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Review

How prokaryotic microbes fix nitrogen?

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ABSTRACT

Over the last one hundred years biological nitrogen fixation processes have been discovered and characterized exclusively in prokaryotes (*Archaea*, *Rhodobacter*, *Cyanobacteria*, *Azotobacter*, *Klebsiella*, *Pseudomonas*, *Azospirillum*, *Rhizobia*, *Paenibacillus* etc.). So far only a few species of higher plants (e.g. legumes) have been found to be able to fix atmospheric nitrogen *via* nitrogen fixing bacteria (e.g. rhizobia). In the past four decades, knowledge obtained from the research work on versatile microbes in this area has been more and more important. In this review we attempt to highlight some key aspects of various prokaryotic microbes including evolution of biological nitrogen fixation, regulation, biochemistry, nitrogenase assembly and several mechanisms required for nitrogenase protection, and related matters.

KEYWORDS: biological nitrogen fixation, diazotroph, nitrogenase, evolution, regulation

INTRODUCTION

Microbes capable of fixing atmospheric nitrogen have evolved dinitrogenases to reduce inert N_2 gas to ammonia. This process has enormous benefits for sustainable agriculture and environment. Mo-dependent nitrogenases dominate the nitrogen fixation kingdom (Fig. 1). However, alternative nitrogenases (V-dependent and Fe-dependent) have been reported as existing at least in *Archaea*, *Cyanobacteria* and *Azotobacter*. All nitrogenases are oxygen sensitive, however, microbes have developed a variety of strategies to deal with reactive oxygen, preventing the damage of such delicate protein catalysts. Nitrogen fixation is a high energy-demanding process. Therefore the expression of nitrogenase genes is tightly regulated *via* various sophisticated mechanisms. It is expected that one day its understanding in combination with innovative biotechnology may help to tackle the problems for sustainable agriculture.

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1. Nitrogen fixation in Archaea

The evolution of biological nitrogen fixation, along with the appearance of photosynthesis apparatus, could be traced back to a very early period, over 4 billion years ago, spanning almost the entire history of life on earth. In fact, these two most basic processes share common ancestry with the key catalysts: nitrogenase and DPOR [1, 2]. It is commonly known that nitrogen fixation appeared before the separation of Archaea and Eubacteria. Therefore its appearance may not be later than photosynthesis. Nitrogen fixation was discovered in Archaea, firstly in the methanogenic species (*Methanosarcina barkeri* and *Methanococcus thermolithotrophicus*) in 1984. Within the Archaea kingdom (the Euryarchaeota and the Crenarchaeota) nitrogen fixation has been found only in the methanogenic Euryarchaeota. However, within the methanogens, nitrogen fixation is widespread in all the three orders: Methanococcales, Methanomicrobiales and Methanobacteriales. Not surprisingly, nitrogen fixation in Archaea fundamentally resembles nitrogen fixation in Bacteria [3].

2. Nitrogen fixation in cyanobacteria

Cyanobacteria have inhabited much of the surface of the earth for over 2.5 billion years and are still

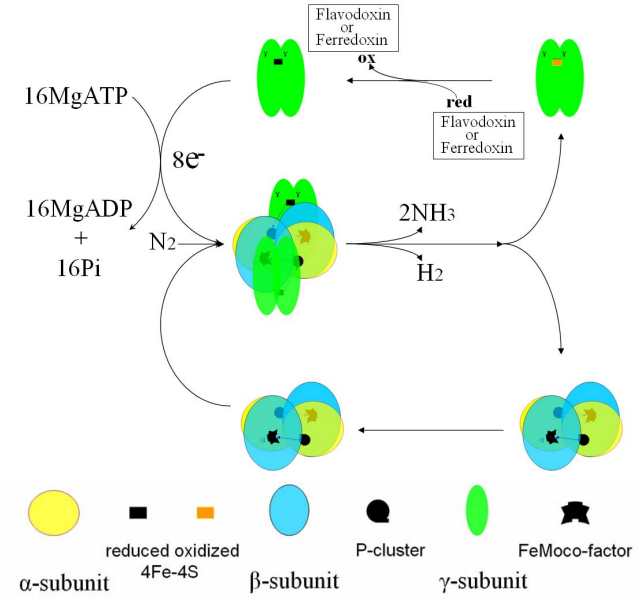

Fig. 1. The mechanism of nitrogenase turnover. The reduction of N_2 occurs on the MoFe protein ($\alpha_2\beta_2$ hetero-tetramer) in a multiple-step reaction with the Fe protein (γ_2 homo-dimer). Electron transfer was six times per N_2 molecule fixed. Nitrogenase also reduces protons to H_2, consuming two electrons. The total cost of N_2 reduction is therefore 16 MgATPs hydrolyzed and 8 electrons transferred [2].

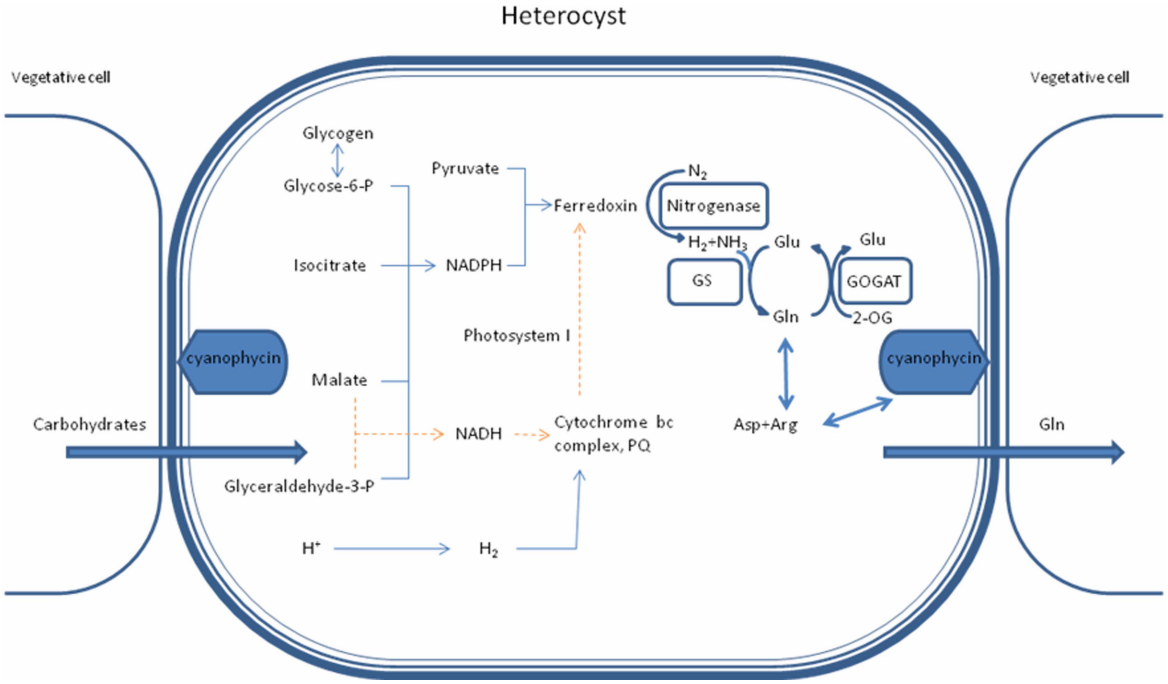

Fig. 2. Heterocyst metabolism and nitrogen fixation. The scheme of a heterocyst with adjacent vegetative cells is shown. Heterocysts import carbohydrates from vegetative cells, with glutamine moving in the opposite direction. In heterocysts, in the light, ferredoxin can be reduced via photosystem I. Alternatively, either NAD(P)H and a dehydrogenase or H_2 and uptake hydrogenase can feed in electrons at the plastoquinone site (or close to it). In darkness, ferredoxin can be reduced by NAD(P)H and NAD(P)H:ferredoxin oxidoreductase (FNR) present in heterocysts and vegetative cells. The reduction of ferredoxin can also be achieved by the pyruvate phosphoroclastic reaction. The ammounium generated by nitrogenase could be assimilated to glutamine by the GS (glutamine synthetase)/GOGAT (glutamate synthase) pathway. Alternatively, glutamine may be converted to arginine which is then incorporated into the cyanophycin granule. This may be degraded by cyanophycinase in a dynamic way depending on the N demand of heterocysts and vegetative cells.

responsible for a significant proportion of the biological fixation of nitrogen [4, 5]. Ancient cyanobacteria were apparently the first oxygenic photosynthetic organisms capable of utilizing water as the ultimate source of electrons for the generation of reductant in photosynthesis. Oxygenic photosynthesis, as evolved in cyanobacteria and evidently inherited by green plants, is the most important mechanism for capturing solar energy on Earth [6]. The existence, in many species of cyanobacteria, of two conflicting metabolic systems, oxygen-evolving photosynthesis and oxygen-sensitive nitrogen fixation, used to be a puzzling paradox. However, studies in both heterocystous and nonheterocystous cyanobacteria have shown that various strategies have been evolved in these organisms to reconcile the incompatibility between nitrogen fixation and oxygenic photosynthesis.

2.1. Heterocystous cyanobacteria

Heterocysts are complex cellular systems produced by the transformation of vegetative cells in cyanobacteria belonging to group sections IV and V and comprising the genera *Anabaena*, *Nodularia*, *Cylindrospermum*, *Nostoc*, *Scytonema*, *Calothrix*, *Fischerella* and *Chlorogloeopsis*. Heterocysts provide a finely regulated heterocystous anaerobic micro-environment for the efficient function and protection of nitrogenase. Heterocyst development results in the distinct spatial separation of the two contrasting metabolic activities of oxygenic photosynthesis and oxygen-sensitive nitrogen fixation. In heterocysts PSII is degraded. PSI remains active and ATP is produced by cyclic photophosphorylation [7, 8, 9]. ATP can also be produced by oxidative phosphorylation, a process that consumes oxygen. Loss of PSII usually means loss of the ability to reduce $NADP^+$ to NADPH photochemically. That indeed occurs in heterocysts. However, an alternate source of NADPH is made available by importing carbohydrates from neighboring vegetative cells [10, 11, 12, 13]. This material, which is still unidentified but possibly a disaccharide such as sucrose or maltose, is converted to glucose-6-phosphate, 6-phosphogluconate and ribose-5-phosphate, producing NADPH in the process [14, 15, 16, 17]. It has been suggested that NADPH provides electrons for N_2 reduction, reducing a special ferredoxin by way of nucleotide-ferredoxin oxidoreductase [5] (Fig. 2). Heterocysts have all the components of an electron transport chain, from reduced pyridine nucleotide to oxygen [18, 19, 20]. Even though N_2 fixation in heterocyst-forming cyanobacteria is dependent largely on the energy and reducing power, generated in light reactions, the nitrogenase reaction *per se* is independent of light [2, 21]. Several strains possess the ability to utilize organic substrates (mainly sugars) by heterotrophic metabolism enabling them to grow and to fix N_2, albeit slowly, in the dark [22, 23, 24]. Even in obligate photoautotrophs, such as *A. cylindrica*, N_2 fixation will continue in the dark until endogenous carbon reserves, produced during the previous period of photosynthesis, are exhausted [25]. Eventually the ammonia produced by nitrogenase is assimilated by reaction with glutamate to yield glutamine (using glutamine synthetase/GOGAT).

2.2. Nonheterocystous cyanobacteria

In nonheterocystous cyanobacteria, a variety of strategies that function more or less efficiently (alone or in combination) to protect the nitrogenase complex against both exogenous (atmospheric) and endogenous (photosynthetic) sources of oxygen, have been found in several nonheterocystous strains: *Gloeothece*, *Synechococcus*, *Plectonema*, *Microcoleus*, *Oscillatoria*, and *Trichodesmium*.

The first proposals regarding possible temporal separation of photosynthetic and nitrogen fixing activities were based on the discovery that these two activities peaked at different stages during growth in continuously illuminated batch cultures [26, 27, 28, 29, 30, 31]. More revealing are studies on the variations in metabolic activity in cultures grown with alternating light-dark periods under conditions that resemble the natural diurnal cycle [32, 33]. When *Gloeothece* cells were incubated under a 12 h light/12 h dark regime, significant nitrogenase activity was detected only during the dark period. This was supported by the catabolic breakdown of reserve carbohydrate accumulated in the previous light period [34, 35, 36, 37, 38, 39]. It has also been found that nitrogen fixation of *Gloeothece* sp. 68DGA, which normally is observed only in the dark phase under LD, proceeded after the dark-to-light transition when the assimilation of ammonia was inhibited by the addition of l-methionine sulfoximine.

The fast-growing (generation time, 20 h) marine *Synechococcus* strains, Miami BG43511 and

Miami BG43522, have been shown to synthesize nitrogenase under conditions of nitrogen depletion and to maintain enzyme activity during aerobic incubation [40, 41]. When synchronous growth was induced (by transfer in the dark and aeration for 20 h) and the synchronized cell material then incubated under alternating 12 h light/12 h dark cycles, metabolic activity and cell growth and division displayed regular cyclic variations. Photosynthesis peaked in the light, whereas maximum nitrogenase activity was observed in the middle of the dark period. The cell carbohydrate content was highest in the middle of the light phase. Its rapid decrease in the dark phase coincided with increased nitrogenase activity. This was thought to imply that nitrogen fixation depended on the degradation of cellular carbohydrate reserves and that energy reductant and possibly protection of nitrogenase from oxygen were provided by means of respiration. It was concluded that temporal segregation of photosynthesis and nitrogen fixation within the cell division cycle enables *Synechococcus* strains to grow on dinitrogen in the natural diurnal cycle [42, 28]. Another, rather slow-growing (generation time, 60 h) epiphytic marine isolate of the genus *Synechococcus*, designated strain SF1, seems to differ in several aspects from the Miami strains, most importantly in its inability to fix N_2 in the dark [40]. The freshwater *Synechococcus* species strain RF1, isolated from a rice field, grows slowly in culture (generation time, 60 h) and can fix N_2 aerobically both when illuminated continuously and when incubated in alternating light-dark cycles (12 h light/12 h dark) [43]. Nitrogenase activity in the latter case was always restricted to the dark period. During growth in a diurnal cycle, the synthesis of both nitrogenase and nitrogenase mRNA occurred in the dark [44, 45, 46]. This cyclic pattern of *nif* gene expression was sustained for several days when the culture was changed to continuous illumination [46, 47]. These results suggest that synthesis of nitrogenase proceeds without diurnal oscillation in the continuous light acclimated cells of *Gloeothece* sp. 68DGA. As the respiration rate in CL-acclimated culture was as high as the maximum rate observed in 12 h light/12 h dark cycles culture, oxygen-uptake mechanism(s) may have been upregulated to maintain low intracellular pO_2.

The filamentous nonheterocystous *Plectonema boryanum* is able to fix N_2 when incubated in the light under microaerobic conditions, as effected by sparging the culture suspension continuously with an oxygen-free gas mixture (N_2-CO_2) [48, 49, 50]. *P. boryanum* is able to grow heterotrophically in the dark in the presence of air and in the presence of suitable organic substrates such as glucose [51]. When sulfide was supplied at a concentration that inhibited CO_2 fixation [50], or when CO_2 was excluded from the culture [52], photoheterotrophic growth on N_2 could be sustained at the expense of organic carbon sources in the medium. Under these conditions, reductant for N_2 fixation is apparently generated by oxidative metabolism while cyclic photophosphorylation may provide the ATP requirement for nitrogenase activity [49]. Limited N_2 fixation can also take place in the dark [48] by utilizing endogenous carbon reserves sufficient to support respiratory metabolism but not to inactivate nitrogenase. Aerobic respiration could not only meet the needs of energy for reductant reaction but could also provide limited protection for nitrogenase by the control of intracellular oxygen tensions.

The filamentous organism *Microcoleus chthonoplastes* was isolated from a marine cyanobacterial mat and has been shown to reduce acetylene under aerobic conditions [53]. *M. chthonoplastes* was able to fix N_2 under ambient atmospheric conditions but reduced oxygen tensions increased its nitrogenase activity in the light. However, in the dark, complete deprivation of oxygen prevented acetylene reduction [53]. These observations and the dependence of nitrogenase activity in the dark on carbon reserves produced by photosynthesis during previous light incubation [54], imply that the energy and reductant required for N_2 fixation are generated by aerobic respiration. Since the highest rates of nitrogenase activity were measured in the light [54], it is assumed that *M. chthonoplastes* must possess adequate mechanisms to protect nitrogenase against inactivation by oxygen released during photosynthesis. The nature of these mechanisms is as yet unknown.

Investigations of N_2 fixation by the marine *Oscillatoria limosa* strain 23 have revealed several features that could be important in the survival of cyanobacteria in the specific benthic environment

of intertidal mats, from where this strain was isolated [55]. The ability of the organism to maintain nitrogenase activity in air was attributed to the existence of several protective mechanisms. Nitrogenase activity was shown to be unaffected by oxygen concentrations up to 0.15 atm (15.19 kPa), but higher oxygen tensions were inhibitory and eventually caused the inactivation of nitrogenase [56]. This was apparently because of the limited capacity of the oxygen-scavenging devices. Distinct sensitivity of nitrogenase to oxygen was indicated by the response to decreased atmospheric and endogenous oxygen tensions. Nitrogenase activity was enhanced 4.5-fold when filaments were incubated under helium, 6-fold when DCMU was added to the culture suspension, and 5-fold when cultures were transferred to the dark [55]. When *O. limosa* was grown with alternating light-dark cycles, nitrogenase activity was measurable mainly during the dark period [57]. This was thought to indicate the temporal separation of photosynthetic and nitrogen fixing activities under such conditions [56]. Changing from light to dark incubation resulted in the cessation of oxygen evolution and increased rates of respiration [57]. Reductant and ATP required for N_2 fixation were apparently generated at the expense of catabolic breakdown of reserve carbohydrate accumulated during the light period. Significant nitrogenase activity was also recorded when *O. limosa* was incubated under dark anaerobic conditions. This was considered to indicate that the requirements for N_2 fixation can be met in this organism through fermentative metabolism [58]. Field measurements of photosynthetic and nitrogenase activities in marine cyanobacterial mats, dominated by *Oscillatoria* or *Microcoleus spp.*, have indicated that temporal separation of the two metabolic process also occurs in natural populations of cyanobacteria [59, 60, 61]. However, the diurnal patterns are influenced by seasonal variations in light intensity, temperature, and dissolved-oxygen concentration [61]. Strains of gas-vacuolated marine planktonic *Oscillatoria* (*Trichodesmium*) are widely distributed in the tropical and subtropical oceans, periodically forming massive blooms under calm water conditions. Research on the physiology of *Trichodesmium spp.*, and on their remarkable ability to maintain nitrogenase activity in seawaters saturated with oxygen, has been hampered until recently by the repeated failure to isolate and grow the organism in axenic culture. Controversially, when *Trichodesmium spp*. were grown under a light-dark regime, nitrogenase activity was significant only in the light, almost disappeared in the dark, and reappeared upon exposure to light. These observations are in agreement with earlier records made with the materials collected in the field [62, 63]. Nitrogenase activity can be detected only when *Trichodesmium spp*. are grown in a medium free from combined nitrogen. The component proteins of the enzyme complex were shown to be present, in an apparently inactive form, in cells grown on nitrate or in the presence of low concentrations of ammonia, but not urea [64]. It is possible that nitrogenase activity in *Trichodesmium spp*., as in certain heterocystous cyanobacteria, is regulated and protected by the reversible modification of the Fe protein, since this modification arises upon exposure to oxygen. Axenic cultures should enable basic physiological and biochemical investigations on the possible presence of mechanisms capable of protecting nitrogenase. Light stimulation of nitrogenase activity in *Trichodesmium spp*. could stem from the direct supply of ATP and reductant generated in the photochemical reaction. Alternatively it could be the result of light-dependent oxygen consumption or photorespiration, which may play an important part in decreasing cellular oxygen tension and thus in protecting nitrogenase against inactivation by oxygen [6].

3. Alternative nitrogenases

Alternative nitrogenases that lack molybdenum were first discovered in *Azotobacter vinelandii* by Bishop and coworkers [65] and an alternative nitrogenase from *Azotobacter chroococcum* was first purified and characterized by Robson *et al.* (1986, 1989) [66, 67]. It has been demonstrated in several laboratories that alternative forms of nitrogenase component I exist which contain either vanadium (V) and Fe, or only Fe, instead of molybdenum (Mo) and Fe. The diazotroph *Azotobacter vinelandii* possesses three nitrogenases that are expressed in response to the presence or absence of Mo or V in the growth medium. Alternative nitrogenases have been found in Archaea [68] and some aerobic gram-negative

nitrogen fixing bacteria [69]: the cyanobacterium *Anabaena variabilis* [70], *Azospirillum brasilense* [71], *Azotobacter salinestris* [72], *Azotobacter paspali* [72], *Azomonas macrocyto* genes [72]; the phototrophic non-sulfur purple bacteria: *Rhodobacter capsulatus* [73, 74], *Rhodospirillum rubrum* [75, 76], *Rhodobacter sphaeroides* [77], *Rhodopseudomonas palustris* [78], *Xanthobacter autophicus* [79]; and the eubacterium: *Clostridium pasteurianum* [80] and *Methanosarcina acetivorans* [81]. It was discovered that diazotrophs with Mo-independent nitrogenases (alternative nitrogenase V and Fe-dependent) can be readily isolated from diverse natural environments [82]. Interestingly, it has been previously suggested that Mo-independent forms of nitrogenase were responsible for nitrogen fixation on early Earth because oceans were Mo-depleted and Fe-rich. However, phylogenetic and structure-based examinations of multiple nitrogenase proteins suggest that such an evolutionary path is unlikely [83]. The evolution of the nitrogenase ancestor and its associated bound metal cluster was controlled by the availability of fixed nitrogen in combination with local environmental factors. Until a point in Earth's geologic time, local environmental factors influenced metal availability where the most desired metal, Mo, became sufficiently bioavailable to bring about and refine the solution (Mo-nitrogenase) we see perpetuated in extant biology.

The *nif*-encoded dinitrogenase proteins are $\alpha_2\beta_2$ tetramers of the *nifDK* gene products, whereas the alternative dinitrogenases (*Azotobacter vinelandii*) contain a third subunit, forming a hexamer, and are encoded by the *vnfDKG* genes and the *anfDKG* genes [84, 85]. Similar to *nifH*, the gene *vnfH* encoding dinitrogenase reductase-2 is required for expression of the vanadium nitrogenase from *Azotobacter vinelandii* [86]. Interestingly, the *nifU*, *nifS* and *nifV* gene products are required for activation of all the three nitrogenases of *Azotobacter vinelandii* [87]. Two species of VnfG were observed in cell-free extracts resolved on anoxic native gels. One is composed of VnfG, associated with the VnfDK polypeptides and the other is a homodimer of the VnfG protein. VnfG is required for processing apodinitrogenase 2 to functional dinitrogenase 2 [88]. *In vivo* studies suggest that NifH supports FeV-co synthesis and that AnfH supports FeMo-co synthesis [84, 89], while *in vitro* experiments show that VnfH can replace NifH both in the biosynthesis of FeMo-co and in the maturation of apo-dinitrogenase 1 [90]. These results suggest that the dinitrogenase reductase proteins do not specify the heterometal incorporated into the cofactors of the respective nitrogenase enzymes. Little is known about the actual biosynthesis of the iron-vanadium and iron only cofactors of nitrogenase 2 and 3. Whereas, in the case of the nitrogenase structural genes, duplicated genes exist for each of the three systems. This appears to be the case for only some of the cofactor genes. The *vnfENX* genes are homologous of *nifENX* and are necessary for synthesis of the nitrogenase 2 and 3 cofactors [91]. *nifH* and *nifQ* mutants are not affected in their nitrogenase 2 and 3 activities. However, no *nifQ* counterparts have yet been found in the other systems. No counterparts appear to exist for *nifB* and *nifV*. Mutations in these genes affect all the three nitrogenase systems [92]. The involvement of NifB in alternative nitrogenase function suggests that a cofactor-specific Fe-S assembly pathway is common to all nitrogenase systems. In *Azotobacter vinelandii*, VnfA and AnfA are required for the expression of the *vnf* and *anf* genes. VnfA is involved in the repression of the *nifHDK* operon in the absence of molybdenum, and VnaA is also involved in the repression of the *anfHDGK* operon in the presence of vanadium. Either NifA or NtrC can activate the expression of *nifM*. Expression of the *anf* operon is thought to have required the NifA transcriptional activator, although there are no NifA binding sites at appropriate locations upstream of *anfH* (or *anfA*) [93]. Surprisingly, although both the alternative Fe proteins appeared to form normal chemical cross-linking complexes with the MoFe protein, neither was able to compete with wild-type Fe protein in an activity assay [94].

Finally, it might be noteworthy that Fe atoms are present in all types of nitrogenases. The importance of using iron as a catalyst to accelerate the reaction of N_2 and H_2 in the Haber-Bosch process might indicate the similar importance of Fe presence in all the three types of nitrogenase in their metallocentres. The industrial catalytic mechanism involves dissociation of N_2 to atomic nitrogen on the active crystal face of the iron catalyst, followed by the reaction with dissociated hydrogen to form ammonia.

4. Nitrogen regulation

The two major signals that regulate nitrogen fixation, in most diazotrophs, are oxygen and ammonia. Bacteria can use both organic and inorganic nitrogen compounds as sole sources of cellular nitrogen. They respond to the environmental nitrogen. The expression of *nif* genes is modulated by the response of NifA and NifL proteins to molecular oxygen and/or fixed nitrogen [95, 96]. The product of the *Klebsiella pneumoniae nifL* gene inhibits NifA-mediated transcriptional activation.

Nitrogen regulation of NifL function occurs at two levels: transcription of the *nifLA* operon is regulated by the general nitrogen regulation (*ntr*) system, and the activity of the NifLA complex is controlled by an unknown nitrogen responsive mechanism. NifL is a redox-sensitive regulatory protein and may represent a type of flavoprotein in which electron transfer is not coupled to an obvious catalytic activity. In addition, the activity of NifL is also responsive to adenosine nucleotides, particularly ADP [97]. In enteric and many other bacteria there is a general Ntr system that controls the expression of many genes concerned with nitrogen metabolism. This *ntr* system is composed of four enzymes: GlnD, PII protein, NtrB and NtrC. GlnD is an uridylyltransferase/uridylyl-removing enzyme (UTase/UR) encoded by the *glnD* gene. Many prokaryotes encode two PII proteins, usually named GlnK and GlnB, and some organisms encode a third PII named GlnJ [98, 99]. A two-component regulatory system is composed of the histidine protein kinase NtrB and the response regulator NtrC [100]. The phosphorylated form of NtrC (NtrC-P) is needed for transcription of the regulatory *nifLA* operon; NtrB controls the phosphorylation state of NtrC. Phosphorylation of NtrC requires NtrB and the uridylylated form of PII. Uridylylation of PII is regulated by GlnD, with nitrogen limitation favouring uridylylation and nitrogen excess conditions favouring removal of the uridylyl residue. PII signal transduction proteins are pivotal players in post-translational control of nitrogenase activity [101]. The intracellular ratio of glutamine (Gln) to 2-oxoglutarate (2OG) acts as a signal of nitrogen status. Non-uridylylated PII supports NtrB-mediated dephosphorylation of NtrC. As a consequence, NtrC-P predominates under conditions of nitrogen limitation and transcription of *nifLA* is favoured (Fig. 3).

NifA is a positive activator of transcription of the other *nif* operons facilitated by bending of the DNA integration by the host factor (IHF) [102]. NifA acts as an enhancer binding protein (EBP) that recognizes sequences (typically TGT-N10-ACA, designated UAS) [103] located upstream of *nif*-specific promoters. Such promoters have a unique consensus sequence at -24/-12, which is regulated by RNA polymerase holoenzyme containing the alternative sigma factor σ54 [104]. NifA proteins are structurally similar to each other and to other EBPs, such as NtrC. In *Azospirillum*, PII is also required to activate NifA by regulating the inhibitory effect of the N-terminal domain of NifA under conditions of nitrogen fixation [105, 106].

The regulation of nitrogen fixation in *Azospirillum brasilense* occurs at both transcriptional and post-translational levels [107, 100]. Unlike regulation seen in *Klebsiella pneumoniae*, transcription of *nifA* does not require *ntrB*/*ntrC* in *A. brasilense* and regulation of *nif* gene expression in response to the level of fixed N is achieved by modulating NIFA activities in response to PII.

Post-translational regulation of nitrogenase activity in *A. brasilense* involves the DRAT/ DRAG regulatory system, which is similar to that of *Rhodospirillum rubrum.* This post-translational regulation has also been found in *Azotobacter chroococcum* [108]. For a long time both DRAT and DRAG activities were regulated *in vivo* but the mechanisms for their regulation are unknown [109]. Recently it was shown for *A. brasilense* and *R. rubrum* that DraG membrane sequestration is involved in the regulatory mechanism. It is dependent on the interaction with small signaling PII-like proteins (such as GlnB, GlnK, GlnZ, GlnJ), which themselves can form complexes with the ammonium transporter protein AmtB [110, 111, 112]. After an ammonium shock, DraG was found to be membrane associated in a PII/AmtB dependent manner. The current model for the regulation of the DraT/DraG-system suggests that the steric separation of DraG from its substrate prevents nitrogenase activation (Fig. 3). The DRAT/DRAG system is also responsive to oxygen. For the photosynthetic purple nonsulfur bacterium, *Rhodospirillum rubrum,* and other members of the α-proteobacteria like *Azospirillum brasilense*, *Azospirillum lipoferum*, and *Rhodobacter capsulatus*, it has been shown

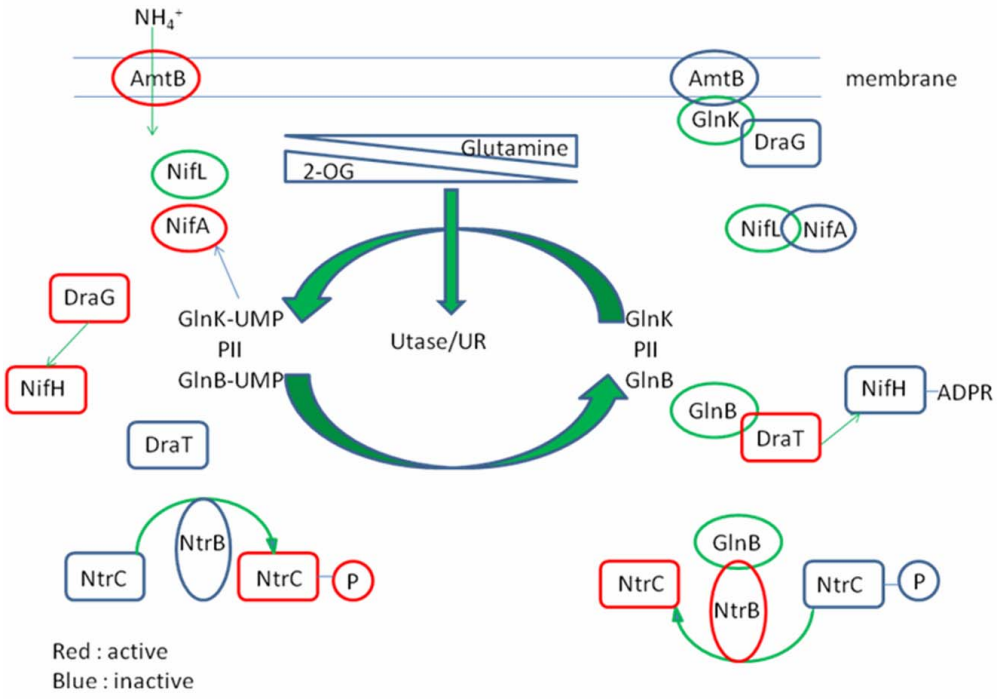

Fig. 3. Nitrogen regulation in free-living diazotroph *K. pneumoniae*. PII signal-transduction proteins are important for communicating the nitrogen status to various regulatory targets. GlnB and GlnK are PII homologues. The intercellular concentration of glutamine is a key signal of nitrogen status. Under nitrogen limiting conditions this is relatively low. UTase/UR uridylylates the PII proteins, altering the conformation of these trimeric proteins and their interaction with targets. GlnB and GlnK are not complexed to DraT and DraG. DraT is inactive and DraG is located in the cytoplasm and active; consequently NifH is not modified, allowing nitrogenase activity. Conversely, under conditions of nitrogen sufficiency, GlnB and GlnK are de-uridylylated (involved proteins and processes are shown in green). GlnK binds avidly to both AmtB and DraG blocking the ammonium transport and promoting DraG inactivation. At the same time, DraT is activated by interaction with de-uridylylated ADP-bound GlnB. GlnB protein controls the activity of the NtrB–NtrC two-component regulatory system. The de-uridylylated form of the PII proteins binds to histidine protein kinase NtrB, to activate its phosphatase activity and consequently control the phosphorylation state of the response regulator NtrC.

that nitrogenase activity is rapidly switched-off, either as a consequence of the addition of ammonium or of energy depletion [113, 114, 115, 116, 117]. One subunit of the nitrogenase F is modified under switch-off conditions, the modification leading to the inactivation of the enzyme. In case of *R. rubrum* and *R. capsulatus* it has been proven that the modifying group is an ADP-ribose moiety on the amino acid residue arginine101 or arginine102 [118,119].

5. Oxygen protection

Oxygen is found to be extremely harmful for nitrogenase. Even in the Haber-Bosch process, oxygen "poisons" the iron catalyst, preventing it from working [120]. All nitrogenases, regardless of source, show a similar extreme sensitivity to oxygen *in vitro* although the oxygen sensitivity of N_2 fixation by diazotrophs *in vivo* varies considerably [6]. All manipulations of the proteins must be performed with an atmosphere of <1 ppm oxygen. In both components solvent exposure of the [4Fe-4S] cluster might be one reason for oxygen sensitivity. The [4Fe-4S] cluster of the Fe protein becomes more exposed upon MgATP binding. This is consistent with the observation that the Fe protein becomes more oxygen sensitive when MgATP is bound [121]. The half-life for Fe protein and MoFe protein are 45 s and 10 min, while the Mo-independent nitrogenases seem even more sensitive to oxygen inactivation [122].

Due to this oxygen sensitivity, nitrogen fixation *in vivo* has to occur under conditions which prevent oxygen damage to nitrogenase. In many diazotrophs various adaptive mechanisms may be present. Obligate anaerobes, such as *Clostridium pasteurianum* and *Desulfovibrio desulfuricans*, are apparently devoid of any specific device to protect their nitrogenase, or indeed any other cell constituents, from the deleterious effects of oxygen. Therefore, they can live and fix nitrogen only in the complete absence of oxygen and are limited in their natural distribution to oxygen-free environments. Facultative bacteria, for example *K. pneumoniae*, *B. polymyxa* and *R. rubrum*, are able to grow on combined nitrogen in both the presence and absence of oxygen but can fix nitrogen only anaerobically. Microaerophilic bacteria, such as *Azospirillum* species, show a preference for subatmospheric levels of oxygen when fixing nitrogen. They are unable to fix nitrogen at high oxygen tensions or under anaerobic conditions. Finally, aerobic bacteria, represented by the *Azotobacter* species, are capable of growth on dinitrogen in air [6]. Although the concentration of oxygen in air (21% v/v, 0.21 atm, 21.2 kPa) is equivalent to a dissolved oxygen concentration of 0.258 mM at 25°C in air-saturated water, this relationship is valid only if it is assumed that complete equilibration exists between atmospheric and dissolved oxygen, which may not necessarily be so. Moreover, solubility of oxygen is dependent on temperature, pressure and composition of the medium. Therefore, there is no substitute for direct measurement of dissolved oxygen and, even then the internal concentration of oxygen may differ markedly from that measured in the growth medium. The internal concentration of oxygen, to which nitrogenase is exposed, is often lower than that measured externally particularly in those diazotrophs most tolerant of oxygen. It therefore follows that many diazotrophs are equipped with mechanisms for limiting access of oxygen to the oxygen–sensitive nitrogenase [123]. Recent studies in *Anabaena variabilis* show that oxygen not only destroys nitrogenase activity but triggers proteolysis of nitrogenase subunits, and saturation of the nucleotide binding sites protects the Fe protein from degradation [124].

5.1. Respiratory protection

Azotobacter spp. and other aerobic diazotrophs are able to respond to increased concentrations of dissolved oxygen by increasing their rate of respiration, thereby maintaining low levels of intracellular oxygen and protecting their nitrogenase from inactivation [125, 126]. At higher oxygen tensions the respiratory response becomes nonlinear [127], which may indicate the involvement of additional protective mechanisms [128, 129]. Under natural conditions, within a limited range of dissolved-oxygen concentration, respiratory protection may be sufficient to scavenge excess oxygen and to maintain nitrogenase in a virtually oxygen-free cellular environment.

The cytochrome bd-type oxidase was found to be involved in respiratory protection of nitrogenase in *A. vinelandii* [130]. The catalytic mechanism of oxygen reduction by cytochrome bd has been studied with both membranes and purified oxidase using a *cydR* mutant which overexpresses cytochrome bd., containing CO and NO probes of the oxygen-binding site(s). However, CO and NO bind to haem d with high affinity and to reduced haem b595 with lower affinity [131]. Respiratory protection has been invoked to explain the prevention of inactivation of oxygen-sensitive nitrogenase in the cytoplasm of *Azotobacter* [132] and of *Rhizobium* and *Klebsiella* under microaerobic conditions [133]. In *A. vinelandii* and *K. pneumoniae*, cytochrome bd appears to terminate the respiratory protective electron transport pathway. Whereas, in *Rhizobium meliloti* and *Bradyrhizobium japonicum* cytochrome cbb' (or cbb3) is critically important [134, 135]. *Azorhizobium caulinodans* appears to be capable of synthesising five terminal oxidases, two of which, cytochrome bd and cytochrome cbb' act as oxidases during growth at low oxygen tensions. Both mechanisms seem to offer sufficient respiratory protection to allow nitrogen fixation at 50% of wild-type levels when the organism grows symbiotically [76].

5.2. Conformational protection

It has been well documented that the protected oxygen-tolerant form of nitrogenase in *Azotobacter spp*. is the result of an association between nitrogenase component proteins and a protective FeSII protein, also called Shethna protein II [136, 137, 138, 139, 140]. Complex formation may depend

on the redox state of FeSII, oxidation and reduction of which is a one electron step involving a flavodoxin whose oxidation state may signal complex formation. Whether this flavodoxin is the same as that which donates electrons to nitrogenase is not clear yet. In *Azotobacter chroococcum* there may be only a single species of flavodoxin [141], while in *Azotobacter vinelandii* there appears to be three flavodoxins [142]. Furthermore, the Fe protein of nitrogenase can also donate electrons to FeSII and it has been suggested that the redox state of nitrogenase itself is important in the formation of the oxygen-stable complex [143]. Whatever the precise mechanism, under oxidizing conditions, and in the presence of Mg^{2+}, FeSII forms an oxygen-stable but catalytically inactive complex with nitrogenase. Restoration of a reducing environment, as a consequence of either removal of oxygen or adaptation of respiration to cope with the elevated concentration of oxygen, causes complex dissociation and restoration of nitrogenase activity [123]. This "conformational protection" has not been found in *K. pneumoniae* and the FeSII protein appears not to protect the V-dependent nitrogenase from oxygen damage in *A. chroococcum* [144] or in *A. vinelandii* [145]. Prevention from oxygen damage to nitrogenase may also occur by a phenomenon termed "autoprotection". The purified Fe protein component of the nitrogenase from *A. chroococcum*, in the presence of either ATP or ADP, can reduce oxygen to H_2O (via H_2O_2) without loss of activity provided that the protein is in a four-fold molar excess over oxygen [146].

5.3. Covalent modification

In a number of diazotrophs, including certain photosynthetic bacteria, *Azospirillum* and possibly in *Azorhizobium* and *Bradyrhizobium*, nitrogenase activity is regulated by reversible covalent ADP-ribosylation of the Fe protein. For example, in the nitrogenase of *A. vinelandii*, the site of ADP-ribosylation is a specific argine residue (Arg 100) on only one of the two identical subunits of the Fe protein. Arg 100 is covalently modified by the enzyme termed dinitrogenase reductase ADP-ribosyl transferase (DRAT) with NAD^+ functioning as the ADP-ribose donor. Removal of ADP-ribose from the Fe protein, which restores nitrogenase activity, is catalyzed by dinitrogenase reductase activating glycohydrolase (DRAG). The relative activities of DRAT/DRAG are controlled, probably post-translationally [147, 115], by a number of external factors including oxygen, though most studies have centered on covalent modification of the Fe protein in response to fixed nitrogen or darkness [148, 123].

5.4. Physical barrier

Alginate is a linear copolymer of 1→4-linked β-D-mannuronic acid and α-L-guluronic acid, where some of the mannuronic acid residues may be acetylated. Sabra *et al.* showed that the alginate capsule of *A. vinelandii* was affected by the oxygen tension [149]. The alginate biosynthetic gene set consists of twelve genes (Avin10860 to Avin10970). The physical organization of this cluster is highly conserved in *A. vinelandii* and in Pseudomonas species able to produce alginate. *A. vinelandii* encodes a set of seven secreted mannuronan C-5 epimerases (AlgE1-7) that modify the polymer outside the cells. Some of the epimerases are responsible for introducing consecutive guluronic acid residues into the polymer, and this structural feature is necessary for forming a gel with divalent cations [150].

In symbiotic systems, diazotrophs invade legume plant roots or stems, forming a nodule architecture which effectively protects nitrogenase within the bacteroids (modified rhizibial cells) which occupy infected plant cells. The nitrogen fixing tissue is surrounded by a cortex of uninfected cells which, in the stem nodules, even contains active chloroplasts (functional PSII evolving oxygen) [151, 152, 153, 154]. Within bacteroids a low intracellular concentration of oxygen is maintained by a high oxygen-consuming respiration rate coupled with a barrier to oxygen diffusion that resides in the inner cortex, a layer of relatively thick-walled cells with numerous intercellular gas spaces between them [155, 156, 157]. Oxygen diffusion into the infected region of the nodule involves a pathway that is partially air-filled and partially water-filled [158, 159]. The air-filled region of the pathway consists of intercellular air spaces. Oxygen diffuses more rapidly through the air-filled portion of the pathway than through the water-filled portion. Hence alterations in the relative proportion of these regions alter the rate

of diffusion of oxygen to the bacteroids. One mathematical model suggests that the variable diffusion barrier is achieved by manipulation of the intercellular air spaces in the inner cortex [157]. Another model suggests that intercellular air spaces become filled with water or glycoprotein [160, 161]. Some of the nodule-specific proteins (nodulins) that are produced during the early stages of nodule development may have a role in limiting oxygen diffusion to the infected cells [162, 163]. In order to sustain optimum rates of N_2 fixation bacteroids require oxygen for respiratory ATP production. However, as with aggregates of free-living diazotrophs, simple diffusion of the oxygen that arrives at the surface of the infected zone of the nodules would allow only the outermost layer of bacteroids to respire [164]. Delivery of oxygen to the entire mass of infected cells is achieved by the oxygen-carrying haem protein leghaemoglobin. A continuous supply of bound oxygen ensures that efficient, though oxygen-limited, respiration can be supported [123].

6. Nitrogen fixation in associative bacteria

Unlike laboratory model free-living nitrogen fixers such as *Klebsiella* and *Azotobacter*, some bacteria fix nitrogen while loosely being associated with plants. Those diazotrophs are usually seen as endophytes. *Pseudomonas stutzeri* A1501, a γ-proteobacteria, was originally isolated from a rice root. It can survive in the soil, colonize the root surface and invade the superficial layers of the root cortex. Therefore, it has been reported as an endophyte. The recent revealing of the complete genome of A1501 and comparative analysis with other *Pseudomonas* species suggested a lateral gene transfer of a clustered 49-kb nitrogen fixation island and several other set of genes reported to play roles in some physiological properties, for instance, osmotolerance [165]. Wild-type strains of *Pseudomonas* usually do not produce the above mentioned alginate under laboratory conditions, while most strains of *A. vinelandii* produce alginate constitutively and use the polymer both for making the vegetative state capsule and for the cyst coat [150]. While in Pseudomonas, AlgR is required for alginate production, *algR* mutants of *A. vinelandii* produce alginate but are unable to form mature cysts. Orthologies of AlgR, AlgB, AlgP, AlgQ and AmrZ, responsible for the regulation of alginate synthesis, are also present in *P. stutzeri* A1501. However, A1501 does not produce alginate [150]. Furthermore, a global transcriptional profiling analysis of nitrogen fixation and ammonium repression in A1501 revealed that a total of 166 genes, including those coding for the global nitrogen regulation (Ntr) and Nif-specific regulatory proteins, were upregulated under nitrogen fixation conditions but rapidly downregulated as early as 10 min after ammonium shock. Among these nitrogen fixation-inducible genes, 95 have orthologs in each of *Azoarcus sp. BH72* and *Azotobacter vinelandii AvoP*. In particular, a 49-kb expression island containing *nif* and other associated genes was markedly downregulated by ammonium shock [166].

Endophytes, like *Bacillus*, are often used as plant growth promoting rhizobacteria (PGPR) rather than nitrogen fixers, although some of them do have nitrogen fixation properties under certain conditions. Compared to plant growth-promoting *Pseudomonas* rhizobacteria, relatively little is known about the lifestyle of plant-associated Bacillus spp., which were originally considered as typical soil bacteria despite their well-established advantages for beneficial action on plant growth and bio-control [167]. Endophytic bacteria residing within the living tissue of plants are of high interest for agrobio-technological applications, such as the improvement of plant growth and health, phytoremediation or even as biofertilizer. Grass endophytes *Azoarcus sp. BH72* and *Gluconacetobacter diazotrphicus* have been shown to supply biologically fixed nitrogen to their hosts kallar grass and sugarcane; thus they may have potential agronomical and ecological importance [168]. The complete genome sequence of *Azoarcus* sp. strain BH72 offers insights into genomic strategies for an endophytic life style and allows identification of various features that may contribute to their interaction with plants.

7. PROSPECTS

The excellent research over the last forty years has provided enormous information on biological nitrogen fixation operation in microbes. Given the pressure of agricultural demand, the major question is which way to go further. There are two possibilities: (1) making non-legume plants have

symbiotic nitrogen fixation by transferring the genetic pathways for symbiotic fixation from legumes [169], (2) making non-legume plants able to fix nitrogen autonomously by direct transfer of the nitrogenase enzyme system to chloroplasts or mitochondria. Both approaches have been encouraged by being highlighted in Nature and Science [170, 171]. It has already been shown that some *nif* genes can be transferred to plants and function in them [172]. A nitrogen fixing plant would require a set of essential genes, making the task difficult, but not impossible [170]. The effort being put into these programs is large. It seems likely that progress will eventually be made. But it is not a risk-free approach. A nitrogen fixing cereal could potentially become a major weed in other crops or invasive in unmanaged ecosystems. If nitrogen fixation were to be transferred to a crop with wild relatives, that ability might become more widespread with wholly unpredictable consequences for natural ecosystems and the global nitrogen cycle [173]. In this respect, a nitrogen fixing chloroplast approach may have the advantage of being maternally inheritable.

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