Molecular and Biochemical Characterization of *nifHDK* Genes in *Klebsiella pneumoniae*

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Abstract: *Nitrogen fixation is carried by an enzyme complex called nitrogenase which consists of two main components, a dinitrogenase that is encoded by nifD and nifK and an iron containing reductase, also called Fe protein which is encoded by nifH. Nitrogen-free medium was used to detect the ability of nitrogen fixation by Klebsiella pneumonia, then DNA was extracted and overlap extension polymerase chain reaction of nifH, nifD and nifK. To obtain nucleotide sequences of these genes, sequencing of the PCR products was one. The reverse sequence of nifH and the forward sequences of nifD and nifK were converted into amino acids using online translation tool. Homology modeling was carried out using SWISS-MODEL. The modeled amino acids sequences was validated using ERRAT and PROCHECK. The modeled sequences were reliable and of quality higher than 90%. The two subunits of Fe protein were constructed and tertiary structure was predicted together with the binding sites for prosthetic group and ADP molecule in Fe protein. The following amino acids Asp11, Lys13, Asn157, Ser158, Val183, Pro184, Arg185, Asp186, Val189, Gln190 and Glu193 seem to participate in the ADP binding. The complexity of this enzyme makes it difficult to be cloned in plants.*

Keywords: *Nitrogenase, Nitrogen fixation, Homology modeling.*

Introduction

Nitrogen is essential for the synthesis of amino acids, proteins and organic molecules for living organisms [28]. Despite of its presence in the atmosphere, it has to be reduced into ammonia through symbiotic and non-symbiotic relationships in order to be exploited by plants [15]. This process is called biological nitrogen fixation which is carried out by 90 different genera of microorganisms [7]. In last three decades, nitrogen fixation has been found in most phyla of bacteria and archaea [34]. The symbiotic nitrogen fixation occurs within vascular plants and includes rhizobia that lives with legumes and Frankia (Actinomycetes) and cyanobacteria [1, 22, 31].

Nitrogen fixation is performed by an enzyme complex referred to as nitrogenase. Nitrogenase consists of two main components, a dinitrogenase which contains molybdenum and iron, and is encoded by *nifD* and *nifK* and an iron containing reductase which is encoded by *nifH* [19]. Component I, or MoFe protein, is a 220 KDa of $\alpha 2\beta 2$ subunits and associated with P-cluster which is composed of Fe8S7 bridged by six cysteine residues between α- and β-subunits. Another cluster called M-cluster or FeMoCo cofactor is composed of MoFe7S9 C-homocitrate and is connected to α-subunits by histidine and cysteine. Component II is Fe protein appear to be 68 KDa dimer bridged by Fe4S4 [11, 12, 25].

Nitrogenase was described at first in *Klebsiella oxytoca* strain as a 24 Kb region and shown to be composed of twenty genes that are organized in several transcriptional units in the order nifJHDKTYENXUSVWZMFLABQ, along the chromosome of *K. pneumoniae* [2, 25]. The genes *nifB, nifQ, nifE, nifX, nifN, nifS, nifV, nifY, nifU* and also *nifH* are required for synthesis of M cluster, *nifS* and *nifU* for production of iron sulfur clusters and *nifW* and nifZ for the maturation of enzyme components [11, 25, 26]. *Klebsiella* also contains the genes *nifF* and *nifJ* for electron transport and *nifLA* genes to control gene expression of the nitrogenase gene cluster [6]. Liu et al. [17] stated that nif genes' cluster encodes to structural proteins, ferredoxins, proteins for metal cofactor biosynthesis and proteins with unknown functions but essential for activity. The gene cluster comprises *nifH, nifDK, nifV, nifB, nifU, nifEN, nifT, nifZ, cysEZ, nifX, nifW, hesA, hesB, feoB2, feoA2, modB, fdxN, fdxH and fdxB*. The aim of this study is to amplify and sequence the *nifH, nifD, nifK* genes in *K. pneumoniae* isolate and homology modeling of the amino acid sequences of these genes.

Materials and methods

Test for N_2 fixation

K. pneumoniae was kindly provided by Prof. Jean-Marc from University of Leicester, UK. Nitrogen-free medium was used to detect the ability of nitrogen fixation. It is composed of glucose, K_2HPO_4, KH_2PO_4, NaCl, $CaSO_4.2H_2O$, $Na_2MoO_4.2H_2O$, $FeSO_4.7H_2O$ an $MgSO_4$ [33]. Bacteria were grown for 6 days at 30 °C. The broth was centrifuged at 10 000 rpm for 2 min. Nessler reagent supplied by Sigma was added to 1 ml of supernatant an yellowish-brown color was developed due to the reaction with the produced ammonia [35].

DNA isolation of K. pneumoniae isolate

DNA extracted according to Neumann et al. [23]. Briefly, lysozyme was used to lyse the bacteria. 1 ml of culture was centrifuged at 13000 rpm for 10 min. The pellet was washed with 400 µl of NaCl-EDTA Tris buffer (STE) and centrifuged again with the supernatant discarded. The precipitate was resuspended with STE and centrifuged again. The precipitate is suspended with 200 µl of 10 mM Tris HCl+ 1 mM EDTA (TE) buffer and 100 µl of saturated phenol, vortexed and centrifuged at 13000 rpm for 10 min. at 40 °C. The last step is repeated three times with the addition of 5 µl of 10 mg/ml RNase. The tube was incubated at 37 °C for 10 min. and 100 µl of phenol was added, vortexed and centrifuged at 13000 rpm at 4 °C for 5 min. The upper layer was transferred into eppendrof and stored in -25 °C until use. The DNA concentration was measured using Nanodrop spectrophotometer (ThermoFischer, USA). The purity was estimated by measuring optical density at 260/280 where a ratio of 1.6 or less indicates protein contamination [4].

Polymerase chain reaction of nifH, nifD and nifK

Overlap extension PCR [18] was used to amplify *nifH, nifD and nifK* using two pairs of primers: P1 5′ GGC GTT ATG ACT GAG GAT CA 3′, P2 5′ GAA GCA GCT CCA GCC TAC ACC CGG ATT TGA GAC CTA TCT CGG 3′, P5 5′ GGA CCA TGG CTA ATT CCC ATG CTT TGT TTT TCG CG ATC GG 3′ and P6 5′ ACT GAG CAA AAT TCG CAG C 3′. Reaction steps were initial denaturation at 94°C for 10 min, 30 cycles of denaturation 95 °C for 45 sec, annealing 55 °C for 45 °C and an extension at 72 °C for 2 min, and final extension at 72 °C for 10 min. Electrophoresis was performed on 1% agarose for 45 min at 120 V using 1 Kb molecular marker.

DNA sequencing of nifH, nifD and nifK

To obtain nucleotide sequences of *nifH, nifD and nifK*, sequencing was performed of the PCR products by Eurofins, Germany. Briefly, DNA bands were cut from the agarose gel. Gel extraction/purification kit (Promega, USA) was used and DNA concentration was measured by NanoDrop to obtain 10 ng/ml of DNA. Primers used for sequencing were: P1 5′ GGC GTT ATG ACT GAG GAT CA 3′, P2 5′ GCT GCG ATT TAC GGT TCG AG 3′, P3 5′ CAG CAG GCC AAC GAA TAT CG 3′, P4 5′ GAA GTG CAT CTG GCG GAA C 3′, P5 5′ GGA ACC TGA CCT CAT CGG C 3′ and P6 5′ ACT GAG CAA AAA TTC GCA GC 3′.

Homology modeling

The reverse sequence of nifH and the forward sequence of *nifD* and *nifK* were converted to amino acids using in silico translation tool at http://insilico.ehu.es/translate/ developed by Bikandi et al. [3] which is maintained by University of the Basque country, Spain. Queries for amino acid sequences were tested by Protein Basic Local Alignment Search Tool (BLAST) from National Center for Biotechnology Information available at https://blast.ncbi.nlm.nih.gov/Blast.cgi. Homology modeling was performed using SWISS-MODEL [32] at http://swissmodel.expasy.org/ at Swiss Institute of Bioinformatics, Biozentrum, University of Basel, Switzerland. The modeled amino acid sequences was validated using ERRAT online tool [5] and PROCHECK [16], at the Molecular Biology Institute, University of California, Los Angeles and available at http://servicesn.mbi.ucla.edu/. RaptorX [14] was used to predict binding site. It is from University of Chicago and available at http://raptorx.uchicago.edu/. GalaxyWEB server was used to predict the binding site [8] available at Computational Biology Laboratory, Department of Chemistry, Seoul National University at http://galaxy.seoklab.org.

Results and discussion

Polymerase chain reaction may be used for fusion of DNA fragments in a method called overlap-extension PCR which is composed of a two-step reaction [18]. The method was used to amplify *nifK* and half of *nifD* by one pair of primers and to amplify *nifH* and the other half of *nifD* by a second pair. The complete sequence of *nifD* and partial sequences of both *nifH* and *nifK* are presented in the Supplement. Nucleotide sequences of part of the *Klebsiella pneumoniae nifD* gene and the complete *nifK* was determined by Holland et al. [9] followed by Ioannidis and Buck [13] who suggested that *nifD* encodes for 483 amino acid residues of α-subunit with a molecular mass of 54156 Da. According to that study, the sequence contains conserved cysteine residues at 63, 89, 155, 184 and 275 positions.

A translate tool was used to convert the nucleotide sequences using the reverse strand of *nifH* and the forward strands of both *nifD* and *nifK*. This resulted in polypeptides of 264, 249 and 243 amino acids residues for *nifH*, *nifD* and *nifK* respectively (Fig. 1). Queries for amino acid sequences by Protein BLAST showed 100%, 99% and 100% identity to nitrogenase Mo reductase and maturation protein NifH of *K. pneumoniae* subsp. *pneumoniae*, nitrogenase MoFe protein α-chain of *K. pneumonia*, nitrogenase MoFe protein subunit β of *K. pneumonia*, respectively.

The binding sites of Fe protein were predicted using RaptorX, a web server for protein structure and function prediction and related services. Four pockets were identified; the first one is for iron-sulfur cluster (Fe_4S_4) which is formed by Cys^{69}, Ala^{70}, Gly^{71} and Cys^{104}. The second pocket for magnesium ion and formed by Asp^{11}, Lys^{13}, Leu^{99} and Gly^{100}. The third pocket is for ADP molecule and formed by Asp^{11}, Lys^{13}, Asn^{157}, Ser^{158}, Val^{183}, Pro^{184}, Arg^{185}, Asp^{186}, Val^{189}, Gln^{190}, Glu^{193}, Gln^{208} and Tyr^{212}. The last pocket is also for

ADP and formed by Glu[126] and Met[128]. GalaxyWEB server also predicted two binding sites; the first is for iron-sulfur cluster and formed by Cys[69], Ala[70], Gly[71], Cys[104] and Gly[106] and the second is for ADP and formed by Lys[13], Asn[157], Ser[158], Arg[159], Pro[184], Arg[185], Asp[186], Val[189], Gln[190] and Glu[193] (Fig. 2).

Fe protein

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VMIVGCDPKADSTRLILHAKAQNTIMEMAAEVGSVEDLELEDVLQIGYGDVRCAES  60
PGVGCAGRGVITAINFLEEEGAYEEDLDFVFYDVLGDVVCGGFAMPIRENKAQEIY  120
GEMMAMYAANNISKGIVKYAKSGKVRLGGLICNSRKTDREDELIIALAEKLGTQMI  180
RDNIVQRAEIRRMTVIEYDPTCQQANEYRQLAQKIVNNTKKVVPTPCTMDELESLL  240
IMEEEDTSIIGKTAAEENAA    264
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A)

α-subunit

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TGERNLALIQEVLEVFPETARKERRKHMMISDPQMESVGKCIISNRKSQPGVMTVR  60
AGSKGVVFGPIKDMAHISHGPIGCGQYSRAGRRNYYTGVSGVDSFGTLNFTSDFQE  120
FGGDKKLTKLIEEMELLFPLTKGITIQSECPVGLIGDDISAVANASSKALDKPVIP  180
GFRGVSQSLGHHIANDVVRDWVLNNREGQPFASTPYDVAIIGDYNIGGDAWASRIL  240
GLRVM  249
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B)

β-subunit

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IDKIHSCYPLFEQDEYQTLFQNKKTLEEAHDAQRVQEVFAWTTTAEYEALNFQREA  60
PAKACQPLGAVLCALGFAGTLPYVHGSQGCVAYFRTYFNRHFKEPVACVSDSMTED  120
GGNNNMNLGLQNASALYKPEIIAVSTTCMAEVIGDDLQAFIANAKKEGFVDDSIAI  180
TPSFIGSHVTGWDNMFEGFAKTFTADYAGQPGKQQKLNLVTGFETYLGNFRVLKRM  240
      243
```

C)

Fig. 1 Amino acid sequences of: (A) one subunit of Fe protein encoded by *nifH*; (B) *α*-subunit coded by *nifD*; (C) *β*-subunit coded by *nifK*.

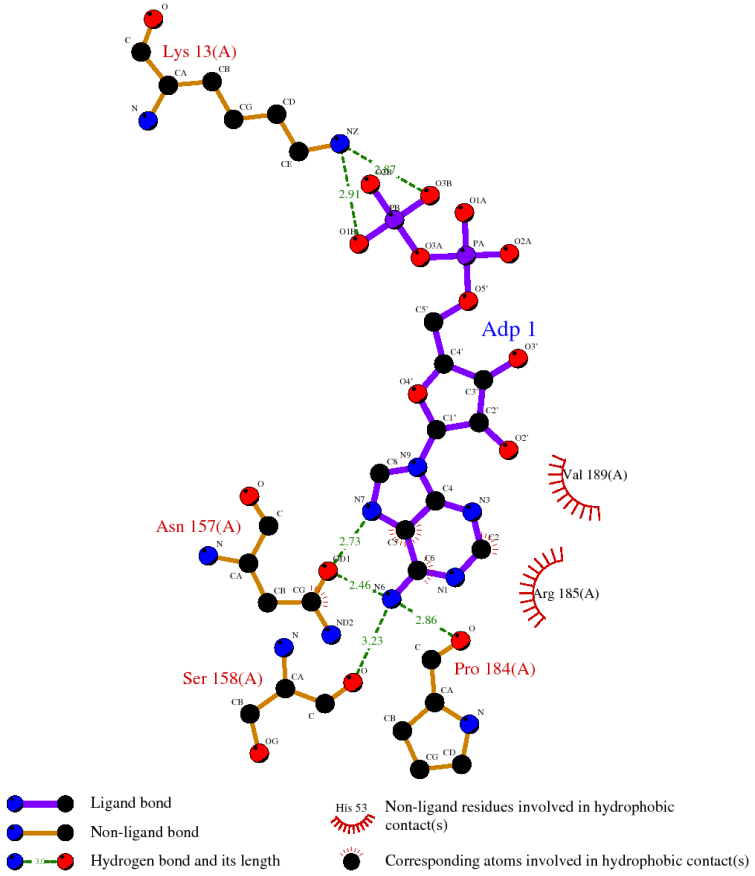
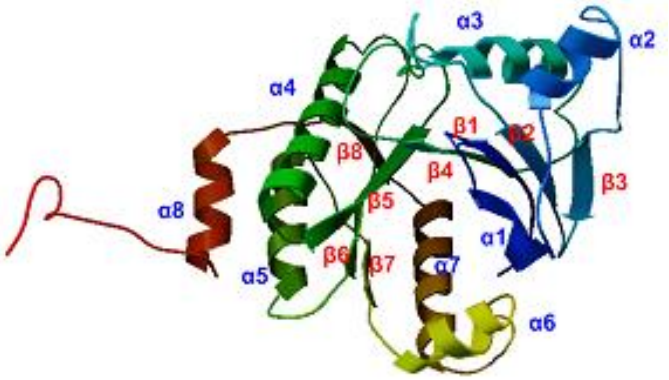


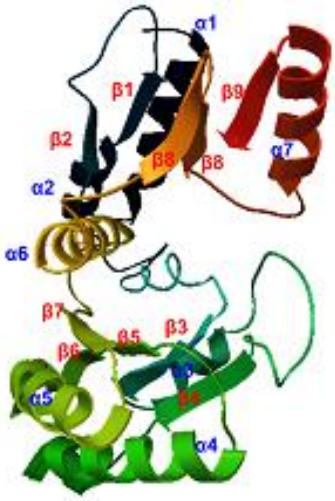
Fig. 2 Binding site prediction for ADP in Fe protein encoded by *nifH* using GalaxyWEB server

The Fe protein is a member of nucleotide utilizing family of proteins; therefore, its primary amino acid sequence possesses a GXGXXG motif as a binding site for Mg-ATP molecule. The crystal structure of this protein is unkown but data suggests a conformational change to occur in the process of ATP binding and hydrolysis [10, 12].

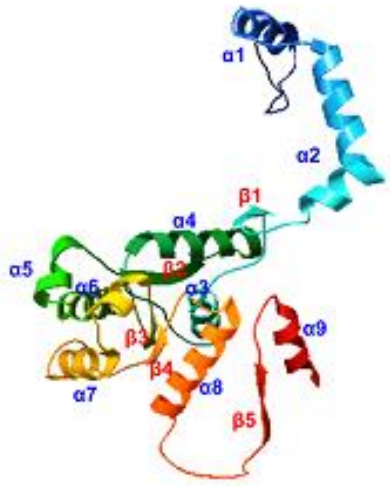
Homology modeling of the amino acid sequences was performed by SWISS-MODEL. Fe protein was modeled using 2.20 Å X-ray diffraction model of nitrogenase iron protein from *Azotobacter vinelandii* [29], PDB ID: 1G5P. *α*-subunit was modeled using 1.9 Å X-ray diffraction model of Mo-Fe nitrogenase protein from *K. pneumoniae* NIFV [20], PDB ID: 1H1L with sequence similarity of 96.37%. *β*-subunit was modeled using 1.6 Å X-ray diffraction model of molybdenum-iron nitrogenase protein, PDB ID: 1QGU whose sequence similarity of 93.33% respectively [21] (Fig. 3). The tertiary structure of the Fe iron as complete structure is illustrated in Fig. 4.



A)

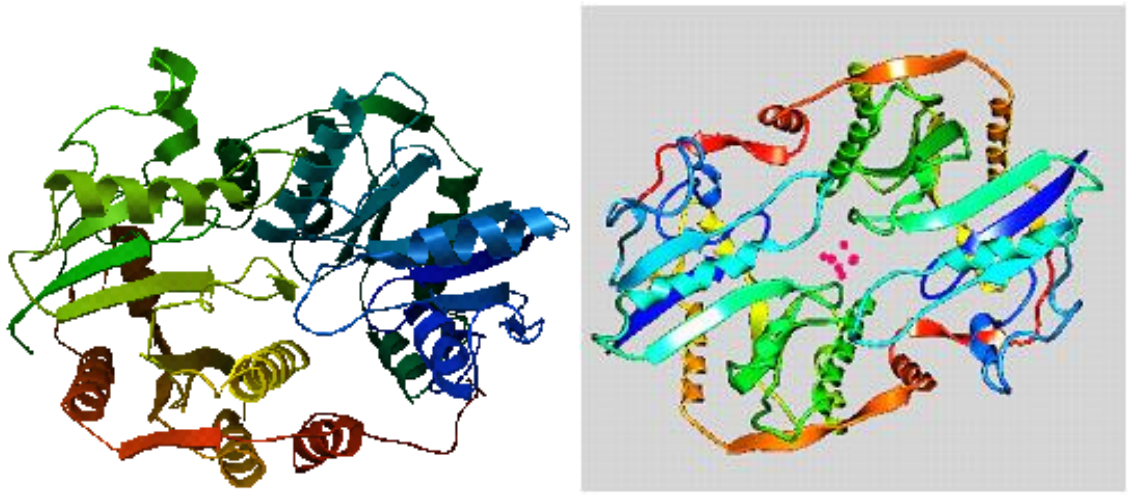


B)



C)

Fig. 3 Tertiary structure of: (A) one subunit of Fe protein encoded by *nifH*; (B) *α*-subunit coded by *nifD*; (C) *β*-subunit coded by *nifK*. Visualized by Python Molecular Viewer [27].



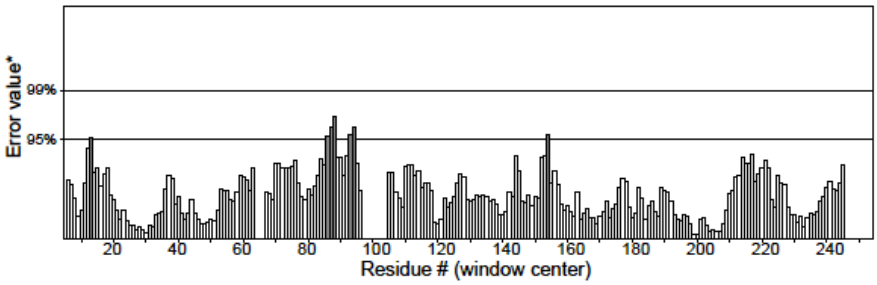
A) B)

Fig. 4 Tertiary structure of: (A) Fe protein encoded by *nifH*, visualized by Python Molecular Viewer [27]; (B) Fe protein with iron-sulfur cluster (red balls) between the two identical subunits, viewed by ArgusLab 4.0.1 [30].

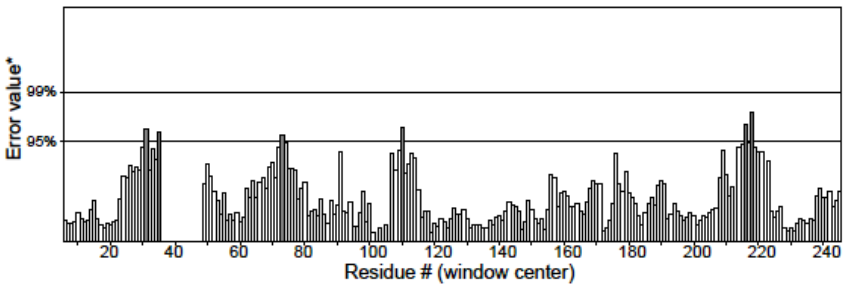
Table 1. Quality assessment of the modeled sequences

Modeled sequence	ERRAT	Ramachandran plot			
		Residues in most favoured regions	Residues in additional allowed regions	Residues in generously allowed regions	Residues in disallowed regions
One subunit of Fe protein	96.9	209 (92.9%)	15 (6.7)	1 (0.4%)	0 (0%)
Fe protein	96.9	413 (91.4%)	35 (7.7%)	4 (0.9%)	0 (0%)
α-subunit	97.3	189 (90.9%)	19 (9.1%)	0 (0%)	0 (0%)
β-subunit	99.0	191 (90.1%)	21 (9.9%)	0 (0%)	0 (0%)

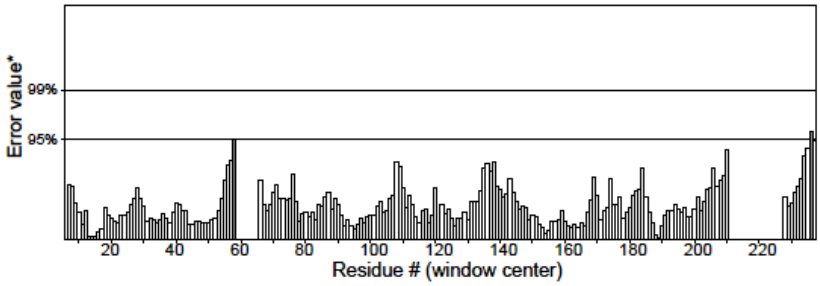
Quality assessment of the models was carried out by ERRAT and PROCHECK (Table 1). ERRAT (Fig. 5) is a program to verify structure of proteins by statistical estimation of the distribution of heavy atom types C, N and O with respect to each other in order to differentiate between correct and not correct regions in the structure [5]. Ramachandran et al. [24] have created two-dimensional plot of the torsion angles ϕ, ψ and ω that are the main determinants of protein folding and this scatter plot is referred to as Ramachandran plot (Fig. 6). According to Laskowski et al. [16], it is recommended that a reliable predicted model should have a more than 90% of the residues lying in the most favored region. Ioannidis and Buck [13] suggested that α- and β-subunits show similarities in secondary structure elements. In α-subunit, α-helices constitute 30% of the subunit secondary structure while β-sheets are 23%. In β-subunit, α-helices constitute 35% of the subunit while β-sheets are 20%. Also, there is a sequence homology between the two subunits at the N-terminal.



A)

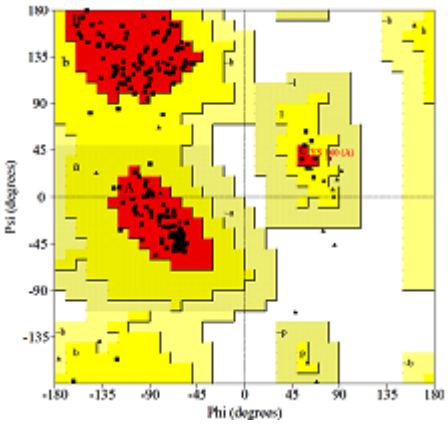


B)

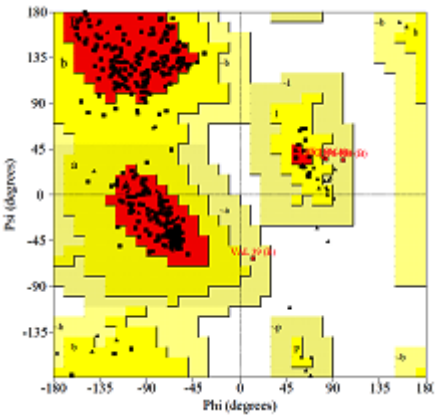


C)

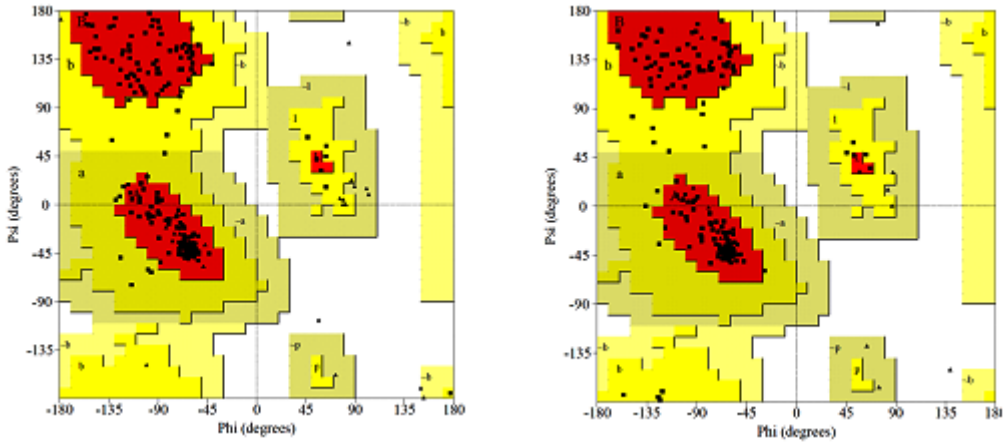
Fig. 5 ERRAT analysis of the models: (A) one subunit of Fe protein encoded by *nifH*; (B) *α*-subunit coded by *nifD*; (C) *β*-subunit coded by *nifK*. On the error axis there are two lines that indicate the confidence in which it is possible to reject regions.



A)



B)



C) D)

Fig. 6 Ramachandran plot of: (A) one subunit of Fe protein encoded by *nifH*; (B) Fe protein, 2 subunits (C) *α*-subunit coded by *nifD* and (D) *β*-subunit coded by *nifK*. The most favored regions are marked as A, B, and L, red-colored areas. The additional allowed regions are marked as a, b, l, and p, yellow-colored areas. Residues that are in generously allowed regions as ~a,~b,~l,~p, light-brown colored areas. All non-glycine and proline residues are shown as filled black squares whereas glycines (non-end) are shown as filled black triangles. Disallowed amino acid residues are red-colored squares.

Conclusion

Nitrogenase enzyme is composed of four subunits with associated prosthetic groups. The tertiary structure of Fe protein is presented in this research as theoretical model with the iron-sulfur cluster. May other microorganisms used in cloning experiments as an attempt to increase N_2 fixation in soil since it is difficult to transfer this property into plants.

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References

1. Andrew J. W., D. Jonathan, R. Andrew, S. Lei, N. N. Katsaridou, S. Mikhail, A. D. Rodionov (2007). Living Without Fur: The Subtlety and Complexity of Iron-responsive Gene Regulation in the Symbiotic Bacterium *Rhizobium* and Other *α*-proteobacteria, Biometals, 20, 501-511.
2. Arnold W., A. Rump, W. Klipp, U. B. Priefer, A. Piihler (1988). Nucleotide Sequence of a 24,206 Basepair DNA Fragment Carrying the Entire Nitrogen Fixation Gene Cluster of *Klebsiella pneumonia*, J Mol Biol, 203(3), 715-38.
3. Bikandi J., R. San Millán, A. Rementeria, J. Garaizar (2004). *In silico* Analysis of Complete Bacterial Genomes: PCR, AFLP-PCR, and Endonuclease Restriction, Bioinformatics, 20, 798-799.
4. Brown T. A. (2010). Gene Cloning and DNA Analysis: An Introduction, 6th Ed., John Wiley and Sons Ltd., UK.
5. Colovos C., T. O. Yeates (1993). Verification of Protein Structures: Patterns of Non-bonded Atomic Interactions, Protein Science, 2, 1511-1519.

6. Dixon R., D. Kahn (2004). Genetic Regulation of Biological Nitrogen Fixation, Nat Rev Microbiol, 2, 621-631.
7. Gaby J. C., D. H. Buckley (2012). A Comprehensive Evaluation of PCR Primers to Amplify the *nifH* Gene of Nitrogenase, PLoS ONE, 7, e42149.
8. Heo L., W.-H. Shin, M. S. Lee, C. Seok (2014). GalaxySite: Ligand-binding Site Prediction by Using Molecular Docking, Nucleic Acid Res, 42, W210-214.
9. Holland D., A. Zilberstein, A. Zamir, J. L. Sussman (1987). A Quantitative Approach to Sequence Comparisons of Nitrogenase MoFe Protein α- and β-subunits Including the Newly Sequenced *nifK* Gene from *Klebsiella pneumoniae*, Biochemical J, 247(2), 277-285.
10. Howard J. B., D. C. Rees (1996). Structural Basis of Biological Nitrogen Fixation, Chem Rev, 96, 2965-2982.
11. Hu Y., A. W. Fay, C. C. Lee, M. W. Ribbe (2007). P-cluster Maturation on Nitrogenase MoFe Protein, Proc Natl Acad Sci, 104, 10424-10429.
12. Hu Y., M. W. Ribbe (2015). Nitrogenase and Homologs, J Biol Inorg Chem, 20, 435-445.
13. Ioannidis I., M. Buck (1987). Nucleotide Sequence of the *Klebsiella pneumoniae nifD* Gene and Predicted Amino Acid Sequence of the Alpha-subunit of Nitrogenase MoFe Protein, Biochemical J, 247, 287-291.
14. Källberg M., H. Wang, S. Wang, J. Peng, Z. Wang, H. Lu, J. Xu (2012). Template-based Protein Structure Modeling Using the RaptorX Web Server, Nat Protoc, 7, 1511-1522.
15. Khan H. R. M, R. Mohiuddin, M. Rahman (2008). Enumeration, Isolation and Identification of Nitrogen-fixing Bacteria Strains at Seedling Stage in Rhizosphere of Rice Grown in Non-calcareous Grey Flood Plain Soil of Bangladesh, J Fac Environ Sci Technol, 13, 97-101.
16. Laskowski R. A., M. W. MacArthur, D. Moss, J. M. Thornton (1993). PROCHECK: A Program to Check the Stereochemical Quality of Protein Structures, J Appl Crystal, 26, 283-291.
17. Liu D., M. Michelle, J. Yu, H. B. Pakrasi, M. Bhattacharyya-Pakrasia (2018). Engineering Nitrogen Fixation Activity in an Oxygenic Phototroph, mBio, 9, e01029-18.
18. Luo W.-G., H.-Z. Liu, W.-H. Lin, M. H. Kabir, Y. Su (2013). Simultaneous Splicing of Multiple DNA Fragments in One PCR Reaction, Biolog Proc Online, 15, 9.
19. Matthew C. J., M. K. Bjorkman, M. K. David, A. M. Saito, P. J. Zehr (2008). Regional Distributions of Nitrogen-fixing Bacteria in the Pacific Ocean, Limnol Oceanogr, 53, 63-77.
20. Mayer S. M., C. A. Gormal, B. E. Smith, D. M. Lawson (2002). Crystollographic Analysis of the MoFe Protein of Nitrogenase from *nifV* Mutant of *Klebsiella pneumoniae* Identifies Citrate as a Ligand to the Molybdenum of Iron Molybdenum Cofactor (FeMoCo), J Biol Chem, 277, 35263.
21. Mayer S. M., D. M. Lawson, C. A. Gormal, S. M. Roe (1999). New Insights into Structure-function Relationships in Nitrogenase: A 1.6 Å Resolution X-ray Crystallographic Study of *Klebsiella pneumoniae* MoFe-protein, J Mol Biol, 292, 871-891.
22. Meeks J. C., J. Elhai (2002). Regulation of Cellular Differentiation in *Filamentous cyanobacteria* in Free-living and Plant Associated Symbiotic Growth States, Microbiol Mol Biol Rev, 66, 94-121.
23. Neumann B., A. Pospiech, H. U. Schairrer (1992). Rapid Isolation of Genomic DNA from Gram-negative Bacteria, Trends Genet, 8, 332-333.
24. Ramachandran G. N., C. Ramakrishnan, V. Sasisekharan (1963). Stereochemistry of Polypeptide Chain Conformations, J Mol Biol, 7, 95-99.

25. Rubio L. M., P. W. Ludden (2008). Biosynthesis of the Iron-molybdenum Cofactor of Nitrogenase, Annu Rev Microbiol, 62, 93-111.
26. Rubio L. M., P. W. Ludden (2005). Maturation of Nitrogenase: A Biochemical Puzzle, J Bacteriol, 187, 405-414.
27. Sanner M. F. (1999). Python: A Programming Language for Software Integration and Development, J Mol Graphics Mod, 17, 57-61.
28. Shridhar B. S. (2012). Nitrogen Fixing Microorganisms, Inter J Microbiol Res, 3, 46-52.
29. Strop P., P. M. Takahara, H. Chiu, H. C. Angove, B. K. Burgess, D. C. Rees (2001). Crystal Structure of the All-ferrous [4Fe-4S]0 Form of the Nitrogenase Iron Protein from *Azotobacter vinelandii*, Biochemistry, 40, 651-656.
30. Thompson M. A. (2004). Molecular Docking Using ArgusLab, an Efficient Shape-based Search Algorithm and Score Based Search Algorithm and the XScore Scoring Function, ACS Meeting, Philadelphia 172, CINF 42 PA.
31. Vessey J. K., K. Pawlowski, B. Bergman (2004). Root-based N_2-fixing Symbiosis: Lequmes, Actinorhizal Plants, Parasponia and Cycads, Plant Soil, 266, 205-230.
32. Waterhouse A., M. Bertoni, S. Bienert, G. Studer, G. Tauriello, R. Gumienny, F. T. Heer, T. A. P. de Beer, C. Rempfer, L. Boroli, R. Lepore, T. Schwede (2018). SWISS-MODEL: Homology Modeling of Protein Structures and Complexes, Nucleic Acids Res, 46, W296-W303.
33. Wilson P. W., S. G. Knight (1952). Experiments in Bacterial Physiology, Burgess Publishing Co., Minneapolis, USA.
34. Young P. (1992). Phylogenetic Classification of Nitrogen-fixing Organisms, In: Biological Nitrogen Fixation, Stacey G., R. H. Burris, H. J. Evans (Eds.), Chapman and Hall Inc., New York, 43-86.
35. Yu S. S., E. P. Kyaw, T. mar Lynn, Z. K. Latt, A. Aung, T. M. Sev, M. T. Nwe, W. W. Mon (2017). The Correlation of Carbon Source and Ammonium Accumulation in Culture Broth by Nitrogen-fixing Bacterial Isolates, J Sci Innov Res, 6, 63-67.

Supplement

nifH nucleotide sequence - forward strand

CAGAAGCGCGTCAGCCCCATTACGAAATCCGGATCGCCGTAGAGGCCGAATTTTTTGCCA
TGCAGCCAGGTGTGGGAATCCAGCATCATGTCGACCAGCCGGCCGCGCTCCAGGGTCAGA
GCGTCGGCGATCGGTTTGCCGGTCAGCTGACTGACGGTCATCAGCAGCGCGTCGGTGGCG
GCCAGGCCCAGCGGAACGGCCACCTCGGTGGCGGGCTGGTTCCACATCTCCTGAACCACC
TTTTTGCTTTTCACCAGCTGCCACGGCTGCAGCAGCAGGGTGTCAATGGCGTCCGGCGCG
GTTTTGATCTCCTGCTGGCTGGTGCCGCCGGCGTACATCCGGTAATGGCCGTCGGCGGGG
GTGTCGAGCACCTCTGATGGGTCGGAGAGCAGGCTGCACGGGACATCCATCTGCGCCATC
ATCCGCTTCAGCACGCGGAAGTTGCCGAGATAGGTCTCAAATCCGGTCACCAGATTGAGC
TTTTGCTGTTTGCCCGGCTGCCCGGCGTAGTAAGCGGTAAAGGTCTTCGCGAACCCTTCG
AACATATTGTCCCAGCCGGTGACATGGCTGCCGATAAAGCTGGGGGTATGGGCGTAAGGA
ATGGCGATGCTGTCGTCAACAAATCCCTCTTTTTTGGCGTTGGCGATAAACGCCTGCAGA
TCGTCGCCGATCACCTCGGCCATGCAGGTGGTGGAGACGGCGATAATCTCGGGTTTATAC
AGCGCGCTGGCATTCTGCAGGCCCAGATTCATGTTGTTGTTGCCGCCGAACACCGCCGCG
TCCTCGGTCATGGAGTCGGAGACGCAGGCGACCGGCTCTTTAAAATGGCGGTTAAAGTAG
GTGCGAAAATAGGCGACGCAGCCCTGGGAGCCGTGCACGTAGGGCAGGGTGCCGGCGAAC
CCCAGCGCGC";

nifH nucleotide sequence -reverse strand

GCTCTCATCTGCGGATCGCTGATCATCATGTGCTTTCTGCGCTCTTTGCGCGCGGTTTCG
GGAAACACCTCCAGGACTTCCTGGATGAGCGCAAGGTTACGTTCGCCTGTTGCGTTGGTC
ATGGTGAGGATCCTGTGCTCAGGCCGCGTTTTCTTCAGCGGCGGTTTTACCAATGATGCT
GGTGTCTTCTTCTTCCATGATGCCGAACTCCATCAGCAGCGATTCCAGCTCGTCCATGGT
GCACGGCGTCGGCACCACTTTTTTGGTGTTATTGACGATCTTCTGCGCCAGTTGACGATA
TTCGTTGGCCTGCTGACAGGTCGGGTCGTACTCGATCACCGTCATCCGGCGGATCTCCGC

GCGCTGCACAATGTTGTCGCGGGGAACGAAGTGGATCATCTGCGTGCCAAGCTTCTCCGC
CAGGGCGATGATCAGTTCGTCTTCCCGGTCGGTTTTGCGCGAGTTACAGATCAGGCCGCC
GAGGCGCACCTTGCCGGATTTGGCGTACTTCACGATCCCCTTGGAGATATTGTTGGCGGC
ATACATCGCCATCATCTCGCCGGAGCAGACGATGTAGATCTCCTGGGCTTTGTTTTCGCG
GATCGGCATGGCGAAGCCGCCGCAGACCACGTCGCCGAGGACGTCATAGAAGACGAAATC
CAAATCTTCCTCATAGGCGCCTTCTTCCTCGAGGAAGTTGATGGCGGTGATCACCCCGCG
TCCGGCGCAGCCGACGCCTGGCTCCGGGCCGCCGGATTCGGCGCAACGGACATCGCCATA
GCCGATTTGCAGCACGTCTTCGAGCTCCAGATCTTCGACCGAGCCCACTTCCGCCGCCAT
CTCCATGATGGTGTTCTGGGCTTTAGCGTGAAGGATCAGACGGGTGGAATCCGCTTTCGG
ATCGCAGCCGACGATCATCACTTTCTTACCCATCTCGGCGAGGGCCGC";

nifD nucleotide sequence - forward strand

ACCAAAAAAGTGGTGCCGACGCCGTGCACCATGGACGAGCTGGAATCGCTGCTGATGGAG
TTCGGCATCATGGAAGAAGAAGACACCAGCATCATTGGTAAAACCGCCGCTGAAGAAAAC
GCGGCCTGAGCACAGGATCCTCACCATGACCAACGCAACAGGCGAACGTAACCTTGCGCT
CATCCAGGAAGTCCTGGAGGTGTTTCCCGAAACCGCGCGCAAAGAGCGCAGAAAGCACAT
GATGATCAGCGATCCGCAGATGGAGAGCGTCGGCAAGTGCATTATCTCGAACCGTAAATC
GCAGCCCGGGGTGATGACCGTGCGCGGCTGCGCCTATGCGGGCTCGAAAGGGGTGGTGTT
TGGGCCAATCAAAGACATGGCCCATATCTCGCACGGCCCCATCGGCTGCGGCCAGTACTC
GCGCGCCGGACGGCGCAACTACTATACCGGCGTCAGCGGTGTCGACAGCTTCGGCACCCT
GAACTTCACCTCTGATTTTCAGGAGCGCGATATTGTTTTCGGCGGCGATAAAAAGCTGAC
CAAACTGATCGAAGAGATGGAGCTGCTGTTCCCGCTGACCAAAGGGATCACCATCCAGTC
GGAGTGCCCGGTGGGCCTGATCGGCGATGACATCAGCGCCGTGGCCAACGCCAGCAGCAA
GGCGCTGGATAAACCGGTGATCCCGGTGCGCTGCGAAGGCTTTCGCGGCGTATCGCAATC
GCTGGGCCACCATATCGCCAACGACGTGGTGCGCGACTGGGTGCTGAACAATCGCGAAGG
GCAGCCGTTTGCCAGCACCCCGTATGATGTTGCCATCATTGGCGATTACAACATCGGCGG
CGACGCCTGGGCCTCGCGCATTCTGCTGGAAGAGATGGGGCTGCGCGTAATGG";

nifD nucleotide sequence - reverse strand

ATATATTTCTCTTTGATCCCGGAGCCGATGAGGTCAGGTTTCAGCGCTTTGACGAAGGCC
TCCAGCTCATAGCTGCTGGCATCGTCAAACAGCAGGGTGCCCTCTTTCAGGTCCGGCAGG
GTGCGGTCGTAATCATCGTTATGGGCAAACTCGTAGCCGGCGGCGATGATCTCCATCCCG
AGATCCTCATAGGCGCCGATGACGTGGCGCGGCCGCAGCCCCCCCATGTACAGCAGCACT
TTGCGCCCCTCCAGTCGCGGGCGATATTTGGCGATGATGGCCGCCATTTGCCCCTCATAT
TTGGCGATCACCGCTTCCGCATTGGCGCGAATGGTGTCATCAAATTGATCGGCGATCTTG
CGCAGCGATTCGGCGATTTTGGTCGGGCCGAAGAAGTTATATTCCATCCACGGGATCTGA
TGTTTCTCCTCCATATGGCGGGCGATATAGTTCATCGAACGGTAGCAGTGGACGAGGTTA
AGCTTAACGAATGGGGTGTTCTCCATCTCCACCAGGGTGCCGTCGCCGGACCACTGCGCC
ACTACGCGCAGCCCCATCTCTTCCAGCAGAATGCGCGAGGCCCAGGCGTCGCCGCCGATG
TTGTAATCGCCAATGATGGCAACATCATACGGGGTGCTGGCAAACGGCTGCCCTTCGCGA
TTGTTCAGCACCCAGTCGCGCACCACGTCGTTGGCGATATGGTGGCCCAGCGATTGCGAT
ACGCCGCGAAAGCCTTCGCAGCGCACCGGGATCACCGGTTTATCCAGCGCCTTGCTGCTG
GCGTTGGCCACGGCGCTGATGTCATCGCCGATCAGGCCCACCGGGCACTCCGACTGGATG
GTGATCCCTTTGGTCAGCGGGAACAGCAGCTCCATCTCTTCGATCAGTTTGGTCAGCTTT
TTATCGCCGCCGAAAACAATATCGCGCTCCTGAAAATCC";

nifK nucleotide sequence - forward strand

TCAGAAATGGGGGTGCCGTTCCGCCAGATGCACTCCTGGGACTATTCCGGCCCCTATCAC
GGCTATGACGGCTTCGCCATCTTTGCCCGCGATATGGATATGACCCTGAACAATCCGGCG
TGGAACGAACTGACTGCCCCGTGGCTGAAGTCTGCGTGATTGCCCCTTCACTGTCCCGTC
AGTCCACCGATTTGTGGCGCGGGAGGAGAACACCATGAGCCAAACGATCGATAAAATTCA
CAGCTGTTATCCGCTGTTTGAACAGGATGAATACCAGACCCTGTTCCAGAATAAAAAGAC
CCTTGAAGAGGCGCACGACGCGCAGCGTGTGCAGGAGGTTTTTGCCTGGACCACCACCGC
CGAGTATGAAGCGCTGAACTTCCAGCGCGAGGCGCTGACCGTCGACCCGGCCAAAGCCTG
CCAGCCGCTCGGCGCCGTACTCTGCGCGCTGGGGTTCGCCGGCACCCTGCCCTACGTGCA
CGGCTCCCAGGGCTGCGTCGCCTATTTTCGCACCTACTTTAACCGCCATTTTAAAGAGCC
TGTCGCCTGCGTCTCCGACTCCATGACCGAGGACGCGGCGGTGTTCGGCGGCAACAACAA
CATGAATCTGGGCCTGCAGAATGCCAGCGCGCTGTATAAACCCGAGATTATCGCCGTCTC
CACCACCTGCATGGCCGAGGTGATCGGCGACGATCTGCAGGCGTTTATCGCCAACGCCAA
AAAAGAGGGATTTGTTGACGACAGCATCGCCATTCCTTACGCCCATACCCCCAGCTTTAT
CGGCAGCCATGTCACCGGCTGGGACAATATGTTCGAAGGGTTCGCGAAGACCTTTACCGC
TGACTACGCCGGGCAGCCGGGCAAACAGCAAAAGCTCAATCTGGTGACCGGATTTGAGAC

CTATCTCGGCAACTTCCGCGTGCTGAAGCGGATGATGGCCCA";

nifK nucleotide sequence - reverse strand

ACTCAACAACAGGAGAAGTCACCATGACCATGCGTCAATGCGCTATCTACGGTAAAGGCG
GTATCGGTAAATCCACCACCACCCAGAATCTCGTCGCGGCCCTCGCCGAGATGGGTAAGA
AAGTGATGATCGTCGGCTGCGATCCGAAAGCGGATTCCACCCGTCTGATCCTCCACGCTA
AAGCCCAGAACACCATCATGGAGATGGCGGCGGAAGTGGGCTCGGTCGAAGATCTGGAGC
TCGAAGACGTGCTGCAAATCGGCTATGGCGATGTCCGTTGCGCCGAATCCGGCGGCCCGG
AGCCAGGCGTCGGCTGCGCCGGACGCGGGGTGATCACCGCCATCAACTTCCTCGAGGAAG
AAGGCGCCTATGAGGAAGATTTGGATTTCGTCTTCTATGACGTCCTCGGCGACGTGGTCT
GCGGCGGCTTCGCCATGCCGATCCGCGAAAACAAAGCCCAGGAGATCTACATCGTCTGCT
CCGGCGAGATGATGGCGATGTATGCCGCCAACAATATCTCCAAGGGGATCGTGAAGTACG
CCAAATCCGGCAAGGTGCGCCTCGGCGGCCTGATCTGTAACTCGCGCAAAACCGACCGGG
AAGACGAACTGATCATCGCCCTGGCGGAGAAGCTTGGCACGCAGATGATCCACTTCGTTC
CCCGCGACAACATTGTGCAGCGCGCGGAGATCCGCCGGATGACGGTGATCGAGTACGACC
CGACCTGTCAGCAGGCCAACGAATATCGTCAACTGGCGCAGAAGATCGTCAATAACACCA
AAAAAGTGGTGCCGACGCCGTGCACCATGGACGAGCTGGAATCGCTGCTGATGGAGTTCG
GCATCATGGAAGAGGAAGA";

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