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Minireview

Nitrogen fixation genes involved in the *Bradyrhizobium japonicum*–soybean symbiosis

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The symbiotic nitrogen fixation genes (*nif, fix*) of *Bradyrhizobium japonicum*, the root nodule endosymbiont of soybean, are organized in at least two separate chromosomal gene clusters. These genes code for proteins of the nitrogenase complex, for proteins involved in their assembly with cofactors and for putative electron transport functions. One gene, *nifA*, codes for a transcriptional regulatory protein that plays a central role in the control of expression of the other genes in response to the cellular oxygen status. Only at low partial pressures of O_2 will the target promoters be activated by NifA.

Gene regulation; Nitrogenase; Nitrogen fixation; Symbiosis; *Bradyrhizobium japonicum*

1. INTRODUCTION

Bradyrhizobium japonicum is a Gram-negative, aerobic soil bacterium capable of inducing the formation of root nodules on its host plant, soybean (*Glycine max* L. Merr.). Within the infected nodule cell *B. japonicum* lives as a true endosymbiont. In this state, the bacterial cells are called bacteroids. Bacteroid metabolism heavily depends on the supply by appropriate, plant-derived nutrients. Bacteroids take up carbon sources of plant origin, for example, and oxidize them using a bacteroid-specific respiratory chain that ingeniously functions with the very low oxygen concentration (in the nanomolar range) prevailing in the infected nodule cells. This condition also favors the induction of the nitrogenase system that catalyzes the reduction of molecular dinitrogen (N_2) to NH_4^+. The major amount of this product is then exported from the bacteroids and assimilated by cytosolic plant enzymes.

In *B. japonicum* and all other diazotrophic bacteria studied to date, the nitrogenase complex consists of two components. Component I carries the active site at which N_2 reduction occurs. It is an $\alpha_2\beta_2$ tetramer containing four [4Fe:4S] clusters, a specific cofactor and additional metal centers. Usually, the cofactor has the composition $MoFe_{6-8}S_{7-9}$ and thus contains molybdenum. Under molybdenum deficiency, however, some diazotrophs may induce the formation of one or two alternative nitrogenases, one of which carries a vanadium-containing cofactor. *Azotobacter vinelandii*, a free-living diazotroph, has been the first example of a bacterium in which alternative nitrogenases were found [1]. Nitrogenase component II, a dimeric protein complexed with one [4Fe:4S] cluster, functions enzymatically as an oxidoreductase accepting electrons from a ferredoxin or flavodoxin and transferring them to component I. This reaction is energy-demanding in that 2 ATP are hydrolyzed per one electron transferred. The ferredoxin or flavodoxin, in turn, obtains electrons either from a fermentation end product such as pyruvate (in anaerobes) or (in aerobes, to which *B. japonicum* belongs) probably from NAD(P)H; consequently, additional nitrogen fixation-specific proteins exist, either a pyruvate-flavodoxin oxidoreductase or a putative NAD(P)H dehydrogenase, respectively [2].

With the given complexity of the nitrogenase system it is conceivable that each diazotrophic bacterium possesses a substantial number of genes involved in determining the N_2 fixation process. Apart from genes for the constituent nitrogenase polypeptides, there are genes encoding the electron transport proteins and proteins concerned with the biosynthesis and incorporation of iron-sulfur cluster and metal cofactors. To date, extensive research is devoted to studying nitrogen fixation genes from several free-living bacteria (e.g. *Klebsiella pneumoniae, Azotobacter* spp., *Rhodobacter cap-*

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sulatus, Anabaena variabilis) as well as from several symbiotic rhizobia (e.g. *Rhizobium meliloti, Bradyrhizobium japonicum, Azorhizobium caulinodans*). This article reviews the current knowledge on the organization, function and regulation of the *nif* and *fix* genes in *B. japonicum*. The term '*nif* genes' refers to genes that are structurally and functionally homologous to the 20 known *nif* genes in *K. pneumoniae* [3] in which they were first found, whereas the '*fix* gene' terminology denotes genes first discovered to be essential for symbiotic N_2 fixation (usually in rhizobial species). At present, the term '*fix* gene' is still used in the widest possible sense, including genes determining essential functions in bacteroid development and metabolism. Many of them will probably be renamed once the precise biochemical functions of their products have become known. For example, some *B. japonicum fix* genes previously identified in our laboratory have turned out to encode cytochromes of the respiratory chain [4]. Genes of that kind are not the subject of this paper.

2. ORGANIZATION AND FUNCTION

B. japonicum nif and *fix* genes are located in at least two separate chromosomal gene clusters (Fig. 1). The distance between the two clusters is not known. Most of the genes are accumulated in cluster I. Three other *nif* and *fix* genes map in cluster II, closely adjacent to at least 10 nodulation (*nod*) genes which are required for functions in the very early steps of root nodule formation; the *nod* genes will not be discussed here. In addition to the identified *nif* and *fix* genes, a few open reading frames have been detected whose functions are unclear (see below): these are numbered in Fig. 1. Many, but not all, *nif* and *fix* genes are grouped in operons, i.e. they form parts of multicistronic transcription units: *nifDKENX, ORF118ORF73nifS, nifB-frxA, nifH, ORF35fixBCX, fixRnifA, fixA* (see Fig. 1).

With regard to the functions of the *B. japonicum* genes and their corresponding products, much has been learned from studies performed with the free-living diazotrophs *K. pneumoniae* and *A. vinelandii*. The inference is made, therefore, that a conserved *nif* gene detected in any other bacterium such as *B. japonicum* plays the same role as in *K. pneumoniae*. Often, there is also additional experimental evidence which corroborates this assumption. The *nifD* and *nifK* genes are the structural genes for the α and β subunits, respectively, of the nitrogenase component I (MoFe protein or dinitrogenase) whereas *nifH* encodes the two identical subunits of component II (Fe protein or dinitrogenase reductase) [5,6]. The *nifB*, *nifE* and *nifN* genes are involved in the biosynthesis of the iron-molybdenum cofactor [7–9] (M. Aguilar, personal communication). Interestingly, *nifE* and *nifN* share a certain sequence similarity with *nifD* and *nifK*, respectively [10]. Moreover, *nifD* and *nifK* share homologies and so do *nifE* and *nifN* [10–12]. Obviously, this reflects the function of all four gene products in the binding of the FeMo cofactor or precursors of it. The *nifS* gene appears to contribute to a full activity or stability of the nitrogenase iron protein (component II) [8,13], but the biochemical function of the NifS protein is not known. Similarly, the role of the *nifX* gene, that was also found recently in *B. japonicum* (H. Hennecke, unpublished), remains obscure. In *K. pneumoniae, nifX* may play a negative regulatory role in the expression of the nitrogenase genes [14]. The *fixB*, *fixC* and *fixX* genes (in cluster I) and the *fixA* gene (in cluster II) appear to be restricted to rhizobia and other microaerobic or aerobic diazotrophs, whereas they are not present in *K. pneumoniae* [15,16]. The fact that *fixX* encodes a ferredoxin-like electron transfer protein [17] and a few other arguments [15,18] have led to the suggestion that these *fix* gene products are involved in an unknown redox reaction essential for nitrogenase activity. Another ferredoxin-like electron transfer protein is encoded by *frxA*; in *B. japonicum*, however, it is not essential for conventional nitrogenase activity [19]. A few open reading frames are also associated with the *B. japonicum nif* and *fix* genes described so far. *ORF35*

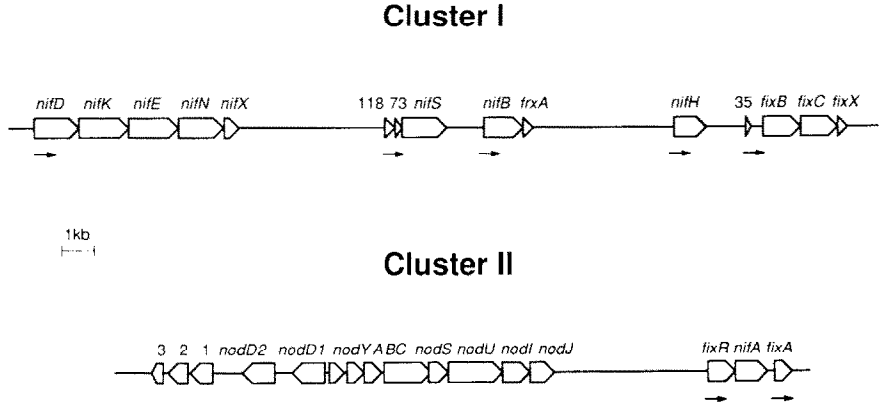

Fig. 1. Organization of the *B. japonicum nif, fix* and *nod* genes in chromosomal clusters I and II. Genes and open reading frames (numbered) are shown as open arrows. Small arrows below the map denote the identified transcription start sites.

codes for a putative polypeptide of 35 amino acids whose translation strongly contributes to the stability of the *ORF35fixBCX* mRNA [17]. Upstream of the *B. japonicum nifS* gene, *ORF118* and *ORF73* have been detected recently [20]. The predicted 118 amino acid polypeptide of *ORF118* is homologous to the product of *ORF6* present in *Azotobacter vinelandii* [21] (Fig. 2A). Although the *ORF*s in both organisms are not essential for the conventional nitrogenase activity, their strong conservation suggests that they have a function. One possibility is that they are required for alternative nitrogenases, which can be induced in *A. vinelandii*; in *B. japonicum*, however, the question of whether alternative nitrogenases are present under molybdenum starvation has not been addressed. The predicted *ORF73*-encoded protein (73 amino acids) shares little, though intriguing, sequence similarity to the *nifU* gene products of *K. pneumoniae* and *A. vinelandii* [22] (Fig. 2B). In the latter organisms the NifU proteins respectively, are 274 and 312 amino acids long and their corresponding genes are located in the same relative position as *ORF73* in *B. japonicum*, i.e. upstream of *nifS*. The precise role of *nifU* is not known. In the case of *B. japonicum*, it cannot be excluded that *ORF73* is just a non-functional, truncated evolutionary relic of a former *nifU*-like gene.

The last genes to be discussed here are the *fixR* and *nifA* genes which form an operon, *fixRnifA*. The *fixR* gene has so far only been found in *B. japonicum* [23]. Its function is not known, but the predicted FixR protein sequence shows it to be homologous to NAD-dependent dehydrogenases [24] which suggests that it is involved in an oxidation/reduction process. The *nifA* gene [25] is conserved in many N_2 fixing bacteria in which its product is a positive regulatory protein for the expression of the other *nif/fix* genes and operons (see section 3).

3. REGULATION

In *B. japonicum*, oxygen appears to be the only environmental signal that governs the expression of *nif* and *fix* genes, i.e. these are expressed only at low partial pressures of O_2 whereas they are not expressed in aerobiosis. At least two levels of control have been identified (Fig. 3): one occurs at the level of the expression of the *nifA* gene, which encodes the positive regulator for all other *nif* and *fix* genes, and the other one occurs at the level of the function of the NifA protein.

The promoters of the six NifA-activatible *B. japonicum nif/fix* operons have been mapped and sequenced: p*nifD*, p*ORF118*, p*nifB*, p*nifH*, p*ORF35*, p*fixA* (Fig. 1). All are similar to the typical $-24/-12$ consensus sequence characteristic for σ^{54}-dependent promoters [26,27]: 5′-TGGYRYR-N_4-TTGCT-3′. Upstream activator sequences (UAS) which are necessary for the enhancement of *nif/fix* gene expression by *nifA*-mediated activation [28] have been found in the 5′ flanking DNA regions of p*nifD* (two copies), p*ORF118* and p*nifB* (one copy each), and p*nifH* (two copies). They all have the consensus sequence 5′-TGT-N_{10}-ACA-3′. Similar sequences, however, have not been detected in front of p*ORF35* and p*fixA*. In spite

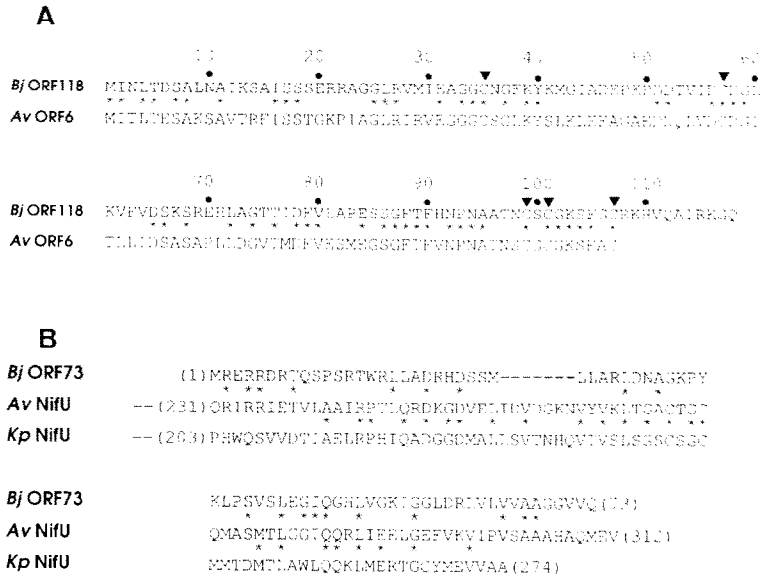

Fig. 2. Amino acid sequence alignments of the predicted *B. japonicum (Bj)* ORF118 protein with the *Azotobacter vinelandii (Av)* ORF6 protein (A), and of the ORF73 protein with the *A. vinelandii* and *Klebsiella pneumoniae (Kp)* NifU proteins (B). The *B. japonicum* sequences are taken from [20]; the corresponding nucleotide sequence has been deposited in the EMBL sequence data library under accession no. X13691. Identical amino acids are connected with asterisks. Four conserved cysteine residues in (A) are marked with arrowheads. In (B) only the homologous part of the NifU proteins is shown. References: *Av* ORF6 [21], *Kp* and *Av* NifU [22].

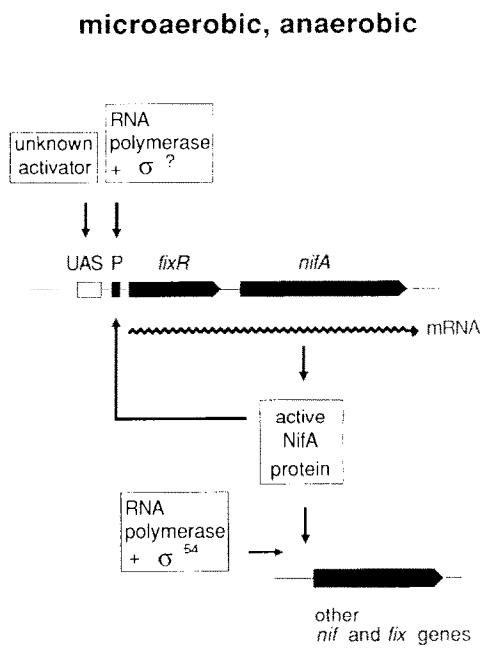

Fig. 3. Model of *nif* and *fix* gene regulation in *B. japonicum*. For a description see text. Abbreviations: P, promoter of the *fixRnifA* operon; UAS, upstream activator sequence of the *fixR* promoter.

of this, the expression of the *fixA* gene and *ORF35fixBCX* operon is strongly dependent on the presence of *nifA* [29]. The presence of two, one or no UAS of the TGT-N_{10}-ACA type in front of the promoters may be responsible for a fine-tuned regulation of expression of the genes resulting in different amounts of the respective gene products [30].

In vivo, both in the homologous *B. japonicum* as well as in the heterologous *E. coli* backgrounds, the activity of the *B. japonicum* NifA protein has been shown to be sensitive to high oxygen concentrations [31]. By contrast, the *Klebsiella pneumoniae* NifA protein per se was not inactivated by oxygen. This functional difference is reflected in structural differences between the *B. japonicum* and *K. pneumoniae* NifA proteins. The *B. japonicum* NifA protein has 4 essential cysteine residues in a domain with the following primary structure: cys-X_{11}-cys-X_{19}-cys-X_4-cys (X = any other amino acid) [32,33]. Three of these cysteines are missing in the *K. pneumoniae* NifA protein. We have proposed previously that the four-cysteine-motif could function as a metal-binding site, because the in vivo activity of the *B. japonicum* NifA protein, but not that of the *K. pneumoniae* NifA protein, was inhibited by chelating agents [32]. It is thus possible that the *B. japonicum* NifA protein is a metalloregulatory protein, in which the bound metal (perhaps Fe^{2+}) must be in the reduced form in order for NifA to be active. Higher oxygen concentrations or high (positive) redox potentials might lead to oxidation of the bound metal and, hence, to inactivation of NifA.

As mentioned, the *B. japonicum nifA* gene is part of the *fixRnifA* operon and thus dependent on transcription from the *fixR* promoter (Fig. 3). This operon is expressed at a basal level even under aerobic conditions. Under microaerobic conditions or when the cells are grown anaerobically with nitrate, the expression of the *fixRnifA* operon is increased by a factor of 5 [34] (Fig. 3). The NifA protein has been shown to autoregulate, directly or indirectly, its own expression.

The aerobic expression from the *fixR* promoter requires the presence of an upstream activator sequence located around position −66 from the transcription start site. This suggests that under aerobic conditions the *fixRnifA* operon is controlled positively [23,24]. The gene for the postulated aerobic activator has yet to be found.

In conclusion, symbiotic nitrogen fixation in *B. japonicum* is regulated by a cascade of at least two gene activation steps. In the first step the *fixRnifA* operon must be activated. Once the NifA protein is synthesized, it can activate the other known *nif/fix* operons. This second activation step is strictly dependent on low oxygen partial pressure. One of the challenging research goals in the future is to elucidate the mechanism whereby the NifA protein ultimately responds to the redox state in the cell.

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