American Journal of Biochemistry and Biotechnology 7 (4): 196-203, 2011
ISSN 1553-3468

Discovery of Evolutionary Divergence of Biological Nitrogen Fixation and Photosynthesis: Fine Tuning of Biogenesis of the NifH and the ChlL by a Peptidyl-Prolyl *Cis/Trans* Isomerase

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Abstract: Problem statement: Despite the structural and functional similarities between the nitrogenase that performs biological nitrogen fixation reaction and the Dark Protochlorphyllide Oxidoreductase (DPOR) that performs chlorophyll-biosynthesis, attempts to substitute nitrogenase-components with DPOR-components have hitherto failed. This investigation was undertaken to test if *Chlamydomonas reinhardtii* protochlorophyllide (Pchlide) reductase (ChlL) that shares some structural similarity with Nitrogenase Reductase (NifH) could complement the functions of NifH in biological nitrogen fixation of *Azotobacter vinelandii*. **Approach:** Genetic complementation studies were performed to test if the *chlL* gene and its mutants cloned under transcriptional control of *nifH* promoter (*nifHp*) in a broad-host range low copy plasmid pBG1380 could render a Nif^+ phenotype to NifH-deficient *A. vinelandii* strains. **Results:** Expression of ChlL could render Nif^+ phenotype to NifH-deficient *A. vinelandii* only in the absence of NifM, a nif-specific PPIase essential for biogenesis of NifH. The ChlL mutants Cys95Thr and Cys129Thr were unable to substitute for NifH. Thus, the conserved cysteine ligands of [4Fe-4S] cluster in ChlL are essential for successful substitution of NifH by ChlL. Since C-termini of NifH and ChlL demonstrated the least similarity and Pro258, a substrate for the PPIase activity of NifM, is located in the C-terminus of NifH, we posited that replacing the C-terminus of NifH with that of ChlL would render NifM-independence to NifH. The NifH-ChlL chimera could support the growth of NifH- and NifM-deficient *A. vinelandii* in nitrogen limiting conditions implying that it has acquired NifM-independence. **Conclusion/Recommendations:** Collectively, these observations suggest that NifM, an evolutionarily conserved nif-specific PPIase, could have contributed to the functional divergence of biological nitrogen fixation and photosynthesis during evolution by virtue of its ability to exert opposing effects on structurally similar substrates, ChlL and NifH.

Key words: Nitrogenase, NifH, ChlL, Dark Protochlorphyllide Oxidoreductase (DPOR), NifM, PPIase

INTRODUCTION

Functional divergence of biological nitrogen fixation and photosynthesis, the two fundamental biological processes that sustain life on earth, is still an enigma. Structural and functional similarities exist between nitrogenase that performs the biological nitrogen fixation reaction and Dark Protochlorphyllide Oxidoreductase (DPOR) that performs reduction of protochlorophyllide to chlorophyllide during chlorophyll-biosynthesis (Brocker *et al*., 2008; Gavini *et al*., 2006; Georgiadis *et al*., 1992; Sarma *et al*., 2008; Tezcan *et al*., 2005; Watzlich *et al*., 2009; Yamamoto *et al*., 2009; 2008; Yamazaki *et al*., 2006a; 2006b; Nomata *et al*., 2006a; 2006b). Both nitrogenase and DPOR are oxygen sensitive two-component systems. However, attempts to substitute nitrogenase components NifH or NifDK by DPOR components have not been successful thus far.

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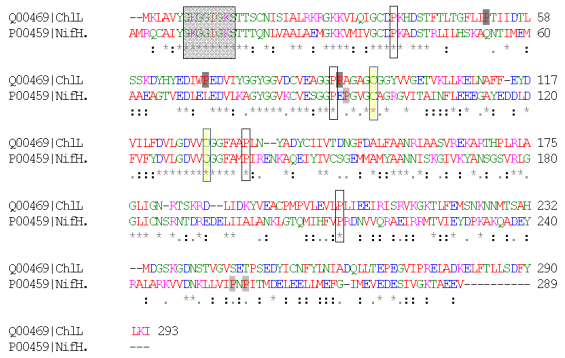


Fig. 1: Comparison of the amino acid sequences of *A. vinelandii* NifH and *C. reinhardtii* ChlL using. ClustalW (http://www.clustal.org/). The conserved cysteine ligands are shown in light yellow box. The ATP binding site is shown in gray box. The four proline residues that are conserved between the NifH, BchL and the ChlL are marked by open box. Three more additional proline residues that are conserved among NifH peptides are highlighted in light gray and the additional proline residues that are conserved among BchL and ChlL (shown in Fig. 2) are highlighted in dark gray

Nitrogenase is dependent on a multitude of nif-specific accessory proteins for its maturation and assembly (Brocker *et al*., 2008; Gavini *et al*., 2006; Georgiadis *et al*., 1992; Yamazaki *et al*., 2006b; Betancourt *et al*., 2008; Chen *et al*., 1994; Christiansen *et al*., 2001; Curatti *et al*., 2007; Gavini and Burgess, 1992; Gavini *et al*., 1994; Finan, 2002; Howard *et al*., 1989; 1986; Howard and Rees, 1996; Jacobson *et al*., 1989a; 1989b; Lei *et al*., 1999; 1998; Peters and Szilagyi, 2006; Petrova *et al*., 2002; Rubio and Ludden, 2005; Robinson *et al*., 1987). The DPOR component analogous to the nitrogenase component NifH is the BchL/ChlL protein encoded by the *bchL/chlL* genes of the photosynthetic bacteria.

Biogenesis of functional NifH is dependent on nif-accessory protein NifM in its natural system or in heterologous system (Finan, 2002; Howard *et al*., 1986; Jacobson *et al*., 1989a; Petrova *et al*., 2002). The NifM is a peptidyl-prolyl cis/trans isomerase (PPIase) belonging to the Parvulin family (Edlich and Fischer, 2006; Rahfeld *et al*., 1994). We have proposed that the NifM-mediated cis-to trans isomerization of one or more of the seven conserved prolines is needed for the generation of the functional NifH (Finan, 2002).

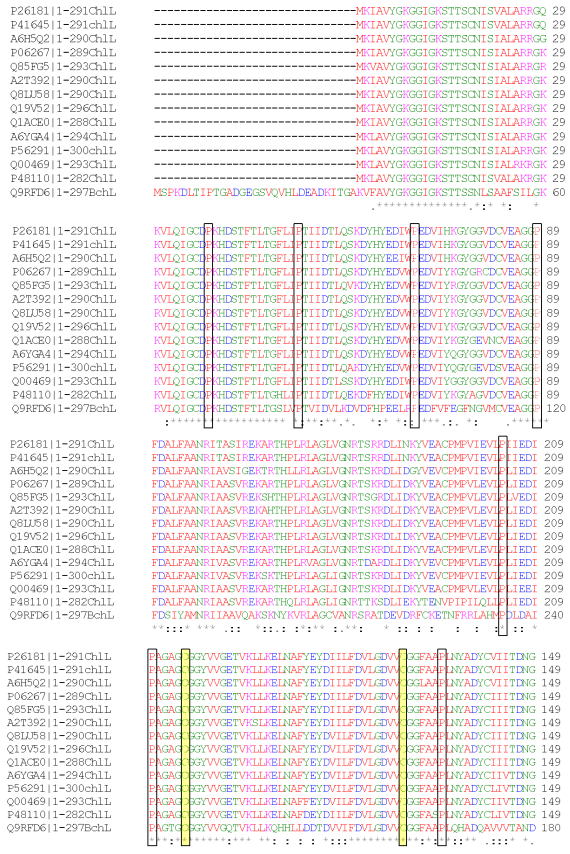


Fig. 2: ClustalW alignment of 13 ChlL peptides with BcHL. The conserved cystein ligands (light yellow box), ATP binding site (gray box) and proline residues (open box) are marked. P26181: *P. contorta*, P41645: *P. thunbergii*, A6H5Q2: *C. taitungensis*, P06267: *M. polymorpha*, Q85FG5: *A. capillus-veneris*, A2T392: *A. evecta*, Q8LU58: *C.* globosum, Q19V52: *C.atmophyticus*, Q1ACE0: *C. vulgaris*, A6YGA4: *L. terrestris*, P56291: *Chlorella vulgaris*, Q00469: *C. reinhardtii*, P48110: *C. paradoxa*, Q9RFD6: *R. sphaeroides*

Other PPIases are unable to substitute for NifM unless their catalytic domain is fused to the N-terminal region of NifM. A comparison of ChlL with NifH shows four conserved prolines (Fig. 1) that are part of the nine conserved prolines among the ChlL peptides (Fig. 2). Additionally, superimposing a predicted model of ChlL onto the NifH template (PDB ID: 1NIP) (Georgiadis *et al*.,1992), using Swiss PDB (Deep View) protein modeling software (Fig. 3) shows that the ChlL protein model thus generated has marked resemblance to the NifH protein.

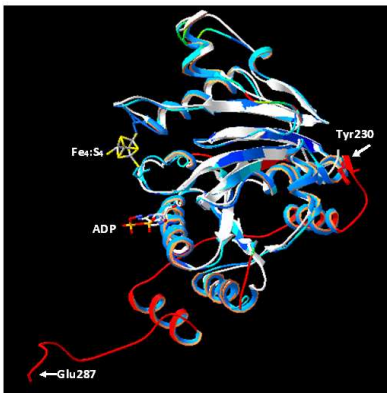


Fig. 3: The NifH monomer structure (based on 1NIP crystallographic structure of the NifH peptide from *A. vinelandii* (Georgiadis *et al*., 1992) colored according to differences with the ChlL model. Deep View color for ChlL was white. The Deep View coloration of NifH was done according to root mean square deviation type of coloration (blue = maximum similarity; red = maximum dissimilarity) in comparison to ChlL. Bottom view shows a particularly dissimilar C-terminal region (red stretch) starting with Tyr230 and ending with Glu287 of NifH. The [4Fe-4S] cluster and the bound MgADP in NifH are indicated

Since the ChlL protein showed such high structural similarity to the NifH protein, we hypothesized that ChlL would substitute for NifH in *A. vinelandii* and that NifM may play a role in modulating the functional properties of the NifH-like proteins such as ChlL/Bchl. The purpose of this study was to determine whether ChlL could substitute for NifH in biological nitrogen fixation in presence and absence of functional NifM.

MATERIALS AND METHODS

Construction of plasmid pBG2400: An 879bp DNA fragment encoding the ORF of *chlL* flanked by EcoRV and HindIII restriction enzyme sites was generated by PCR amplification using *C. Reinhardtii* chromosomal DNA as template and cloned into the pCR2.1 TOPO vector to create plasmid pBG1382 (Suh, 2002). This fragment was then subcloned into the EcoRV and HindIII digested broad-host range expression vector pBG1380 that contained the *nifHp* (Gavini *et al*., 2006) to generate pBG2400. Thus, the plasmid pBG2400 had the *chlL* gene under the transcriptional regulation of the *nifHp*.

Construction of ChlL mutants: PCR-mutagenesis with 'QuikChange™ Site Directed Mutagenesis Kit' (Stratagene Products, La Jolla, CA) was used to generate these mutants according to manufacturer's protocol. Plasmid pBG2400 was used as template. To generate Cys95Thr amino acid replacement, the forward primer was: 5'-CCACCTGCCGGTGCGGGGACCGGTGGTTATGTTG TAGGT-3' and reverse primer was 5'-ACCTACAACATAACCACCGGTCCCCGCACCGGC AGGTGG-3'. To generate Cys129Thr amino acid replacement, the forward primer was: 5'-GTTTTAGGTGATGTTGTTACCGGTGGCTTTGCTG CTCCA-3' and reverse primer was 5'-TGGAGCAGCAAAGCCACCGGTAACAACATCACC TAAAAC-3'.

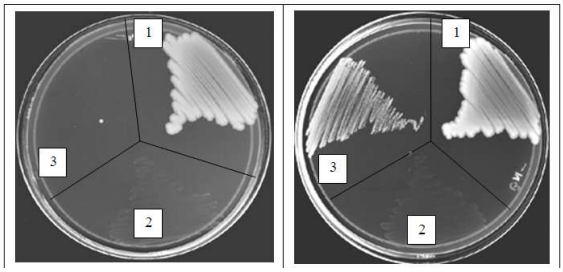
Construction of NifH-ChlL chimera: The 873bp DNA fragment encoding *nifH* ORF was PCR amplified using pDB6 (Jacobson *et al*., 1989b) as the template and initially cloned into the pCR2.1 TOPO vector. The EcoRV- HindIII fragment encoding *nifH* ORF was subcolned into EcoRV- HindIII digested pBG1380 to generate pBG2434 that carries *nifH* gene under the transcriptional control of *nifH* promoter (*nifHp*). The129bp region that carries the last 42 amino acids at the C-terminus of NifH was removed via Sal1 digestion. Next, the DNA containing the last 55 codons of the *chlL* was PCR-amplified using a 5' primer that carries a SalI site (5'-GTCGACAATTCTACAGTAGGAGTGTC-3') and a 3' primer with a HindIII site (5-AAGCTTTTAAATTTTAAGATAGAAATC-3'). The resultant PCR product encoding the C-terminal region of ChlL protein (55 amino acids at the C-terminal end) was cloned into the SalI-HindIII digested pBG2434 (carrying the N-terminus of *nifH*) to generate a *nifH-chlL* chimeric gene in which the DNA encoding the C-terminal region of NifH (bp745-873) was replaced by the DNA encoding C-terminal region of ChlL (bp718-882).

RESULTS AND DISCUSSION

ChlL can substitutes for NifH in biological nitrogen fixation reaction only in the absence of NifM: Two NifH-deficient *A. vinelandii* strains, one NifM-positive (*nifM*$^+$ *A. vinelandii* DJ54 (Gavini *et al*., 1994; Robinson *et al*., 1987) and one NifM-negative (*nifM::kan A. vinelandii* BG98 (Gavini *et al*., 2006) respectively, were used to test the ability of the ChlL to substitute for the NifH in nitrogen fixation reaction by *A. vinelandii*. Both strains were transformed with pBG2400 that carries the *C. reinhardtii chlL* gene cloned under the transcriptional regulation of the *nifHp* and the ability of the transformants to grow under nitrogen limiting conditions was assessed as follows.

Table 1: Replacing the cysteine ligands Cys95 and Cys129 with Thr inhibits the ability of the ChlL to substitute for the NifH in NifH-deficient NifM-minus *A. vinealndii* BG98

Strain	Plasmid	Codon/amino acid for position 95 of ChlL	Codon/ amino acid for position 129 of ChlL	Growth on BN^+ medium with Chloramphenicol	Growth on BN^- medium
A. vinelandii DJ54 (*ΔnifH*) $NifM^+$	pBG2400	TGT/Cys	TGT/Cys	+++	---
A. vinelandii BG98 *nifM*$^-$ NifH-deficient	pBG2400	TGT/Cys	TGT/Cys	+++	+++
A. vinelandii BG98 *nifM*$^-$ NifH-deficient	pBG2406	ACC/Thr	TGT/Cys	+++	---
A. vinelandii BG98 *nifM*$^-$ NifH-deficient	pBG2428	TGT/Cys	ACC/Thr	+++	---
A. vinelandii DJ54 (*ΔnifH*) $NifM^+$	pBG2406	ACC/Thr	TGT/Cys	+++	---
A. vinelandii DJ54 (*ΔnifH*) $NifM^+$	pBG2428	TGT/Cys	ACC/Thr	+++	---



(A) (B)

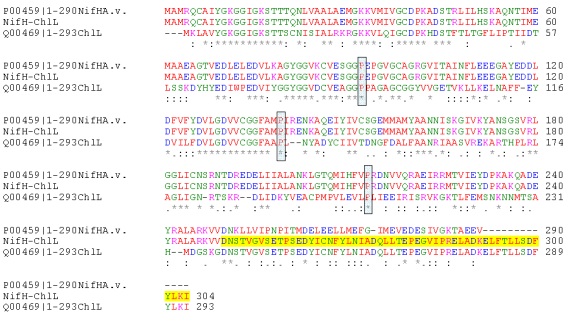
Fig. 4: Comparative growth of NifH-deficient *A. vinelandii* strains carrying plasmids with or without *chlL* gene on Burk's nitrogen free (BN^-) agar plate is shown. A) *nifM*$^+$ *DnifH A. vinelandii* DJ54 carrying pBG1380 that does not harbor *chlL* (marked 2) or pBG2400 that harbors *chlL* (marked 3); B) *nifM*$^-$ (*nifM* gene interrupted by insertion of Kan^R) *A. vinelandii BG98* that expresses a non-functional NIfH and harboring pBG1380 (marked 2) or pBG2400 (marked 3). Wild type *A. vinelandii* was used as control (marked as 1) on both plates. The pBG1380 is a parental plasmid that harbors the *nifH* promoter[2]. The pBG2400 carries the *chlL* under the transcriptional control of the *nifHp* of pBG1380, therefore, the *chlL* is expressed under nitrogen limiting conditions. The presence of pBG2400 allowed the growth of NifH-deficient *nifM::kan A. vinelandii* BG98, on BN^- agar plates (B 3), while it did not support the growth of NifH-deficient *nifM*$^+$ *A. vinelandii* DJ54 on BN^- agar (A 3). Transformations were repeated at least six times

Since the plasmid carries a chloramphenicol resistance (Cm^R) marker, the transformants were originally selected on Burk's Nitrogen plus (BN^+) (Strandberg and Wilson, 1968) medium supplemented with 72 μg mL^{-1} of chloramphenicol. Then, the ability of the *chlL* gene to render Nif^+ phenotype to *A. vinelandii* strains DJ54 and BG98 was monitored by growing the transformants on Burk's nitrogen free (BN^-) media containing molybdenum (Strandberg and Wilson, 1968) that imits expression of alternate nitrogenases (Betancourt *et al*., 2008).

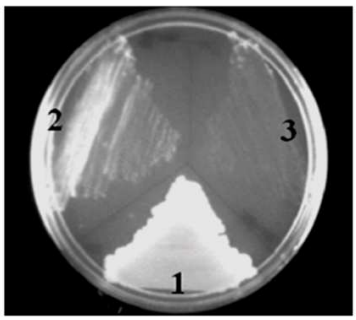
It was found that pBG2400 could not render a Nif^+ phenotype to the *A. vinelandii* DJ54 (Fig. 4a), but it rendered Nif^+ phenotype to the *A. vinelandii* BG98 (Fig. 4b).

Since the difference between the two strains is that DJ54 has an intact *nifM*, whereas BG98 has a disrupted *nifM*, we concluded that the ChlL could restore nitrogenase activity in the absence of functional NifH- but only if NifM was also absent. In summary, the ChlL can replace the NifH-function in biological nitrogen fixation by *A. vinelandii*. However, the NifM, the accessory PPIase essential for biogenesis of functional NifH, has a negative effect on the compensatory ability of ChlL. This interpretation is consistent with the observations that (a) the *chlL* gene could not complement the Δ*nifH* of *nifM*$^+$ *A. vinelandii* DJ54 and (b) the BchL protein (similar in structure and function to the ChlL) isolated from *nifM*$^+$ *A. vinelandii* was unable to substitute for the NifH protein in nitrogenase assay (Sarma *et al*., 2008).

Cys95 and Cys129 of the ChlL are required for substitution of NifH by ChlL in biological nitrogen fixation reaction: The cysteine ligands Cys97 and Cys132 of the NifH peptide are conserved in the ChlL peptide (Cys95 and Cys129 of the ChlL peptide respectively; Fig. 1). Replacing these conserved cysteines of NifH impairs its function (Howard *et al*., 1989). We posited that the Cys95 and Cys129 of the ChlL have a similar role in the ability of the ChlL to participate in nitrogen fixation reaction. Therefore we generated ChlL mutants Cys95Thr and Cys129Thr. In both mutants a TGT to ACC conversion was made that resulted in the codon change TGT (Cys) to ACC (Thr) and created a new *PinA*1 restriction enzyme site that facilitated identification of these mutants. Locations of the mutations were confirmed by nucleotide sequencing. As shown in Table 1, neither of the mutant *chlL* genes was able to support the growth of *nifM*$^-$ NifH-deficient *A. vinelandii* BG98 on BN^- medium.



(A)



(B)

Fig. 5: (A) Comparison of the amino acid sequence of the NifH-ChlL chimera with the NifH and the ChlL. Conserved proline residues are marked by the open box. The C-terminal region of the NifH-ChlL chimera that corresponds to that of the ChlL peptide is highlighted in yellow. (B) Growth of the NifH-deficient *nifM*$^-$ *A. vinelandii* strain BG98 carrying the parental plasmid pBG1380 (marked 2) and pBG1380-derivative expressing the nifH-chlL chimeric gene (marked 3) on BN$^-$ medium is shown. Wild type *A. vinelandii* (marked 1) served as control. Thus, the NifH-ChlL chimera could support growth of *nifM*$^-$ *A. vinelandii* strain BG98 in nitrogen limiting conditions indicating that replacement of the C-terminal region of the NifH with that of the ChlL resulted in partial relief from NifM-dependence. Experiments were repeated at least six times

These observations suggested that the Cys95 and Cys129 of the ChlL could play roles analogous to that of Cys97 and Cys132 of NifH in stabilizing the [4Fe-4S] cluster of ChlL.

A *nifH-chlL* chimera could render Nif$^+$ phenotype to *A. vinelandii* BG98: As shown in Fig. 1 and 3, the C-termini of the NifH and the ChlL are highly dissimilar. Therefore, the fact that NifH is not functional in the absence of NifM while ChlL is not functional in the presence of NifM might be traced to this region. We have shown previously that the Pro258 located in the C-terminus of the NifH is one of the substrates for the PPIase activity of NifM (Gavini *et al*., 2006).

Because the C-terminus of the ChlL is dissimilar to that of the NifH and the ChlL could substitute the NifH in the absence of the NifM in nitrogen fixation, it is conceivable that the C-terminus of the ChlL would render NifM-independence to the NifH. To test this idea, we analyzed the effect of replacing the C-terminus of NifH with that of ChlL. The DNA encoding the C-terminal region of NifH (bp745-873) was replaced by the DNA encoding C-terminal region of ChlL (bp718-882) to construct the *nifH-chlL* chimeric gene. Therefore, the resulting NifH-ChlL chimera did not contain Pro258 of the NifH. Amino acid sequence of the NifH-ChlL chimera is shown in Fig. 5a. *A. vinelandii* BG98 transformants expressing the *nifH-chlL* chimeric gene were capable of growing on BN$^-$ medium (Fig. 5b). Thus, the *nifH-chlL* chimera could render partial NifM-independence to *A. vinelandii* BG98.

CONCLUSION

Our results show that the NifM, a NifH-specific PPIase that is essential for biogenesis of the NifH protein, has a role in disabling structurally similar ChlL from participating in the biological nitrogen fixation reaction. Significance of PPIase-substrate interactions are particularly highlighted in many pathological conditions. For example, overexpression of human Pin1 is implicated in the formation of Lewy bodies in Parkinson's Disease, while the same protein has a beneficial effect in Alzheimer's disease, since it regulates amyloid precursor protein processing and amyloid beta production (Pastorino *et al*., 2006; Ryo *et al*., 2006). Similarly, Macrophage Infectivity Potentiators (MIPs) are PPIases expressed by bacterial pathogens, however, they interact with host-cell proteins and alter their functions to establish infection (Kohler *et al*., 2003). These examples show that the molecular interactions between PPIases and proteins that share structural similarity to their natural substrates result in pathogenesis. The example that has emerged from this study is that of a PPIase which could have contributed to the functional divergence of two fundamental biological processes (nitrogen fixation and photosynthesis) during evolution. This is because this PPIase prototype (NifM) would render functionality to one substrate (NifH) and hinder the function of the other structurally similar substrate (ChlL) so that

nitrogen fixation is favored under conditions that lead to NifM expression (such as nitrogen limitation). These findings represent a unique example of an accessory protein playing a vital part in the evolutionary divergence of biological processes.

The observation that ChlL mutants Cys 95Thr and Cys129Thr were unable to substitute for NifH further extends the structure-function similarity of the NifH and ChlL related to their mechanistic involvement in nitrogen fixation. Although the structure of ChlL is not yet solved, these observations strengthen the similarities in the role of Cys ligands of the (4Fe-4S) cluster of ChlL in electron transfer by the ChlL to that of the NifH. On the other hand, our studies also highlight the dissimilarity of the C-termini of the NifH and the ChlL. The C-terminus of the NifH is involved in the NifM-dependence of the NifH due to the presence of Pro258 (Gavini *et al*., 2002). In contrast, the C-terminus of the ChlL could render NifM-independence to the NifH as shown by the functional NIfH-ChlL chimera (Fig. 5). It is conceivable that a protein similar to the NifH-ChlL chimera could have served as a common ancestor for the NifH and the ChlL before the functional divergence of biological nitrogen fixation and photosynthesis during evolution.

ACKNOWLEDGMENT

We thank members of Gavini/Pulakat laboratories for valuable comments and technical help. Funding for this research was provided by NSF grant # MCB-1041718 to LP.

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