Review

Methanogens: a window into ancient sulfur metabolism

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Methanogenesis is an ancient metabolism that originated on the early anoxic Earth. The buildup of O_2 about 2.4 billion years ago led to formation of a large oceanic sulfate pool, the onset of widespread sulfate reduction and the marginalization of methanogens to anoxic and sulfate-poor niches. Contemporary methanogens are restricted to anaerobic habitats and may have retained some metabolic relics that were common in early anaerobic life. Consistent with this hypothesis, methanogens do not utilize sulfate as a sulfur source, Cys is not utilized as a sulfur donor for Fe–S cluster and Met biosynthesis, and Cys biosynthesis uses an unusual tRNA-dependent pathway.

Hydrogenotrophic methanogens, present day surrogates of early metabolisms

Methanogenesis, or CH_4 production, may have been one of the oldest biological processes on Earth and probably played a major role in the evolution of Earth's atmosphere. Earth evolved from an ancient anoxic atmosphere to an oxygenated one (the so-called 'Great Oxidation Event') about 2.4 billion years ago (Ga) [1]. Methanogenesis probably dates from earlier than 3.4 Ga, well before the rise of O_2 [2,3]. On the anoxic Earth, methanogenesis resulted in atmospheric CH_4 levels 100–1500 times higher than today's level of 1.8 parts per million (ppm) [4,5]. Atmospheric CH_4 during the Archean eon impacted Earth's evolution in at least two major areas. First, CH_4 played a dominant role in the greenhouse effect, which kept the early Earth from freezing when the Sun was 20–25% less luminous than it is today [6]. Second, CH_4 contributed to the oxidation of the atmosphere. After the emergence of oxygenic photosynthesis before 2.7 Ga, Earth's atmosphere remained anoxic for at least another 300 million years, possibly because the early environment was sufficiently reducing to scavenge O_2 [5]. Biogenic CH_4 is decomposed to C + 4H in the upper atmosphere by UV radiation, and H can rapidly escape to space. Such reductant loss or 'hydrogen escape' could have facilitated the irreversible oxidation of Earth's surface environment [5]. Although methanogenesis does not directly produce free O_2, it may have helped make Earth habitable for aerobic life.

Contemporary methanogens are microorganisms that form methane as a major product of their energy metabolism [7,8]. They share several features: (i) they are all Archaea and members of the Euryarchaeota phylum; (ii) they are obligate CH_4-producers and obtain all or most of their energy for growth from CH_4 production; (iii) they are obligate anaerobes and are restricted to strictly anoxic environments; and (iv) they utilize a limited number of substrates for methanogenesis, mostly $H_2 + CO_2$ or formate (hydrogenotrophic methanogens), methyl-containing C–1 compounds (methylotrophic methanogens) and acetate (aceticlastic methanogens) [7,8].

Hydrogenotrophic methanogens (including *Methanobacteriales*, *Methanococcales*, *Methanomicrobiales*, *Methanopyrales* and *Methanocellales*) appear to be more ancient than methylotrophic and aceticlastic methanogens (*Methanosarcinales*) [9]. Evidence that they retain some metabolic features resembling those that were common on the anoxic Earth include the following. (i) The utilization of H_2 as the major fuel for energy metabolism is considered an ancient trait. The predicted H_2 concentrations in an early anoxic atmosphere are on the order of 1000 ppm [10], which is well above the threshold for methanogenesis and the modern levels. Phylogenetic analyses of hydrogenases suggest that they were present in the last universal common ancestor (LUCA) [11]. (ii) Cytochromes and other proteins common in aerobic respiratory systems, which apparently evolved after the buildup of atmospheric O_2, are generally absent in hydrogenotrophic methanogens (except *Methanocellales*) [12]. (iii) Many enzymes involved in methanogenesis are extremely O_2-labile, which may have prevented them from adapting to oxidizing environments. (iv) Likewise, many biosynthetic enzymes are also very O_2-sensitive, including the CO dehydrogenase/acetyl-coenzyme A (CoA) synthase, pyruvate oxidoreductase and other ferredoxin-dependent oxidoreductases involved in CO_2 fixation [13], indolepyruvate oxidoreductase involved in aromatic amino acid biosynthesis [14], and acetohydroxy acid synthase and dihydroxy acid dehydratase involved in branched-chain amino acid biosynthesis [15,16]. Presumably, these O_2-sensitive enzymes represent forms that were common before the atmosphere became aerobic. (v) Methanogens also possess a large number of Fe–S cluster-containing proteins as indicated by a bioinformatics analysis of the frequency of the [4Fe–4S] binding motif [17] and by a direct measurement of the intracellular acid-labile sulfur and iron contents [18]. Because Fe–S clusters can spontaneously assemble in the presence of high amounts of iron and sulfide, which may have been abundant on the anoxic Earth, they may represent the earliest inorganic catalysts. In modern organisms, the presence of Fe–S clusters is strongly correlated with anaerobiosis [17]. Presumably, when aerobes evolved, they

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preferentially replaced Fe–S cluster enzymes with cluster-free enzymes that were more resistant to O_2. For example, pyruvate oxidoreductase would have been replaced by pyruvate dehydrogenase that reduces NAD^+ in aerobes [19]; and fumarases A and B would have been replaced by enzymes such as the cluster-free fumarase C in *Escherichia coli* that is used during oxidative stress [20]. (vi) Many hydrogenotrophic methanogens can grow autotrophically using CO_2 as the sole carbon source. They fix CO_2 via the modified reductive acetyl-CoA or Ljungdahl-Wood pathway [21], which is potentially an ancestral pathway in the Euryarchaeota [9]. Although controversial, a chemolithoautotrophic origin of life has been proposed assuming that life started from hydrothermal vent systems with metal sulfide surfaces or compartments acting as catalysts [22,23]. In this scenario, the reductive acetyl-CoA pathway closely resembles the ancestral abiotic carbon fixation because of the extensive utilization of Fe–S clusters [24,25]. Furthermore, because this pathway produces acetyl-CoA, which can be converted to acetate with the formation of ATP, it intimately links carbon fixation and energy conservation. Therefore, the reductive acetyl-CoA pathway is an ideal route for primordial metabolism [26,27]. Overall, these ancestral traits of hydrogenotrophic methanogens suggest that they may have retained metabolic features of ancient anaerobic microorganisms. Therefore, studying the physiology and biochemistry of hydrogenotrophic methanogens may provide a living window into the biology of ancient microbial life.

Evolution of the sulfur cycle and modern sulfur assimilation processes

Recent geochemical evidence suggests that the ancient and modern sulfur cycles may have been fundamentally different (reviewed in [28–30]). These studies take advantage of the isotopic fractionations of sulfur by biochemical reactions that discriminate against the heavy isotopes of sulfur (^{34}S) and result in mass-dependent fractionation of sulfur isotopes. The sedimentary sulfides before 2.4 Ga are much less depleted in ^{34}S when compared with minerals formed afterwards, suggesting that a profound and persistent reorganization of the global sulfur cycle occurred along with the rise of atmospheric O_2 [31–34]. This reorganization is highlighted by two features. First, photochemical reactions, reflected by mass-independent fractionation (MIF) of sulfur isotopes, played major roles in the Archean and early Proterozoic sulfur cycle. By contrast, biological oxidation and reduction of sulfur became geochemically more important later on. Second, the oceanic sulfate concentration was less than 200 μM (less than 1% of the present concentration of 28 mM) before 2.4 Ga [34]. Presumably, the Great Oxidation Event led to the increase of the oceanic sulfate pool and the subsequent onset of widespread bacterial sulfate reduction. In agreement with the isotopic record, the phylogenetic analyses based upon whole genome sequences also estimate that the modern lineages of sulfate-reducing bacteria and archaea originated after the rise of O_2, at about 2.3–2.4 Ga [9,35,36].

In a mechanism similar to microbial dissimilatory sulfate reduction, the eight electron reduction from sulfate to sulfide is the most common and widespread process for modern sulfur assimilation. In plants and most microorganisms, sulfate is taken up by membrane-bound proteins, activated with ATP to form adenosine-5′-phosphosulfate (APS), further activated with another ATP to form 3′-phosphoadenylsulfate (PAPS), reduced to sulfite with thioredoxin as the electron donor, and finally reduced to sulfide with NADPH as the electron donor [37]. Sulfide is then incorporated into L-cysteine, which serves as a central organic sulfur donor for the biosynthesis of L-methionine, glutathione, Fe–S clusters, CoA, lipoic acid, thiamine, molybdopterin, thionucleotides and other organic sulfur compounds (Figure 1) [38,39]. Given the limited availability of sulfate on the anoxic Earth, the relatively late evolution of the sulfate reduction pathway, and the significant consumption of energy to assimilate sulfate, the sulfate assimilation pathways that are common and widespread now were not necessarily so in the past.

Sulfur metabolism in methanogens

Sulfur sources for methanogens

In contrast to most other microbes, methanogens are generally unable to assimilate sulfate; instead, all known methanogens can use sulfide as the sole sulfur source, which may have been abundant on the early anoxic Earth. Moreover, members of the *Methanococcales* usually inhabit anaerobic environments with high levels of sulfide and are normally cultivated with 3–5 mM Na_2S [40,41]. Under neutral pH, a third of the sulfide remains in the non-ionized form (H_2S) and may freely diffuse across cell membranes. Therefore, the intracellular sulfide concentrations of methanococci are likely to be in the millimolar range [18]. Methanococci are adapted to growth with high levels of sulfide because they lack many targets for sulfide toxicity, such as the α,β-unsaturated fatty acid biosynthetic intermediates and cytochromes.

Most methanogens also use S^0 as a sulfur source [42]. In the anoxic and geothermally-influenced marine environments of the Archean Earth, S^0 may have been the dominant sulfur species, and sulfur reduction may represent a very early means of energy conservation [43]. Many methanogens produce high levels of sulfide from S^0 in the presence of H_2 [42,44]. Although abiotic S^0 disproportionation at high temperatures (>80 °C) can produce significant amounts of H_2S [45], the rates of H_2S production in *Methanothermococcus thermolithotrophicus* cultures at 65 °C (~5 μmol/ml/h) are more than three orders of magnitude higher than those of the abiotic reaction [42]. Similarly, mesophilic methanogens also reduce S^0 at well above the abiotic rate. Therefore, methanogens reduce S^0 enzymatically with H_2 serving as the ultimate electron donor. Although this reduction is exergonic, it does not support the growth of methanogens. In fact, S^0 inhibits both growth and methanogenesis, presumably from the accumulation of large quantities of H_2S [42,46]. Although the S^0 reductase activity has been identified in all of the major groups of methanogens [42,44], the enzyme catalyzing S^0 reduction has not yet been elucidated.

Some methanogen species (e.g. *Methanocaldococcus jannaschii*, *Methanothermococcus thermolithotrophicus*, *Methanothermobacter thermautotrophicus*, *Methanobrevibacter ruminantium* and *Methanosarcina barkeri*) can also

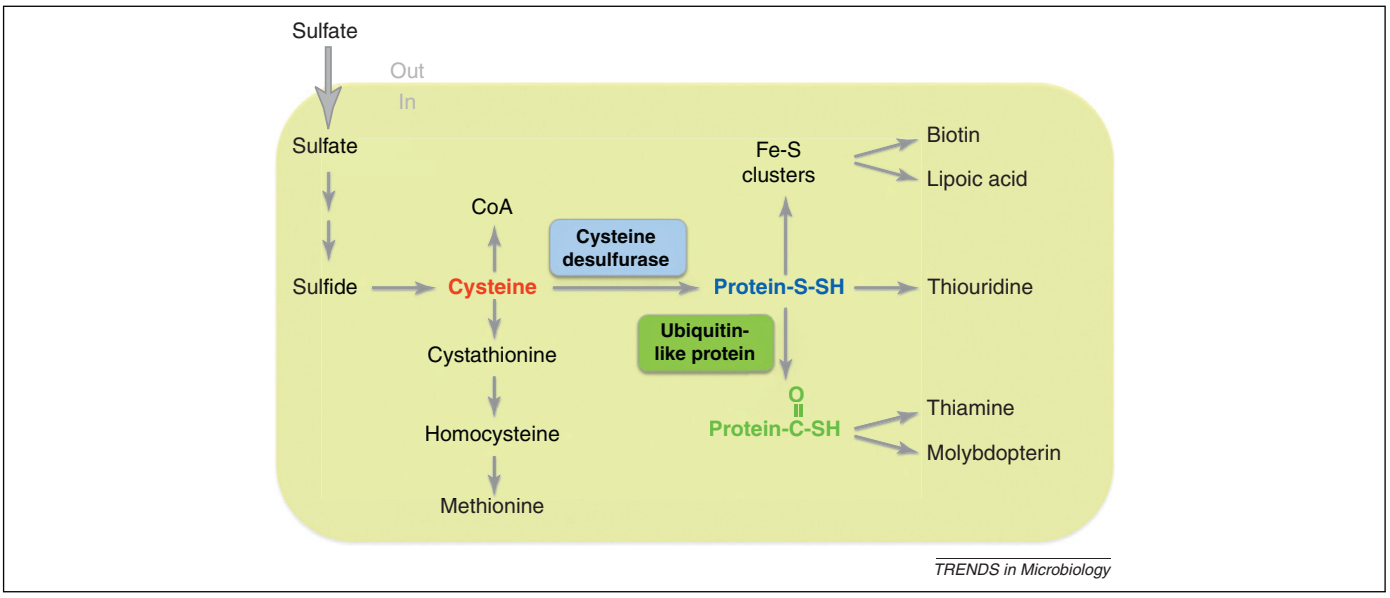

Figure 1. Sulfur assimilation scheme in *Escherichia coli*. Sulfate is transported into the cell and then reduced to sulfide. Sulfide reacts with *O*-acetylserine to generate cysteine, which serves as the central sulfur donor for the biosynthesis of other sulfur compounds. For methionine biosynthesis, cysteine reacts with *O*-succinylhomoserine to produce cystathionine, which is then cleaved to homocysteine and pyruvate. Homocysteine then receives a methyl group from N^5-methyltetrahdrofolate to generate methionine. For the biosynthesis of other sulfur compounds, cysteine donates sulfur to a conserved Cys residue of cysteine desulfurase to generate a persulfide group, which then functions as the proximal sulfur donor for the biosynthesis of Fe–S clusters and thionucleotides (i.e. 4-thiouridine and 2-thiouridine in tRNAs). Fe–S clusters function as the sulfur donor for the biosynthesis of biotin and lipoic acid. For the biosynthesis of some vitamins, the persulfide group of cysteine desulfurase donates sulfur to the C-terminal Gly of ubiquitin-like proteins to generate a thiocarboxylate group, which functions as the proximal sulfur donor for the biosynthesis of thiamine and molybdopterin. Abbreviation: CoA, coenzyme A.

use sulfite or thiosulfate as alternative sulfur sources [47,48]. These sulfur species were probably common on the anoxic Earth, but their role in ancient metabolism is unclear [43]. Thiosulfate and/or sulfite reductase activities have been studied in some methanogens [44], and two types of sulfite reductase have been characterized. The P590 sulfite reductase was isolated from *M. barkeri*. It is a 23-kDa protein that contains one Fe-siroheme and one [4Fe–4S] cluster per polypeptide chain [49,50]. P590 reduces sulfite to sulfide *in vitro* using the artificial electron donor methyl viologen. The physiological significance of this protein has not been determined. The Fsr sulfite reductase was first isolated from *Methanocaldococcus jannaschii*, and its homologs have been found in *Methanopyrus kandleri* and *M. thermautotrophicus*. Fsr is a 70-kDa protein with an N-terminal H_2F_{420} dehydrogenase domain and a C-terminal dissimilatory-type siroheme sulfite reductase domain [51]. The electron donor is reduced coenzyme F_{420} (H_2F_{420}), which is an abundant deazaflavin in methanogens. Heterologous expression of Fsr in *Methanococcus maripaludis* allows this organism to use sulfite as a sole sulfur source [52].

Several additional sulfur compounds (e.g. sulfate, Cys, Met and mercaptans) can be assimilated by some methanogen species, although utilization of these sulfur sources is not a widespread attribute among methanogens. Only *M. thermolithotrophicus* and *M. ruminantium* are able to use sulfate as a sole sulfur source [47,48]. In the presence of low levels (20–30 μM) of sulfide, some strains of *M. thermolithotrophicus*, *M. jannaschii* and *M. thermautotrophicus* are able to assimilate methanethiol, ethanethiol, *n*-propanethiol, *n*-butanethiol, methyl sulfide, dimethyl sulfoxide, ethyl sulfide or CS_2 [48]. These sulfur compounds are presumably converted to sulfide for biosynthesis, although the mechanism and enzymes involved are not known.

Biosynthesis of sulfur-containing compounds: a continuing puzzle

Methanogenic archaea synthesize a wide variety of organic sulfur compounds. In addition to the sulfur-containing amino acids Cys and Met, sulfur is also present in cofactors and vitamins such as Fe–S clusters, thiamine, molybdopterin, CoA and biotin. Methanogens also have two thiol coenzymes used in the methanogenesis pathway, coenzyme M (CoM) and coenzyme B (CoB). The cellular levels of some of the sulfur-containing compounds in *Methanococcus maripaludis* are listed in Table 1. Although genomic sequences and biochemical investigations (mostly performed with *M. maripaludis* as the model organism) have provided clues to their biosynthesis, how sulfur is incorporated into these compounds is still an enigma.

Methanogens use a unique tRNA-dependent pathway for *de novo* Cys biosynthesis (Figure 2). Previous studies showed that the canonical Cys-tRNA synthetase (CysRS)

Table 1. Levels of selected sulfur compounds in *Methanococcus maripaludis* and *Escherichia coli*

	Amount in the cell (nmol/mg protein)	
Chemicals	***M. maripaludis***	***E. coli***
Inorganic sulfide	45 ± 9[a]	2.5 ± 0.4[a]
Cys residues	102 ± 7[a]	158[b]
Met residues	202 ± 11[a]	265[b]
Coenzyme M[c]	0.7 ± 0.1[a]	–[d]

[a]Values from [18].
[b]Values from [64].
[c]This value represents the intracellular free coenzyme M pool size.
[d]Absent in *E. coli*.

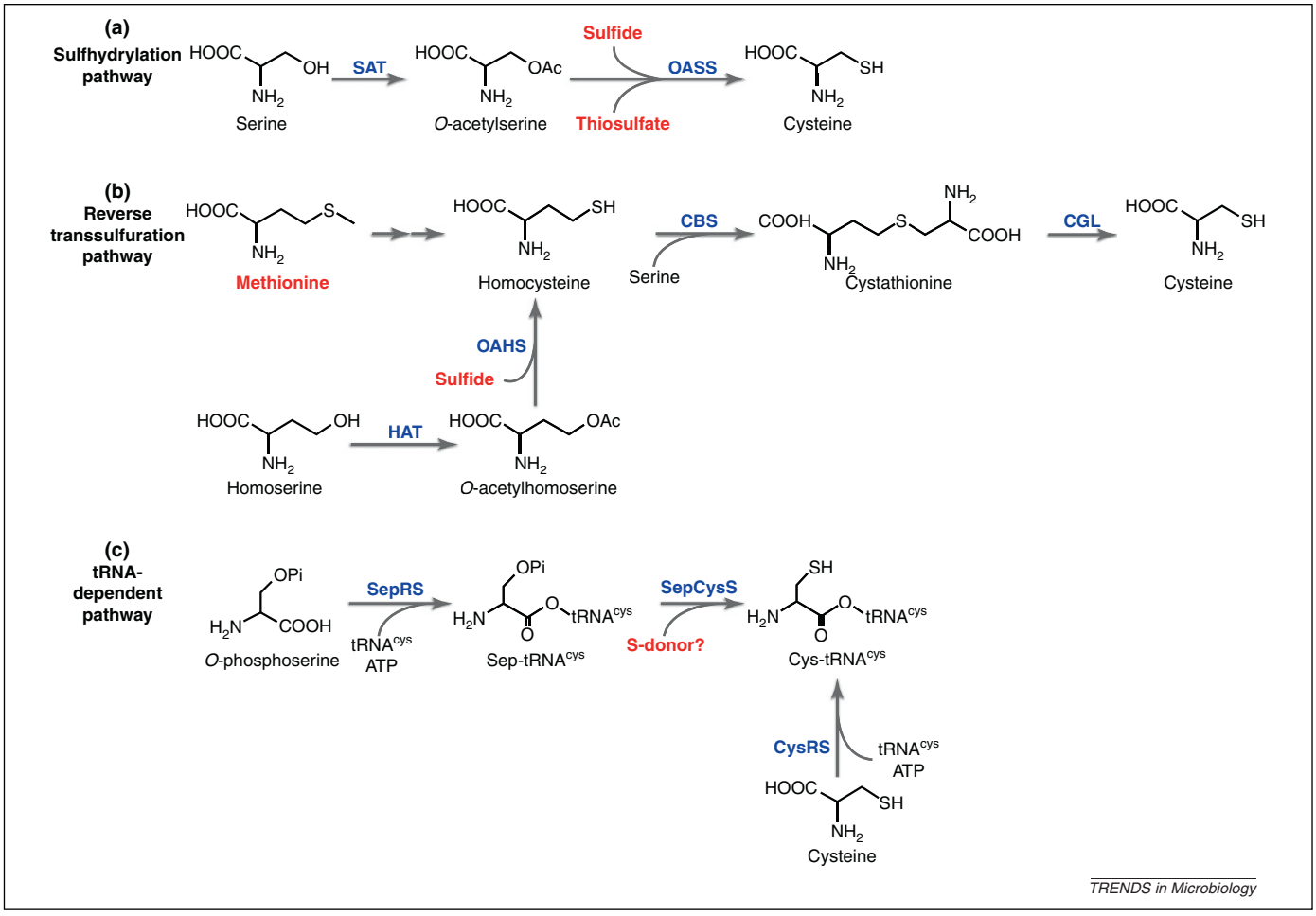

Figure 2. Cys biosynthesis pathways. **(a)** The Ser sulfhydrylation pathway is utilized by higher plants and many bacteria, such as enteric bacteria and actinobacteria. In this pathway, Ser is activated in the form of *O*-acetylserine and then converted to Cys by OASS. Most bacteria use sulfide as the direct sulfur donor; in enteric bacteria, either sulfide (under aerobic condition) or thiosulfate (under anaerobic condition) is the sulfur donor [65]. **(b)** The reverse transsulfuration pathway is utilized by mammals, *Saccharomyces cerevisiae*, and some bacteria, such as *Lactococcus lactis* [66] and *Pseudomonas putida* [67]. In this pathway, the intermediate cystathionine is synthesized from homocysteine and Ser by CBS and then converted to Cys by CGL. In mammals and some bacteria, the sulfur group in Cys is derived from Met, which is converted to homocysteine through the intermediate *S*-adenosylmethionine; in *S. cerevisiae*, the overall sulfur donor is sulfide, which reacts with *O*-acetylhomoserine to form homocysteine by OAHS [68]. **(c)** The tRNA-dependent pathway is utilized by methanogenic archaea. In this pathway, $tRNA^{Cys}$ is aminoacylated with *O*-phosphoserine (Sep) by SepRS, and then Sep-$tRNA^{Cys}$ is converted to Cys-$tRNA^{Cys}$ by SepCysS with an unknown sulfur donor [54]. Abbreviations: SAT, serine *O*-acetyltransferase; OASS, *O*-acetylserine sulfhydrylase; CBS, cystathionine β-synthase; CGL, cystathionine γ-lyase; HAT, homoserine *O*-acetyltransferase; OAHS, *O*-acetylhomoserine sulfhydrylase; SepRS, *O*-phosphoseryl-tRNA synthetase; SepCysS, Sep-tRNA:Cys-tRNA synthase; CysRS, cysteinyl-tRNA synthetase.

and other bacterial or eukaryotic enzymes involved in Cys biosynthesis are not encoded in the genomes of many hydrogenotrophic methanogens (Table 2); CysRS is present but nonessential in *M. maripaludis* [53]; and two non-canonical enzymes, *O*-phosphoseryl-tRNA synthetase (SepRS) and Sep-tRNA:Cys-tRNA synthase (SepCysS), are required for the generation of Cys-$tRNA^{Cys}$ [54]. These enzymes catalyze Cys biosynthesis in two steps. First, $tRNA^{Cys}$ is aminoacylated with *O*-phosphoserine (Sep) by SepRS. Next, the Sep moiety of Sep-$tRNA^{Cys}$ is converted to Cys with an unknown sulfur donor by SepCysS (Box 1). Many *Methanococcales* and thermophilic *Methanobacteriales* species contain only the tRNA-dependent pathway for *de novo* Cys biosynthesis (Table 2). Moreover, the deletion of SepRS (Δ*sepS*) in *M. maripaludis*, which also contains a canonical CysRS, resulted in Cys auxotrophy. Thus, the tRNA-dependent pathway is the primary pathway for Cys biosynthesis in methanococci [18,54]. However, the *Methanosarcinales* and the *Methanomicrobiales* species also possess either the bacterial or the eukaryotic enzymes for Cys biosynthesis in addition to the tRNA-dependent pathway (Table 2); the redundancy of multiple Cys biosynthesis pathways in these organisms has not been investigated.

Phylogenetic analyses suggest that the tRNA-dependent Cys biosynthesis pathway is ancient and was already present at the time of LUCA [55]. Given that Cys is a relatively late addition to the genetic code [56], the indirect charging of $tRNA^{Cys}$ (aminoacylation with Sep and then conversion to Cys) may represent an ancient route of Cys-$tRNA^{Cys}$ formation that evolved before the LUCA. Similarly, it is likely that CysRS was absent in the ancestor of the archaea and eukaryotes and was horizontally transferred from bacteria through multiple independent events [55,57,58]. Thus, CysRS and the biosynthesis of free cysteine were also probably absent in LUCA. Homologs of SepRS and SepCysS always coexist and are encoded in almost all sequenced contemporary methanogens (except *Methanosphaera* and *Methanobrevibacter* species) and *Archaeoglobus* species, which are sulfate-reducing archaea that possess many enzymes of the methanogenesis pathway. The selection pressures for methanogens to retain the tRNA-dependent Cys biosynthesis pathway and for non-methanogenic

Table 2. Presence (+) or absence (−) of Cys biosynthesis enzymes in methanogens[a]

Organism	OASS/CBS[b]	CGL/OAHS[c]	CysRS[d]	SepRS[e]	SepCysS[f g]
Methanobacteriales					
Methanothermobacter thermautotrophicus	−	−	−	+	+
Methanobrevibacter smithii	+	+	+	−	−
Methanosphaera stadtmanae	+	+	+	−	−
Methanococcales					
Methanocaldococcus jannaschii	−	−	−	+	+
Methanococcus aeolicus	−	−	+	+	+
Methanococcus maripaludis	−	−	+	+	+
Methanococcus vannielii	+	+	+	+	+
Methanococcus voltae	−	−	+	+	+
Methanomicrobiales					
Methanocorpusculum labreanum	−	+	−	+	+
Methanoculleus marisnigri	+	−	−	+	+
Methanospirillum hungatei	+	+	+	+	+
Candidatus 'Methanoregula boonei'	+	+	+	+	+
Candidatus 'Methanosphaerula palustris'	+	+	+	+	+
Methanosarcinales					
Methanococcoides burtonii	+	+	−	+	+
Methanosarcina acetivorans	+	+	+	+	+
Methanosarcina barkeri	+	+	+	+	+
Methanosarcina mazei	−	+	+	+	+
Methanosaeta thermophila	−	−	−	+	+
Methanopyrales					
Methanopyrus kandleri	−	−	−	+	+
Methanocellales					
Methanocella sp. RC-I	−	+	+	+	+

[a]The shaded area corresponds to the methanogens missing gene homologs for both the sulfhydrylation and the transsulfuration Cys biosynthetic enzymes.

[b]OASS and CBS are closely related enzymes, and homology searches do not differentiate between them. Homologs were identified using BLASTp with *E. coli* OASS (CysK, b2414; CysM, b2421) and *Saccharomyces cerevisiae* CBS (YGR155W) as queries.

[c]CGL, OAHS and CGS are closely related enzymes, and homology searches do not differentiate between them. Homologs were identified using BLASTp with *S. cerevisiae* CGL (YAL012W) and OAHS (YLR303W) as queries.

[d]CysRS homologs were determined using BLASTp with *M. maripaludis* CysRS (MMP1060) as the query.

[e]SepRS homologs were determined using BLASTp with *M. maripaludis* SepRS (MMP0688) as the query.

[f]SepCysS homologs homologs were determined using BLASTp with *M. maripaludis* SepCyS (MMP1240) as the query.

[g]Abbreviations: OASS, *O*-acetylserine sulfhydrylase; CBS, cystathionine β-synthase; CGL, cystathionine γ-lyase; OAHS, *O*-acetylhomoserine sulfhydrylase; CGS, cystathionine γ-synthase, CysRS, cysteinyl-tRNA synthetase, SepRS, *O*-phosphoseryl-tRNA synthetase; SepCysS, Sep-tRNA:Cys-tRNA synthase.

Box 1. Sulfur donor for tRNA-dependent Cys biosynthesis in methanogens

The nature of the physiological sulfur donor for the tRNA-dependent Cys biosynthesis remains unknown. Sodium sulfide, thiophosphate or Cys can function as sulfur donors *in vitro*, and sodium sulfide provides the highest activity [54,69]. However, the catalytic efficiency (k_{cat}/K_m) of SepCysS with sulfide is about 500-fold lower than that of SepRS for phosphoserylation of $tRNA^{Cys}$; therefore, sulfide is unlikely to be the sulfur donor *in vivo* [69].

Although the sulfur delivery mechanism of SepCysS remains unclear, a persulfide group is proposed to be formed on SepCysS, mainly based upon the fact that SepCysS is closely related to cysteine desulfurase in terms of primary sequence [55] and overall structure [72]. Cysteine desulfurase catalyzes sulfur transfer by mobilizing the thiol group of free Cys, yielding a persulfide enzyme adduct and Ala [38]. The persulfidic sulfur (R–S–SH), which is covalently linked to a conserved Cys residue of cysteine desulfurase, is then donated to other sulfur carrier proteins for the biosynthesis of Fe–S clusters, thionucleotides or sulfur-containing vitamins [39]. In analogy to cysteine desulfurase, SepCysS may mobilize sulfur from a sulfur source, form a persulfide group on a Cys residue and then donate the sulfur to Sep-$tRNA^{Cys}$ to generate Cys-$tRNA^{Cys}$. Indeed, three conserved Cys residues are located at the active site of SepCysS, and two of them are essential for forming a persulfide [70]. If the persulfide group of SepCysS functions as the sulfur donor for Cys biosynthesis, then it needs to be generated with a free Cys-independent approach (Figure 3).

archaea to replace it with the bacterial pathways are unclear. Interestingly, methanogens and *Archaeoglobus* possess high Cys content (an average of ~1.3% of the entire proteome according to genomic sequences) when compared to non-methanogenic archaea (~0.7%) [59]. Therefore, the loss of the tRNA-dependent Cys biosynthesis pathway in non-methanogenic archaeal lineages may have correlated with a reduction in Cys contents.

The tRNA-dependent Cys biosynthetic pathway raises the following questions: (i) is an intracellular free Cys pool present in methanogens; and (ii) if so, can Cys donate sulfur for the biosynthesis of other sulfur-containing compounds as in bacteria and eukaryotes? To answer the first question, the intracellular free Cys was quantified in *M. maripaludis*. Although the free Cys pool size in this organism (~20 μM) is approximately 5–10 times lower than normally observed in bacteria (100–200 μM) [18], the involvement of free Cys in the biosynthesis of sulfur-containing compounds cannot be excluded. The free Cys is probably derived from protein degradation, Cys-$tRNA^{Cys}$ hydrolysis and/or other trivial pathways of Cys biosynthesis. To answer the second question, the *M. maripaludis* Δ*sepS* mutant strain, which cannot synthesize Cys *de novo*, was grown with ^{34}S-labeled sulfide and unlabeled Cys, and

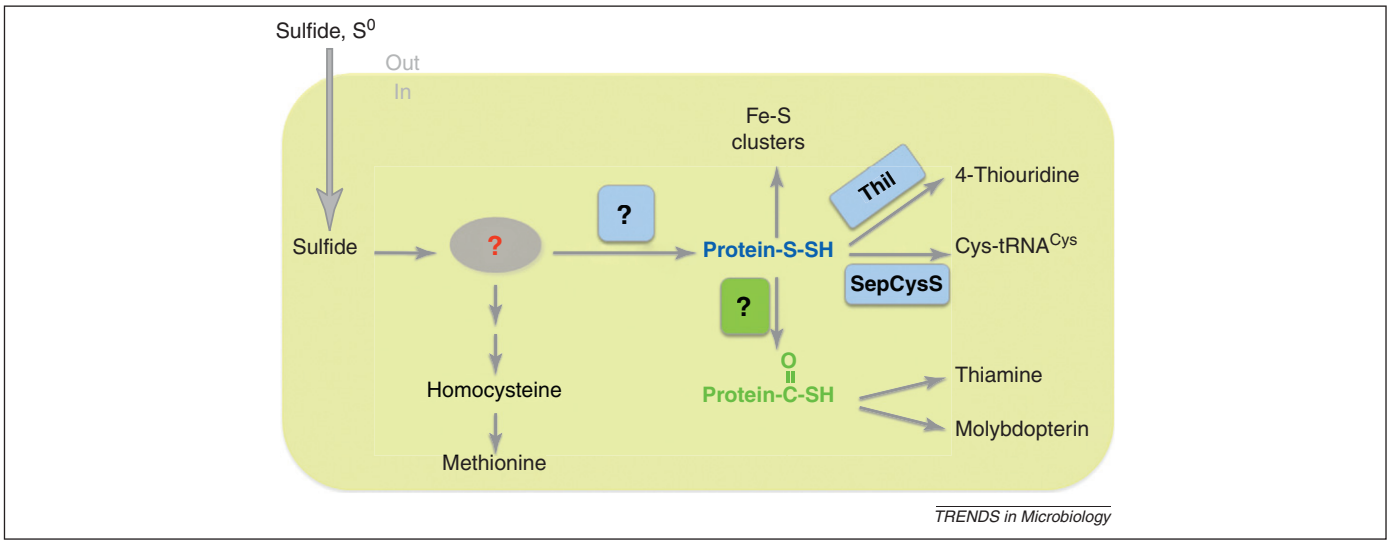

Figure 3. A proposed model for sulfur assimilation in methanogens. Sulfide is generally used as the sole sulfur source for growth. For Cys biosynthesis, a persulfide (with one sulfane sulfur) group on Sep-tRNA:Cys-tRNA synthase (SepCysS) is likely to be the proximal sulfur donor for the conversion of Sep-tRNA^{Cys} → Cys-tRNA^{Cys} [69,70]. However, the processes of sulfide oxidation to sulfane sulfur and sulfane sulfur transfer to SepCysS are not known. For Met biosynthesis, L-homocysteine is probably an intermediate [18]. Methionine synthase, which catalyzes the methylation of homocysteine to form Met, is conserved in methanogens. Although two activities leading to homocysteine (i.e. sulfhydrylation of *O*-phosphohomoserine [71] and β-cleavage of cystathionine [18]) have been proposed in methanococci, the discovery of the complete pathway requires further investigation. For Fe–S cluster biosynthesis, Cys does not serve as a sulfur donor. Whether sulfur-carrier protein(s) are involved in Fe–S cluster assembly remains to be understood. For tRNA thiolation, Thil, the enzyme that catalyzes the sulfur incorporation into 4-thiourindine through a persulfide intermediate, is conserved in methanogens.

then the distribution of S isotopes was examined in Fe–S clusters and Met [18]. As a result, Fe–S clusters and Met were highly enriched with ^{34}S, indicating that Cys is not the sulfur donor for Fe–S cluster and Met biosynthesis in methanogenic archaea [18]. This observation suggested that, in addition to the unusual tRNA-dependent Cys biosynthesis pathway, methanogens also use unique pathways for sulfur incorporation into other sulfur compounds (Figure 3).

Iron–sulfur proteins are abundant in methanogens [18], and methanogen genomes encode more Fe–S cluster motifs per open reading frame (ORF) than most other archaea or bacteria [17]. However, the Fe–S cluster biosynthesis pathway in methanogens is not yet known. Only three proteins of the known Fe–S cluster assembly and transport machineries are conserved in all methanogens: SufB, a scaffold protein for Fe–S cluster assembly; SufC, an ABC-type ATPase that might facilitate the transfer of Fe–S clusters from scaffold proteins to target proteins; and ApbC/Nbp35, a carrier protein that could be involved in Fe–S cluster transport [60]. *E. coli* possesses the ISC (iron–sulfur cluster) and the SUF (mobilization of sulfur) machineries for Fe–S cluster assembly; the SUF machinery is activated with oxidative or heavy metal stress or iron deficiency [61,62], suggesting that SUF acts primarily for Fe–S cluster repair in *E. coli*. In some other microorganisms including mycobacteria and cyanobacteria, SUF is the sole machinery for Fe–S cluster assembly, suggesting that SUF is fully functional for Fe–S cluster biosynthesis. Although SufB and SufC are conserved in archaea, many methanogens lack a homolog of the cysteine desulfurase (SufS) [18], which is the key enzyme for the mobilization and transfer of sulfur from Cys to scaffold proteins (Figure 1) [63]. The absence of SufS and the remaining SUF components (SufE, SufU and SufA) suggests that methanogens utilize unknown biosynthetic enzymes or exploit known enzymes with novel functions for the biosynthesis of Fe–S clusters.

Concluding remarks

Sulfur is an essential element required for the growth of all known organisms and is present in a wide variety of molecules with distinctive physiological functions. Although genomic sequences of methanogens provide a general idea about their metabolism, many essential enzymes of established sulfur assimilation pathways are missing. Recent studies indicated the presence of a tRNA-dependent pathway for Cys biosynthesis in methanogens, but how sulfur is incorporated into Cys is still unknown. Moreover, the biosynthesis of other sulfur compounds, e.g. Fe–S clusters and Met, remains to be elucidated. Box 2 lists questions that address a few of the many possible avenues for future research. Given the antiquity of methanogenesis and the obligately anaerobic lifestyle of

Box 2. Outstanding questions

- How do methanogens assimilate S^0 as a sulfur source for growth? What are the enzymes responsible for S^0 reduction? What is the physiological and ecological significance of S^0 reduction by methanogens?
- How do methanogens regulate sulfur metabolism in response to the speciation and availability of sulfur sources?
- Why do only methanogens retain the tRNA-dependent Cys biosynthesis pathway? Is this pathway redundant in methanogens that also contain the bacterial Cys biosynthesis pathways?
- What is the sulfur donor for the tRNA-dependent Cys biosynthesis? What is the reaction mechanism of SepCysS, the enzyme converting Sep-tRNA^{Cys} to Cys-tRNA^{Cys}?
- How do methanogens synthesize Fe–S clusters and other sulfur-containing cofactors in the absence of cysteine desulfurase?
- How do methanogens synthesize Met?

methanogens, studying the sulfur metabolism of methanogens may lead to a better understanding of ancient sulfur metabolism.

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