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# **Production of methanethiol and volatile sulfur compounds by the archaeon “*Ferroplasma acidarmanus*”**

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## **Abstract**

Acidophiles are typically isolated from sulfaterich ecological niches yet the role of sulfur metabolism in their growth and survival is poorly defined. Studies of heterotrophically grown “*Ferroplasma acidarmanus*” showed that its growth requires a minimum of 100 mM of a sulfate-containing salt. Headspace gas analyses by GC/ MS determined that the volatile sulfur compound emitted by active “*F. acidarmanus*” cultures is methanethiol. In “*F. acidarmanus*” cultures grown either heterotrophically or chemolithotrophically, methanethiol was produced constitutively. Radiotracer studies with <sup>35</sup>S-labeled methionine, cysteine, and sulfate showed that all three were used in methanethiol production. Additionally, <sup>3</sup>H-labeled methionine was incorporated into methanethiol and was probably used as a methyl-group donor. Methanethiol production in whole cell lysates supplied with SO<sub>3</sub><sup>2-</sup> indicated that NADPH-dependant sulfite reductase and methyltransferase activities were present. Cell lysates also contained enzymatic activity for methionine-c-lyase that cleaved the side chain of either methionine to form methanethiol or cysteine to produce H<sub>2</sub>S. Since methanethiol was detected from the degradation of cysteine, it is likely that sulfide was methylated by a thiol methyltransferase. Collectively, these data demonstrate that “*F. acidarmanus*” produces methanethiol through the metabolism of methionine, cysteine, or sulfate. This is the first report of a methanethiol-producing acidophile, thus identifying a new contributor to the global sulfur cycle.

**Keywords:** Cysteine, Methanethiol, Methionine, Sulfate, Sulfite reductase, Thiol methyltransferase

## **Abbreviations**

APS Adenosine 50-phosphosulfate reductase  
CGS Cystathionine-c-synthase  
CPB Citrate phosphate buffer  
dH<sub>2</sub>O Deionized water  
DMDS Dimethyldisulfide  
Dsr Dissimilatory sulfite reductase  
DTT Dithiothreitol  
GC Gas chromatography  
mfer Chemolithotrophic growth medium for “*Ferroplasma acidarmanus*” strain fer1  
LSC Liquid scintillation counting  
MGL Methionine-c-lyase  
MPN Most probable number  
MT Methanethiol  
NoS No sulfate growth medium (6 N HCl was used as acidulant and the only source of sulfate was from

added Na<sub>2</sub>SO<sub>4</sub> or MgSO<sub>4</sub>)  
ORFs Open reading frames  
PAPS Phosphoadenosine 50-phosphosulfate reductase  
PFPD Pulsed flame photometric detector  
PLP Pyridoxal 50-phosphate hydrate  
PTFE Polytetrafluoroethylene  
SAM S-adenosyl-L-methionine  
TCA Trichloroacetic acid  
TMT Thiol methyltransferase  
VOSC Volatile organic sulfur compound  
VSC Volatile sulfur compound

### **Introduction**

Sulfur is one of the more common elements in the biosphere that is essential to life. It is present in the amino acids cysteine and methionine, some vitamins, enzymes, and coenzymes. Furthermore, sulfide is essential to iron-sulfur (Fe-S) clusters that are present in metalloproteins, like ferredoxin and cytochrome C. Sulfate (SO<sub>4</sub><sup>2-</sup>), the most oxidized form of sulfur, is utilized as a sulfur source by plants, fungi, and prokaryotes through the assimilatory sulfate reduction pathway. In comparison, sulfate-reducing bacteria generate energy from the reduction of sulfate to H<sub>2</sub>S by the dissimilatory sulfate reduction pathway while other prokaryotes can utilize reduced forms of sulfur as an energy source. Dissimilatory sulfate-reducing microorganisms have a high demand for sulfate, with freshwater bacteria requiring SO<sub>4</sub><sup>2-</sup> levels ranging from 100–250 μM, and marine microorganisms needing ~25 mM (Nealson 1997). Although not typically mentioned, acidic biotopes have the highest sulfate concentrations on Earth yet the metabolism and production of sulfur compounds by organisms inhabiting these sites have not been thoroughly investigated.

The Richmond mine in Iron Mountain, CA contains acidic waters with some of the lowest pH (–3.7) values ever reported (Nordstrom and Alpers 1999). The acidic waters of the Richmond mine have reported sulfate concentrations ranging from 657–786 mM (Druschel et al. 2004). This site has been the subject of recent research that resulted in the isolation of unique archaeal, bacterial, and eukaryotic organisms (Edwards et al. 2000; Tyson et al. 2005; Baker et al. 2003; Baker et al. 2004). Archaea are of particular interest because of their relatively recent description and placement in a separate phylogenetic domain. Many archaea are extremophiles but others are found in an array of environments including the ocean where they have been found in high numbers (Giovannoni and Stingl 2005). Due to their presence in extreme environments and unidentified nutritional requirements, basic molecular tools and culture methods are lacking for many archaeal species, which has hampered progress on the studies of these organisms. Of the isolates from the Richmond mine, the archaeon “*F. acidarmanus*” stands out as an attractive candidate for the study of sulfur metabolism because it has an unusually high (~100 mM) sulfate requirement for growth (Baumler et al. 2005), and its genome (~1.94 Mb) has been sequenced (Allen et al. 2007). A metabolic reconstruction of a “*F. acidarmanus*” cell model based on genome annotation predicts that SO<sub>4</sub><sup>2-</sup> is transported into the cytoplasm but its role in metabolism is unknown (Allen et al. 2007).

Additionally, Ferrer et al. (2007) revealed that *F. acidiphilum* has metabolic machinery that is dominated by iron-containing proteins, many with Fe–S centers, that adds to the body of information on the importance of sulfur and sulfur metabolism in *Ferroplasma* species and the appropriateness of studying sulfur metabolism in “*F. acidarmanus*”.

Initial studies on the production of volatile sulfur gases by “*F. acidarmanus*” were conducted because of the foul odor associated with cultures. The headspace gases were directed into a zinc acetate trap where a white precipitate formed. Analysis of the precipitate for sulfide by the formation of methylene blue (Cline 1969) was negative but ion coupled plasma-optical emission spectroscopy detected the presence of zinc and sulfur (Baumler et al. 2005). This was the first report of the production of a volatile sulfur compound (VSC) by a *Ferroplasma* spp. In the current study the VSC produced by “*F. acidarmanus*” was identified as the volatile organic sulfur compound (VOSC) methanethiol (MT). Dimethyldisulfide (DMDS) was also detected and is likely the dimerized form of MT that is produced in the presence of oxygen (Lomans et al. 2002; Nordmann et al. 1994). <sup>35</sup>S- and <sup>3</sup>H-labeled substrates (methionine, cysteine, and sulfate) as well as enzyme assays with cell lysates were used to determine precursors and probable intermediates involved in the MT production pathway. Since VOSCs surpass H<sub>2</sub>S as the major biogenic sulfur carriers to the atmosphere (Kelly et al. 1994; Kiene 1996), this study identified a new contributor to the biogeochemical cycling of sulfur in one of the most sulfur-rich ecological niches on Earth.

## **Materials and methods**

### Strains and growth conditions

*Archaeoglobus fulgidus*-ATCC 49558 was grown anaerobically in 1775 *Archaeoglobus* medium (DSM 399) with dithiothreitol (Sigma, St. Louis, MO) used as reductant. “*F. acidarmanus*” strain fer1 was grown in either chemolithotrophic mfer medium (Baumler et al. 2005) or in a newly defined medium termed NoS (No Sulfate, 6N HCl was used as acidulant and the only source of sulfate was from added Na<sub>2</sub>SO<sub>4</sub> or MgSO<sub>4</sub>). Freshly prepared mfer was inoculated as described (Baumler et al. 2005), while NoS medium was inoculated with a 14-day culture of “*F. acidarmanus*” grown in NoS. All growth studies were conducted with static cultures [10% (v/v)] at 37°C. Growth measurements were determined spectrophotometrically (OD<sub>492</sub>), and viable cell counts were determined using a most probable number (MPN) technique in mfer medium as previously described (Baumler et al. 2005).

### **Media composition and preparation**

For chemolithotrophic growth studies, mfer medium was prepared as previously described (Baumler et al. 2005). For heterotrophic growth, NoS medium was comprised of the following per liter: Na<sub>2</sub>SO<sub>4</sub>, 350 mM; NH<sub>4</sub>Cl, 10 mM; NiCl<sub>2</sub>, 2 mM; KH<sub>2</sub>PO<sub>4</sub>, 3 mM; MgCl<sub>2</sub>, 0.65 mM; MnCl<sub>2</sub>, 0.05 mM; ZnCl<sub>2</sub>, 0.51 mM; CoCl<sub>2</sub>, 0.04 mM; H<sub>3</sub>BO<sub>3</sub>, 0.5 mM; Na<sub>2</sub>MoO<sub>4</sub>, 0.35 mM; and 0.1% (w/v) yeast extract

(Becton Dickinson, Sparks, MD). Chemicals were obtained from Sigma/Aldrich chemical company except KH<sub>2</sub>PO<sub>4</sub> (Mallinckrodt Inc., Hazelwood, MO), and H<sub>3</sub>BO<sub>3</sub> (EM Science, Gibbstown, NJ).

NoS medium was adjusted to pH 1.0 using concentrated HCl (6 N), and prepared by adding ingredients to deionized water (dH<sub>2</sub>O), except Na<sub>2</sub>SO<sub>4</sub> and yeast extract, and adjusted to a final volume of 1 l with dH<sub>2</sub>O (basal salts). The basal salts solution and yeast extract concentrate (10% w/v) were sterilized at 121 °C for 25 min at 17 psi. To eliminate precipitate that results from the acidification of yeast extract, Na<sub>2</sub>SO<sub>4</sub> was added to the yeast extract solution (10 ml), adjusted to the appropriate pH, then added to 190 ml of the autoclaved basal salts solution, and filtered through a 0.2-µm pore membrane filter (Fisher). This filtered yeast extract and Na<sub>2</sub>SO<sub>4</sub> solution (200 ml) was added to 800 ml of sterile basal salts solutions to make the completed NoS medium. All pH measurements were made as previously described (Macalady et al. 2004).

#### Headspace analysis by gas chromatography/mass spectroscopy

Headspace gas (250 µl) was sampled from either *A. fulgidus* (3 days) or “*F. acidarmanus*” (7 or 14 days) cultures grown in mfer in 125 ml headspace vials (Wheaton, Millville, NJ) with a gas-tight syringe (Hamilton Company, Reno, NV) and analyzed by GC/MS (Hewlett Packard 6890 gas chromatograph connected to an Agilent 5973 network mass detector). The instrument was under the control of Chemstation software, and analyses conducted in selected ion monitoring mode programmed for all known biotically produced VOSCs (Lomans et al. 2002). A Supelco Supel-Q Plot column (30 m · 0.32 mm) was used and running conditions were 50 °C (1 min) to 230 °C at a rate of 10 °C/min. The injector temperature was 250 °C. Helium was used as a carrier gas at a flow rate of 3 ml/min. The mass spectrometer operated in the electron ionization mode (70 eV) at a source temperature of 250 °C.

#### Gas chromatography, pulsed flame photometric detection of MT

Headspace gas (250 µl) from cultures was extracted using a gas-tight syringe and analyzed using a GC-PFPD (Varian, Walnut Creek, CA). A CP8510 fused silica capillary column (15 m · 0.25 mm, 1.0 µm film, Varian) was used with the following running conditions: 50 °C (1 min) to 230 °C at a rate of 10 °C/min. The injector was operated in a splitless mode with a temperature of 250 °C. Helium was used as the carrier gas at a flow rate of 3 ml/min. The pulsed flame photometric detector (PFPD) was held at 240 °C with the following flow rates: air 1 at 18 ml/min and H<sub>2</sub> at 14 ml/min, and air 2 at 10 ml/min. Detector response signals were integrated using computer software (Star Workstation 6.2, Varian). MT and DMDS were quantified by comparison to gas standards (Sigma). A standard curve was generated using methanethiol-nitrogen calibration mixtures in Tedlar gas-sampling bags (SKC Inc., Eighty Four, PA). Mean values from three independent trials are reported with error bars representing the standard error of the means.

#### 35S and 3H studies

In 3H and 35S studies, cultures of “*F. acidarmanus*” were grown heterotrophically in 50 ml NoS containing 350 mM Na<sub>2</sub>SO<sub>4</sub> and harvested by centrifugation (10 min, 12,200·g at 21°C) following 7 days of incubation. The cell pellet was resuspended in 5 ml of NoS medium with either 150 ICi of 3H-methionine, 35S-methionine, 35S-cysteine, or 1 mCi Na<sub>2</sub> 35SO<sub>4</sub> (ICN Biomedicals, Irvine, CA) and incubated for 4 h at 37°C. Then, 5 ml of the initial culture supernatant was added and incubated for 20 h. The remaining supernatant was added to a final volume of 50 ml, and incubated for 56 days at 37°C. The headspace gas was directed into zinc-acetate traps, and since “*F. acidarmanus*” is microaerophilic (Baumler et al. 2005), 60 cc of air was injected every 3.5 days into the culture flask which forced headspace gases into the zinc-acetate trap. The precipitate was collected and resuspended in a liquid scintillation vial with 5 ml Instagel (Packard Instruments, Meriden, CT) and analyzed with a LS 6500 multi-purpose scintillation counter (Beckman Coulter, Fullerton, CA).

### Cell lysates

The cell lysates for enzyme assays were prepared from 400 ml of a 14-day “*F. acidarmanus*” culture grown in mfer and harvested by centrifugation (12,200·g, 10 min at 21°C). The supernatant was discarded and the cell pellets were resuspended in 3.0 ml of 1% (w/v) NaCl acidified to pH 1.0 with H<sub>2</sub>SO<sub>4</sub>. The resuspended cell mixture was transferred to two 1.5 ml microcentrifuge tubes and centrifuged (12,200·g for 10 min at 21°C) and the supernatants discarded. Next, the surface of the cell pellets were gently washed with 750  $\mu$ l dH<sub>2</sub>O to remove any residual traces of pH 1.0 solution. One of the cell pellets was resuspended in 750  $\mu$ l of 0.2 M Na<sub>2</sub>HPO<sub>4</sub> which was then used to resuspend the second cell pellet. The cell suspension was transferred to a 15 ml conical tube, and 213  $\mu$ l of 0.2 M Na<sub>2</sub>HPO<sub>4</sub> and 1.5 ml dH<sub>2</sub>O were added and vortexed for 15 s. Next, 300  $\mu$ l of 0.1 M citric acid and 237  $\mu$ l dH<sub>2</sub>O were added. The final crude lysate (3 ml) had a pH of 6.2. For assays conducted at pH 6.8, 6.5, or 5.5, citric acid (0.1 M) and dH<sub>2</sub>O were added at the following volumes 120 and 417  $\mu$ l, 220 and 317  $\mu$ l, or 440 and 97  $\mu$ l, respectively. Cell lysis occurred as the membrane lipids of “*F. acidarmanus*” dissociate at neutral pH. Lysis efficiency was determined at this step by enumerating the viable cells/ml using the MPN procedure (n = 3), and compared to MPN’s taken following the first resuspension step [3.0 ml of 1% (w/v) NaCl acidified to pH 1.0 with H<sub>2</sub>SO<sub>4</sub>]. Lysis efficiency was determined and indicated that less than 10<sup>3</sup> viable cells remained of the \*10<sup>10</sup> cells used. Protein concentrations of the crude lysates were determined using the BCA protein assay kit (Pierce, Rockford, IL) with BSA used as standard.

### Sulfite reductase activity

The lysate (3 ml) was placed into 10 ml-headspace vials (Wheaton) and EDTA (1mM), DTT (5 mM), and Na<sub>2</sub>SO<sub>3</sub> (25 mM) added. Finally, 105.2  $\mu$ l citrate phosphate buffer (CPB) was added to the reaction mixture (pH 6.2, final volume of 3.2 ml). Vials were flushed with nitrogen gas, and crimp-sealed with PTFE-silicone headspace seals (Wheaton). The vial was incubated at 37°C and the reaction started by injecting NADPH

to a final concentration of 100  $\mu$ M. At each timepoint, 250  $\mu$ l of headspace was sampled and the amount of MT monitored using GC/MS. Mean values from five independent trials are reported with error bars representing the standard error of the means.

#### S-adenosylmethionine-dependent thiol methyltransferase activity

Cell extracts (0.5 ml) were assayed for thiol methyl transferase (TMT) activity using a radiometric assay that measures the transfer of the  $^3$ H-methyl group from [methyl- $^3$ H]S-adenosyl-L-methionine (SAM) to sulfide as described by Drotar et al. (1987). The reaction was started by addition of 0.1 mM [methyl- $^3$ H]SAM and incubation at 37°C for 30 or 60 min. The reaction was terminated by addition of 0.25 ml NaOH (10 M). Toluene (Sigma) was added (6 ml) to the reaction mixture and the tube was mixed by inversion ten times, and 4.5 ml of the organic layer was added to 5 ml of instagel and analyzed by LSC. Three trials of the assay were performed and the data are presented as the mean values  $\pm$  the standard error of the mean.

#### Methionine-c-lyase activity

Cell lysates were prepared as described above, with the exception that the pH was adjusted to 6.8 instead of 6.2. The final reaction mixture contained 0.2 mM pyridoxal 50-phosphate hydrate (PLP) and either 5 mM L-methionine (Sigma) or 5 mM L-cysteine hydrochloride monohydrate (Sigma) in a final volume of 1.0 ml. The reaction mixture was placed in 2 ml GC headspace vials (National Scientific Co., Rockwood, TN), sealed, and the reaction started by incubation at 37°C. Mean values from three independent trials are reported with error bars representing the standard error of the mean.

## Results

Headspace gases of “*F. acidarmanus*” cultures were analyzed for sulfur gases by GC/MS using the selective ion monitoring mode for the detection of VSCs [ $\text{H}_2\text{S}$ ,  $\text{SO}_2$ ,  $(\text{CH}_3)_2\text{S}$ ,  $(\text{CH}_3)_2\text{S}_2$ ,  $\text{CH}_3\text{SH}$ ,  $\text{CS}_2$ , or  $\text{COS}$ ]. The headspace from chemolithotrophically grown “*F. acidarmanus*” (7 or 14 day cultures) contained MT ( $\text{CH}_3\text{SH}$ ) and some DMDS [ $(\text{CH}_3)_2\text{S}_2$ ] with elution times of 3.9 and 8.9 min, respectively (Fig. 1). MT and DMDS were also detected in “*F. acidarmanus*” cultures grown heterotrophically (7 or 14 days) in either NoS with 350 mM  $\text{Na}_2\text{SO}_4$  or in mfer ( $\text{FeSO}_4$  replaced with 100 mM  $\text{MgSO}_4$ ). The low levels of DMDS in the samples were most likely due to the conversion of MT to DMDS in the presence of oxygen (Lomans et al. 2002; Nordmann et al. 1994). In the headspace of sealed “*F. acidarmanus*” cultures (14 days),  $\text{O}_2$  levels decreased,  $\text{CO}_2$  levels increased, and  $\text{N}_2$ ,  $\text{CH}_4$ , and  $\text{H}_2$  remained unchanged compared to cell-free controls sealed for 14 days (data not shown).

Since some sulfate-reducing prokaryotes can degrade methionine with the release of MT (Kiene and Visscher 1987; Taylor and Kiene 1987) and in order to compare the production of VSC in “*F. acidarmanus*” with a bona fide sulfate-reducing prokaryote, the headspace gases of *Archaeoglobus fulgidus*, a well-characterized sulfatereducing archaeon, was examined. In the headspace gases of *A. fulgidus*,  $\text{H}_2\text{S}$  and some  $\text{SO}_2$  were

detected but not MT or DMDS (data not shown). Pilcher et al. (2003) reported that MT can be formed by the transfer of methyl groups from the organic constituents of the culture medium to sulfides such as H<sub>2</sub>S (Visscher and van Gernerden 1993; Visscher et al. 2003). To address this possibility, a 1.0 M NaS solution was injected into a sealed headspace vial containing uninoculated mfer as a control. GC/MS analysis revealed the presence of H<sub>2</sub>S and SO<sub>2</sub> but no MT or DMDS (data not shown). Thus, the presence of MT in the headspace gas of “*F. acidarmanus*” cultures was a result of microbial metabolism and not abiotic reactions among medium constituents and sulfide ions.

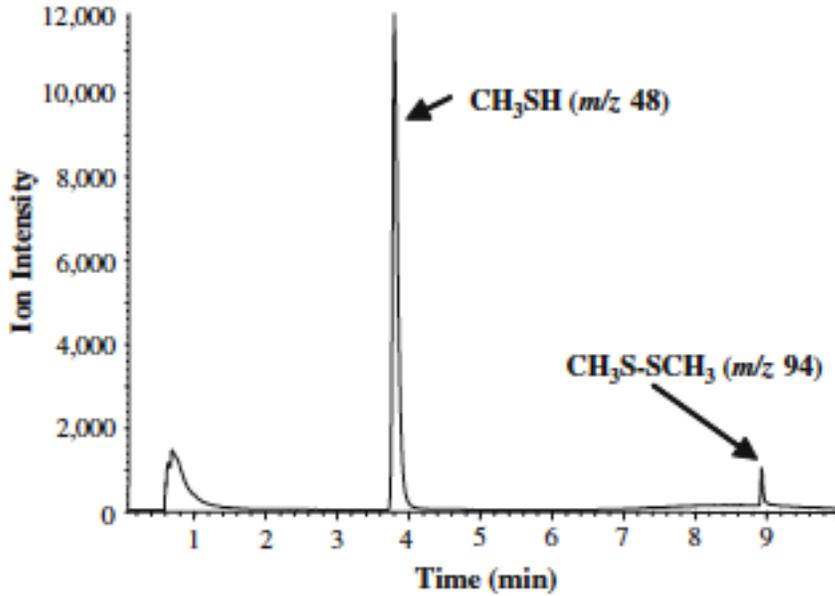


Fig. 1 Volatile organic sulfur compounds detected in the headspace of “*F. acidarmanus*” cultures using GC/MS. The profile shows peaks for methanethiol (m/z 48) and dimethyldisulfide (m/z 94)

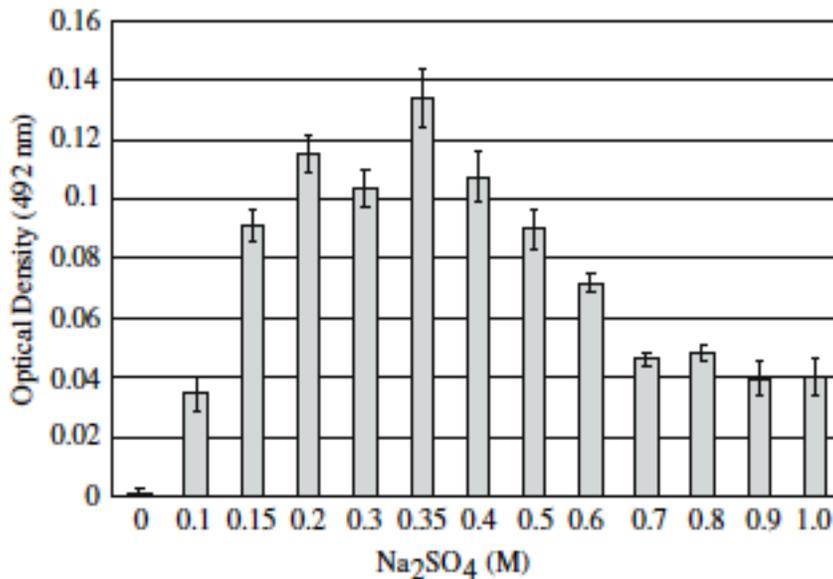


Fig. 2 Sulfate-dependent growth of “*F. acidarmanus*”. The optimal concentration of Na<sub>2</sub>SO<sub>4</sub> for growth was determined spectrophotometrically using HCl-acidified NoS (pH 1.0) medium supplemented with concentrations of Na<sub>2</sub>SO<sub>4</sub> ranging from 0 to 1 M and incubation for 14 days at 37 °C. Background absorbance of uninoculated NoS medium was subtracted from optical density values. All data represent the mean from three trials. Error bars represent the standard error of the mean

As a precursor to experiments with <sup>35</sup>S-labeled substrates and to control for the level of SO<sub>4</sub><sup>2-</sup> in the medium, the growth of “*F. acidarmanus*” at different sulfate concentrations was evaluated in NoS medium with Na<sub>2</sub>SO<sub>4</sub> added as the sole sulfate source. After 14 days of incubation, growth (OD<sub>492</sub>[0.05]) was detected in the range of 150–600 mM Na<sub>2</sub>SO<sub>4</sub>. The highest cell density was achieved at 350 mM (Fig. 2). The growth of “*F. acidarmanus*” in NoS medium with sulfate added as MgSO<sub>4</sub>, resulted in a similar trend with growth occurring between 150 and 500 mM and the greatest optical density obtained at 500 mM (data not shown). To test if the absence of growth at 700 mM Na<sub>2</sub>SO<sub>4</sub> or 500 mM MgSO<sub>4</sub> was due to toxic levels of the cationic group or SO<sub>4</sub><sup>2-</sup>, growth studies in NoS medium containing an equimolar mixture of Na<sub>2</sub>SO<sub>4</sub> and MgSO<sub>4</sub> were added in the range of 0–1.0 M SO<sub>4</sub><sup>2-</sup>. “*F. acidarmanus*” growth (OD<sub>492</sub>[0.05]) occurred at SO<sub>4</sub><sup>2-</sup> concentrations up to and including 1 M, indicating that SO<sub>4</sub> did not inhibit growth at this level. Inhibitory concentrations of Na<sup>+</sup> and Mg<sup>2+</sup> were 1.2 M and 500 mM, respectively. A concentration of 350 mM SO<sub>4</sub><sup>2-</sup> was used in subsequent studies with NoS medium.

To determine if the production of MT was associated with a particular phase of growth or whether it is a constitutive process, viability and MT production during chemolithotrophic and heterotrophic growth of “*F. acidarmanus*” were monitored. Chemolithotrophic and heterotrophic cultures produced MT during both log and stationary phases of growth with the greatest level of production occurring at day 8 and 12, respectively (Fig. 3). The quantities of MT decreased slightly or remained steady for 4 days after reaching maximum levels and declined thereafter. Cultures grown in the presence of FeSO<sub>4</sub> (chemolithotrophic) produced MT more rapidly and at greater quantities than heterotrophic cultures. These findings indicated that MT production is constitutive and linked with active cell metabolism.

MT is typically produced by microbes that metabolize the sulfur-containing amino acids methionine and cysteine (Bentley and Chasteen 2004). The growth media for “*F. acidarmanus*” contain some methionine and cysteine in the added yeast extract as well as SO<sub>4</sub><sup>2-</sup>. To identify the sulfur source(s) used for MT production, <sup>35</sup>S-labelled amino acids were utilized. Headspace gases from cultures grown in NoS supplemented with <sup>35</sup>S-methionine or <sup>35</sup>S-cysteine were directed into zinc acetate traps that resulted in the formation of a white metallothiol precipitate. Following the addition of radiolabeled substrate, 7 days of incubation were necessary before precipitates were visible in the zinc acetate traps. The harvested precipitate contained <sup>35</sup>S activity with the greatest level present 21 days after the addition of either labeled amino acid. The level of precipitate and <sup>35</sup>S present decreased during the next 35 days of incubation (Fig. 4a, b). The quantity of <sup>35</sup>S recovered from methionine was approximately six times greater than that

with cysteine when maximum recoveries (day 21) were compared. These data showed that both methionine and cysteine could serve as precursors to the production of MT but production from methionine metabolism was significantly greater.

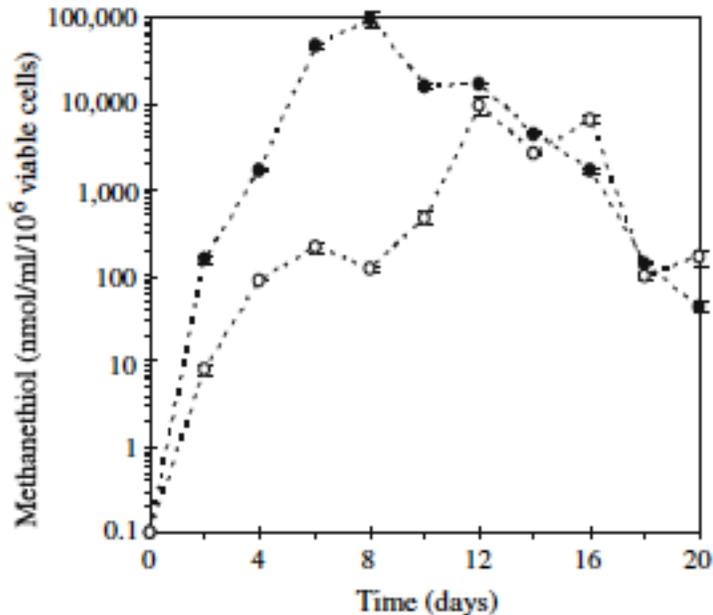


Fig. 3 Methanethiol production during growth of “*F. acidarmanus*”. Cells were grown chemolithotrophically (filled circle) or heterotrophically (open circle) in sealed headspace vials and incubated for 20 days at 37°C. At the indicated timepoints, the concentration of methanethiol was determined by GC/PFPD and viable cells enumerated by a most probable number method. The data are mean values from three independent trials. Error bars represent the standard error of the mean

The terminal methyl group of methionine can be cleaved as a methyl–thiol group (SCH<sub>3</sub>) or function as a substrate for methyl transfer (Bentley and Chasteen 2004). Therefore, studies with <sup>3</sup>H-methionine were conducted to determine if “*F. acidarmanus*” cleaves or utilizes the terminal methyl group of methionine in the production of MT. <sup>3</sup>H-activity was detected in the harvested metallothiol precipitate in zinc acetate traps throughout the 56-day experiment (Fig. 4c); however, the maximum amount of activity was recovered at day 35, 2 weeks after the peak recovery with <sup>35</sup>S-methionine. These results demonstrated that the methyl group of methionine contributes to the formation of MT by “*F. acidarmanus*” but probably by a different pathway from that used by the sulfur of methionine.

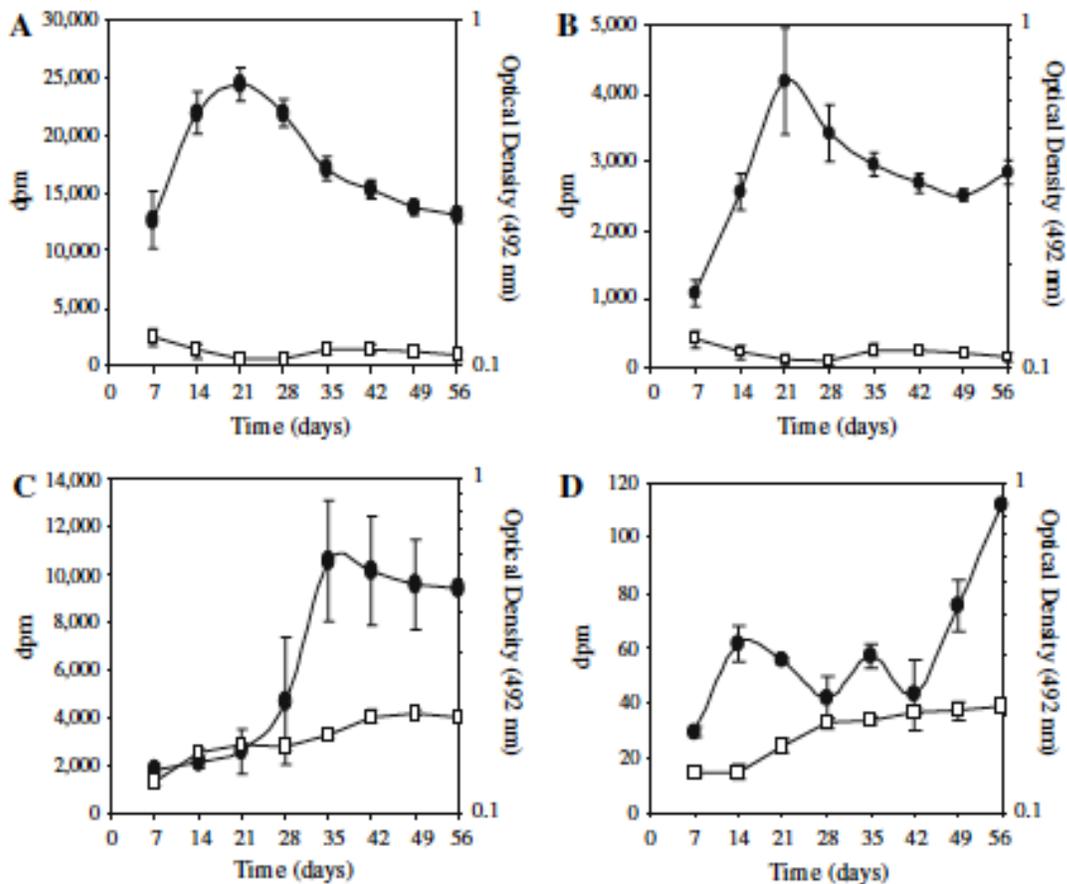


Fig. 4 Utilization of methionine, cysteine, and sulfate in methanethiol production. Log-phase cells were resuspended in NoS medium supplemented with either 150 ICi 35S-methionine (a), 150 ICi 35Scysteine (b), 150 ICi [methyl-3H]methionine (c), or 1 mCi of Na<sub>2</sub> 35SO<sub>4</sub> (d) and incubated for 4 h at 37°C. After incubation, 5 ml of supernatant from the original culture was added and the culture incubated for an additional 20 h. The remaining supernatant from the original culture medium was then added (50 ml final volume) and incubated for 56 days at 37°C. The headspace gas was directed into a zinc-acetate trap (10% w/v). Sixty cubic centimeter of air was injected every 3.5 days into the culture headspace to sustain “*F. acidarmanus*” growth. The metallothiol precipitate was collected and resuspended in 5 ml instagel and analyzed by LSC (filled circle). Background counts from cultures with no radiolabeled substrate added were subtracted from the data presented. The optical densities of cultures are also shown (open square)

Initial experiments with cultures pulsed in NoS medium supplemented with 150 ICi Na<sub>2</sub> 35SO<sub>4</sub> and 350 mM Na<sub>2</sub>SO<sub>4</sub> revealed no radioactive activity (above background) in the harvested metallothiol precipitate throughout the 56-day study. Based on the background radiation and on the concentrations of non-radiolabeled SO<sub>4</sub><sup>2-</sup>, the level of detection for 35SO<sub>4</sub><sup>2-</sup> required that the ratio of labeled:unlabeled sulfate to be approximately 1:5 · 10<sup>7</sup>. The ratio in our initial experiment was \*1:1 · 10<sup>8</sup>. To determine if the negative results from our initial 35SO<sub>4</sub><sup>2-</sup> experiment was a result of the detection limit, an attempt was made to

improve the labeled:unlabeled sulfate ratio by lowering the amount of Na<sub>2</sub>SO<sub>4</sub> in the NoS medium to 100 μM. However, “*F. acidarmanus*” cells did not survive at this level of sulfate [MPN assay revealed a 108 decrease in viable cells (data not shown)]. Consequently, a third attempt was undertaken where the amount of <sup>35</sup>S<sub>2</sub>O<sub>4</sub><sup>2-</sup> was increased from 150 μCi to 1 mCi, which provided a labeled:unlabeled ratio of 1:2 · 10<sup>7</sup>. In this experiment, a low level of radioactivity was detected in the metallothiol precipitates after corrections for background, indicating that “*F. acidarmanus*” was capable of using sulfate to produce MT (Fig. 4d).

At the end of experiments with radiolabeled substrate, TCA-insoluble material was collected and tested for the presence of <sup>35</sup>S or <sup>3</sup>H. The detection of radioactivity in TCA-insoluble cellular components coupled with its removal by proteinase K treatment demonstrated that <sup>35</sup>S-methionine, <sup>3</sup>H-methionine, and <sup>35</sup>S-cysteine were incorporated into protein. In most cases, the proteinase K treatment removed over 50% of the total radioactivity from the TCA-insoluble fraction (Table 1). Experiments with <sup>35</sup>S<sub>2</sub>O<sub>4</sub><sup>2-</sup> indicated that some sulfate was assimilated into protein, most likely through the production pathways for cysteine and methionine.

MT can be produced via cleavage of the methyl-thiol (-SCH<sub>3</sub>) group of methionine by methionine-c-lyase (MGL) (Tokoro et al. 2003). When “*F. acidarmanus*” lysates (pH 6.8) were incubated with 0.2 mM pyridoxal 5-phosphate (PLP) and 5 mM L-methionine, increasing amounts of MT were detected with time throughout the 90 min incubation (Fig. 5). When 5 mM L-cysteine was added to cell lysates with 0.2 mM PLP instead of methionine, H<sub>2</sub>S (data not shown) was detected in addition to a significant (P<0.0001) amount of MT compared to either lysates without L-cysteine or to boiled cell preparations (Fig. 5). Control cell lysates with 0.2 mM PLP but no added methionine or cysteine produced a low level of MT that increased with incubation time. This likely represented the degradation of endogenous methionine and cysteine present in cell lysates. The amount produced was significantly less than the reactions with added L-methionine or L-cysteine. Boiled lysates containing 5 mM of either L-methionine or L-cysteine produced negligible amounts of MT (Fig. 5). Overall, these results indicated that “*F. acidarmanus*” lysates possessed MGL-like activity (Tokoro et al. 2003). The formation of MT from cysteine may proceed by the cleavage of HS- followed by methylation involving a thiol methyltransferase (TMT).

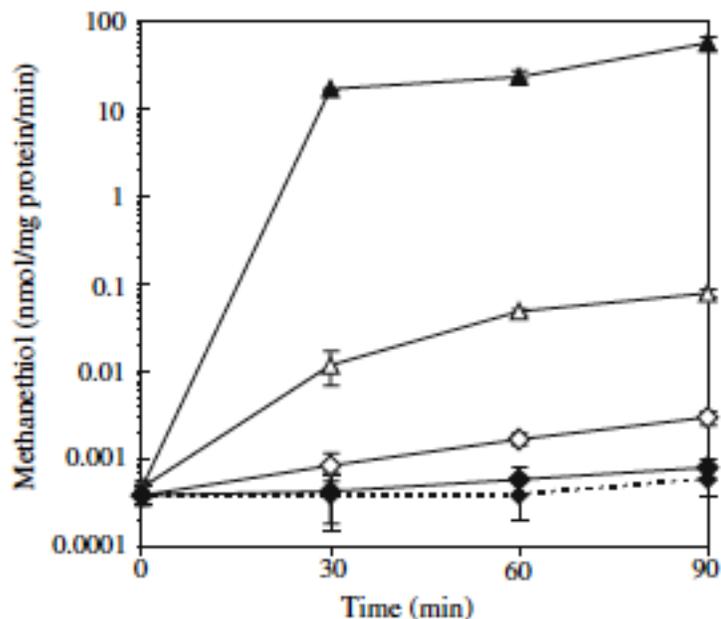


Fig. 5 Methanethiol production from methionine and cysteine by cell lysates. The cell lysates were prepared as previously described. Reaction mixtures were comprised of 1.0 ml of citrate phosphate buffer (pH 6.8), 0.02 mM pyridoxal 50-phosphate hydrate, and 5 mM of either methionine (filled triangle) or cysteine (open triangle). The reaction mixtures were incubated at 37°C and headspace samples (250  $\mu$ l) analyzed by GC/PFPD every 30 min. Boiled cell lysates containing 5 mM of methionine (filled diamond) or cysteine (filled diamond in dotted line) and lysates incubated without methionine or cysteine (open diamond) served as controls. The data points are mean values from three independent trials. Error bars represent the standard error of the mean

Since the  $^{35}\text{SO}_4^{2-}$  experiments indicated that sulfate was used to produce MT and incorporated into protein, most likely by the assimilatory sulfate reduction pathway, a cell lysate assay was developed to test for the presence of sulfite reductase activity. Cell lysates were incubated in headspace vials under nitrogen at 37°C and samples analyzed every 30 min by GC/PFPD. Elution times for  $\text{H}_2\text{S}$ , MT, and DMDS were 1.8, 4.2, and 6.2 min, respectively. Under the cell lysate assay conditions,  $\text{H}_2\text{S}$  was not detected when lysates were incubated with  $\text{SO}_3^{2-}$ , but rather MT was produced (Fig. 6). One possible explanation is that enzymes for both reduction of  $\text{SO}_3^{2-}$  to  $\text{HS}^-$  and subsequent methylation to MT were present and active in the cell lysates. Further evidence to support the existence of a sulfite reductase was the requirement for NADPH. No MT was detected when lysates were incubated with alternative electron donors such as NADH or thioredoxin, or when lysates were incubated without  $\text{SO}_3^{2-}$ . MT was not detected with boiled cell extracts, which indicated that the production of MT was not the result of non-enzymatic side reactions with  $\text{SO}_3^{2-}$ . These results provided evidence for a NADPH-dependent sulfite reductase activity and TMT activity in cell lysates of “*F. acidarmanus*.”

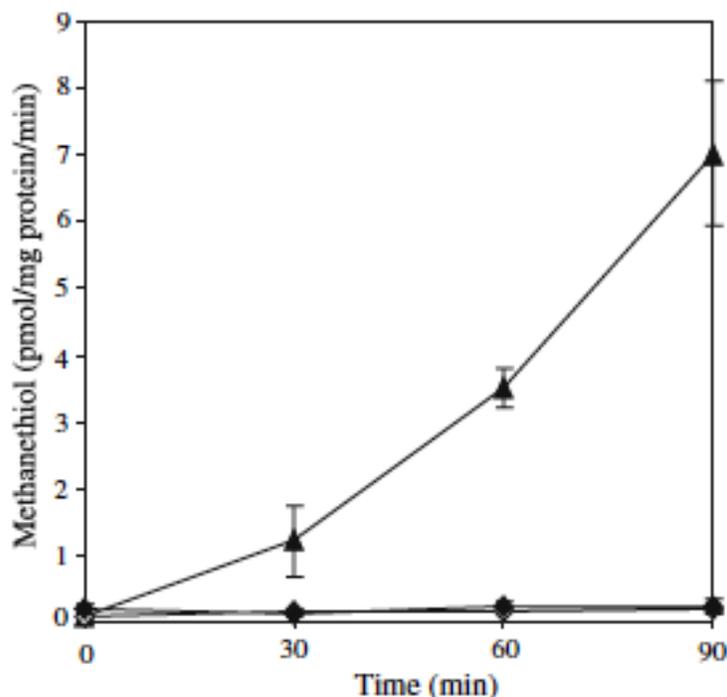


Fig. 6 Methanethiol production from sulfite by cell lysates. The cell lysates were transferred to a nitrogen-flushed vial and sealed with a teflon-coated septum cap. Standard reactions were performed in 3.2 ml citrate phosphate buffer, pH 6.2, and containing 1.0 mM EDTA and 5.0 mM DTT. NADPH (100  $\mu$ M) and Na<sub>2</sub>SO<sub>3</sub> (25 mM) were added to the lysates (filled triangle) and incubated at 37°C and headspace samples (250  $\mu$ l) analyzed by GC/PFPD every 30 min. Boiled (15 min) cell lysates (filled diamond) and lysates incubated without sodium sulfate (open diamond) served as controls. The data presented are the mean values from five independent trials. Error bars represent the standard error of the mean

The results with whole cells demonstrating the utilization of the terminal methyl group of 3H-methionine in MT formation as well as the production of MT from SO<sub>3</sub><sup>2-</sup> by cell lysates indicated the possible presence of a TMT. However, tests for the presence of a SAM-dependent methyltransferase in cell lysates adjusted to pH 6.5 by a radiometric method (Drotar et al. 1987) did not detect significant activity. Similar results were obtained with lysates adjusted to pH 5.5 (data not shown). It is possible that the enzymatic assay conditions were not optimal or an alternative methyl donor is used.

## Discussion

Due to the absence of genetic methods and the difficulty associated with the propagation of many extremophilic archaea, this study utilized a biochemical approach to address the following objectives pertaining to sulfur metabolism in “*F. acidarmanus*”: (1) identify the VSC present in headspace gases, (2) discern precursors to VSC production, (3) develop and utilize an assay for detection of enzyme activities, and (4) begin to elucidate the significance of sulfur compounds in

the physiology of this unique archaeon. Results from this study determined that “*F. acidarmanus*” constitutively produce the VOSC MT and is the first report of such activity in an acidophilic archaeon. Considering the sulfate requirements and the abundance of sulfate and other sulfur-containing compounds in the habitats occupied by this organism, it is likely that the production of MT and sulfur metabolism are central to its physiology.

The development and use of NoS medium where  $\text{SO}_4^{2-}$  was added as  $\text{Na}_2\text{SO}_4$  or  $\text{MgSO}_4$  permitted the determination of  $\text{SO}_4^{2-}$  quantities necessary for growth and survival. Growth occurred in the  $\text{SO}_4^{2-}$  range of 150 mM to 1 M, and  $\frac{1}{3}$  mM was required for survival (data not shown). The high level of sulfate required for optimal growth and survival of “*F. acidarmanus*” is unique. Previously, we reported that heterotrophic growth required the addition of 100 mM of a  $\text{SO}_4^{2-}$  containing salt to mfer medium (Baumler et al. 2005). mfer contains  $\text{SO}_4^{2-}$  in the basal salts and the acidulate ( $\text{H}_2\text{SO}_4$ ) which amounts to 96 mM plus either  $\text{FeSO}_4$  (72 mM) or a  $\text{SO}_4^{2-}$  containing salt (100 mM) that results in a final  $\text{SO}_4^{2-}$  concentration of 168 mM and 196 mM, respectively. These values are within the range of sulfate concentrations that resulted in “*F. acidarmanus*” growth in the current study. The optimal growth concentration of 350 mM  $\text{SO}_4^{2-}$  is the highest reported for a prokaryote. The Richmond mine, where isolation of “*F. acidarmanus*” occurred, was reported to have  $\text{SO}_4^{2-}$  concentrations that fluctuated between 657 and 786 mM when monitored through 1999–2002 (Druschel et al. 2004). Therefore, growth in the range of 150 mM to 1 M  $\text{SO}_4^{2-}$  is not surprising. In comparison, sulfate-reducing prokaryotes from freshwater and marine environments grow optimally at sulfate levels of 100–250 mM and around 25 mM, respectively (Nealson 1997). The importance of sulfate and sulfur compounds metabolism in acidophiles occupying ecological niches high in sulfate may have been over-looked in the past due to the difficulties associated with detecting isotopic tracers when ambient levels of sulfate are high.

The incorporation of 35  $\text{SO}_4^{2-}$  into proteins indicated that “*F. acidarmanus*” imports sulfate and carries out assimilatory sulfate reduction. Additionally, once the ratio of hot:cold sulfate particles was adjusted for detection limits, 35  $\text{SO}_4^{2-}$  was found in metallothiol precipitates from headspace gases of cultures fed with radiolabeled sulfate. Further, cell lysate assays found MT after the addition of sulfite. The absence of PLP in this assay eliminated the possibility of MT production by methionine-*c*-lyase (MGL) where MT is produced from the cleavage of methionine because PLP is a required cofactor for MGL. Thus, it appears that “*F. acidarmanus*” is capable of producing MT via at least two different pathways.

The production of MT by the methylation of sulfide, including sulfide that was produced as a result of the reduction of sulfate, has been proposed (Drotar et al. 1987; Soda et al. 1983; Bak et al. 1992; Grech-Mora et al. 1996; Lomans et al. 2001). However, the activity of the most common class of thiol methyltransferase, the SAM-dependent methyltransferase, was not detected in the assay conditions employed in this study. This may mean that either the methylation of sulfide is

mediated by a methyltransferase that does not utilize SAM, or that the SAM-dependent methyltransferase in “**F. acidarmanus**” has a different characteristic than those previously reported.

While both L-methionine and L-cysteine are used to produce MT in “**F. acidarmanus**,” they are not utilized in identical fashions. After 21 days of incubation with the radiolabeled amino acids, when peak production of MT was detected, cultures fed with <sup>35</sup>S-methionine produced approximately six times more activity in <sup>35</sup>S-containing metallothiol precipitates than cultures fed with <sup>35</sup>S-cysteine. These results may reflect a greater uptake of methionine, a preference for cysteine incorporation into protein, or enzyme specificity (i.e., MGL). Alternatively, the amount of methionine and cysteine present in yeast extract (not determined) may affect the rate of MT production from <sup>35</sup>S-labeled methionine or cysteine. In comparison, the peak activity in the precipitate from cultures fed with <sup>3</sup>H-methionine (<sup>3</sup>H-methyl group of methionine) occurred at day 35 rather than day 21 as noted with the <sup>35</sup>S-labeled amino acids. This suggested that the methyl group of methionine was incorporated into MT by a pathway other than directly from methionine and involving a carbon-sulfur lyase (i.e., MGL). It is possible that methylase activity that uses methionine as a methyl donor shunts the labeled methyl group to other cellular constituents until they are degraded to release MT; however, it is currently unclear what pathway might be involved or why the peak activity with <sup>3</sup>H-methionine occurs 2 weeks after the maximum activity noted with <sup>35</sup>S-methionine.

In cell lysate assays supplemented with the cofactor PLP and either L-methionine or L-cysteine, MT alone or H<sub>2</sub>S and MT were produced, respectively. These findings indicated that the cell lysate contains MGL-like activity. The binding site for PLP (prosite PD0C00677) was used to identify candidate ORFs for the enzymes in the transsulfuration pathway in the genome of “**F. acidarmanus**” (cystathionine-**c**-lyase, cystathionine-**c**-synthase, cystathionine-**b**-lyase, and MGL). Only two PLP-binding candidates for cystathionine-**c**-synthase (GenBank Accession Number ZP\_00609085 and ZP\_00610186) were identified. In the work of Tokoro et al. (2003), only two PLP binding candidates in **Entamoeba histolytica** were found. When these loci were cloned and the enzymes characterized, both were found to function as MGLs. Phylogenetic analysis of these two loci suggested a horizontal gene transfer event from an archaeon. The activity indicative of MGL in “**F. acidarmanus**” warrants further investigation to determine the precise functions of the two PLP-binding protein candidates.

Examinations of the complete genome of “**F. acidarmanus**” also shed light on other aspects of sulfate metabolism. The typical assimilatory sulfate reduction pathway consists of three key enzymes: the sulfate adenylyltransferase (EC: 2.7.7.4), the 30-phosphoadenosine 50-phosphoadenosine (PAPS) reductase (EC: 1.8.4.8), and the assimilatory sulfite reductase (EC: 1.8.1.2). The annotated genome of “**F.**

**acidarmanus** ” contains a putative match for the first enzyme (GenBank Accession Number ZP\_00609637). Another locus has been annotated as the APS/PAPS reductase (GenBank Accession Number ZP\_00609636), with the conserved motif (KRT)ECG(LI)H and the catalytically active cysteine residue typical of this enzyme present (Berendt et al. 1995 ; Weber et al. 2000 ). This predicted enzyme also contains two conserved cysteine pairs. This characteristic has been used in some cases to distinguish between the assimilatory versus the dissimilatory sulfate reduction pathway (Kopriva et al. 2002 ). The significance of these cysteine pairs in this predicted “**F . acidarmanus** ” enzyme is still unclear and its true nature remains a subject for further investigation. The third enzyme, responsible for the key step of the six-electron reduction of sulfite to sulfide, is sulfite reductase (Odom and Peck 1984 ). Despite the characterization of sulfite reductase from multiple bacteria and two archaea (Dahl et al. 1993 ; Hatchikian and Zeikus 1983 ; Lee and Peck 1971 ; Lee et al. 1973 ; Trudinger 1970 ; Johnson and Mukhopadhyay 2005), a candidate for this enzyme was not found in the “**F . acidarmanus** ” annotated genome. Since experimental evidence incontrovertibly indicated the presence of an enzyme that can carry out this function, the absence of a candidate gene in the annotated genome suggested that the function of this enzyme is carried out by another enzyme that is heretofore unconnected with sulfate reduction. Whatever the identity of this enzyme is, data suggested that its function is dependent on the cofactor NADPH. The identification and characterization of this enzyme should prove invaluable to the understanding of both the sulfur metabolism in general as well as its role in this acidophile.

Collectively, the results from this study provide evidence for multiple MT pathways in “**F . acidarmanus** ” (Fig. 7 ). The significance of MT production is underscored in a study of the Lower Kane Cave in Wyoming, where the association of VOSC and the acidic cave biotope was established (Engel et al. 2004 ). The findings from our studies have identified a new contributor to the global cycling of sulfur. This is the first report of an acidophilic MT-producing archaeon. The constitutive production of MT by “**F . acidarmanus** ” provides new insight into acidic communities since the production of MT may function in a general detoxification system and/or serve as substrate or a chemotactic signal to associated members of the acidic community (Edwards et al. 2000 ; Tyson et al. 2005 ; Baker et al. 2003 ; Baker et al. 2004 ). The uptake from the pH 1.0 growth medium and incorporation of 35 S-methionine and 35 S-cysteine into protein has broader implications since it establishes a protein-labeling method for application in studies to identify proteins induced in response to changes in the environment or nutrients (Macario et al. 1999).

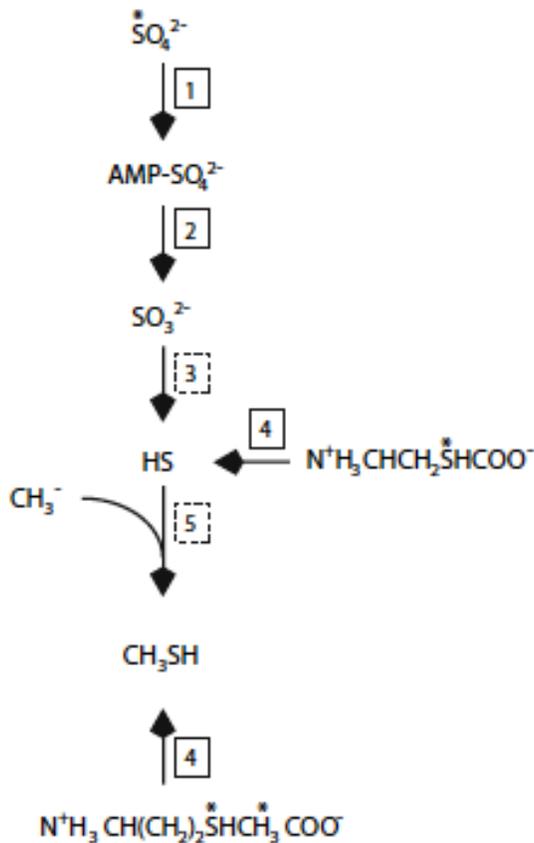


Fig. 7 Proposed pathways of methanethiol production by “*F. acidarmanus*”. **1** sulfate adenylyltransferase, **2** adenosine 50-phosphosulfate reductase, **3** sulfite reductase, **4** methionine-c-lyase, **5** thiol S-methyltransferase. **Solid boxes** represent annotated open reading frames of the genome. **Dashed-line boxes** represent enzymes with no significant sequence similarity identified in the genome. **Asterisks** represent labeled substrates used in experiments

Likewise, the generation of cell extracts suitable for use in enzyme assays should facilitate elucidation of the metabolism of other related genera that also have a completed genome sequence through genome annotation and protein identification and characterization. The in vitro production of MT from sulfite suggests the presence of a sulfite reductase that likely produces sulfide that is methylated to form MT; however, additional work is required to identify and isolate candidate enzymes. Considering the optimal level of sulfate required for growth and survival of “*F. acidarmanus*”, it is likely that future studies will provide new insights into the fundamental role of sulfur in the physiology of acidophiles as well as their contributions to the global sulfur cycle and acidic biotopes.

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