Research Journal of Microbiology

ISSN 1816-4935

Academic Journals Inc.

www.academicjournals.com

Research Journal of Microbiology 5 (10): 1011-1021, 2010
ISSN 1816-4935 / DOI: 10.3923/jm.2006.319.329

The Carboxyl Terminus of NifK Protein is Involved in Formation of a Stable Nitrogenase Complex under Acidic Growth Conditions in *Azotobacter vinelandii* *

Hanqing Dong, Preeti Patil, Lakshmi Pulakat and Nara Gavini
Department of Biological Sciences, Mississippi State University,
Mississippi State, MS-39762, USA

Abstract: The enzyme nitrogenase, which catalyzes nitrogen fixation in *Azotobacter vinelandii*, consists of two components, the Fe protein and the MoFe protein. The MoFe protein is an $\alpha_2\beta_2$ heterotetramer encoded by the *nifD* and *nifK* genes respectively. NifD, which consists of 492 amino acids, contains the FeMo cofactor; while the 523 amino acid β-subunit NifK is involved in electron transfer and protein-protein interaction. NifK contains several highly conserved residues implicated in its functions throughout the protein. However, the carboxyl terminus of NifK is not implicated in any of the known functions of this protein. Therefore, the present study explored the role of carboxyl terminal region of NifK by constructing a truncated NifK in which the last 58 amino acids were deleted by insertion of a stop codon at position 465. The results of growth analysis in standard Burke's nitrogen media showed that the mutant strain exhibited a growth pattern similar to that of the wild type strain. However, when the media was adjusted to be slightly acidic, the strain that expressed the mutated NifK yielded a 15% lower growth compared to the wild type. These observations implied that the carboxyl terminus of the NifK contributes to the formation of a stable nitrogenase complex when *A. vinelandii* is grown in acidic environment.

Key words: *Azotobacter vinelandii*, NifK, nitrogen fixation, nitrogenase complex

Introduction

Nitrogen fixation is catalyzed by the enzyme nitrogenase in diazotrophs (Howard and Rees, 1996; Rees *et al*., 1993). In *A. vinelandii*, there are three types of nitrogenases distinguished by different metal centers (Loveless and Bishop, 1999a) including molybdenum, iron and vanadium (Loveless *et al*., 1999b; Burgess and Lowe, 1996; Rajagopalan and Johnson, 1992). The molybdenum-dependent nitrogenase is most thoroughly elucidated (Burgess and Lowe, 1996; Rajagopalan and Johnson, 1992; Christiansen *et al*., 2001); it consists of two components, the Fe protein and the MoFe protein (Kim and Rees, 1992; Mayer *et al*., 2002; Schlessman *et al*., 1998; Strange *et al*., 2003). The Fe protein is a highly conserved homodimer with a molecular weight of approximately 60 kDa. Both subunits are bridged by a 4Fe:4S cluster (Kim and Rees, 1994). Coupled by *in vivo* ferredoxin reduction and MoFe protein-accompanied ATP hydrolysis, the Fe protein repeats the process of association and dissociation multiple times with the MoFe protein, thus donating electrons to the MoFe protein (Schindelin *et al*., 1997; Thorneley and Lowe, 1984). The MoFe protein has a molecular mass of approximately 240 kDa and is a tetramer with two α subunits of 492 amino acids encoded by *nifD* and two β subunits of 523 amino acids encoded by *nifK* (Jang *et al*., 2000). The structural features of the MoFe protein are elucidated by X-ray crystallography (Kim and Rees, 1992). Two types of metal clusters, the FeMo cofactor (FeMoco) and the P cluster are found in either αβ dimer.

Corresponding Author: Nara Gavini, Department of Biological Sciences, Mississippi State University, Mississippi State, MS-39762, USA Tel: (662) 325 7573 Fax: (662) 325 7939

*Originally Published in Research Journal of Microbiology, 2006

The FeMoco is buried within the α subunit and it has 4Fe:3S and 1Mo:3Fe:3S clusters bridged by three non-protein ligands (Howard and Rees, 1996; Kim and Rees, 1992). The FeMoco is the substrate reduction site and destination of the electron flow (Igarashi *et al*., 2004). This is confirmed by the fact that isolated or *in vitro* synthesized FeMoco complements the non-functional apoenzyme (Dean *et al*., 1993; Gavini *et al*., 1994). The P cluster, a likely intermediate participant of the electron transport from the Fe protein to the FeMoco, however, is located at the interface between α and β subunits. It consists of two identical 4Fe: 4S clusters bridged by two cysteine thiol ligands (Howard and Rees, 1996; Kim and Rees, 1992).

The β subunit, or NifK protein is the less conserved subunit of the MoFe protein complex compared to the NifD protein. A number of residues from NifK contribute to maintaining the enzyme function and structure. Mutated strains constructed previously in our lab containing disruptions at various positions of *nifK* gene have resulted in complete abolishment of nitrogenase activity. The only metal center that is structurally related to NifK protein is the P cluster and studies have indicated that cysteine residues at β70, β95 and β153 serve as putative P cluster ligands (Kim and Rees, 1992). All three residues are interspecifically conserved. Cysβ95 bridges the two 4Fe: 4S subunits of the cluster while the other cysteines form ligands with remaining Fe atoms (Kim and Rees, 1992). Substitutions of these residues by different amino acids either decrease or completely eliminate nitrogenase activity (Kim and Rees, 1992).

Interactions between the Fe protein and the MoFe protein are essential for nitrogenase catalysis because each association of the two components manages to transfer one electron (Gavini *et al*., 1994) while the reduction of dinitrogen requires more. Furthermore, neither of the metal centers in the MoFe protein is exposed accessibly on the surface, hence interactions between the two components of dinitrogenase have to be precisely directed. Most recently, it was demonstrated by cocrystallization that the surface of the MoFe protein is somehow inflexible, while Fe protein can have diverse conformations so that it exclusively docks to three different regions of the MoFe protein (Tezcan *et al*., 2005), among which Lysβ400 has been well explained to form a carbodiimide crosslink with Gluγ112 of the Fe protein (Willing and Howard, 1990).

As mentioned earlier, previous studies have dealt with the importance of various regions of NifK protein, however, there are no reports indicating that the carboxyl terminus region of NifK protein contributes to nitrogenase activity. Interspecies sequence analysis of NifK protein from various organisms as well as AnfK and VnfK of *A. vinelandii* (Fig. 1) also shows that the carboxyl terminus domains exhibit less homology than the entire NifK proteins. To analyze whether this less conserved carboxyl terminus domain of NifK plays any significant role in the function of the protein, we constructed a strain containing mutated *nifK* gene encoding the NifK protein with a truncated carboxyl terminal region. The study found out that the carboxyl terminus of NifK protein in *A. vinelandii,* although not significant in nitrogen fixation under normal conditions, plays a pivotal role in growth regulation under pH stresses.

Materials and Methods

Bacterial Strains, Plasmids and Growth Conditions

The bacterial strains and plasmids used for this study are listed in Table 1. *Escherichia coli* strains were grown at 37°C in 2YT media (Sambrook and Maniatis, 1992) and for antibiotic selections, final concentration of ampicillin was adjusted to 50 μg mL^{-1}. *A. vinelandii* strains were cultured at 30°C in modified Burk's nitrogen-free (BN^-) media (Strandberg and Wilson, 1968; Page and Tigerstrom, 1979). When it was necessary to include fixed nitrogen, ammonium acetate was added to a final concentration of 200 mM. Also for antibiotic selections, final concentrations of ampicillin and kanamycin were adjusted to 50 and 2.5 μg mL^{-1}, respectively.

Construction of Plasmids Carrying Truncated A. vinelandii nifK

The QuikChange® Site-Directed Mutagenesis Kit (Stratagene Inc., La Jolla, CA) (QuikChange® Site-Directed Mutagenesis Kit Instruction Manual. Invitrogen) was used to construct a *nifK* mutant strain with a stop codon insertion at position 465 so that translation of the last 58 amino acids of NifK protein was inhibited. The template was the nifK gene present in the plasmid pBG1341. pBG1341 is a derivative a pUC18 which carries partial *nifD* gene and the entire *nifKTY* genes (Table 1). Catalyzed by *Pfu*Turbo DNA polymerase, the primers were extended during temperature cycling. This series of cycles resulted in a nicked plasmid with the mutation incorporated. The mixture of the mutated DNA and the template DNA was subjected to treatment by *Dpn*I in order to digest the methylated parental DNA [QuikChange® Site-Directed Mutagenesis Kit Instruction Manual. Invitrogen]. *E. coli* TG1 competent cells were then transformed with the mixture of the parental DNA and the mutant DNA and the plasmids were harvested from the transformants.

Construction of the A. vinelandii Mutant Strain

The plasmid extracted from the *E. coli* strain was a derivative of pUC18, which does not replicate in *A. vinelandii*. However, because of the high homologous recombination frequency in *A. vinelandii* (Lei *et al*., 1999), we could integrate the mutated gene into the genome. In our experiment, we used *A. vinelandii* strain BG1720, in which the *nifK* gene is interrupted by a kanamycin resistance gene insertion, as the transformation target. Due to the disruption in the *nifK* gene, this strain does not express a functional MoFe protein, thus it does not grow on BN^- plates. The *A. vinelandii* transformation was carried out as described previously (Page and Tigerstrom, 1979). When the mutated *nifK* was incorporated into the genome, a different NifK protein was expressed and selection was made upon the change of Nif phenotype by allowing the cells to grow on BN^- plates. Only the mutant strain that expressed the truncated NifK protein was capable of biological nitrogen fixation and the mutant strain was named *A. vinelandii* BG3801.

Growth Characteristics of A. vinelandii Mutant and Wild Ttype Strains

Growth analysis of *A. vinelandii* wild type and the mutant strain BG3801 were carried out in both BN^+ and BN^- media. Overnight cultures were prepared in BN^+ to attain satisfactory growth. They were then resuspended and used for inoculating 30 mL of BN^+ and BN^- media with various pH ranging from 6.75 to 7.2 in 250 mL sidearm flasks. Using a Klett-Summerson Colorimeter (Klett Manufacturing Inc., New York, NY), the data was recorded every two hours over a 14 h period.

Results and Discussion

The NifK protein in *A. vinelandii* serves two major functions: it together with the NifD protein stabilizes the P-cluster with three cysteine ligands which are conserved over a variety of species and several less important residues (Howard and Rees, 1996; Kim and Rees, 1992); also, the protein provides dinitrogenase reductase with a few residues functioning as docking sites so that the electron flow which is originating from the 4Fe:4S cluster of the Fe protein can be facilitated (Tezcan *et al*., 2005). Mutations of the residues that are involved in either function group lead to either diminution or abolishment of nitrogenase activity entirely (Howard and Rees, 1996; Kim and Rees, 1992). However, there has been no research conducted so far concerning the carboxyl terminus of NifK protein. Also, in the last 58 amino acids of NifK protein that were truncated, only three of them are conserved (Fig. 1). It is not known if this region of NifK protein plays a significant role in the functions of nitrogenase. Therefore we explored whether the carboxyl terminus domain of NifK protein is flexible enough to accommodate drastic structural changes; and whether such changes would have any influences on the functions of nitrogenase. Thus the strategy was employed to generate a mutant *A. vinelandii* strain expressing a truncated NifK protein in the carboxyl terminus domain.

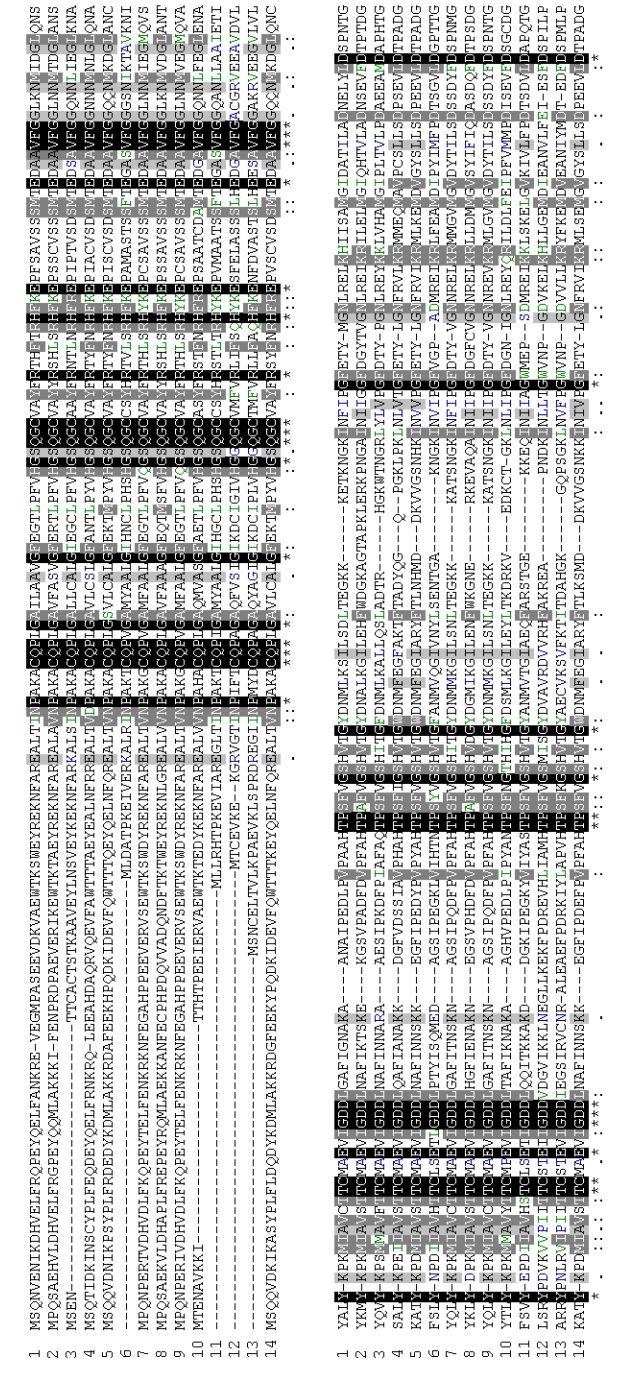

Fig. 1: Continued

Fig. 1: Amino acid sequence comparison of NifK from various organisms. This clustalW result reflects the whole sequence comparison. All amino acid sequences were obtained from GenBank (http://www.ncbi.nlm.nih.gov). The following proteins/organisms were included: 1. NifK of *Lyngbya majuscula*; 2. NifK of *Bradyrhizobium japonicum*; 3. NifK of *Heliobacterium chlorum*; 4. NifK of *Klebsiella pneumoniae*; 5. NifK of *Pseudomonas stutzeri*; 6. NifK of *Clostridium pasteurianum*; 7. NifK of *Nostoc* sp. PCC 7120; 8. NifK of *Rhizobium etli*; 9. NifK of *Anabaena variabilis*; 10. NifK of *Desulfuromonas acetoxidans*; 11. NifK of *Chlorobium limicola*; 12. AnfK of *Azotobacter vinelandii*; 13. VnfK of *Azotobacter vinelandii*; 14. NifK of *Azotobacter vinelandii*. The data was uploaded to San Diego Supercomputer Center (http://workbench.sdsc.edu) for further analyses. Maximum gap and mismatch penalties were opted for when the comparison was performed. In all NifK proteins a number of residues labeled by asterisks/black shades were fully conserved including three P-cluster cysteine ligands. Some labeled by semicolons/dark gray shades were highly conserved; the ones labeled by periods/light gray shades were semi conserved. The other residues without any labels were not conserved. The carboxyl termini domain of NifK proteins appeared to be less conserved compared to complete proteins

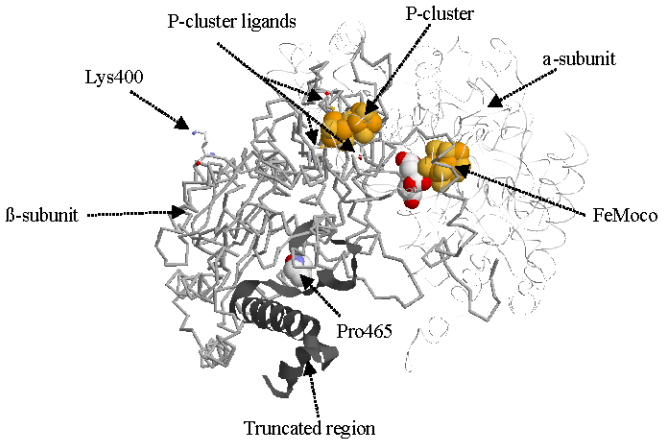

Fig. 2: Crystallographic structure of one αβ dimer of dinitrogenase and the mutated NifK protein superimposed on wild type NifK. The complete crystallized protein structure was obtained from RSCB protein data bank (http://www.rcsb.org). The protein structure with highlighted structural motifs shown in the figure was generated using RasTop (http://www.geneinfinity.org/rastop). The figure reflects one αβ dimer of nitrogenase: NifD (α-subunit) in light gray thin ribbon, NifK (β-subunit) in gray backbone and the NifK carboxyl terminal region truncated in charcoal wide ribbon; P-cluster, FeMo cofactor and Proβ465 in spacefill; P-cluster ligands Cysβ70, Cysβ95, Cysβ153 and Lysβ400 in stick

Based on the X-ray crystallographic structure of the MoFe protein (Fig. 2) (Howard and Rees, 1996; Kim and Rees, 1992), the carboxyl terminus domain of the NifK protein appears to be comparatively irrelevant to the more important structural motifs found in the NifK protein including both the P-cluster ligands and the Fe protein docking sites. They are so distant from each other (>3nm) that no possible interactions between them should be expected. Recently, it was shown that a NifD-K fusion protein encoded by fused *nifD* and *nifK* genes, which is a homodimeric protein, functions equivalently to the wild type tetramer protein that is encoded by separate *nifD* and *nifK* genes (Lahiri *et al*., 2005; Suh *et al*., 2003). This discovery indicated that the dinitrogenase tetramer structure can actually be regarded as two dimeric structures and the putative interactions between the carboxyl terminus domain of the NifK protein from one of the dimers and structural motifs from the other dimer structure are minor for nitrogenase activities.

Construction of A. vinelandii Mutant Strain Expressing Truncated NifK and its Growth Characteristics

In the NifK protein of *A. vinelandii*, the region following immediately after Pro465 is mainly composed of an α-helix. As the proline residue that is closest to the carboxyl terminus of NifK, Pro465 drew most attention. It is known that proline is the only common proteinogenic amino acid carrying a secondary amine group. When forming a peptide bond with another amino acid, its lack of hydrogen results in that the proline cannot play the role as the hydrogen donor in the formation of a hydrogen bond. Thus proline tends to disrupt protein structures: in α-helices proline either bends them slightly or disturbs the helix structures completely; in β-sheets proline functions as turns. In the $\alpha_2\beta_2$ heterotetramer, based on the distances between the residues, this helix does not interact with the NifD protein that is co-localizing the P-cluster; it only possibly interacts with the other NifD protein and this interaction is unimportant according to the crystal structure. Therefore we decided to truncate the NifK protein at this proline residue.

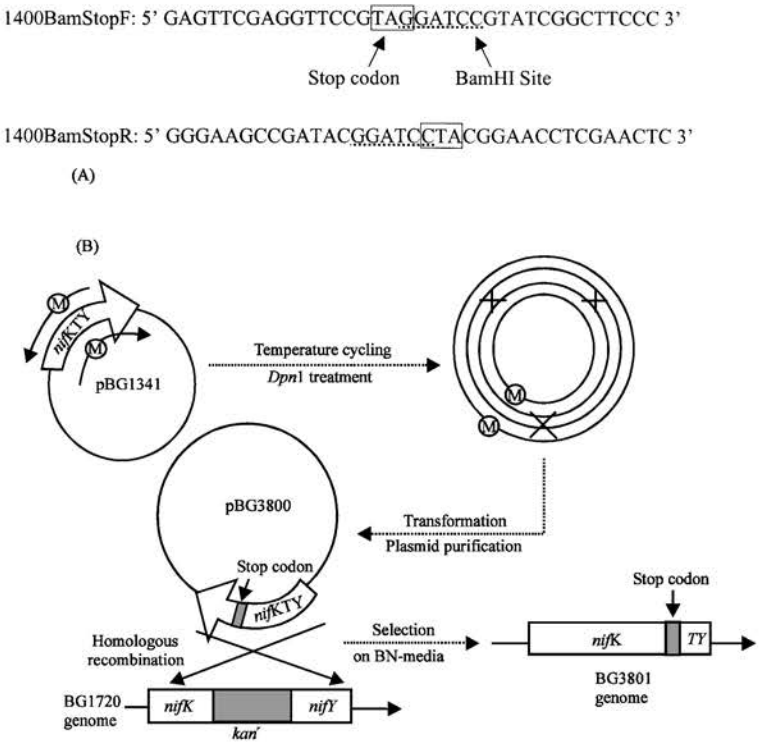

Fig. 3: Cloning strategy by which the NifK mutant *A. vinelandii* BG3801 was generated. (A) The primers for mutagenesis were designed with the stop codon immediately followed by a new *Bam*HI site. The stop codon inserted was shown as boxed text; the novel *Bam*HI site was underlined by dotted line. (B) The primers containing the mutation annealed to the template DNA. After a series of temperature cycling, a new plasmid pBG3800 was generated and the maternal DNA was digested by *Dpn*I treatment. The mixture was used for transformation and purified plasmids were verified by *Bam*HI digestion. The plasmid containing the mutation was further used to transform *A. vinelandii* strain BG1720, which was not capable of nitrogen fixation due to the *nifK* gene interruption by a kanamycin resistance cassette. The incorporation of the mutation into the genome of the cell, because of homologous recombination, led to the change of the phenotype of BG1720 from Nif$^-$ to Nif$^+$, which indicated that the truncated NifK protein functioned in the same manner as the wild type

When the primers for mutagenesis were designed, not only did a stop codon have to be included in the primers, but also for the purpose of verification, a new restriction site had to be incorporated into the gene encoding the NifK protein containing the mutation (Fig. 3A). It would be convenient that the insertion of the stop codon could be confirmed by exhibiting a different restriction digestion pattern. As a result of comparisons and analyses, the DNA sequence immediately following the codon of Pro465 is similar to that of the recognition site of *Bam*HI, which meant when mutation was to be generated at this locus, minimal structural alterations would be introduced into the gene other than the stop codon as well as the restriction enzyme recognition site. Thus we chose to insert a stop codon at position 465 so that the last 58 amino acids of NifK would be truncated. To verify the presence of mutation in the *nifK* gene, we performed *Bam*HI digestions on the plasmids isolated from the transformants. Experiments carried out on the mutated plasmids yielded a fragment with the size of

Table 1: Bacterial strains and plasmids used in this study

Strain/plasmid	Relevant characteristics and description	Source/reference
E. coli TG1	K-12 Δ(lac-pro) supE thi hsd-5/F′ traD36 *proA*$^{+}$*B*$^{+}$ *lacI*^{q} *lacZΔM15*	Amersham Biosciences, Piscataway, NJ
A. vinelandii DJWT	Wild type, Nif^{+}, soil bacterium	(Suh *et al*., 2003)
pBG1341	Derivative of pUC18 containing partial *nifD* and entire *nifK*, *nifT* and *nifY* cloned	Chaturvedi, V., Mississippi State University
A. vinelandii BG1720	Nif^{-}, containing disrupted nifKTY, a kanamycin cassette was inserted between the *Sal*I site of *nifK* and the one in *nifY*	Lahiri, S., Mississippi State University
pBG3800	Derivative of pBG1341, encoding a NifK protein with a stop codon insertion at position 465	Present study
A. vinelandii BG3801	Nif^{+}, BG1720 transformed with pBG3800	Present study

approximately 1400 bp while no such fragments were released when the mutation was not generated and the parental DNA remained undigested after *Dpn*I treatment (Fig. 3B). The plasmid carrying the mutant *nifK* gene was designated pBG3800 and was used for further experiments. The mutated plasmid was verified by *Bam*HI digestion.

The verified plasmid pBG3800 was introduced into *A. vinelandii* strain BG1720, in which the insertion of a kanamycin resistance within *nifK* cassette led to complete loss of nitrogenase activity (Table 1). The resulting *A. vinelandii* strain BG3801 was capable of growth on BN^{-} plates and this phenotype alteration suggested that the truncated NifK protein could function in a similar manner to that of full-length NifK. Furthermore, the growth analysis also showed that the mutant strain could grow in BN^{-} media at the same rate as that of the wild type *A. vinelandii* strain DJWT (Fig. 4A). These observations strongly indicated that a truncated NifK protein, which has a stop codon insertion at position 465, is functional in biological nitrogen fixation.

Carboxyl Terminus of NifK is Involved in pH Stress Regulation

Despite the fact that carboxyl terminus domain of NifK is not essential for cell growth or nitrogenase activity as was shown in the growth pattern exhibited by *A. vinelandii* strain BG3801, this region of the MoFe protein has remained intact over vast evolutionary periods amongst a variety of diazotrophic species. If this region is non-functional completely, more mutations throughout the genes such as gaps or residue alterations should be expected. According to this observation, we therefore proposed that the carboxyl terminus domain of NifK must function in an important yet unidentified way.

As a bacterium that is generally found in the soil, *A. vinelandii* confronts numerous environmental pressures, pH stress being one of them. Multiple possibilities were explored by monitoring the growth patterns of the NifK truncation strain in various stress conditions and it was discovered that the mutant *A. vinelandii* strain BG3801 was yielding a lower growth rate in more acidic media in contrast to the wild type. Therefore, we compared the growth patterns of wild type *A. vinelandii* DJWT and the mutant strain *A. vinelandii* BG3801; pH stress was selected as the limiting factor and growth analysis was conducted on both the wild type *A. vinelandii* strain and the mutant strain *A. vinelandii* BG3801. To comprehend the overall impact of pH on the bacterial strains, the growths of both *A. vinelandii* BG3801 and the wild type strain were first monitored on a wider pH spectrum using BN^{-} plates with pH gradients. The gradient plate technique was developed in 1950s (Sacks, 1956) and was originally employed for antibiotic gradients. The usage of this technique in generating pH gradients was one of the major adaptations derived from the initial concept. With a combination of different potassium phosphate salts (monobasic and dibasic in this case) the pH of the agar plates ranged from 5.5 at one end of the petridish to 7.5 at the other. The pH values were confirmed by non-bleeding pH indicating strips (EM Science Inc., Gibbstown, NJ) and the bacterial cells were transferred to the pH gradient plates by either direct streaking or a sequence of swift placing and removing of filter paper

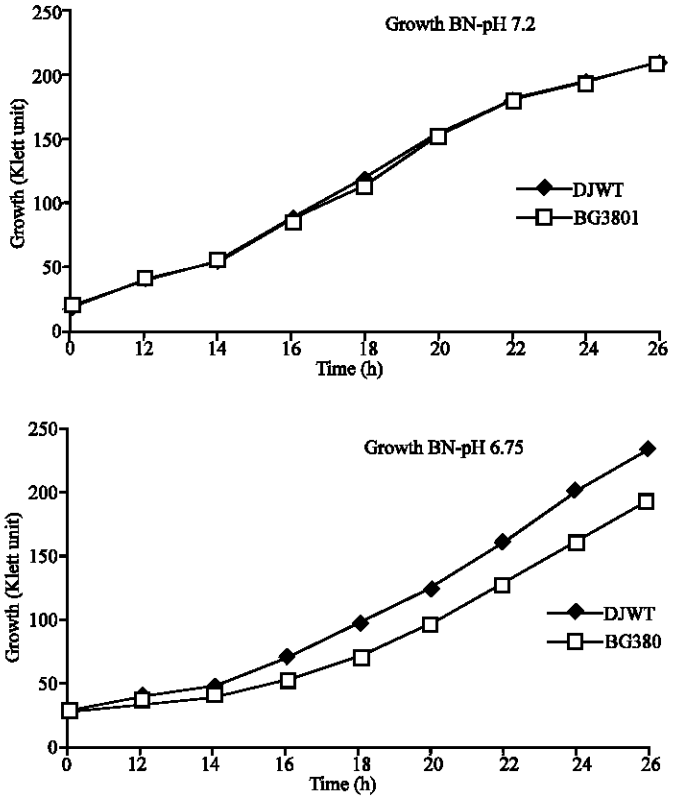

Fig. 4: Growth characteristics of *A. vinelandii* BG3801 compared to wild type under normal and acidic conditions. The growths of the different strains were recorded in Klett units using 250 mL side arm flasks. The experiments were performed a minimum six times (triplicates each time) for accuracy and reproducibility. The growth data shown in the graphs reflected an average value of independent observations. In standard media (BN^- pH7.2) both wild type and mutant strain exhibited similar growth patterns (A); while in acidic media, mutant strain that expresses the truncated NifK showed approximately 15% lower growth compared to wild type strain (B)

strips that had been immersed in the cultures for a brief period of time. It was discovered that the growth of the mutant strain decreased significantly when the media became more acidic (<6.75), while that of the wild type decreased at a much slower rate. To further elucidate the impact of pH, a growth curve analysis was carried out in liquid BN^- media with pH 6.75 (Fig. 3B). When adjusting the pH of the BN^- media, hydrochloric acid was used to finalize the media pH value at 6.75; readings were taken and verified with the help from the Accumer® AB15 pH meter (Fisher Inc., Pittsburgh, PA). The media were inoculated with DJWT as well as *A. vinelandii* BG3801 and the initial concentrations of the cultures were adjusted within the range of 20 to 25 Klett units using Klett-Summerson colorimeter. No monitoring was applied for the first 12 h because the cells were still in lag phase. After that, readings were carried out every two hours for 14 consecutive hours.

As was indicated in the graphs, the wild type strain and the mutant strain exhibited identical growth patterns in BN^- media with normal pH. However, when the pH of the media was adjusted to 6.75, the mutant strain showed slower growth by approximately 15% lower than the wild type. This observation implied that the existence of the carboxyl terminus domain of NifK protein is crucial because this region of the protein contributes to a stable nitrogenase structure when environmental pH stress is present.

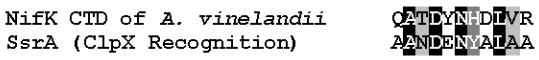

Fig. 5: Amino acid sequence comparison of carboxyl terminus domain of NifK protein with SsrA tag. SsrA tag, an 11 amino acid long sequence, is the putative recognition site for ClpX, which is an ATPase that regulates responses to environmental stress to certain extent. The protein sequences were obtained from GenBank and the clustalW was performed with the help of San Diego Supercomputer Center. In the NifK carboxyl terminus of *A. vinelandii*, we found that this region exhibited similarity to SsrA tag: 4 out of 11 amino acids labeled by black shades were fully conserved; highly conserved residues were labeled by dark gray shades while the weakly conserved residues were labeled by light gray shades. Unlabeled residues were not conserved. This suggested this region may function in similar manners

Carboxyl Terminus of NifK Shares Homology to ClpX Binding Domain

Amino acid comparisons were conducted between the sequences of the NifK carboxyl terminus with a number of other proteins in order to understand its possible function. It was indicated that the last 11 amino acids of NifK protein shares sequence homology with SsrA tag (Fig. 5), the known recognition site for the ClpX protein. ClpX has been reported to be an ATPase that contributes to growth regulation under environmental pressures including pH, salt concentration and others (Kruger *et al*., 2000). Since the carboxyl terminus domain of NifK protein shares homology with the SsrA tag, it is possible that this region may function in a similar manner to a ClpX binding site. Taken together, the observations that the carboxyl terminus contributes to the formation of an optimum-functioning nitrogenase at acidic pH and that it contains a ClpX-binding site-resembling domain further support the role of this region in the functionality of nitrogenase under pH stress.

Acknowledgements

Part of this study was done at Bowling Green State University, Ohio. This study is supported by NSF and NIH to NG and LP. We thank members of Gavini/Pulakat laboratory for helpful discussions and technical help.

References

Burgess, B.K. and D.J. Lowe, 1996. Mechanism of molybdenum nitrogenase. Chem. Rev., 96: 2983-3011.

Christiansen, J., D.R. Dean and L.C. Seefeldt, 2001. Mechanistic features of the Mo-containing nitrogenase. Annu. Rev. Plant Physiol. Plant Mol. Biol., 52: 269-295.

Dean, D., J.T. Bolin and L. Zheng, 1993. Nitrogenase metalloclusters: Structures, organization,and synthesis. J. Bacteriol., 175: 6737-6744.

Gavini, N., L. Ma, G. Watt and B.K. Burgess, 1994. Purification and characterization of a FeMo cofactor-deficient MoFe protein. Biochemistry, 33: 11842-11849.

Howard, J.B. and D.C. Rees, 1996. Structural basis of biological nitrogen fixation. Chem. Rev., 96: 2965-2982.

Igarashi, R.Y., P.C. Dos Santos, W.G. Niehaus, I.G. Dance, D.R. Dean and L.C. Seefeldt, 2004. Localization of a catalytic intermediate bound to the FeMo-cofactor of nitrogenase. J. Biol. Chem., 279: 34770-34775.

Jang, S.B., L.C. Seefeldt and J.W. Peters. 2000. Insights into nucleotide signal transduction in nitrogenase: Protein with MgADP bound. Biochemistry, 39: 14745-14752.

Kim, J.S. and D.C. Rees, 1994. Nitrogenase and biological nitrogen-fixation. Biochemistry, 33: 389-397.

Kim, J.S. and D.C. Rees, 1992. Structural models for the metal centers in the nitrogenase molybdenum-iron protein. Science, 257: 1677-1682.

Kruger, E., E. Witt, S. Ohlmeier, R. Hanschke and M. Hecker, 2000. The clp proteases of *Bacillus subtilis* are directly involved in degradation of misfolded proteins. J. Bacteriol., 182: 3259-3265.

Lahiri, S., L. Pulakat and N. Gavini, 2005. Functional NifD-K fusion protein in *Azotobacter vinelandii* is a homodimeric complex equivalent to the native heterotetrameric MoFe protein. Biochem. Biophys. Res. Commun., 337: 677-684.

Lei, S., L. Pulakat and N. Gavini, 1999. Regulated expression of the nifM of *Azotobacter vinelandii* in response to molybdenum and vanadium supplements in Burk's nitrogen-free growth medium. Biochem. Biophys. Res. Commun., 264: 186-190.

Loveless, T.M. and P.E. Bishop, 1999a. Identification of genes unique to Mo-independent nitrogenase systems in diverse diazotrophs. Can. J. Microbiol., 45: 312-317.

Loveless, T.M., J.R. Saah and P.E. Bishop, 1999b. Isolation of nitrogen-fixing bacteria containing molybdenum-independent nitrogenases from natural environments. Applied Environ. Microbiol., 65: 4223-4226.

Mayer, S.M., C.A. Gormal, B.E. Smith and D.M. Lawson, 2002. Crystallographic analysis of the MoFe protein of nitrogenase from a *nifV* mutant of *Klebsiella pneumoniae* identifies citrate as a ligand to the molybdenum of iron molybdenum cofactor (FeMoco). J. Biol. Chem., 277: 35263-35266.

Page, W. and M. Tigerstrom, 1979. Optimal conditions for transformation of *Azotobacter vinelandii*. J. Bacteriol., 139: 1058-61.

Rajagopalan, K.V. and J.L. Johnson, 1992. The pterin molybdenum cofactors. J. Biol. Chem., 267: 10199-10202.

Rees, D.C., M.K. Chan and J.S. Kim, 1993. Structure and function of nitrogenase. Adv. Inorg. Chem., 40: 89-119.

Sacks, L., 1956. A pH gradient agar plate. Nature, 178: 269-270.

Sambrook, J.F. and T. Maniatis, 1992. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Schindelin, N., C. Kisker, J.L. Schlessman, J.B. Howard and D.C. Rees, 1997. Structure of ADP•AlF_4^--stabilized nitrogenase complex and its implications for signal transduction. Nature, 387: 370-376.

Schlessman, J.L., D. Woo, L. Joshua-Tor, J.B. Howard and D.C. Rees, 1998. Conformational variability in structures of the nitrogenase iron proteins from *Azotobacter vinelandii* and *Clostridium pasteurianum*. J. Mol. Biol., 280: 669-685.

Strandberg, G. and P.W. Wilson, 1968. Formation of the nitrogen-fixing enzyme system in *Azotobacter vinelandii*. Can. J. Microbiol., 14: 25-31.

Strange, R.W., R.R. Eady, D. Lawson and S.S. Hasnain, 2003. XAFS studies of nitrogenase: the MoFe and VFe proteins and the use of crystallographic coordinates in three-dimensional EXAFS data analysis. J. Synchrotron. Radiat., 10: 71-75.

Suh, M., L. Pulakat and N. Gavini, 2003. Functional expression of a fusion-dimeric MoFe protein of nitrogenase in *Azotobacter vinelandii*. J. Biol. Chem., 278: 5353-5360.

Tezcan, F.A., J.T. Kaiser, D. Mustafi, M.Y. Walton, J.B. Howard and D.C. Rees, 2005. Nitrogenase complexes: Multiple docking sites for a nucleotide switch protein. Sci., 309: 1377-1380.

Thorneley, R. and D.J. Lowe, 1984. The mechanism of *Klebsiella pneumoniae* nitrogenase action. Simulation of the dependences of H_2-evolution rate on component-protein concentration and ratio and sodium dithionite concentration. Biochem. J., 224: 903-909.

Willing, A. and J.B. Howard, 1990. Cross-linking site in *Azotobacter vinelandii* complex. J. Biol. Chem., 265: 6596-6599.