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Characterization of DsrK and DsrO from *Allochromatium vinosum* and other Proteobacteria Using the Amino Acid Sequences.

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Research Article

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ABSTRACT

Sulfur metabolism is one of the oldest known redox geochemical cycles in our environment. One of the operons involved in this process is called *dsr* operon. The operon is involved in the balancing and utilization of environmental sulphur compounds. One of the central players of this operon is DsrMKJOP complex. DsrO is a periplasmic protein and binds FeS clusters which is mainly responsible for electron transfer to DsrP. DsrP could be involved in electron transfer from DsrP to DsrM. DsrM would then donate electrons to DsrK, the catalytic subunit of this complex. In the present study we tried to analyze the probable molecular details of DsrO and DsrK proteins from a diverse set of microbial species using only their sequence information. There are certain mutations present in the conserved domain of the protein. Those mutations confer some additional functionality to the protein. Sequences of the DsrO and DsrK proteins from different proteobacterial species have been analyzed to study the effects of mutations in the sequences and a phylogenetic relationship between the organisms has been drawn. We analyzed the secondary structural patterns of the DsrK and DsrO proteins also. There are no previous reports that deal with the bioinformatic analysis of the DsrO and DsrK protein. This is so far the first report of its kind. Our study would therefore pave the pathway to future genetic and mutational studies using DsrK and DsrO proteins that would lead to illumination of the biochemical mechanism of sulphur metabolism.

INTRODUCTION

Sulfur cycle is one of the important biogeochemical cycles in the world. Sulfur has a wide range of oxidation states viz., +6 to -2 and this makes the element capable of taking part in a number of different biological processes. Sulfur based chemo or photolithotrophy is one of such processes involving the transfer of electrons from reduced sulfur compounds. The sulfur metabolism is meadiated by a diverse set of microorganisms. The different sulfur anions that are abundant in nature and are used by these sets of microorganisms are sulfide, polysulfide, thiosulfate, as well as elemental sulfur. Only very little is known about the molecular mechanisms of this ecologically as well as industrially important process and about the microorganisms. One of them is *Allochrmatium vinosum*, dominant member of purple sulfur bacteria uses reduced sulfur compounds as electron donors for anoxygenic photosynthesis [1]. Recent studies with *Allochrmatium vinosum* revealed that a multiple gene cluster comrpising of genes *dsrA*, *dsrB*, *dsrE*, *dsrF*, *dsrH*, *dsrC*, *dsrM*, *dsrK*, *dsrL*, *dsrJ*, *dsrO*, *dsrP*, *dsrN*, *dsrS* and *dsrR* is involved in the process [2]. The organism *A. vinosum* has been used in different industrial processes, viz., waste remediation and removal of toxic compounds, e.g. odorous sulfur compounds like sulfide or even explosives [1]. This organism has also been used in the production of industrially relevant organo-chemicals such as vitamins or bio-polyesters and the production of biohydrogen [1]. From the currently available litteratures it was revealed that in sulfur oxidizer *A. Vinosum* the degradation of sulfur globules is strictly dependent on the presence of the DsrMKJOP proteins [2]. DsrJ, periplasmic protein, may be involved in the oxidation of a putative sulfur substrate in the periplasm and the released electrons would be transported across the membrane via the other components (DsrO, DsrP, DsrM, DsrK successively) of the DsrMKJOP complex [2]. DsrO is a periplasmic iron-sulfur, ferredoxin-like protein [2]. DsrP and DsrM are both quinone-inter-acting transmembrane proteins, and the heme b present in DsrP could be involved in electron transfer from DsrP to DsrM [2]. DsrM may work as a quinol oxidase donating electrons

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to DsrK. DsrK is proposed to be a cytoplasmic iron sulfur protein, and the catalytic subunit of the DsrMKJOP protein complex that it acts as a (hetero) disulfide reductase, and that the protein DsrC might be its substrate [2]. So, DsrO and DsrK were both experimentally proven to be FeS-containing proteins [2] take part into the electron transfer. In our study we made an attempt to characterize DsrO and DsrK protein at the sequence level. We analyzed the amino acid sequences of these two proteins from different proteobacteria. We predicted the putative secondary structures and as well as conserved domains present in those proteins. Multiple sequence analyses of these proteins revealed the presence of certain mutations in the protein. We also predicted the effects of those mutations present in the conserved domain of DsrK and DsrO protein and correlated the effects of mutations with the taxonomical distributions of the microorganisms. Till date there are no such reports that deal with the analyses of the mutations in these two proteins using bioinformatics approach. This work is therefore first of its kind. Since there are no previous reports regarding the molecular details of DsrO and DsrK proteins our work would therefore be important to analyze the biochemical details of the *dsr* operon.

MATERIALS AND METHODS

Sequence Homology Search and Pair Wise Alignment of Sequences

From NCBI protein database, *Allochromatium vinosum* DSM 180 used as input for BLAST. BLAST program [3] is run using the algorithm Blastp (protein-protein BLAST), non redundant (nr) protein sequences database. The blast result is sort by maximum identity, 29 organisms are selected for study which have greater than 50% identity. We removed the uncultured bacterial species and redundancies from the collected sequences. Only those amino acid sequences were chosen for which there were clear annotations and no ambiguities were present. NCBI refseq was selected for collecting our required sequences because it provides comprehensive, integrated, non-redundant and a well-annotated set of sequences. The name of the DsrO and DsrK proteins of the selected microorganisms were listed below.

Table1: List of organisms of DsrO protein having identity more than 50%. This list is obtained from BLAST DsrO

Sequence number	Accession code	Organism	Identity (%)
S1	Gb|ADC62200.1|	*Allochromatium vinosum* DSM180	100
S2	Gb|EGV31404.1|	*Thiorhodococcus drewsli* AZ1	77
S3	Gb|AFL72613.1|	*Thiocystis violascens DSM 198*	81
S4	Gb|EGV17846.1|	*Thiocapsa marina 5811*	79
S5	Gb|AGA89028.1|	*Thioflavicoccus mobilis 8321*	73
S6	Gb|EIC21190.1|	*Thiorhodovibrio sp. 970*	67
S7	Gb|EGV22420.1|	*Marichromatium purpuratum 984*	76
S8	Gb|EGW53661.1|	endosymbiont of *Tevnia jerichonana (vent Tica)*	63
S9	Gb|EHR72134.1|	*Burkholderiales bacterium JOSHI_001*	59
S10	Gb|AGA32540.1|	*Thioalkalivibrio nitratireducens DSM 14787*	62
S11	Gb|EGZ34775.1|	*Thioalkalivibrio thiocyanoxidans* arh 4	60
S12	Gb|ACL73275.1|	*Thioalkalivibrio sulfidophilus* HL-ebgr7	58
S13	Gb|AAZ98428.1|	*Thiobacillus denitrificans* ATCC 25259	59
S14	Dbj|GAB72886.1|	*Sulfuricella denitrificans* skb26	55
S15	Gb|EGV52271.1|	endosymbiont of *Riftia pachyptila (vent Ph05)*	66
S16	Gb|ABI57009.1|	*Alkalilimnicola ehrlichii* MLHE-1	61
S17	Gb|ADE11909.1|	*Sideroxydans lithotrophicus* ES-1	56
S18	Gb|EIJ36278.1|	*Thiothrix nivea* DSM 5205	52
S19	Emb|CAM75798.1|	*Magnetospirillum gryphiswaldense* MSR-1	55
S20	Gb|ADP69869.1|	*Rhodomicrobium vannielii* ATCC 17100	53
S21	Gb|ABL02576.1|	*Candidatus Ruthia magnifica* str. Cm (*Calyptogena magnifica*)	51
S22	Dbj|BAF61891.1|	*Candidatus Vesicomyosocius okutanii* HA	51
S23	Ref|WP_010645608.1|	endosymbiont of *Bathymodiolus sp*	51
S24	Ref|WP_009869254.1|	*Magnetospirillum magnetotacticum*	54
S25	Gb|EME69575.1|	*Magnetospirillum sp.* SO-1	53
S26	Gb|ABM62725.1|	*Halorhodospira halophila* SL1	56
S27	Gb|EDN71423.1|	*Beggiatoa sp.* PS	66
S28	Dbj|BAE52181.1|	*Magnetospirillum magneticum* AMB-1	54

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Table2: List of organisms of DsrK protein having identity more than 50%. This list is obtained from BLAST DsrK

Sequence no.	Accession code	Organism	% identity
seq1	gb|ADC62197.1|	*Allochromatium vinosum DSM 180*	100
seq2	gb|EGV31407.1|	*Thiorhodococcus drewsii AZ1*	93
seq3	gb|EGV22417.1|	*Marichromatium purpuratum 984*	92
seq4	gb|AFL72610.1|	*Thiocystis violascens DSM 198*	91
seq5	gb|EGV17849.1|	*Thiocapsa marina 5811*	91
seq6	gb|AGA89025.1|	*Thioflavicoccus mobilis 8321*	83
seq7	gb|EIC21193.1|	*Thiorhodovibrio sp. 970*	84
seq8	gb|EGV52268.1|	*endosymbiont of Riftia pachyptila (vent Ph05)*	78
seq9	gb|ACL73272.1|	*Thioalkalivibrio sulfidophilus HL-EbGr7*	77
seq10	gb|EIJ36275.1|	*Thiothrix nivea DSM 5205*	75
seq11	gb|AGA32543.1|	*Thioalkalivibrio nitratireducens DSM 14787*	76
seq12	gb|EGZ34778.1|	*Thioalkalivibrio thiocyanoxidans ARh 4*	76
seq13	gb|EDN71419.1|	*Beggiatoa sp. PS*	79
seq14	gb|ADP69872.1|	*Rhodomicrobium vannielii ATCC 17100*	74
seq15	dbj|BAE52178.1|	*Magnetospirillum magneticum AMB-1*	73
seq16	emb|CAM75801.1|	*Magnetospirillum gryphiswaldense MSR-1*	70
seq17	gb|AAZ98431.1|	*Thiobacillus denitrificans ATCC 25259*	69
seq18	gb|EIJ41167.1|	*Beggiatoa alba B18LD*	68
seq19	dbj|GAB72883.1|	*Sulfuricella denitrificans skB26*	68
seq20	gb|EHR72138.1|	*Burkholderiales bacterium JOSHI_001*	69
seq21	ref|WP_010645602.1|	*endosymbiont of Bathymodiolus sp*	66
seq22	dbj|BAF61894.1|	*Candidatus Vesicomyosocius okutanii HA*	67
seq23	gb|ABI57006.1|	*Alkalilimnicola ehrlichii MLHE-1*	68
seq24	gb|ADE11912.1|	*Sideroxydans lithotrophicus ES-1*	66
seq25	gb|ABL02579.1|	*Candidatus Ruthia magnifica str. Cm(Calyptogena magnifica)*	66
seq26	gb|ABK42579.1|	*Magnetococcus marinus MC-1*	66
seq27	gb|ABM62722.1|	*Halorhodospira halophila SL1*	64
seq28	ref|WP_009867036.1|	*Magnetospirillum magnetotacticum*	73
seq29	gb|EDN72653.1|	*Beggiatoa sp. SS*	72

These sequences were used as inputs to run the program BLAST using the default parameters, in order to find out the conserved domains in those proteins. The BLAST results again produced the same set of sequences as obtained previously. This could be considered as a double check of our initial results of downloading the sequences.

Prediction of Secondary Structure

The secondary structures of the DsrO and DsrK proteins of 28 and 29 different organisms respectively were predicted from their amino acid sequences using the Network Protein Analysis Server (NPS@). We used the following methods: SOPM , HNN , DPM , DSC , GOR I , GOR III , PHD , PREDATOR. The secondary structures were classified as alpha helix, random coil, extended strand and others.

Prediction of Conserved Domain

The conserved functional domains of the DsrO and DsrK protein were predicted from the outputs of Pfam [4]. For Pfam search we used the full amino acid sequence of those proteins.

Multiple Sequence Alignment (MSA)

In order to study the sequence conservations among the 28 and 29 proteins of DsrO and DsrK proteins respectively we generated a sequence profile by MSA, using the default parameters in the software tool ClustalW [5].

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We used the full sequence of DsrO and DsrK from all the organisms as mentioned in the section 2.1. From the results of MSA the presence of mutations in the sequences were detected.

Mutation analysis in the conserved domain

The significant mutations found in the conserved domain of the two proteins by MSA were analyzed using Pfam. The Pfam search enabled us to detect the presence of additional sub domains in those proteins. These additional sub domains are involved in different metabolic processes as required by the organisms depending on their habitat.

Distance Matrix Calculation and Construction of Phylogenetic Tree

A distance matrix was generated using MEGA [6]. This tool used the Maximum Composite Likelihood (MCL) method to estimate the evolutionary distances between sequences. The MCL approach was different from the existing approaches for evolutionary distance estimation, where each distance was estimated independent of others, either by analytical formulae or by likelihood methods.

Result

Predicted Secondary Structure

The consensus secondary structural patterns of DsrO and DsrK proteins of *A. vinosum* as obtained from the averaging of the predicted secondary structures by the methods mentioned in the 2.2 section of the manuscript was compared in Table 3 and presented in Fig. 1a and 1b respectively.

Table3: Comparative result of Predicted Secondary Structure Patterns of DsrO and DsrK

Protein Name	Alpha helix (%)	Extended strand (%)
DsrO	23.51	19.40
DsrK	39.12	8.21

Figure 1a

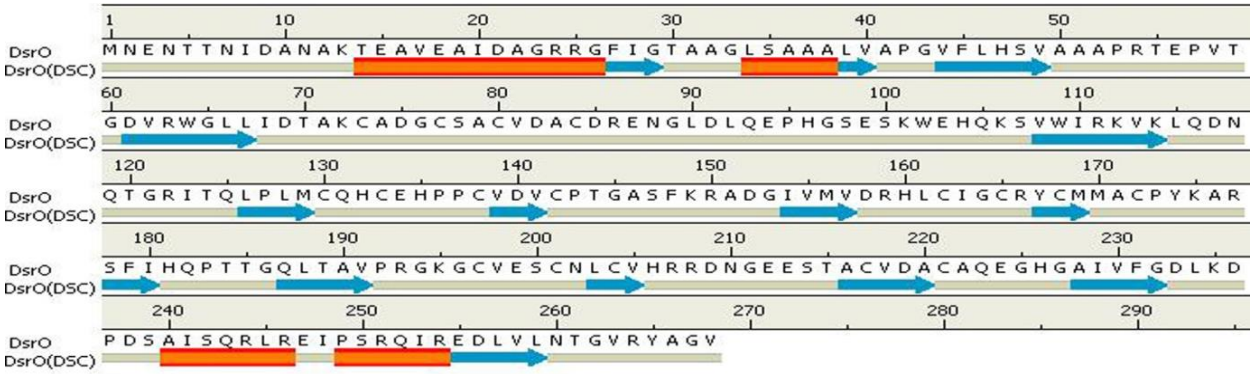
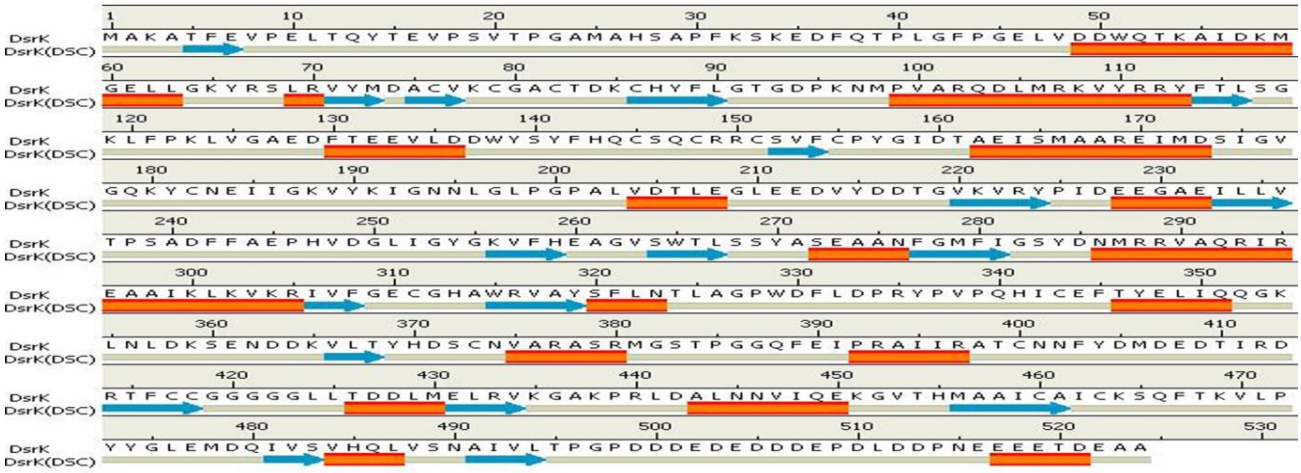


Figure 1b



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Secondary structure of DsrO (Fig.1a) and DsrK (Fig.1b) of *Allochrmatium vinosum*. Colour represents in the figure: Alpha helix (Orange), Extended strand (Blue), Random coil (colourless).

Predicted Conserved Domain

The full length sequence of DsrO protein revealed a significant protein family, Fer4_11 i.e. 4Fe-4S discluster domain (PF13247) at the amino acid residues 125-224 and another insignificant family, Fer2_11i.e. 4Fe-4S binding domain (IPR001450) at the amino acid position 64-84. This superfamily includes proteins containing domains which bind to iron-sulphur clusters. Members include bacterial ferredoxins, various dehydrogenases, and various reductases. Structure of the domain is an alpha-antiparallel beta sandwich. Domain contains two 4Fe4S clusters. Based upon sequence analysis, it is previously reported that DsrK contains two classical $CX_2CX_2CX_3C$ binding sites for [4Fe-4S] clusters and one C-terminal CCG domain with the sequence $CX_{43}CCGX_{40}CX_2C$ that may bind an additional putatively catalytic [FeS] cluster [2]. In our Pfam result of the full sequence of DsrK showed significant two protein families Fer 4_8 (4Fe-4S discluster domain, PF13183) and CCG (Cystein-rich domain, IPR004017) at amino acid position 73-159 and 368-467 respectively. The CCG protein family contains up to four conserved cysteines. The group includes proteins characterised as: heterodisulphide reductase, subunit B (HrdB); succinate dehydrogenase, subunit C; Fe-S oxidoreductase; glycerol-3-phosphate dehydrogenase subunit C; and glycolate oxidase iron-sulphur subunit (GlcF) [7]. Another insignificant match found at amino acid residues 128-163 of protein family named alpha-hel2 (Alpha-helical domain 2, PF14456). An alpha-helical domain found in gene neighbourhoods encoding genes containing bacterial homologs of components of the ubiquitin modification pathway such as the E1, E2, Ub and JAB peptidase proteins. These conserved functional domains of the two proteins ensure their experimental evidence as Fe-S binding domain through bioinformatics approach. But all of their significant presence in related to electron transfer through MKJOP complex is still not known to us. So, any mutations occurring in these conserved domains helping them to form any functional specificity was our next study topic.

Mutation Study within Conserved Domain

The amino acid sequences of DsrO and DsrK proteins from all those organisms were analysed from the sequence profiles that were generated by MSA using the software tool ClustalW. The MSA results of DsrO showed the presence of 45 conserved and 25 synonymous substitutions in the global alignment; whereas in DsrK 95 conserved and 64 synonymous substitutions were noted. The remaining positions of the alignment have undergone some kind of significant mutations. We analyzed those significant mutations using Pfam and obtained the presence of some new sub domains. The significant sub domains analyzed from Pfam were described here in the following paragraphs.

In DsrO protein, a new subdomain similar to TAT (twin-arginine translocation) pathway signal sequence protein family (IPR019546) was found in a few organisms at amino acid residue position 8-30. This protein transports folded protein across energy transducing membrane [8]. In *Thioalkalivibrio sulfidophilus* HL-EbGr7 mutation found at amino acid sequence position 37. There following two new sub-domains were found: CytB6-F_Fe-S (PF08802) (IPR014909) and Rubredoxin (IPR024935) (PF00301). The cytochrome B6-F complex present in amino acid sequence spanning 7-28, which mediates electron transfer between photosystem II (PS II) and photosystem I (PS I), cyclic electron flow around PSI, and state transitions. This domain corresponds to the alpha helical transmembrane domain of the cytochrome B6-F complex Rieske iron-sulphur subunit. Rubredoxin protein domain spans in the amino acid sequence range 37-62. It is a low molecular weight iron-containing bacterial protein involved in electron transfer sometimes replacing ferredoxin as an electron carrier [9]. Rubredoxins possess a 45- to 55-residue domain containing one iron atom tetrahedrally coordinated to four cysteinyl residues. Structural analysis of the domains have shown them to be folded into a short three-stranded antiparallel beta-sheet and a number of loops. Mutation in 52 (I) of endosymbiont of *Bathymodiolus sp*. revealed insignificant match at amino acid position 44-79 with Iron_permease (PF04120) (IPR007251), i.e. low affinity iron permease. It is originally identified as a low-affinity iron (II) permease [10]. Fet4 has since been shown to import several other transition metal ions, including copper and zinc.

In DsrK few sub-domains were found which are described here. The group of serine protease inhibitors (IPR004094) belong to MEROPS inhibitor family. They inhibit serine peptidases of the S1 family [11] and are characterised by a well conserved pattern of cysteine residues. It is found at amino acid position 145-161. Glycine reductase complex selenoprotein A (GRDA) protein domain family (IPR006812) found in *Thiocapsa marina* 5811and endosymbiont *of Riftia pachyptila (vent Ph05)* at the amino acid sequence spanning 188-214. This protein contains one active site selenocysteine and catalyses the reductive deamination of glycine, which is coupled to the esterification of orthophosphate resulting in the formation of ATP [12]. In *Thiorhodovibrio sp.* 970 at amino acid position 53 mutation showed new domain spanning 30-63 named Enterotoxin_b (IPR001835). It is a heat-labile enterotoxin is a bacterial protein toxin with an AB5 multimer structure, in which the B pentamer has a membrane-binding function and the A chain is needed for enzymatic activity [13]. From experimental analysis it was

also found that DsrK has some membrane bound region and it is the catalytic subunit of MKJOP complex [2]. Adenylate cyclase associated (CAP) N terminal (CAP_N) (IPR013992) has been found in *Beggiatoa sp.* PS and *Alkalilimnicola ehrlichii* MLHE-1 at amino acid sequence spanning 225-275. This N-terminal domain is responsible for adenylate cyclase activation. *Candidatus Vesicomyosocius okutanii* HA at amino acid position 183 showed mutation whichrevealed new domain from 169-209, i.e. SAM like domain present in kinase suppressor RAS 1 (KSR1-SAM) (IPR025561). This SAM-like domain is found in kinase suppressor of Ras proteins, which are location-regulated scaffolding proteins connecting MEK to RAF. Peptidase M16C associated (M16C_assoc) (IPR013578) found in *Candidatus Ruthia magnifica str. Cm (Calyptogena magnifica*) at amino acid spanning 208-234. It is found near the C terminus of metalloproteases required for catalysis. Another significant domain found in N-6 DNA Methylase (N6_Mtase) (IPR003356) in *Thiobacillus denitrificans* ATCC 25259 at amino acid position 417. This domain spanning from 397-438 is found in N-6 adenine-specific DNA methylase from Type I and Type IC restriction systems. These enzymes are responsible for the methylation of specific DNA sequences in order to prevent the host from digesting its own genome via its restriction enzymes.

In this new domain study helps us to study the functional importance of the DsrO and DsrK protein. Additional functionality is observed by Pfam showed the presence of domains that are peptidase or kinase associated protein families required for catalysis in DsrK protein. Now, in *A.vinosum* how these taxonomically related microorganisms' mutation help to form Fe-S binding protein DsrK and DsrO can be understand.

Phylogenetic analysis

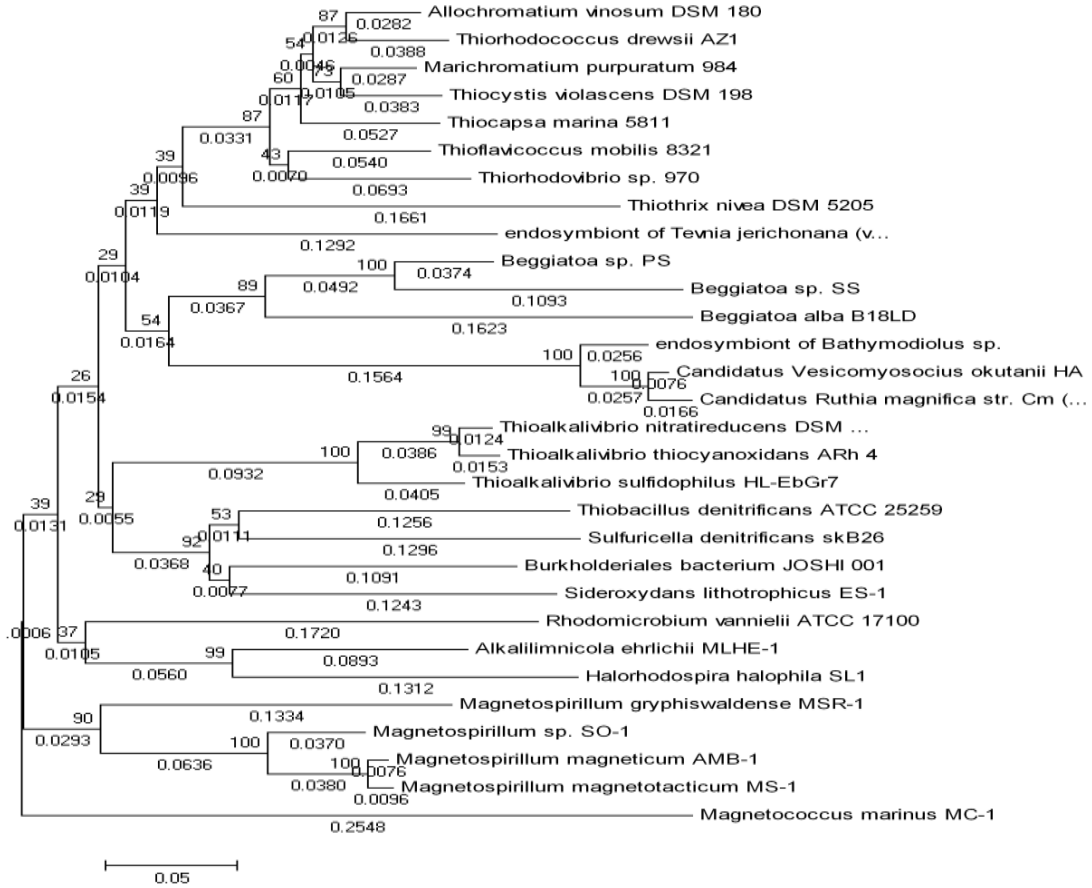

Figure 2 Phylogenetic tree drawn using Neighbor Joining method with 1000 bootstrap. Model: Amino: Poisson Correction

The phylogenetic study of DsrO was already reported [14], so we concentrated on the phylogenetic relationship to DsrK. We used Multiple Sequence Alignment (MSA) to detect the sequence conservation/variations in the DsrK proteins in all the 29 different organisms. In order to derive a phylogenetic relationship between these proteins a phylogenetic tree comprising the 29 different proteobacterial species (belonging to the classes Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteia) was constructed (Fig.2). In the top of the branch of the tree *Allochromatium vinosum* and *Thiorhodococcus drewsii* strain AZ1 were clubbed together. Both of these micro-organisms are non-sporulating, mesophilic and belonging to marine or alkaline habitat. They have intracellular vesicular membranes and are capable of using hydrogen sulfide, sulfur, thiosulfate, and molecular hydrogen as electron donors during anaerobic phototrophic growth. Elemental sulfur globules were transiently

stored inside the cells of these micro-organisms [1; 15]. With them *Marichromatium purpuratum* 984, *Thiocystis violascens* DSM 198, *Thiocapsa marina* 5811, *Thioflavicoccus mobilis* 8321, *Thiorhodovibrio sp.* 970 formed a subgroup. All these organisms belong to the Kingdom: Bacteria Phylum: Proteobacteria Class: Gammaproteobacteria Order: Chromatiales Family: Chromatiaceae [14; 16]. Therefore their grouping together was very much significant. Similarly, the next branch showed similar physiological characteristic organisms *Beggiatoa alba* B18LD, *Beggiatoa sp.* PS and *Beggiatoa sp.* SS belonging Kingdom: Bacteria; Phylum: Proteobacteria; Class: Gammaproteobacteria; Order: Thiotrichales; Family: Thiotrichaceae; Genus: Beggiatoa. The same trend followed throughout the phylogenetic tree. At the bottom of the tree had Magnetospirillum *gryphiswaldense* MSR-1, *Magnetospirillum magnetotacticum* MS-1, *Magnetospirillum magneticum* AMB-1, *Magnetospirillum sp.* SO-1 belong to the Kingdom: Bacteria; Phylum: Proteobacteria; Class: Alphaproteobacteria; Order: Rhodospirillales; Family: Rhodospirillaceae; Genus: Magnetospirillum. Magnetospirillum is a Gram-negative, microaerophilic genus of magnetotactic bacterium with spirillar or helical morphology; motile; and has peculiar capacity to orient itself according to Earth's magnetic field, named magnetotaxis which is achieved through the special organelles called magnetosomes [17]. So, they formed a group altogether. The importance of lateral gene transfer for the distribution of the *dsr* genes is underscored by the fact that the clade of sulfide oxidizers contains members of the phylogenetically distantly related species. Therefore it can be easily concluded that the phylogenetic arrangements of the bacterial species follow their taxonomic chronology.

DISCUSSION

In this work we tried to analyze the details of DsrO and DsrK proteins of the *dsr* operon at the sequence level. Both proteins are the central players of the *dsr* operon and take part in the electron transfer process. We analyzed the secondary structural patterns of those proteins. The sequence analyses of these proteins revealed the presence of conserved domains. There are certain mutations present inside the conserved domain of the protein. Those mutations confer some additional functionality to the protein. Mutation analysis confirmed that DsrO is a FeS containing protein binding to iron-sulphur clusters. DsrK also is a FeS containing protein binding to iron-sulfur clusters and has a cysteine rich domain. Finally we analyzed the DsrK protein using phylogenetic trees. Our study is the first of its kind. Till date there are no previous reports regarding the in depth analyses of DsrO and DsrK proteins from their amino acid sequences. Our study would therefore pave the pathway to future genetic and mutational studies using these two proteins that would lead to illumination of the biochemical mechanism of sulfur metabolism.

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