

# Activities of methionine- $\gamma$ -lyase in the acidophilic archaeon “*Ferroplasma acidarmanus*” strain fer1

M A Khan<sup>1</sup>  
 Madeline M López-Muñoz<sup>2</sup>  
 Charles W Kaspar<sup>3</sup>  
 Kai F Hung<sup>1</sup>

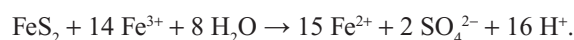
<sup>1</sup>Department of Biological Sciences, Eastern Illinois University, Charleston, IL, USA; <sup>2</sup>Department of Biology, Universidad de Puerto Rico, Mayaguez, Puerto Rico; <sup>3</sup>Bacteriology Department, University of Wisconsin, Madison, WI, USA

**Abstract:** Biogeochemical processes on exposed pyrite ores result in extremely high levels of sulfuric acid at these locations. Acidophiles that thrive in these conditions must overcome significant challenges, including an environment with proton concentrations at pH 3 or below. The role of sulfur metabolism in the archaeon “*Ferroplasma acidarmanus*” strain fer1’s ability to thrive in this environment was investigated due to its growth-dependent production of methanethiol, a volatile organic sulfur compound. Two putative sequences for methionine- $\gamma$ -lyase (EC 4.4.1.11), an enzyme known to carry out  $\alpha,\gamma$ -elimination on L-methionine to produce methanethiol, were identified in fer1. Bioinformatic analyses identified a conserved pyridoxal-5'-phosphate (PLP) binding domain and a partially conserved catalytic domain in both putative sequences. Detection of PLP-dependent and L-methionine-dependent production of  $\alpha$ -keto compounds and thiol groups in fer1 confirmed the presence of methionine- $\gamma$ -lyase activity. Further, fer1 lysate was capable of processing related substrates, including D-methionine, L-cysteine, L-cystathionine, and L/D-homocysteine. When the two putative fer1 methionine- $\gamma$ -lyase gene-coded proteins were expressed in *Escherichia coli* cells, one sequence demonstrated an ability to carry out  $\alpha,\gamma$ -elimination activity, while the other exhibited  $\gamma$ -replacement activity. These fer1 methionine- $\gamma$ -lyases also exhibited optimum pH, substrate specificity, and catalytic preferences that are different from methionine- $\gamma$ -lyases from other organisms. These differences are discussed in the context of molecular phylogeny constructed using a maximum likelihood algorithm based on methionine- $\gamma$ -lyase sequences from a diverse selection of organisms.

**Keywords:** acidophiles, archaea, methionine- $\gamma$ -lyase, L-methionine metabolism, volatile organic sulfur compound

## Introduction

Acidophiles are organisms that not only survive, but actually thrive in acidic environments (pH < 3) whereas most other organisms perish. Some of the sites from which acidophiles, including archaea, bacteria, fungi, and protists, have been successfully cultured include hydrothermal sites, bioleaching reactors, and environments rich in pyrite (FeS<sub>2</sub>), such as abandoned mines.<sup>1-16</sup> At these mine sites, pyrite is converted to sulfuric acid in the presence of water and oxygen in the following reaction:



When chemolithotrophic microbes that utilize Fe<sup>2+</sup> ions as electron donors are present, this process results in accumulation of high proton concentrations.<sup>1</sup>

Correspondence: Kai F Hung  
 Room 3028, Life Sciences Building,  
 600 Lincoln Avenue, Charleston, IL  
 61920, USA  
 Tel +1 217 581 2500  
 Fax +1 217 581 7141  
 Email khung@eiu.edu

This biogeochemical process creates extremely acidic conditions, with naturally occurring pH as low as  $-3.6$  being recorded.<sup>17</sup> These highly acidic waters also contain unusually high levels of dissolved metals, with up to 200 g/L reported, creating an acid mine drain that impacts the ecology surrounding these sites.<sup>18</sup>

The highly acidic environment poses two important challenges for acidophiles, ie, how to utilize the readily available proton gradient to generate ATP and how to prevent denaturation of biomacromolecules both on the cell surface and within the cytoplasm. Studies on the archaeon “*Ferroplasma acidarmanus*” strain fer1 (fer1), which was isolated from an abandoned mine in California, have begun to shed some light on these challenges.<sup>1</sup> This archaeon appears to use tetra-ether-linked lipids as one mechanism of resisting acidification of their cytoplasm.<sup>19</sup> Further, they exhibit resistance to high levels of copper and arsenic, two heavy metals found at acid mine drain sites.<sup>20,21</sup> Previous work has also tied fer1’s ability to metabolize sulfate from their surroundings to the cellular ATP level, suggesting that sulfate metabolism may play an important role in this organism’s ability to thrive in its habitat.<sup>22</sup> This idea is supported by other data showing that optimal growth of fer1 under heterotrophic conditions requires a minimum of 100 mM sulfate.<sup>23</sup> In contrast, the closest evolutionary relative that carries out dissimilatory sulfate reduction, *Archaeoglobus fulgidis*, requires only 14 mM sulfate for growth, suggesting additional metabolic fates for sulfate in fer1.<sup>24</sup> Radiotracer experiments had previously established that one fate for the sulfate is assimilation into proteins.<sup>23</sup> Another observation concerning the sulfur metabolism of fer1 is the formation of a volatile organic sulfur compound, methanethiol ( $\text{CH}_3\text{SH}$ ), during active growth of fer1 cultures.<sup>25</sup> However, the link between assimilatory sulfate reduction and methanethiol production is still missing.

In some organisms, the L-methionine- $\gamma$ -lyase enzyme (EC 4.4.1.11) catalyzes production of methanethiol from L-methionine.<sup>26</sup> L-Methionine- $\gamma$ -lyase (MGL) is a multifunctional catalytic enzyme that requires pyridoxal-5'-phosphate (PLP) as a coenzyme. This enzyme typically functions as a tetramer to catalyze the  $\alpha,\gamma$ -elimination or  $\gamma$ -replacement of L-methionine and related substrates, like L-cystathionine.<sup>26</sup> Some forms of MGL can also process L-cysteine using  $\alpha,\beta$ -elimination and  $\beta$ -replacement, as well.<sup>27,28</sup> So far, MGL activity has been characterized in several organisms, including bacteria, plants, and fungi.<sup>29–31</sup> In addition to enzymatic characterizations, crystallographic analyses of the MGLs from *Pseudomonas putida*, *Citrobacter freundii*,

and *Entamoeba histolytica* have shed light on the structural properties of this enzyme, identifying key amino acid residues in this PLP-dependent enzyme.<sup>32–36</sup>

Examination of fer1 MGL will provide not only a better understanding of the biological role of sulfate metabolism in this acidophile, but may also offer other novel insights. For example, because extremophiles like fer1 can harbor enzymes possessing properties different from their counterparts in regular microbes, a study of the fer1 MGL may lead to discovery of a new variant of the enzyme.<sup>37–39</sup> An MGL with new enzymatic properties can offer advantages in several fields. For instance, because MGL is a target for treatment of protist infections and certain types of cancers, having a new source for this enzyme with potentially novel properties may prove useful in therapeutic applications.<sup>29,40–42</sup> In addition, a better understanding of the biology of acidophiles regarding methionine and sulfate metabolism could prove beneficial when they are utilized in bioleaching applications. Moreover, volatile organic sulfur compounds are important flavoring compounds in microbiological applications involved with food, such as ripening of different types of cheese, which will make MGL with different pH tolerance a useful tool in designing specific applications.<sup>43</sup> Finally, volatile organic sulfur compounds are also significant contributors to the nucleation process of clouds, which affect the albedo component of global temperature regulation.<sup>44,45</sup>

In this study, MGL activity in fer1 cells was confirmed, and the activities of putative fer1 MGL orthologs that were heterologously expressed in *E. coli* were examined. These results paved the way for further analyses of fer1 MGL, which may have a significant impact on our understanding of acidophiles and on applications ranging from cancer therapy to bioleaching.

## Methods and materials

### Growth and culture

Strain fer1 was cultured as previously described at 35°C in mfer medium.<sup>19</sup> Unless otherwise stated, *E. coli* and *C. freundii* strains were propagated using Luria-Bertani medium supplemented with ampicillin 100  $\mu\text{g}/\text{mL}$  as needed under standard conditions (35°C either on 1.5% [w/v] agar plates or with 110 rpm shaking in liquid broth culture).

### Bioinformatics

Putative MGL coding sequences from fer1 were identified using the *P. putida* W619 sequence as the query in a BLAST search against the complete fer1 genome.<sup>46</sup> MGL protein sequences from different organisms were retrieved from

GenBank (Table 1). Analyses of fer1 MGL sequences were carried out using ProtParam at the ExPASy site.<sup>47</sup> Modeling comparisons were carried out using the Phyre2 algorithm.<sup>48</sup> Multiple sequence alignment for protein regions was carried out using the ClustalW2 algorithm.<sup>49</sup> A phylogenetic tree for MGL proteins was generated using PhyML, with 300 iterations for bootstrap analysis, and the result was rendered using TreeDyn hosted at the Phylogeny.fr site.<sup>50–52</sup>

## Gene cloning

Forward primers incorporating an *Nde*I site and reverse primers incorporating an *Not*I site created for each of the two putative fer1 *mgl* loci to generate inframe translational fusion for 3' 6 × His addition in the pET21b vector (EMD Millipore, Billerica, MA, USA) were ordered from Integrated DNA Technology Inc (Coralville, IA, USA). Polymerase chain reaction was carried out following the standard manufacturer's protocol using the KoD enzyme (EMD Millipore). Purification of DNA fragments from agarose gel was carried out using Wizard® SV Gel and the PCR Clean-up Kit (Promega, Fitchburg, WI, USA). Ligation was carried out using T4 DNA ligase (Promega). The ligated

product was transformed into competent BL21(DE3) *E. coli* cells and transformants were selected for and then propagated. Selected clones were then sequenced for the inserts from both directions in duplicate to confirm sequence integrity.

## Cell lysis and protein quantification

To prepare cells for the assays, BL21(DE3) *E. coli* strains and *C. freundii* control were grown to mid-log phase and then expressions of cloned proteins were induced by addition of 1 mM isopropyl- $\beta$ -D-1-thiogalactopyranoside for 3 hours at 20°C with 150 rpm shaking. One hundred mL of overnight cultures were pelleted, rinsed, and then resuspended in 800  $\mu$ L of 100 mM Tris-HCl (pH 8.0). Lysis was achieved by adding 200  $\mu$ L 10 × FastBreak® (Promega buffer supplemented with Halt™ Protease Inhibitor Single-Use Cocktail EDTA-Free [Thermo Fisher Scientific, Waltham, MA, USA]) and DNaseI (Promega) and then incubated at 10°C for 30 minutes with 150 rpm shaking. Following lysis, the samples were centrifuged at 13,000× *g* for 12 minutes at 4°C and the supernatant was aliquoted for assay. For fer1, cell lysis was carried as reported before.<sup>23</sup> Total protein was measured using Coomassie Plus Reagent (BioRad, Hercules,

**Table 1** List of methionine- $\gamma$ -lyase sequences and accession numbers

Abbreviation	Species	Classification	Accession no
Ach	<i>Acidianus hospitalis</i> W1	Crenarchaeota	YP_004459337.1
Aep	<i>Aeropyrum pernix</i> K1	Crenarchaeota	NP_147803.1
Agb	<i>Agaricus bispora</i> var. <i>burnettii</i> JB137-S8	Fungus	EKM76873.1
Art	<i>Arabidopsis thaliana</i> (Col)	Dicotyledon plant	NP_176647.1
Bah	<i>Bacillus halodurans</i> C-125	Gram-positive bacteria	BAB04518.1
Bad	<i>Batrachochytrium dendrobatidis</i> JAM81	Fungus	EGF82431.1
Brl	<i>Brevibacterium linens</i>	Gram-positive bacteria	AAV54600.1
Cif	<i>Citrobacter freundii</i> ATCC29063	Gram-negative bacteria	AAO46884.1
Eh1	<i>Entamoeba histolytica</i> HM-1:IMSS	Protozoan	BAC75877.2
Eh2	<i>Entamoeba histolytica</i> HM-1:IMSS	Protozoan	BAC75878.2
Fa1	" <i>Ferroplasma acidarmanus</i> " strain fer1	Euryarchaeota	ZP_05571061.1
Fa2	" <i>Ferroplasma acidarmanus</i> " strain fer1	Euryarchaeota	ZP_05570711.1
Hav	<i>Haloferax volcanii</i> DS2	Euryarchaeota	YP_003536951.1
Mtc	<i>Metallosphaera cuprina</i> Ar-4	Crenarchaeota	YP_004409978.1
Msa	<i>Methanosarcina acetivorans</i> C2A	Crenarchaeota	NP_617435.1
Msb	<i>Methanosarcina barkerii</i> strain Fusaro	Crenarchaeota	YP_306497.1
Php	<i>Physcomitrella patens</i> subsp. <i>Patens</i>	Moss	XP_001761633.1
Pit	<i>Picrophilus torridus</i> DSM9790	Euryarchaeota	YP_023880.1
Psp	<i>Pseudomonas putida</i> W619	Gram-negative bacteria	ACA71420.1
Py1	<i>Pyrococcus yayanosii</i> CH1	Euryarchaeota	YP_004623865.1
Py2	<i>Pyrococcus yayanosii</i> CH1	Euryarchaeota	YP_004623825.1
Sac	<i>Saccharomyces cerevisiae</i> S288c	Yeast	NP_009390.1
Sus	<i>Sulfolobus solfataricus</i> P2	Crenarchaeota	NP_343729.1
Sut	<i>Sulfolobus tokodaii</i> strain 7	Crenarchaeota	BAK54311.1
Tcb	<i>Thermococcus barophilus</i> MP	Euryarchaeota	YP_004071583.1
Tpn	<i>Thermoproteus neutrophilus</i> V24Sta	Crenarchaeota	YP_001793642.1
Trv	<i>Trichomonas vaginalis</i> G3	Protozoan	XP_001309488.1
Zem	<i>Zea mays</i>	Monocotyledon plant	DAA50062.1

CA, USA) with a standard curve generated using bovine serum albumin (Sigma-Aldrich, St Louis, MO, USA) protein standards.

## Enzyme assays

The activity of MGL was determined using either 3-methyl-2-benzothiazolinone ([MBTH] Sigma-Aldrich), which reacts with  $\alpha$ -keto compounds, or 5,5'-dithio-bis(2-nitrobenzoic acid) ([DTNB] Sigma-Aldrich), which reacts with thiol groups.<sup>53,54</sup> For both assays, cell lysate (40  $\mu$ L) was combined with 20  $\mu$ L of 10 mM PLP (Thermo-Fisher) and incubated for 5 minutes at 35°C, followed by addition of reaction substrate and incubation for 25 minutes at 35°C. Substrates, which were dissolved in 50 mM Tris-Cl adjusted to different pH as specified, included: 50 mM L-methionine, 50 mM D-methionine, 50 mM L-cysteine, 5 mM L-cystathionine, and 10 mM DL-homocysteine (final concentrations). All chemicals were purchased from either Sigma-Aldrich or Thermo-Fisher. For the MBTH assays, the reaction was adjusted to a final volume of 1 mL. After incubation with the substrate, 100  $\mu$ L of 50% (w/v) trichloroacetic acid solution was added and mixed to stop the reactions. Two mL of 1 M sodium acetate (pH 5) and 800  $\mu$ L of 0.1% (w/v) MBTH were then added and incubated as specified. Production of azine derivatives (extinction coefficient 15.7/mM/cm) indicative of MGL activity were detected from 1 mL of the reaction using spectrophotometric measurement at 320 nm. For DTNB assay, 40  $\mu$ L of lysate, 5  $\mu$ L of 1 mM DTT, substrate, and 100  $\mu$ L of 1 mM DTNB in 2.5 mM sodium acetate solution (pH 2.5) were combined, brought to a final volume of 1 mL, vortexed, and incubated at 25°C for 25 or 30 minutes. Products with an extinction coefficient of 13.6/mM/cm were detected using spectrophotometric measurement at 412 nm. Spectrophotometric measurements were taken using a GenesysII reader (Thermo-Fisher). For both assays, values of samples were corrected by assay blanks containing only the buffer. Enzyme activity ( $\mu$ mol/min) was then calculated using the extinction coefficient and duration of the enzymatic reaction, and corrected for the volume of the reaction actually quantified in the case of the MBTH assay. The specific activity level was calculated by dividing enzyme activity by the amount of protein in the cell lysates ( $\mu$ mol/min/mg).

## Statistical analyses

Statistical significance was determined using a two-tailed *t*-test and reported as the *P* value. Averages, standard errors,

and *t*-test values were calculated using Microsoft Excel software (Microsoft Corporation, Redmond, WA, USA).

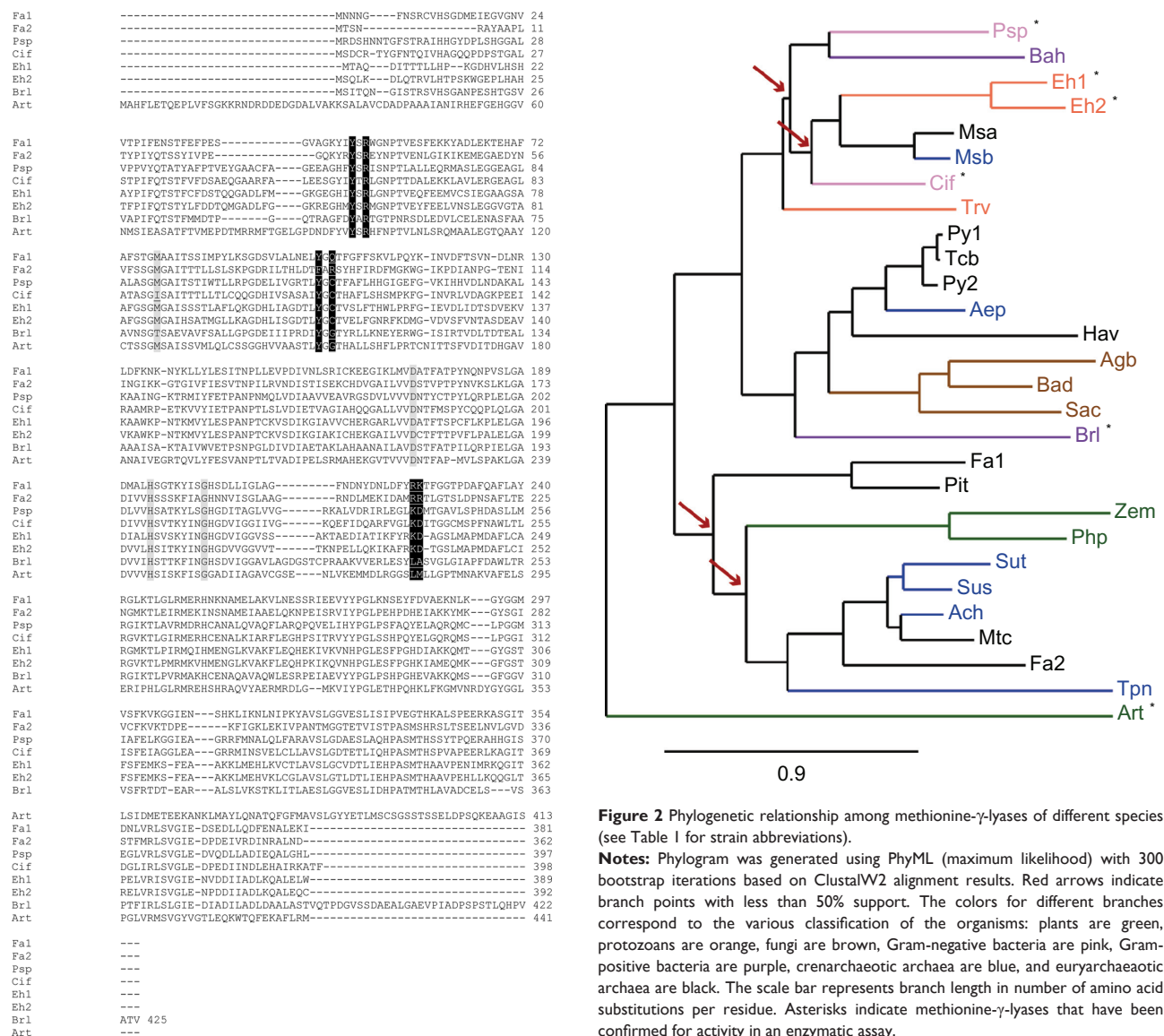
## Results

### In silico analyses

Similar to the protozoan *Entamoeba histolytica*, the completed fer1 genome contains two sequences with significant homology to other PLP-requiring enzymes involved in methionine/cysteine metabolism, ie, ZP\_05571061 (fer1MGL1) and ZP\_05570711 (fer1MGL2, Table 1). Compared with the amino acid sequence of the functionally well characterized MGL from *P. putida*, fer1MGL1 shares 37% identity and 57% similarity, while fer1MGL2 shares 32% identity and 57% similarity.<sup>46</sup> The two fer1 sequences are significantly alike but not identical (38% identity, 54% similarity): fer1MGL1 is predicted to be 42.3 kDa with a pI of 5.34, whereas fer1MGL2 is predicted to be 39.9 kDa with a pI of 6.11.<sup>47</sup>

The two putative PLP-dependent protein sequences were identified as candidates for methionine- $\gamma$ -lyase based on ClustalW2 alignment with five MGL sequences that have been previously characterized enzymatically and/or with crystallography, from *P. putida* (amino acid positions used as reference), *C. freundii*, *Brevibacterium linens*, *E. histolytica*, and *Arabidopsis thaliana* (Table 1).<sup>49</sup> The results show that the four residues known to be critical to binding the PLP cofactor required for enzymatic activity, ie, Met<sup>90</sup>, Asp<sup>187</sup>, His<sup>207</sup>, and Gly<sup>215</sup>, are present in both fer1MGL1 and fer1MGL2 with the correct spacing between amino acids, suggesting that both proteins from fer1 require PLP as a coenzyme (Figure 1). This prediction is further corroborated by protein-folding modeling using the Phyre2 algorithm, where both sequences exhibited significant structural similarities to PLP-binding regions.<sup>48</sup> Of the six residues that are implicated in catalytic activity (Tyr<sup>59</sup>, Arg<sup>61</sup>, Tyr<sup>114</sup>, Cys<sup>116</sup>, Lys<sup>240</sup> and Asp<sup>241</sup>), both fer1MGL1 and fer1MGL2 showed conservation at the Tyr<sup>59</sup> and Arg<sup>61</sup> residues. The fer1MGL1 also shares Tyr<sup>114</sup>, but fer1MGL2 has a Phe<sup>114</sup> instead, substituting a polar residue for a nonpolar one, although both contain a ring structure. Noticeably, the Cys<sup>116</sup> residue that is critical for catalytic activity in *P. putida* was not conserved in either of the putative fer1MGL orthologs.<sup>55</sup> At that same residue, *B. linens* and *A. thaliana* both carry a glycine residue instead of cysteine, whereas fer1MGL1 and fer1MGL2 carry glutamine and arginine, respectively. At the last two of the six catalytic residues, Lys<sup>240</sup> and Asp<sup>241</sup>, fer1MGL1 carries arginine and lysine while fer1MGL2 carries two tandem arginine residues. Compared with *P. putida*, the two putative fer1 orthologs have conserved a basic residue by substituting Arg<sup>240</sup> with





**Figure 1** ClustalW2 alignment of methionine- $\gamma$ -lyases protein sequences (see Table 1 for strain abbreviations and accession numbers).

**Notes:** Residues involved in PLP binding are highlighted in gray, whereas residues involved in catalytic reactions are highlighted in black. In both regions, residues that differ from the reference sequence are underscored.

Lys<sup>240</sup>. However, at the second basic residue Arg<sup>241</sup>, they carry Asp<sup>241</sup>, which is an acidic residue. Interestingly, the lysine-asparagine doublet is not conserved in *B. linens* or in *A. thaliana*. Given the extent of substitutions and deviations from the conserved catalytic residues, the catalytic characters of these two fer1 enzymes are likely to differ significantly from the ones that have been previously characterized.

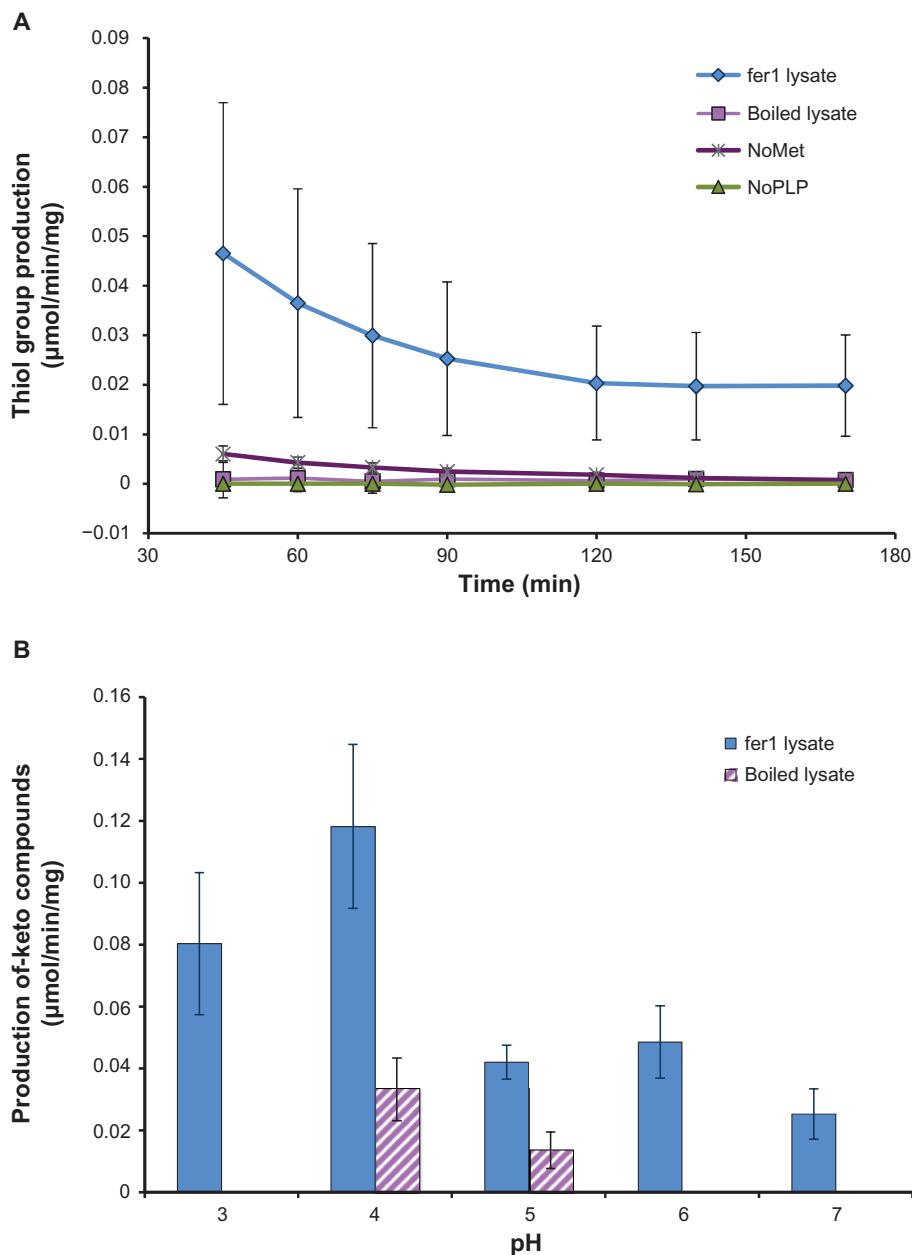
To place fer1MGL1 and fer1MGL2 in the evolutionary context of MGL sequences, a phylogenetic analysis using the maximum likelihood method (PhyML) was carried out with an alignment file generated from the ClustalW2 algorithm consisting of organisms from each major

evolutionary lineage (Figure 2).<sup>49,50</sup> While four branch points had low bootstrap values (<50%), most of the rest were well supported.<sup>56</sup> The results show that although the three fungal sequences examined formed a monophyletic group, the other sequences did not typically align with organismal phylogeny. The crenarchaeal and euryarchaeal sequences were distributed in different branches, intermingling with Gram-positive and Gram-negative bacteria as well as with protozoans. Several archaeal MGL sequences from both crenarchaeal and euryarchaeal members, including fer1MGL1 and fer1MGL2, share higher homology with plant MGLs than they do with bacterial, protozoan, or other archaeal sequences. Notably, fer1MGL1 and fer1MGL2 are not the closest neighbors to each other, reflecting the significant sequence divergence already described. This placement of the two fer1 orthologs is of particular

interest in comparison with the close placement for the two putative MGLs from the archaeon *Pyrococcus yayanosii* (a euryarchaeote) and from the protozoan *E. histolytica*. In those two organisms, it appears that the two copies of MGL might have resulted from evolutionary divergence following gene duplication, whereas in *fer1* the two sequences might have different origins, or it might indicate that they diverged in function much sooner than their counterparts did in the other two organisms.

## Methionine- $\gamma$ -lyase activities in *fer1*

The presence of MGL activity in *fer1* was assayed using DTNB with L-methionine as a substrate. Compared with control conditions in which *fer1* lysate was denatured at 100°C for 15 minutes, untreated lysate showed significantly higher activity during the assay ( $P < 0.01$ , Figure 3A). Furthermore, when PLP or the substrate L-methionine was omitted from the assay mixtures, the *fer1* cell lysate generated lower levels of thiol products ( $P < 0.05$ ), confirming the PLP-dependence



**Figure 3** Methionine- $\gamma$ -lyase (MGL) activity is present in *fer1* and varies with pH. **(A)** Production of free thiol groups from L-methionine by *fer1* cell lysate in 5,5'-dithio-bis(2-nitrobenzoic acid) assay was quantified using spectrophotometric measurement at A412. **(B)** Optimal pH for *fer1* MGL activity was assayed by quantifying production of  $\alpha$ -keto compounds from L-methionine in 2-methyl-3-benzothiazolinone assays.

**Note:** Results represent data from at least three independent trials and vertical bars represent standard errors.

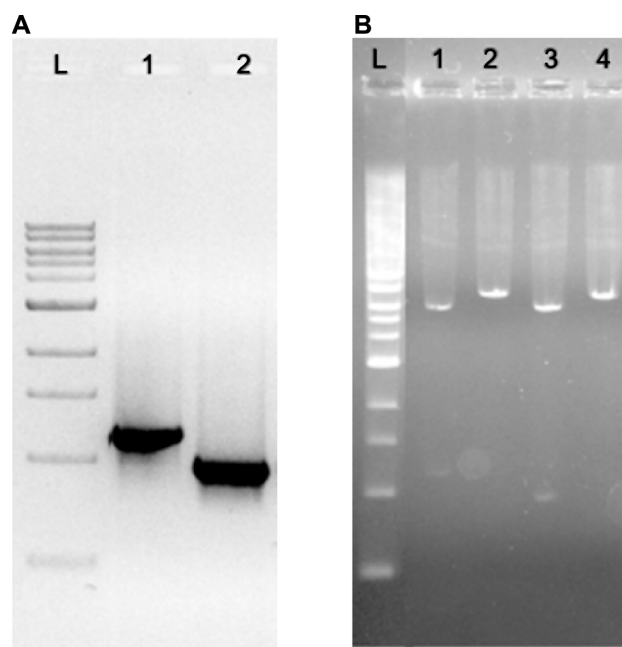
and substrate-dependence of *fer1* lysate, respectively. The effects of pH on the enzymatic activity of MGL were then examined by quantifying the production of  $\alpha$ -keto compounds from L-methionine in MBTH assays (Figure 3B). The highest specific activity ( $0.12 \pm 0.02 \mu\text{mol}/\text{min}/\text{mg}$ ) was detected when the assay buffer was adjusted to pH 4. Furthermore, the substrate specificity of the *fer1* lysate was also examined in MBTH assays. Results from three independent trials showed that there was no statistically significant difference in *fer1* lysate activity between L-methionine ( $3.97 \pm 0.16 \times 10^{-2} [\mu\text{mol}/\text{min}/\text{mg}]$ ), D-methionine [ $3.87 \pm 0.60 \times 10^{-2} \mu\text{mol}/\text{min}/\text{mg}$ ], L-cysteine [ $5.17 \pm 0.37 \times 10^{-2} \mu\text{mol}/\text{min}/\text{mg}$ ], L-cystathionine [ $3.53 \pm 0.56 \times 10^{-2} \mu\text{mol}/\text{min}/\text{mg}$ ], and LD-homocysteine [ $6.06 \pm 0.97 \times 10^{-2} \mu\text{mol}/\text{min}/\text{mg}$ ,  $P > 0.05$ ]. In contrast, lysate inactivated by boiling showed less activity ( $0.60 \pm 0.14 \times 10^{-2} \mu\text{mol}/\text{min}/\text{mg}$ ,  $P < 0.01$ ) and a positive control using whole cell lysate from *C. freundii* showed more activity ( $40.49 \pm 4.21 \times 10^{-2} \mu\text{mol}/\text{min}/\text{mg}$ ,  $P < 0.01$ ).

## Cloning of *fer1* MGLs

Primers for each of the two *fer1* MGLs were designed to incorporate *NotI* (5' primers) and *NdeI* (3' primers) at appropriate positions to create inframe translational fusions in the pET21b vector where the coding region is fused with 6 $\times$  His tag at the carboxyl terminus and is expressed under the control of a promoter that is inducible by isopropyl  $\beta$ -D-1-thiogalactopyranoside. Polymerase chain reactions using a high-fidelity polymerase generated amplicons of appropriate lengths (Figure 4A). To clone the polymerase chain reaction fragments into the pET21b vector, both vector DNA and polymerase chain reaction fragments were first digested by *NotI* and *NdeI* simultaneously and then separated by agarose gel electrophoresis. Vector backbone DNA and polymerase chain reaction fragments were then purified from agarose gel slices and mixed for ligation. Chemically competent BL21(DE3) *E. coli* cells were prepared and used as hosts for ligated products using a heat-shock transformation protocol.<sup>57</sup> Plasmids from survivors were isolated and then digested with *NotI* and *NdeI* to confirm the length of the inserted polymerase chain reaction product (Figure 4B). The integrity of the cloned inserts was confirmed by sequencing reactions in duplicate from both ends of the cloning sites.

## Methionine- $\gamma$ -lyase activity of *fer1*MGL1 and *fer1*MGL2

The MGL activity of heterologously expressed *fer1*MGL1 and *fer1*MGL2 was assayed to confirm the functional



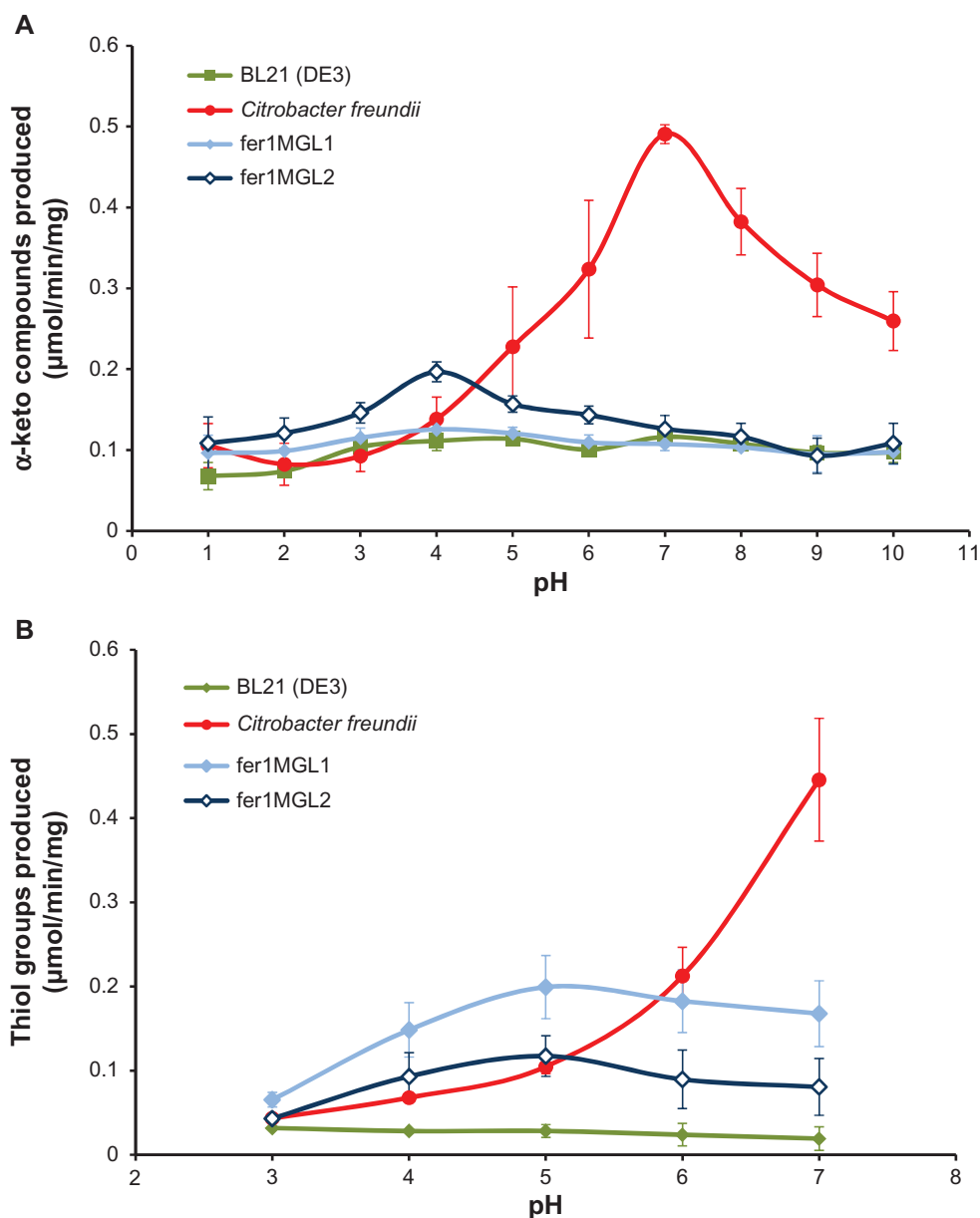
**Figure 4** PCR amplification and cloning of *fer1*MGL1 and *fer1*MGL2. **(A)** PCR amplification of *fer1*MGL1 (lane 1) and *fer1*MGL2 (lane 2) produced amplicons of appropriate lengths, 1146 and 1089 bp, respectively, as visualized by 0.8% (w/v) agarose gel electrophoresis. Lane L contains molecular weight marker, where the 4 smallest bands are 500 bp, 1000 bp, 1500 bp, and 2000 bp, with the higher weight bands each at 1000 bp apart. **(B)** Confirmation of PCR fragment insertion into pET21b was carried out using digestion with *NotI* and *NdeI* (lanes 1 and 3).

**Notes:** Control digestions with identical plasmids using only *NdeI* were performed as well (lanes 2 and 4). Fragment of correct length was detected for both putative clones of *fer1*MGL1 (lanes 1 and 2) and *fer1*MGL2 (lanes 2 and 4). Lane L contains molecular weight marker, where the 4 smallest bands are 500 bp, 1000 bp, 1500 bp, and 2000 bp, with the higher weight bands each at 1000 bp apart.

**Abbreviations:** MGL, methionine- $\gamma$ -lyase; PCR, polymerase chain reaction.

predictions of these two loci using MBTH assays, which detect the ability of these cell lysates to produce  $\alpha$ -keto compounds from L-methionine (Figure 5A). The results showed that the *fer1*MGL2 cell lysate exhibited activity above control conditions using BL21(DE3) cells without any vectors when assays were carried out at pH values from 2 to 6 ( $P < 0.05$ ). In comparison, the *fer1*MGL1 cell lysate did not show significant activity under identical conditions, nor at the other pH values tested ( $P > 0.05$ ). The validity of the assay was confirmed using *C. freundii* as a positive control, which showed significant activity at pH values from 5 to 9 ( $P < 0.05$ ). Further, *fer1*MGL2 showed a significant increase in specific activity when compared with identical lysates that had been inactivated by boiling at 100°C for 15 minutes ( $P < 0.05$ , data not shown).

When the same set of strains were examined for production of thiol groups using DTNB assays adjusted to different pH values, *fer1*MGL1 exhibited more activity than the BL21(DE3) control at pH 5 ( $P < 0.05$ ), while *fer1*MGL2 exhibited the same specific activity as BL21(DE3) at all pH



**Figure 5** Heterologously expressed fer1MGL1 and fer1MGL2 exhibited MGL activities in DTNB and MBTH assays. **(A)** Production of  $\alpha$ -keto compounds from L-methionine by fer1MGL1 and fer1MGL2 expressed in *Escherichia coli* was measured in the MBTH using spectrophotometric measurement at A320. BL21(DE3) is the *E. coli* host which did not contain any pET21b vectors. Results represent data from three independent trials and vertical bars represent standard errors. **(B)** Production of thiol compounds from L-methionine by fer1MGL1 and fer1MGL2 expressed in *E. coli* was measured in the DTNB assays using spectrophotometric measurement at A412. BL21(DE3) is the *E. coli* host which did not contain any pET21b vectors.

**Note:** Results represent data from two independent trials and vertical bars represent standard errors.

**Abbreviations:** DTNB, 5,5'-dithio-bis(2-nitrobenzoic acid); MBTH, 2-methyl-3-benzothiazolinone assays; MGL, methionine- $\gamma$ -lyase.

values assayed ( $P > 0.05$ , Figure 5B). The positive *C. freundii* control showed significant activity at pH values from 5 to 7 ( $P < 0.05$ ).

## Discussion

The genome of *F. acidarmanus* strain fer1, which has been completely sequenced, contains only two putative open reading frames with a significant match to PLP-dependent proteins. The automated genome annotation

assigned the two loci as cystathionine- $\gamma$ -lyase (CGL) and a “hypothetical protein” based on sequence homology. However, the degree of amino acid sequence homology between the two sequences (42% identity, 61% similarity for fer1MGL1 and 36% identity, 59% similarity for fer1MGL2) and the previously characterized CGL from the bacterium *Lactobacillus reuteri* (YP\_001270900) is comparable with their sequence homology to MGL.<sup>58</sup> Further, CGL typically produces hydrogen sulfide from L-cysteine, but actively



growing *fer1* cultures released methanethiol ( $\text{CH}_3\text{SH}$ ) and not hydrogen sulfide.<sup>25</sup> Moreover, in a study of CGL from the archaeal organism *Methanocaldococcus jannaschii*, incubation with  $\text{Fe}^{2+}$  solution can restore lost activity from purified enzymes that had been exposed to air.<sup>59</sup> In contrast, *fer1* whole cell lysates showed depressed activities in DTNB assays when  $\text{Fe}^{2+}$  solutions were added as a supplement (data not shown). In consideration of these discrepancies, as well as the flexibility in substrate binding exhibited by many of the PLP-dependent enzymes, it is not surprising that both the *fer1* lysate and the two putative proteins exhibited MGL activities when they were functionally assayed.<sup>60</sup> Moreover, since MBTH assays on *fer1* lysate demonstrated activities using L-cystathionine as a substrate, it is possible that the two MGL proteins from *fer1* are also capable of carrying out CGL activities. However, confirmation of CGL activities will require further testing.

The ability of *fer1* lysate to convert L-methionine to thiol compounds was confirmed using DTNB assays and was shown to be PLP-dependent. When *fer1* cell lysate was examined in MBTH assays, it produced  $\alpha$ -keto compounds from L-methionine, D-methionine, L-cysteine, L-cystathionine, and L/D-homocysteine at a comparable rate. The most likely explanation for these results is that the *fer1* cell lysate carried out the  $\alpha,\gamma$ -elimination activity of MGL on L-methionine, producing methanethiol (detected by DTNB), ammonia, and 2-oxobutanoate (detected by MBTH). Interestingly, when *E. coli* cells heterologously expressing *fer1MGL1* were examined, positive results were detected in DTNB assays but not in MBTH assays. Because  $\gamma$ -replacement reaction on L-methionine produces substituted thiol groups but no  $\alpha$ -keto acids, the result suggest that *fer1MGL1* might have a catalytic preference for  $\gamma$ -replacement over  $\alpha,\gamma$ -elimination reactions. This difference in catalytic preference echoes the different substrate preference for the two versions of MGL in *E. histolytica* and may reflect the divergence in amino acid sequences at the catalytic sites between *fer1MGL1* and *fer1MGL2* (Figure 1).<sup>28</sup> In comparison, *fer1MGL2* appeared to exhibit activity in MBTH assays but not in DTNB assays (Figure 5A). This could be due to the sensitivity of the assay systems or interference from other cellular components.

Another interesting observation is that, contrary to all the cases where MGL activity had been studied, *fer1* cell lysate was able to process D-methionine under DTNB assay conditions.<sup>26,30,61,62</sup> This may reflect the new catalytic residues found in *fer1MGL1* and *fer1MGL2*, which do not match the residues of the other sequences examined in this study. This discovery is in agreement with other cases where *fer1*

enzymes, such as a DNA repair protein, a DNA helicase, and a DNA ligase, exhibited unusual activity when compared with similar enzymes.<sup>37–39</sup> Moreover, the pH optimum for the *E. coli* lysate expressing *fer1MGL1* and *fer1MGL2* was around pH 5, which is significantly different from the pH optimum reported for the pH 7 value typically reported for other MGLs.<sup>29</sup> This pH optimum also matches the estimated internal pH for *fer1*, which is  $4.98 \pm 0.48$ .<sup>19</sup> These new characteristics of *fer1* MGLs may be useful in the design of an MGL suitable for therapeutic use in treating certain types of amoebiasis or as anticancer agents against cells that rely on L-methionine for proliferation.<sup>40–42</sup>

The phylogenetic alignment of MGLs from various organisms using a maximum likelihood model showed a surprisingly mixed evolutionary relationship for this enzyme, even when branch points not well supported by bootstrap values (<50%) were disregarded, eg, crenarchaeal and euryarchaeal versions of MGL were found in mixed lineages with each other (Figure 2). This heterogeneity of protein molecular character may be an artifact of using sequence data that have not been functionally confirmed in many of the species examined. Alternatively, this could point to either a high frequency of lateral gene transfer events or to the flexibility of many of the PLP-dependent enzymes concerning their substrate-binding capacities.

Regarding substrate specificity, among the MGLs that have been enzymatically characterized, only MGLs from *E. histolytica* and *Trichomonas vaginalis*, both protozoans, showed the same pattern of exhibiting comparable levels of activity towards both L-methionine and L-cysteine. In contrast, the other MGLs assayed all showed significantly reduced activity towards L-cysteine when compared with L-methionine.<sup>29,30</sup> However, when the six catalytic residues and phylogenetic relationships were examined, *fer1MGL1* and *fer1MGL2* showed less similarity to these protozoan MGLs than they did to plant MGLs. Fortuitously, in a study that examined the role of specific amino acid residues in *E. histolytica* MGL enzymatic activities, a mutation that changed the Tyr<sup>114</sup> residue to Phe<sup>114</sup> was created, mimicking the same substitution found in *fer1MGL2*.<sup>28</sup> In the *E. histolytica* mutation, the MGL activity for the Y114F mutant was significantly reduced in the MBTH assay. In *fer1*, no activity was detected for *fer1MGL1* under MBTH assay conditions, but it did exhibit  $\gamma$ -replacement activity under DTNB assay conditions.

Growth of *fer1* requires an unusually high level of sulfate ( $\geq 100$  mM).<sup>23</sup> While the sulfate level in *fer1* has been shown to correlate with the cellular ATP level, it remains unclear what other roles sulfate may play in the survival of *fer1*.

Because the  $\alpha,\gamma$ -elimination of L-methionine carried out by MGL creates methanethiol, ammonia, and 2-oxobutanoate, one positive outcome of MGL activity might be to help stabilize cellular pH by producing ammonia. Alternatively, removal of six protons in the reduction of sulfite to sulfide during sulfate assimilation may serve to remove excess protons from the cytoplasm, with the eventual release of sulfur atoms in methanethiol. Moreover, sulfate assimilation and methanethiol production may serve to maintain a correct balance of redox carriers. With the activity of MGL demonstrated and the connection between sulfate assimilation and methanethiol production established, future studies could further explore the metabolic roles of sulfate and methanethiol in *fer1* to elucidate the pH homeostasis mechanisms of this acidophile.

In summary, using whole-cell lysate of *fer1*, PLP-dependent and L-methionine-dependent production of  $\alpha$ -keto compounds and thiol groups was demonstrated, thus indicating the presence of MGL in this acidophilic archaeon. Enzymatic activity was also detected in lysates of *E. coli* cells induced to express cloned *fer1* MGL orthologs. These new features of *fer1*MGL1 and *fer1*MGL2 in their pH optimum, substrate specificity, and catalytic preferences will shed light on the mechanism of sulfur metabolism in this organism, and possibly lead to a better understanding of this organism's ability to thrive in highly acidic environments. Further experiments using purified *fer1*MGL1 and *fer1*MGL2 will be required to characterize these enzymes better and to elucidate their possible contribution to other applications, like therapeutic uses and production of volatile organic sulfur compounds. Studies aiming to elucidate the regulatory aspects of these two MGLs will also be valuable in understanding the role of sulfur metabolism in this acidophile. Finally, more work is needed to reconcile the phylogenetic relations of MGLs amongst organisms of various evolutionary lineages.

## Disclosure

The authors report no conflicts of interest in this work.

## References

- Druschel GK, Baker BJ, Gihring TM, Banfield JF. Acid mine drainage biogeochemistry at Iron Mountain, California. *Geochem Trans.* 2004;5:13–20.
- Huang L-N, Zhou W-H, Hallberg KB, Wan C-Y, Li J, Shu W-S. Spatial and temporal analysis of the microbial community in the tailings of a Pb-Zn mine generating acidic drainage. *Appl Environ Microbiol.* 2011;77(15):5540–5544.
- Bruneel O, Pascault N, Egal M, et al. Archaeal diversity in a Fe-As rich acid mine drainage at Carnoulès (France). *Extremophiles.* 2008;12(4):563–571.
- Tan G-L, Shu W-S, Hallberg KB, et al. Culturable and molecular phylogenetic diversity of microorganisms in an open-dumped, extremely acidic Pb/Zn mine tailings. *Extremophiles.* 2008;12(5):657–664.
- Xiao S, Xie X, Liu J, He Z, Hu Y. Compositions and structures of archaeal communities in acid mineral bioleaching systems of Dongxiang copper mine and Yinshan lead-zinc mine, China. *Curr Microbiol.* 2008;57(3):239–244.
- Sánchez-Andrea I, Rodríguez N, Amils R, Sanz JL. Microbial diversity in anaerobic sediments at Rio Tinto, a naturally acidic environment with a high heavy metal content. *Appl Environ Microbiol.* 2011;77(17):6085–6093.
- Golyshina OV, Yakimov MM, Lünsdorf H, et al. *Acidiplasma aeolicum* gen. nov., sp. nov., a euryarchaeon of the family Ferroplasmaceae isolated from a hydrothermal pool, and transfer of *Ferroplasma cupricumulans* to *Acidiplasma cupricumulans* comb. nov. *Int J Syst Evol Microbiol.* 2009;59(Pt 11):2815–2823.
- Hawkes RB, Franzmann PD, O'Hara G, Plumb JJ. *Ferroplasma cupricumulans* sp. nov., a novel moderately thermophilic, acidophilic archaeon isolated from an industrial-scale chalcocite bioleach heap. *Extremophiles.* 2006;10(6):525–530.
- Aguilera A, Gomez F, Lospitao E, Amils R. A molecular approach to the characterization of the eukaryotic communities of an extreme acidic environment: methods for DNA extraction and denaturing gradient gel electrophoresis analysis. *Syst Appl Microbiol.* 2006;29(7):593–605.
- Russo G, Libkind D, Sampaio JP, Van Broek MR. Yeast diversity in the acidic Rio Agrio-Lake Cavihue volcanic environment (Patagonia, Argentina). *FEMS Microbiol Ecol.* 2008;65(3):415–424.
- Brown PB, Wolfe GV. Protist genetic diversity in the acidic hydrothermal environments of Lassen Volcanic National Park, USA. *J Eukaryot Microbiol.* 2006;53(6):420–431.
- Gadanhho M, Libkind D, Sampaio JP. Yeast diversity in the extreme acidic environments of the Iberian pyrite belt. *Microb Ecol.* 2006;52(3):552–563.
- Golyshina OV, Pivovarova TA, Karavaiko GI, et al. *Ferroplasma acidiphilum* gen. nov., sp. nov., an acidophilic, autotrophic, ferrous-iron-oxidizing, cell-wall-lacking, mesophilic member of the Ferroplasmaceae fam. nov., comprising a distinct lineage of the Archaea. *Int J Syst Evol Microbiol.* 2000;50(Pt 3):997–1006.
- Baker BJ, Hugenholtz P, Dawson SC, Banfield JF. Extremely acidophilic protists from acid mine drainage host Rickettsiales-lineage endosymbionts that have intervening sequences in their 16S rRNA genes. *Appl Environ Microbiol.* 2003;69(9):5512–5518.
- Baker BJ, Lutz MA, Dawson SC, Bond PL, Banfield JF. Metabolically active eukaryotic communities in extremely acidic mine drainage. *Appl Environ Microbiol.* 2004;70(10):6264–6271.
- Dopson M, Baker-Austin C, Hind A, Bowman JP, Bond PL. Characterization of *Ferroplasma* isolates and *Ferroplasma acidarmanus* sp. nov., extreme acidophiles from acid mine drainage and industrial bioleaching environments. *Appl Environ Microbiol.* 2004;70(4):2079–2088.
- Nordstrom DK, Alpers CN, Ptacek CJ, Blowes DW. Negative pH and extremely acidic mine waters from Iron Mountain, California. *Environ Sci Tech.* 2000;34:254–258.
- Nordstrom DK, Alpers CN. Negative pH, efflorescent mineralogy, and consequences for environmental restoration at the Iron Mountain Superfund site, California. *Proc Natl Acad Sci U S A.* 1999;96(7):3455–3462.
- Macalady JL, Vestling MM, Baumler D, Boekelheide N, Kaspar CW, Banfield JF. Tetraether-linked membrane monolayers in *Ferroplasma* spp: a key to survival in acid. *Extremophiles.* 2004;8(5):411–419.
- Gihring TM, Bond PL, Peters SC, Banfield JF. Arsenic resistance in the archaeon "*Ferroplasma acidarmanus*": new insights into the structure and evolution of the *ars* genes. *Extremophiles.* 2003;7(2):123–130.
- Baker-Austin C, Dopson M, Wexler M, Sawers RG, Bond PL. Molecular insight into extreme copper resistance in the extremophilic archaeon "*Ferroplasma acidarmanus*" *Fer1*. *Microbiology.* 2005;151(Pt 8):2637–2646.

22. Baumler DJ, Hung K-F, Jeong KC, Kaspar CW. Molybdate treatment and sulfate starvation decrease ATP and DNA levels in "*Ferroplasma acidarmanus*." *Archaea*. 2008;2(3):205–209.
23. Baumler DJ, Jeong KC, Fox BG, Banfield JF, Kaspar CW. Sulfate requirement for heterotrophic growth of "*Ferroplasma acidarmanus*" strain fer1. *Res Microbiol*. 2005;156(4):492–498.
24. Stetter KO, Lauerer G, Thomm M, Neuner A. Isolation of extremely thermophilic sulfate reducers: evidence for a novel branch of Archaeobacteria. *Science*. 1987;236(4803):822–824.
25. Baumler DJ, Hung KF, Jeong KC, Kaspar CW. Production of methanethiol and volatile sulfur compounds by the archaeon "*Ferroplasma acidarmanus*." *Extremophiles*. 2007;11(6):841–851.
26. Tanaka H, Esaki N, Yamamoto T, Soda K. Purification and properties of methioninase from *Pseudomonas ovalis*. *FEBS Lett*. 1976;66(2):307–311.
27. Lockwood BC, Coombs GH. Purification and characterization of methionine  $\gamma$ -lyase from *Trichomonas vaginalis*. *Biochem J*. 1991;279(Pt 3):675–682.
28. Sato D, Yamagata W, Harada S, Nozaki T. Kinetic characterization of methionine gamma-lyases from the enteric protozoan parasite *Entamoeba histolytica* against physiological substrates and trifluoromethionine, a promising lead compound against amoebiasis. *FEBS J*. 2008;275(3):548–560.
29. El-Sayed AS. Microbial L-methioninase: production, molecular characterization, and therapeutic applications. *Appl Microbiol Biotechnol*. 2010;86(2):445–467.
30. Goyer A, Collakova E, Shachar-Hill Y, Hanson AD. Functional characterization of a methionine gamma-lyase in *Arabidopsis* and its implication in an alternative to the reverse trans-sulfuration pathway. *Plant Cell Physiol*. 2007;48(2):232–242.
31. El-Sayed AS. Purification and characterization of a new L-methioninase from solid cultures of *Aspergillus flavipes*. *J Microbiol*. 2011;49(1):130–140.
32. Sato D, Yamagata W, Kamei K, Nozaki T, Harada S. Expression, purification and crystallization of L-methionine gamma-lyase 2 from *Entamoeba histolytica*. *Acta Crystallogr Sect F Struct Biol Cryst Commun*. 2006;62(Pt 10):1034–1036.
33. Sato D, Karaki T, Shimizu A, Kamei K, Harada S, Nozaki T. Crystallization and preliminary X-ray analysis of L-methionine gamma-lyase 1 from *Entamoeba histolytica*. *Acta Crystallogr Sect F Struct Biol Cryst Commun*. 2008;64(Pt 8):697–699.
34. Nikulin A, Revtovich S, Morozova E, et al. High-resolution structure of methionine gamma-lyase from *Citrobacter freundii*. *Acta Crystallogr D Biol Crystallogr*. 2008;64(Pt 2):211–218.
35. Revtovich SV, Morozova EA, Khurs EN, et al. Three-dimensional structures of noncovalent complexes of *Citrobacter freundii* methionine  $\gamma$ -lyase with substrates. *Biochemistry*. 2011;76(5):564–570.
36. Kudou D, Misaki S, Yamashita M, et al. Structure of the antitumor enzyme L-methionine gamma-lyase from *Pseudomonas putida* at 1.8 Å resolution. *J Biochem*. 2007;141(4):535–544.
37. Kanugula S, Pauly GT, Moschel RC, Pegg AE. A bifunctional DNA repair protein from *Ferroplasma acidarmanus* exhibits O6-alkylguanine-DNA alkyltransferase and endonuclease V activities. *Proc Natl Acad Sci U S A*. 2005;102(10):3617–3622.
38. Pugh RA, Lin Y, Eller C, Leesley H, Cann IKO, Spies M. *Ferroplasma acidarmanus* RPA2 facilitates efficient unwinding of forked DNA substrates by monomers of FacXPD helicase. *J Mol Biol*. 2008;383(5):982–998.
39. Ferrer M, Golyshina OV, Belouqui A, et al. A purple acidophilic di-feric DNA ligase from *Ferroplasma*. *Proc Natl Acad Sci U S A*. 2008;105(26):8878–8883.
40. Kappes B, Tews I, Binter A, Macheroux P. PLP-dependent enzymes as potential drug targets for protozoan diseases. *Biochim Biophys Acta*. 2011;1814(11):1567–1576.
41. Cavuoto P, Fenech MF. A review of methionine dependency and the role of methionine restriction in cancer growth control and life-span extension. *Cancer Treat Rev*. 2012;38(6):726–736.
42. Stone E, Paley O, Hu J, Ekerdt B, Cheung N-K, Georgiou G. De novo engineering of a human cystathionine- $\gamma$ -lyase for systemic L-methionine depletion cancer therapy. *ACS Chem Biol*. 2012;7(11):1822–1829.
43. Arfi K, Amarita F, Spinnler H-E, Bonnarme P. Catabolism of volatile sulfur compounds precursors by *Brevibacterium linens* and *Geotrichum candidum*, two microorganisms of the cheese ecosystem. *J Biotechnol*. 2003;105(3):245–253.
44. Lomans BP, Pol A, Op den Camp HJ. Microbial cycling of volatile organic sulfur compounds in anoxic environments. *Water Sci Technol*. 2002;45(10):55–60.
45. Bentley R, Chasteen TG. Environmental VOCs – formation and degradation of dimethyl sulfide, methanethiol and related materials. *Chemosphere*. 2004;55(3):291–317.
46. Altschul SF, Madden TL, Schäffer AA, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res*. 1997;25(17):3389–3402.
47. Gasteiger E, Hoogland C, Gattiker A, et al. Protein identification and analysis tools on the ExpASY server. In: Walker JM, editor. *The Proteomics Protocols Handbook*. Totowa, NJ: Humana Press Inc; 2005.
48. Kelley LA, Sternberg MJE. Protein structure prediction on the web: a case study using the Phyre server. *Nat Protoc*. 2009;4(3):363–371.
49. Larkin MA, Blackshields G, Brown NP, et al. ClustalW and ClustalX version 2. *Bioinformatics*. 2007;23(21):2947–2948.
50. Guindon S, Dufayard J-F, Lefort V, Anisimova M, Hordijk W, Gascuel O. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst Biol*. 2010;59(3):307–321.
51. Chevenet F, Brun C, Bañuls A-L, Jacq B, Christen R. TreeDyn: towards dynamic graphics and annotations for analyses of trees. *BMC Bioinformatics*. 2006;7:439.
52. Dereeper A, Guignon V, Blanc G, et al. Phylogeny. fr: robust phylogenetic analysis for the non-specialist. *Nucleic Acids Res*. 2008;36(Web Server issue):W465–W469.
53. Soda K. A spectrophotometric microdetermination of keto acids with 3-methyl-2-benzothiazolone hydrazone. *Agric Biol Chem*. 1967;31(9):1054–1060.
54. Laakso S, Nurmi V. A spectrophotometric assay for demethylating activity. *Anal Biochem*. 1976;72:600–605.
55. Kudou D, Misaki S, Yamashita M, Tamura T, Esaki N, Inagaki K. The role of cysteine 116 in the active site of the antitumor enzyme L-methionine  $\gamma$ -lyase from *Pseudomonas putida*. *Biosci Biotechnol Biochem*. 2008;72(7):1722–1730.
56. Soltis DE, Soltis PS. Applying the bootstrap in phylogeny reconstruction. *Stat Sci*. 2003;18(2):256–267.
57. Inoue H, Nojima H, Okayama H. High efficiency transformation of *Escherichia coli* with plasmids. *Gene*. 1990;96(1):23–28.
58. Lo R, Turner MS, Barry DG, Sreekumar R, Walsh TP, Giffard PM. Cystathionine gamma-lyase is a component of cystine-mediated oxidative defense in *Lactobacillus reuteri* BR11. *J Bacteriol*. 2009;191(6):1827–18237.
59. Tchong S-I, Xu H, White RH. L-cysteine desulfidase: an [4Fe-4S] enzyme isolated from *Methanocaldococcus jannaschii* that catalyzes the breakdown of L-cysteine into pyruvate, ammonia, and sulfide. *Biochemistry*. 2005;44(5):1659–1670.
60. Aitken SM, Lodha PH, Morneau DJK. The enzymes of the transsulfuration pathways: active-site characterizations. *Biochim Biophys Acta*. 2011;1814(11):1511–1517.
61. Nakayama T, Esaki N, Lee W-J, Tanaka I, Tanaka H, Soda K. Purification and properties of L-methioninase gamma-lyase from *Aeromonas* sp. *Agric Biol Chem*. 1984;48:2367–2369.
62. Kreis W, Hession C. Isolation and purification of L-methionine- $\alpha$ -deamino- $\gamma$ -mercaptomethane-lyase (L-methioninase) from *Clostridium sporogenes*. *Cancer Res*. 1973;33(8):1862–1865.

**Research and Reports in Biology****Dovepress****Publish your work in this journal**

Research and Reports in Biology is an international, peer-reviewed, open access journal publishing original research, reports, editorials, reviews and commentaries on all areas of biology including animal biology, biochemical biology, cell biology, ecological studies, evolutionary biology, molecular biology, plant science and botany. The

manuscript management system is completely online and includes a very quick and fair peer-review system. Visit <http://www.dovepress.com/testimonials.php> to read real quotes from published authors.

Submit your manuscript here: <http://www.dovepress.com/research-and-reports-in-biology-journal>