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A Functional Genomics Approach To Analyze Two Methionine-[Gamma]-Lyase Orthologs In "Ferroplasma Acidarmanus" Using In Vitro Enzyme Assays

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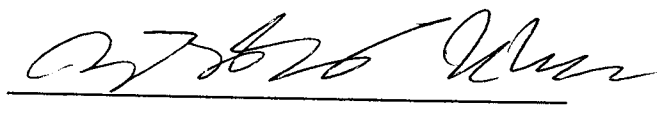

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A Functional Genomics Approach to Analyze Two Methionine-γ-Lyase Orthologs in "Ferroplasma acidarmanus" Using in vitro Enzyme Assays

(TITLE)

BY

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THESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

Master's in Biological Sciences

IN THE GRADUATE SCHOOL, EASTERN ILLINOIS UNIVERSITY
CHARLESTON, ILLINOIS

2011

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A Functional Genomics Approach to Analyze Two Methionine-γ-Lyase Orthologs in "*Ferroplasma acidarmanus*" Using *in vitro* Enzyme Assays

Master's Thesis Research, Fall 2009 - Summer 2011

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List of figures

List of tables

Page no.

Abstract

Functional genomics predicts that fer1 genome has two candidate genes for the production of two methionine-γ-lyase (MGL) orthologs, which are thought to be pivotal for the production of methanethiol. These orthologs show a high level of protein sequence identity (~35%) and similarity (~60%) when compared to known MGLs. In addition, ClustalW2 alignment of fer1 MGL orthologs and other known MGLs showed a high level of similarity in amino acid residues, which are involved for the stabilization of 3D structure of MGL and in forming active site for the substrates. To confirm the predictive functions of these two orthologs, enzymatic assays using fer1 cell lysate as well as *E. coli* BL21 (DE3) cell lysates having these orthologs were carried out. The break down products of L-methionine by MGL includes methanethiol and and/or α-keto butyrate. In the first assay, DTNB (5, 5'-dithiobis-(2-nitrobenzoic acid) was used to detect compounds containing thiol (-SH) group, such as methanethiol. The second assay uses MBTH (3-methyl-2-benzothiazolinone) to detect the presence of alpha-keto acids. In DTNB assays, fer1 lysate produced thiol-containing compounds in a time-temperature dependent reaction (N=4). The lack of activity in heat-treated lysate controls showed that the reaction was enzymatic. Moreover, these activities were dependent on both pyridoxal phosphate (PLP), a coenzyme of previously reported MGLs from other sources, and on the addition of L-methionine as substrate. Using the fer1 cell lysates, the highest specific activity (0.14 μmol/mg/min, N=5) was detected at pH 4.0 in MBTH assay. As with the L-methionine, substrate specificities of fer1 lysate were studied. D-methionine, L-cysteine, L-cystathionine and DL-homocysteine were degraded to 97.5%, 130.2%, 88.9%, 152.5% respectively of the level of activity on L-Met. In order to study individually, these MGL candidates have been cloned in pET21b vectors and transferred in E. coli BL21 (DE3). The cell lysates of *E. coli* expressing these orthologs were tested for MGL

activities. For MBTH and DTNB assays, the highest specific activity of MGL1, 0.19 μmol/mg/min (N=4 and N=2, respectively) was detected at pH 4 and at pH 5, respectively.

Introduction

According to the current classification scheme, Archaea, Bacteria and Eukarya constitute the three domains of life, where Archaea have been incorporated as the third domain by Carl Woese *et al* in 1977 (1). Archaea are unicellular, prokaryotic microorganisms. Although they share some bacterial (energy generation and gene organization) and eukaryotic (DNA and RNA polymerases) traits, they possess many unique characteristics, such as their reliance on ether lipids in their cell membranes, unique rRNA structure etc. (2, 3, 4).

According to rDNA sequencing, there are a few phyla under the domain Archaea. Most of the cultivable and well-investigated species of Archaea have been included among the two main phyla: Euryarchaeota and Crenarchaeota. Two other phyla have been tentatively created. A species, *Nanoarchaeum equitans,* has been given its own phylum, the Nanoarchaeota (5). A new phylum, Korarchaeota, has also been proposed, which contains a small group of unusual thermophilic species that shares features of both of the main two phyla, but is more closely related to the Crenarchaeota (6). Although some archaea were isolated from normal environments, most of them are found in extreme habitats, such as hot springs, salt lakes, hydrothermal vents, acid mine drainage, etc. (7).

These extremophiles not only survive, but also require these extreme conditions for their propagation. Therefore the study of archaea may help us better understand biology at extreme conditions. Since these extreme environments are also comparable to the conditions of early Earth and some extraterrestrial locations such as Mars, study of the physiology and molecular biology of archaea might shed light on the evolution of life and astrobiology (8). In addition,

extremophiles could be a potential source of novel enzymes with many biotechnological applications, which are capable of performing catalytic activities under hostile conditions.

Microorganisms, which require very low pH, typically less than pH 4, for growth are called acidophiles. In the evolutionary tree of life, there are two groups of acidophilic archaea including the orders Sulfolobales and Thermoplasmatales (Figure 1). The former one belongs to the Crenarchaeota branch of archaea that typically contains polyextremophilic members (8).

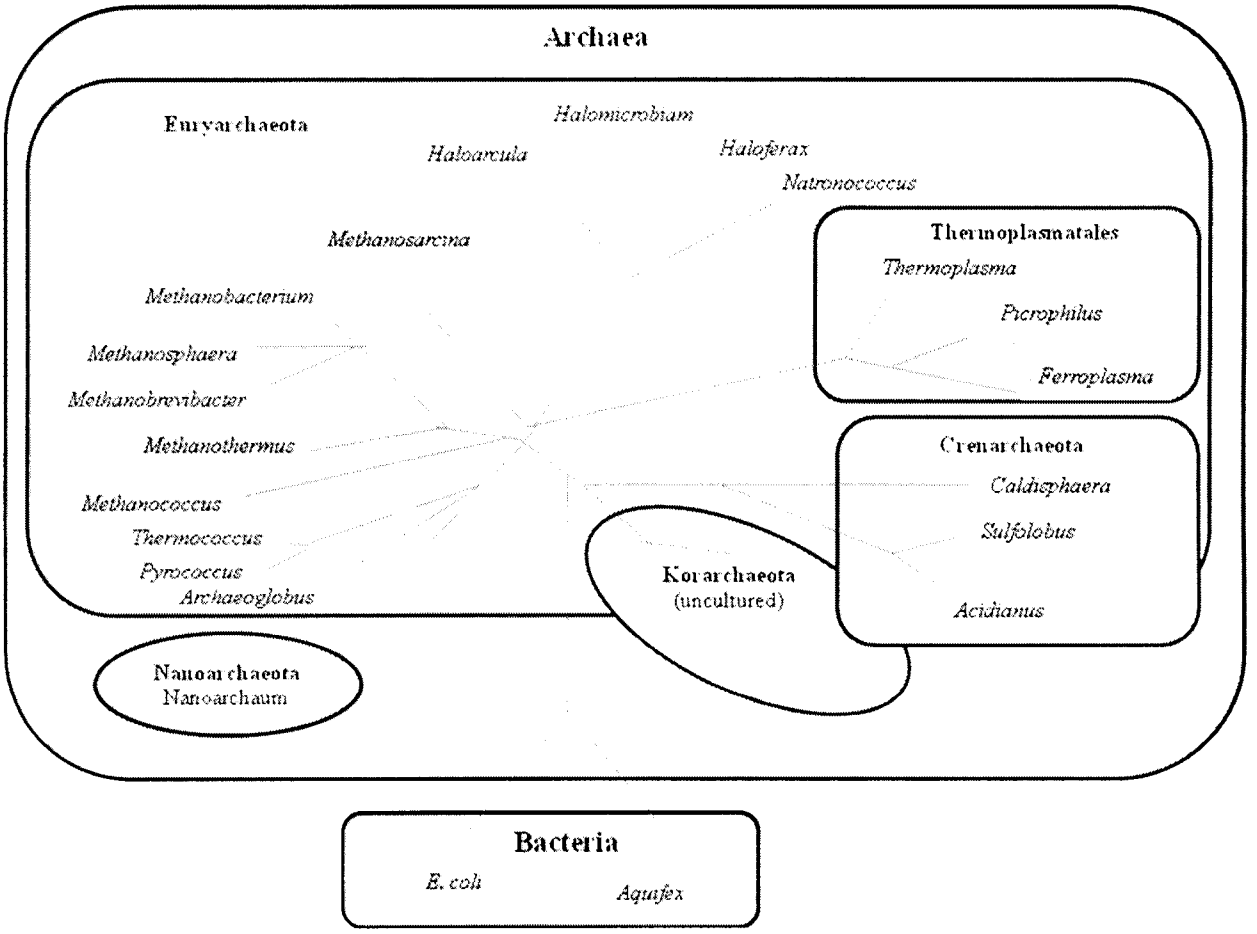


Figure 1 Originally the Bacteria and Archaea were thought to be one large diverse family of prokaryotes until Carl Woese and others investigated the evolutionary tree of ribosomal RNAs and found that there were three distinct founding evolutionary domains, then named Bacteria, Archaea along with the Eukaryota (9).

These members are hyperthermophilic, with optimum growth temperature 70-90°C, and acidophilic, with optimum growth pH 1.5 - 4.5. Most of the Sulfolobales members are capable of oxidizing elemental sulfur and producing sulfuric acid (8). The latter group, Thermoplasmatales, members of which populate highly acidic, hot and metal-rich environments, belongs to Euryarchaeota branch (10, 11). This order is also known for having single layer membrane and no cell wall (5). This group contains three families with each having one genus. One of the members is *Ferroplasma,* which has two species, namely "*F. acidarmanus*" strain fer1 and *F. acidiphilum.* Although fer1 is phylogenitically similar to *F. acidiphilum*, there are many physiological dissimilarities between them (12). Both fer1 and *F. acidiphilum* require yeast extract for growth but fer1 can also grow on yeast extract heterotrophically, whereas *F. acidiphilum* cannot. fer1 grows between pH 0.0 and 2.5 with an optimum of 1.2. On the other hand, *F. acidiphilum* grows between pH 1.3 and 2.2 with an optimum of 1.7 (12).

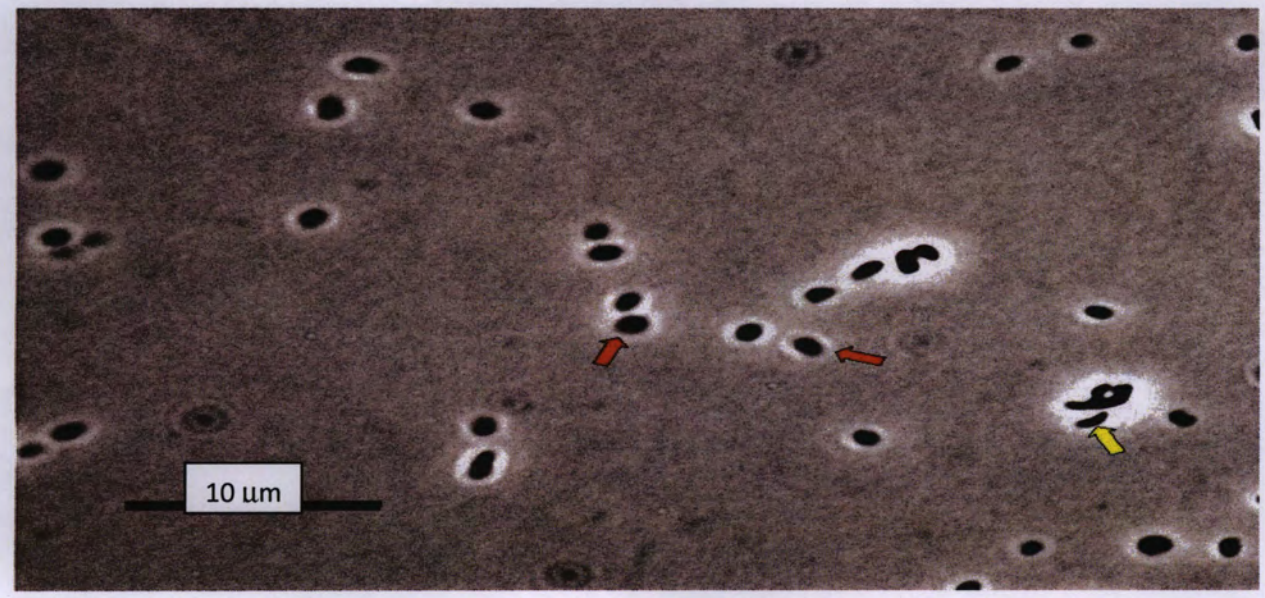


Figure 2 Pleamorphic archaeon, "*Ferroplasma acidarmanus*" strain fer1 (13). fer1 cells were observed under 1,000x magnification on a Nikon Eclipse TE300 inverted microscope (phase contrast). Images were recorded electronically using a digital camera (Hamamatsu, Hamamatsu-City, Japan; model #C4742-95-10NR) and a personal computer equipped with MetaMorph software v. 4.5r6 (Universal Imaging Corporation, West Chester, PA).

The cells of fer1 are pleomorphic, appearing in a multiple number of shapes (Figure 2). A few of the shapes observed are irregular cocci (red arrows) and comma shape (yellow arrow). Cells range in size from 0.48 to 1.08 μm long and 0.48 to 0.85 μm wide. *"F. acidarmanus"* grows much slower with a generation time of ~2 days when grown at pH 1.0, whereas *Escherichia coli* in the laboratory which has a generation or doubling time of 15 to 20 min (13). One of the important traits of fer1 is that its cytoplasmic membrane is tetra-ether linked (14). fer1 is a facultatively anaerobic archaeon and can be grown both chemolithotrophically and heterotrophically (15, 16).

"*F. acidarmanus*" strain fer1 was isolated from the Richmond mine at Iron Mountain, California (12). This strain of *Ferroplasma* is estimated to be 85% of the biofilm population of that site (Kaspar, unpublished data). This site has a temperature of 35-50°C and a pH of -3.7 to 1.0, the lowest pH ever reported (17). The low pH conditions at this mountain dissolve many metal ions, making the solution metal rich. For example, iron accounts for over 111 g/L, and copper, arsenic, zinc, cadmium all were measured in the tens to tenths of grams per liter range (18, 19). The tetraether-linked cell membrane of fer1 contributes to the defense against these high ion concentration gradients, including proton gradients (14). The low pH in the acidic mine water is due to the oxidative dissolution of pyrite ore, where ferric ions (Fe^{3+}) oxidizes pyrite ore (FeS_2) in the presence of water. This reaction produces ferrous ions (Fe^{2+}) and sulfuric acid, and reaches equilibrium when the concentrations of the two iron species become equal. By oxidizing Fe^{2+}, fer1 is able to shift the equilibrium to the right side to increase the amount of sulfuric acid.

$$FeS_2 + 14Fe^{3+} + 8H_2O \xrightarrow[\text{microbes}]{} 15Fe^{2+} + 2SO_4^{2-} + 16H^+$$

Figure 3 The oxidative dissolution of pyrite continues in the presence of microbes.

Therefore, fer1 plays a major role in the breakdown of pyrite and formation of acid mine drainage (AMD), which is detrimental to almost all life forms (Figure 3; 14). The ability of fer1 to oxidize ferrous ions contributes to the global sulfur and iron cycle. At Iron Mountain, fer1 accelerates the rate of pyrite dissolution through the regeneration of ferric ions, where much immobilized sulfur becomes accessible to the living organisms, which would otherwise be impossible in such an acidic and metal rich habitat by other organisms (12). In addition, fer1 could be an ideal source of identifying and cloning of acid tolerant enzymes, which would have applications in industrial processes such as bioleaching and biomining to recover valuable metals such as gold and copper from low grade ores. Moreover, methanethiol, a byproduct of fer1 growth, is the predominant source of assimilated sulfur for marine microbes and can act as a cloud nucleating particle, which in turn can contribute to the global weather such as amount of precipitation, reducing the radiation from the sun (20).

Sulfur is an essential element to all forms of life. It could be taken up into cells as inorganic compounds such as sulfate, sulfite and sulfide or as sulfur containing amino acids (SAA) i.e., cysteine and methionine (21). In mammals, SAAs are mainly degraded via oxidative cysteine catabolism, leading to the production of hypotaurine, taurine, pyruvate, and sulfate (22). In bacteria and plants having a methionine biosynthetic pathway, *in vitro* assays have showed that cysteine is also degraded by cystathionine-γ-lyase (EC. 4. 4. 1. 8) to thiocysteine, pyruvate, and ammonia (23, 24). On the other hand, a few groups of organisms, mainly microorganisms, have a unique pathway, in which SAAs are converted to α-keto acids, ammonia, and volatile thiols by methionine-γ-lyase (MGL, EC. 4. 4. 1. 11) (21). Sulfate, the most oxidized form of sulfur, is utilized by plants, fungi and prokaryotes by assimilatory sulfate reduction pathway. In comparison, sulfate-reducers, mainly bacteria, reduce sulfate to sulfide to conserve energy by a

dissimilatory sulfate reduction pathway (21). Consequently, dissimilatory sulfate reducers require high sulfate, e. g., freshwater bacteria require sulfate ranging 100-250 μM and marine microbes require about 25 mM (25, 26). The acidic water at Richmond mine from where fer1 has been isolated has sulfate concentrations ranging from 657-786 mM and a previous study reported that fer1 requires over 100 mM of sulfate with an optimum of 350 mM for growth, the highest sulfate requirements by a microbe known to date (16, 25). Compared to the sulfate requirements of other sulfate reducers, fer1 sulfate requirements have drawn much attention and have demanded scientific investigation into it. Previous *in vitro* study revealed that fer1 can take up sulfate into cytoplasm, which then can be funneled into L-methionine and/or L-cysteine that are ultimately catabolized into methanethiol, a volatile organic sulfur compound (VOSC) (26, 27). The functional genomics study also predicts that fer1 genome has two candidates of MGL and it is thought that these are involved in the production of methanethiol from SAAs. These candidates are called MGL orthologs, as they are predicted to have similar function in methionine-cysteine metabolism but their evolutionary history is still unknown. However, there are multiple possible routes for the production of methanethiol in fer1; one of them does not even require MGL (26). Two of them are MGL dependent where L-methionine and L-cysteine are broken down to methanethiol, and within the third one, which is MGL independent, sulfate is reduced down to sulfide that is then methylated to form methanethiol. This study will focus mainly on the characterization of these MGL candidates (MGL1, MGL2) in the degradation of L-methionine and L-cysteine, and some of their analogs.

Enzymological properties of a typical MGL

Methionine-γ-lyase is ubiquitous almost in all forms of life, including bacteria, fungi, plants and animals, except mammals (28). It can carry out an α, γ-elimination reaction using L-methionine and its derivatives such as L-homocysteinie, L-ethionine and L-selenomethionine to produce α-keto butyrate, methanethiol and ammonia (Figure 4; 21). It can also catalyze an α, β–elimination reaction using L-cysteine and its analogs such as s-methyl-L-cysteine as substrates to produce pyruvate, ammonia and hydrogen sulfide (21).

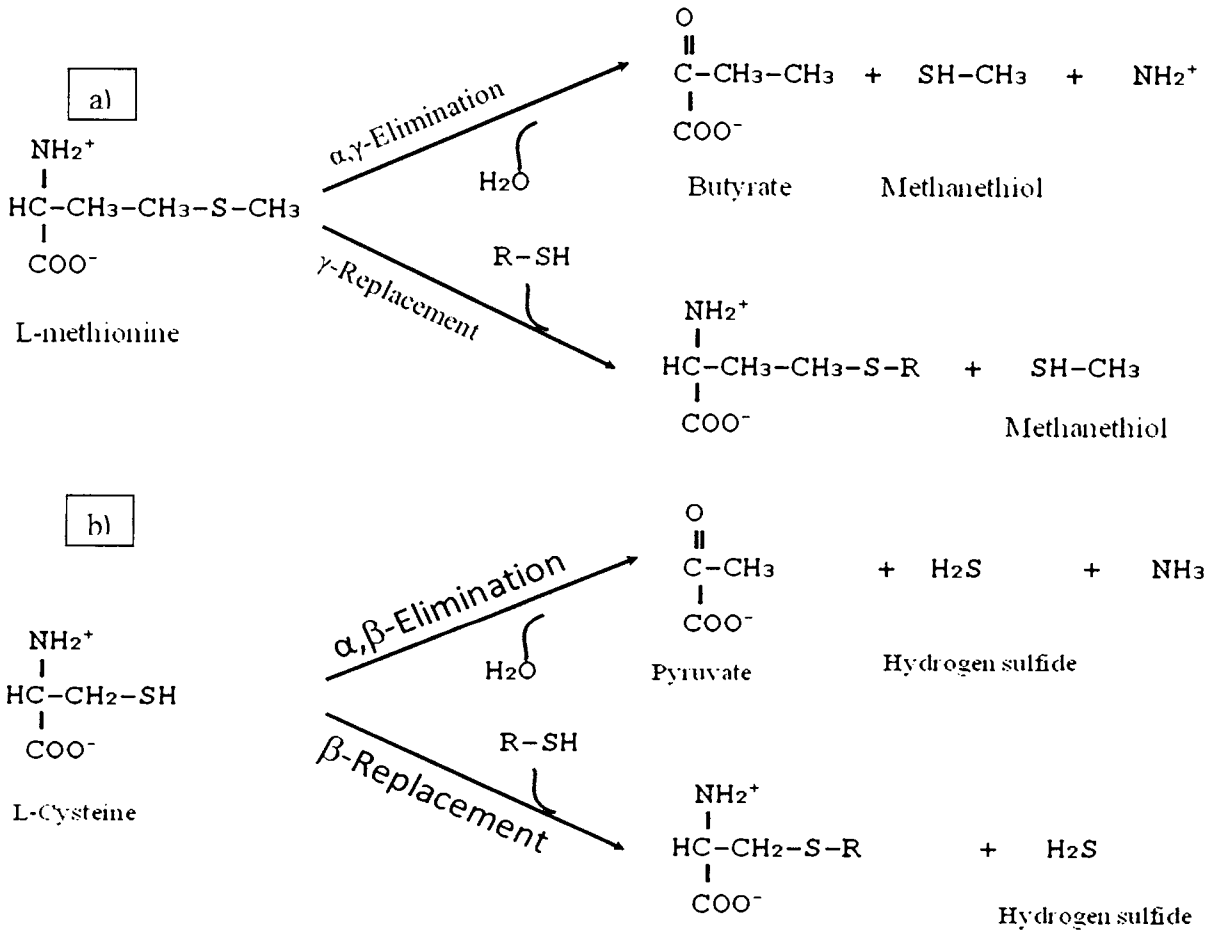


Figure 4 The dual catalytic roles of typical methionine-γ-lyases on L-methionine: α, γ-elimination and γ-replacement (a), and on L-cysteine: α, β-elimination and β-replacement (b) (21).

Alternatively, this enzyme can carry out β– / γ-replacement reactions, where the sulfur or oxygen atom at the β– or γ-position of the substrate is substituted with a reactant having thiol group (21). In enzymology, the rate of a reaction catalyzed by an enzyme is typically described by specific activity i.e., the production of a product in micromolar concentration per milligram of enzyme per min of time at a specific temperature, usually 35°C (21). The unit of specific activity is thus μmol/mg/min.

Biochemical properties of MGLs

Depending on the origin of the enzyme, the nucleotide sequences of the open reading frame for MGL range from 1,170-1,179 base pairs and the number of amino acids varies from 389-441 with predicted molecular weights 42-45 KDa (21, 28). According to the National Center for Biotechnology Information (NCBI) database, ferl MGL1 is 381 amino acid long with a molecular weight of 42.215 KDa; ferl MGL2 is 362 amino acid long with a molecular weight of 39.749 KDa (29). The physical properties of MGLs from different microorganism known to date are relatively identical (28). For instance, pH optima range from 7.0-8.0, pH stability from 6.0-9.0 and optimum temperature is around 30°C (28). Typically, MGL is a tetrameric enzyme, with each of the tetramers containing one pyridoxal phosphate (PLP), a coenzyme form vitamin B_6 (21). The active MGL tetramer is composed of two sets of dimers that are tightly associated and the active site is at the interface of two neighboring subunits (30, 31).

The amino acid residues involved in the substrate specificity and catalysis as well as the spacing of these residues are highly conserved among all MGL isozymes (31). For example, sequence analyses of *Pseudomonas putida* and *Entamoeba histolytica* MGLs revealed that a group of six

amino acid residues, Tyr^{59}, Arg^{61}, Tyr^{114}, Cys^{116},Lys^{240}, Asp^{241} and Tyr^{53}, Arg^{55}, Tyr^{108}, Cys^{110}, Arg^{233}, Lys^{234} respectively, are involved in the substrate binding pocket and catalysis (21). One line of evidence showed that Cys^{116} of *P. putida* (corresponding to Cys^{110} of *E. histolytica* MGL) MGL takes part in the unique enzymatic reactions of MGL (31). The mutational studies of *E. histolytica* and *Trichomonas vaginalis* MGL isozymes also demonstrated that the corresponding cysteine residue directly contributes to the substrate specificity (reviewed in 21). In addition, a group of four amino acids (Met^{90}, Asp^{187}, His^{207} and Gly^{215} in *P. putida*) are found to be highly conserved, among all the PLP-dependent enzymes and are considered to be enzyme stabilizers (Table 3; 32).

Objective of the study

Bioinformatics tools can predict, but not confirm, the molecular function of enzymes based on sequence analyses. After getting the prediction of the fer1 MGL orthologs, this study was then aimed to provide functional confirmation of the predictions using L-methionine, L-cysteine and some of their derivatives and analogs as substrates.

To analyze the byproducts, biochemical approaches were adopted to accomplish the following objectives regarding the involvement of two MGL orthologs in sulfate metabolism in fer1: (1) to determine the *in vitro* optimum parameters for the reactions catalyzed by fer1 MGLs, (2) to validate the functional genomics predictions on fer1 MGL orthologs, (3) to characterize them biochemically in the purified forms, (4) to individually determine the reaction type catalyzed by two orthologs, (5) to determine the substrate specificity of the orthologs, and (6) to determine the overall role of these orthologs in sulfate metabolism in fer1.

Method and Materials

Bioinformatics and functional genomics

Predictions of fer1 MGL orthologs and their functions were conducted using the following tools: Kyoto Encyclopedia of Genes and Genomes (KEGG), Integrated Microbial Genomes (IMG), ClustalW2 and Basic Local Alignment Search Tool (BLAST). The KEGG pathway and IMG databases were used to derive the methionine-cysteine metabolic pathway in fer1 and the roles of two fer1 MGL orthologs in the metabolic process. The BLAST tool was employed to obtain the overall similarities and identities of fer1 MGL orthologs with other known MGLs. A comprehensive amino acid alignment of fer1 MGLs and other MGLs were constructed using ClustalW2. The phylogenetic tree based on amino acid compositions of MGLs was also obtained using ClustalW2.

In these analyses, the sequences of MGL were obtained from the NCBI including fer1 MGL1 (GenBank Accession Number ZP_05571061) and fer1 MGL2 (GenBank Accession Number ZP_05570711) as well as MGLs from different organisms including Gram-positive bacteria such as *Bacillus halodurans* c-125 (GenBank Accession Number BAB04518) and *Brevibacterium linens* (GenBank Accession Number AAV54600); Gram-negative bacteria such as *Pseudomonas fluorescens* SBW25 (GenBank Accession Number YP_002872146), *P. putida* W619 (GenBank Accession Number ACA71420); protozoa such as *Entamoeba histolytica* (GenBank Accession Number BAC75877) and plant such as *Arabodopsis thaliana* (GenBank Accession Number NP_176647).

Media composition and growth conditions of "*Ferroplasma acidarmanus*" strain fer1

"*Ferroplasma acidarmanus*" strain fer1 was grown routinely in chemolithotrophic medium called "mfer" medium. This medium was prepared as previously described (18). fer1 was grown microaerobically at pH 1.0 and 35°C in mfer. At first basal medium of mfer1 was prepared. The basal medium is comprised of the following inorganic salts per liter: 800 mg $(NH_4)_2SO_4$, 800 mg $Ni(NH_4)_2(SO_4)_2.6H_2O$, 400 mg KH_2PO_4, 160 mg $MgSO_4.7H_2O$, 85 mg $Na_2MoO_4.2H_2O$, 70 mg $ZnCl_2$, 31 mg H_3BO_3, 10 mg $MnCl_2.4H_2O$, 10 mg $CoCl_2.6H_2O$. In 900 ml of deionized water, all the ingredients of basal medium were dissolved and adjusted to pH 1.0 using concentrated H_2SO_4 (10 N) as acidulant. The volume was adjusted to 1000 ml by adding deionized water. The basal salts solution was then transferred into two 1000-ml bottles with each bottle having 500 ml and autoclaved for 15 min at 121°C and 15 lb/in^2.

From each sterilized medium, 100 ml was then transferred into a small flask. Yeast extract, 0.5 g and $FeSO_4.7H_2O$, 10 g were then added to basal medium and sterilized by filtration using a 0.2-μm pore membrane filter. Following the transfer of 400 ml basal medium and 100 ml of $FeSO_4.7H_2O$ and yeast extract solution into a sterile 2.5-L flask, the medium was inoculated with 10 ml of 3-week old fer1 culture. The mouth of the 2.5-L flask was then masked with sterile aluminium foil and was then incubated at 35°C for growth without shaking.

To maintain the growth of fer1, 10 ml of 3-week old fer1 culture was transferred into a newly made mfer medium of 500 ml every third week.

Preparation of fer1 lysates for enzymatic studies

A 500 ml volume of 20-25 day old fer1 culture was equally transferred into 4 centrifuge bottles. The bottles were then centrifuged at 6500*g* for 10 minutes. Using 1 M NaCl (pH 1.0) buffer, cells were washed twice and then resuspended in a total of 500 µl buffer before being transferred into a sterile 1.5-ml microcentrifuge tubes and centrifuged at 13,300*g* for 10 minutes. After discarding the supernatant, 700 µl of sterile deionized water was added to the tube and discarded without disturbing the pellet to minimize carry-over of acidic medium to the following steps. Using 800 µl of the Na_2PO_4 buffer (0.2 M Na_2HPO_4, pH 9.2), the centrifuged cells were resuspended and incubated at room temperature for about 25 minutes to lyse the cells. After the incubation, the microcentrifuge tube was centrifuged at 13, 000*g* for 10 min at room temperature. The supernatant was then transferred to a new autoclaved 1.5-ml microcentrifuge tube without disturbing the cell debris. The supernatant was then placed on ice until further use.

To genearate a negative control, a portion of fer1 lysate was denatured by heat treatment at 100°C for 30 min.

Quantification of soluble proteins in a 96-well microplate

Using Coomassie Plus (Bradford) Assay Kit (Thermo Scientific, Morris Plains, NJ), total soluble protein in the supernatant was quantified. At the beginning of protein quantification, different concentrations of bovine serum albumin (BSA) protein standards (0, 50, 100, 150, 250, 500, 750, 1000 µg /ml) were prepared using 100 mM tris-HCL (pH 8.0) as diluent. To quantify the total protein in fer1 lysate, 10 µl of the supernatant, protein standards and Na_2HPO_4 (0.2 M Na_2HPO_4, pH 9.2) as negative control were added to the designated wells of a 96-well plate. According to

the protocol, 300 µl of the Coomassie Plus Reagent was added to each well, incubated for 15 minutes at room temperature and the plate was then read at 562 nm by a spectrophotometer. A standards curve of protein standards was derived on Excel (Microsoft, Redmond, WA).

Enzymatic assays using fer1 cell lysate

The α, γ-elimination reaction using L-methionine as substrate catalyzed by MGL is as follow:

L-Methionine ---------- MGL ----------> Methanethiol + α-Keto butyrate + ammonia

The products of the reaction, thiol group (-SH) containing methanethiol and keto group (=CO) containing α-keto butyrate, were determined quantitatively by two spectrophotometric assays, 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and 3-methyl-2-benzothiazolinone (MBTH), respectively.

DTNB assay of methionine-γ-lyase: Determination of thiol group

Principle

DTNB reagent (Thermo-Scientific, Rockford, IL) reacts with free thiol group, e.g., methanethiol that is a byproduct of an MGL catalyzed reaction, in a reducing atmosphere and produces a yellow compound 5-Mercapto-2-nitrobenzoic acid, which can be spectrophotometrically measured at 412 nm. The millimolar extinction coefficient of this reaction product is 12.74 cm^{-1} (33).

Reagent

1 mM DTNB in 2.5 mM sodium acetate solution (pH 2.5). It was stored at 4°C.

$[-SC_6H_3(NO_2)CO_2H]_2$ + R-SH = 2 OC(=O)C1=CC(S)=CC=C1[N+]([O-])=O + R-S-S-R

DTNB 5-Mercapto-2-nitrobenzoic acid

Figure 5 The oxidation reaction of thiol-group and DTNB in DTNB assay. In the presence of thiol compounds colorless DTNB is converted into a yellow 5-Mercapto-2-nitrobenzoic acid, which absorbs light maximally at 412 nm.

Procedure

In a sterile 1.5-ml microcentrifuge tube, 20 µl of 10 mM PLP and 40 µl of fer1 cell lysate were added, mixed and incubated at 35°C for 5 minutes. A negative control was also included that contained 40 µl of 0.2 M Na_2HPO_4 (pH 9.2) instead of cell lysate. After the short incubation, 5 µl of 1 mM DTT, 835 µl of Reagent A (from MBTH assay) and 100 µl of DTNB reagent were added, vortexed and incubated at 35°C for 25 minutes.

The test and control solutions were transferred to suitable cuvettes and recorded the A_{412} using spectrophotometer.

Calculation

Specific activity = A_{412} x (Total volume/Sample volume) / (13.6 x duration of incubation x protein concentration)

Total Volume/Sample volume = 1 ml / 0.04 ml = 25; Millimolar extinction coefficient of this reaction product = 13.6 cm^{-1}.

MBTH Assay of methionine-γ-lyase: Determination of α-keto group

Principle

α-keto acid, a product of an MGL catalyzed reaction reacts sensitively with nucleophilic MBTH reagent (Sigma-Aldrich, St. Louis, MO) to form a complex azine derivative. Characteristically, this derivative gives an ultraviolet absorption that is highest at 320 nm. The millimolar extinction coefficient of the azine derivative is 15.74 cm^{-1} (34).

Reagents

A. 50 mM tris-HCl buffer with 50 mM L-methionine; Reagent A was made in a wide range of pHs (1-10) and substrates, such as D-methionine (50 mM), L-cysteine (50 mM), L-cystathionine (5 mM), and DL-homocysteine (10 mM), in addition to L-methionine. They were stored at 4°C.
B. 50% (w/v) trichloroacetic acid solution (TCA). It was stored at 4°C.
C. 1 M sodium acetate buffer, adjusted to pH 5.0 at 25°C with HCl (6 N). It was stored at room temperature.
D. 0.1% (w/v) 3-methyl-2-benzothiazolinone hydrazone (MBTH). It was stored at -20°C to avoid the formation of breakdown products.

$C_8H_9N_3S \cdot HCl$ + =CO = Complex azine compound

MBTH

Figure 6 Reaction of an α-keto group (formaldehyde) and MBTH in MBTH assay (35). The complex compound that is formed absorbs light maximally at 320 nm.

Procedure

In step 1, 40 µl of fer1 cell lysate and 20 µl of 10 mM PLP were mixed in a 15-ml conical tube and incubated for 5 minutes at 35°C. Thereafter, 940 µl of Reagent A was added, vortexed and incubated at 35°C for 25 minutes. A negative control was also included that contained 40 µl of 0.2 M Na_2HPO_4 (pH: 9.2) instead of cell lysate. Following incubation, 100 µl of Reagent B was added and mixed by inversion few times to stop the reactions. In step 2, 2 ml of Reagent C and 0.8 ml of D were added and mixed by inversion few times. Then the tubes were incubated at 50°C for exactly 30 minutes and then at room temperature for another 30 minutes. The test and control solutions were transferred to suitable cuvettes and the A_{320} recorded using spectrophotometer.

Calculation

Specific activity = A_{320} x (Total volume/Sample volume) / (15.74 x duration of incubation x protein concentration)

Total Volume/Sample volume = 3.8 ml / 0.04 ml = 95

Millimolar extinction coefficient of the azine derivative = 15.74 cm^{-1}

Validation of enzymatic assays

To validate the DTNB assay, five different concentrations of a thiol group containing compound, L-cysteine were made using sterile water as a diluent. The concentrations were 0.1 mM 0.2 mM, 0.5 mM, 1 mM and 2 mM. Sterile water was included in this experiment as blank.

To validate the MBTH assay, five different concentrations of a keto group containing compound, pyruvate were made using sterile water as a diluent. The concentrations were 0.1 mM 0.2 mM, 0.5 mM, 1 mM and 2 mM. Sterile water was included in this experiment as blank.

fer1 *mgl* orthologs in vector pET21b

An *E. coli* B strain, BL21 (DE3) was used as host for a T7 promoter driven vector pET21b carrying fer1 MGL orthologs. Coding sequences for fer1 *mgl*1 and *mgl*2 were inserted into pET21b vector using NdeI and NotI, placing the sequences under the control of an IPTG-inducible promoter to create a translational fusion with poly his at the C-terminal. The *E. coli* cell lines containing MGL1 and MGL2 in vectors were called FRIK2601 and FRIK2609, respectively. The strain that does not have any insert in the vector is FRIK2600.

Confirmation study of the fer1-*mgl* inserts in pET21b vectors

Preparation of cell lysates

Three *E. coli* BL21 (DE3) cell lines, FRIK2600, FRIK2601 and FRIK2609, were grown in 4 ml Luria-Bertuni (LB) media supplemented with 100 μg/ml ampicillin, as appropriate, for about 18 hours at 150 rpm at 35°C. For all types of cell lines, a few colonies were used as inocula. After incubation, 1.5 ml of each type of cell lines was used prepare the cell lysate according to the protocol given by PureYield™ Plasmid purification kit (Promega, Madison, WI) .

Plasmid DNA Digestion and Electrophoresis

In an autoclaved microcentrifuge tube, the following reagents are mixed for Master Mix preparation (45 μl). For double digestion, the following reagents were mixed: 10 μl BSA, 10 μl

buffer, 0.5 µl NotI, 0.5 µl NdeI and 39 µl deionized water. For single digestion the following reagents were mixed: 10 µl BSA, 10 µl buffer, 0.5 µl NotI, and 39.5 µl deionized water.

15 µl of master mix and 10 µl of plasmid DNA were mixed in an autoclaved microcentrifuge tube and incubate at 35° C for 2 hours.

Electrophoresis

0.8% (w/v) of 40 ml agarose gel with 2 µl of GelRed™ (Phenix Research Product, Candler, NC) was prepared. 15 µl of digested plasmid DNA with 2 µl of 6x loading dye was mixed and added to wells of the gel. Undigested DNA of each type and 5 µl ladder (New England BioLabs, Ipswich, MA) as marker were also included in the gel. The gel was then run for 120 minutes at 90 volts and viewed under UV light. 1x TBE buffer was used as running buffer.

Long term storage of cultures

The cultures that had the correct inserts were stored at –70°C. At first, the cultures were grown at 35°C and 150 rpm in 3 ml LB medium supplemented with 100 µl/ml ampicillin until the OD_{600} reached 0.4. Then 900 µl of culture was mixed thoroughly with 100 µl of sterile glycerin in a cryotube and stored at –70°C.

Preparation of culture lysates for enzymatic studies

Determination of the growth curves of E. coli BL21 (DE3) cultures and Citrobacter freundii, a well known MLG producer

Determination of growth curve is very important as the mid-point of exponential phase is considered as the appropriate time for induction. At first, FRIK2601 and FRIK2609 cultures containing the pET21b vector were grown in 3 ml LB media with 100 µl /ml ampicillin selection for about 18 hours at 35°C and 150 rpm in 15-ml conical tubes. FRIK2600 culture, which does not have the vector, and *Citrobacter freundii* were also grown in LB without ampicillin at the same manner. The cultures were then diluted to 1,000 fold in 100 ml LB media in 250-ml erlenmeyer flasks. Ampicillin selection was also included for FRIK2601 and FRIK2609 cultures.

Starting from time 0 hour, OD_{600} values of each culture were recorded every hour until growth reached the stationary phase. LB medium without culture was included in this experiment as a negative control.

Cultures preparation and protein expression

Cultures were grown from small scale to large scale in a way similar to growth curve preparation, except that the three *E. coli* BL21 (DE3) cultures in 100 ml volumes were induced by IPTG (1 mM) at the mid-exponential phase. At the same time, L-methionine (1 mM) was added to the medium containing *Citrobacter freundii*. During the course of induction for 3 hours, the temperature was dropped down to 20° C.

Concentration of cell volume by centrifugation

100 ml of cultures were then transferred into 200-ml centrifuged bottles. Prior to centrifugation, each bottle was set to the same weight by adding and/or removing a few milliliter of culture. The bottles were then centrifuged at 6,500*g* for 10 minutes. After washing twice with 100 mM tris-HCL (pH 8.0) buffer and centrifuging, 400 µl of the same buffer was added to each bottle to resuspend the culture thoroughly. Thereafter, resuspended culture was pipetted, transferred to appropriately labeled 1.5-ml microcentrifuge tube and then kept on ice until use.

Preparation of cell lysates using FastBreak™ Cell Lysis Reagent/DNase I

10x FastBreak™ Cell Lysis Reagent (Promega Corporation, Madison, WI) contains a proprietary nonionic detergent to facilitate lysis of bacterial cells. FastBreak™ Reagent/DNase I was prepared according to the standard protocol, which was provided in the kit, in 1.5-ml microcentrifuge tube and then transferred in 200 µl volume in 500–µl microcentrifuge tube. This solution can be stored at -20°C for 6 months and is stable for up to 5 freeze-thaw cycles.

In each 800 µl concentrated culture and 800 µl of 100 mM pH 8.0 tris-HCL (as a negative control), 200 µl of FastBreak™ Reagent/DNase I solution and 10 µl of 100x Halt™ Protease Inhibitor Single-Use Cocktail EDTA-Free (Thermo Scientific, Morris Plains, NJ) were added to lyse the cells and to avoid the protein of interest being degraded simultaneously. The tube containing the buffer but no culture was included as negative control in this experiment. The tubes were then incubated at 15°C for 30 minutes with shaking at 150 rpm.

Collection of supernatant by centrifugation

After the incubation, the tubes having cell lysates and negative control were centrifuged at 13,300*g* for 12 minutes in cold room (4°C). The supernatant supposed to have the protein of interest was then transferred to a new autoclaved 1.5-ml microcentrifuge tube without disturbing the cell debris. The supernatant was then placed on ice until further use.

Confirmation of the protein of interest (MGL)

Expression of protein is confirmed using sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE). This gel is a 10% tris-glycine Gel (PAGEr Gold Precast Gels, Lonza, Rockland, ME). 10 µl of supernatant and 10 µl of Sample Buffer (New England BioLabs, Ipswich, MA) were mixed in an autoclaved microcentrifuge tube and then the entire volume was transferred to the well. 10 µl of ladder (BIO-RAD, San Diego, CA) was also included. 1x TE buffer was used as a running buffer. The gel was then run at 200 volts until the lightest band of the ladder reached close to the end of gel. After staining and destaining of the gel using Staining and Destaining Reagents (BIO-RAD, Hercules, CA), the gel was viewed under visible light.

Quantification of soluble proteins in 96-well microplate

At first, protein standards were made as described before (pages 15-16). To quantify the total protein of the cell lysates, 10 µl of the supernatant from each culture type, protein standards and negative control were added to the designated wells. Then 300 µl of the Coomassie Plus Reagent was added to each well and mixed gently by shaking with hands for 30 seconds. The plate was the incubated for 10 minutes at room temperature. The plate was then read at 562 nm by a plate reading spectrophotometer. The values were then subtracted by the value of negative control.

Statistical analysis

Statistical analyses were carried out using Excel (Microsoft, Redmond, WA).

Results

Bioinformatics and functional genomics studies

A comparative analysis of amino acid sequences of MGLs and some other PLP-dependent enzymes involved in cysteine-methionine metabolism was conducted using bioinformatics tools. BLAST revealed that fer1 MGL orthologs share a high level of overall protein sequence identity (~35%) and similarity (~60%) with other known MGLs from a variety of sources (Table 1). Here, identity means having the same amino acid at the corresponding position and similarity means having a different amino acid but with similar property.

Source of MGL	Identity / similarity with MGL1 of fer1	Identity / similarity with MGL2 of fer1
Citrobacter freundii	39% / 60%	37% / 59%
Pseudomonas putida	37% / 57%	32% / 57%
P. fluorescens	37% / 55%	31% / 53%
Brevibacterium linens	34% / 53%	36% / 57%
Bacillus halodurans	37% / 58%	32% / 55%
Arabidopsis thaliana	34% / 53%	30% / 52%
Entamoeba histolytica	40% / 60 %	34% / 57%

Table 1 BLAST results of fer1 MGL orthologs showed the overall identity and similarity based on amino acid sequences of MGLs.

However, there are much more similarities and identities of fer1 orthologs observed with some other PLP-dependent enzymes, which are also involved in the methionine-cysteine metabolism (Table 2). For example, fer1 MGL1 shares 57% identity and 77% similarity with no gap when compared to cystathionine-β-lyase (CBL, EC 4.4.1.8) of an archaeon *Thermoplasma acidophilum*, which is evolutionarily closely related to *Ferroplasma* (Figure 1). On the other hand, fer1 MGL2 sequence best matches with cystathionine-γ-synthase (CGS, EC 2.5.1.48) of another closely related archaeon *Picrophilus torridus*, where it shares 59% identity and 76%

similarity with only 2% gap. In these BLAST analyses, expectation value or E value is a parameter that describes the number of hits obtained by random. The higher the E values the lower the chance that the hit is biologically significant and vice versa. Typically, an E value of less than e-20 is considered significant. Although overall sequence similarity predicts about the functional similarity, but not necessarily, as not all the amino acids in the protein are involved in forming internal bonds essential for stabilization and in forming active sites for a substrate and other structural analogs. As sequence identity at the key sites conserved for functional significance, therefore, it warrants specific amino acids studies among MGLs, which are crucial for the stabilization and catalysis.

Ref. Microbe	Enzyme (EC)	MGL1		MGL2	
		Similarity/ Dissimilarity	E value	Similarity/ Dissimilarity	E value
Thermoplasma acidophilum	Cystathionine –β-lyase (EC 4.4.1.8)	38% / 63%	1e-68	57% / 77%	3e-126
E. coli BL21 (DE3)	Cystathionine –β-lyase (EC 4.4.1.8)	30%/51%	4e-38	25%/50%	4e-33
Picrophilus torridus	Cystathionine –γ-lyase (EC 4.4.1.1)	51% / 69%	5e-109	37% / 60%	8e-68
Picrophilus torridus	Cystathionine –γ-synthase (EC 2.5.1.48)	59% / 76%	1e-134	39% / 60%	6e-69

Table 2 Comparative analysis of protein sequence homology of the two fer1 MGL orthologs based on their enzymatic activity with their evolutionary relatives using BLAST.

In MGL of *Pseudomonas putida*, previous study has shown that four amino acids (Met^{90}, Asp^{187}, His^{207}, Gly^{215}) are involved in the stabilization of 3D structure and six other amino acids (Tyr^{59}, Arg^{61}, Tyr^{114}, Cys^{116}, Lys^{240} and Asp^{241}) are involved in substrate specificity and catalysis. Using ClustalW2, the amino acid sequences of fer1 MGLs and MGLs from a variety of sources were

aligned together using *P. putida* MGL as a reference (Tables 3, 4). The numbers on the superscript position of three-lettered amino acids represent the position of amino acid in their primary structures, when it is counted from N-terminal. The alignments provide information that each amino acid in each column is assumed to be located at the same position in their spatial arrangements in MGLs and involved critically in interacting with other amino acids in the chain for the stabilization of protein and forming the active site for a substrate.

Using ClustalW2, the four conserved residues for stabilizing 3D protein structure of ferl MGLs and MGLs from other sources were aligned together (Table 3). It seemed that amino acids in each position are highly conserved, with some variations at the first position. Instead of having Met in the first position, ferl MGL2, *B. linens* MGL and *C. freundii* MGL have Tyr, Ile and Thr respectively. The spacing between the next amino acids in the polypeptide is also highly conserved, even though there is a slight variation between second and third position in ferl MGLs and MGLs from *A. thaliana* and *E. histolytica* (Table 3).

Pseudomonas putida	**Met^{90}**	**Asp^{187}**	**His^{207}**	**Gly^{215}**
ferl MGL1	Met^{78}	Asp^{175}	His^{194}	Gly^{202}
ferl MGL2	Tyr^{62}	Asp^{159}	His^{178}	Gly^{186}
Citrobacter freundii	Ile^{89}	Asp^{186}	His^{206}	Gly^{214}
P. fluorescens	Met^{90}	Asp^{188}	His^{208}	Gly^{216}
Brevibacterium linens	Thr^{81}	Asp^{178}	His^{198}	Gly^{206}
Bacillus halodurans	Met^{87}	Asp^{184}	His^{204}	Gly^{212}
Arabidopsis thaliana	Met^{126}	Asp^{223}	His^{244}	Gly^{252}
Entamoeba histolytica	Met^{84}	Asp^{181}	His^{201}	Gly^{209}

Table 3 Amino acid alignment of MGLs of various sources that are involved in stabilizing tetrameric MGL, where MGL of *P. putida* is used as a reference.

The six conserved residues of ferl MGLs and MGLs for substrate specificity from other sources were also aligned together using ClustalW2 (Table 4). It also seemed that amino acids in each position are highly conserved with a few variations in fourth, fifth and sixth positions in both ferl MGLs and other MGLs. In the fourth position, ferl MGLs contain Gln and Arg, whereas other MGLs contain Cys. In the fifth position, the most common amino acid is Lys, however, ferl MGLs and *E. histolytica* MGL have another amino acid, Arg, of similar function. The amino acid in the sixth position seems very flexible, wherein ferl MGLs contain Lys and Arg. On the other hand, the spacing between first and second, second and third positions are exactly the same among all MGL polypeptide chains, however, much variations were observed among the other positions. These variations in residues and spacing predict the variations in substrate specificities among different MGLs.

Pseudomonas putida	**Tyr^{59}**	**Arg^{61}**	**Tyr^{114}**	**Cys^{116}**	**Lys^{240}**	**Asp241**
ferl MGL1	Tyr^{47}	Arg^{49}	Tyr^{102}	Gln^{104}	Arg^{224}	Lys^{225}
Ferl MGL2	Tyr^{31}	Arg^{33}	Tyr^{86}	Arg^{88}	Arg^{209}	Arg^{210}
Citrobacter freundii	Tyr^{58}	Arg^{60}	Tyr^{113}	Cys^{115}	Lys^{239}	Asp^{241}
Pseudomonas fluorescens	Tyr^{59}	Arg^{61}	Tyr^{114}	Cys^{116}	Lys^{241}	Asp^{242}
Brevibacterium linens	Tyr^{50}	Arg^{52}	Tyr^{105}	Cys^{107}	Leu^{237}	Ala^{238}
Bacillus halodurans	Tyr^{56}	Arg^{58}	Tyr^{111}	Cys^{113}	Lys^{236}	Asp^{241}
Arabidopsis thaliana	Tyr^{95}	Arg^{97}	Tyr^{150}	Cys^{152}	Lys^{170}	Met^{180}
Entamoeba histolytica	Tyr^{53}	Arg^{55}	Tyr^{108}	Cys^{110}	Arg^{223}	Lys^{234}

Table 4 Amino acid alignment of MGLs of various sources that are involved in substrate specificity and catalysis.

Using ClustalW2, phylogenetic tree of MGLs from different sources was constructed that showed the differentiation in their amino acid compositions (Figure 7). In this tree, there are three clades: firstly, fer1-*Arabidopsis-Entamoeba-Brevibacterium* group; secondly, *Pseudomonas-Citrobacter* group and thirdly, *Bacillus* group.

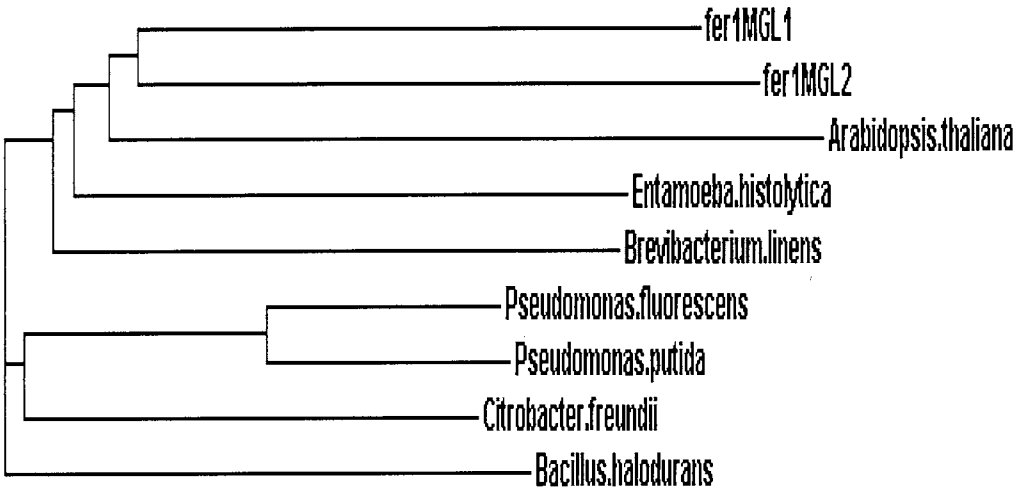
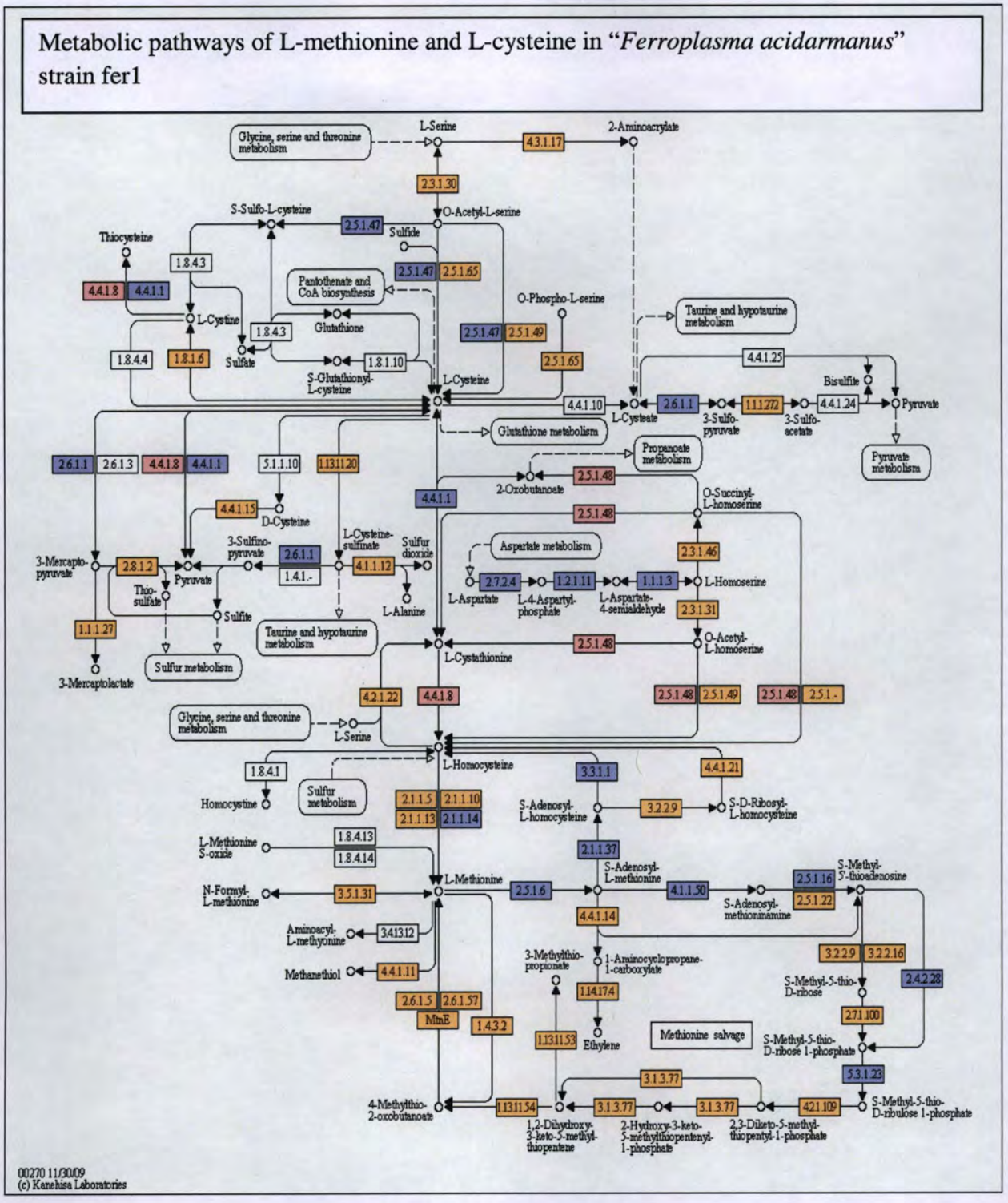


Figure 7 Phylogenetic relation of MGLs from different organisms based on their amino acid compositions. The vertical position is related to the distance of the enzyme from the different organisms.

A tentative cysteine-methionine metabolic pathway in "*Ferroplasma acidarmanus*" strain fer1 has been obtained using KEGG pathway and IMG databases (Figure 8), where fer1 MGL orthologs play the major role. In this pathway, fer1 MGL orthologs have been recognized as multifunctional enzymes having the functions of cystathionine-β-lyase (EC 4.4.1.8), cystathionine-γ-synthase (EC 2.5.1.48) and cystathionine-γ-lyase (EC 4.4.1.1). But these databases did not specify which ortholog performs which enzymatic action(s) in this pathway.



Current Gene (fer1 MGL1); Positional Cluster Gene; Other genes in "*Ferroplasma acidarmanus*" *strain* fer1; Genes found in other genomes

Figure 8 KEGG pathway and IMG databases prediction of methionine-cysteine metabolism in fer1.

Validation of enzymatic assays

A curve was obtained by plotting the values of different concentrations of L-cysteine to the X-axis and OD_{412} to the Y-axis (Figure 9).

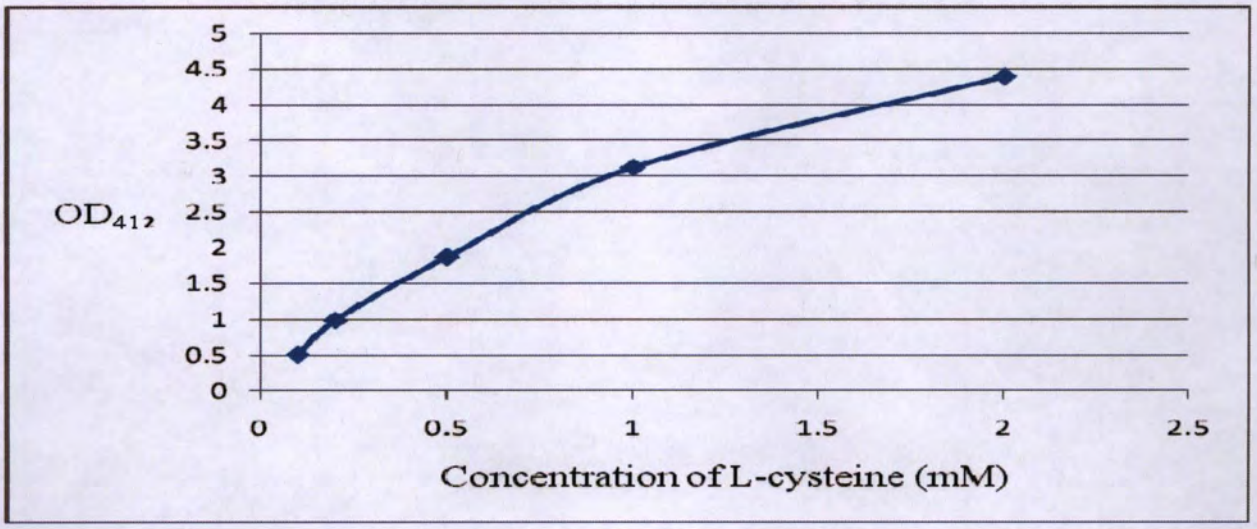


Figure 9 The curve represents the linear relationship between the concentration of thiol group containing compound, L-cysteine and OD_{412} (N=1).

A curve was also obtained by plotting the values of different concentrations of pyruvate to the X-axis and OD_{320} to the Y-axis (Figure 10).

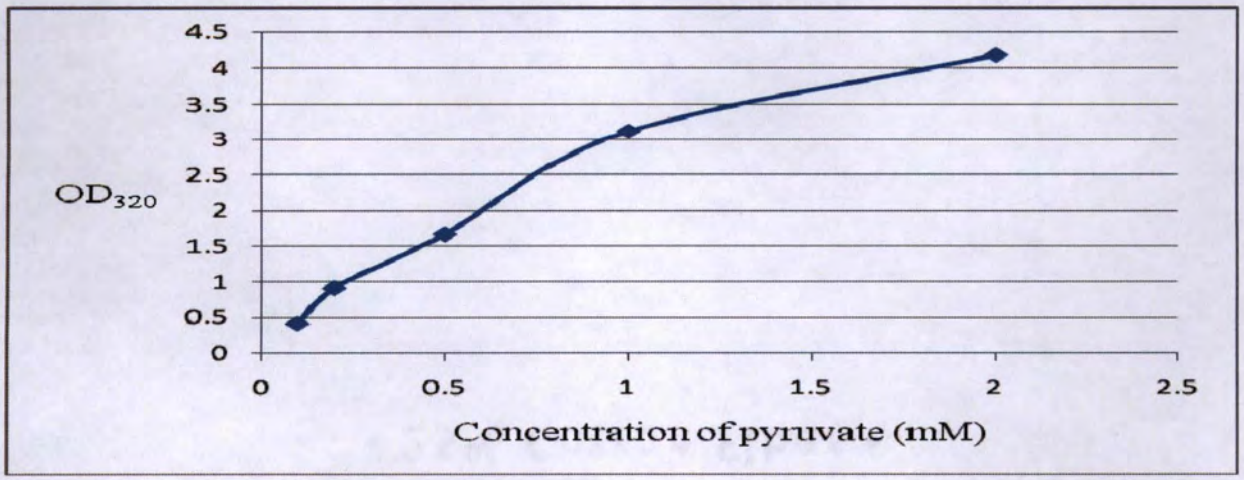


Figure 10 The curve represents the linear relationship between the concentration of keto group containing compound, pyruvate and OD_{320} (N=1).

Preliminary test: MGL activity of fer1 lysate in DTNB assay

The production of thiol group (-SH) containing compound(s) by fer1 lysate in a time-temperature dependent manner was observed in DTNB assay at pH 8.0 (Figure 11). Here, L-methionine (~40 mM) was used as substrate and PLP (0.2 mM) was used as coenzyme. In this assay it was observed that the –SH group production by the lysate increased as temperature increased to 55°C (55° Celsius zone in the figure). All of the values were statistically significant when compared to heated lysate control (N=4, $p < 0.05$). In the reactions without PLP (No PLP control) as well as in reactions without substrate (Substrate Blank control, no L-methionine), there were very little activity, if any, was found and was not statistically significant when compared to heated lysate control. However, the No PLP control curve started to rise and proceeded in parallel with the fer1 lysate curve after 135 min.

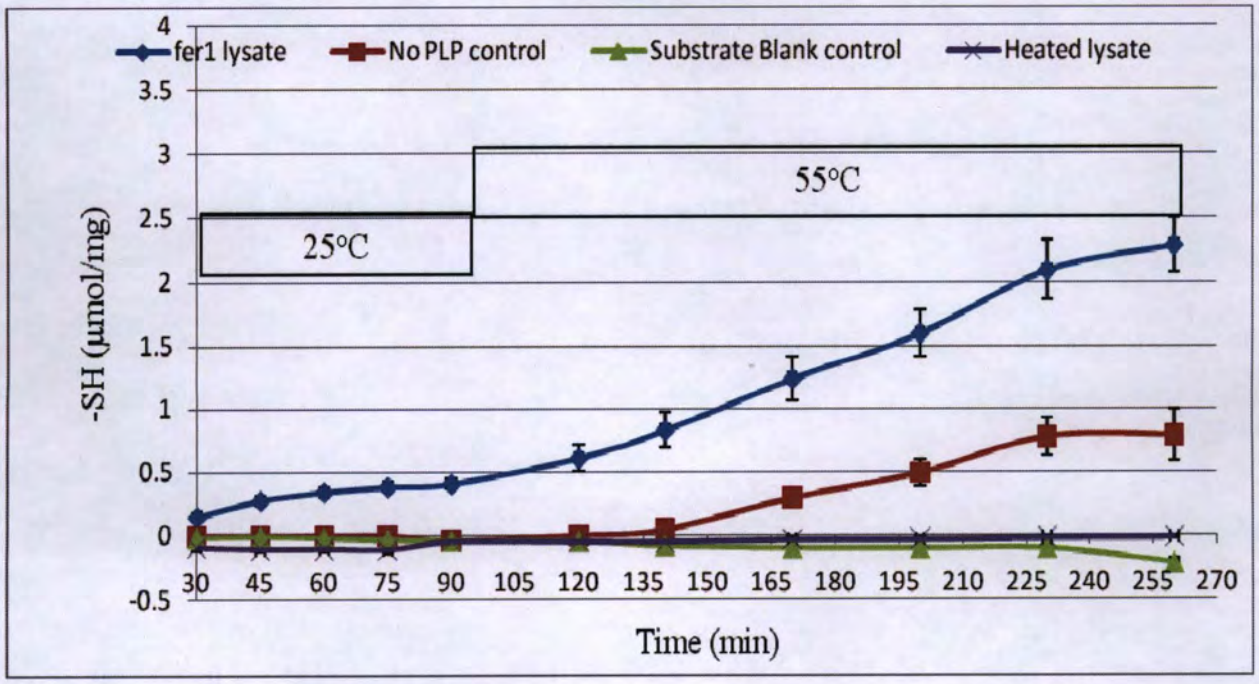


Figure 11 Preliminary MGL activity of fer1 lysate using DTNB assay (N=4, $p < 0.05$). Vertical bars represent the standard error.

Determination of optimum parameters for the enzymatic reaction by fer1 lysate in MBTH assay

Quantitative determination of total protein concentration in fer1 lysate

The OD_{562} values of BSA protein standards were measured using a spectrophotometer. Then a standard curve was derived by plotting the BSA protein standards to the X-axis and the corresponding OD_{562} values to the Y-axis (Figure 12). Using Excel (Microsoft, Redmond, WA), the slope (m) and intercept (C) of the curve were determined to be 0.00101 and 0.08119, respectively.

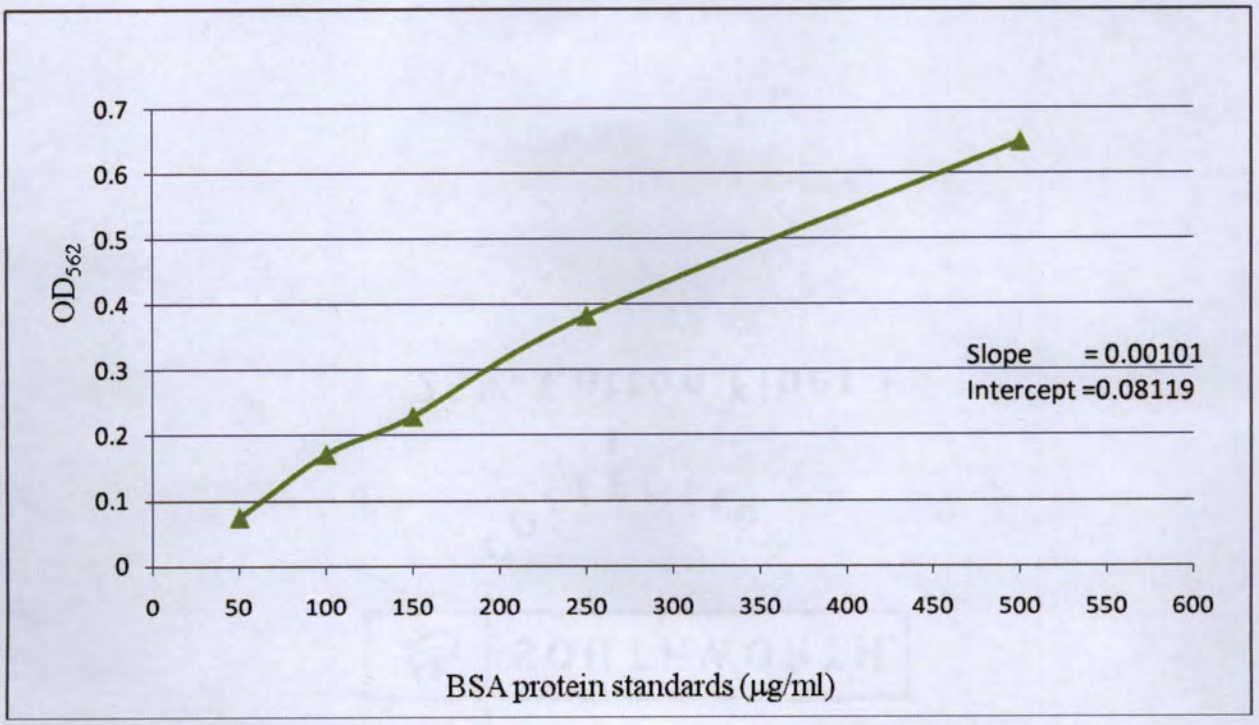


Figure 12 Protein standard curve.

To determine the total soluble protein in the fer1 lysate, OD_{562} values of fer1 lysate and negative control (0.2 M Na_2HPO_4) were measured. The corrected OD_{562} value for fer1 lysate was obtained by subtracting the negative control value (0.364) from the lysate value. Then the values of the slope and intercept derived from the curve were used to derive the total protein concentration.

Determination of optimum pH at 35°C for 30 minutes in MBTH assay

In MBTH assay, optimum pH was also attempted to be determined using fer1 lysate. The production of α-keto group (=CO) containing compound(s) by fer1 lysate was quantitatively determined at A_{320} in five independent trials, where L-methionine (47 mM) was used as substrate and PLP (0.2 mM) was used as coenzyme (Figure 13). Total soluble protein concentration in fer1 lysate was quantified as described. The values of the total protein concentration and A_{320} were then used to derive the specific activities at 35°C for 30 minutes. The highest specific activity of fer1 lysate, 0.14 µmol/mg/min, was detected at pH 4.0 (N=5). Heat treated lysate was included as negative control and no activity was found (not shown).

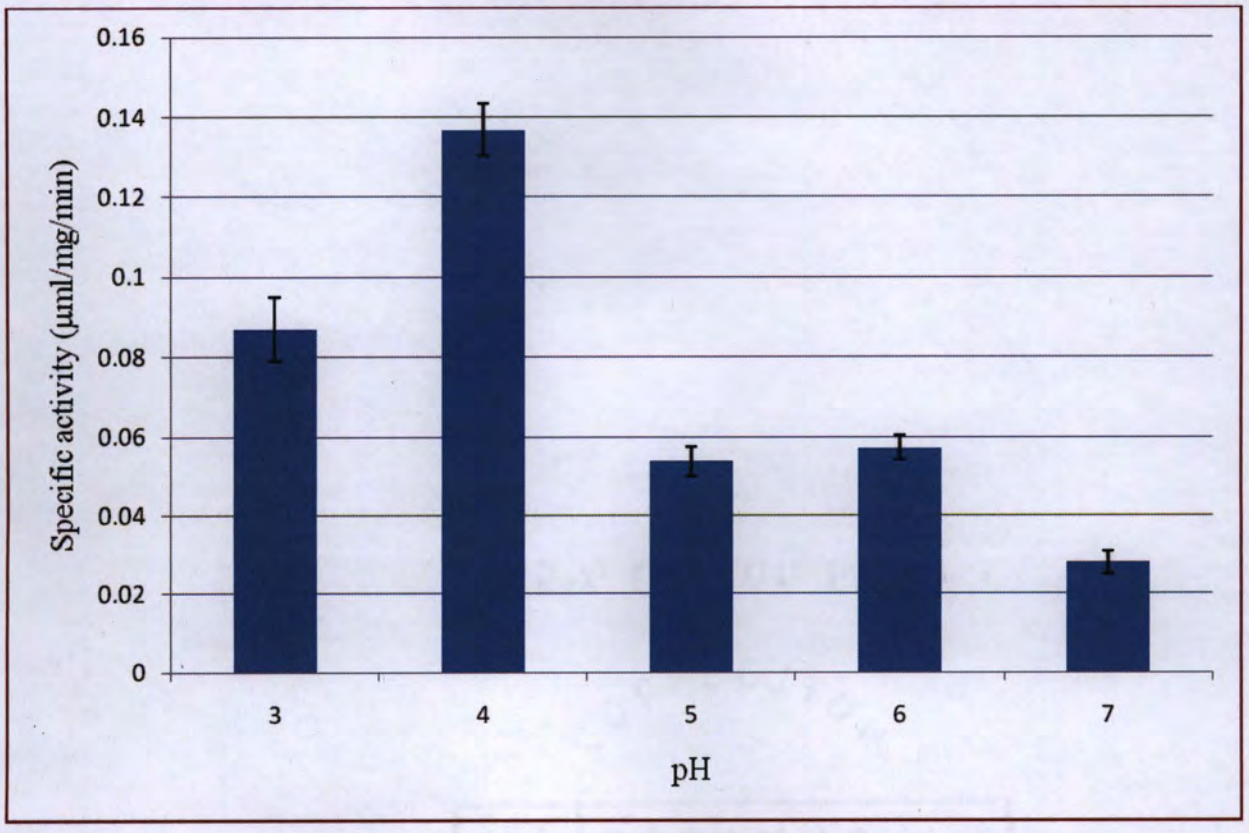


Figure 13 Specific activities of fer1 lysate at different pHs (3.0 - 7.0) and at 35°C for 30 minutes in MBTH assay, which were statistically significant when compared to heated lysates (N=5, $p < 0.05$). Vertical bars represent the standard error.

Substrate specificity of fer1 lysate at pH 5 in MBTH assay

The substrate specificity of fer1 cell lysate was also studied at pH 5.0 using MBTH assay (Figure 14). The production of α-keto group (=CO) containing compound(s) by fer1 lysate was quantitatively determined at A_{320} (N=3), where L-methionine (47 mM), L-cystathionine (5 mM), L-cysteine (47 mM), D-methionine (47 mM) and DL-homocysteine (10 mM) were used as substrates and PLP (0.2 mM) was used as coenzyme. Specific activities were determined at 35°C for 30 minutes. The specific activity of fer1 lysate using L-methionine was detected, 0.04 μmol/mg/min. This value of specific activity was arbitrarily considered as 100%. The specific activities of other substrates were 0.035 μmol/mg/min for L-cystathionine (88.9%), 0.061 μmol/mg/min for DL-homocysteine (152.5%), 0.052 μmol/mg/min for L-cysteine (130.2%), 0.039 μmol/mg/min for D-methionine (97.5%). Heated fer1 lysate and *Citrobacter freundii* lysate (CF) were included as negative and positive controls, respectively.

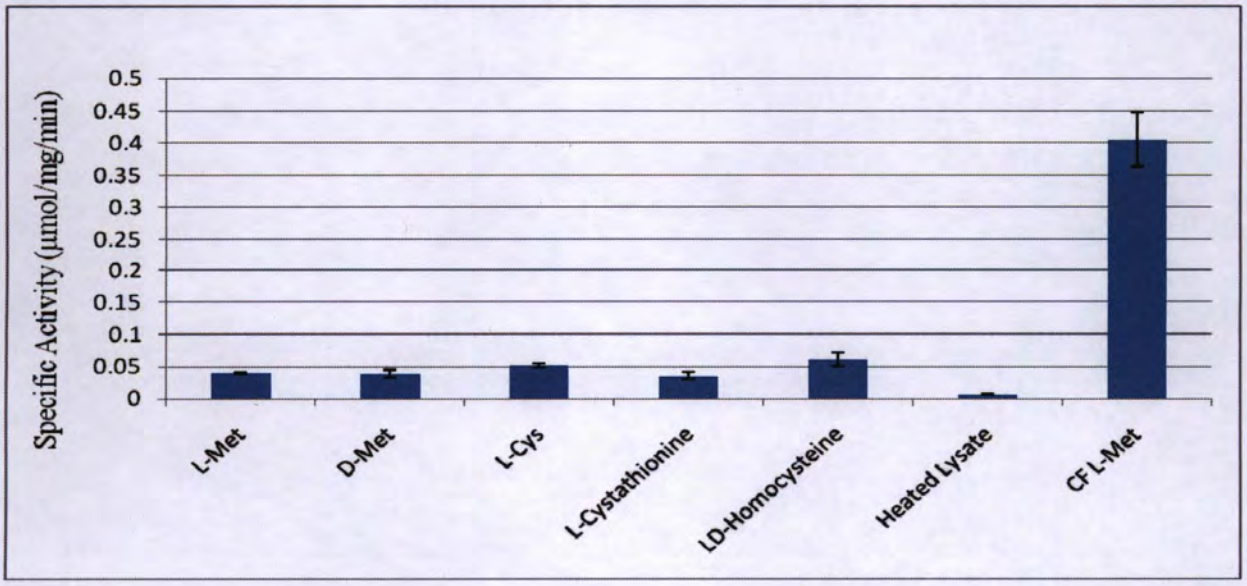
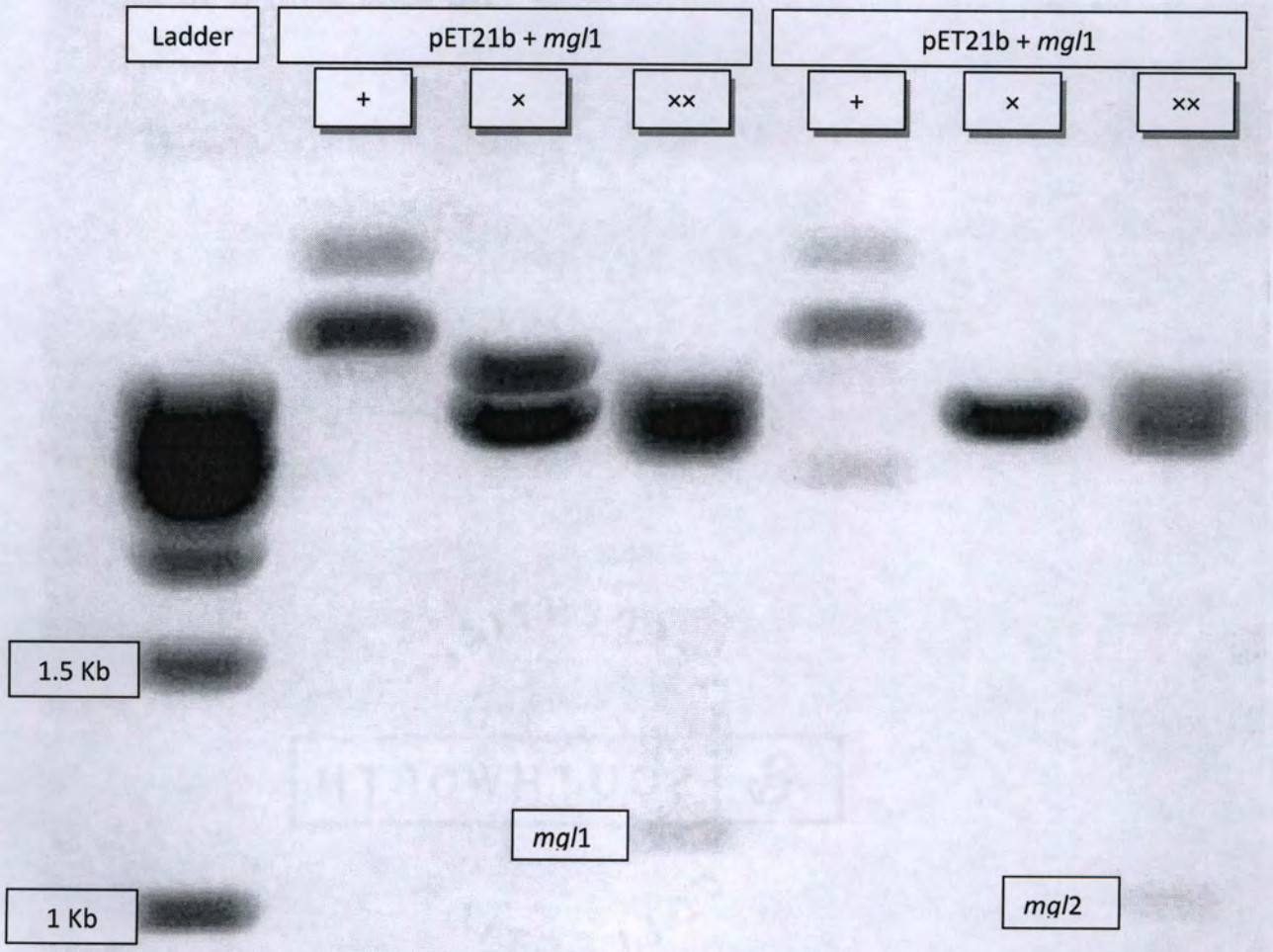


Figure 14 Substrate specificities of fer1 lysate at pH 5.0 in MBTH assay, which were statistically significant when compared to heated lysates (N=3, $p < 0.05$). Vertical bars represent the standard error.

Confirmation study of the fer1-*mgl* inserts in pET21b vectors

An attempt was taken to determine if the *E. coli* BL21 (DE3) cell lines had the correct inserts in their vector pET21b using agarose gel electrophoresis. The results showed the inserts, which were within 1 and 1.5 Kb long. Both inserts appeared to be the correct predicted size (Figure 15).



'+' indicates no digestion; 'x' indicates single digestion by NotI; 'xx' indicates double digestion by NotI and NdeI.

Figure 15 Agarose gel electrophoresis showed the digested and undigested products of the vector pET21b inserted with mgl1 and mgl2 orthologs.

Determination of growth curves of *E. coli* BL21 (DE3) cell lines and *Citrobacter freundii*

An attempt was taken to determine the growth curve, mainly mid-exponential phase, of *E. coli* BL21 (DE3) cell lines and *Citrobacter freundii* (Figure 16). At first, the cultures were grown in 100 ml sterile LB media at 35°C and OD_{600} was taken at every hour until 11 hours. It was found that lag phase continued until 3 hours for all the cultures, however, FRIK2600 culture took about one more hour. The exponential phase for all of the cultures lasted roughly until 10 hours, wherein 0.6 of OD600 was determined to be the mid-exponential phase, which appeared after about 7 hours. Then all the cultures entered into the stationary phases. In this experiment, 100 µg/ml ampicillin was added to both FRIK2601 and FRIK2609 cultures. LB medium was included as negative control.

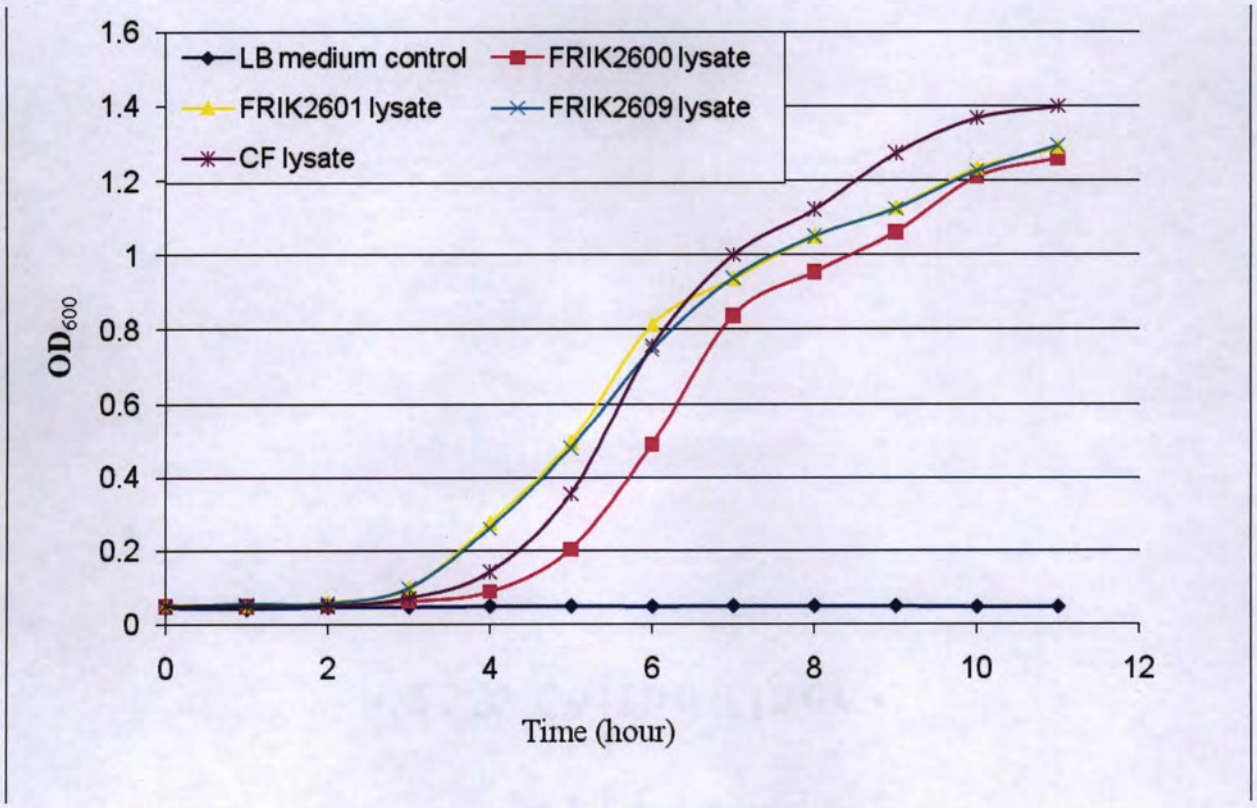


Figure 16 The growth curve of *E. coli* BL21 (DE3) cell lines and *Citrobacter freundii* (CF).

Heterologously expressed fer1 MGL activity in DTNB assay

Specific activities using cell lysates of E. coli cell lines and Citrobacter freundii (CF)

The production of thiol group (-SH) containing compound(s) by *E. coli* BL21 (DE3) cell lines and *C. freundii* were quantitatively determined at A_{412} in two independent trials using DTNB assay, where L-methionine (~40 mM) was used as substrate and PLP (0.2 mM) was used as coenzyme (Figure 17). Total soluble protein concentrations were quantified as described. The values of the total protein concentration and A_{412} derived in DTNB assay were used to derive the specific activities at different pHs and 35°C for 30 min. The highest specific activities of FRIK2601 lysate, 0.197 µmol/mg/min and FRIK2609 lysate, 0.103 µmol/mg/min, respectively were obtained at pH 5.0 and were statistically significant when compared to heated lysates (N=2, $p < 0.05$). In this assay *C. freundii* (CF) lysate and FRIK2600 lysate (pHs 3.0 to 5.0) were included as positive and negative controls, respectively.

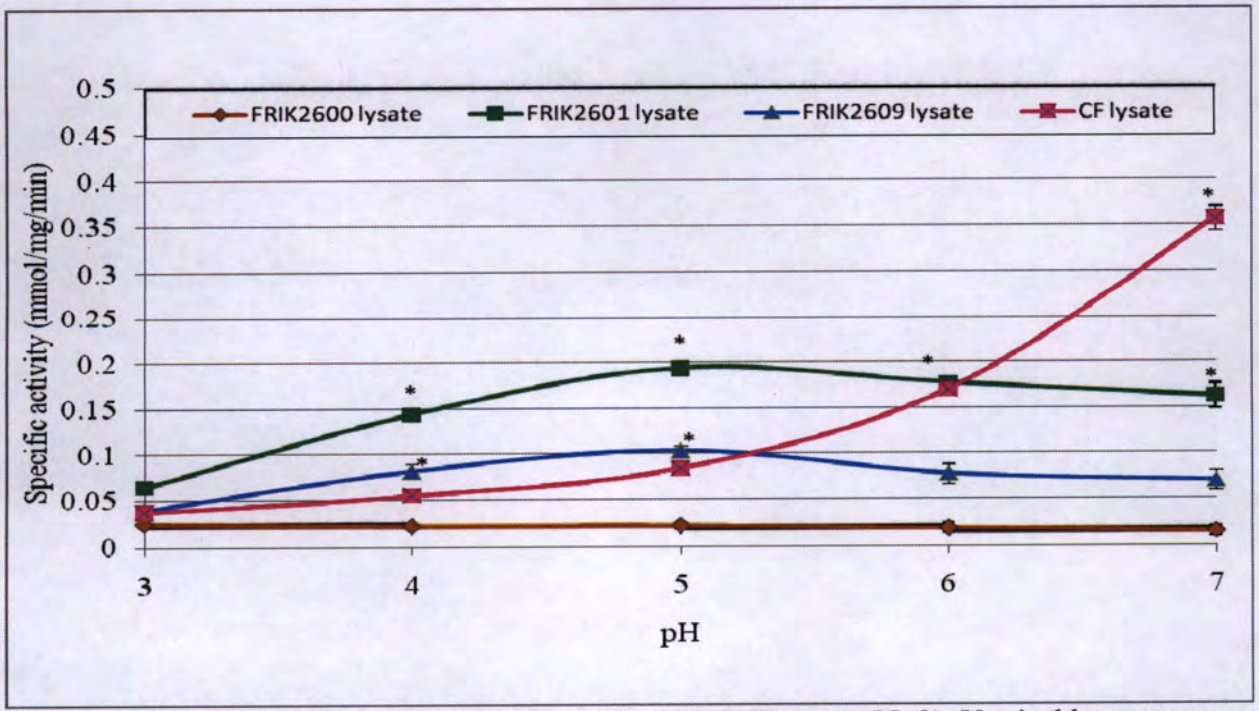


Figure 17 Heterologously expressed MGL activity in DTNB assay (N=2). Vertical bars represent the standard error. * denotes statistical difference ($p < 0.05$) when compared to heated lysates.

Heterologously expressed fer1 MGL Activity in MBTH assay

Specific activities using cell lysates of E. coli cell lines and Citrobacter freundii (CF)

The production of α-keto group (=CO) containing compound(s) by *E. coli* BL21 (DE3) cell lines and *C. freundii* were quantitatively determined at A_{320} in four independent trials using MBTH assay, where L-methionine (47 mM) was used as substrate and PLP (0.2 mM) was used as coenzyme (Figure 18). Total soluble protein concentrations were quantified as described. The values of the total protein concentration and A_{320} derived in MBTH assay were used to derive the specific activities at different pHs and 35°C for 30 min. In this assay, the highest specific activity of FRIK2609 lysate, 0.197 μmol/mg/min, was obtained at pH 4.0 (N=4), whereas no significant activity was observed at every pH point using FRIK2601 lysate. In this assay *C. freundii* (CF) lysate and FRIK2600 lysate were also included as positive and negative controls, respectively.

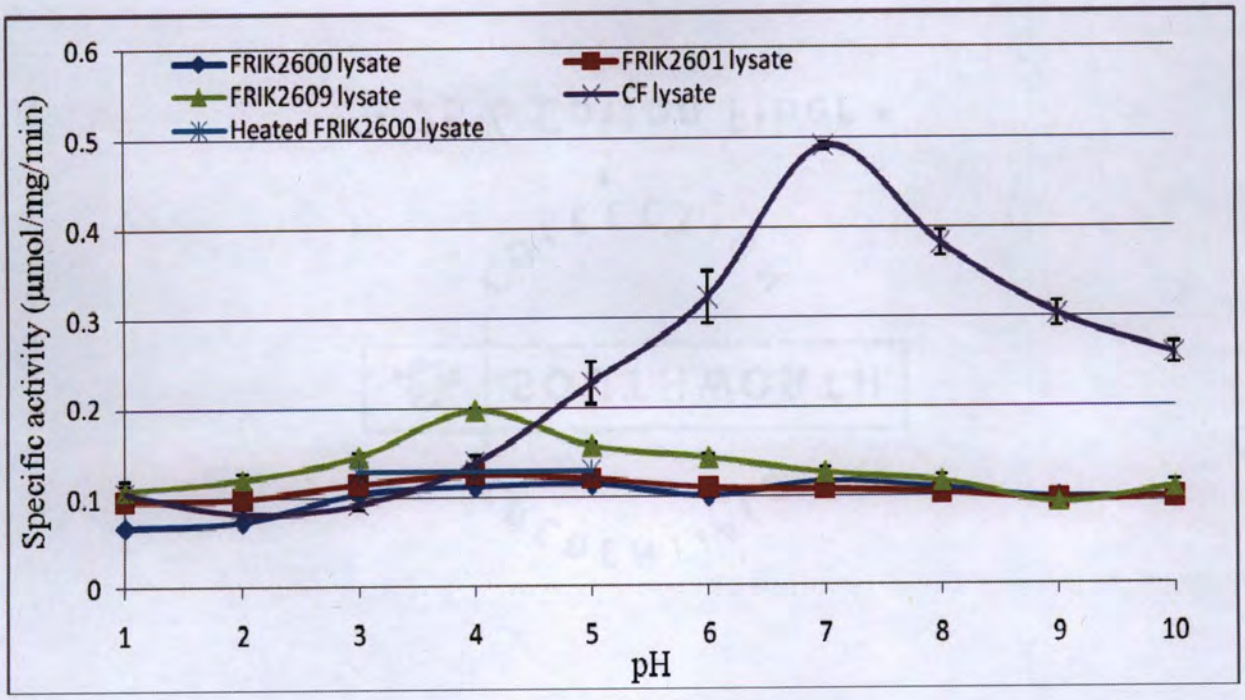


Figure 18 Heterologously expressed MGL activities in MBTH assay. Vertical bars represent the standard error.

In this assay, heated FRIK2600 lysate (pHs 3.0 to 5.0) was included as a negative control, which did not give statistically significant activity when compared to regular FRIK2600 lysate (N=4, $p > 0.05$), where as all other values were statistically significant when compared to heated lysate control (N=4, $p < 0.05$).

Discussion

Bioinformatics: Comparative analysis of fer1 MGL orthologs with other MGLs

According to BLAST, fer1 MGL orthologs share a high level of overall protein sequence identity (~35%) and similarity (~60%) when compared to other known MGLs (Table 1). The fer1 MGL orthologs also share about the equal level of overall identity (38%) and similarity (59%) between themselves (data not shown). In addition, these two orthologs share a very reasonable, sometimes higher overall identity and similarity with some other PLP-dependent enzymes, which were selected from some evolutionarily closely related archaea and *E. coli* BL21 (DE3), that are also involve in the metabolism of methionine-cysteine (Table 2). For example, fer1 MGL1 shares 57% identity and 77% similarity with cystathionine-β-lyase (CBL) of *Thermoplasma acidophilum*, which are much higher than those with MGLs from different sources. Similarly, fer1 MGL orthologs have very high overall identity and similarity with cystathionine-γ-lyase (CGL) and cystathionine-γ-synthase (CGS) (Table 2). Therefore, current annotation shows these two fer1 MGL sequences as CBL, CGS and CGL. However, these are putative and may not reflect their actual roles in methionine-cysteine metabolism.

Using *Pseudomonus putida* MGL as reference, a comprehensive amino acid alignment was conducted to explore the structural identity of fer1 MGL orthologs compared to MGLs from different organisms including Gram-positive and Gram-negative bacteria, protozoa and plant. Among all the PLP-dependent enzymes including MGL, Met^{90}, Asp^{187}, His^{207} and Gly^{215} (the numbers on the superscript of amino acids were based on MGL in *P. putida*) were found to be highly conserved and considered as enzyme stabilizers (Table 3; 32). However, instead of having Met in the first position of fer1 MGL2, *B. linens* and *C. freundii* MGL, they have Tyr, Ile and Thr respectively (Table 3). Interestingly, all of these amino acids are either polar neutral (Tyr

and Thr) or non-polar (Met, Ile), and are thus typically involved in the forming same kinds of bond in stabilizing 3D structures of protein.

On the other hand, a network of hydrogen bonding among six amino acids (Tyr^{59}, Arg^{61}, Tyr^{114}, Cys^{116}, Lys^{240} and Asp^{241}) in their spatial arrangements has been found to be critical in forming the substrate binding pocket and catalysis (Table 4; 36, 37). Among them, study conducted with various suicide inhibitors revealed that Cys^{116}, as the nucleophilic catalytic residue, plays the pivotal role in the enzyme activity of *P. putida* (38, 39). As opposed to having Cys at the corresponding position, fer1 MGL orthologs have nucleophilic Gln (MGL1) and electrophilic Arg (MGL2). However, the affinity of MGL for β-elimination reaction was apparently enhanced if Cys^{116} was replaced with another electrophilic amino acid, His, which may be due to the change in electrostatic interaction, conformational modification and geometric arrangement (36, 37). Therefore, fer1 MGL2 might have higher affinity to L-cysteine than L-methionine, as it has Arg at the corresponding position, which has similar property as His. MBTH assay showed that fer1 lysate had about 1.3 times higher affinity for L-cysteine compared to L-methionine, which actually supports this bioinformatics prediction. However, until now, the complete picture of the fluctuation of the enzyme affinity due to change in amino acids towards various substrates is not fully understood.

The sequence similarity of MGLs among different organisms is clearly differentiated, as depicted in the phylogenetic tree (Figure 7). Since evolutionarily Archaea are closer to Eukarya than Bacteria, it is reasonable to find higher sequence similarities among fer1 MGL orthologs and MGLs from eukaryotic *E. histolytica* and *A. thaliana* than those of fer1 with bacterial MGLs.

This eukaryotic nature of fer1 MGLs may enhance the likelihood of finding an application for prospective therapeutic use in cancers.

The KEGG pathway and IMG databases revealed a tentative pathway of cysteine-methionine metabolism in fer1 (Figure 8). Although most of the enzymes are apparently absent in fer1 genome, there might be some unique enzymes in fer1 cells that are involved to carry out this metabolic pathway. However, it seems that MGL orthologs may be able to catalyze multiple steps in this transsulfuration process. This tentative pathway therefore predicts the typical versatile nature of MGLs. However, these predictions require enzymatic assays to validate.

Enzymatic assays of fer1 lysate

The first step of the study was to validate whether the MBTH and DTNB assays using keto group containing pyruvate and thiol group containing L-cystheine. The experiments confirmed that the amount of the thiol group and keto group were reflected in the optical density (OD) values in DTNB and MBTH assays, respectively (Figures 9 and 10). Even though the curves in both cases were not straight lines but showed high level of linearity.

The next step was to confirm whether fer1 exhibits MGL activity. In the DTNB assay, fer1 lysate produced thiol group containing compound(s) in a time-temperature dependent reaction (Figure 11). The production of thiol group containing compound(s) was gradually increasing over time and expedited at higher temperature (55°C), which were detected at OD_{412} at different time points. However, fer1 cannot grow at 55°C. A few controls were included in this assay to validate not only the authenticity of the assay but also cell lysate activity. Very low activity was detected in the absence of either PLP or substrate in the reaction mixture. This preliminary assay

showed that ferl cell lysate has enzymatic activity that is PLP and L-methionine dependent. This finding supports the previous study, where methanethiol was detected using gas chromatography in headspace gases evolved from a reaction mixture containing PLP, ferl lysate and L-methionine/-cysteine, but not from heated lysate and no substrate controls (26). However, after about 135 minutes of this experiment, the curve for No PLP control started to rise and followed in parallel with the regular lysate curve. This finding pointed to two possibilities. One would be that the production of thiol group containing homocysteine from L-methionine in three reaction steps, which require PLP-independent enzymes (EC 2.5.1.6, EC 2.1.1.37 and EC 3.3.1.1) (Figure 8). The other possibility would be that the gradual acquisition of PLP in the MGLs from the cell lysate, which should some PLP, made the enzymes active and functional later in the reaction. In addition to the No PLP and substrate blank controls, heat treated lysate (100°C for 30 min) was also included as the negative control. The lack of activity in heat treated lysate control showed that the reaction was enzymatic. Generally, the optimum temperature of all known MGLs is about 25-35°C and activity decreases greatly as temperature increases (40). For example, MGL of *B. linens* has highest activity at 25°C and no activity was detected at and above 35°C (40). The high temperature requirements of ferl lysate for MGL activity might be due to the need to overcome the effects of some factors such as pH and ionic concentrations, which might not be optimal during the reaction. In this case, where the pH was set to 8.0, the high temperature (55°C) might be needed to mobilize the reacting molecules to a rate that would have been achievable at 25-35°C, if all other parameters were at their optima. Therefore, ferl lysate warrants the determination of optimum parameters to conduct the *in vitro* experiments for MGL activity.

One confounding factor for the DTNB assay is that it includes thiol group containing reducing agent DTT while trying to assay for thiol group containing compound(s). Therefore, a small change in DTT concentration in either the control and test mixtures causes significant difference in OD_{412}. Consequently, a second spectrophotometric assay, MBTH assay, was adopted and preferred to determine the optimum parameters (temperature, time and pH) with fer1 lysate. In four different time-temperature combinations, the highest specific activity was determined to be 0.03 μmol/mg/min at 55°C for 30 min at pH 8.0, which was much lower than the range (0.26-46 μmol/mg/min) reported for other microbial MGL (data not shown; 40). However, at 35°C, a reasonably higher value, 0.0125 μmol/mg/min, was determined for 30 min at pH 8.0. Due to the slow growth of fer1 and time restraints, this experiment was restricted to just few time-temperature combinations, and considering the optimum growth temperature of fer1, the rest of the experiments were performed at 35°C for 25-30 min. At 35°C for 25 minutes, the highest specific activity (0.14 μmol/mg/min, N=5, $p < 0.05$) was detected at pH 4.0, however, the internal pH of fer1 was determined around 5.6 by NMR (Figure 13; 14). According to the definition, specific activity is only applicable to the purified enzyme, not the crude cell lysate. Since only one/two enzymes in the lysate are involved in MGL catalyzed reaction(s) while current calculation used total protein of lysate, the actual specific activities could be hundred(s) times higher than they were determined.

As with the L-methionine, substrate specificities of fer1 lysate were studied using MBTH assays. D-methionine, L-cysteine, L-cystathionine and DL-homocysteine were degraded to 97.5%, 130.2%, 88.9%, and 152.5% respectively of the level of activity on L-Met (Figure 14). The metabolism of different substrates by both α, β- and α, γ-elimination reactions showed the multifunctional nature of fer1 lysate, typical of MGL activity. The reason for highest activity on

DL-homocysteine is unknown, but variations in the amino acid in the fourth, fifth and sixth positions might create an active site that suits best for DL-homocysteine (Table 4), or fer1 cell lysate might contain other enzymes that works on homocysteine and produces α-keto product(s). Although fer1 MGL1 shares higher identity and similarity with cystathionine-γ-lyase (CGL) of *P. torridus* and other CGLs than with MGLs from different sources, fer1 lysate showed less activity using L-cystathionine than with L-methionine. The reason might be due to the absence of one or more critical amino acids that are used for α, γ-elimination, or fer1 MGL1 would only carry out γ-replacement reaction. Since, fer1 MGL1 shares even higher identity and similarity to cystathionine-γ-synthase (CGS) of *P. torridus*, the study of CGS activity using fer1 MGL1 would be worthy trying in future. On the other hand, fer1 MGL2 shares very high similarity and identity, and zero percent gap to cystathionine-β-lyase (CBL) of *T. acidophilum*, but fer1 lysate did not show better activity for the reaction catalyzed by CBL. However, the higher activity of fer1 lysate on L-cysteine supports bioinformatics prediction, which might be due to MGL2. From both of the assay types, it is now clear that fer1 cell lysate has MGL activity, but not clear what type(s) of reaction is carried out by fer1 MGLs, since MGL can carry out multiple reactions.

Enzymatic assay of heterologously expressed fer1 MGL orthologs

Since the cell lysate contains hundreds of different types of enzymes for maintenance of cellular metabolism, it was not possible to determine whether the MBTH results were due to the two putative MGL orthologs. In addition, like other archaea, fer1 contains many genes with unknown functions or harbors proteins with new functions, such as a bifunctional DNA repair protein that exhibits O6-alkylguanine-DNA transferase and endonuclease V activities (41). Therefore, assays

using purified proteins were attempted. As previously mentioned, fer1 genome contains two candidate genes for MGL. To understand their roles in methionine-cysteine metabolism better, they were cloned individually in vector pET21b and then transferred in *E. coli* BL21 (DE3), creating cell lines FRIK2601 and FRIK2609. FRIK2600, which does not have vector and is sensitive to ampicillin, was used as negative control, while *C. freundii*, a well known MGL producer, was used as positive control. The vector digestion and purification experiment using agarose gel electrophoresis confirmed the absence of vector in control *E. coli* strain and presence of inserts of correct size from other two strains, where the *mgl*1 insert was longer than *mgl*2 (Figure 15).

According to the Integrated Microbial Genomes (IMG) database, *E. coli* BL21 (DE3) genome contains gene for PLP-dependent cystathionine-β-lyase (CBL, ~43 KDa; EC 4.4.1.8), which is also involved in cysteine-methionine metabolism and has reasonably good percentage of overall identity and similarity in amino acid sequences with fer1 MGL orthologs (Table 2). SDS-PAGE analysis revealed that all three cell lines of *E. coli* BL21 (DE3) lysates contain the same thick bands at around 41 KDa positions, which is roughly the molecular weight of MGLs and CBL (data not shown). All three cell lines showed the same type of bands at around 41 KDa positions, when they all were induced and uninduced with IPTG (data not shown). Thus it was not clear if the bands were due to the production of fer1 MGLs or housekeeping CBL in vector containing *E. coli* BL21 (DE3) cultures. One critical downside of working with *E. coli* BL21 (DE3) lysates would be that all the three cell lines might give the same type of byproduct(s) using L-methionine, L-cysteine and their analogs and derivatives. However, the control cell line, FRIK2600, was sensitive to ampicillin and other two were resistant to it. In addition, heated FRIK2600 lysate did not give statistically significant activity when compared to regular

FRIK2600 lysate (Figure 18). In the future, knock-out strain of *E. coli* BL21 (DE3) host with CBL eliminated should be constructed to allow for a more precise assay.

The spectrophotometric assays were conducted for MGL activities that are considered very specific, reliable and quantitative. Before the assays were conducted, mid-exponential phase of all *E. coli* cell lines were determined around 0.6 at OD_{600}, which is considered the best time for the cells to be induced (Figure 16). In DTNB assay, the highest specific activity of FRIK2601 lysate, 0.197 μmol/mg/min (N=2, $p < 0.05$) was detected at pH 5.0 (Figure 17), whereas in MBTH assay, FRIK2601 lysate did not show any statistically significant activity at every tested pH value (N=4, $p > 0.05$) (Figure 18). On the other hand, the highest specific activity of FRIK2609 lysate, 0.197 μmol/mg/min (N=4, $p < 0.05$) was detected in MBTH assay at pH 4.0 and 0.103 μmol/mg/min (N=2, $p < 0.05$) in DTNB assays at pH 5 (Figures 17, 18). Results from MBTH assays of heterologously expressed orthologs showed that FRIK2609 lysate, but not FRIK2601 lysate, catalyzes the production of α-keto group containing product(s). This suggested that FRIK2609 lysate, but not FRIK2601 lysate, causes α, γ-elimination of L-methionine (Figure 4), but it was not sure with the DTNB assays if FRIK2609 lysate can cause the γ-replacement reaction or not. But DTNB assay of heterologously expressed orthologs suggested that FRIK2601 lysate catalyzes γ-replacement reaction. Results from both assay types revealed that α, γ-elimination is carried out optimally at pH 4.0 and γ-replacement is at pH 5.0, which might be a result of pH preference of two MGL orthologs. In both of the assays, *Citrobacter freundii*, a well known MGL producer, was included as the positive control to make sure the assays were working. In both cases, the highest specific activity was detected at pH 7.0. Control *E. coli* strain and boiled cell lysates were included as negative controls. Whereas boiled cell lysates did not give statistically significant activities compared with the regular cell lysates having vectors, in

both DTBN and MBTH assays, the control *E. coli* strain showed MGL-like activity, especially at the neutral and near neutral pHs, even though those were not statistically significant for the most part (data not shown).

To eliminate the confounding effect of using whole cell lysates, purification of His-tagged ferl MGL orthologs was attempted. However, but none of the elutes gave detectable level of MGL activity when standard manufacturer's protocol was followed. Different factors have been manipulated to optimize the results such as induction time (2 - 4 hours) and temperature (15 - 35°C), different concentrations of FastBreak reagent, DNase I and protease inhibitor cocktail, cell lysis time and temperature, shaking rate of the incubating shaker, amount of Ni-resin, changing the concentration of imidazole (50-1000 mM) in the Wash/Binding and elution buffer, addition of glycerol, 2-mercaptoethanl, PLP in the elutes right after the column being eluted etc. None of these changes resulted in detectable MGL activity (data not shown). However, in all cases, the protein concentrations in the elutes were usually high, even higher than cell lysate protein concentration in both Lowry and Bradford methods. Nonetheless, none of them showed the expected band in SDS-PAGE, even though some faint unwanted bands were visible in some cases (data not shown). The reason for it is still unknown.

The reasons for not getting the recombinant proteins purified would be many. The induction time might be short for the production of sufficient amount of purified proteins at 25°C. Thus time could be extended, such as for 15 hours or so, or 25 hours at 15°C. The poly His-tag might get into an interior position when proteins fold in to their active structure. In order to confirm that, the protein could be denatured with urea that will expose the poly His-tag and then passed through the column. The denatured protein can be reverted into their native structure by dialysis.

Other possibilities would be the inserts might have become mutated or might require different vector and/or a eukaryotic expression system like *Saccharomyces cerevisiae*, as archaeal physiology is more eukaryotic than prokaryotic (2, 3, 4). For example, some tRNAs that are essential for the translation of fer1 MGL orthologs might be absent in *E. coli* BL21 (DE3) but present in *S. cerevisiae*. At this point, some immunological testing could be performed to observe if the recombinant protein is being synthesized or not, such as Western blotting using anti-His antibody. Further, a different protein purification kit, such as the magnetic system, could be used.

In summary, fer1 has been shown to contain MGL activity in both MBTH and DTNB assays that are PLP and substrate dependent. The degradation of different substrates by both α, β- and α, γ-elimination reactions showed the multifunctional nature of fer1 lysate, typical of MGL activity. For both predicted orthologs of MGL, enzymatic assay results confirmed functions predicted by genomic annotation. However, specific characteristics of these orthologs in the purified forms, such as K_m, V_{max}, and substrate specificity, remain to be determined. Nonetheless, results from these experiments suggested that each step of the transsulfuration pathway would be amenable to similar analyses to confirm functional predictions. Full elucidation of the methionine-cysteine pathway will eventually shed light on the sulfur metabolism of this archaeal acidophile.

Studying on MGL may open the door in the treatment of certain types of cancer such as prostate, breast and gastric, as well as protozoan infections (28). The elevated requirement of L-methionine by some type of cancer cells opens the door of using MGL as a potential therapeutic agent in treating those cancers. The treatment with MGL causes depletion of L-methionine from the blood plasma (42). The efficiency of MGL was extensively documented over the following

cancer types: breast, gastric, kidney, colon, lung, and prostate cell lines (28). Moreover, some protozoan infections are also L-methionine dependent and MGL would also be applied in their treatments (21).

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