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In-Silico Analysis of Flavin Mononucleotide Binding Domain of Nitroreductase from *Pseudomonas aeruginosa* PAO1

Mithil Mahale and Razia Kutty*

Department of Microbiology, Savitribai Phule Pune University, Pune, India

***Corresponding author:** Razia Kutty, Department of Microbiology, Savitribai Phule Pune University, Pune 411 007, India, Tel: 305-799-753; E-mail: razkutty@yahoo.co.in

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Abstract

Objective: To carry out *in-silico* sequence and structural analysis of flavin mononucleotide binding domain (FMN) of *Pseudomonas aeruginosa* nitroreductase enzyme which is of biotechnological significance and Comparison with its structural analogues

Methods: The FMN domain from *Pseudomonas aeruginosa* nitro reductase gene was cloned and sequenced. The sequence was subjected to multiple alignment, homology modeling and Ramchandran analysis with close structural homologues of nitro reductase as well as with that of phylogenetically related species.

Results: FMN binding site in case of Pseudomonas aeruginosa nitro reductase is more like the structural analogues *B. subtilis*, *T. thermophilus* and *S. pneumoniae* nitro reductases; conservation scores are mostly>6. However, the dimer interface sites are observed to be variable in nature. The phylogenetically related and diverse FMN domains were also analyzed with respect to conservation and variation of sequence

Conclusions: The conservation or variations of amino acids at the dimer interface would have implications in varied conformations of FMN binding domain inferring difference in function and kinetics of catalysis

Keywords: Nitro reductase; Flavoproteins; Flavin mononucleotide; Homology modelling

Introduction

Nitro reductase enzyme are flavoproteins found in all kingdoms. They catalyze the reduction of nitro compounds using NADPH as source of reducing power and FMN as redox co-factor [1-4]. Nitro reductase enzymes possesses broad substrate specificity and follows ping-pong mechanism. The isoalloxazine ring in FMN can accept and donate one or two electrons in biological reactions. The conserved domain analysis shows present of a single domain which has the FMN binding domain, NADPH binding domain and interface region where the homodimeric unit interacts [5-8]. The FMN co-factor incorporated in flavoproteins as a posttranslational modification it might be of significance in oxidoreductase functions. To study the molecular mechanism involved in flavoproteins, site directed mutagenesis is adopted as a favored technique which minimally disrupts the interaction between FMN, NADPH and the apoprotein. However, mutation of the same amino acids in similar enzymes do not cause the same functional changes. There is no universal rule which can be accepted for mutation studies. Substitution of residues cannot be based only on chemical properties the confirmation and the folding requirements play a major role [9]. Hence comparison of domains of structures of reported proteins with respect to confirmation or flexibility will give insights to structure function correlation of flavoproteins **(Figure 1)**.

Figure 1 Three-dimensional structure of FMN domain showing alpha-beta-alpha helix.

This study attempts to address the significance of the variations observed in the FMN binding and dimer interface sites in case of *Pseudomonas aeruginosa* PAO1 nitro reductase.

Materials and Methods

The work done is comprised of sequence analysis, multiple sequence alignment, 3-D structure using homology modeling and characterization of secondary structures of the proteins of closely related species along with their Ramchandran analysis to identify the protein folds.

Construction of phylogenetic tree and selection of clade

The protein query sequence of FMN domain was subjected to BLAST analysis using NCBI-PSI BLAST [10]. Phylogenetic tree was constructed based on the sequence similarity up to 40%. Later, a clade was selected from this tree for further analysis. *Methylobacter* spp. BBA5.1 was taken as an outlier. Multiple Sequence Alignment was done of those protein sequences.

Homology modeling and secondary structure prediction

The sequences obtained by MSA were then used for determining the 3-D structure of the proteins in those clades. This was done using Protein Homology/Analogy Recognition Engine 2 (PHYRE 2) software [11]. The individual sequences were also converted into PDB format to view their 3-D structure using RasMol or CHIMERA. PHYRE 2 software also provided the information regarding homology modeling between the protein structures of the organisms under study. The secondary structure determination was also performed using this software that clarified the region of conserved amino acid residues in the individual protein.

Ramchandran analysis

Ramchandran plot was constructed of the proteins under study by putting the amino acid sequence in the RAMPAGE software that gave an idea about the torsional folds and the conformation between the angles of amino acids in those proteins [12].

Results and Discussion

The FMN binding domain from nitro reductase gene was amplified by PCR and cloned in PUC18 vector and further sequenced **(Figures 2 and 3)**. Secondary structure analysis by PHYRE2 shows alpha helix (59%) and Beta strand (9%) **(Figure 4)**. The 3D structure based on homology modelling shows the characteristic alpha beta alpha structure of nitro reductase family **(Figure 4)**.

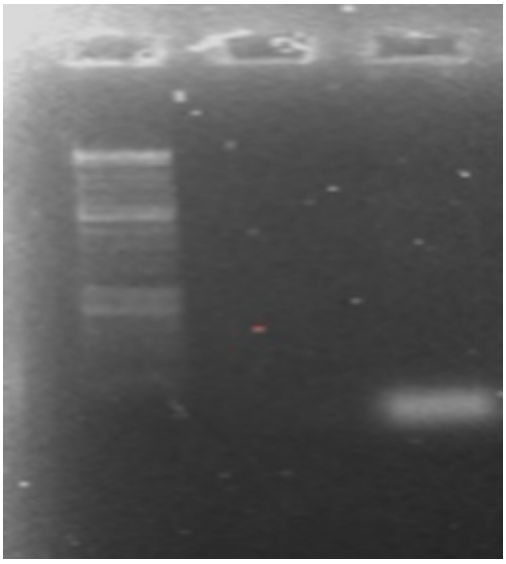

Figure 2 PCR amplified 100 bp.

Asn (N) 2	1.4%
Asp (D) 13	8.8%
Cys (C) 3	2.0%
Gln (Q) 12	8.1%
Glu (E) 8	5.4%
Gly (G) 8	5.4%
His (H) 3	2.0%
Ile (I) 2	1.4%
Leu (L) 16	10.8%
Lys (K) 3	2.0%
Met (M) 5	3.4%
Phe (F) 3	2.0%
Pro (P) 5	3.4%
Ser (S) 9	6.1%
Thr (T) 4	2.7%
Trp (W) 3	2.0%
Tyr (Y) 3	2.0%
Val (V) 14	9.5%

Figure 3 Amino acid composition of FMN domain.

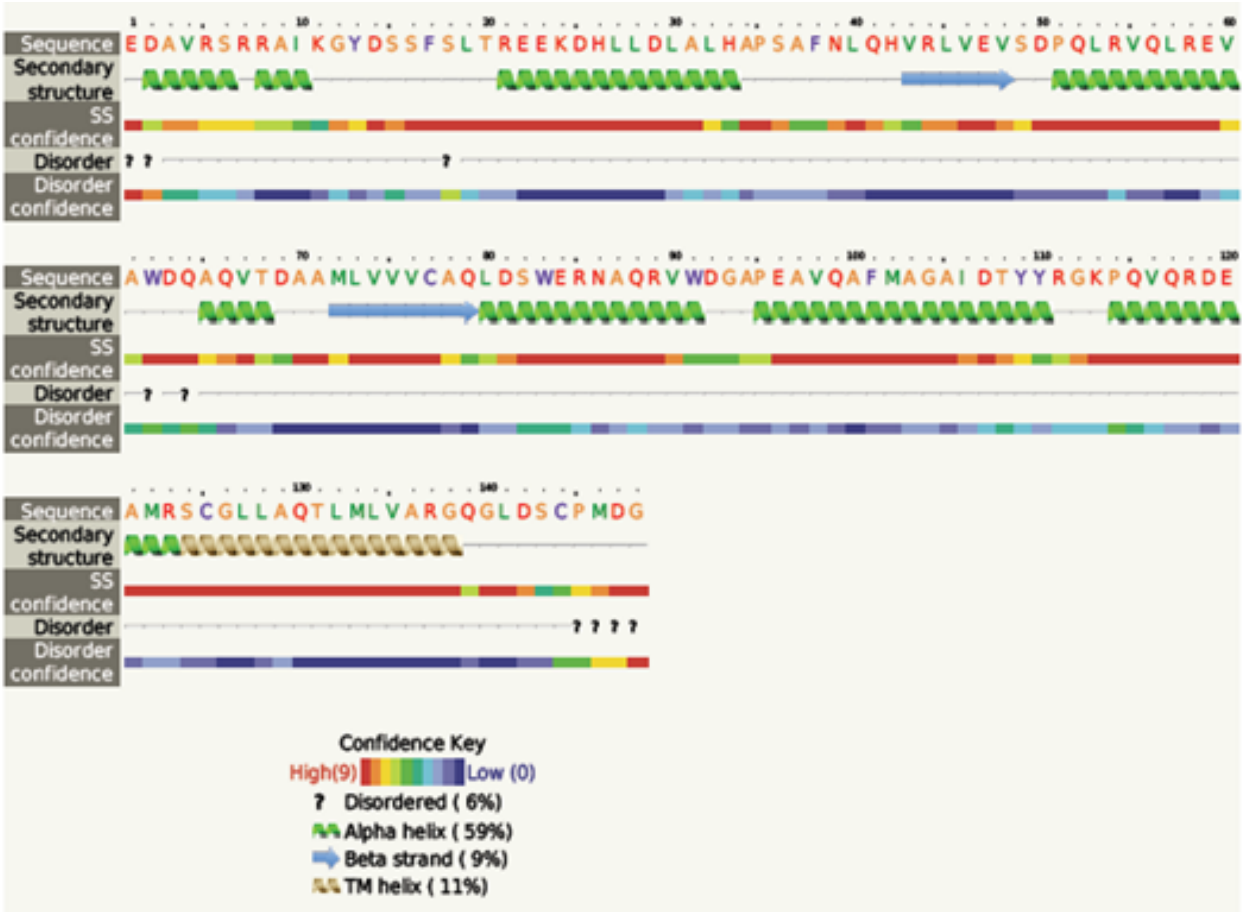

Figure 4 Secondary Structure of FMN domain.

In this study an *In-silico* analysis of the FMN binding domain of nitro reductase enzyme from *Pseudomonas aeruginosa* and its close structural analogues (obtained from database) has been done to understand structure function correlation with well-studied homologue NADH Oxidase (PDB ID 1NOX) **(Table 1) (Figure 5)**. Comparison of residues from the four homologues in the present study indicated that the FMN binding site in case of PaNR is more similar as observed in case of *B. subtilis*, *T. thermophilus* and *S. pneumoniae* nitro reductase; conservation scores are mostly>6 **(Table 1)**. However, the dimer interface sites are observed to be variable in nature **(Table 2)**. As previously studied the stretch of residues binding to FMN changes its conformation in both reduced and oxidized states which is responsible for electron transfer and rebinding of FMN to the apoprotein [13-17]. Thus, the variation in residues could have implications in binding of FMN to the monomers and changes in enzyme activity. FMN plays diverse role, its effect on antioxidant activity in *Deinococcus radiodurans* under H_2O_2 stress has been documented [18,19]. This residue can be categorized into different types of motifs. These variations are correlated with modeled structures of FMN domain in terms of Ramchandran analysis parameters. The phylogenetically close FMN domain can show significant variation in allowed and disallowed residues **(Table 3)**. Analysis of residues present in disallowed main chain confirmation or multiple conformations can be investigated further for the redox characteristics of proteins.

Figure 5 Protein sequence alignment of Nitro reductase with two close structural homologues 1NOX and 3BEM. The regions which are highly conserved are highlighted with an asterisk. The regions with yellow highlighted region are comprises FMN binding domain. The residues 10-14 are conserved and reported to be involved in FMN binding. Asterisks indicate the residues which are fully conserved. Two dots indicate the conservation of strong groups and single dot indicate the conservation of weak groups. The shaded residues comprise the putative FMN binding site, amino acids corresponding to Arg203 and Arg208 of *E. coli* NfsA.

Table 1 Comparison of PaNR sequence with four structural homologs (Single column fitting Table).

PDB ID	Source	%Query Coverage	%Similarity	%Identity	Resolution in Angstrom
3BEM	*B. subtilis*	94	50	29	1.65

1NOX	*Thermus thermophilus*	96	49	34	1.59
2B67	*Streptococcus pneumoniae*	82	45	25	2.05
1ICV	*E. coli*	92	43	25	2.4

Table 2 The residues in the FMN binding site and dimer interface among the four Nitroreductase sequence (PaNR, 3BEM, 1NOX, 2B67 and 1ICV) along with the conservation is listed. The sites conserved among all the four sequences are highlighted in red while semi-conserved sites are shown in blue. (2 Column fitting Colored Table).

Site	Residue position as per 3BEM	3BEM	1NOX	2B67	1ICV	Pseudo_NR	Conservation score	Other variable residues
FMN binding	11	R	R	R	R	R	9	H, A, R
	13	S	S	A	S	A	9	A, S, T, D
	39	P	P	P	P	P	9	S, A, P
	40	S	S	S	S	S	9	A, S, T, D
	41	A	A	A	S	A	9	H, A, S, P, G, V
	43	N	N	N	N	N	8	A, F, S, N, K, Y, H, M, D, G
	70	K	H	Q	K	Q	6	F, A, W, N, K, Y, V, H, Q, M, R, G, L
	137	L	I	L	L	L	7	H, A, M, C, I, L
	154	C	V	N	V	C	8	A, T, N, C, I, G, V
	155	P	P	I	P	P	6	S, F, A, W, T, P, Y, V, M, I
	157	I	L	L	E	D	7	A, S, T, I, G, L, E
	158	G	G	G	G	G	8	A, S, P, G, V
	159	F	F	F	F	F	6	A, F, T, K, I, Y, L, V
Dimer interface	7	L	A	L	V	A	5	S, A, T, E, V, M, D, I, L
	8	V	A	N	A	V	5	A, F, N, Y, V, M, I, L
	11	R	R	R	R	R	9	H, A, R
	29	N	R	R	E	D	1	S, A, T, N, K, E, H, Q, M, D, R
	36	A	L	T	Q	L	6	A, T, N, K, V, Q, M, D, R, I, G, L
	37	L	R	L	Y	H	3	A, S, F, T, W, N, K, Y, Q, M, C, R, L
	38	A	A	A	S	A	9	S, A, T, G, V
	43	N	N	N	N	N	8	A, F, S, N, K, Y, H, M, D, G

	45	Q	Q	Q	Q	Q	8	S, T, Y, E, H, Q, C, R
	49	Y	I	F	F	L	7	F, I, Y, V
	50	V	V	V	I	V	7	F, T, C, I, Y, L, V
	51	T	V	V	V	E	7	F, W, T, N, Y, V, R, I, G, L
	53	L	R	R	S	S	5	S, T, N, K, E, H, Q, M, D, R, L
	54	D	D	E	T	D	7	S, T, D, N, E
	55	Q	P	K	E	P	2	S, A, N, K, P, E, V, Q, M, D, R, G
	136	S	Y	G	Y	G	7	S, A, T, P, G, Y
	137	L	I	L	L	L	7	H, A, M, C, I, L
	144	L	L	L	L	L	6	A, H, M, T, I, L, V
	145	S	L	A	S	V	8	S, A, M, T, G, L
	148	A	A	D	A	G	8	S, A, T, N, V, Q, C, D, R, G, L
	168	L	L	L	F	I	8	F, N, G, L, V
	169	N	G	E	G	N	1	S, F, A, N, K, E, V, Q, D, R, I, G, L
	195	Y	H	Y	S	G	6	S, T, N, K, P, E, Y, V, H, D, R, L
	196	R	R	R	R	K	9	A, N, R, E, V
	197	K	L	L	L	L	5	S, P, K, Y, E, Q, D, I, R, L
	199	V	L	V	Q	E	1	A, F, K, E, Y, V, H, Q, I, G, L
	200	N	E	D	N	D	4	A, S, N, K, E, H, Q, D
	201	E	R	E	I	E	3	A, T, K, E, V, H, Q, M, D, R, I, G, L
	202	F	V	I	T	N	7	F, A, W, I, L, V

Table 3 Ramchandran analysis of FMN domain of organisms containing Ntr gene. Note: P1 refers to first species of *Pseudomonas aeruginosa* from the clade under study and likewise. The outlier selected was *Methylobacter* sp. BBA5.1 (>WP_051961828)

Sr. no	Query Sequences	Ramchandran analysis		
		No. of residues in favoured region (~98% expected)	No. of residues in allowed region (~2% expected)	No. of residues in Outlier region
1	FMN domain	132 (96.4%)	5 (3.6%)	0

2	P1 (>WP_074207593)	131 (90.3%)	13 (9.0%)	1 (0.7%)
3	P2 (>WP_034026955)	132 (91%)	12 (8.3%)	1 (0.7%)
4	P3 (>WP_024917716)	132 (91%)	12 (8.3%)	1 (0.7%)
5	P4 (>WP_058178814)	133 (91.7%)	10 (6.9%)	2 (1.4%)
6	P5 (>WP_003153367)	131(90.3%)	13 (9.0%)	1 (0.7%)
7	P6 (>WP_043158434)	127 (90.7%)	11 (7.9%)	2 (1.4%)
8	P7 (>WP_058162654)	135 (93.1%)	9 (6.2%)	1 (0.7%)
9	P8 (>WP_086340576)	129 (94.2%)	6 (4.4%)	2 (1.5%)
10	Methylobacter sp. BBA5.1 (>WP_051961828)	136 (93.8%)	7 (4.8%)	2 (1.4%)

Comparative analysis is also done from phylogenetically close and distant species in order to gain information of evolutionary patterns of FMN domain as documented earlier [20] **(Figures 6 and 7)**. This analysis demonstrates close relation with *Pseudomonas clade* followed by multispecies and *Methylobacter*.

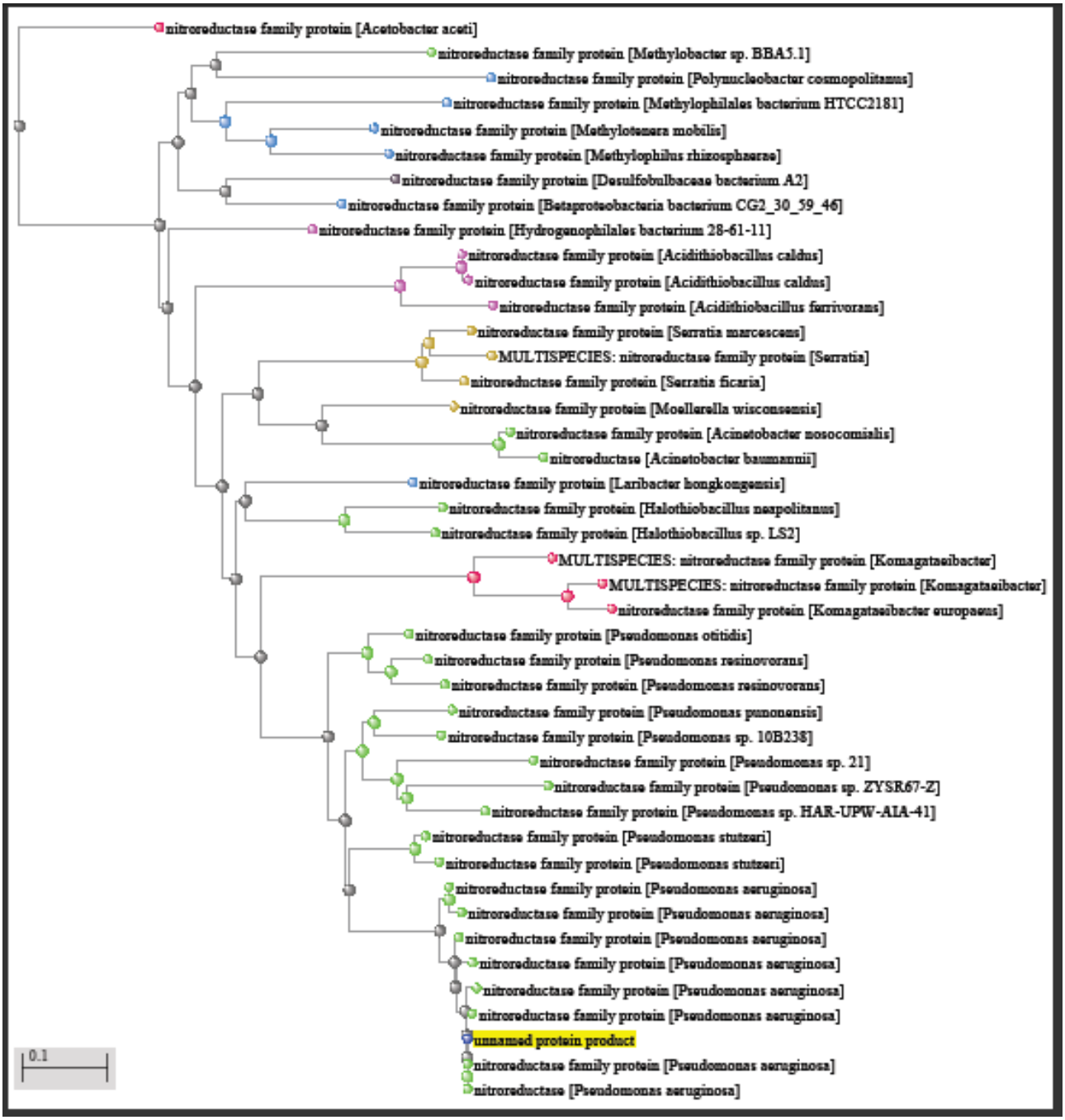

Figure 6 Phylogenetic tree of FMN domain of Nitro reductase gene. Bar denotes number of base substitutions per nucleotide position. Clade selected on the basis phylogeny and protein structure modelling of ntr gene sequences. *Methylobacter* sp. BBA5.1 was used as an outlier.

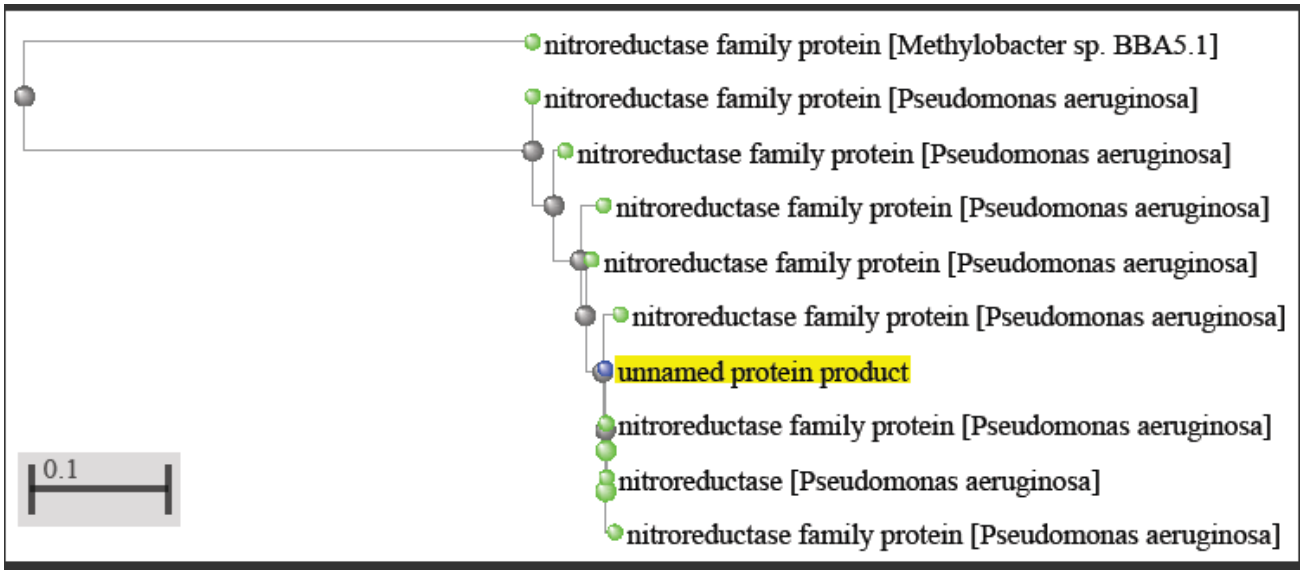

Figure 7 Clade selected on the basis phylogeny and protein structure modelling of ntr gene sequences. The bar denotes number of base substitutions per nucleotide position. ntr gene of *Methylobacter* sp. BBA5.1 was used as an outlier.

Flavoproteins are the proteins containing flavin moiety viz. FMN or FAD as the redox co-factor which functions as electron carrier during enzymatic reaction. Nitro reductases, CytochromeP450 monooxygenases, amine oxidase are flavoproteins of relevance in biological reactions. The energy producing reactions are dependent on flavins as they can convert two electrons process to one electron reduction. The oxidoreduction reaction is mediated by the interconversion of the isoalloxazine moiety of co-factor in oxidized and reduced state. Due to this redox property flavins are involved in many biochemical reactions. The flavins are mostly bound to the apoproteins by non-covalent bonds. There are few flavoproteins with flavins bound covalently. The studies reporting the mechanism of flavoproteins have shown that the FMN cofactor undergoes changes in protonation state and affects the conformation of FMN binding domain. Whereas the affinity of flavoproteins to the flavins depends on the amino acid residues as demonstrated by mutation studies.

The conservation or variations of amino acids would be reflected in varied conformational changes of FMN binding domain and hence different binding affinity among enzymes [19]. The experimental validation of the conformational changes and redox potential will imply the significance of this variations observed.

Conclusions

The FMN domain of nitro reductase from *Pseudomonas aeruginosa* PAO1 was cloned and sequenced. The conservation of amino acid residues with structural analogues of nitro reductase and variations at dimer interface can be explored for functional differences among structural analogues. The 3D structure of the domain shows characteristic alpha beta alpha helix like nitro reductase. This information would have implications in varied redox potentials of FMN binding domain.

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