Review

P_{II} signal transduction proteins: pivotal players in post-translational control of nitrogenase activity

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The fixation of atmospheric nitrogen by the prokaryotic enzyme nitrogenase is an energy-expensive process and consequently it is tightly regulated at a variety of levels. In many diazotrophs this includes post-translational regulation of the enzyme's activity, which has been reported in both bacteria and archaea. The best understood response is the short-term inactivation of nitrogenase in response to a transient rise in ammonium levels in the environment. A number of proteobacteria species effect this regulation through reversible ADP-ribosylation of the enzyme, but other prokaryotes have evolved different mechanisms. Here we review current knowledge of post-translational control of nitrogenase and show that, for the response to ammonium, the P_{II} signal transduction proteins act as key players.

Introduction

Biological nitrogen fixation, the reduction of atmospheric N₂ to NH₃ by nitrogen-fixing bacteria, is a key step in the nitrogen cycle. This process is catalysed by nitrogenase, the most common form of which is the molybdenum nitrogenase, composed of dinitrogenase (MoFe protein or NifDK), an $\alpha_2\beta_2$ tetramer encoded by the *nifD* and *nifK* genes, respectively, and dinitrogenase reductase (Fe protein or NifH), a γ_2 homodimer encoded by the *nifH* gene. The NifH protein is responsible for ATP-hydrolysis-driven electron transport to the NifDK protein, which contains the site for the reduction of dinitrogen to ammonium (Seefeldt *et al.*, 2009). The reduction of N₂ to two molecules of ammonium is an energy-expensive process requiring the hydrolysis of 16 ATPs.

To avoid energy wastage, diazotrophs have evolved both transcriptional and post-translational mechanisms to shut-down nitrogen fixation when ammonium is available in the environment. Post-translational control of nitrogenase activity has been found in a range of diazotrophs and affords a rapid and reversible mechanism by which the organism can respond to transient changes in the environment. Here we review current knowledge of the different mechanisms of post-translational control of nitrogenase. We focus on the two best-described systems: ADP-ribosylation of NifH, which occurs in proteobacteria, and the interaction of Nifl regulatory proteins with NifDK in archaea, which potentially also operates in some anaerobic diazotrophic bacteria.

Regulation of nitrogenase activity by reversible ADP-ribosylation

Historical perspective

The process of metabolic inactivation of nitrogenase in response to ammonium was first described in *Azotobacter vinelandii* (Burris & Wilson, 1946); this phenomenon was later identified in other prokaryotes and named nitrogenase 'switch-off' (Zumft & Castillo, 1978). Different mechanisms are used to regulate nitrogenase post-translationally depending on the organism. The best-studied mechanism operates through reversible ADP-ribosylation of NifH (reviewed by Nordlund, 2000). This system responds to not only the presence of ammonium but also a decrease in the availability of cellular energy, in response to either darkness in phototrophs such as *Rhodospirillum rubrum* or anaerobiosis as seen in *Azospirillum brasilense*.

Researchers at the University of Wisconsin demonstrated that nitrogenase switch-off in *R. rubrum* occurred due to the addition of an ADP-ribosyl group to the arginine 101 residue of one subunit of the NifH protein (Pope *et al.*, 1985). This residue is located in the docking site between NifH and NifDK during the electron transfer cycle of nitrogenase (Schindelin *et al.*, 1997). ADP-ribosylation of this residue presumably disrupts the contact between the nitrogenase components, blocking the electron transfer reaction necessary to reduce N₂.

The enzymes involved in nitrogenase modification in *R. rubrum* were purified and characterized *in vitro* (Pope *et al.*, 1986; Saari *et al.*, 1986). ADP-ribosylation of NifH is

catalysed by dinitrogenase reductase ADP-ribosyltransferase (DraT) and the process is reversed by a different enzyme, dinitrogenase reductase ADP-ribosyl glycohydrolase (DraG). Some years later, the same group cloned, sequenced and analysed the function of the structural genes for these enzymes, *draT* and *draG*, from *R. rubrum* (Fitzmaurice *et al.*, 1989).

Distribution of the nitrogenase ADP-ribosylation system in prokaryotes

The *draTG* genes have also been sequenced and characterized in *A. brasilense* (Zhang *et al.*, 1992), *Azospirillum lipoferum* (Fu *et al.*, 1990b; Inoue *et al.*, 1996), *Rhodobacter capsulatus* (Masepohl *et al.*, 1993) and in *Azoarcus* sp. (Oetjen & Reinhold-Hurek, 2009). The genes usually constitute an operon and are presumably co-transcribed.

A BLASTX analysis identified 29 different bacterial genera coding for DraT orthologues, including members of the alpha-, beta-, gamma- and deltaproteobacteria, verrucomicrobia, deferribacteres, chrysiogenetes and some unclassified bacteria (Table 1). DraG orthologues are widespread in nature (see below). As all the species listed in Table 1 also

have NifH orthologues, we predict that these organisms regulate nitrogenase through ADP-ribosylation. In most cases, *draT* and *draG* are adjacent to each other; in some organisms like *Azoarcus* sp. they are approximately 6 kb apart. *draT* is usually located near the nitrogenase structural genes or genes involved in nitrogenase co-factor biosynthesis.

Protein ADP-ribosylation has been reported to perform a variety of functions, and genes coding for ADP-ribosyltransferases are found in organisms ranging from viruses to humans. DraT is classified as part of the R-S-E 'cholera toxin-like' group of ADP-ribosyl-transferases, in which R-S-E identifies a conserved catalytic triad (Hottiger *et al.*, 2010). However, DraT has low sequence similarity to other members of the R-S-E group and should therefore be considered as a particular subgroup. In contrast, DraG belongs to the ADP-ribosyl-hydrolase family, and shares a much higher sequence and structural similarity to all members of this family, including orthologues in archaea, bacteria and eukarya (Koch-Nolte *et al.*, 2008). DraG orthologues are widely distributed in prokaryotes, including organisms that do not encode DraT or NifH proteins (Oetjen & Reinhold-Hurek, 2009), suggesting that these orthologues have evolved different functions.

Table 1. Organisms with DraT orthologues

The *A. brasilense* DraT was used as a query to search orthologues in the NCBI nr database using BLASTX. All hits reported have an E-value $< 3 \times 10^{-21}$.

Alphaproteobacteria <i>Azospirillum brasilense</i> <i>Azospirillum lipoferum</i> <i>Azospirillum</i> sp. B510 <i>Magnetospirillum magneticum</i> AMB1 <i>Magnetospirillum magnetotacticum</i> MS-1 <i>Magnetospirillum gryphiswaldense</i> MSR-1 <i>Rhodobacter capsulatus</i> SB 1003 <i>Rhodobacter sphaeroides</i> ATCC 17025 <i>Rhodopseudomonas palustris</i> BisB5 <i>Rhodopseudomonas palustris</i> BisB18 <i>Rhodopseudomonas palustris</i> HaA2 <i>Rhodopseudomonas palustris</i> CGA009 <i>Rhodopseudomonas palustris</i> TIE-1 <i>Rhodopseudomonas palustris</i> BisA53 <i>Rhodospirillum rubrum</i> ATCC 11170 Gammaproteobacteria <i>Acidithiobacillus ferrooxidans</i> ATCC 23270 <i>Acidithiobacillus ferrooxidans</i> ATCC 53993 <i>Allochromatium vinosum</i> DSM 180 <i>Methylobacter tundripaludum</i> SV96 <i>Methylococcus capsulatus</i> str. Bath <i>Methylomonas methanica</i> MC09 <i>Teredinibacter turnerae</i> T7901 <i>Tolomonas auensis</i> DSM 9187 Deferribacteres <i>Calditerrivibrio nitroreducens</i> DSM 19672 <i>Denitrovibrio acetiphilus</i> DSM 12809	Deltaproteobacteria <i>Anaeromyxobacter</i> sp. Fw109-5 <i>Anaeromyxobacter</i> sp. K <i>Desulfobacterium autotrophicum</i> HRM2 <i>Desulfuromonas acetoxidans</i> DSM 684 <i>Geobacter bemidjensis</i> Bem <i>Geobacter lovleyi</i> SZ <i>Geobacter metallireducens</i> GS-15 <i>Geobacter</i> sp. M21 <i>Geobacter</i> sp. FRC-32 <i>Geobacter uraniireducens</i> Rf4 <i>Geobacter sulfurreducens</i> PCA <i>Pelobacter carbinolicus</i> DSM 2380 <i>Pleobacter propionicus</i> DSM 2379 Betaproteobacteria <i>Accumulibacter phosphatis</i> UW1 <i>Azoarcus</i> sp. BH72 <i>Dechloromonas aromatica</i> RCB <i>Sideroxydans lithotrophicus</i> ES-1 <i>Rubrivivax benzoatilyticus</i> JA2 Unclassified Proteobacteria <i>Magnetococcus</i> sp. MC-1 Verrucomicrobia <i>Coralimargarita akajimensis</i> DSM 45221 <i>Verrucomicrobiae bacterium</i> DG1235 Chrysiogenetes <i>Desulfurispirillum indicum</i> S5
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The regulation of DraT and DraG activities

DraT and DraG activities are subject to opposing regulation *in vivo* according to the prevailing nitrogen or energy levels (Kanemoto & Ludden, 1984; Liang *et al.*, 1991; Zhang *et al.*, 1993). The model proposed by the Wisconsin group (reviewed by Zhang *et al.*, 1997) suggested that under nitrogen-fixing conditions, DraT is inactive and DraG is active. When ammonium is added or when the energy levels decrease, DraT becomes active and DraG is inactivated; DraT remains active for a short period while DraG remains inactive until consumption of the ammonium by bacterial metabolism. After ammonium exhaustion or restoration of the energy levels, DraG is reactivated to restore nitrogenase activity (Fig. 1). The same regulation model was assumed to occur in darkness-induced nitrogenase switch-off.

The major question posed by this model was to understand how the signal of the prevailing environmental ammonium or cellular energy levels could be transduced to change DraT and DraG activities. The signalling pathway is likely to be similar in different organisms, as demonstrated by the fact that *A. brasilense draTG* genes in *draT/G* mutants of *R. rubrum* re-establish NifH ADP-ribosylation in response to ammonium or energy depletion and vice versa (Zhang *et al.*, 1995). Furthermore, heterologous expression of *dra* genes in organisms that do not regulate nitrogenase by ADP-ribosylation led to regulated ammonium nitrogenase switch-off (Fu *et al.*, 1990a; Inoue *et al.*, 1996; Yakunin *et al.*, 2001). Hence, the ammonium-sensing components of the system were likely to be part of a ubiquitous bacterial nitrogen control system.

Role of P_{II} and AmtB proteins in the ammonium-elicited nitrogenase ADP-ribosylation pathway

P_{II} protein function

Nitrogen metabolism in prokaryotes is regulated by a highly conserved family of trimeric proteins named P_{II} (Forchhammer, 2008; Leigh & Dodsworth, 2007). Many prokaryotes encode two P_{II} proteins, usually named GlnK and GlnB, and some organisms encode a third P_{II} named GlnJ. The *glnK* genes are defined by their transcriptional association with the ammonium transporter gene, *amtB*. The *glnB* genes are usually linked to the glutamine synthetase (*glnA*) or the NAD⁺ synthetase (*nadE*) genes (Sant'Anna *et al.*, 2009). There are some exceptions to these rules, for instance in *A. brasilense* the *glnZ* gene (orthologous to *glnK*) is not linked to *amtB* (de Zamaroczy, 1998). A different subgroup of P_{II} is the Nif₁ and Nif₂ proteins, which are found in methanogenic archaea and in strictly anaerobic bacteria. The *nifl* genes are located in the vicinity of nitrogenase structural genes (Sant'Anna *et al.*, 2009).

P_{II} proteins bind small molecules such as ATP, ADP and 2-oxoglutarate (2-OG), known as the P_{II} effectors. ATP and ADP bind to the same site in the lateral clefts between each

subunit of the P_{II} trimers in a competitive manner (Xu *et al.*, 1998, 2001; Jiang & Ninfa, 2007). Each trimer can bind up to three adenine nucleotide molecules, thus the *in vivo* population of P_{II} proteins can have different combinations of bound ATP/ADP (Jiang & Ninfa, 2009; Jiang & Ninfa, 2007). Three 2-OG binding sites are also located in the lateral clefts between each subunit of P_{II} (Truan *et al.*, 2010; Fokina *et al.*, 2010).

Many P_{II} proteins are subject to reversible covalent modification in a flexible loop, the T-loop, that projects from each subunit of the P_{II} trimer. In proteobacteria, P_{II} is uridylylated and the modification status is determined by the activity of a bifunctional uridylyl-transferase/uridylyl-removing enzyme (GlnD) whose activity is regulated by the intracellular glutamine pool. High intracellular glutamine indicates high nitrogen levels and triggers de-uridylylation of P_{II}. Conversely, under low nitrogen, glutamine levels are reduced and P_{II} is uridylylated (Jiang *et al.*, 1998).

The signal transduction function of P_{II} depends on conformational changes determined by its post-translational modification status and the effectors that are bound to the three lateral clefts of the trimer. The intracellular availability of each P_{II} effector defines the prevailing conformation of P_{II} and thereby regulates its interaction with a variety of different P_{II} target proteins. The balance between ATP/ADP signals the cellular energy status, and 2-OG availability signals not only the cellular carbon status but also nitrogen availability, because 2-OG is a key intermediate in ammonium assimilation.

It is believed that the most ancient protein target regulated by P_{II} proteins is the ammonium transport protein AmtB (Javelle & Merrick, 2005). Some prokaryotes encode more than one Amt protein, and in nearly all cases *amt* genes are co-transcribed with P_{II} coding genes (Sant'Anna *et al.*, 2009). AmtB is a trimeric integral membrane protein with each monomer forming a membrane-spanning pore which conducts ammonia (Khademi *et al.*, 2004). Depending on the uridylylation status of P_{II}, and the intracellular levels of ATP, ADP and 2-OG, P_{II} can form a complex with AmtB on the membrane, the T-loop of P_{II} acting as a plug to block ammonia flux through the transporter (Conroy *et al.*, 2007; Gruswitz *et al.*, 2007; Radchenko *et al.*, 2010). Interaction between Amt and P_{II} is widespread in prokaryotes and has been described in several bacteria and also in archaeal species (Tremblay & Hallenbeck, 2009).

Genetic evidence identified P_{II} and AmtB proteins as part of the ammonium signalling pathway

Given the key role of P_{II} proteins in nitrogen metabolism, these proteins were obvious candidates to control DraT and DraG activities. The first indication that P_{II} could regulate DraT and DraG was reported in *R. capsulatus* where a *glnB* mutant showed defective ammonium-induced nitrogenase inactivation (Hallenbeck, 1992).

Fig. 1. Regulation of nitrogenase activity by reversible ADP-ribosylation. (a) In response to the presence of ammonium or energy depletion, DraT is activated and covalently links one ADP-ribosyl group (ADPR) to one subunit of the NifH, turning it to an inactive form. When the ammonium is consumed by the cellular metabolism or when the energy levels return to normal, DraG removes the ADPR attached to NifH reactivating nitrogenase. (b) The post-translational regulation of nitrogenase can be followed by measurement of the nitrogenase activity itself using the acetylene reduction method. The arrows labelled switch-off and switch-on indicate the times at which the negative stimulus for nitrogenase (ammonium or energy depletion) was added or removed, respectively. The intensity of the switch-off effect varies according to the organism and to the type and amount of the negative stimulus. Some organisms inactivate nitrogenase at the metabolic level as shown in (b), without NifH ADP-ribosylation. (c) The ADP-ribosylation of NifH can be followed by separation of the unmodified and ADP-ribosylated NifH (NifH-ADPR) by Western blot; NifH-ADPR shows a slower migration rate. The activities of DraT and DraG are subjected to opposing regulation during nitrogenase switch-off. Active enzyme (+); inactive enzyme (-).

Further studies supported the participation of P_{II} proteins in the regulation of DraT/DraG activities in *R. rubrum* (Zhang *et al.*, 2001b), *A. brasilense* (Klassen *et al.*, 2001, 2005; Huergo *et al.*, 2005), *R. capsulatus* (Drepper *et al.*, 2003) and *Azoarcus* sp. (Martin & Reinhold-Hurek, 2002). All these studies reported defects in nitrogenase switch-off and/or ADP-ribosylation in P_{II} or *glnD* mutant backgrounds.

An important breakthrough in the field was the observation that the *amtB* gene was necessary for ammonium-induced ADP-ribosylation of nitrogenase in *R. capsulatus* (Yakunin & Hallenbeck, 2002). Ammonium uptake was not affected in the *amtB* mutant owing to the presence of alternative uptake pathways, and hence it was suggested that AmtB might play a role in the ammonium sensing

mechanism (Yakunin & Hallenbeck, 2002). The same response was later observed in other organisms.

The ammonium regulation model in *A. brasilense*

A. brasilense encodes only two P_{II} proteins (GlnB and GlnZ) and one Amt protein (AmtB), and therefore provides a simpler genetic background than *R. capsulatus* and *R. rubrum* in which to study the role of P_{II} and Amt proteins in NifH ADP-ribosylation. Below we describe the current knowledge of the ammonium signalling pathway in this organism and then discuss how the resulting model can be applied to other bacteria.

The first insight into the ammonium signalling pathway in *A. brasilense* came from the observation that *ntrBC* mutant

strains do not ADP-ribosylate NifH in response to ammonium (Zhang *et al.*, 1994). In this organism, *amtB*, *glnZ* and *glnB* are all under NtrC transcriptional control (de Zamaroczy *et al.*, 1993; de Zamaroczy, 1998; van Dommelen *et al.*, 1998; Huergo *et al.*, 2003), suggesting that alteration in the expression of these genes could be the cause of the observed phenotype in an *ntrC* mutant. A *glnZ* mutant showed only partial recovery of ammonium-induced switch-off (Klassen *et al.*, 2001) and NifH modification persisted for a longer period after ammonium addition (Huergo *et al.*, 2006b). A *glnB* mutant showed neither ammonium-induced nitrogenase switch-off (Klassen *et al.*, 2005) nor NifH ADP-ribosylation (Huergo *et al.*, 2006b), whilst GlnB overexpression resulted in NifH modification under nitrogen-fixing conditions (Huergo *et al.*, 2005). These results supported the hypothesis that GlnB and GlnZ might be involved in the regulation of DraT and DraG activities, respectively.

As had been shown previously in *E. coli* (Coutts *et al.*, 2002), P_{II} proteins in *A. brasilense* are sequestered to the cytoplasmic membrane in response to an ammonium shock in an AmtB-dependent fashion (Huergo *et al.*, 2006b). A time-course analysis of GlnB/GlnZ uridylylation, membrane sequestration and NifH ADP-ribosylation cycles in response to ammonium showed that these processes are synchronized (Huergo *et al.*, 2006b). An *A. brasilense amtB* mutant did not ADP-ribosylate NifH in response to ammonium, but showed wild-type GlnB/GlnZ uridylylation cycles in response to ammonium, suggesting that ammonium uptake was not affected in the absence of AmtB. However, as expected, the *amtB* mutant failed to sequester GlnB and GlnZ to the membrane, supporting the notion that this event could be part of the NifH modification signalling pathway (Huergo *et al.*, 2006b).

Cellular localization studies showed that DraG behaves exactly as the P_{II} proteins do, in that upon an ammonium shock it is also reversibly associated with the membrane in an AmtB-dependent manner. The movement of DraG to the membrane is synchronized with NifH modification, supporting the idea that membrane binding could inactivate DraG after ammonium addition (Huergo *et al.*, 2006b). This model would explain why NifH is not modified in an *amtB* mutant, as DraG remains in the cytosol and thus active after ammonium addition. So why is membrane sequestration of DraG AmtB-dependent? One possibility is that DraG interacts with AmtB indirectly through the formation of a ternary complex involving a P_{II} protein.

To study whether DraT and/or DraG could interact with P_{II} *in vivo*, strains expressing His-tagged versions of these enzymes were constructed and used to perform pull-down experiments in which cells were collected under nitrogen-fixing conditions and after an ammonium shock (Huergo *et al.*, 2006a). DraT interacted with de-uridylylated GlnB, and DraG interacted with both uridylylated and de-uridylylated GlnZ, though the DraG–GlnZ interaction was stronger with non-uridylylated GlnZ (Huergo *et al.*,

2006a). The roles of the P_{II} effectors and P_{II} uridylylation in the regulation of DraT–GlnB or DraG–GlnZ complex formation were studied *in vitro*. Both complexes were more stable when P_{II} was de-uridylylated and when the ATP/ADP ratio and the 2-OG concentrations were low (Huergo *et al.*, 2009). The same response was observed for the AmtB–GlnB and AmtB–GlnZ complexes. A ternary complex between AmtB–GlnZ–DraG could be formed *in vitro* in the presence of ADP, explaining the role of AmtB in DraG sequestration to the membrane after an ammonium shock (Huergo *et al.*, 2007).

Based on metabolomics data from *E. coli*, it is expected that the 2-OG concentrations will drop and that the glutamine levels will increase seconds after ammonium addition to a nitrogen-starved culture (Yuan *et al.*, 2009; Radchenko *et al.*, 2010). The increase in glutamine concentration promotes de-uridylylation of GlnB and GlnZ, catalysed by GlnD (Araújo *et al.*, 2008), whilst the drop in 2-OG levels facilitates the exchange of ATP for ADP in the nucleotide-binding sites of the P_{II} proteins (L. F. Huergo, unpublished data), thereby stabilizing the DraT–GlnB and the DraG–GlnZ–AmtB complexes (Huergo *et al.*, 2009) (Fig. 2). In this scenario, DraT is activated by binding to de-uridylylated ADP-bound GlnB, and DraG is inactivated by binding to the membrane-bound AmtB–GlnZ complex (Fig. 2) (Huergo *et al.*, 2009).

According to this model (Fig. 2), DraG would be inactivated when bound to the AmtB–GlnZ complex on the membrane, and hence DraG should not be inactivated in a *glnZ* mutant. Surprisingly, NifH ADP-ribosylation upon ammonium addition was observed in this mutant despite the fact that DraG was not sequestered to the membrane (Huergo *et al.*, 2006b). It is possible that, in the absence of GlnZ, GlnB could partially substitute for GlnZ function. Indeed, GlnB and GlnZ are 67 % identical in sequence and the structure of the DraG–GlnZ complex shows that only three of the nine GlnZ amino acid residues involved in polar contacts with DraG differ in GlnB (Rajendran *et al.*, 2011).

Structural analysis of the DraG–GlnZ complex revealed that upon GlnZ binding, the active site of DraG would be spatially hindered from binding to ADP-ribosylated NifH. However, a simple model where formation of a DraG–GlnZ complex would inactivate DraG is not supported by the lack of DraG regulation in an *amtB* mutant, indicating that DraG inactivation requires the membrane-binding event. Our current hypothesis is that the DraG–GlnZ complex is not stable enough to effectively inactivate DraG; however, the binding of the DraG–GlnZ complex to AmtB further stabilizes the DraG–GlnZ interaction and provides effective DraG inactivation by steric hinderance of the active site (Rajendran *et al.*, 2011).

Previous studies using purified *R. rubrum* DraT and DraG showed that both enzymes are active *in vitro*, suggesting that the enzymes would be regulated by loosely bound negative effectors *in vivo* (Saari *et al.*, 1986; Lowery & Ludden, 1988). Whilst our model supports the proposal that DraG is negatively regulated, we suggest that DraT is

Fig. 2. Role of the AmtB and P_{II} proteins in the regulation of DraT and DraG activities in response to ammonium. (a) Under nitrogen-fixing conditions GlnB and GlnK are fully uridylylated, located in the cytoplasm and presumably saturated with ATP and 2-OG (denoted by ⁺), therefore are not complexed to DraT and DraG. Under this condition, DraT is inactive and DraG is located in the cytoplasm and active; consequently NifH is not modified, allowing nitrogenase activity. (b) Upon an ammonium shock, ammonium assimilation by the glutamine synthetase/glutamate synthase increases the intracellular glutamine and decreases the 2-OG pools. The rise in glutamine activates the uridylyl-removing activity of GlnD, thus GlnB and GlnK are de-uridylylated. The decrease in the 2-OG pool facilitates the exchange of ATP bound to P_{II} to ADP; consequently, GlnB and GlnK are bound to ADP (denoted by *). 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.

activated by interaction with de-uridylylated ADP-bound GlnB. Interestingly, SDS-PAGE analysis of purified DraT from *R. rubrum* indicated a contaminant band with the molecular mass expected for a P_{II} monomer. The authors also noted that attempts to remove this contaminant resulted in loss of DraT activity. Furthermore, DraT was only active if ADP was present in all purification steps, which, according to our model, would stabilize the active DraT–GlnB complex (Lowery & Ludden, 1988).

Ammonium regulation in *R. rubrum*

R. rubrum has three P_{II} and two AmtB coding genes, named *glnB*, *glnKamtB1* and *glnJamtB2*; the latter two pairs are organized in operons (Zhang *et al.*, 2001b).

Ammonium-induced NifH ADP-ribosylation analysis indicated a normal response in *glnB*, *glnK* and *glnBK* mutants, but little modification was seen in *glnBJ* and *glnBKJ* mutants, suggesting that GlnB or GlnJ are necessary for the correct regulation of DraT and DraG (Zhang *et al.*, 2001). Expression of a constitutively de-uridylylated GlnB (Y51F mutation) led to DraT activation under nitrogen-fixing conditions (Zhang *et al.*, 2000), and DraT–GlnB interaction was detected using the yeast two-hybrid system (Zhu *et al.*, 2006). These data are consistent with the *A. brasilense* model (Fig. 2) where DraT is activated by forming a complex with de-uridylylated GlnB after an ammonium shock.

In *R. rubrum*, DraG was also found associated with the membrane after an ammonium shock, a phenomenon that

required the presence of AmtB1 and GlnJ or GlnB (Wang *et al.*, 2005; Zhang *et al.*, 2006). Similarly, NifH ADP-ribosylation in this bacterium required the presence of those proteins. *In vitro* assays showed that all three P_{II} proteins interact with AmtB1-containing membranes though the interaction was stronger with GlnJ and was stabilized by low ATP/ADP ratio and low 2-OG concentrations (Wolfe *et al.*, 2007; Teixeira *et al.*, 2008). Cross-linking experiments using membrane preparations of *R. rubrum* and purified DraG showed the presence of an adduct cross-reacting with DraG, AmtB and P_{II} antibodies (Akentieva, 2008), suggesting that *R. rubrum* DraG is targeted to the membrane via the AmtB1–GlnJ complex and, similarly to the model proposed for *A. brasilense*, it is thereby inactivated (Fig. 2).

The crystal structure of *R. rubrum* DraG was solved in both unbound and ADP-ribose-bound forms (Berthold *et al.*, 2009). This work revealed the structure of a putative reaction intermediate analogue and suggested a mechanism of catalysis for ADP-ribose removal (Berthold *et al.*, 2009). The structure of *R. rubrum* DraG is very similar to the *A. brasilense* orthologue (Li *et al.*, 2009), further supporting that they share similar regulatory mechanisms.

Ammonium regulation in *R. capsulatus*

R. capsulatus has two P_{II} proteins encoded by *glnB*, which is co-transcribed with the glutamine synthetase gene (*glnA*), and *glnK*, which is co-transcribed with *amtB* (Zinchenko *et al.*, 1994; Drepper *et al.*, 2003). A *glnBglnK* double mutant failed to inactivate and ADP-ribosylate NifH upon an ammonium shock (Drepper *et al.*, 2003). Later, it was shown that both P_{II} proteins were necessary for the proper regulation of ammonium-induced nitrogenase switch-off and NifH ADP-ribosylation (Tremblay *et al.*, 2007). Furthermore, interactions between the two P_{II} proteins and DraT were detected by yeast two-hybrid analysis (Pawlowski *et al.*, 2003).

Expression of a non-uridylylatable form of GlnK (Y51F mutant) in the *glnK* mutant was sufficient to re-establish ammonium-induced NifH ADP-ribosylation, suggesting that GlnK uridylylation is not critical to its function (Tremblay *et al.*, 2007). This is consistent with the *in vitro* analysis of the GlnZ–DraG, GlnB–DraT interactions in *A. brasilense* where the major regulators of these interactions were shown to be the ATP/ADP ratio and 2-OG levels rather than P_{II} uridylylation (Huergo *et al.*, 2009). Both *R. capsulatus* GlnB and GlnK are sequestered to the membrane after an ammonium shock in an AmtB-dependent manner (Tremblay *et al.*, 2007). Interestingly, *amtB* is required for ammonium-induced NifH ADP-ribosylation but not for ammonium uptake (Yakunin & Hallenbeck, 2002), and AmtB variants that do not form a complex with GlnK fail to complement NifH ADP-ribosylation in the *amtB* mutant (Tremblay & Hallenbeck, 2008). All these data support the concept that movement of P_{II} to the membrane is critical for NifH regulation,

presumably to inactivate DraG by membrane sequestration as proposed in *A. brasilense* (Fig. 2).

Strikingly, transport-incompetent AmtB variants that form the AmtB–GlnK complex upon an ammonium shock fail to properly regulate nitrogenase. This result was interpreted as an indication that ammonia transport and/or the occupation of the AmtB pore by ammonia could be essential for nitrogenase regulation (Tremblay & Hallenbeck, 2008). It is possible that ammonia transport through AmtB, whose mechanism is still under debate, could alter the intracellular levels of metabolites such as ATP/ADP, thus eliciting the protein–protein interactions required for NifH ADP-ribosylation.

Ammonium regulation in *Azoarcus* sp. strain BH72

Azoarcus sp. strain BH72 has one *draT* gene and two unlinked *draG* genes, and the nitrogenase NifH protein is subject to ADP-ribosylation in response to either ammonium addition or oxygen deprivation (Oetjen & Reinhold-Hurek, 2009). However, whilst a *draT* mutant fails to ADP-ribosylate NifH, it still shows nitrogenase switch-off in response to either ammonium addition or anaerobiosis. Hence, nitrogenase switch-off is independent of ADP-ribosylation and is presumed to be mediated by a second unknown mechanism (Oetjen & Reinhold-Hurek, 2009). *Azoarcus* sp. strain BH72 has three P_{II} and two AmtB-coding genes – *glnB*, *glnKamtB* and *glnYamtY* – the latter two pairs are organized in operons (Martin *et al.*, 2000). Ammonium-induced nitrogenase switch-off requires AmtB and GlnK, but not GlnB or GlnY, whilst NifH ADP-ribosylation requires the presence of GlnB, GlnK and AmtB (Martin & Reinhold-Hurek, 2002).

Surprisingly, GlnK was found associated with the membrane irrespective of the nitrogen status of the cell, and P_{II} membrane binding also occurred independently of AmtB (Martin & Reinhold-Hurek, 2002). As already discussed, in all other organisms studied, P_{II} interaction with the membrane is controlled by nitrogen levels and is AmtB-dependent. Hence, despite the fact that in *Azoarcus* sp. strain BH72 P_{II} and AmtB participate in both metabolic nitrogenase inactivation and NifH ADP-ribosylation, it is possible that this organism has evolved a distinct nitrogen signalling system.

The elusive energy depletion ADP-ribosylation signalling pathway

ADP-ribosylation of nitrogenase can be triggered by energy depletion, in response to either darkness in phototrophs such as *R. rubrum* or anaerobiosis as seen in *A. brasilense*. However, very little is known about the signalling mechanism responsible for regulation of DraT and DraG in response to energy depletion. Pioneer studies in *R. rubrum* indicated that DraG was located in the membrane fraction of the cells and could be removed by salt washes (Ludden & Burris, 1976). DraG purification used membrane

preparations as the starting material (Ljungström *et al.*, 1989; Saari *et al.*, 1984) and because large volumes were used, the cells were probably subjected to anaerobic/darkness conditions, and hence energy depletion, during cell harvesting. Conversely, when small volumes of *A. brasilense* are collected and quickly cooled, thereby avoiding energy depletion, DraG is predominantly found on the cytosol (Huergo *et al.*, 2006b). These data led to the hypothesis that DraG is negatively regulated by membrane binding after energy depletion, though this has not been proved by systematic experiments.

In *R. rubrum*, either GlnB or GlnJ is necessary for darkness-induced NifH ADP-ribosylation (Zhang *et al.*, 2001b). Analysis of nitrogenase activity in a *glnD* mutant suggested that DraT is activated in response to darkness but DraG is not reactivated upon exposure to light. Hence, DraG reactivation requires uridylylation of P_{II} proteins (Zhang *et al.*, 2005). An *R. rubrum* DraG N100K variant did not show inactivation by darkness (Kim *et al.*, 2004), and as the structure of the *A. brasilense* DraG–GlnZ complex (Rajendran *et al.*, 2011) showed that N100 is part of the P_{II} interaction surface, this supports the hypothesis that DraG–P_{II} interaction might play a role in darkness-induced switch-off.

Another study showed that in the absence of *amtB1*, *R. rubrum* had only a partial response to darkness (Zhang *et al.*, 2006); analysis of an *amtB1draG* double mutant led to the conclusion that DraG is not properly inactivated in the *amtB* background. Western blot analysis revealed that in response to darkness, GlnJ and GlnB are de-uridylylated and GlnJ associates with the membrane in an AmtB-dependent manner (Zhang *et al.*, 2006; Teixeira *et al.*, 2010). The signal triggering P_{II} protein de-uridylylation in response to darkness is not known, though a slight increase in glutamine levels has been reported (Kanemoto & Ludden, 1987).

The data reviewed so far support the proposal that darkness-induced NifH ADP-ribosylation in *R. rubrum* might share at least some of the same signalling mechanisms as ammonium-dependent inactivation (Fig. 2). However, this hypothesis is challenged by several sets of experimental data. In *R. rubrum*, neither GlnB nor GlnJ are de-uridylylated under darkness switch-off when cells are cultivated using N₂ as nitrogen source instead of glutamate (Teixeira *et al.*, 2010). Hence, as fully uridylylated P_{II} cannot interact with AmtB (Rodrigues *et al.*, 2011), AmtB–P_{II} complex formation seems dispensable for the darkness response in *R. rubrum* (Teixeira *et al.*, 2010). Furthermore, AmtB is not required for darkness-induced NifH ADP-ribosylation in *R. capsulatus* (Yakunin & Hallenbeck, 2002) or for anaerobically induced NifH ADP-ribosylation in *A. brasilense* (L. F. Huergo, unpublished data) and *Azoarcus* sp. strain BH72 (Martin & Reinhold-Hurek, 2002).

So, it is possible that distinct signalling mechanisms might operate to transduce the levels of ammonium and energy to the DraT/DraG system. The DraT and DraG enzymes have

opposite specificities for NifH bound to MgADP or MgATP: DraT is more active against the MgADP–NifH form and DraG against the MgATP–NifH (Saari *et al.*, 1984; Lowery & Ludden, 1988). Consequently, a decrease in the ATP/ADP ratio could lead to the energy depletion switch-off response. However, measurements suggest that darkness-induced ATP/ADP fluctuations are probably insufficient to account for the regulation (Paul & Ludden, 1984), and furthermore a reduction in the cellular ATP levels did not significantly affect the post-translational regulation of nitrogenase in *R. rubrum* (Zhang *et al.*, 2009).

The activities of *R. rubrum* DraT and DraG are also affected by the redox status of NifH in opposing ways. DraT is more active against oxidized NifH whilst DraG is more active using reduced NifH as substrate (Halbleib *et al.*, 2000a, b). Cellular GTP and NAD⁺ levels have also been suggested to regulate nitrogenase switch-off but neither of these signals has been subjected to a systematic evaluation (Norén & Nordlund, 1994, 1997; Norén *et al.*, 1997; Halbleib & Ludden, 1999).

In conclusion, although some studies in *R. rubrum* suggest that AmtB and P_{II} proteins participate in energy depletion switch-off, this dependence seems to occur only when cells are cultivated using glutamate but not using N₂ as nitrogen source. In *A. brasilense*, the data available so far suggest that AmtB and P_{II} are not involved in anaerobic switch-off.

Alternative nitrogenase switch-off mechanisms in bacteria

Whilst NifH ADP-ribosylation is the best characterized and probably the major ammonium-induced switch-off mechanism in bacteria, amongst the model organisms studied, it is only in *R. rubrum* that it appears to be the only form of nitrogenase inactivation. In that organism, substitution of the arginine residue at the ADP-ribosylation site of NifH by tyrosine (R101Y) abolishes nitrogenase inactivation (Zhang *et al.*, 1996).

By contrast, ammonium-induced nitrogenase inhibition without detectable NifH ADP-ribosylation has been reported in many diazotrophs, including *R. capsulatus* (Pierrard *et al.*, 1993), *A. brasilense* (Zhang *et al.*, 1993), *A. vinelandii* (Laane *et al.*, 1980), *Azospirillum amazonense* (Hartmann *et al.*, 1986), *Rhodobacter sphaeroides*, *Methylosinus trichosporium* (Yoch *et al.*, 1988), *Herbaspirillum seropedicae* (Fu & Burris, 1989), *Gluconacetobacter diazotrophicus* (Burris *et al.*, 1991), *Pseudomonas stutzeri* A1501 (Desnoues *et al.*, 2003) and *Azoarcus* sp. strain BH72 (see above). Despite these observations, little is known about the mechanism(s) involved in these organisms. Several authors have suggested that the addition of ammonium could decrease the ATP pool and/or divert electrons from nitrogenase, thus reducing its activity. However, systematic studies to examine such hypotheses are lacking.

Studies of this phenomenon in model organisms are limited to date. *R. capsulatus* strains expressing mutant

forms of the NifH protein in which the ADP-ribosylation site (R102) was substituted, still showed nitrogenase inactivation in response to ammonium (Pierrard *et al.*, 1993). In *A. brasilense*, the substitution of the equivalent arginine residue (NifH R101) by tyrosine, or knock out of the *draT* gene, also displayed partial ammonium-induced switch-off without NifH ADP-ribosylation (Zhang *et al.*, 1993). In *A. brasilense*, neither P_{II} nor AmtB seem to be necessary for ADP-ribosylation-independent switch-off, since an *ntrC* knockout strain still shows partial inactivation of nitrogenase in response to ammonium at levels comparable to that found in a *draT* mutant (Zhang *et al.*, 1994).

In *H. seropedicae*, knockout of either *glnK* or *amtB* partially abolished ammonium-induced nitrogenase switch-off (Noindorf *et al.*, 2006). In this organism, GlnK is sequestered to the cell membrane by AmtB a few minutes after ammonium addition (Huergo *et al.*, 2010). These results suggest that the ADP-ribosylation-independent nitrogenase switch-off in *H. seropedicae* could be controlled by formation of an AmtB–GlnK complex in the membrane (Noindorf *et al.*, 2011).

Post-translational control of nitrogenase activity in archaea by the Nifl proteins

Metabolic inactivation of archaeal nitrogenase in response to ammonium was first described in *Methanosarcina barkeri* (Lobo & Zinder, 1988). The inactivation was reversible and dependent on the amount of ammonium added. However, no electrophoretic alteration in NifH migration was detected by Western blotting, suggesting a mechanism other than NifH ADP-ribosylation (Lobo & Zinder, 1990; Kessler *et al.*, 2001).

Most studies on nitrogenase activity control in archaea have been performed in *Methanococcus maripaludis*. This organism has five P_{II} protein-coding genes, two of which, *nifl₁* and *nifl₂*, are located between the *nifH* and *nifD* genes (Kessler & Leigh, 1999). Deletion of *M. maripaludis nifl₁* or *nifl₂* abolished reversible inactivation of nitrogenase by ammonium (Kessler *et al.*, 2001), and the nitrogenase activity in cellular extracts of a $\Delta nifl_1 nifl_2$ mutant was higher than that in wild-type cell extracts. Addition of 2-OG increased nitrogenase activity in *M. maripaludis* wild-type, but not in $\Delta nifl_1 nifl_2$ cell extracts, and the intracellular levels decreased after an ammonium shock, supporting the hypothesis that 2-OG is sensed by Nifl proteins which, in turn, regulate nitrogenase activity (Dodsworth *et al.*, 2005).

The inhibitory effect of Nifl₁ and Nifl₂ on nitrogenase occurs via direct interaction of a Nifl₁/Nifl₂ heteromer with the nitrogenase NifDK component. This interaction is disrupted in the presence of 2-OG, which causes further oligomerization of the Nifl₁/Nifl₂ heteromer (Dodsworth & Leigh, 2006). The binding of the Nifl₁/Nifl₂ heteromer to NifDK prevents the association of the latter with NifH,

thereby disrupting the nitrogenase electron transfer mechanism (Dodsworth & Leigh, 2007). Based on these results, a model for regulation of nitrogenase activity in *M. maripaludis* was proposed (Leigh & Dodsworth, 2007) (Fig. 3). Under nitrogen-fixing conditions, the intracellular concentration of 2-OG is high; 2-OG binds to Nifl forming higher order Nifl₁/Nifl₂ oligomers, possibly a dodecamer, which cannot interact with NifDK. Upon ammonium shock, the intracellular 2-OG concentration drops; the Nifl₁/Nifl₂ heteromer is converted into a lower mass oligomer (possibly an hexamer), which interacts avidly with NifDK abrogating the interaction of the latter with NifH and resulting in nitrogenase inactivation (Fig. 3).

The occurrence of *nifl* genes adjacent to *nif* genes is observed in all nitrogen-fixing archaea and in several anaerobic bacteria including members of the gamma-proteobacteria, firmicutes, chlorobi and chloroflexi (Table 2). This distribution suggests that the nitrogenase regulatory mechanism described in *M. maripaludis* is probably wide-spread among diazotrophs (Dodsworth & Leigh, 2006).

The presence of *nifl* genes in both archaea and bacteria suggests an early origin of the *nifl* genes. Hence, the Nif₁/Nif₂-dependent nitrogenase control is likely to be the most ancient mode of nitrogenase post-translational regulation. An analysis of the molecular evolution of *nif* clusters carrying *nifl₁* and *nifl₂* showed that in the *nif* operons of the firmicute *Helicobacterium chlorum* and of the deltaproteobacterium *Desulfotobacterium hafniense*, the *nifl* genes are located upstream of *nifH*. These organisms are placed in an intermediate position between organisms with *nif* operons carrying *nifl* genes downstream of the *nifH* gene (found in archaea and anaerobic bacteria) and those with *nif* operons lacking *nifl* (typically found in aerobic bacteria) (Enkh-Amgalan *et al.*, 2006).

Phylogenetic analysis of the *nifDK* operon confirmed that the *nifDK* genes from deltaproteobacteria and *Helicobacterium* are indeed placed between the group of organisms including archaea and anaerobic bacteria listed in Table 2 and those proteobacteria carrying *draT* listed in Table 1 (Hartmann and Barnum, 2010). Interestingly, some deltaproteobacteria contain *nifl* orthologues (Table 2), while others contain *draT* orthologues (Table 1) and *Desulfobacterium autotrophicum* contains both *nifl* and *draT*. It is therefore tempting to speculate that DraT/DraG nitrogenase regulation evolved in an anaerobic proteobacteria ancestor containing *nifl* which was lost during the evolution to aerobic nitrogen fixation, while *draT* was kept in the proteobacteria lineage as a result of vertical inheritance combined with gene loss and/or lateral gene transfer. One apparent advantage of DraT/DraG over Nifl is that the former system can also respond to other signals such as the cellular energy levels. Further extensive phylogenetic analysis is necessary to clarify the evolution of these nitrogenase regulatory systems.

Fig. 3. Model for ammonium-induced nitrogenase switch-off in archaea. Under nitrogen-fixing conditions (a) the intracellular 2-OG pool is high; 2-OG binds to the Nif₁Nif₂ heteromer promoting the formation of a high Nif₁Nif₂ oligomer which cannot interact with NifDK, thus allowing nitrogenase activity. Upon an ammonium shock (b), the ammonium assimilation reduces the intracellular 2-OG pool; the Nif₁Nif₂ heteromer is converted into a lower oligomer that binds avidly to NifDK, blocking the docking site and thus electron transfer between the nitrogenase components, NifDK and NifH, thereby promoting nitrogenase inactivation.

Nitrogenase post-translational modification in cyanobacteria

Analysis of the electrophoretic migration of NifH in cyanobacteria revealed that it could be resolved into two bands, and the larger form was assumed to be modified, possibly by ADP-ribosylation. However, systematic studies using ³²P *in vivo* labelling and anti-ADP-ribose antibodies showed that the larger NifH band is not ADP-ribosylated in *Anabaena variabilis* or *Gloeotheca* (Durner *et al.*, 1994; Gallon *et al.*, 2000). Furthermore, *draT* orthologues have not been found in any cyanobacteria to date.

The appearance of the larger form of NifH was shown to be regulated by light in *Synechococcus* sp. and was inversely correlated with nitrogenase activity, suggesting that it could be a modified inactive form of NifH (Chow & Tabita, 1994). Incubation of cultures of *Gloeotheca* with ³H-labelled palmitic acid resulted in the accumulation of radioactivity in the larger form of NifH, suggesting palmitoylation; however, the functional significance of this modification was not investigated (Gallon *et al.*, 2000). Two isoforms of NifH were present in 2D gels of an unidentified cyanobiont of *Azolla*, and MS analysis suggested that one NifH isoform is modified, by an unidentified group, in the same portion of NifH that is subjected to ADP-ribosylation in proteobacteria. Thus, it is likely that the modified NifH represents an inactive form of the protein (Ekman *et al.*, 2008). Three isoforms of NifH were identified in 2D gels of the *Nodularia spumigena*, two lighter forms with different pIs and one heavier form. The putative modification of the lighter form varied under light

and darkness regimes but did not vary in response to different nitrogen regimes. The heavier form appeared to be constantly modified and membrane-bound; the nature and the function of the modifications remains unknown (Vintila *et al.*, 2011). More studies are necessary to determine the functional significance of these reported NifH modifications and whether P_{II} proteins participate in the regulation of nitrogenase activity in cyanobacteria.

Concluding remarks

Several nitrogen-fixing prokaryotes have evolved mechanisms to reversibly inactivate nitrogenase when the environmental conditions are not favourable for nitrogen fixation. These mechanisms are diverse and include (i) covalent modification of NifH in proteobacteria, (ii) interaction of the NifI inhibitory proteins with NifDK in archaea and possibly also in other anaerobic bacteria, and (iii) other unknown mechanisms that have been reported in diazotrophs. Such diversity suggests that these mechanisms appeared independently during evolution, supporting the concept that nitrogenase post-translational regulation prevents unnecessary use of ATP to drive N₂ reduction when ammonium is available, thereby offering a metabolic advantage. Despite this diversity, P_{II} proteins, which are now recognized as key signal transduction proteins in the regulation of prokaryotic nitrogen metabolism, are emerging as the universal regulators of ammonium-induced nitrogenase inactivation in those systems where it has been well studied.

Table 2. Organisms with *nifl* orthologues adjacent to the nitrogenase structural genes

Organisms carrying *nifl* orthologues adjacent to the nitrogenase structural genes were retrieved from the paper by Sant'Anna *et al.* (2009) and also by using the String 9.0 gene neighbourhood analysis tool (<http://string-db.org>).

Archaea	Firmicutes
<i>Methanobacterium ivanovii</i>	<i>Alkaliphilus metalliredigens</i>
<i>Methanococcus aeolicus</i> Nankai-3	<i>Caldicellulosiruptor saccharolyticus</i>
<i>Methanococcus maripaludis</i> S2	<i>Clostridium acetobutylicum</i> ATCC 824
<i>Methanococcus maripaludis</i> C5	<i>Clostridium beijerinckii</i>
<i>Methanococcus maripaludis</i> C7	<i>Clostridium beijerinckii</i> NCIMB 8052
<i>Methanococcus vannielii</i> SB	<i>Clostridium beijerinckii</i> NCIMB 8052
<i>Methanosarcina acetivorans</i> C2A	<i>Clostridium kluyveri</i> DSM 555
<i>Methanosarcina barkeri</i>	<i>Desulfitobacterium hafniense</i> Y51
<i>Methanosarcina barkeri</i> str. Fusaro	<i>Desulfitobacterium hafniense</i> DCB-2
<i>Methanosarcina mazei</i> Go1	<i>Desulforudis audaxviator</i>
<i>Methanothermobacter marburgensis</i>	<i>Heliobacterium chlorum</i>
<i>Methanothermococcus thermolithotrophicus</i>	<i>Heliobacterium gestii</i>
Clorobi	<i>Moorella hermoacetica</i> ATCC 39073
<i>Chlorobaculum parvum</i>	Cloroflexi
<i>Chlorobium chlorochromatii</i> CaD3	<i>Dehalococcoides ethenogenes</i> 195
<i>Chlorobium ferrooxidans</i> DSM 13031	Deltaproteobacteria
<i>Chlorobium limicola</i> DSM 245	<i>Desulfatibacillum alkenivorans</i>
<i>Chlorobium phaeobacteroides</i> BS1	<i>Desulfobacterium autotrophicum</i>
<i>Chlorobium phaeobacteroides</i> DSM 266	<i>Desulfomicrobium baculatum</i>
<i>Chlorobium phaeovibrioides</i>	<i>Desulfovibrio magneticus</i>
<i>Chlorobium tepidum</i> TLS	<i>Desulfovibrio salexigens</i>
<i>Pelodictyon luteolum</i> DSM 273	<i>Desulfovibrio vulgaris</i> Hildenborough
<i>Pelodictyon phaeoclathratiforme</i> BU-1	<i>Desulfovibrio vulgaris</i> Myazaki
<i>Prosthecochloris aestuarii</i> DSM 271	<i>Desulfovibrio vulgaris</i> DP4
<i>Prosthecochloris vibrioformis</i> DSM 265	<i>Syntrophobacter fumaroxidans</i> MPOB

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