

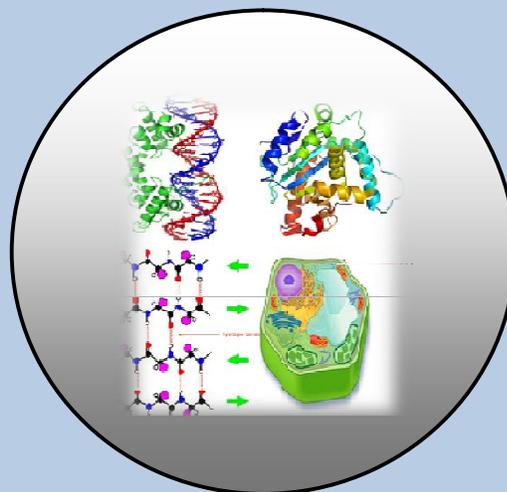
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ISSN 0970-4973 (Print)

ISSN 2319-3077 (Online/Electronic)

J. Biol. Chem. Research  
Volume 31 (1) 2014 Pages No. 192-201



**Journal of  
Biological and  
Chemical Research**

(An International Journal of Life Sciences and Chemistry)

Published by Society for Advancement of Sciences®



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REVIEW ARTICLE

Received: 13/10/2013

Revised: 21/11/2013

Accepted: 03/01/2014

# Relationship between Photosynthesis and Nitrogen Fixation

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### ABSTRACT

*A Rhodobacter capsulatus reporter strain, carrying a constitutively expressed nifA gene and a nifH-lacZ gene fusion, was used for random transposon Tn5 mutagenesis to search for genes required for the NtrC-independent ammonium repression of NifA activity. A mutation in hvrA, which is known to be involved in low-light activation of the photosynthetic apparatus, released both ammonium and oxygen control of nifH expression in this reporter strain, demonstrating a regulatory link of nitrogen fixation and photosynthesis via HvrA. In addition, a significant increase in bacteriochlorophyll a (BChl<sub>a</sub>) content was found in cells under nitrogen-fixing conditions. HvrA was not involved in this up-regulation of B Chl a. Instead, the presence of active nitrogenase seemed to be sufficient for this process, since no increase in B Chl a content was observed in different nif mutants.*

**Key words:** Nitrogen Fixation, Nif Mutants and B Chl a.

### INTRODUCTION

Under anaerobic conditions in the light, the phototrophic purple bacterium *Rhodobacter capsulatus* is able to fix atmospheric dinitrogen. A large number of genes involved in synthesis and regulation of the molybdenum nitrogenase and the alternative, heterometal-free nitrogenase have been identified and characterized in detail (Masepohl and Klipp, 1996). Both nitrogenase systems are only expressed under conditions of ammonium depletion, and a regulatory cascade resembling the general nitrogen regulation (*ntr*) system of enteric bacteria has been proposed for *R. capsulatus* (Foster et. al., 1993, Hubner et. al. 1994, Kranz and Foster, 1990 and Kranz et al. 1990). Homologs of NtrB (NifR2), NtrC (NifR1), and GlnB (NifR5) are responsible for sensing and responding to the intracellular concentration of fixed nitrogen.

Under nitrogen limitation the phosphorylated form of NtrC activates the transcription of a number of genes, including *nifA1/nifA2* and *anfA*, which encode the transcriptional activators of the molybdenum and the alternative nitrogenase, respectively. However, in contrast to all known members of the NtrC family, *R. capsulatus* NtrC does not activate its target genes in concert with the RNA polymerase containing  $\zeta^{54}$  (Foster et. al. 1994, Foster and Kranz, 1992, Hubner et. al. 1993, Kranz and Foster, 1990). The *R. capsulatus rpoN (nifR4)* gene encoding  $\zeta^{54}$  is part of the nitrogen fixation regulon and forms an autoregulatory circuit activated by NifA and AnfA, respectively (Cullen et. al. 1994 and Masepohl and Klipp, 1996). These transcriptional activators in turn stimulate the expression of all other nitrogen fixation genes in a  $\zeta^{54}$ -dependent manner. However, nitrogen fixation in *R. capsulatus* is not only regulated at the transcriptional level but also at the posttranslational level. In response to ammonium and darkness, the *draTG* gene products control the activity of dinitrogenase reductase of both nitrogenase systems by reversible, covalent ADP-ribosylation (Masepohl et. al. 1998). In addition, the NifA-dependent transcriptional activation is negatively regulated at the posttranslational level by ammonium in an NtrC-independent manner (Hubner et. al. 1993). In *Klebsiella pneumoniae* and *Azotobacter vinelandii*, an additional regulatory protein, NifL, is responsible for modulation of NifA activity in response to oxygen and fixed nitrogen. However, there is no evidence for a *nifL*-like gene in *R. capsulatus* (Masepohl et. al. 1996). In addition, the domain structure of *R. capsulatus* NifA differs from NifA of *K. pneumoniae* and *A. vinelandii* (Masepohl et al., 1988) and corresponds to the rhizobial type of oxygen-sensitive NifA proteins (Fischer, 1994), which in general seem to be independent of regulation by NifL homologs. In phototrophic bacteria the high energy demand of the nitrogen fixation process can be fulfilled by photosynthesis. Genes encoding the reaction center, the light-harvesting complex and proteins involved in synthesis of the corresponding pigments, are only expressed under anaerobic conditions. In addition, synthesis of the photosynthetic apparatus is regulated by light intensity (Bauer, 1995). Regulatory genes involved in this dual control are clustered in one region of the chromosome and include a two-component regulatory system (*regA-regB*) responsible for oxygen regulation (Joshi and Tabita, 1996, Mosley et. al. 1994, Qian and Tabita, 1996 and Sganga and Bauer, 1992) and two genes (*hvrA* and *hvrB*) necessary for low-light activation (Buggy et. al., 1994 and Buggy and Bauer, 1994). Recently, the *regA-regB* systems in *Rhodospirillum rubrum* and *Rhodobacter sphaeroides* were shown not only to regulate photosystem biosynthesis and transcription of genes required for CO<sub>2</sub> fixation but also to control the ability of these bacteria to grow under nitrogen-fixing conditions. (Joshi and Tabita, 1996.) In this paper we present evidence that HvrA, which was previously shown to be responsible for light regulation of the photosynthetic apparatus, is also involved in the NtrC-independent ammonium control of *nif* gene expression. The observed increase of the bacteriochlorophyll *a* (BChl*a*) content under conditions of nitrogen fixation further corroborates the hypothesis of a regulatory link of photosynthesis and nitrogen fixation.

### Identification of an *R. capsulatus* gene (*hvrA*) involved in NtrC-independent ammonium regulation of *nif* gene expression.

Contrary to earlier models of *nif* gene regulation, constitutive expression of *nifA1* did not result in ammonium-independent expression of a *nifH-lacZ* reporter gene fusion in *R. capsulatus* (Hubner et. al. 1993). This repression by high ammonium concentrations was not affected by mutations in *ntrC* or *glnB* (Hubner et. al. 1993). However, constitutively expressed *nifA* genes from *K. pneumoniae* and *Rhizobium meliloti* complemented an *R. capsulatus nifA1 nifA2* double mutant, but neither of the heterologous *nifA* gene products was affected by ammonium, indicating that the ammonium sensitivity is a specific property of *R. capsulatus* NifA (Masepohl and Klipp, 1996).

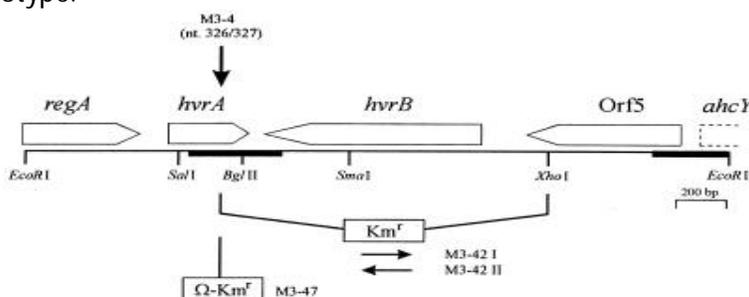
To identify genes involved in ammonium repression of NifA activity, we performed a random transposon Tn5 mutagenesis (Klipp et. al. 1988) of *R. capsulatus* W491/R3721 (a derivative of *R. capsulatus* B10S (Klipp et. al. 1988) carrying a constitutively expressed *nifA1* gene [*nifA1*(Con)] and a chromosomally integrated *nifH-lacZYA* reporter gene fusion (Bauer, 1995). Tn5-induced mutants (kanamycin resistant) were screened for the ability to form deep blue colonies in the presence of ammonium on solid RCV medium supplemented with X-Gal (5-bromo-4-chloro-3-indolyl- $\beta$ -d-galactopyranoside) under anaerobic phototrophic growth conditions. Expression of the *nifH-lacZ* fusion in mutant strains identified by this procedure was quantified by measuring  $\beta$ -galactosidase activity of cells grown in liquid RCV medium containing either ammonium or serine as nitrogen source. Among 70,000 kanamycin-resistant mutants, three strains (M3-4, M7-1, and M8-20) were identified, which showed significant expression of the *nifH-lacZ* fusion in the presence of ammonium (Table 1.)

Tn5-containing *EcoRI* fragments from these mutant strains were cloned and analyzed by DNA sequencing. Tn5 insertions in mutant strains M7-1 and M8-20 mapped within the *nifH* promoter region at distances of 52 and 55 bp upstream of the *nifH* start codon, respectively. It seemed reasonable that insertion of Tn5 at these positions created promoters constitutively expressing the *nifH-lacZ* fusion. In general, Tn5 insertions result in polar mutations, but nonpolar Tn5 insertions have also been described for the *nifR3-R2-R1* (*nifR3-ntrBC*) gene region (Jones et. al. 1989). Since promoter sequences are not yet well defined in *R. capsulatus*, these artificial promoters created by fusion of Tn5 and target sequences in the *nifH* promoter region were not obvious from the DNA sequence.

**Table 1. *R. capsulatus* Tn5 mutants showing an increased expression of *nifH-lacZ* in the presence of ammonium.**

Strain	Localization of Tn5 <sup>a</sup>	Size of cloned <i>EcoRI</i> fragment <sup>b</sup>	$\beta$ -Galactosidase activity <sup>c</sup>	
			NH <sub>4</sub> <sup>+</sup>	Serine
Parental strain <sup>d</sup>			30	1200
M3-4	<i>hvrA</i> (nt. 326/327)	2.8 kb	680	1980
M7-1	<i>P<sub>nifH</sub></i> (nt. 1237/1238)	9.8 kb	1150	900
M8-20	<i>P<sub>nifH</sub></i> (nt. 1234/1235)	9.8 kb	790	640

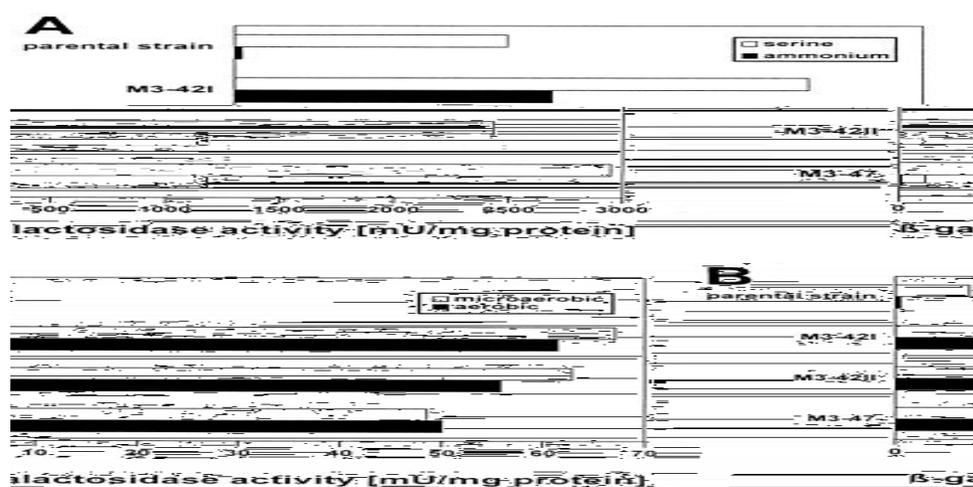
As shown in Fig. 1, the Tn5 insertion in M3-4 was localized within the *hvrA* gene, which is part of a regulatory gene cluster involved in the oxygen and light regulation of the photosynthetic apparatus (Buggy et. al. 1994). To ensure that the phenotype observed in M3-4 did not result from a secondary mutation, we reconstructed the *hvrA* mutation in the background of the parental strain W491/R372I. For this purpose, mutant strains M3-47, M3-42I, and M3-42II were constructed (Fig 1.). For the construction of M3-47, the Tn5-containing *EcoRI* fragment from mutant M3-4 was first cloned into the mobilizable plasmid pWKR102A (Colonna et. al., 1990) prior to replacement of the Tn5-internal 3.4-kb *HindIII* fragment by a 2-kb *HindIII* fragment carrying a  $\Omega$ -Km cassette. The resulting hybrid plasmid was introduced into the parental strain W491/R372I, and marker rescue resulted in M3-47, carrying the  $\Omega$ -Km cassette at the original site of Tn5 insertion. Mutant strains M3-42I and M3-42II, carrying a deletion encompassing the 3' end of *hvrA*, the *hvrB* gene, and part of Orf5, were constructed in a similar manner. Mutants M3-42I and M3-42II are distinguished by the orientation of the Km resistance gene. Correct homogenotization of interposons was verified in each case by Southern hybridization (data not shown). As found for the original Tn5 mutant, the reconstructed interposon mutant strains M3-47, M3-42I, and M3-42II formed deep blue colonies on X-Gal plates in the presence of ammonium (data not shown), corroborating the conclusion that the mutation of *hvrA* indeed was responsible for the observed phenotype.



Organization of regulatory photosynthesis genes in *R. capsulatus* and localization of mutations affecting NifA-dependent expression of *nif* genes. The physical and genetic organization of the photosynthesis gene cluster has been described previously (Buggy et. al. 1994.)

#### Disruption of *hvrA* leads to *nifH* expression in the presence of ammonium and oxygen.

For a more detailed study of the ammonium regulation of *nifH-lacZ* expression in mutant strains M3-47, M3-42I, and M3-42II, in comparison to the parental strain W491/R372I [*nifA*(Con) *nifH-lacZYA*], all strains were grown phototrophically in the presence of either ammonium or serine as nitrogen source under anaerobic conditions. In contrast to the parental strain, which showed little *nifH* expression in the presence of ammonium, the mutants strongly expressed the *nifH-lacZ* fusion under these conditions (Fig. 2A), and the levels of  $\beta$ -galactosidase activities were in the range of the maximum activity obtained with the parental strain under nitrogenase-derepressing conditions (serine as nitrogen source (Masepohl et. al., 1988) Moreover, in the presence of serine, the mutant strains showed about two times greater  $\beta$ -galactosidase activities than the parental strain.



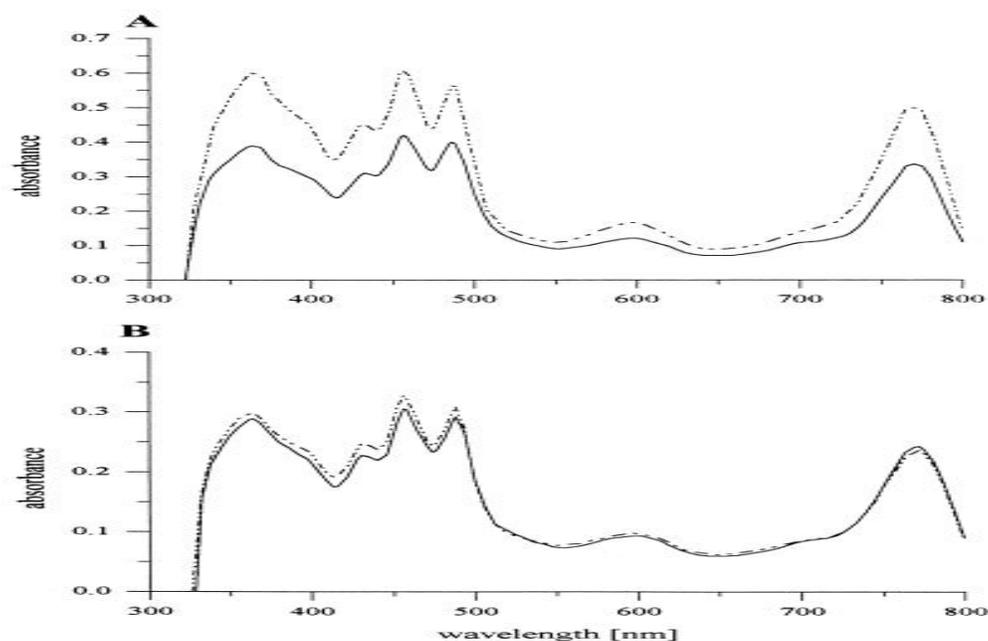
**Fig 2.** Influence of *hvrA* mutations on NifA-dependent expression of *nifH-lacZ*. Mutant strains M3-42I, M3-42II, and M3-47 contain the *hvrA* insertion or deletion mutations outlined in the legend to Fig. 1, respectively, in the background of the parental ...

Since *R. capsulatus* NifA belongs to the class of interdomain linker-containing NifA proteins, which were shown to be regulated by oxygen (Fischer, 1994), the effect of *hvrA* mutations on this regulation was studied (Fig.2 B). All strains were cultivated in RCV medium containing serine under either aerobic or microaerobic conditions. Aerobic growth was achieved by growing 20-ml cultures in 500-ml flasks with shaking (150 rpm). For growth under microaerobic conditions, 70-ml cultures were incubated in 100-ml flasks with shaking (150 rpm). After aerobic or microaerobic growth only negligible  $\beta$ -galactosidase activities were detected for the parental strain W491/R372I. In contrast, all mutant strains expressed the *nifH-lacZ* fusion in the presence of oxygen. No significant differences in the level of  $\beta$ -galactosidase activities could be observed between high- and low-oxygen cultures (Fig. (Fig.2 B). Whereas strictly anaerobic conditions resulted in a further, about 40-fold increase of *nifH* expression (Fig. 2, A).

No significant differences in ammonium or oxygen control between the *hvrA* insertion mutant M3-47 and the *hvrA-hvrB*-Orf5 deletion mutants M3-42I and M3-42II were obvious, indicating that neither *hvrB* nor Orf5 contributed to the observed phenotype. In conclusion, HvrA is not only involved in low-light activation of the photosynthetic apparatus (3) but also influences the modulation of NifA activity by oxygen and ammonium.

#### **Induction of active nitrogenase is paralleled by an increase in pigment synthesis.**

To fulfill the high energy demand imposed by nitrogen fixation, photosynthetic bacteria can use photosynthesis to create ATP and low potential redox equivalents. This energetic link between photosynthesis and nitrogen fixation was reflected by a significantly increased pigment content found in nitrogen-fixing cultures of *R. capsulatus* wild type B10S compared to ammonium-grown cells (Fig. 3A). In contrast, mutant strain M3-47 [*nifA*(Con) *nifH-lacZ* *hvrA*:: $\Omega$ -Km] lacked the ability to increase photopigment synthesis under nitrogenase-derepressing conditions (Fig. 3 B).



**Fig 3. Spectral analysis of photopigments of *R. capsulatus* wild-type B10S (A) and mutant strain M3-47 (B). M3-47 contains the *hvrA* mutation outlined in the legend to Fig. 1, in the background of strain W491/R372I [*nifA*(Con) *nifH-lacZYA*]. ...**

To determine whether the observed differences in phenotypes were a consequence of *hvrA* disruption or constitutive expression of *nifA* or were due to lack of an active, energy-consuming nitrogenase, we measured BChla content and nitrogenase activities of mutant strains affected in each of the corresponding genes (Table, 2). Neither a single *hvrA* mutation (B10S-47) nor a constitutively expressed *nifA* gene (W491) affected the increase of BChla content under nitrogenase-derepressing conditions compared to nitrogen-replete conditions. In contrast, *R. capsulatus* R372I carrying a chromosomal *nifH-lacZ* fusion showed no increase in BChla content. Since the integration of the *nifH-lacZ* fusion into the chromosome abolished the expression of *nifHDK* and thus prevents synthesis of nitrogenase apoproteins, it was tempting to assume that the lack of active nitrogenase was the major reason for the observed phenotype. To test this hypothesis, strains carrying lesions in *nifR1* and *nifR4* encoding NtrC and  $\zeta^{54}$ , two major regulatory components necessary for *nif* gene expression, were analyzed. In addition, a *nifQ* mutant strain, which is able to synthesize active nitrogenase only in the presence of 1 mM  $\text{Na}_2\text{MoO}_4$  (Moreno et al. 1989), was tested. As shown in Table, 2, the ability to synthesize active nitrogenase was directly paralleled with the increase of BChla content, whereas HvrA did not influence this regulation. These results indicated that the high demand for ATP and/or redox equivalents of nitrogenase, which are necessary to reduce nitrogen to ammonia, are involved in the up-regulation of the photosynthetic apparatus.

**Table 2. B Chl *a* content and nitrogenase activities of *R. capsulatus* wild type and mutant strains.**

Strain	Relevant genotype	BChl <i>a</i> content <sup>a</sup>		Nitro- genase activity <sup>b</sup>
		NH <sub>4</sub> <sup>+</sup>	Serine	
B10S	Wild type	13.0 ± 1.4	24.0 ± 2.3	66
B10S + Mo <sup>c</sup>	Wild type	10.0 ± 1.7	18.0 ± 0.5	40
M3-47	<i>nifA1</i> (Con) <i>nifH-lacZYA hvrA::Ω-Km</i>	13.4 ± 1.7	8.9 ± 1.1	0
B10S-47	<i>hvrA::Ω-Km</i>	17.1 ± 1.2	28.0 ± 1.0	84
W49I	<i>nifA1</i> (Con)	12.0 ± 2.0	28.0 ± 1.0	64
R372I	<i>nifH-lacZYA</i>	13.8 ± 0.2	15.0 ± 0.5	0
W49I/R372I	<i>nifA1</i> (Con) <i>nifH-lacZYA</i>	9.7 ± 0.7	8.0 ± 1.0	0
PBK2I	<i>nifR1::Ω-Km</i>	17.4 ± 0.8	15.4 ± 2.2	0
KSHIAII	<i>nifR4::Km</i>	10.2 ± 0.4	9.5 ± 0.1	0
R477I	<i>nifQ::Km</i>	8.3 ± 1.3	8.9 ± 0.8	2
R477I + Mo <sup>c</sup>	<i>nifQ::Km</i>	7.9 ± 1.3	14.5 ± 1.2	56

It is worth noting that the *hvrA* mutant strain B10S-47 exhibited an increased specific nitrogenase activity compared to the wild type (Table, 2). This would be in line with the finding that *nifH* expression was also significantly increased under nitrogenase-derepressing conditions in *hvrA* mutant strains (Fig. 2). These results indicate that HvrA might directly or indirectly act as a general negative effector of the nitrogen fixation process.

## CONCLUSIONS

Disruption of the *hvrA* gene allowed NifA-dependent *nif* gene expression in the presence of ammonium and oxygen, but an *hvrA* mutation did not affect photosynthetic growth or nitrogenase activity under the high-light conditions used in this study. Therefore, it is unlikely that the observed phenotype was due to altered transport of ammonium or to a reduced photosynthetic capacity resulting in an altered energy charge of the cells. One might speculate that HvrA modulates the activity of a not yet identified signal-transducing cascade involved in the posttranslational regulation of NifA. Since HvrA belongs to the class of histone-like, DNA-binding proteins, it seems more likely that HvrA either affects binding of NifA to its upstream activator sequence or influences DNA bending between the upstream activator sequence and the  $\zeta^{54}$ -RNA polymerase binding site. However, since it is known that histone-like proteins are involved in the regulation of a large number of genes in other bacterial species, it could not be excluded that indirect effects are responsible for the observed phenotypes.

## ACKNOWLEDGMENTS

This work was supported by financial grants from Semnan University Iran.

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