintegRev and oselDintegRev/oNpacintegFor was used, which target the junctions between the chromosomal locus and the *pacN* cassette (Fig. 1).

Genomic lesions introduced into *M. maripaludis* by double homologous recombination were also analysed by DNA hybridization according to Southern (1975) using digoxigenin-labelled DNA probes as described (Pritchett *et al*., 2004). For detection of the upstream region of the gene encoding selenophosphate synthetase, the 902 bp HindIII/SpeI fragment of pTS0904 was used; for detection of the respective coding region, a 304 bp fragment obtained with PCR from *M. maripaludis* S2 chromosomal DNA with primers oSelD-probe/For and oSelD-probe/Rev was used; for detection of *E. coli selD*, a 813 bp PvuI/NcoI fragment of pWLSTselD was used; for detection of the *pacN* cassette, a 570 bp NdeI/BstXI fragment of pNPAC was used.

Genotypic changes in the region surrounding the gene encoding the HesB-like protein of *M. maripaludis* were analysed by DNA hybridization using an 823 bp HindIII/SpeI fragment of pKoHesB as probe, which corresponds to the upstream region of the gene in *M. maripaludis* S2. A 260 bp fragment derived from PCR with primers oS20252.1/forw and oS20252.1/rev comprising a portion of MMP0252 was used to detect the wild-type allele. As deletion/disruption using pKoHesB removed only 90 bp from the genome, loss of this fragment could not be verified with this probe; instead, PCR

with the primers ohesBintegFor and ohesBintegRev, targeting a genomic region of 2027 bp including the structural gene, was conducted, which allows to detect both the wild-type allele and the one disrupted by the *pacN* cassette.

Reverse transcription PCR on M. maripaludis *RNA*

Cultures of *M. maripaludis* S2 grown on $H_2 + CO_2$ to late exponential phase under selenium-limited conditions, i.e. in the absence of added selenium, were harvested by centrifugation and lysed in distilled water. Total RNA was isolated using the High Pure RNA Isolation Kit (Roche) according to the manufacturer's instructions. After extensive DNaseI treatment, the RNA was purified with the RNA-Clean-up Kit (Macherey-Nagel, Düren). First strand cDNA was synthesized with M-MLV reverse transcriptase (Promega) using statistical oligonucleotide hexamers (Promega) as primers. Subsequent amplification by PCR of fragments corresponding to mRNAs of the selenoproteins and of the respective cysteine-containing isoforms from *M. maripaludis* S2 with gene-specific primers (Table S1) was carried out using GoTaq Polymerase (Promega) according to the manufacturer's instructions. The specificity of the individual primer pairs was verified by sequencing the respective PCR fragments. Parallel experiments with chromosomal DNA and total RNA as template were included in the analysis to verify the size of the PCR fragment and absence of contaminating DNA in the RNA preparation.

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