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Research Article

Evolutionary Analysis of unique membrane protein gene family of *Acidithiobacillus ferrooxidans*

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ABSTRACT

Acidithiobacillus ferrooxidans is a gram negative chemolithoautotropic proteobacterium that lives in high concentration of soluble ferrous and ferric iron, making it an interesting model for understanding biological mechanisms of bioleaching and bio mining in extremely acidic conditions. This bacteria has fascinating challenges for cells that use energy from bioleaching to grow. In this study we focus on the role of bioinformatics and its tools used in analysis of various unique uncharacterized membrane proteins in this bacterium that helps in explaining the activity of *Acidithiobacillus ferroxidans* in industrial bioleaching and its role as a primary producer in acidic environment.

Keywords: *Acidithiobacillus ferrooxidans;* Bioleaching; Phylogenetic Analysis.

INTRODUCTION

Acidithiobacillus ferroxidans is a chemolithoautotropic, γ-proteobacterium which uses energy from the oxidation of iron- and sulfur-containing minerals for growth. It survives in mineral rich, acidic environments optimally at 30ºC at extremely low pH of 1-2 which in turn fixes nitrogen and carbon from the atmosphere. They also solubilise other metals from rocks and plays a significant role in biogeochemical cycle in the atmosphere (Harrison 1984). This extremophile is mostly used for the bioleaching or bio-mining as the outer membrane of this bacterium is able to withstand acidic environments of pH 2–3 generate in the mining area (Guiliani & Jerez 2000). This outer membrane of *Acidithiobacillus ferroxidans* acts as a molecular sieve that acts as a passage channel for all ions and small hydrophilic organic molecules. This property of outer membrane is due to the presence of numerous speciali zed proteins which provide the porous nature to the membrane (Jap & Walian 1996; Koebnik et al. 2000; Nikaido 2003). The outer membrane of *A. ferrooxidans* helps bacteria not only as molecular sieve but also assists in the redox reaction activity like oxidation of ferrous iron or reduction of inorganic sulfur compounds for obtaining energy (Manchur et al. 2011). Therefore, studies of the structure and function of the outer membrane proteins of *Acidithiobacillus ferrooxidans* and their association with other membrane proteins is not fully understood.

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The membrane studies may reveal description of mechanisms involved in the fundamental biological reactions of bioleaching (Manchur et al. 2011).

Bioleaching is a hydrometallurgical process in which metal is solubilized by chemolithotrophs. Chemolithotrophs like *Acidithiobacillus ferrooxidans, Acidithiobacillus thiooxidans, Leptospirillus ferrooxidans* plays a major role in the extraction of metals from its ores. These organisms attach to the surface of the minerals and enhances the bioleaching. The attachment of the organisms with the minerals is facilitated by chemotaxis (Harneit et al. 2006). *Acidithiobacillus ferrooxidans* is the most important organism in bioleaching. It is a gram negative γ-proteobacterium has an optimum growth pH of 2 and optimum growth temperature of 30° C (Valdés, Pedroso, Quatrini & Holmes 2008). It obtains it energy from inorganic sources and fixes i ts own carbon. It is capable of oxidizing ferrous iron to ferric iron and sulphur and reduced sulphur compounds to sulphuric acid (Devasia & Natrajan K. A. 2004). It is widely studied because of its ability to recover metals like copper, gold, zinc cobalt from its ores. It is also important in the formation of acid mine drainage (Zhao et al. 2013).

During growth of *Acidithiobacillus ferrooxidans* on elemental sulphur, ferrous iron or sulfidic ores, membrane proteins play a key role in the transfer of electron (Zhang et al. 2002). The abundance or the activity of these membrane proteins may be up regulated due to the presence of solid substrate like sulphur and down regulated during the growth in iron containing solution (Ramírez et al. 2004).

There are two mechanisms involved in bioleaching by which *Acidithiobacillus ferrooxidans* oxidises the minerals 1. Indirect and 2. Direct mechanism. The indirect

mechanism is based on the Fe^{2+} to Fe^{3+} conversion cycle where Fe^{3+} is a strong oxidizing agent and able to oxidize metal sulphides to metal sulphates and also mono sulphides and disulphides to thiosulphates. But direct mechanism is based on the attachment of the organism on the ore surface. After the attachment the bacteria enzymatically attack the metal sulphides forming leached pits (Devasia & Natrajan K. A. 2004), (Xia et al. 2013), (Zhao et al. 2013)).

Outer membrane proteins (OMPs) which are synthesized in bacterial cytoplasm acts as (i) adhesins, (Aeckersberg et al. 2001) (ii)pores or channels for passive diffusion of metal ions, (Freitag, Neupert, and Benz 1982) (iii) protein translocation pores and (iv) enzymes *e.g.* lipases, proteases..etc (Nikaido 2003). There arises many challenges on passage of compounds through periplasmic membrane to the outer membrane due to the hydrophobic nature of phospholipid layer (Mogensen & Otzen 2005).

The structure and function of the proteins are studied by performing experiments which are laborious and time consuming. The *in-silico* approach saves time and reduces redundancy thereby helping to understand the issues which are not possible through conventional and traditional molecular methods.

The extensive literature search has revealed the lacuna in the scientific arena about evolution of membrane proteins of extremophiles. This work was designed to understand the similarity in membrane proteins and choose the unique proteins in the cell membrane of *Acidithiobacillus ferrooxidans* enabling it to survive in the extreme environments.

MATERIAL AND METHODS

Database search

The gene sequences of outer membrane proteins of *Acidithiobacillus ferroxidans* ATCC 23270 were collected from Gen-Bank/ EMBL/DDBJ (CP001219) (Valdés, Pedroso, Quatrini, Dodson, et al. 2008). The obtained accession number of the protein were submitted to SWISSPORT search engine to retrieve the FASTA sequence.

Selection of extremophiles

In order to get more subtle and accurate proteins of extremophiles the results obtained from BLASTP were further condensed by considering the percentage of maximum identity.

Phylogenetic analysis of protein families

Phylogenetics is a graphical representation, aimed at describing sequences similarity through evolutionary relationships among various organisms depending on the computational methods (Stillman et al. 2009).

The sequences of proteins obtained from other extremophiles were used for BLASTP search to retrieve the sequences in FASTA format. All the FASTA sequence of proteins of extremophiles were obtained, aligned and submitted in CLUSTALW for multiple sequence alignment. The construction of Phylogenetic tree was done using CLUSTAL W.

Multiple sequence alignment

Multiple sequence alignment of input query sequence were assumed to have an evolutionary relationship by which they share a common ancestor. This can also give conserved regions from which we can predict the secondary, tertiary structures and even individual amino acids and nucleotides. A list of all conserved regions were chosen for Phylogenetic analysis. The initial alignment were edited to produce a cladogram whi ch was used to find the closely related organisms.

Structure prediction

For protein analysis, information in protein databases can be used to predict certain properties about a protein, which can be useful for its empirical investigation. Primary, secondary tertiary structures of the unique proteins were predicted using the tools available online.

Primary structure

ProtParam was used for primary structure prediction and compute pI/Mw was used for the prediction of isoelectric point of the target protein sequence and molecular weight. These are free tools available at www.expasy.org. These tools uses extensive annotations available in the Swiss -Prot database mainly the position-specific annotations taking the posttranslational modifications in account (Gasteiger et al. 2005).

Secondary structure

The Secondary structure was predicted using the tools GOR4 and SOPMA. The output of the program contains 2 parts in which the first one consist of the predicted secondary structure in rows, Helix, extended or beta strands, and coils and the second one consist of the probability values for each secondary structure at each amino acid position (Garnier et al. 1996), (Geourjon C 1995).

Tertiary structure

The tertiary structure prediction was done with the tool SWISS-MODEL. It is an automated modelling system to determine the 3D structures of protein from its amino acid sequences using homology modelling techniques. It uses the friendly web interface for getting the input sequence and also to display the output. I -TASSER server was also used for the structure prediction (Biasini et al. 2014) (Zhang 2008; A Roy, A Kucukural 2010; Yang et al. 2015).

RESULTS

To investigate whether a protein family is evolved by duplication or mutation in common ancestors. We have analysed protein family of *Acidithiobacillus fer-*

rooxidans and other neighbouring families phylogenetically. Specifically we wanted to find out whether these protein family were closely or distantly related to the neighbouring organisms. The protein family were analysed phylogenetically in which species of *Acidithiobacillus ferrooxidans* were used as outgrowth to provide relative results. Identification of the evolutionary relationships of the unique membrane proteins from *Acidithiobacillus ferrooxidans* among other related organisms across taxa provides evolutionary insight for the genetic distance and function. Phylogenetic trees were constructed for the five different proteins and results were interpreted.

FASTA of all protein sequences included in this family were collected and submitted in BLASTP search tool in which one protein had nearly 100 hits which represents closely and distantly related species based on the E-value and this was used in the Phylogenetic analysis

Table 1: 65 Membrane proteins of *Acidithiobacillus ferrooxidans;* Out of 65 proteins identified, 38 proteins that are non-extremophiles were excluded based on 50% maximum identity.

Table 2: 27 Proteins Showing Complete Extremophilic Activity Considering the Stable Hit Value Of 50% Maximum Identity;The sequences were further filtered to 5 proteins with complete extremophiles taken in to consideration of 35% maximum identity.

Table 3: 5 Proteins Showing Complete Extremophilic Activity Considering the Stable Hit Value Of 35% Maximum Identity

MSA and phylogenetic tree for serine protease, DO/DEQQ family

Multiple sequence alignment performed in this protein showed highly conserved region (**LGSGF, INPGNS)** residues in *Acidithiobacillus ferrooxidans, A. caldus, A. thiooxidan, A. ferrivorans* and also few other closely related extremophiles. This conserved regions can be used for structure and function prediction. The following are the closely related organisms having this protein with our organism.

Iron-sulphur cluster-binding protein:

Only a partial conserved regions were showed with this protein in multiple sequence alignment but the closely organism in the tree are *Acidithiobacillus ferrooxidans, A. thiooxidans, A. caldus, A. ferrivorans, Thioalkalvibrio sp, T. sulphidophilus, T. nitratireducens, Ectothiorhodospira, T. sibirica, H. denitrificans.*

Putative uncharacterized protein

The results of this protein also show a partial conserved region and the closely related organisms are *A. ferrooxidans, Acidithiobacillus* GG1221*, A. ferrivorans, Acidiphilum* (fig. C). Among all closely related species the distance of *Acidithiobacillus* GG1221 is much higher.

Thioredoxin protein family

This protein shows less conserved regions and a closely related organism shows the same species and different genus which might have the similar functions to our query organism when compared to other organisms (fig. D). The related organisms are *A. ferrooxidans, A. ferrivorans, A. caldus*, *Acidithiobacillus* GG1221

Transcriptional regulator, MerR family

This protein shows more partially conserved regi ons and closely related organisms shows the same species and different genus which might have a similar function to the query organism. The related organism are *A. ferrovirans, A. thiooxidans, A. caldus, T. intermedia, Nitrococcus mobilis, Thiorhodococcus drewsii, Thioflavicoccus mobilis.*

Structure prediction

Primary structure

In the primary structure prediction number of amino acids, its molecular weight and the isoelectric points of the unique membrane proteins have been identified. Among all the 5 proteins the Serine protease (AFE_1396) have been found to have highest number of amino acids (479) and also higher molecular weight (48673). The Is-electric points of the 5 proteins are at neutral except for Iron-Sulfur Binding protein (AFE_2555).

Secondary structure

The secondary structure was computed with online tools GOR and SOPMA. In GOR prediction out of 5 proteins 4 have around 90 to 100 amino acids used for the formation of alpha helices and the proteins AFE_2867 has less amino acids used for alpha helix formation. The use of amino acids in extended strands and random coils were more in AFE_1396 and AFE_2463 when compared to other three proteins. There were no significant beta turns observed.

In SOPMA prediction the proteins with id AFE_0373 and AFE_1396 have used around 90 to 100 amino acids used for the formation of alpha helices whereas in AFE_2463 and AFE_2867 amino acids used for the formation of alpha helices were found to be less. But the protein with id AFE_2555 has used more amino acid for the alpha helices (151 amino acids). The extended strands are more in AFE_1396 and AFE_2463 and other proteins have used considerably less amino acids. In AFE_1396 and AFE_2463 proteins 46 and 22 amino acids respectively were used for formation of beta turns. Random coils were also more in AFE_1396 and AFE_2463 when compared to other proteins.

Tertiary structure

The tertiary structure was predicted by using SWISS-MODEL and I_TASSER online tools. The tools predicts more than one model for each protein. The model 1

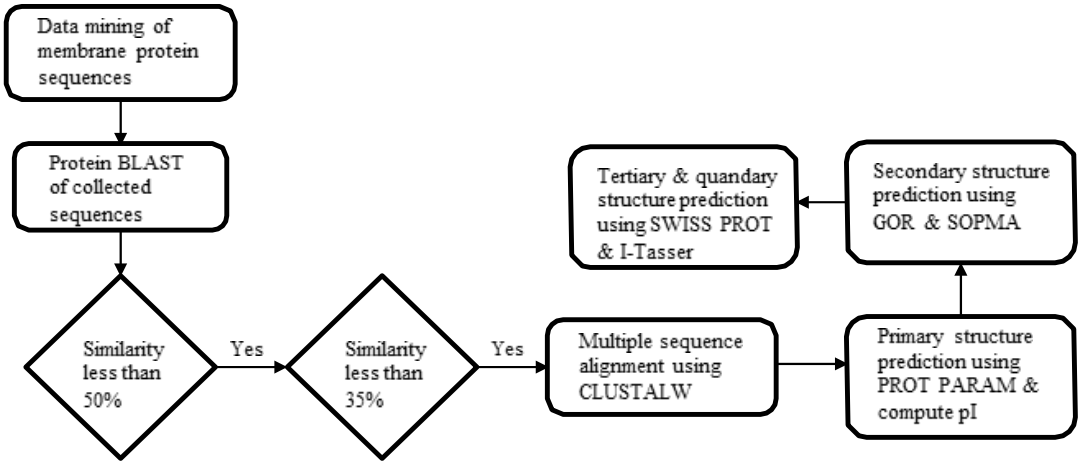

Figure 1: Overall flow of the work

Figure 2: Phylogenetic tree analysis serine protease

Table 1: 65 Membrane proteins of *Acidithiobacillus ferrooxidans*

Si No	Accession Number	Protein Name
1	AFE_0174	Exodeoxyribonuclease III
2	AFE_0848	2-dehydro-3-deoxyphosphooctonate aldolase
3	AFE_0169	Conserved domain protein
4	AFE_0343	50S ribosomal protein L18
5	AFE_0560	Putative uncharacterized protein
6	AFE_0075	Putative uncharacterized protein
7	AFE_0076	Polysaccharide biosynthesis protein
8	AFE_0783	Putative uncharacterized protein
9	AFE_0158	Toluene ABC transporter, ATP-binding protein
10	AFE_0297	Riboflavin biosynthesis protein RibD
11	AFE_0745	Acetolactate synthase
12	AFE_0850	Cell division protein FtsB
13	AFE_0851	Elongation factor P
14	AFE_0259	Biotin synthase
15	AFE_0373	Transcriptional regulator, MerR family
16	AFE_0008	Spore coat protein
17	AFE_0378	Outer membrane protein transport protein, OMP
18	AFE_0831	Putative uncharacterized protein
19	AFE_0847	CTP synthase
20	AFE_0555	Lipoprotein signal peptidase
21	AFE_1151	Putative uncharacterized protein
22	AFE_1648	Heat shock protein, Hsp20 family
23	AFE_1054	Putative uncharacterized protein
24	AFE_1590	Putative uncharacterized protein
25	AFE_1256	Putative uncharacterized protein
26	AFE_1586	DNA-binding protein, HU family
27	AFE_1587	Putative uncharacterized protein
28	AFE_1632	Putative uncharacterized protein
29	AFE_1685	Carboxysome shell peptide
30	AFE_1154	Putative uncharacterized protein
31	AFE_1396	Serine protease, DO/DeqQ family
32	AFE_2463	Putative uncharacterized protein
33	AFE_2957	Sensor histidine kinase
34	AFE_2438	Putative uncharacterized protein
35	AFE_2437	Cyclopropane-fatty-acyl-phospholipid synthase
36	AFE_2436	Putative uncharacterized protein
37	AFE_2435	Putative uncharacterized protein
38	AFE_2865	Putative uncharacterized protein
39	AFE_2866	Putative uncharacterized protein
40	AFE_2867	Thioredoxin family protein
41	AFE_2868	Putative uncharacterized protein
42	AFE_2975	Capsule polysaccharide export protein, BexD/C
43	AFE_2979	WbbD domain protein
44	AFE_2654	DNA polymerase IV
45	AFE_2655	Probable ribonuclease VapC
46	AFE_2555	Iron-sulfur cluster-binding protein
47	AFE_2556	Putative uncharacterized membrane protein
48	AFE_2579	Putative uncharacterized membrane protein
49	AFE_2748	ATP-dependent protease ATPase subunit HslU
50	AFE_2953	Maf-like protein AFE_2953
51	AFE_2722	Peptidase, U32 family
52	AFE_2087	Putative uncharacterized membrane protein
53	AFE_2445	Putative uncharacterized membrane protein

54	AFE_2621	NADH-quinone oxidoreductase, J subunit
55	AFE_2516	Putative uncharacterized membrane protein
56	AFE_2493	Putative uncharacterized membrane protein
57	AFE_2996	Putative uncharacterized membrane protein
58	AFE_2607	Transcriptional regulator, MerR family
59	AFE_2266	Acetyltransferase, GNAT family
60	AFE_2974	Putative uncharacterized protein
61	AFE_3103	Phosphoribosyl transferase domain protein
62	AFE_3018	Phosphoenolpyruvate-protein phosphotransferase
63	AFE_3179	Putative uncharacterized protein
64	AFE_3180	Putative uncharacterized membrane protein
65	AFE_3186	Mrr restriction system protein

Table 2; 27 proteins showing complete extremophilic activity considering the stable hit value of 50% maximum identity

Sl.no	Accession no	Protein
1	AFE_0848	2-dehydro-3-deoxyphosphooctonate aldolase
2	AFE_0169	Conserved domain protein
3	AFE_0075	Putative uncharacterized protein
4	AFE_0158	Toluene ABC transporter, ATP-binding protein,
5	AFE_0297	Riboflavin biosynthesis protein RibD
6	AFE_0373	Transcriptional regulator, MerR family
7	AFE_0378	Outer membrane protein transport protein, OMP
8	AFE_0847	CTP synthase
9	AFE_0555	Lipoprotein signal peptidase
10	AFE_1685	Carboxysome shell peptide
11	AFE_1396	Serine protease, DO/DeqQ family
12	AFE_2463	Putative uncharacterized protein
13	AFE_2957	Sensor histidine kinase
14	AFE_2867	Thioredoxin family protein
15	AFE_2868	Putative uncharacterized protein
16	AFE_2975	Capsule polysaccharide export protein, BexD/C
17	AFE_2654	DNA polymerase IV
18	AFE_2655	Probable ribonuclease VapC
19	AFE_2555	Iron-sulfur cluster-binding protein
20	AFE_2556	Putative uncharacterized protein
21	AFE_2748	ATP-dependent protease ATPase subunit HslU
22	AFE_2722	Peptidase, U32 family
23	AFE_2621	NADH-quinone oxidoreductase, J subunit
24	AFE_2516	Putative uncharacterized protein
25	AFE_2266	Acetyltransferase, GNAT family
26	AFE_2974	Putative uncharacterized protein
27	AFE_3018	Phosphoenolpyruvate-protein phosphotransferase

Table 3: 5 Proteins Showing Complete Extremophilic Activity Considering the Stable Hit Value Of 35% Maximum Identity

Sl.no	Accession no	Protein
1	AFE_0373	Transcriptional regulator, MerR family
2	AFE_2555	Iron-sulfur cluster-binding protein
3	AFE_1396	Serine protease, DO/DeqQ family
4	AFE_2867	Thioredoxin family protein
5	AFE_2463	Putative Uncharecterised Protein

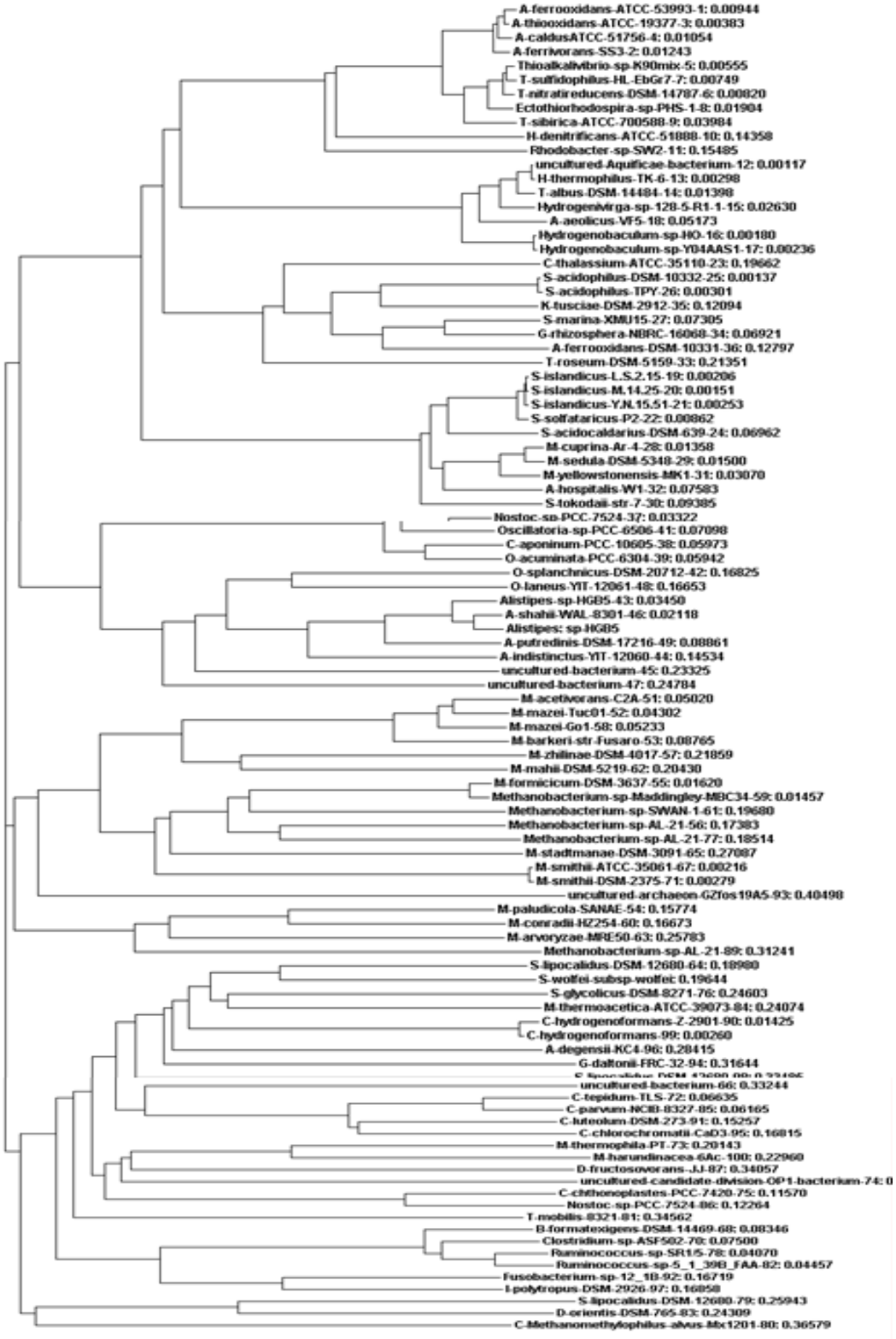

Figure 3: Phylogenetic tree analysis Iron sulphur cluster binding protein

Table 4: Primary structure prediction of unique membrane proteins

Si. No.	Gene	No. of amino acids	Molecular weight	Iso-electric Point
1	AFE_0373	158	17489	7.66
2	AFE_1396	479	48673	7.14
3	AFE_2463	396	42614	7.82
4	AFE_2555	228	26494.2	4.58
5	AFE_2867	87	9521.2	7.76

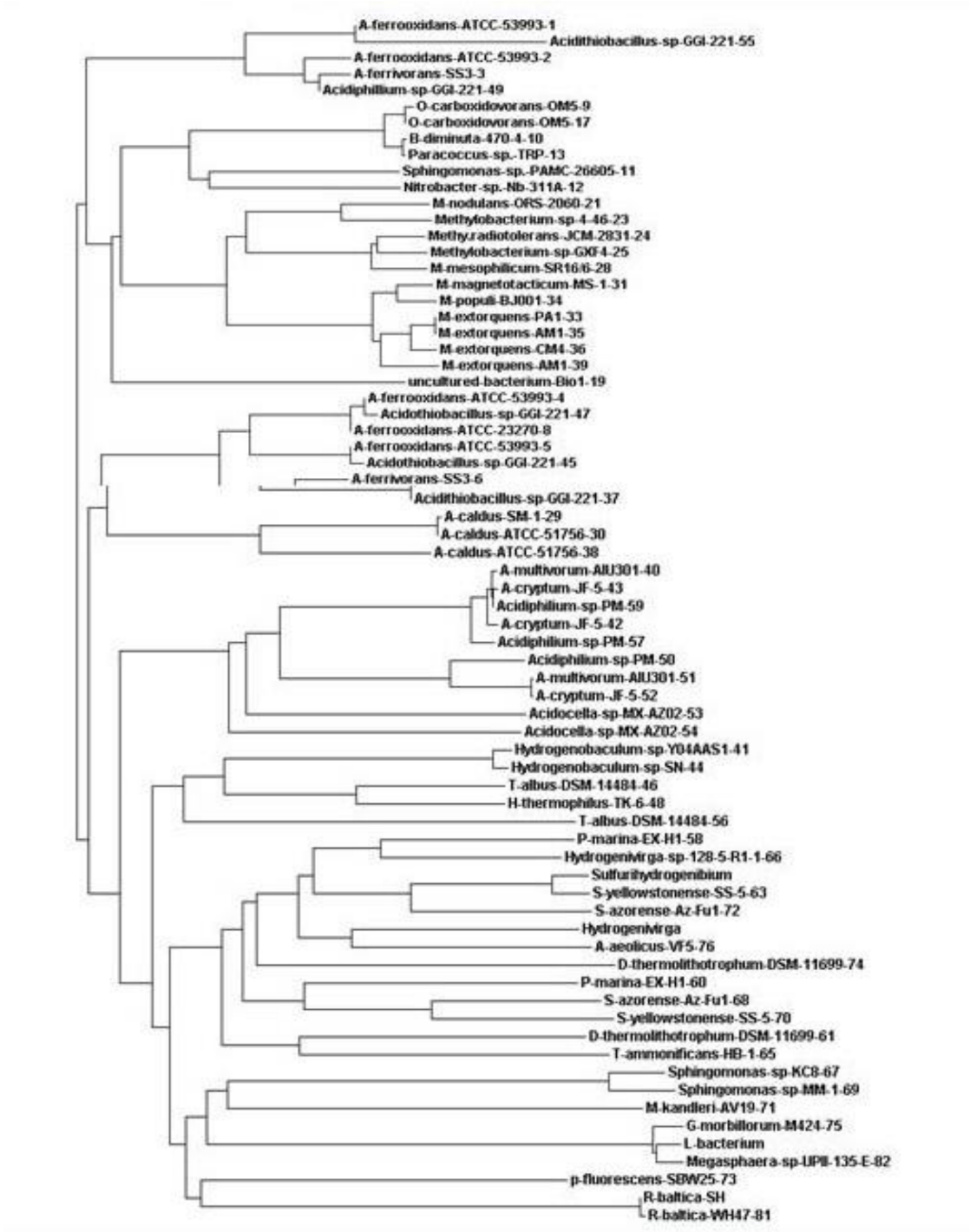

Figure 4: Phylogenetic tree analysis Putative uncharacterised protein

Table 5: Secondary structure prediction of unique membrane proteins using GOR tool

Si. No.	Gene	GOR4									
		Alpha helix	3_{10} helix	Pi helix	Beta bridge	Extended strand	Beta turn	Bend region	Random coil	Ambiguous states	Other states
1	AFE_0373	88	0	0	0	18	0	0	52	0	0
2	AFE_1396	93	0	0	0	130	0	0	256	0	0
3	AFE_2463	92	0	0	0	73	0	0	231	0	0
4	AFE_2555	101	0	0	0	35	0	0	92	0	0
5	AFE_2867	25	0	0	0	20	0	0	42	0	0

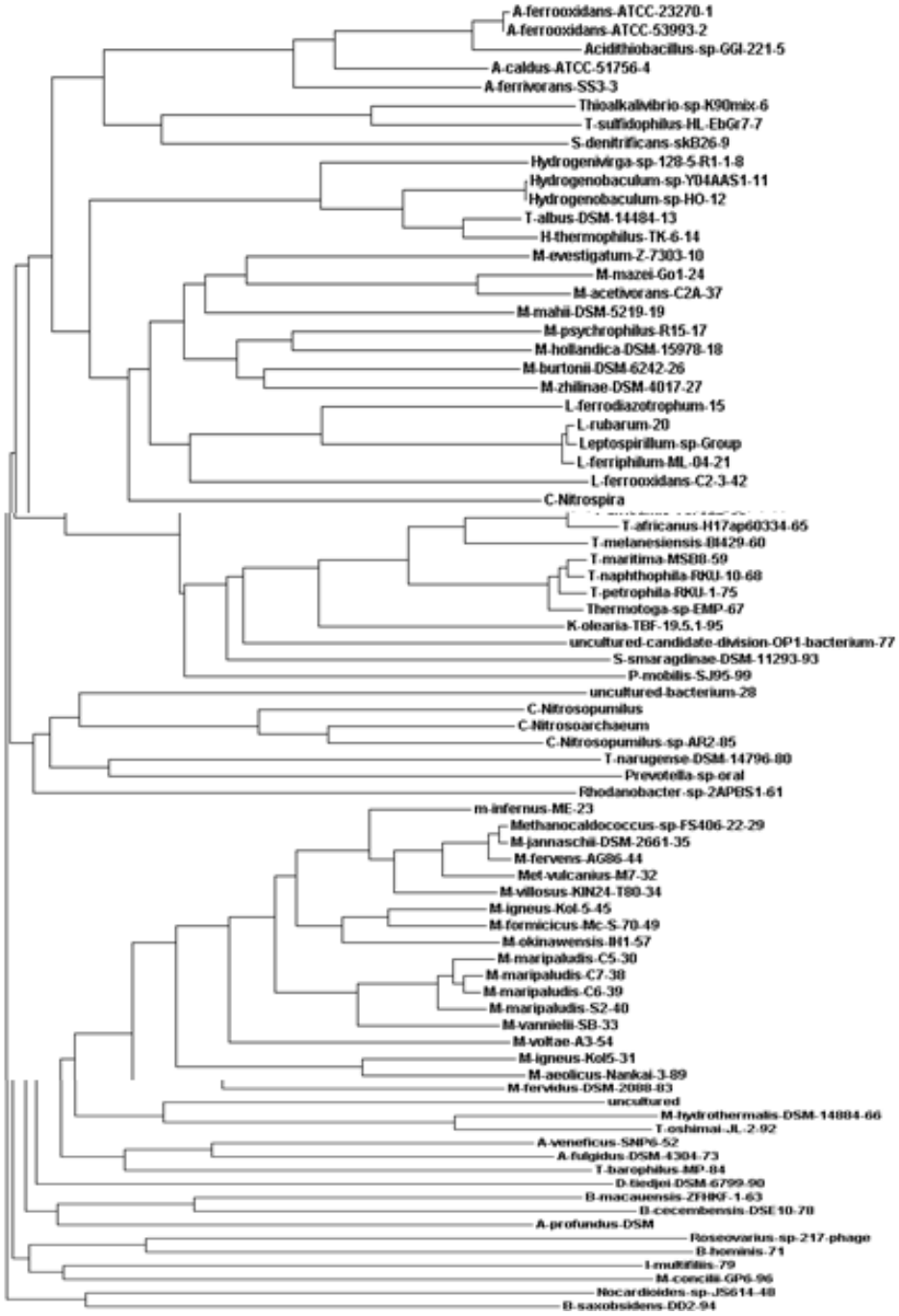

Figure 5: Phylogenetic tree analysis of Thioredoxin protein family

Table 6: Secondary structure prediction of unique membrane proteins using SOPMA tool

Si. No.	Gene	SOPMA									
		Alpha helix	3_{10} helix	Pi helix	Beta bridge	Extended strand	Beta turn	Bend region	Random coil	Ambiguous states	Other states
1	AFE_0373	98	0	0	0	2	6	0	52	0	0
2	AFE_1396	93	0	0	0	127	46	0	213	0	0
3	AFE_2463	52	0	0	0	112	22	0	210	0	0
4	AFE_2555	151	0	0	0	7	9	0	61	0	0
5	AFE_2867	28	0	0	0	22	4	0	33	0	0

Figure 6: Phylogenetic tree analysis of Transcriptional regulator, merr family

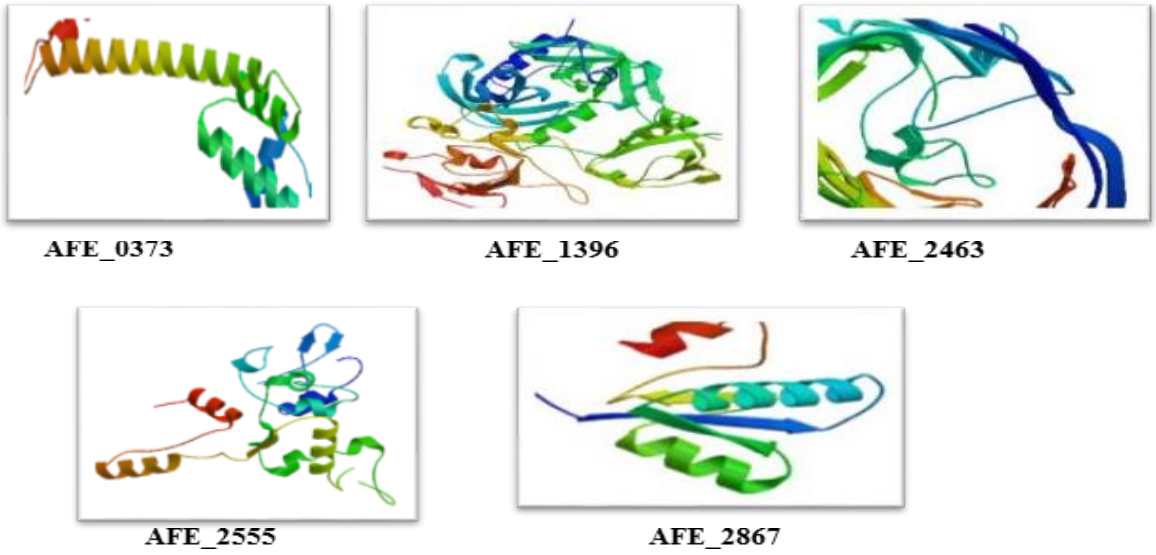

Figure 7: SWISS_MODEL structure prediction of 5 unique membrane proteins

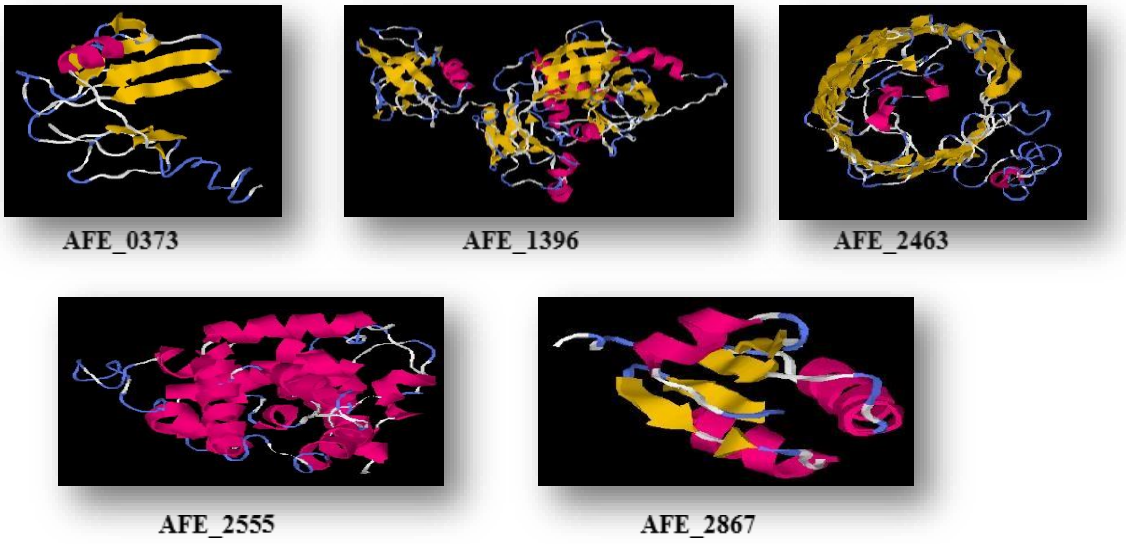

Figure 8: T-Tasser structure prediction of 5 unique membrane proteins

predicted by each tool has been taken as the best fit based on its RMSD value.

DISCUSSION

Finally, the functions of all these closely related organisms were identified. These functions were as follows . *Acidithiobacillus ferrooxidans* usually lives in pyrite deposits, which metabolises ferric and sulphur group to produce sulphuric acid. *Acidithiobacillus thiooxidans* takes up sulphur and produces sulphuric acid. *Thioalkalivibrio* is completely a salt tolerance bacterium which also produces sulphuric acid by consuming thesulphur group. *T. nitratireducens* is a thermophilic bacterium which reduces nitrate to nitric acid. *Ectothiorhodospira* is a gram negative phototrophic bacterium which also has the same function thereby reducing sulphur group. Hence the extremophilic organisms which we have isolated almost have a similar function (i.e.) all these organisms are involved in the reduction of nitrate to nitric acid and sulphur to sulphuric acid. So these organisms can be utilized in the process such as bioleaching, bio mining, etc.

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