

GENETIC REGULATION OF BIOLOGICAL NITROGEN FIXATION

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Some bacteria have the remarkable capacity to fix atmospheric nitrogen to ammonia under ambient conditions, a reaction only mimicked on an industrial scale by a chemical process that requires high temperatures, elevated pressure and special catalysts. The ability of microorganisms to use nitrogen gas as the sole nitrogen source and engage in symbioses with host plants confers many ecological advantages, but also incurs physiological penalties because the process is oxygen sensitive and energy dependent. Consequently, biological nitrogen fixation is highly regulated at the transcriptional level by sophisticated regulatory networks that respond to multiple environmental cues.

DIAZOTROPHIC ORGANISMS

A nitrogen-fixing organism that is capable of growth on atmospheric nitrogen as the sole nitrogen source.

CHEMOLITHOTROPH

An organism that is capable of using CO, CO₂ or carbonates as the sole source of carbon for cell biosynthesis and that derives energy from the oxidation of reduced inorganic compounds.

ACTINORHIZAL

Symbiotic associations of plants that have the capacity to form root nodules with nitrogen-fixing actinomycetes.

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Biological nitrogen fixation — the conversion of atmospheric nitrogen into ammonia by symbiotic, associative and free-living bacteria — is of tremendous importance to the environment and to world agriculture. Nitrogen fixation is an important part of the nitrogen cycle as it replenishes the overall nitrogen content of the biosphere and compensates for the losses that are incurred owing to denitrification. The availability of fixed nitrogen is frequently the limiting factor for crop productivity, making demands on global agriculture to provide food security as the world's population increases in the twenty-first century. The increased use of chemical fertilisers, which constitutes the largest human interference in the nitrogen cycle, has prompted concerns regarding the increased emissions of nitrogen oxides, soil acidification and water eutrophication. The fixed nitrogen that is provided by biological nitrogen fixation is less prone to leaching and volatilization as it is utilized *in situ* and therefore the biological process contributes an important and sustainable input into agriculture. Biological nitrogen fixation also has an important role in the marine nitrogen cycle and can influence the capacity of the ocean biota to sequester atmospheric CO₂¹.

Although nitrogen fixation is not found in eukaryotes, it is widely distributed among the Bacteria and the Archaea, revealing considerable biodiversity among DIAZOTROPHIC ORGANISMS. The ability to fix nitrogen is found in most bacterial phylogenetic groups, including

green sulphur bacteria, Firmibacteria, actinomycetes, cyanobacteria and all subdivisions of the Proteobacteria. In Archaea, nitrogen fixation is mainly restricted to methanogens. The ability to fix nitrogen is compatible with a wide range of physiologies including: aerobic (for example, *Azotobacter*), facultatively anaerobic (for example, *Klebsiella*) or anaerobic (for example, *Clostridium*) heterotrophs; anoxygenic (for example, *Rhodobacter*) or oxygenic (for example, *Anabaena*) phototrophs; and CHEMOLITHOTROPHS (for example, *Leptospirillum ferrooxidans*). Diazotrophs are found in a wide variety of habitats including free-living in soils and water, associative symbioses with grasses, symbiotic associations in termite guts, ACTINORHIZAL associations with woody plants, cyanobacterial symbioses with various plants and root–nodule symbioses with legumes.

Nitrogenases

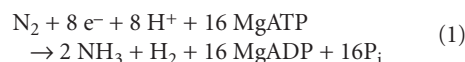
Nitrogenases, which catalyse the biological reduction of dinitrogen to ammonia, are complex metalloenzymes with conserved structural and mechanistic features^{2,3}. These enzymes contain two components that are named according to their metal composition. The smaller dimeric component, known as the iron (Fe) protein, functions as an ATP-dependent electron donor to the larger heterotetrameric component, known as the molybdenum–iron (MoFe) protein, which contains the enzyme catalytic site.

TRANSITION-STATE ANALOGUE

A substrate designed to mimic the properties or the geometry of the transition state of a reaction. This is an intermediate state in which the enzyme has reached a geometric and energetic state necessary to overcome the activation energy required for the reaction.

All diazotrophs have a molybdenum–iron nitrogenase system, but under conditions of molybdenum depletion, some organisms — for example, *Azotobacter vinelandii*, and *Rhodobacter capsulatus* — induce the synthesis of alternative nitrogenases containing vanadium–iron or iron–iron co-factors⁴. Both of the nitrogenase-component proteins are extremely oxygen sensitive. The oxygen sensitivity of the Fe protein is conferred by a surface-exposed [4Fe–4S] cluster that bridges the two subunits of the dimer. The MoFe protein contains two types of metal centres: the P cluster (an [8Fe–7S] cluster); and the FeMo cofactor (MoFe₇S₉-homocitrate), which is the site of substrate reduction^{5,6} (FIG. 1a). The

overall stoichiometry of dinitrogen reduction under optimal conditions is as follows (equation 1):



The enzyme mechanism requires reduction of the Fe protein by electron donors such as ferredoxin and flavodoxin, transfer of single electrons from the Fe protein to the MoFe protein (which is dependent on MgATP hydrolysis) and, finally, internal electron transfer in the MoFe protein by the P cluster to the FeMo cofactor substrate-binding site. Each electron-transfer step requires an obligatory cycle of association of the Fe and MoFe proteins to form a complex, after which the two components dissociate⁷ (FIG. 1b). Nitrogenase is a relatively slow enzyme with a turnover time of ~5 s⁻¹, and dissociation of the complex is the rate-limiting step⁸. Complex formation has a crucial role in the enzyme mechanism as it is required for the coupling of ATP hydrolysis to electron transfer.

X-ray crystallographic structures of the **Fe protein**, **MoFe protein** and **stabilized complexes** of the two proteins have been obtained. The overall architecture of the Fe protein is similar to other nucleotide-binding proteins, such as Ras p21, but it has particular structural homology to two other ATPases, **ArsA** and **MinD**, which function in oxyanion extrusion and spatial regulation of cell division, respectively⁹. Although these proteins have diverse functions, their structural similarity is likely to reflect a common requirement for coupling ATP hydrolysis to the formation of a transient protein complex. Other enzymes with functional similarity to nitrogenase include 2-hydroxyglutaryl-CoA dehydratase and benzoyl-CoA reductase, which also catalyse ATP-dependent electron transfer^{10,11}. Analysis of nitrogenase complexes stabilized with ADP–AlF₄⁻ — a **TRANSITION-STATE ANALOGUE** of ATP hydrolysis — indicates that conformational changes in the Fe protein upon ATP turnover are coupled to repositioning of the [4Fe–4S] cluster. This brings the cluster in closer proximity to the MoFe protein, thereby facilitating inter-protein electron transfer from the Fe protein to the MoFe protein¹² (FIG. 1a).

Physiology and genetic regulation

The minimal stoichiometry of two MgATP hydrolysed per electron that is transferred at each step in the catalytic cycle of nitrogenase is a considerable energetic input. Moreover, the relatively slow turnover time of the enzyme requires diazotrophs to synthesize large quantities of nitrogenase (up to 20% of the total protein in the cell) to use dinitrogen as a sole nitrogen source. Therefore, the synthesis of both molybdenum and the molybdenum-independent nitrogenases is stringently regulated at the transcriptional level in response to the availability of fixed nitrogen.

The oxygen sensitivity of nitrogenase also imposes considerable physiological constraints on diazotrophy as there is an obligation to protect the enzyme from oxygen damage. An aerotolerant nitrogenase has been reported in the chemolithotrophic thermophile

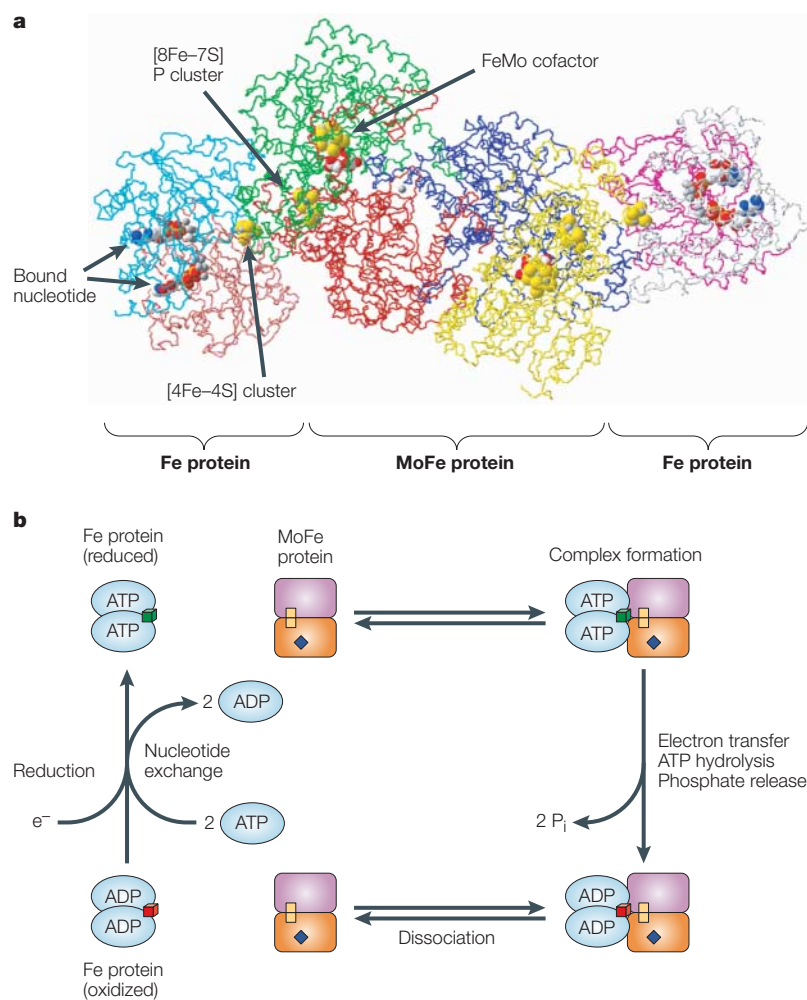
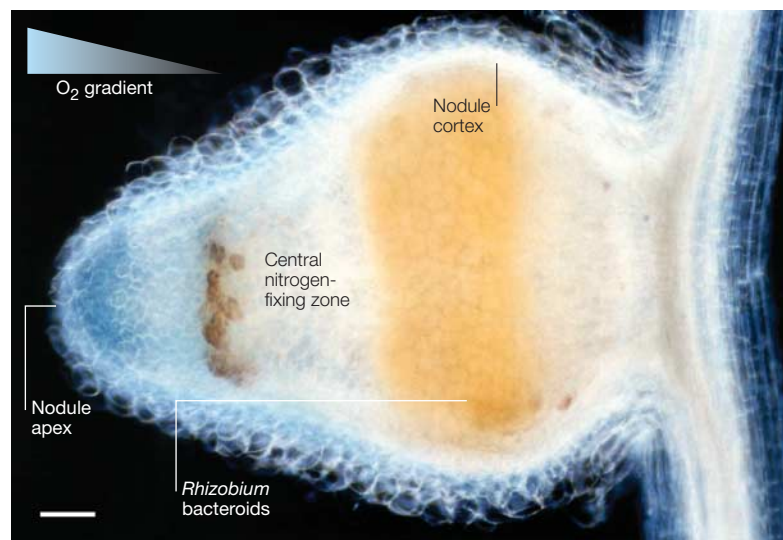


Figure 1 | **Nitrogenase — structure and turnover cycle.** **a** | Structure of the complex that is formed between the component Fe and MoFe proteins of *Azotobacter vinelandii* nitrogenase, stabilized by the transition-state analogue for ATP hydrolysis, ADP–AlF₄⁻ (REFS 12,77). The subunits of the two Fe protein dimers are coloured in cyan, brown, magenta and grey. The α-subunits of the MoFe protein are coloured in green and yellow, with β-subunits in red and blue. The oxygen-sensitive metalloclusters and bound nucleotides are shown in space-fill. For simplicity, arrows indicating these features are only shown on one half of the symmetrical complex. **b** | Schematic representation of the nitrogenase Fe protein cycle. The Fe protein dimer is shown in light blue with the cube representing the [4Fe–4S] cluster coloured green to indicate the reduced form and red to represent the oxidized form. The α and β subunits of the MoFe protein are depicted as orange and pink, respectively, the yellow squares represent the P cluster and the blue diamond represents the FeMo cofactor. Changes in the oxidation state of the MoFe protein are not shown.

Box 1 | Distribution of oxygen in symbiotic nitrogen-fixing nodules

The nitrogen-fixing nodule hosts symbiotic *Rhizobium* bacteroids, which function as specialized nitrogen fixing organelles that exchange fixed nitrogen for photosynthates. A physiological paradox arises from the aerobic requirements of bacteroid metabolism compared with the oxygen sensitivity of nitrogenase in the absence of a dedicated bacterial protective system. Protection against oxygen is provided by the nodule environment through a cortical diffusion barrier so that the main route of oxygen diffusion is through the nodule apex, which generates a longitudinal oxygen gradient (see the figure of a starch-stained alfalfa nodule; scale bar represents 200 μm). As a result, the free oxygen concentration drops to less than 50 nM in the central nitrogen-fixing zone containing *Rhizobium* bacteroids. Oxygen diffusion is facilitated in the central zone by a high concentration of leghaemoglobin, and bacteroid respiration is made possible by the induction of a high affinity *cbb*₃ oxidase^{66,67}. Therefore, *Rhizobium* bacteroids fix nitrogen in a microaerobic, nitrogen-rich environment, which explains why oxygen, not nitrogen, is the main physiological regulatory factor for *nif* gene induction during symbiosis. Figure reproduced with permission from REF 81 © (1997) Cold Spring Harbor Laboratory Press.



Streptomyces thermoautotrophicus but this is exceptional as the optimal level of nitrogen fixation by this system occurs at high temperatures and might involve novel chemistry¹³. In most diazotrophs, a diverse array of physiological strategies are used to provide protection from oxygen, including simple avoidance of oxygen through anaerobic growth, consumption of excess oxygen by respiration, oxygen diffusion barriers, or compartmentation of the enzyme spatially or temporally. The legume–root nodule symbiosis provides an elegant example of such a strategy in which the nodule cortex is an oxygen diffusion barrier. In addition, reversible binding of oxygen by the NODULIN leghaemoglobin facilitates oxygen diffusion at low free-oxygen concentrations, supporting BACTEROID respiration by a high-affinity terminal oxidase (BOX 1). The difficulties of reconciling nitrogen fixation with the presence of oxygen are also reflected at the level of nitrogenase expression, as transcriptional regulation of NITROGEN FIXATION GENES (*nif*) in diazotrophs is tightly controlled in response to the external oxygen concentration.

In addition to regulation at the transcriptional level, nitrogenase activity is also subject to post-translational regulation in some diazotrophs — for example in

Rhodospirillum rubrum, *Rhodobacter capsulatus* and *Azospirillum brasilense*. This is mediated by ADP-ribosylation of the Fe protein of nitrogenase by DraT (dinitrogenase reductase ADP-ribosyltransferase), which inactivates nitrogenase in response to ammonium and light intensity. Covalent modification of nitrogenase is reversed by DraG (dinitrogenase reductase activating glycohydrolase), which removes the ADP-ribose moiety under conditions that are appropriate for nitrogen fixation^{14,15}.

The necessity to respond to the concentrations of fixed nitrogen and external oxygen, and to provide sufficient energy for nitrogen fixation, imposes common regulatory principles among diazotrophs. In the diazotrophic proteobacteria this is reflected by common regulatory components and the use of similar regulatory networks. However, there is considerable plasticity in the regulatory networks, which differ among microorganisms and are dependent on host physiology. Moreover, important switches in the activities of regulatory cascades are evident in symbiotic organisms on transition from the free-living to the symbiotic state. For example, the regulation of *nif* genes in *Azorhizobium caulinodans*, which is a remarkable rhizobium that is able to fix nitrogen in both the free-living and symbiotic states, is responsive to both environmental nitrogen and oxygen concentrations *ex planta*, but in the symbiotic state, the predominant regulatory factor is the free-oxygen concentration^{16,17}. Control of nitrogen fixation in response to the nitrogen status is less important in symbiotic bacteroids as these cells are committed to provide fixed nitrogen for the benefit of the plant.

The modular domain structure of regulatory proteins in the cascades also allows fine-tuning to adapt regulatory communications to the physiology of the host organism. In this review, we will consider the regulatory events that control transcription of the *nif* genes in both free-living and symbiotic diazotrophs. As regulatory networks and detailed interactions are diverse amongst nitrogen-fixing microorganisms, we will focus on the diazotrophic representatives of the Proteobacteria, illustrating general principles with specific examples.

Regulation by NifA

In the Proteobacteria *nif* genes are invariably subject to transcriptional activation by NifA (a member of the enhancer-binding protein (EBP) family), together with the RNA polymerase sigma factor, σ^{54} . NifA has a domain architecture that is similar to other members of the EBP family in which a central, conserved AAA⁺ ATPase domain is flanked by an amino-terminal regulatory domain and a carboxy-terminal DNA-binding domain, which contains a helix–turn–helix motif that is required for recognition of the UAS enhancer-like elements (BOX 2). The N-terminal regions of NifA proteins contain a GAF domain (FIG. 2), a ubiquitous signalling module that is found in all kingdoms of life and which, in some cases, has been shown to bind small molecules including cyclic nucleotides and formate¹⁸. Although the GAF domains of different NifA proteins have been shown to have a role in regulating NifA activity, in many

NODULIN

A plant protein that is specifically found, or strongly induced, in root nodules.

BACTEROID

A differentiated intracellular form of a rhizobial cell, specialized in nitrogen fixation.

NITROGEN FIXATION GENES

These are found in both free-living and symbiotic nitrogen-fixing bacteria. They include the structural genes for nitrogenase, genes that are required for nitrogenase biosynthesis and regulatory genes.

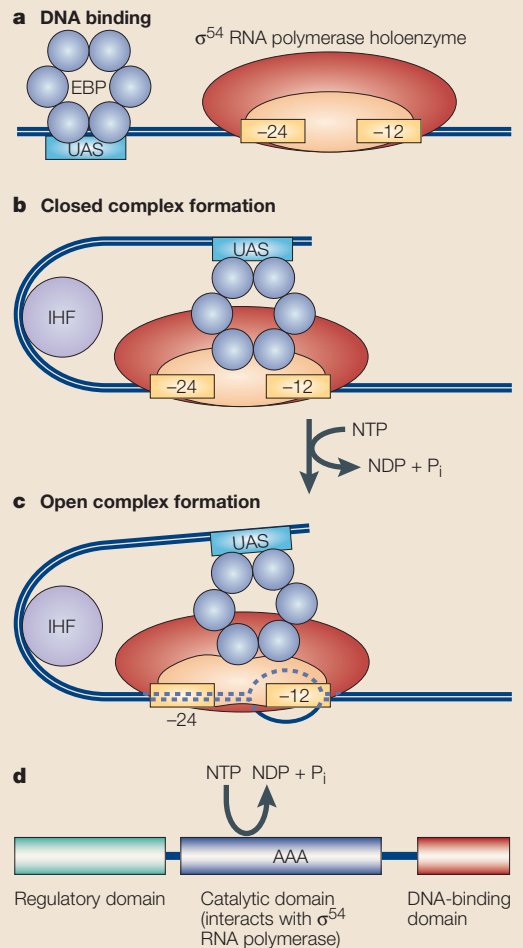
UAS

Upstream activator sequences that provide specific recognition sequences for the NifA protein in the vicinity of σ^{54} -dependent *nif* promoters.

Box 2 | **Enhancer-binding proteins**

Enhancer-binding proteins (EBPs) are a unique class of prokaryotic transcriptional activators that interact with the RNA polymerase sigma factor, σ^{54} . This sigma subunit (also known as RpoN, NtrA and σ^N) has no detectable homology with other known sigma factors, for example, the σ^{70} family. σ^{54} reversibly associates with the core RNA polymerase to recognize promoters with consensus sequences at -12 and -24 bp relative to the transcription start site and transcription initiation is dependent on interaction with a member of the EBP family. Interaction of the EBP with the σ^{54} RNA polymerase holoenzyme (see the figure part a) is facilitated by the binding of the activator (shown as a blue oligomer) to DNA sequences (upstream activator sequences, UAS) usually located at least 100 bp upstream of the transcription initiation site. DNA looping is required to establish productive interactions between the DNA-bound activator and the polymerase. In some cases this is assisted by other DNA-binding proteins, such as integration host factor (IHF) (see the figure part b). In the absence of the EBP, σ^{54} RNA polymerase holoenzyme forms closed promoter complexes (in which the promoter DNA is double-stranded) that rarely undergo spontaneous ISOMERIZATION to form open complexes (in which the DNA strands surrounding the transcription start site are locally denatured). Nucleotide hydrolysis by the activator promotes remodelling of the closed complex through a series of protein–protein and protein–DNA interactions that favour conversion to the open promoter complex (see the figure part c)⁶⁸.

EBPs have a modular domain structure (see the figure part d), which frequently comprises at least three domains: one or more amino-terminal regulatory domains (which often belong to the GAF, PAS or response-regulator families), a highly conserved central domain that is required for the nucleotide-dependent interactions that drive open complex formation by σ^{54} RNA polymerase, and a carboxy-terminal DNA-binding domain with a helix–turn–helix motif that is required for recognition of upstream activator sequences⁶⁹. The conserved central domain, also known as the σ^{54} -interaction module, belongs to the AAA⁺ superfamily of ATPases that function as molecular machines to remodel their substrates. Members of the AAA⁺ family commonly function as oligomers, frequently as hexameric ring structures in which switches in conformation are promoted by nucleotide-dependent interactions^{70,71}. Nucleotide binding influences PROTOMER–protomer interactions, consistent with the observation that the presence of nucleotides alters the oligomerization state of EBPs. In addition to other conserved features found in the AAA⁺ superfamily, the EBPs contain a signature motif, GAFTGA, which has a direct role in the interaction with σ^{54} and the coupling of ATP hydrolysis to open-complex formation⁷². Stable binding of σ^{54} to NifA and PspF, another member of the EBP family, has been detected in the presence of the transition-state analogue ADP–AlF₄⁻ (REF 73). The GAFTGA motif is indispensable for this interaction and it is likely that this σ^{54} -binding surface changes conformation on nucleotide hydrolysis. The recently determined crystal structure of the isolated AAA⁺ domain of the EBP **NtrC1** from *Aquifex aeolicus*, reveals that this protein is a ring-shaped heptamer in which the GAFTGA motif is located on an ordered loop in each subunit, projecting out into a central pore⁷⁴. These loops might form an interaction surface that couples ATP hydrolysis to interactions with the σ^{54} RNA polymerase.



cases the precise mechanism of regulation is not clear and this domain seems to have a diverse role in various diazotrophs. For example, the GAF domain of *Azotobacter vinelandii* NifA binds 2-oxoglutarate to modulate the activity of the AAA⁺ domain in response to a second regulatory protein, NifL (see below). However, binding of 2-oxoglutarate to the GAF domain of *Klebsiella pneumoniae* NifA has not been observed¹⁹. By contrast, the GAF domains of NifA proteins from other diazotrophic proteobacteria — for example, *A. brasilense* — seem to regulate NifA activity in response to the concentration of fixed nitrogen and might interact with the signal-transduction protein GlnB^{20,21} (see below).

Regulatory cascades

The co-dependence of σ^{54} -RNA polymerase and NifA for open-complex formation and transcription initiation at *nif* promoters ensures that transcription of the *nif* genes is stringently regulated. NifA is the master

regulator of nitrogen fixation and, although regulatory cascades differ, each regulatory circuit ultimately results in regulation of NifA expression or modulation of its activity in response to oxygen and/or fixed nitrogen.

In the diazotrophic proteobacteria, the two-component **NtrB–NtrC** regulatory system, which provides global control in response to the nitrogen source, commonly controls NifA expression. Like NifA, NtrC is a σ^{54} -dependent EBP (FIG. 2, BOX 2). In symbiotic diazotrophs, transcription of *nifA* and *fix* genes is predominantly controlled by the oxygen-responsive two-component **FixL–FixJ** system, together with **FixK**, which is a member of the Crp–Fnr superfamily, or by the redox-sensing system RegS–RegR (FIGS 2,3). Different strategies are used to control the activity of NifA to provide a second level of the regulatory hierarchy (FIG. 3). In many diazotrophic proteobacteria, NifA activity is regulated in response to the nitrogen source and seems to be intrinsically oxygen sensitive.

GAF
A domain that was named after three proteins that contain it: cGMP-stimulated phosphodiesterases, *Anabaena* adenyl cyclase and *Escherichia coli* FhlA.

ISOMERIZATION
Describes the step in which the DNA sequence in the RNA polymerase–promoter complex is unwound.

PROTOMER
A subunit from which a larger protein structure is built.

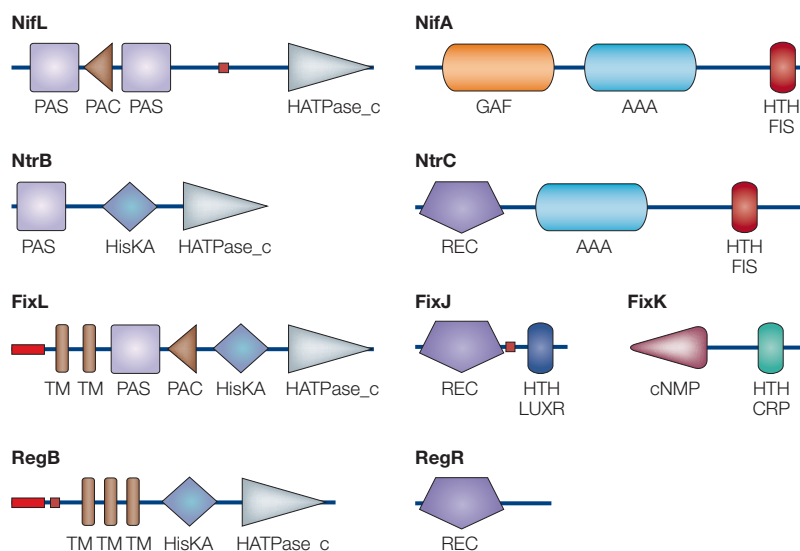


Figure 2 | Domain modules found in proteins that mediate transcriptional control of nitrogen fixation (*nif*) genes. Domains were assigned using SMART (see the online links box) using the following Swiss-Prot/TrEMBL sequences as input: NifL (NifL_azovi), NifA (NifA_azovi), NtrB (NtrB_klepn), NtrC (NtrC_klepn), FixL (FixL_rhime), FixJ (FixJ_rhime), FixK (FixK_rhime), RegS (O86124) and RegR (O86125). PAS domains are shown in light purple. PAC motifs (brown triangles) are found carboxy-terminal to the PAS motifs and are likely to contribute to the PAS structural fold⁷⁸. The GAF domain of NifA is shown in orange. Blue diamonds represent dimerization and phospho-acceptor domains of histidine kinases (HisKA domains). Grey triangles represent HATPase_c domains that are found in the histidine kinase-like ATPases of to the GHKL superfamily. The receiver domains of response regulators (REC) contain a phospho-acceptor site that is phosphorylated by histidine kinase homologues and are shown as purple polygons. AAA⁺ ATPase domains are shown in light blue. Vertical brown bars indicate transmembrane segments in FixL and RegS (also known as PrrB, RegB or ActS in different systems). cNMP (pink) indicates a cyclic nucleotide-monophosphate-like binding domain found in FixK and the Crp–Fnr family. Four distinct HTH (helix–turn–helix) motif-containing domains, which are required for DNA binding, are found in these proteins, as detected in ProDom (see the online links box): a Fis-like DNA-binding domain found in NtrC and NifA, a LuxR-like domain in FixJ, an Fnr/Crp-like domain in FixK and the RegR output domain (not detected by SMART).

FIX GENES

Genes in addition to *nif* genes that are required for nitrogen fixation in symbiotic bacteria. Homologues of some of the *fix* genes are also present in bacteria that do not fix nitrogen.

MICROOXIC

A condition in which oxygen is present at subsaturating concentrations.

PAS

This domain was named after three eukaryotic proteins — PER, ARNT and SIM — in which it is found. In these proteins, the domain detects signals through an associated cofactor.

In the γ -subdivision of the Proteobacteria and in *Azoarcus spp.*, *nifA* is co-transcribed in an operon with *nifL*, which encodes an anti-activator protein that regulates NifA activity in response to oxygen and fixed nitrogen. Unlike classical two-component systems, NifL does not regulate NifA activity by a phospho-transfer mechanism, but interacts with NifA to form an inhibitory complex under conditions that are unsuitable for nitrogen fixation²². When activated in response to environmental cues, NifL inhibits the ATPase activity of the AAA⁺ domain of NifA. Inhibition by NifL requires the GAF domain of NifA, which indicates that the GAF domain controls the activity of the AAA⁺ domain in response to NifL^{23,24}. The overriding functions of these cascades are to control transcription of the *nif* genes in a hierarchical manner. In free-living diazotrophs, the first level of the cascade allows transcription of *nif*-specific regulatory genes in nitrogen-limiting conditions, whereas the second level enables more stringent sensing of the nitrogen concentration in addition to oxygen/redox sensing (FIG. 3). The second step of the cascade therefore allows integration of the antagonistic signals of oxygen and fixed nitrogen.

By contrast, the symbiotic cascade provides hierarchical regulation in response to different oxygen concentrations or the redox environment. In *Bradyrhizobium japonicum*, the FixL–FixJ system induces expression from target promoters at up to 5% oxygen in the gas phase, whereas NifA-dependent targets are only induced effectively at 0.5% oxygen. It is proposed that expression of FixL–FixJ targets allows the bacteria to adapt their respiratory metabolism to the MICROOXIC environment of the nodule, whereas the oxygen sensitivity of NifA is compatible with the low-oxygen conditions that are required for nitrogenase activity in particular zones of the nodule²⁵.

Oxygen sensing

Four proteins that regulate nitrogen fixation are oxygen/redox sensors: the histidine kinase FixL, the histidine kinase RegB (also known as PrrB, RegS or ActS in different systems), the anti-activator NifL and the σ^{54} -dependent activator NifA (which is only oxygen-sensitive in those diazotrophs that lack NifL) (FIG. 2). The domain structures of FixL and NifL are analogous, although as explained above, NifL is not a histidine kinase. Both of these proteins contain N-terminal PAS domains, which are commonly found in prokaryotic and eukaryotic sensors of oxygen, redox, voltage or light²⁶. The sensing method is different in each case, however, as the FixL PAS domain contains a haem prosthetic group, whereas NifL contains flavin-adenine dinucleotide (FAD)^{27,28}. Reversible binding of oxygen to the haem moiety of FixL regulates kinase activity and, consequently, the phosphorylation state of FixJ. In the absence of oxygen, FixL autophosphorylates and transfers the phosphoryl group to FixJ²⁹. Binding of oxygen to the haem moiety deactivates the kinase activity. Structures of unliganded and various ligand-bound forms of the *B. japonicum* and *Sinorhizobium meliloti* FixL haem domains confirm that these domains adopt a PAS fold and undergo a conformational change on ligand binding. The current structural model for the ligand-induced conformational switch in the FixL *B. japonicum* haem domain involves movement of a conserved arginine residue, Arg220, which, on oxygen binding, is released from a salt bridge with the haem propionate and moves into the haem pocket to form a hydrogen bond with the bound oxygen molecule, concomitant with a shift in the loop that joins α -helix F with β -sheet G (the so-called FG loop)^{30,31} (FIG. 4). However, the allosteric changes that result in control of FixL autophosphorylation are not understood. On phosphorylation, the FixJ receiver domain (FixJN) undergoes a conformational change that has been characterized at atomic resolution, demonstrating a reshaping of the $\alpha 4$ – $\beta 5$ face by displacement of helix $\alpha 4$ and rotation of residue Phe101 towards the inside of the receiver domain^{32,33}. This results in FixJ activation, both through the weakening of an inhibitory interaction between FixJN and the transcriptional activator domain, and by allowing FixJ dimerization³⁴ (FIG. 5).

The FAD-containing PAS domain of NifL is required for redox sensing and regulates the ability of NifL to

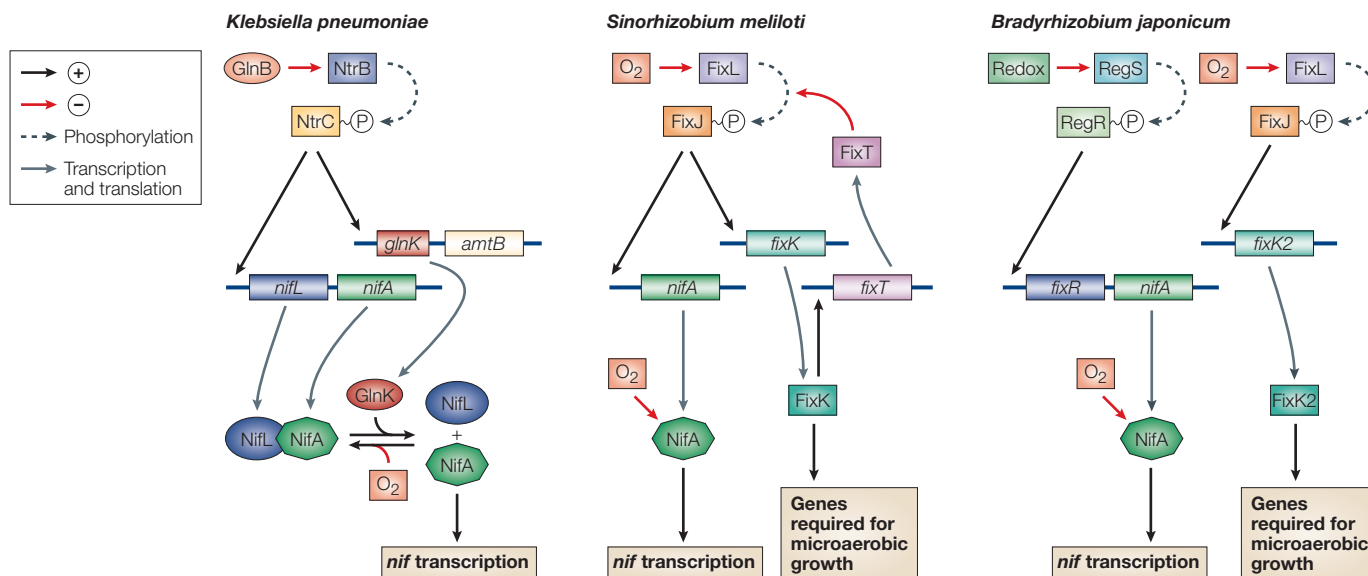


Figure 3 | Comparison of regulatory cascades controlling *nif* transcription in the free-living diazotroph *K. pneumoniae* and in the symbiotic diazotrophs *S. meliloti* and *B. japonicum*. The first level of the cascade involves the nitrogen-regulated NtrB–NtrC two-component system in *Klebsiella pneumoniae*, which is antagonized by the interaction of the PII-like protein GlnB in response to the concentration of fixed nitrogen. By contrast, in *Sinorhizobium meliloti* the first level of the cascade involves the oxygen-responsive FixL–FixJ two-component system and in *Bradyrhizobium japonicum* both the oxygen-responsive FixL–FixJ and the redox-responsive RegS–RegR systems. In *K. pneumoniae* the second level of the cascade involves regulation of NifA activity by the redox-responsive anti-activator protein NifL in response to oxygen and to fixed nitrogen through interaction with the PII-like protein GlnK. NifL-like proteins are not present in *S. meliloti* or *B. japonicum* and, unlike *K. pneumoniae*, NifA activity is responsive to oxygen. The role of FixK in the regulatory cascade varies amongst different symbiotic diazotrophs and, in *Azorhizobium caulinodans* for example, FixK rather than FixJ directly activates NifA expression. In *S. meliloti*, FixK expression is negatively regulated by FixT, which inhibits the activity of FixL^{79,80}. NtrC and NifA are enhancer-binding proteins (EBPs) that interact with σ^{54} -RNA polymerase to activate transcription.

inhibit NifA activity in response to the oxygen status. When the flavin moiety is reduced to the dihydroquinone (FADH₂), *A. vinelandii* NifL is not competent to inhibit NifA activity, but the oxidized form of NifL prevents NifA from catalysing the formation of open promoter complexes²⁸ (BOX 2). The C-terminal region of *A. vinelandii* NifL contains a HATPase_c domain and belongs to the superfamily of GHKL (Gly–His–Lys–Leu) ATPases, which includes the histidine protein kinases, DNA gyrase and Hsp90 (REF. 35) (FIG. 2). Although the corresponding domain of NifL does not hydrolyse ATP, it binds adenosine nucleotides and is required for the interaction with NifA³⁶. It is not yet known whether conformational changes in the PAS domain that occur on changes in redox status can be communicated to the C-terminal domain of NifL. The redox potential of the FAD_{red}/FAD_{ox} couple varies from ~–225 mV (in the case of *A. vinelandii*) to ~–275 mV (in the case of *K. pneumoniae*)^{37,38}. Oxygen is likely to be a physiological oxidant as the reduced form of NifL rapidly oxidizes on exposure to air. The reduced quinone pool has been proposed to be the physiological electron donor to *K. pneumoniae* NifL and reduction of the protein is coupled to reversible membrane association^{39,40}. Under anaerobic conditions, NifL is primarily located in the membrane fraction, but is released to the cytoplasm under oxidizing conditions. As NifA is located in the cytoplasm independent of environmental conditions, membrane sequestration of NifL would provide a

mechanism for releasing NifA from inhibition by NifL under appropriate reducing conditions.

In diazotrophic proteobacteria that lack NifL, NifA proteins have a slightly different domain structure to those that are subject to NifL inhibition. These NifA proteins have an additional linker region of up to 100 residues between the AAA⁺ and DNA-binding domains that contains an invariant Cys–X₄–Cys motif. Proteins of this class also contain an additional invariant cysteine residue in the AAA⁺ domain⁴¹. The presence of these cysteine residues seems to correlate with the oxygen sensitivity of these proteins. All of the conserved cysteines are indispensable for NifA activity, and metal ions, particularly Fe²⁺, are required for *in vivo* activity in *B. japonicum*⁴². This might suggest a model in which metal ion coordination to the cysteine residues controls the activity of these proteins in response to the redox status.

The global redox-responsive RegB–RegA two-component system, which was first identified in purple photosynthetic bacteria, directly regulates *nif* transcription in some diazotrophic members of the α -Proteobacteria. The response regulator RegA functions in conjunction with NtrC to activate transcription from the *nifA2* promoter in *R. capsulatus* and mutants lacking RegA or RegB have reduced levels of nitrogen fixation⁴³. In *B. japonicum*, homologues of RegB and RegA (known as RegS and RegR, respectively; FIGS 2,3) control *nifA* expression during the symbiosis^{44,45}.

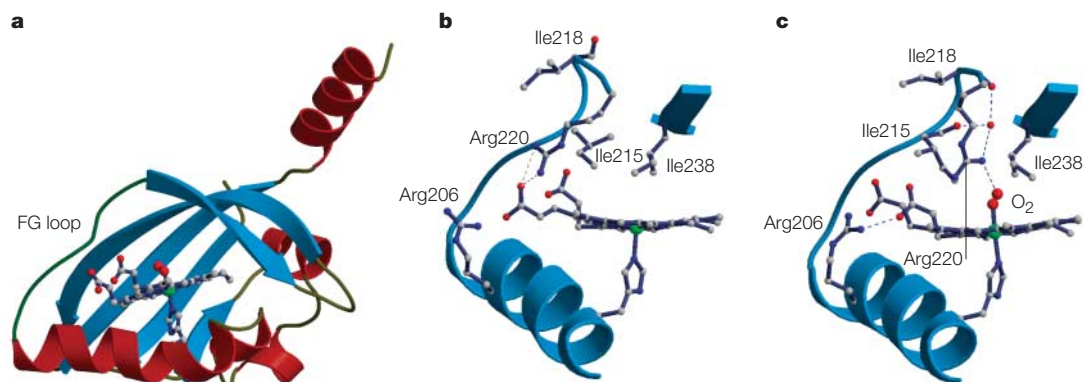


Figure 4 | Conformational change of the FixL haem-containing PAS domain on oxygen binding. **a** | Overall structure of the *Bradyrhizobium japonicum* FixL PAS domain showing haem in the left-handed 'glove' structure and the FG loop. **b** | Detail of the haem-binding pocket in the unliganded ferric state, which is similar to the ferrous state structure³⁰. Note the hydrogen bonding of the Arg220 guanidinium group to haem propionate. **c** | Oxygen binding induces a flattening of the haem followed by hydrogen bonding of Arg220 to the oxygen ligand and a major shift of the FG loop, which is important for signal transduction³¹. Reproduced with permission from REF. 82 © (2000) American Chemical Society.

The histidine kinase RegB and its homologues contain a conserved cysteine residue that is located in a linker region between the autophosphorylation site and the ATP-binding GHKL domain. This conserved cysteine is essential for redox sensing and on oxidation, participates in the formation of an intermolecular disulphide bond that converts the kinase from an active dimeric form to an inactive tetrameric state. Disulphide-bond formation in RegB is metal-ion dependent and the metal-ion cofactor, which *in vivo* is likely to be copper, is likely to have a structural role in the redox mechanism⁴⁶. The activity of PrrB, which is a RegB homologue in *Rhodobacter sphaeroides*, is regulated by electron flow through the high-affinity *cbb*₃-type cytochrome oxidase. Under high-oxygen conditions, the electron flow through the oxidase generates an inhibitory signal that increases the phosphatase activity of PrrB but does not influence kinase activity⁴⁷. It therefore seems likely that the activity of RegB (and its homologues) is regulated

both by interaction of the transmembrane domain with the *cbb*₃ oxidase and also by redox control through the redox active cysteine residue that is located in the cytoplasmic domain.

Nitrogen regulation

PII signal-transduction proteins are important for communicating the nitrogen status to various regulatory targets to control *nif* gene transcription in response to the availability of fixed nitrogen. The availability of more than one homologue of PII enables hierarchical cascades in which, for example, one or more PII targets can control the expression of a second PII-like signal-transduction protein, which in turn can interact with different targets (BOX 3). This is the case in some diazotrophs, for example, *K. pneumoniae*, in which the GlnB protein controls the activity of the NtrB–NtrC two-component regulatory system and the level of phosphorylated NtrC controls expression of the

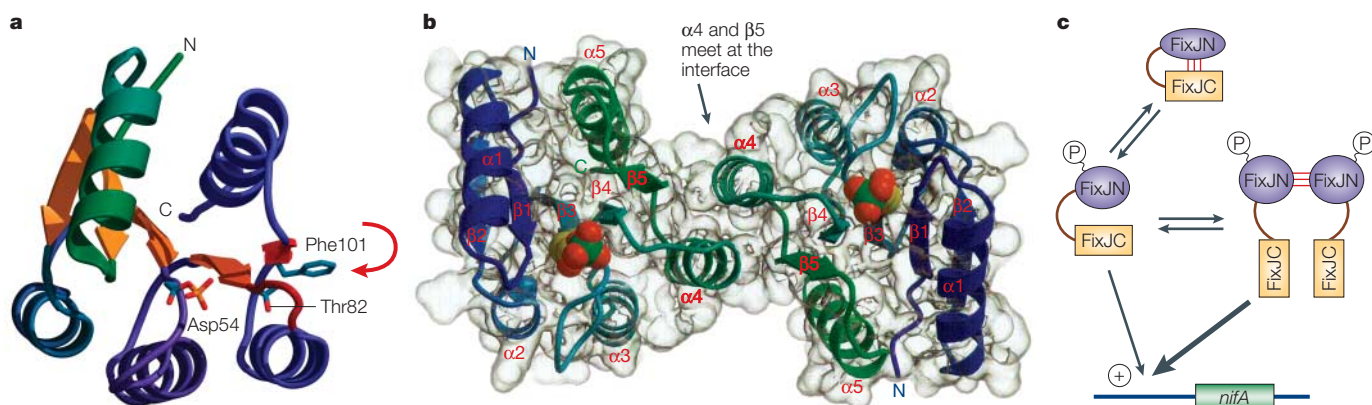
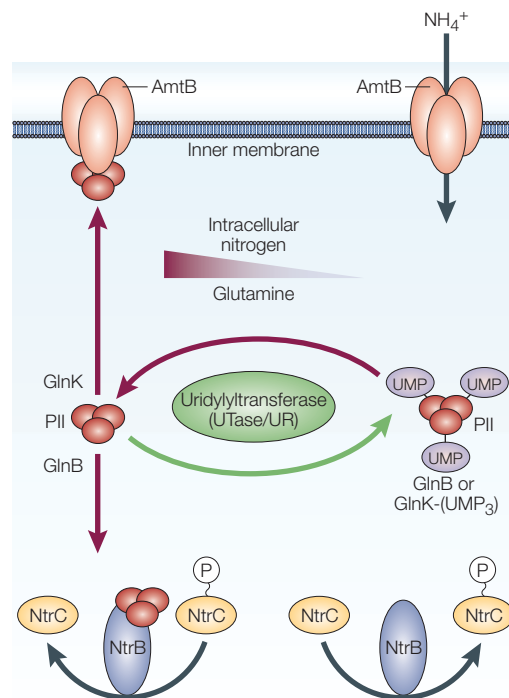


Figure 5 | FixJ structure and activation. **a** | Overall structure of the FixJ receiver domain (FixJN) with the active site Asp54 phosphorylated *in silico*³². After phosphorylation, the Phe101 side chain rotates towards the inside of the receiver domain, shaping the FixJN dimerization interface³³. Reproduced with permission from REF. 32 © (2002) Cold Spring Harbor Laboratory Press. **b** | View of the FixJN-phosphate dimer along the two-fold axis showing the phosphorylated aspartates as spheres. The dimerization interface involves helix $\alpha 4$ and strand $\beta 5$, seen from their amino-terminal and carboxy-terminal ends, respectively. Reproduced with permission from REF. 33 © (1999) Cell Press. **c** | General scheme for FixJ activation. In the native protein the FixJN receiver domain inhibits activity of the FixJC transcriptional activator domain. The conformational change induced by phosphorylation relieves this inhibition and allows FixJ dimerization via FixJN³⁴. Thickness of the arrows reflects DNA-binding activity and efficiency of transcriptional activation.

Box 3 | Sensing the nitrogen status

PII signal-transduction proteins are ubiquitous trimeric proteins found in bacteria, Archaea and plants that integrate metabolic signals of carbon and nitrogen status to control assimilation of fixed nitrogen. Many Proteobacteria contain at least two PII homologues, often designated GlnB and GlnK, that are subject to covalent modification by the uridylyltransferase/uridylyl-removing enzyme (Urase/UR) encoded by the *glnD* gene (see figure). Under nitrogen-limiting conditions, when the intracellular concentration of glutamine — a key signal of the nitrogen status — is relatively low, Urase/UR uridylylates the PII proteins, altering the conformation of these trimeric proteins and their interaction with targets. Conversely, under conditions of nitrogen sufficiency, when the concentration of glutamine is relatively high, binding of glutamine to the Urase/UR causes the enzyme to switch activity in favour of de-uridylylation of the PII proteins, thereby changing PII-receptor interactions^{75,76}. The interaction of PII signal proteins with their targets is also modulated by two other metabolic signals: the energy signal, ATP, and the carbon signal, 2-oxoglutarate (not shown in the figure). Two well-characterized targets for PII proteins are shown in the figure. 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, an enhancer-binding protein that is a key regulator of nitrogen-regulated genes. The non-covalently modified form of the GlnK protein interacts with the ammonium transporter AmtB to inhibit ammonium uptake under conditions of fixed nitrogen excess. Under nitrogen-limiting conditions, covalent modification of GlnK inhibits the interaction with AmtB enabling the uptake of ammonium.



alternative PII protein **GlnK** and the NifL and NifA proteins²² (FIG. 3). A relatively high concentration of phosphorylated NtrC, which is only present under nitrogen-limiting conditions when GlnB is fully uridylylated (BOX 3), is required to activate the σ^{54} -dependent *nifLA* and *glnK amtB* promoters. The non-uridylylated form of GlnB, which predominates under nitrogen excess conditions, interacts with the C-terminal GHKL domain of the histidine protein kinase NtrB to inhibit autophosphorylation and activate its phosphatase activity⁴⁸. This leads to dephosphorylation of NtrC and significant decreases in the level of expression of the NifL, NifA, GlnK and AmtB proteins. In the second level of this cascade, GlnK has an essential role in releasing NifA from inhibition by NifL under anaerobic, nitrogen-limiting conditions (FIG 3). Curiously, the uridylylation state of GlnK does not seem to be important for this interaction, and therefore nitrogen control of the NifL–NifA system at the post-translational level is mediated by the availability of GlnK^{49,50}. However, covalent modification of GlnK does control its interaction with the ammonium transporter AmtB, which is a membrane protein that is co-expressed with GlnK from the *glnK–amtB* operon. In response to micromolar changes in external ammonium concentrations, when GlnK becomes de-uridylylated AmtB sequesters GlnK to the membrane, thereby decreasing the cytoplasmic concentration of this PII protein^{51,52}. So, the amount of GlnK that is available for interaction with NifL and NifA

in *K. pneumoniae* is controlled both at the level of transcription of the *glnK–amtB* promoter and at the post-translational level by membrane sequestration.

Both *A. vinelandii* and *K. pneumoniae* have similar NifL–NifA systems in which NifL controls NifA activity in response to the nitrogen source. However, the mechanisms by which this is achieved in conjunction with PII-like signal-transduction proteins are very different. Like many other Proteobacteria, *A. vinelandii* encodes proteins that are required for general nitrogen control, including a PII-like protein, uridylyltransferase/uridylyl-removing enzyme (Urase/UR) and the NtrB–NtrC two-component regulatory system. In contrast to *K. pneumoniae*, there is only a single PII-like protein, GlnK, that is expressed from the *glnK–amtB* operon and is not subject to NtrC-dependent activation⁵³. NtrC is not required for activation of *nifLA* transcription in *A. vinelandii*, so the first level of the nitrogen cascade in *K. pneumoniae* is absent from *A. vinelandii*. The uridylylation status of GlnK has an important role in nitrogen regulation of transcription of the *nif* genes. Mutations in *glnD* that decrease uridylyltransferase activity prevent activation of *nif* transcription, which results in a Nif phenotype. These mutations can be suppressed by second-site mutations in *nifL*, indicating that NifL constitutively inactivates NifA in *glnD* mutants^{54,55}. Furthermore, a mutant form of *glnK*, *glnK-Y51F*, which encodes a protein that cannot be uridylylated by GlnD, also results in constitutive inhibition of NifA activity by NifL⁵⁶. *In vitro*

SURFACE PLASMON RESONANCE
An optical phenomenon that occurs when light is reflected by thin metal films. The technology is used to monitor the progress of biomolecular interactions (for example, protein–protein interactions) in real-time.

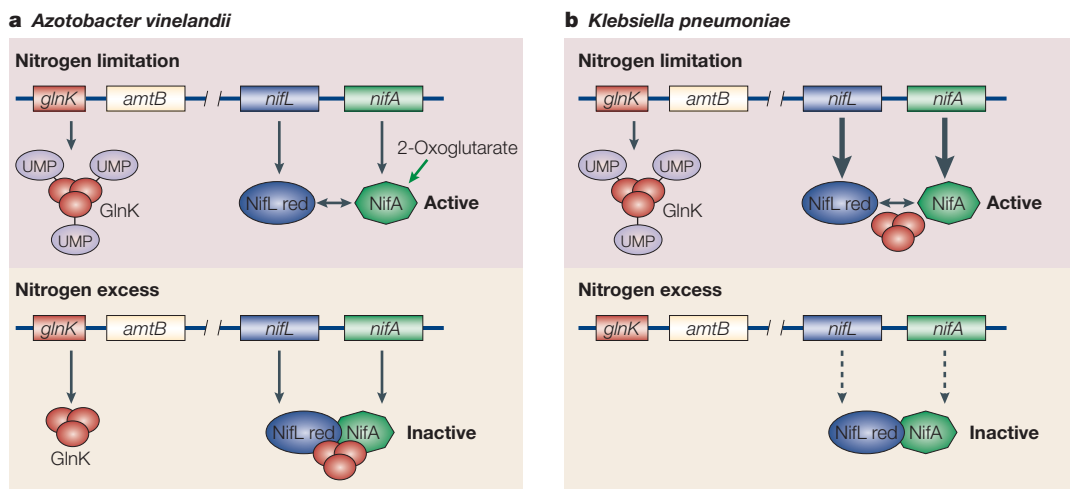


Figure 6 | Schematic to illustrate the different mechanisms by which the nitrogen signal is conveyed to regulate the NifL–NifA systems of *A. vinelandii* and *K. pneumoniae*. Transcription of the *glnK–amtB* and *nifL–nifA* operons is not subject to nitrogen regulation in *Azotobacter vinelandii* (a), whereas in *Klebsiella pneumoniae* (b), both operons are subject to nitrogen regulation by the NtrB–NtrC system. Consequently, under conditions of nitrogen excess, when the concentration of phosphorylated NtrC is relatively low, transcription of these operons decreases significantly in *K. pneumoniae*. In both organisms NifA is active under conditions of nitrogen limitation. In *A. vinelandii* this is promoted by binding of 2-oxoglutarate to NifA, which prevents inhibition by NifL, and also by uridylylation of GlnK, preventing its interaction with NifL. By contrast, in *K. pneumoniae* GlnK is required to prevent interaction of NifL and NifA under nitrogen-limiting conditions. The presence of GlnK is sufficient to prevent inhibition by NifL irrespective of uridylylation status. Under conditions of excess fixed nitrogen NifA is inactive in both *A. vinelandii* and *K. pneumoniae*. In *A. vinelandii* the non-modified form of GlnK interacts with NifL to promote the formation of a GlnK–NifL–NifA ternary complex under such conditions. By contrast, in *K. pneumoniae* there is insufficient GlnK under nitrogen excess conditions to prevent the interaction of NifL with NifA. Sequestration of non-modified GlnK by the membrane transporter AmtB reduces the concentration of cytoplasmic GlnK. Thick and dashed arrows indicate strong and low levels of *nifLA* expression, respectively. red, reduced.

pull-down assays and SURFACE PLASMON RESONANCE experiments have shown that the non-uridylylated form of GlnK interacts with NifL to inhibit NifA activity and that this interaction is prevented when GlnK is fully uridylylated⁵⁷. The GlnK–NifL interaction has also been detected *in vivo* using yeast two-hybrid assays⁵⁶. The N-terminal PAS domain of *A. vinelandii* NifL is not required for nitrogen sensing and GlnK can interact with the C-terminal GHKL domain to promote the formation of a GlnK–NifL–NifA ternary complex⁵⁷.

The metabolic signals ATP and 2-oxoglutarate, which control the interactions of PII-signal proteins with their targets, are also important modulators of the *A. vinelandii* NifL–NifA interaction. The stability of the NifL–NifA binary complex is increased by the binding of adenosine nucleotides to the C-terminal domain of NifL, which favours inhibition of NifA activity^{36,58}. This is countered by the binding of 2-oxoglutarate to the GAF domain of *A. vinelandii* NifA which relieves inhibition by NifL in the absence of GlnK^{19,24,59}. The response of the NifL–NifA system to 2-oxoglutarate is within the physiological concentration range of this ligand, which increases from ~100 μ M under carbon-limiting, nitrogen excess conditions to 1 mM under conditions of nitrogen-limitation. This allows integration of the nitrogen signal that is received through glutamine-dependent uridylylation of GlnK with the carbon signal that is received through the binding of 2-oxoglutarate to the GAF domain of NifA. So, under nitrogen-limiting conditions, when GlnK is uridylylated, the carbon status controls the NifL–NifA

interaction by the interaction of 2-oxoglutarate with the GAF domain of NifA. Conversely, under conditions of nitrogen excess, the non-modified form of GlnK interacts with NifL to promote the ternary GlnK–NifL–NifA complex, even at high 2-oxoglutarate concentrations and therefore the nitrogen signal overrides the carbon signal.

There are therefore several differences in the mechanisms by which the NifL–NifA systems from *K. pneumoniae* and *A. vinelandii* respond to fixed nitrogen. In *K. pneumoniae*, GlnK is required to prevent NifL from inhibiting NifA under nitrogen-limiting conditions. Conversely, in *A. vinelandii*, the non-uridylylated form of GlnK is required for NifL to inhibit NifA under conditions of nitrogen excess. Under conditions of nitrogen limitation, the binding of 2-oxoglutarate to *A. vinelandii* NifA is required to prevent inhibition by NifL, whereas in the *K. pneumoniae* system, the interaction with GlnK seems to provide this function (FIG. 6).

In some of the diazotrophic proteobacteria that do not have NifL, there is evidence that PII signal-transduction proteins either directly or indirectly modulate the activity of NifA through the GAF domain. In *A. brasilense*, *Herbaspirillum seropedicae* and *R. rubrum* the *glnB* gene is essential for nitrogen fixation and the uridylylated form of GlnB seems to be required to activate NifA when fixed nitrogen is limiting^{20,60,61}. By contrast, in *R. capsulatus* and *A. caulinodans*, the PII-like proteins GlnB and GlnK are required for inhibition of NifA activity under conditions of fixed nitrogen excess^{62,63}. This indicates that only the non-uridylylated forms of

these proteins are competent to modulate NifA activity. Direct interaction of GlnB and GlnK with *R. capsulatus* NifA proteins has been demonstrated in yeast two-hybrid experiments⁶⁴. So, in common with the *K. pneumoniae* and *A. vinelandii* systems, the mechanism by which PII signal-transduction proteins regulate NifA activity differs from organism to organism.

Conclusions

Comparison of the regulatory systems that control nitrogen fixation in different proteobacterial species reveals variations around two common themes. The first theme concerns domain arrangements of key regulatory proteins. Different combinations of PAS domains, histidine-kinase domains, receiver domains, AAA⁺ domains and helix–turn–helix domains provide a variety of molecular functions that are involved in responses to oxygen (FixL–FixJ), redox (RegS–RegR) or nitrogen (NtrB–NtrC) (FIG. 2). The second concerns regulatory network architecture, because regulatory responses depend on how different conserved regulatory elements interact with each other (FIG. 3). Moreover,

such interactions depend subtly on protein–protein interactions that can vary significantly, as shown by the various interactions exhibited by the PII-like proteins GlnB and GlnK (see, for example, FIG. 6). The combinatorial design of regulatory proteins and regulatory cascades, together with the plasticity of protein–protein interactions, generates considerable plasticity in these systems. As a consequence it is extremely difficult to generalize observations across regulatory systems, even at relatively close evolutionary range (compare, for example, the regulatory schemes controlling nitrogen fixation in *S. meliloti* and *B. japonicum* in FIG. 3). This plasticity underlies what seems to be the relatively fast evolution of regulatory networks, which provides a mechanism for adaptation to a wide range of physiological constraints. The picture would have been even more diverse if we had included other groups of diazotrophs, for example, the cyanobacteria in this review. Indeed unlike Proteobacteria, cyanobacteria do not seem to use NifA for *nif* regulation and they use phosphorylation, rather than uridylylation, to modulate the activity of PII signal-transduction proteins⁶⁵.

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Competing interests statement

The authors declare that they have no competing financial interests.

Online links

DATABASES

The following terms in this article are linked online to:

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Azotobacter vinelandii | *Rhodospirillum rubrum*
SwissProt: <http://www.ca.expasy.org/sprot/>
 ArsA | DraG | DraT | FixJ | FixK | FixL | GlnB | GlnK | MinD | NifA | NifL | NtrB | NtrC
The Protein Data Bank: <http://www.rcsb.org/pdb/>
 Fe protein | FixJ receiver domain | FixL haem domain | MoFe protein | NtrC1 | stabilized complexes

FURTHER INFORMATION

Azotobacter database: <http://www.azotobacter.org/>
Bradyrhizobium japonicum genome project: <http://www.kazusa.or.jp/rhizobase/Bradyrhizobium/index.html>
Sinorhizobium meliloti genome: <http://bioinfo.genopole-toulouse.prd.fr/annotation/iANT/bacteria/rhime/>
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