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IN SILICO ANALYSIS OF POTENTIAL METALLOTHIONEINS IN *PSEUDOMONAS*

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ABSTRACT: Microorganisms are intimately involved in metal biogeochemistry through a variety of resistance mechanisms, which include efflux, reduction, oxidation, precipitation, extracellular sequestration and intracellular bioaccumulation. Intracellular metal bioaccumulation and homeostasis in cell cytosol often involves cysteine rich metallothionein proteins. The first family of bacterial metallothioneins is the BmtA family (a zinc and cadmium-binding SmtA from the cyanobacterium *Synechococcus* PCC 7942). Many features of SmtA are different from metallothioneins belonging to other organisms. Homologues for SmtA have been identified in some of the bacterial genomes. In comparison to metallothioneins reported in other bacterial strains, *Pseudomonas* metallothioneins are relatively less explored. Despite the progress made in understanding the role of various metal resistance determinants in maintaining metal homeostasis in this genus, many questions remain if we consider the role of metallothioneins. Based on information from database and sequence analysis a close association of some metallothioneins from *Pseudomonas* with *Synechococcus* and other Cyanobacterial SmtA indicates the role of horizontal gene transfer in the acquisition of this gene. Sequence analysis of SmtB (a Zn(II)-responsive transcriptional repressor, named for *Synechococcus* PCC7942 SmtB) like regulator protein from members of genus *Pseudomonas* revealed interesting variability leading to presence or absence of dimerization domain and Zn binding domain. Thus, indicating differences in regulation and mode of action of *smtB* like regulatory gene in *Pseudomonas.*

KEYWORDS: Metallothioneins; *Pseudomonas*; Heavy-metal, Cysteine.

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1. INTRODUCTION

Microorganisms play an important role in metal biogeochemistry through a variety of resistance mechanisms, which include efflux, reduction, oxidation, precipitation, extracellular sequestration and intracellular bioaccumulation [1, 2]. Cysteine rich metallothionein proteins play an important role in immobilization of toxic heavy metals within the bacterial cell; thereby protecting their enzyme catalyzed metabolic processes [3, 4, 5]. Metallothioneins are ubiquitous low molecular weight proteins of extremely high metal and cysteine content having an important role in chelation, intracellular distribution, storage and detoxification of metals and defense against oxidative stress. Metallothioneins include a protein superfamily of 15 families from several animal, plants, fungi, and bacteria. Although eukaryotic metallothioneins have been extensively characterized, bacterial metallothioneins are rare and comparatively less studied [6]. Bacterial metallothioneins first reported in mid of the 1980s was the BmtA family (a zinc and cadmium-binding SmtA from the cyanobacterium *Synechococcus* PCC 7942). The second type of bacterial metallothionein was identified in *Mycobacteria*, which encoded a copper binding gene product named as MymT [7, 8]. Many features of bacterial SmtA are different from metallothioneins belonging to other eukaryotic organisms. Homologues for SmtA having common zinc finger fold have been identified in some of the bacterial genomes. However, these homologous protein sequences show interesting variation especially in terms of the metal ligand binding position and identity [6, 9, 10, 11]. In comparison to metallothioneins reported in other bacterial strains, metallothioneins in versatile genus *Pseudomonas* are less explored. The genus *Pseudomonas* exhibits varied metabolic capacities; which allow it to survive in diverse ecological niches. This metabolically adaptable group has received much attention because of their pathogenicity and drug resistance, their associations with plants, and due to their ability to use diverse carbon sources. Several studies reported pseudomonads inhabiting heavy metal polluted environments and heavy metal homeostasis is maintained by critical processes that include metal sensing, chelation, and transport [12, 13]. Despite the progress made in understanding the role of various metal resistance determinants in maintaining metal homeostasis in this genus, many questions remain if we consider the role of metallothioneins. In this attempt, this study focuses on studying metallothioneins reported in *Pseudomonas* and further looks at the possibilities for discovering new metallothioneins in *Pseudomonas* genomes.

Overview of Bacterial Metallothioneins

Analogous to the way the first mammalian metallothioneins were discovered i.e. as cadmium-binding proteins, the first indications for the existence of prokaryotic metallothionein came from experiments in which bacteria were cultured in the presence of Cd. Metallothionein like proteins was first identified in the marine cyanobacterium *Synechococcus* sp. with the exception of high Cys content, this protein differed considerably from previously characterized metallothioneins, and was

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shown to contain aromatic residues, including histidine residues. The first family of bacterial metallothioneins discovered was named as BmtAs. In the early 1990s, the advent of the polymerase chain reaction and other major advances in molecular biology allowed the cloning and the sequencing of the gene. Subsequently, this work led to the characterization of the entire *smt* operon comprising the gene *smtA* (encoding metallothionein from *Synechococcus* PCC7942) and another gene (*smtB)* for the metal-responsive transcription factor. Further studies revealed that *smt* operon repressor is a metal regulated homodimeric repressor belonging to ArsR subfamily of helix-turn-helix bacterial transcription regulatory proteins [7]. This family includes several proteins including SmtB that appear to dissociate from DNA in the presence of metal ions. Regulation of *smtA* gene is under control of *smtB* gene. Active gene product (SmtB regulatory protein) binds at four sites to form a tetrameric complex at *the smtA* promoter in absence of Zn. At low concentrations of Zn, active SmtB (apo) binds as a monomer or dimer, which allows a low level of transcription. In presence of Zn SmtB is inactive and will not contact the *smtA* promoter [14, 15]. Since discovery of *smt* in *Synechococcus*, homologues for *smtA* have been identified in different bacterial genomes (cyanobacteria, pseudomonads, alphaproteobacteria, gammaproteobacteria, and firmicutes). Four representative proteins from *Anabaena* PCC 7120, *Pseudomonas aeruginosa*, *Pseudomonas putida* KT2440, and *Escherichia coli* have been cloned, overexpressed, purified, and characterized [4]. ‘‘Pseudothionein"CdBP1 from *P. putida* was characterized by ^{1}H and ^{113}Cd NMR spectroscopy, and a contribution of His residues to Cd binding was suggested [16]. The sequences of the pseudothioneins were never determined, owing to difficulties with isolating sufficient amounts of protein. The metallothionein from *P. aeruginosa* PAO1 contains 79 amino acids, among which 10 are cysteine residues. However, homologous sequences from pseudomonads show metallothioneins up to 87 amino acids. Considering regulation of metallothioneins in *Pseudomonas* no reports are available whether a similar SmtB like regulatory protein has any role. Few studies have reported metallothioneins from this genus, such as PmtA in human pathogen *P. aeruginosa* which binds 3 zinc or 4 cadmium molecules. Unlike *Synechococcus* SmtA, but similar to mammalian MT, zinc bound by PmtA from *P. aeruginosa* can be displaced by cadmium. The PmtA from *P. aeruginosa* was found to be slightly less stable in comparison to SmtA from *Synechococcus* PC7942, and is more prone to oxidation and aggregation [17, 18]. In another study the role of metallothionien from *P. aeruginosa* strain WI-1isolated from estuary surface water in lead resistance have been reported [13].

Function and Importance

Since the discovery of metallothioneins, the exact physiological function of this protein is still a question. It is essentially known to have an important role in controlling the intracellular concentration of Zn, Cu and also neutralizes the influence of toxic metals such as Cd and Hg. This protein uses sulfhydryl groups present on cysteine residues to bind toxic and essential heavy metals

and to neutralize ROS (reactive oxygen species). Whereas, in the absence of any stress condition, it acts as a reservoir for essential heavy metals such as zinc and copper. It regulates their flow to their definite cellular destinations such as donation of these metals to apoenzymes and zinc-finger transcription factors that require these essential metals to function. Thus, metallothioneins have a potential role in providing metal cofactors for apo proteins and regulate gene via association with zinc fingers [19, 20]. Metallothionein in a zinc bound state is very stable which makes it an ideal reservoir for intracellular zinc. Under cellular stress from toxic heavy metals, such as cadmium and mercury, the cell increases metallothionein expression. This protein releases some essential metals in order to sequester toxic heavy metals and protects cell damage [21]. It is generally accepted that metallothioneins have an important role in defense against the detoxification of non-essential metals like cadmium and mercury, however, the more important role of this protein is the control of the cellular Zn distribution [9, 22, 23]. Recent report on interactions between the cyanobacterial metallothionein SmtA and uranyl suggests that metallothioneins also protect bacteria against the chemical toxicity of uranium [24]. Due to metal detoxification property bacteria possessing metallothioneins are considered an ideal tool for bioremediation of heavy metal contaminated environments. New possibilities for designing novel multipurpose bacterial metallothioneins with dual ability to sequester both soft metal ions (Cu^{+}, Zn^{2+}, Cd^{2+}, Hg^{2+}, and Pb^{2+}) and hard, high-oxidation state heavy metals such as U(VI) can be undertaken. Thus simultaneous protection from the chemical toxicity of uranium may be valuable for the development of bacterial strains for bio-remediation [24]. Apart from bioremediation, the early induction of metallothionein by trace metals, namely cadmium, in different species also makes this protein a potential biomarker which may be useful to assess the ecotoxicological significance of non-essential (Cd, Hg) and essential, but potentially toxic (Cu), trace metals [7].

2. MATERIALS AND METHODS

Sequence retrieval and alignments

Protein sequences were downloaded from protein databases at the National Center for Biotechnology Information (NCBI) and ‘‘universal protein resource’’ database UNIPRO. *Pseudomonas* specific sequences were downloaded from *Pseudomonas* genome database (http://www.Pseudomonas.com) using SmtA and SmtB protein sequence from *Synechoccous* PCC 7942 as a query. Conserve domain check was performed in web CD search tool in NCBI. This search tool classifies sequences at superfamily level, predicting the occurrence of functional domains. Sequence alignments were generated using ClustalW [25], and were manually adjusted wherever necessary. Molecular phylogenetic trees were constructed using MEGA 4 with the neighbor-joining method with 1000 bootstrap replicates.

3. RESULTS AND DISCUSSION

Information from databases for metallothioneins (SmtA)

All metallothionein sequences were obtained from the Universal Protein Resource (UNIPROT) knowledgebase and reference clusters (http://www.uniprot.org) or the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Targets of this search are sequences of proteins which are expected to be 'metallothioneins' for which detailed studies are not reported. These sequences should have conserved Cys residues to allow the formation of metal–thiolate clusters, and a relatively small size. Number of HITS in UNIPROT was 470 for search 'bacteria metallothioneins'. Finally, 101 Protein sequences were selected from both the databases for further analysis. Conserved domain search was done for selected metallothioneins sequences using a Web CD search tool. Web CD search tool revealed conserve domains in 82 sequences whereas, no conserve domain was detected in 19 sequences. Among 82 sequences further analysis of conserved domain revealed that in 2 sequences S-adenosylmethyltransferase domain was detected. The abbreviation ''smta" also stands for S-adenosylmethyltransferase, this coincidence has led to incidence where an S-adenosyl-methyl transferase protein comes with a description as a putative metallothionein, thus misannotation can lead to mistaken identity. Finally, 80 sequences showing superfamily cl03430 (prokaryotic metallothionein) were used for further multiple sequence alignment and phylogenetic analysis. UNIPROT showed 125 hits for *Pseudomonas* metallothioneins, all unreviewed in SWISS PROT database. Web CD search tool revealed that among these, 3 sequences were from *Escherichia coli* and 5 with mistaken identity to *Pseudomonas putida* were actually sequences from *Arthrobacter siderocapsulatus*. Representative sequences of *Pseudomonas* metallothioniens were selected from UNIPROT database for further analysis.

Multiple sequence alignment and phylogenetic analysis of selected representatives for metallothioneins

Sequence alignments were generated using ClustalW and manually adjusted wherever necessary. Multiple sequence alignment was done for selected representative from genus *Pseudomonas* and standard metallothionein sequences from P9WK08 (*Mycobacterium tuberculosis*) (strain CDC 1551), AKE65862.1 (*Microcystis aeruginosa* NIES-2549), CCQ60692.1 (*Crocosphaera watsonii* WH 0401), (*Synechococcus elongates* PCC7942), P30565 (*Thermosynechococcus vulcanus*), AHB87419.1 (*Thermosynechococcus* sp. NK55a), and AFZ52844.1 (*Cyanobacterium aponinum* PCC 10605). Selected representative sequences for metallothioneins BmtAs along with *Pseudomonas* metallothioneins are presented in Fig. 1. Considering SmtA from *S. elongates* PCC7942 as the standard sequence, following features could be observed, K-C-A-C-$(X)_2$-C-L-C a defining feature is detected in each sequence. In several cases, His 49—is replaced with Cys, Asp, Met, or no appropriate metal-binding amino acid residue at all. It has been suggested earlier that in the case of His-to-Cys replacement or Cys/His mutations zinc finger protein often suffer from

folding and stability damage [6]. If we consider *Pseudomonas* metallothioneins following points could be noticed, the difference in length of protein sequences, variable from 52 to 86 amino acids. Most *Pseudomonas* sequences have a 19–24-residue extension in comparison to BmtAs from other bacteria. Common first CXC motif, His at 40th is conserved in all *Pseudomonas* and *Synechococcus* but is replaced by Gln and Thr in BmtA from other strains. Whereas, at 49 th position His is most variable and it is replaced by Gln, Asp, Met, Ser, and Ala. In order to find proteins even with distant similarity to BmtAs, one elegant way forward may be to employ HMMs (Hidden Markov model). The profiles shown in Fig. 2 are the result of HMMs that are based on multiple sequence alignments. The image illustrates the level of conservation of various residues thus it confirms that the initial alignment was correct. These sequences are relatively rich in Cys (8-10) and His (seven or eight, 10 in *Pseudomonas*) residues. HMMs results using SmtA from *S. elongates* PCC 7942 and representative sequences from genus *Pseudomonas* revealed 100% conserved His at 40th position, whereas His at 49 positions was variable for *Pseudomonas* sequence showing the probability of 0.059. Serine at 32 positions in *S. elongates* was replaced with Cys in most of the *Pseudomonas* sequences showing the probability of 0.853. It has been observed that one of the most peculiar features of BmtAs is the participation of histidine residues in zinc coordination [28]. Mammalian metallothioneins for which crystal structure has been characterized (mammalian MT-I, MT-II, and MT-III) all 20 sulfur ligands in the two clusters are provided by cysteine residues from the protein chain, whereas in case of *Synechococcus* SmtA metal cluster consists of four Zn(II) ions, nine cysteine thiolate sulfurs, and two histidine imidazole nitrogens [7, 29]. In the case of *Pseudomonas* metallothioneins we observe that though cysteine and histidine are the two prevalent zinc binding residues, thermodynamics and dynamics of zinc binding to protein sites containing these residues have not been studied for this genus. Thus, prokaryotic SmtA is a protein that provides an excellent model to study the effects of cysteine and histidine residues on metalloprotein structure and dynamics.

Fig.1

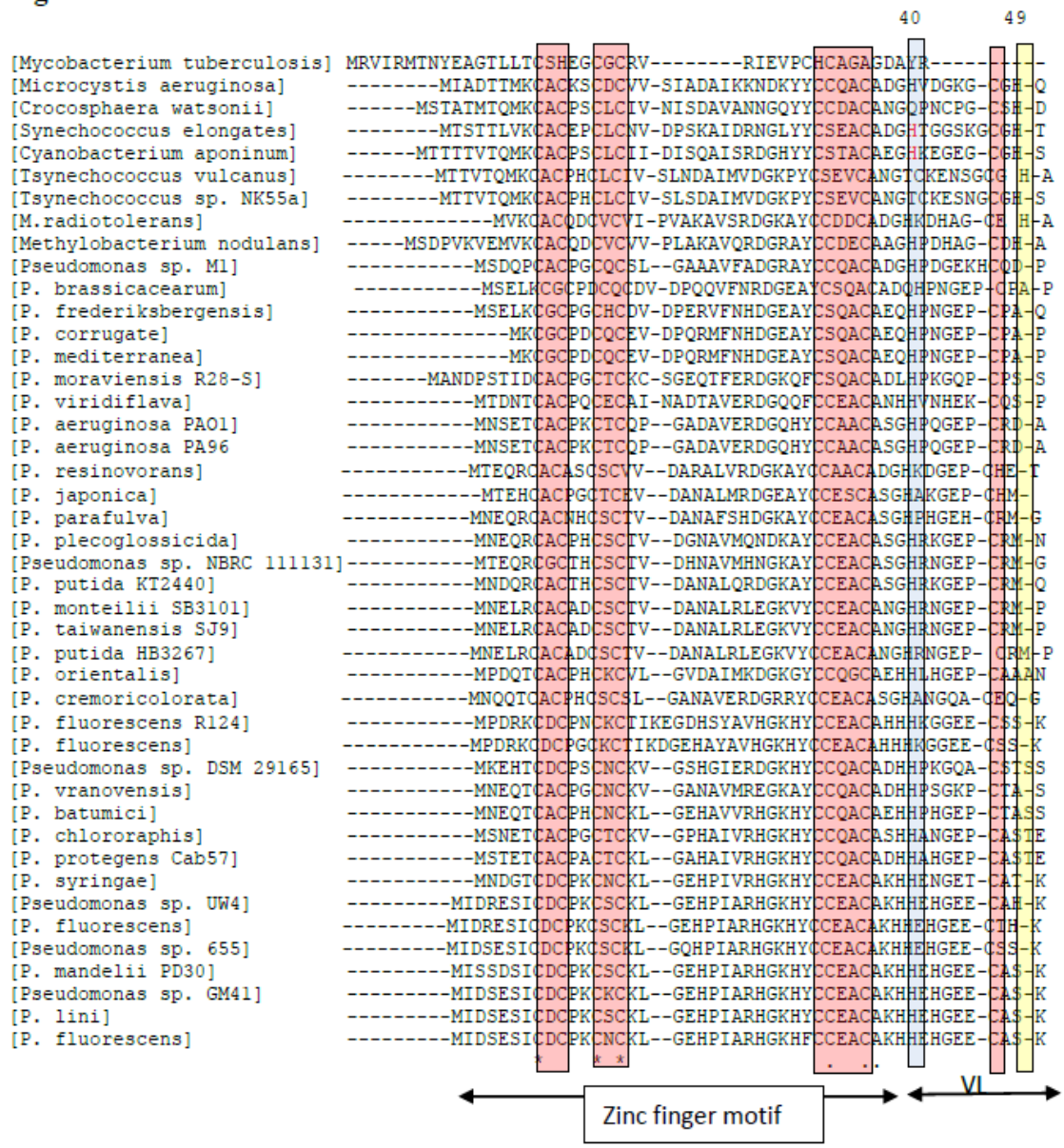

Fig.1. Multiple sequence alignment showing selected representative sequences of bacterial metallothioneins of (*Mycobacterium tuberculosis* strain CDC 1551), AKE65862.1 (*Microcystis aeruginosa* NIES-2549), CCQ60692.1 (*Crocosphaera watsonii* WH 0401), (*Synechococcus elongates* PCC7942), P30565 (*Thermosynechococcus vulcanus*), AHB87419.1 (*Thermosynechococcus* sp. NK55a), and AFZ52844.1(*Cyanobacterium aponinum*) and selected representatives from genus *Pseudomonas*. The zinc finger ligands (Cys 9, Cys14, and Cys16) and two Cys in C-terminal tether region are fully conserved metal ligands. High level of conservation is observed at Cys 47, in Variable loop region (VL), His 49 shows extreme variability among different *Pseudomonas* whereas His 40 is more conserved, the two Cys52 and Cys 54 is conserved in C-terminal tether region (CT).

Fig. 2.

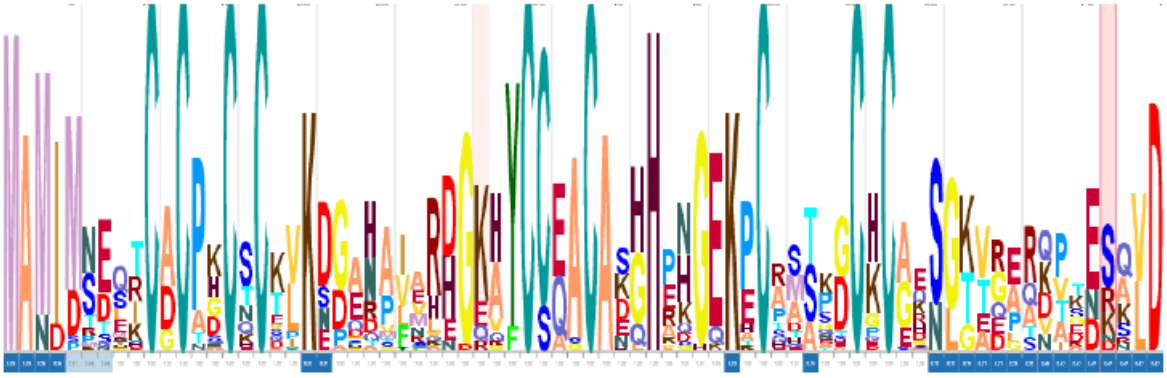

Fig.2. Sequence profiles for metallothioneins. A Hidden Markov model (HMM) profile Metallothio_Pro super family. This HMM is based on an alignment of 34 sequences, including *Synechococcus elongatus* and representatives from genus *Pseudomonas* which accounts for 100% conservation of nine Cys residues at position (9, 11, 14, 16, 32, 36, 47, 52 and 54) and His 40. Cys 33 is conserved among all the *Pseudomonas* but is replaced by S in *S. elongates.*

Phylogenetic analysis of metallothionein

Molecular phylogenetic trees were constructed using MEGA 4 with the neighbor-joining method with 1000 bootstrap replicates. As evident from the neighbor-joining phylogenetic tree (Fig. 3), SmtA protein sequences from various species of *Pseudomonas* along with representative sequences from other bacteria [*Methylobacterium radiotolerens* (WP 043075008.1), *Microcystis aeruginosa* (AKE65862.1), *S. elongates P30331*, *Thermosynechococcus elongates* (NP 681807.1), *Thermosynechococcus sp. NK55a* (AHB87419.1) and *Synechococcus sp. WH 7803* (CAK23271.1)] were grouped into three distinct clusters. Cluster 1 includes the sequences exclusively from genus *Pseudomonas.* Interestingly, in cluster 2 it was observed that SmtA protein from CAK 23271.1 *Synechoccous* sp. WH 78032 was grouped along with metallothioneins derived from different species of *Pseudomonas*. Metallothionein sequence from *P. resinivorans* (WP 016492599.1) lies in a separate cluster along with SmtA sequences from other cyanobacterial strains indicating its close phylogenetic lineage to metallothioneins derived from this group. As evident from cluster 2 and 3, a close association of some metallothioneins from *Pseudomonas* with *Synechococcus* and other Cyanobacterial SmtA indicates the role of horizontal gene transfer in the acquisition of this gene.

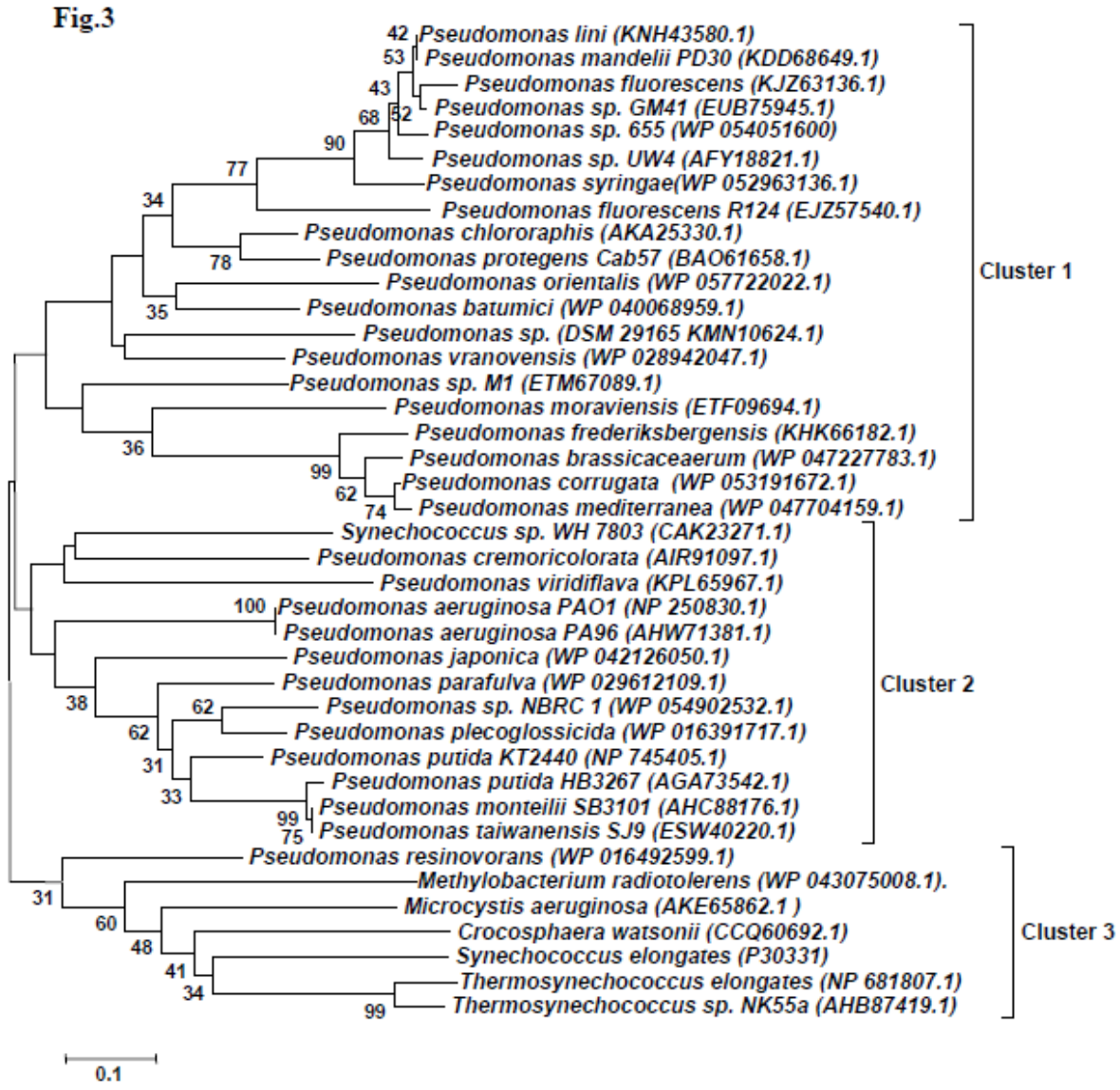

Fig.3. Neighbor-joining tree showing phylogenetic affiliation of metallothionein sequences from representative bacteria along with similar protein sequences from genus *Pseudomonas.*

Information from databases for SmtB like regulator protein

One family of homologous metal sensor proteins is the SmtB/ArsR family, named for *Synechococcus* PCC7942 SmtB, a Zn(II)-responsive transcriptional repressor that negatively regulates the transcription of *the smtA* gene [29]. In an attempt to look for the role of any repressor protein in regulation of metallothionein gene in *Pseudomonas*, SmtB protein sequence from *Synechococcus* PCC7942 was used as a query to retrieve similar transcription regulator in genus *Pseudomonas.* Initially using the query sequence BLAST search was done in NCBI as well as in UNIPROT database but highly similar SmtB protein from other bacteria was found in results. Therefore, to look exclusively for SmtB like the regulator in genus *Pseudomonas*, a BLAST search was done in *Pseudomonas* genome database (http://www.Pseudomonas.com/) using SmtB from *Synechococcus* PCC7942 as a query sequence. Search results revealed about 30-47% identity of

query SmtB to ArsR family transcriptional regulator present in different species of *Pseudomonas*.

Multiple sequence alignment and phylogenetic analysis of selected representatives of SmtB/ArsR

Sixteen representative sequences were selected and further analysis was done in CDD search tool and multiple sequence alignment was performed in clustal W. Conserved domain search tool revealed that all sequences belong to helix turn helix (HTH) superfamily and ArsR subfamily of bacteria transcription regulator producing a repressor protein which dissociates from DNA in presence of metal ions. Helix turn helix superfamily (304362, cl21459) is a large family of mostly alpha-helical protein domains with a characteristic fold; most members function as sequence-specific DNA binding domains, such as in transcription regulators. Apart from DNA binding domain, other domains present in ArsR family are dimerization domain and Zn binding domain. Among the selected *Pseudomonas* sequences it was observed that Zn binding domain was absent in *P. stutzeri* DSM 10701, *Pseudomonas* sp. MT-1, *P. chloraphis* PA23, *P. syringae* B301D, and *P. resinivorans* NBRC 106553 which point to the fact that this regulator protein may not respond to the presence of metal ions. Whereas, interestingly in some other members such as *P. denitrificans* ATCC 13867, *P. fluoresecens* NCIMB 11764, *P. stuzeri* RCH2, *P. synthaxa* BG33R and *P. simiae* WCS417 dimerization domain were missing but DNA binding and Zn binding domain was present, which again indicates that mode of regulation is different from as already reported in SmtB like proteins for Cyanobacterial strains. Thus these observations suggest the variability in the regulation of *smtB* like regulatory gene in *Pseudomonas*. Early reports on ArsR corroborates this hypothesis since substitution of one or both cysteines with non-metal liganding residues in the 30-ELCVCD-35 motif inhibited the ability of arsenate or other metals salts to dissociate ArsR/SmtB from the ars O/P [30]. Selected sequences representing SmtB like Ars regulator from *Pseudomonas* and other bacterial strains were aligned in clustal W and a phylogenetic tree was constructed with the neighbour joining method (Fig 4). Five clusters were observed; the 1st cluster contains sequences from actinobacteria in clade 1, whereas in clade 2 clustering of Ars like regulator from *Pseudomonas* with gamma Proteobacteria *Legnionella* were observed. Cluster 2 shows SmtB like protein from diverse bacterial groups like Cyanobacteria, Proteobacteria and Firmicutes. The third cluster reveals the close association of Bigr protein from *Xyellala fastidiosa* with Ars regulator from some members of genus *Pseudomonas*. Cluster 4 contains exclusively sequences only from *Pseudomonas*, whereas, cluster 5 includes representatives from Cyanobacteria, firmicutes and gamma proteobacteria. As evident from this tree, none of *Pseudomonas* sequences show linage to Cyanobacterial SmtB or to any other group as observed for metallothioneins in Fig. 2. However, a close linage of some *Pseudomonas* sequences to BigR protein from *X. fastidiosa* was observed whereas, some *Pseudomonas* sequences had a close affinity to *Leginonella*. A closer look at conserved domain present in sequence grouped in cluster three revealed that all representatives

from *Pseudomonas* had Zn binding domain and DNA binding domain but the dimerization interface as observed in cyanobacterial SmtB was missing, whereas those showing close affinity to *Legionella* had all the three domains. Similarly in BigR from *X. fastidiosa* dimerization interface was missing along with Zn binding sites. Previous studies showed that BigR belongs to winged-helix transcriptional factors which play important roles in the control of gene expression in many organisms. In the plant pathogens *X. fastidiosa* and *Agrobacterium tumefaciens*, the winged-helix protein BigR, a member of the ArsR/SmtB family of metal sensors, regulates transcription of the *bigR* operon involved in bacterial biofilm growth. Previous studies showed that BigR represses transcription of its own operon through the occupation of the RNA polymerase-binding site; however, activity and the biological function of its operon are still not known. This protein is a homodimer similar to metal sensors but it functions as a novel redox switch that derepresses transcription upon oxidation. Crystal structures of reduced and oxidized BigR reveal that formation of a disulfide bridge involving two critical cysteines induces conformational changes in the dimer that remarkably alter the topography of the winged-helix DNA-binding interface, precluding DNA binding. This structural mechanism of DNA association-dissociation is novel among winged helix factors [31]. Due to sequence similarities to bacterial metal sensors, BigR was designated a member of the ArsR/SmtB protein family. Nevertheless, the sequence homology to metalloregulatory repressors is restricted to the HTH DNA-binding domain, and BigR does not preserve the metal-binding sites usually found in the metal sensors [29]. These findings suggested that BigR and a related group of uncharacterized ArsR like proteins comprise a new subfamily of winged-helix repressors. Thus a close lineage of *Pseudomonas* ArsR to BigR indicates that metallothionein gene in these bacteria may be regulated by a similar mechanism which is unlike as reported for Cyanobacterial strains.

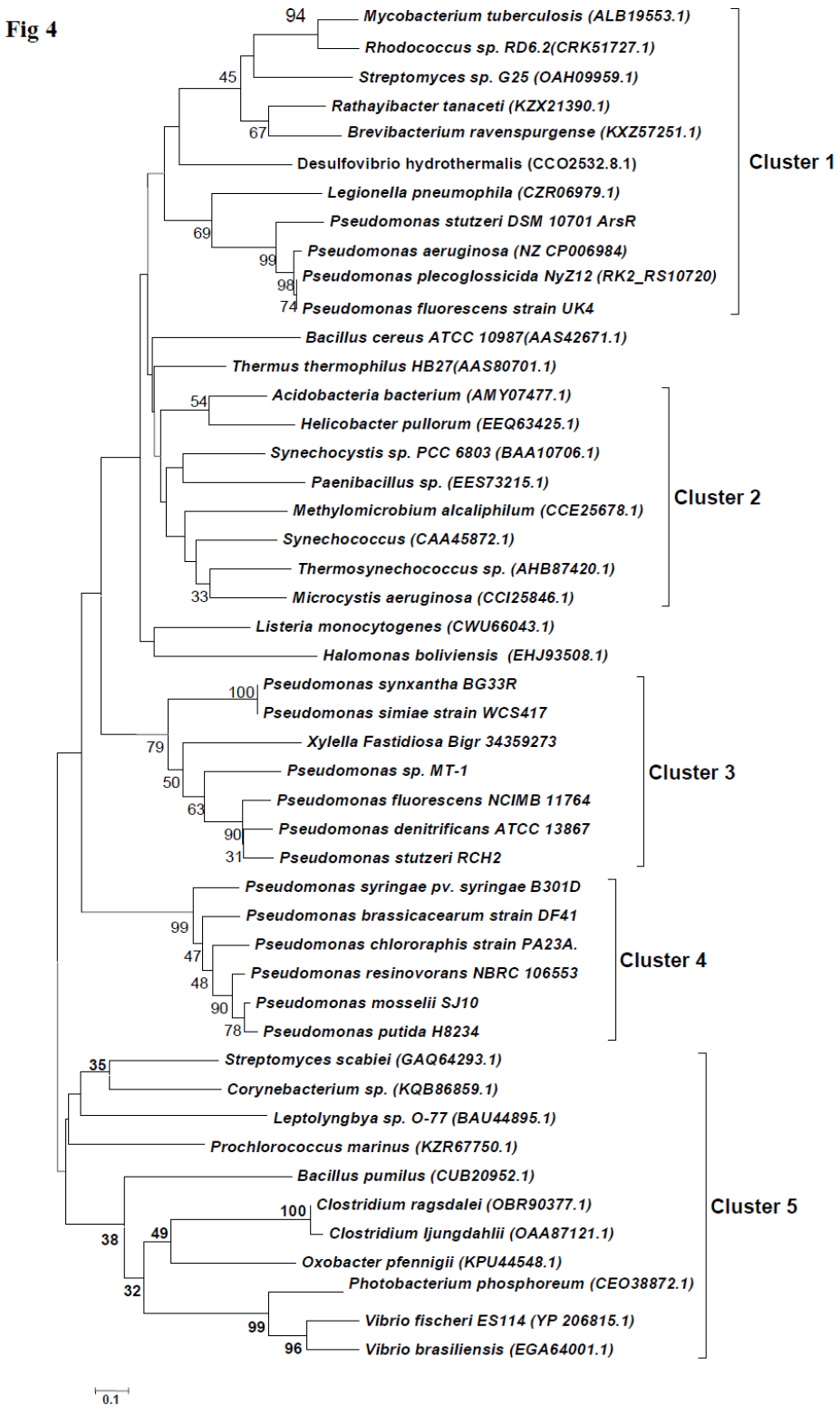

Fig.4. Neighbor-joining tree showing phylogenetic affiliation of smtB/ArsR sequences from representative bacteria along with similar protein sequences from genus *Pseudomonas.*

4. CONCLUSION

The present study on *Pseudomonas* metallothioneins suggests that there may not always be an evolutionary relationship for important genes involved in metal detoxification/ resistance. As evident from cluster 2 and 3 (Fig. 2) a close association of some metallothioneins from *Pseudomonas* with *Synechococcus* and other Cyanobacterial SmtA indicates the role of horizontal gene transfer in the acquisition of this gene. Thus, it is difficult to comment whether evolutionary relationships between metallothioneins from different phyla always exist. Regulation of metallothionein is under control of metal sensor protein SmtB/ArsR family a Zn(II)-responsive transcriptional repressor. Sequence analysis of SmtB like protein from different members of genus *Pseudomonas* revealed interesting variability in the sequences leading to presence or absence of dimerization domain and Zn binding domain. Thus, indicating the difference in regulation and mode of action of SmtB like regulatory gene in *Pseudomonas.* A close lineage of *Pseudomonas* ArsR to BigR from *X. fastidiosa* indicates that metallothioneins gene in these bacteria may be regulated by a similar mechanism which is unlike as reported for
cyanobacterial strains.

CONFLICT OF INTEREST

Author declares no conflict of interest.

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