Structural and gene expression analyses of uptake hydrogenases and other proteins involved in nitrogenase protection in *Frankia*

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The actinorhizal bacterium *Frankia* expresses nitrogenase and can therefore convert molecular nitrogen into ammonia and the by-product hydrogen. However, nitrogenase is inhibited by oxygen. Consequently, *Frankia* and its actinorhizal hosts have developed various mechanisms for excluding oxygen from their nitrogen-containing compartments. These include the expression of oxygen-scavenging uptake hydrogenases, the formation of hopanoid-rich vesicles, enclosed by multi-layered hopanoid structures, the lignification of hyphal cell walls, and the production of haemoglobins in the symbiotic nodule. In this work, we analysed the expression and structure of the so-called uptake hydrogenase (Hup), which catalyses the *in vivo* dissociation of hydrogen to recycle the energy locked up in this 'waste' product. Two uptake hydrogenase syntons have been identified in *Frankia*: synton 1 is expressed under free-living conditions while synton 2 is expressed during symbiosis. We used qPCR to determine synton 1 *hup* gene expression in two *Frankia* strains under aerobic and anaerobic conditions. We also predicted the 3D structures of the Hup protein subunits based on multiple sequence alignments and remote homology modelling. Finally, we performed BLAST searches of genome and protein databases to identify genes that may contribute to the protection of nitrogenase against oxygen in the two *Frankia* strains. Our results show that in *Frankia* strain ACN14a, the expression patterns of the large (HupL1) and small (HupS1) uptake hydrogenase subunits depend on the abundance of oxygen in the external environment. Structural models of the membrane-bound hydrogenase subunits of ACN14a showed that both subunits resemble the structures of known [NiFe] hydrogenases (Volbeda *et al.* 1995), but contain fewer cysteine residues than the uptake hydrogenase of the *Frankia* DC12 and Eu1c strains. Moreover, we show that all of the investigated *Frankia* strains have two squalene hopane cyclase genes (*shc1* and *shc2*). The only exceptions were CcI3 and the symbiont of *Datisca* glomerata, which possess *shc1* but not *shc2*. Four truncated haemoglobin genes were identified in *Frankia* ACN14a and Eu1f, three in CcI3, two in EANpec1 and one in the *Datisca glomerata* symbiont (Dg).

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1. Introduction

Frankia species are Gram-positive fermentative actinobacteria that are capable of living in symbiotic relationships or as free-living organisms. As a symbiont, the bacterium thrives in association with various dicotyledonous plants that are known as actinorhizal plants. The plants that are able to form symbioses with *Frankia* fall into three different phylogenetically related groups: Cucurbitales, Fagales and Rosales (Berry *et al.* 2011). *Frankia* sp is distributed across the world, and suitable host plants grow in climates ranging from the arctic tundra to subtropical regions (Benson and Silvester 1993). It has been suggested that *Frankia* exhibits high levels of variation in genome size and enzymatic activities as a consequence of the adaptation of different populations to specific host plants, giving rise to a varied geographical distribution. This hypothesis was

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confirmed by studies that examined the genome sequences of three *Frankia* species isolated from different host plants: ACN14a isolated from *Alnus* sp., CcI3 isolated from *Casuarina* sp. and EAN1pec isolated from *Elaeagnus* sp. EAN1pec has the largest genome and CcI3 the smallest (Normand *et al.* 2007a, b). This finding prompted the authors to suggest that genetic isolation of the host plant causes reductions in the size of the *Frankia* genome, and that major genome expansions as well as reductions can occur in facultative symbiotic soil bacteria as they respond to new environments in their symbioses (Normand *et al.* 2007a, b). In addition, several new *Frankia* genomes have recently been sequenced and annotated (Grigoriev *et al.* 2012). The genome sequences provide a sound basis for new studies into the role of *Frankia* as an actinomycete and the ecological relationship between nitrogen-fixing bacteria and their hosts.

Hydrogenases enable micro-organisms to exploit hydrogen under both anaerobic and aerobic conditions. They catalyse one of the simplest possible chemical reactions: the dissociation of one molecule of hydrogen into two protons and two electrons: $H_2 \Leftrightarrow 2H^+ + 2e^-$. Because they catalyse the production and oxidation of hydrogen, they play a key role in energy conservation (Robson 2001; Vignais 2007; Vignais and Billoud 2007).

To date, approximately 1000 hydrogenase gene sequences have been determined (Vignais and Billoud 2007) and 3 main classes of hydrogenases have been identified; (i) [Fe]-hydrogenases, (ii) [NiFe]-hydrogenases and (iii) [FeFe]-hydrogenases (Baffert *et al.* 2008). [NiFe] hydrogenases are heterodimeric metalloenzymes, with a large subunit of approximately 60 kDa and a small subunit of approximately 30 kDa.

It has been shown that nitrogenase catalyses the reduction of molecular atmospheric nitrogen to ammonia. This process is accompanied by the concomitant reduction of protons to form molecular hydrogen according to the empirical formula:

$$N_2 + 8e^- + 8H^+ + 16\ ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i.$$

Because nitrogenases can be inhibited by oxygen, they are localized in vesicles that are formed during nitrogen deprivation under aerobic conditions, emerging from enlarged hyphal tips of free-living *Frankia* (Meesters *et al.* 1987). Nitrogenases have been detected in vesicles of symbiotic *Alnus-Frankia* (Huss-Danell and Bergman 1990) and in the hyphae of *Frankia* populations growing in symbiosis with *Casuarina* (Sellstedt and Mattsson 1994). The vesicles are surrounded by layered hopanoid-containing phospholipid envelopes whose numbers increase as the oxygen tension rises (Parsons *et al.* 1987). Squalene hopene cyclases (SHCs) are the key enzymes in hopanoid biosynthesis (Siedenburg and Jendrossek 2011). Hopanoids are integrated into the vesicle membranes, where their rigid polycyclic nature has a condensing effect on the phospholipid layers, reducing their permeability of the membrane (Siedenburg and Jendrossek 2011). Most *Frankia* strains produce these specialized vesicles when growing under free-living conditions, but not when they are growing in nodules such as those formed in symbiosis with *Casuarina*, where the bacteria retain their hyphal forms. Interestingly, endophytic *Frankia* populations seem to induce the deposition of lignin in the walls of infected cells of *Casuarina glauca* (Berg and McDowell 1988), but no such effect has been observed in symbioses with *Alnus*. Lignin deposition is thought to reduce oxygen resistance of the cell (Berg 1983).

Haemoglobins (Hb) are common in eukaryotic cells, and three types of truncated Hbs (trHbs) have been identified in higher plants: truncated, or class I haemoglobins (trHbs); non-symbiotic, or class II HBs; and symbiotic or class III Hbs (Gupta *et al.* 2011). Symbiotic Hb have been shown to facilitate the supply of oxygen to the nitrogen-fixing symbionts in the nodules of legumes (Appleby 1969a, 1969b, 1981) and also in the non-legume, *Parasponia*, as well as in actinorhizal plants (Fleming *et al.* 1987; Sellstedt *et al.* 1991a, b). An additional trHb-like gene (trHbO) was identified in the *Frankia* genome; it encodes a protein whose O_2 binding kinetics suggests that it may facilitate oxygen diffusion (Tjepkema *et al.* 2002).

However, it is also known that both anaerobic and aerobic microorganisms produce uptake hydrogenases that enable them to exploit hydrogen that could be used as a source of energy. *Frankia* is known to produce Hup, a Ni-Fe hydrogenase that converts the hydrogen produced by nitrogenase activity into electrons and protons. This enables the bacterium to recycle the energy locked inside the energy 'waste' that nitrogenase produces together with hydrogen. In addition, the hydrogenase can act as an oxygen scavenger, converting it into harmless water.

It has previously been shown that Hup is expressed in parallel with nitrogenase in *Frankia* (Mattsson and Sellstedt 2000), Furthermore, the *Frankia* Hup enzyme is expected to share certain structural and functional features with Hup enzymes from other organisms. Genes that encode the two hydrogenase subunits are clustered in all *Frankia* genomes; the gene that encodes the small subunit is located upstream of the large subunit (Leul *et al.* 2009).

Analyses of the crystal structures of several Ni-Fe uptake hydrogenases have shown that they consist of large and small subunits that combine to form heterodimers (Volbeda *et al.* 1995; Montet *et al.* 1997; Ogata *et al.* 2010). The catalytic centre contains Ni and Fe atoms that are bound to the large subunit via four cysteine thiolate ligands. The importance of the corresponding four cystein residues in [Ni-Fe]-hydrogenase was discussed by Liebgott *et al.* (2011) and they have been suggested to play a key role in maintaining the bacterium's oxygen tolerance. The Fe atom has also two CN ligands and one bound molecule of CO

(Pierik *et al.* 1999). The small subunit contains either one [3Fe-4S] or two [4Fe-4S] iron–sulphur clusters that mediate electron transfer to and from the catalytic site (Volbeda *et al.* 1995).

The study reported herein was conducted to shed light on the way that the nitrogen-fixing bacterium solve the oxygen dilemma, which arises from the contradiction between the oxygen-intolerance of nitrogenase and the need for a high oxygen flux to maintain respiratory activity. This was done by (1) investigating the expression of the synton 1 uptake hydrogenase in two *Frankia* strains under aerobic and anaerobic conditions, (2) predicting the structures of the large (HupL) and small (HupS) subunit of *Frankia* using CcI3 hydrogenase as a model, (3) determining the abundance of key genes in hopanoid synthesis genes (shc) in both strain and (4) determining the abundance of genes encoding truncated hemogobins (trHbs) in both strains.

2. Materials and methods

2.1 *Frankia strains culture and growth measurement*

Two *Frankia* stains, ACN14a and CCI3, were used for the uptake hydrogenase gene expression study. Both strains were cultured using the methods described by Mohapatra *et al.* (2004). In brief, bacterial cultures were established by growing cells in approximately 50 mL of PUM medium supplemented with ammonium chloride as a nitrogen source for 8 days. The cultures were incubated at 27°C under constant shaking. The established cultures were then transferred to PUM medium with no nitrogen source and incubated for an additional 7 days before harvesting. To analyze gene expression in cultures grown under aerobic and anaerobic conditions, the bacteria were pre-incubated as described above, and then transferred to either argon- or oxygen-aerated PUM medium to produce anaerobic or aerobic cultures, respectively.

2.2 *Genome and protein data analysis*

The bacterial genomes were analyzed using BLAST (Basic Local Alignment Search Tool) (Altschul *et al.* 1990). Conserved protein sequences were identified using the web-based multi-alignment application muscle, MUSCLE (Edgar 2004). Genes of interest were identified in the sequenced *Frankia* genomes by BLAST searches of the conserved amino acid and nucleotide sequences.

The genomes of *Frankia* sp.CcI3, *Frankia alni* ACN14a and *Frankia* sp. Ean1pec were visualized using the MaGe tool in conjunction with the MicroScope interface (Vallenet *et al.* 2006, 2013). The *Frankia* EuI1c and *Frankia* EuN1f genomes were viewed on the JGI homepage (Grigoriev *et al.* 2012).

2.3 *Primer design*

Primers for the qRT-PCR experiments were designed (this study) using the Primer3 program (Rozen and Skaletsky 2000) with default setting and a length of 20 bp (table 1) based on genome sequences obtained from the NCBI GenBank (Pruitt *et al.* 2007; Benson *et al.* 2011)

2.4 *RNA extraction, cDNA synthesis and semi-quantitative RT PCR*

Prior to RNA isolation, all *Frankia* cultures were treated with RNA Protect Bacteria Reagent (Qiagen, Hilden, Germany) using a protocol supplied by the manufacturer, in order to stabilize the bacterial RNA and eliminate all active RNAses within the medium. After harvesting, the bacterial cultures were shock-frozen in liquid nitrogen and their cell walls were disrupted by grinding with a mortar and pestle. The resulting material was processed according to protocol 7 from the RNA Protect Bacteria Handbook. After mechanical disruption, we used the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), to isolate the total RNA from

Table 1. Sequences of *Frankia* HupL1, HupS1 and 16S primers used for gene expression qRT-PCR

Name	Forward primer (5′-3′)	Reverse primer (3′-5′)
HupL1-ACN14a	ACACCAGGTTGTCCTGGAAG	GTGTTCATGAAGGGGAAGGA
HupS1-ACN14a	GTTGTGCCGCCACCTCGGCTC	TGCGACGGCGACACGGTCTCG
HupL1-CcI3	GGGACAACCACACCACCT	ATGATCCATTCCGCCATC
HupS1-CcI3	ATTCCGAACGAGAACATCCA	ATCCACCAGTTCAGGGTCAG
16S ribosomal DNA (CcI3)	GATTTATCGGCTCGGGATG	GTAGGAGTCTGGGCCGTGT

16S control derived from the 16S ribosomal subunit of CcI3. Primers designed (this study) using the Primer3 program (Rozen and Skaletsky 2000) with default setting and a length of 20 bp based on genome sequences obtained from the NCBI GenBank (Pruitt *et al.* 2007; Benson *et al.* 2011).

the bacterial samples according to the manufacturer's protocol. Immediately after isolation, the bacterial RNA was treated with reagents from the DNA free (Invitrogen, CA, USA) for 30 min at 37°C, to remove DNase and divalent cation. The integrity of the resulting RNA was tested by electrophoresis on a 1.5% agarose gel with visualization of the 18S and 23S RNA subunits under UV illumination.

The iScript cDNA Synthesis Kit was used (Bio-Rad, CA USA) to synthesize cDNA from approximately 800–1000 ng of RNA according to the manufacturer's protocol. The efficiency of the designed primers were verified and optimal template/primer ratios were determined by performing PCR analyses of a primer and template cDNA dilution series. qPCR analyses were then performed using the IQ SYBR Green Supermix (Bio-Rad, CA, USA) with optimized quantities of the relevant primers and template DNA. The 16S ribosomal subunit of CcI3 was used as a control gene; the specific primers for the control and the genes of interest are listed in table 1. All qPCR experiments were performed using a CFX96 instrument (Bio-Rad, CA USA), using a thermal cycle involving a initial 3 min denaturation period at 95°C that also served to activate the hot start polymerase used in the reaction mixture, followed by 40 cycles of 15 s of denaturation at 95°C, 30 s of annealing at 58°C and 30 s of extension at 72°C. Melting curves were prepared for each sample by collecting data while heating from 55°C to 95°C in increments of 0.5°C every 10 s.

After the qRT-PCR run had been completed, the resulting data were analysed with the CFX manager 3.0 software package (Bio-Rad, CA USA), using the parameters reported by Pfaffl (2001). The levels of expression for the genes of interest were normalized against those for the control gene and three technical replicate experiments were performed for each biological sample (n=3). Means and standard deviations were calculated based on all three replicates in each sample. A single plate was used to perform direct comparison of target gene expression under aerobic and anaerobic conditions in both *Frankia* strains ACN14a and CcI3.

2.5 *Tertiary structure prediction*

The tertiary structures of the small and large subunit of the Hup protein were predicted using model 3.2 of the CPH protein homology modeling server (Nielsen *et al.* 2010). This online tool achieves template recognition based on profile-profile alignment guided by secondary structure and exposure predictions. The resulting protein database (pdb) output files was then fed into PyMol (Schrödinger 2010), a molecular graphics system to depict the 3D structures of our Hup subunits. The large and small subunits for each Hup protein were then fused *in silico*. To facilitate visual analysis, the α-helixes were displayed as ribbons and the β-sheets as arrows, with the key cysteine residues being shown as colored spheres.

3. Results and discussion

3.1 *The oxygen dilemma*

It is well known that nitrogenases are irreversibly inhibited by oxygen with typical half-decay times in air of 30 to 45 s (Dixon and Wheeler 1986). However, high oxygen fluxes are required to sustain respiratory activity in the cells of aerobic or microaerophilic bacteria. Several mechanisms for coping with the oxygen dilemma have therefore been developed during evolutionary history. Only some of these strategies are discusses in this work, including the expression of various uptake hydrogenases, the formation of specialized vesicles, presence of shc genes, the manipulation of cell wall composition in the hyphae of *Casuarina* symbioses, and presence of truncated haemoglobin genes. *Frankia* cells produce special nitrogen-fixing structures known as vesicles in which the nitrogenase enzyme is sequestered (Huss-Danell and Bergman 1990; Sellstedt and Mattsson 1994). Vesicles are necessary for nitrogenase activity are formed under aerobic conditions during nitrogen-depletion in all free-living *Frankia* strains (Lechevalier and Lechevalier 1990), with the exeption of *Frankia* R43, which also form some vesicles in nitrogen-containing media. Previous investigations have demonstrated that vesicle formation and the activation of the mechanisms for protecting nitrogenase from oxygen are regulated by ambient partial pressure of O_2 in *Frankia* strain HFPCcI3 (Murry *et al.* 1985). Ultrastructural studies of vesicles have revealed the presence of a multilayered envelope that acts as a sort of internal 'cell-wall', reducing the oxygen permeability of the compartment (Newcomb *et al.* 1987; Newcomb and Wood 1987; Parsons *et al.* 1987; Abeysekera *et al.* 1990; Harriott *et al.* 1991; Berg 1994). The envelope was shown to be rich in two hopanoids, bacteriohopanetetrol and bacterio-hopanotetrol phenylacetate monoester (Berry *et al.* 1991, 1993). A subsequent study by Dobritsa et al. (2001) identified DNA sequences from *Frankia* sp that are homologous to a coding region of the *shc* gene. Recently, by performing BLAST searches of sequenced *Frankia* genomes, we were able to show that two genes for enzymes that catalyse the production of hopanoids, biosynthesis, i.e. squalene hopene cyclase (Siedenburg and Jendrossek 2011), are present in the genomes of *Frankia* ACN14a, Eu1c, EAN1pec and Eun1F (table 2). However, only one such gene, *shc1*, is present in *Frankia* CcI3. This gene is believed to be responsible for the *shc* activity in the free-living *Frankia* colonies. It has previously been shown that the vesicle function as a protective barrier that keeps oxygen away from nitrogenase in the free-living state (Parsons *et al.* 1987). Interestingly, Abeysekera

Table 2. Describtion of occurrence of squalene hopene cyclase (*shc)* coding genes in *Frankia* Acn14a, CcI3, Eu1c, Symbiont of *Datisca glomerata*, EAN1pec and Eu1f

GENE	ACN14a	CcI3	Eul1c	Symb of Datisca	EAN1pec	EUN1f
shc1	FRAAL1432	Francci3_0823	FraEuI1c_2682	DAGLOv2_1439	Franean1_5713	FrEUN1fDRAFT_6284
shc2	FRAAL2491	no homol. Gen	FraEuI1c_5917	no homol. Gen	Franean1_4758	FrEUN1fDRAFT_4539

et al. (1990) and Kleemann *et al.* (1994) showed that the same occurs in symbioses, demonstrating that the vesicle envelope presents a robust barrier to the ingress of oxygen (Harris and Silvester 1992).

As mentioned above, *Frankia* does not form differentiated vesicles when in symbiosis with *Casuarina* sp. It is therefore not immediately apparent how its nitrogenase is protected in this case. Berg and McDowell (1988) have demonstrated that lignin layers are deposited in the cell walls of *Casuarina* that harbour *Frankia* symbionts, a finding that was corroborated by subsequent studies on different symbioses between *Casuarina* and *Frankia* (Sellstedt *et al.* 1991a, b). It was clearly shown that the walls of infected *Casuarina* sp. cells differentiate in a way that differs from the standard pattern for actinorhiza, such as *Alnus*; histochemical studies (based on autofluorescense and phloroglucinol staining) and cytochemical analyses revealed extensive lignification of the cell walls following penetration by the endophyte (Berg and McDowell 1988). In addition, infected cells that are adjacent to an infected cell are stimulated to produce additional lignin deposits along those parts of their cell wall that border the infected cell.

Another important component of the oxygen protection mechanism in some symbioses is the production of haemoglobins (Hbs) in some symbioses. It is known that symbiotic plant Hbs are present in plant tissues that house symbiotic bacteria and that they facilitate oxygen diffusion (Arredondo-Peter *et al.* 1998). In addition, Hbs have been detected in actinorhizal plants (Tjepkema 1983) and actinorhizal nodules (Tjepkema and Asa 1987; Sellstedt *et al.* 1991a, b; Suharjo and Tjepkema 1995). Three groups of truncated haemoglobins (trHbs) have been identified in bacteria; trHbOs, trHbNs, and trHbPs. Interestingly, plant trHbs represent a distinct subgroup within the group II of trHbs (Wittenberg *et al.* 2002). Truncated haemoglobins are known to have diverse function. For example, one trHb produced by the pathogen *Mycobacterium tuberculosis* (trHbN) helps to protect against NO generated by nitric oxide synthase II in response to infection (Nathan and Shiloh 2000; Ouellet *et al.* 2002). Nitric oxide (NO) is detoxified in *M. tuberculosis* by its oxidation to nitrate via a reaction with the oxygenated heme group (Ouellet *et al.* 2002). The other trHb produced by *M. tuberculosis*, trHbO, has been proposed to act as a facilitator of oxygen diffusion that maintains a steady oxygen supply to terminal oxidases under the hypoxic conditions found in granulomas (Pathania *et al.* 2002; Liu *et al.* 2004). Since Hbs genes have been detected in *Frankia* (Tjepkema *et al.* 2002) and were detected in four *Frankia* genomes in this work (table 3), they may play an important role in protecting nitrogenase from oxygen exposure in this species. However, there is currently no conclusive experimental evidence to support this hypothesis and more research in this area is needed.

3.2 *Hydrogenase*

Hydrogenases can also be regarded as oxygen scavenging enzymes, since they can catalyse the reaction of molecular hydrogen to form water: $H_2 + 1/2O_2 \rightarrow H_2O$. In this work we predicted the tertiary structure for the Hup protein of *Frankia alni* to verify its proposed identity as a Ni-Fe hydrogenase. Our results demonstrated that the uptake hydrogenase [FeNi] of ACN14a is heterodimeric proteins consisting of small (S) and large (L) subunits (figure 1). The large

Table 3. Description of haemoglobin (*hb*) coding genes in *Frankia* Acn14a, CcI3, Eu1f, EAN1pec and Dg1

Gene	ACN14a	CcI3	Ean1pec	EUN1f	Dg1
trHbO	FRAAL1844	Francci3_1149	Franean1_5317	FrEUN1fDRAFT_3583	DAGLOv2_1833
trHbN	FRAAL2475	Francci3_2581	Franean1_3681	FrEUN1fDRAFT_0664	no homol. Gen
gene,not ann	FRAAL3308	no homol. Gen	no homol. Gen.	FrEUN1fDRAFT_4092	no homol. Gen
trHbP	FRAAL5017	Francci3_2519	no homol. Gen.	FrEUN1fDRAFT_5809	no homol. Gen

The roles they have are *trHbO* (haemoglobin-like protein HbO, involved in stress reactions), *trHbN* (haemoglobin-like protein HbN, involved in adaptation to stress), gene not ann (gene not annotated) (flavohaemoglobin, hypothetical protein, similar to truncated haemoglobins) and *trHbP* (flavohemoprotein, haemoglobin-like protein, involved in respiration). 'no homol. Gen' means that no homologous gene is found (reference for Dg1: Pawlowski *et al*. 2007).

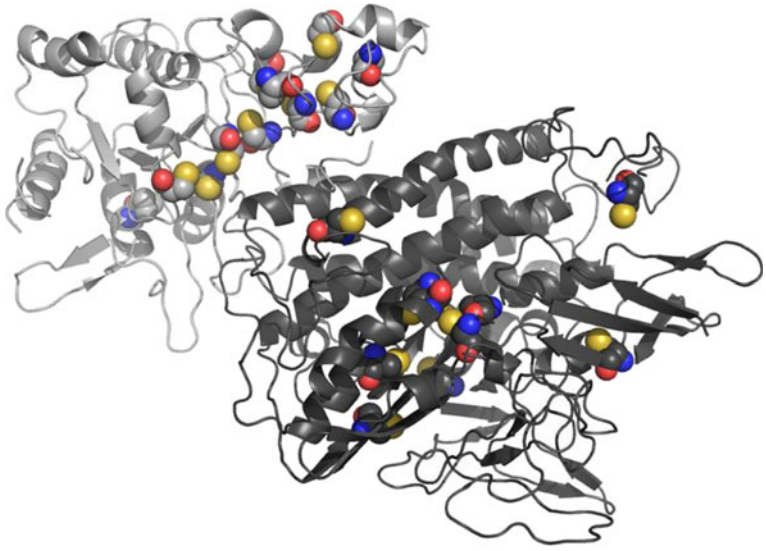

Figure 1. 3D model of the small (light grey) and large (dark grey) Hup subunit of *Frankia* alni ACN14a, predicted by remote homology modelling using structure guided sequence profiles. Conserved cysteines are shown as coloured spheres.

subunit shown in dark grey contains the active site, a [NiFe] (nickel-iron) centre anchored by four cysteine thiolates (shown as spheres). The substrate accesses the active site via a so-called molecular tunnel, which determines how gas substrates and products circulate inside the protein (Volbeda *et al.* 1995; Volbeda and Fontecilla-Camps 2003). The small subunit (light grey) contains three or four [FeS]-clusters that mediate electron transfer between the active site and the protein surface. The Fe ion in the active site is coordinated by cysteine thiolates from the large subunit and also has three non-protein ligands: two cyanide ions and one molecule of carbon monoxide. It has been suggested that the CO and CN ligands enable the metal ions of the active site to exist in low oxidation states, i.e. Fe(II) and Ni(II), without paying an excessive energetic penalty (Grigoriev *et al.* 2012). The oxidation of H_2 is thought to proceed via an intermediate, with an Fe-H^--Ni structure, and ultimately yields 2 electrons and 2 protons.

Table 4. Strain sequence, stat number, protein signature identity, cystein numbers and identity to reference strain (Frankia ACN14a)

Strain sequence/ Stat number	Number of cysteines	% signature ID to reference
ACN14a/F597	11	100
QA3/P597	10	96
CcI3/F597	11	91
CN3/P594	10	81
Ean1/F589	13	69
BCU/P587	13	69
DC12/P586	14	69
Eu1c/F586	14	69
BMG5/P589	12	68
EuN1f/F591	12	60
Dg1/F523	8	27

A previous investigation conducted using immunological techniques (Mattsson and Sellstedt 2000) showed that the mature HupL1 subunits are only found only in the membrane extracts, proving that the large subunit of synton 1 is membrane bound. This made the prediction of its structure via remote homology modelling of the large subunit rather difficult, leading to a predicted protein sequence relatively poor quality, with per-residue (Q Mean Z-scores ranging from 5.6 to 6.5. However, both subunits were amenable to homology modelling and a predicted structure (figure 1) was obtained in which both the small as well as the large (synton1) Hup subunit had structures to those reported for other [NiFe] hydrogenases (Garcin *et al.* 1999; Volbeda and Fontecilla-Camps 2003; Ogata *et al.* 2010).

Our results show that the large subunit of ACN14a Hup contains 11 cysteine residues (figure 1). Of these it is believed that the conserved cysteine ligands in the Ni-Fe active site site are C77, C569, C88 and C572, with the formed two being the terminal Ni-ligating residues and the latter two being the Ni-Fe bridging residues, respectively. This would be in keeping with the results of previous investigations into their postion in *Desulfovibrio gigas* (Matias *et al.* 2001, Volbeda and Fontecilla-Camps 2003). Interestingly, the three studied genomes had *HupL* genes of different studies; *Frankia* CcI3 has the smallest (1293 nucleotides), that of *Frankia* ACN14a is intermediate in size (1614 nucleotides), and *Frankia* Eanpec1 has the largest at 1722 nucleotides

(this study). A comparison of all the *Frankia* genomes that have been sequenced to date revealed that DC12 and Eu1c have the highest total numbers of cysteins (table 4). This is interesting because supernumerary cysteines are known to have a positive effect on oxygen tolerance in [Ni-Fe] hydrogenases (Liebgott *et al.* 2011).

The ACN *Frankia* strain has two sets of Hup genes: free-living bacteria express *HupS1* and *HupL1*, whereas *HupS2* and *HupL2* are expressed when they are living in symbiotic associations (Leul *et al.* 2007; Volbeda *et al.* 1995). In this work, we investigated the expression patterns of both genes encoding *HupS* and *HupL* from synton 1 under anaerobic and aerobic condition. Our results clearly showed that the genes encoding the small and the large subunits are expressed constitutively in *Frankia* CcI3. Conversely, their counterparts in *Frankia* ACN14a are facultative, i.e. only transcribed when needed (figure 2A and B). We also analysed the expression of the *Frankia* uptake hydrogenase under aerobic and anaerobic conditions. These studies demonstrated that the expression of the HupL1 and HupS1 subunits of *Frankia alni* ACN14a increases by a factor of 10–12 under aerobic conditions as compared to anaerobic (figure 2A). Conversely, in CcI3 the expression of the Hup genes increases by around 30% under anaerobic relative to aerobic conditions (figure 2B). It therefore seems that CcI3 strain reacts much less to the anaerobic conditions than ACN14a. The relation between these differing expression patterns and the oxygen tolerance of the strains will be examined in future investigations. Our results seem to corroborate those of Alloisio *et al.* (2010), who reported on high induction of the symbiotic gene *Hup2* under symbiotic condition, but no induction of *Hup1*, which would imply that

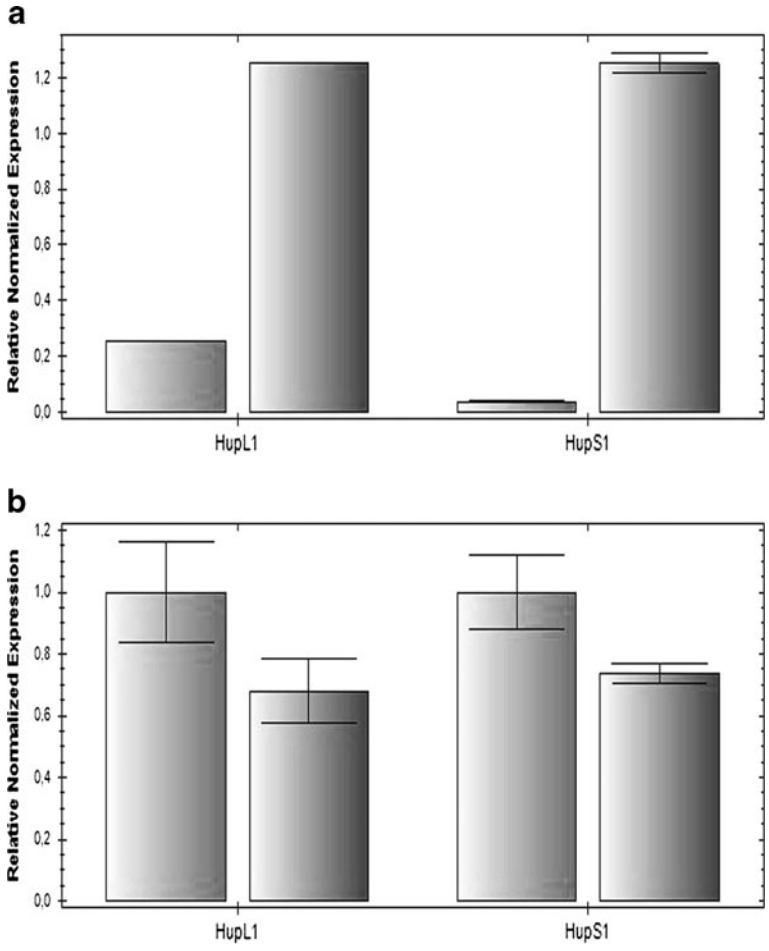

Figure 2. Profiles of relative HupL1 and HupS1 expression in *Frankia alni* ACN14a (**a**) depicted with HupL1 under anaerobic conditions to the left, followed by HupL1 under aerobic conditions, HupS1 under anaerobic conditions, and the last column to the right represents HupS1 under aerobic conditions) and *Frankia* sp. CcI3 (**b**) depicted with HupL1 under anaerobic conditions to the left, followed by HupL1 under aerobic conditions, HupS1 under anaerobic conditions, and the last column to the right represent HupS1 under aerobic conditions. 16S ribosomal subunit of CcI3 was used as housekeeping gene and analysed as a control. Graph data show relative normalized expression ($\Delta\Delta$Cq) relative to zero and error bars reflect the standard deviation.

Hup1 is expressed only under free-living condition. This is also consistent with the findings of Leul *et al.* (2007).

In conclusion, previous studies have demonstrated that different *Frankia* strains have diverse mechanisms for protecting their nitrogenase enzyme from oxygen. These include sequestration in hopanoid-rich vesicles, the synthesis of truncated haemoglobins and the induced lignification of host cell walls when growing in symbiosis with *Casuarina.* In addition, the hydrogenase-encoding synton 1 genes of *Frankia* were shown to have high levels of sequence similarity with known Ni-Fe hydrogenase genes. Furthermore, the predicted structures of the small and the large subunits of the hydrogenase resemble those of known [Ni-Fe] hydrogenases. We have also shown that genes for HupS and HupL hydrogenase subunits are constitutively expressed in CcI3, but differentially expressed in *Frankia alni* ACN14a.

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References

Abeysekera RM, Newcomb W, Silvester WB and Torrey JG 1990 A freeze-fracture electron-microscopic study of *Frankia* in root-nodules of alnus-incana grown at 3 oxygen-tensions. *Can. J. Microbiol*. **36** 97–108

Alloisio N, Querioux C, Fournier P, Pujic P, Normand P, Vallenet D, Medigue C, Yamaura M, Kakoi K and Kucho K-I 2010 The *Frankia alni* transcriptome. *MPMI* **23** 593–607

Altschul SF, Gish W, Miller W, Myers EW and Lipman DJ 1990 Basic local alignment search tool. *J. Mol. Biol.* **215** 403–410

Appleby CA 1969a Electron transport systems of rhizobium japonicum .2. rhizobium haemoglobin cytochromes and oxidases in free-living (cultured) cells. *Biochim. Biophys. Acta* **172** 88–105

Appleby CA 1969b Electron transport systems of rhizobium japonicum. I. Haemoprotein P-450 other co-reactive pigments cytochromes and oxidases in bacteroids from N2-fixing root nodules. *Biochim. Biophys. Acta* **172** 71–87

Appleby CA, Bergersen FJ, Gibson AH, Gresshoff PM and Trinick MJ 1981 Rhizobium cytochrome function in the presence and absence of leghemoglobin. *Proc. Aust. Biochem. Soc*. **14** 117–117

Arredondo-Peter R, Hargrove MS, Moran JF, Sarath G and Klucas RV 1998 Plant hemoglobins. *Plant Physiol*. **118** 1121–1125

Baffert C, Demuez M, Cournac L, Burlat B, Guigliarelli B, Bertrand P, Girbal L and Leger C 2008 Hydrogen-activating enzymes: Activity does not correlate with oxygen sensitivity. *Angewandte Chemie* **47** 2052–2054

Benson DA, Karsch-Mizrachi I, Lipman DJ, Ostell J and Sayers EW 2011 GenBank. *Nucleic Acids Res*. **39** D32–D37

Benson DR and Silvester WB 1993 Biology of *Frankia* strains, actinomycete symbionts of actinorhizal plants. *Microbiol. Rev.* **57** 293–319

Berg RH 1983 Preliminary evidence for the involvement of suberization in infection of Casuarina. *Can. J. Bot*. **61** 2910–2918

Berg RH 1994 Symbiotic vesicle ultrastructure in high pressure-frozen, freeze-substituted Actinorhizae. *Protoplasma* **183** 37–48

Berg RH and Mcdowell L 1988 Chemistry of the cell wall of infected cells in Casuarina Actinorhizae. *Can. J. Bot*. **66** 2038–2047

Berry AM, Harriott OT, Moreau RA, Osman SF, Benson DR and Jones AD 1993 Hopanoid lipids compose the *Frankia* vesicle envelope, presumptive barrier of oxygen diffusion to nitrogenase. *Proc. Nat. Acad. Sci. USA* **90** 6091–6094

Berry AM, Mendoza-Herrera A, Guo YY, Hayashi J, Persson T, Barabote R, Demchenko K, Zhang SX, *et al.* 2011 New perspectives on nodule nitrogen assimilation in actinorhizal symbioses. *Funct. Plant Biol*. **38** 645–652

Berry AM, Moreau RA and Jones AD 1991 Bacteriohopanetetrol - abundant lipid in *Frankia* cells and in nitrogen-fixing nodule tissue. *Plant Physiol*. **95** 111–115

Dixon RO D and Wheeler CT 1986 Biochemistry of nitrogen fixation; in *Nitrogen fixation in plants* (eds) ROD Dixon and CT Wheeler (Glasgow, UK: Blackie & Son Ltd)

Dobritsa SV, Potter D, Gookin TE and Berry AM 2001 Hopanoid lipids in *Frankia*: identification of squalene-hopene cyclase gene sequences. *Can. J. Microbiol.* **47** 535–540

Edgar RC 2004 MUSCLE: Multiple sequence alignment with improved accuracy and speed. *2004 IEEE Computational Systems Bioinformatics Conference, Proceedings* 728–729

Fleming AI, Wittenberg JB, Wittenberg BA, Dudman WF and Appleby CA 1987 The purification, characterization and ligand-binding kinetics of hemoglobins from root-nodules of the non-leguminous Casuarina-Glauca-Frankia symbiosis. *Biochim. Biophys. Acta* **911** 209–220

Garcin E, Vernede X, Hatchikian EC, Volbeda A, Frey M and Fontecilla-Camps JC 1999 The crystal structure of a reduced [NiFeSe] hydrogenase provides an image of the activated catalytic center. *Structure* **7** 557–566

Grigoriev IV, Nordberg H, Shabalov I, Aerts A, Cantor M, Goodstein D, Kuo A, Minovitsky S, *et al.* 2012 The genome portal of the Department of Energy Joint Genome Institute (JGI). *Nucleic Acids Res.* **4** 26–32

Gupta KJ, Hebelstrup KH, Mur LAJ and Igamberdiev AU 2011 Plant hemoglobins: Important palyers at the crossroads between oxygen and nitric oxide. *FEBS Lett*. **585** 3843–3849

Harriott OT, Khairallah L and Benson DR 1991 Isolation and structure of the lipid envelopes from the nitrogen-fixing vesicles of *Frankia* Sp strain Cpi1. *J. Bacteriol*. **173** 2061–2067

Harris SL and Silvester WB 1992 Oxygen controls the development of *Frankia* vesicles in continuous culture. *New Phytol*. **121** 43–48

Huss-Danell K and Bergman B 1990 Nitrogenase in *Frankia* from root-nodules of Alnus-Incana (L) Moench - immunolocalization of the Fe-proteins and Mofe-proteins during vesicle differentiation. *New Phytol*. **116** 443–455

Kleemann G, Alskog G, Berry AM and Huss-Danell K 1994 Lipid-composition and nitrogenase activity of symbiotic *Frankia* (Alnus-Incana) in response to different oxygen concentrations. *Protoplasma* **183** 107–115

Lechevalier MP and Lechevalier HA 1990 Systematics, isolateion, and culture of Frankia; in *The biology of Frankia and actinorhizal plants* (eds) CR Schwintzer and JD Tjepkema (New York: Academic Press, Inc) pp 35–60

Leul M, Normand P and Sellstedt A 2007 The organization, regulation and phylogeny of uptake hydrogenases genes in *Frankia*. *Physiol. Plant*. **130** 464–470

Leul M, Normand P and Sellstedt A 2009 The phylogeny of uptake hydrogenases in *Frankia*. *Int. Microbiol.* **12** 23–28

Liu C, He Y and Chang Z 2004 Truncated hemoglobin o of Mycobacterium tuberculosis: the oligomeric state change and the interactionwith membrane components. *Biochem. Biophys. Res. Commun.* **316** 1163–1172

Liebgott PP, deLacey AL, Burlat B, Cournac L, Richaud P, Brugna M, Fernandez VM, Guigliarelli B, Rousset M, Le´ger C and Dementin S 2011 Original design of an oxygen-tolerant [Ni-Fe] hydrogenase: major effect of a valine-to-cystein mutation near the active site. *J. Am. Chem. Soc*. **133** 986–997

Matias PM, Soares CM, Saraiva LM, Coelho R, Morais J, Le Gall J and Carrondo MA 2001 [NiFe] hydrogenase from Desulfovibrio desulfuricans ATCC 27774: gene sequencing, three-dimensional structure determination and refinement at 1.8 angstrom and modelling studies of its interaction with the tetrahaem cytochrome c(3). *J. Biol. Inorg. Chem*. **6** 63–81

Mattsson U and Sellstedt A 2000 Hydrogenase in *Frankia* KB5: Expression of and relation to nitrogenase. *Can. J. Microbiol*. **46** 1091–1095

Meesters TM, Vanvliet WM and Akkermans ADL 1987 Nitrogenase is restricted to the vesicles in *Frankia* strain Ean1pec. *Physiol. Plant*. **70** 267–271

Mohapatra A, Leul M, Mattsson U and Sellstedt A 2004 A hydrogen-evolving enzyme is present in *Frankia* sp R43. *FEMS Microbiol. Lett*. **236** 235–240

Montet Y, Amara P, Volbeda A, Vernede X, Hatchikian EC, Field MJ, Frey M and Fontecillacamps JC 1997 Gas access to the active site of Ni-Fe hydrogenases probed by X-ray crystallography and molecular dynamics. *Nat. Struct. Biol*. **4** 523–526

Murry MA, Zhongze Z and Torrey JG 1985 Effect of O2 on vesicle formation, acetylene reduction, and O2-uptake kinetics in *Frankia* sp. HFPCcI3 isolated from Casuarina cunninghamiana. *Can. J. Microbiol*. **31** 804–809

Nathan C and Shiloh MU 2000 Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. *Proc. Nat. Acad. Sci. USA* **97** 8841–8848

Newcomb W, Jackson SL and Wood SM 1987 Morphogenesis of nitrogen-fixing actinorhizal and leguminous root-nodules. *Endocytobiosis Cell Res*. **4** 356–356

Newcomb W and Wood SM 1987 Morphogenesis and fine-structure of *Frankia* (Actinomycetales) - the microsymbiont of nitrogen-fixing actinorhizal root-nodules. *Int. Rev. Cytol***109** 1–88

Nielsen M, Lundegaard C, Lund O and Petersen TN 2010 CPHmodels-3.0-remote homology modeling using structure-guided sequence profiles. *Nucleic Acids Res*. **38** W576–W581

Normand P, Lapierre P, Tisa LS, Gogarten JP, Alloisio N, Bagnarol E, Bassi CA, Berry AM, *et al.* 2007a Genome characteristics of facultatively symbiotic *Frankia* sp strains reflect host range and host plant biogeography. *Genome Res.* **17** 7–15

Normand P, Queiroux C, Tisa LS, Benson DR, Rouy Z, Cruveiller S and Medigue C 2007b Exploring the genomes of *Frankia*. *Physiol. Plant*. **130** 331–343

Ogata H, Kellers P and Lubitz W 2010 The crystal structure of the [NiFe] hydrogenase from the photosynthetic bacterium *Allochromatium vinosum*: Characterization of the oxidized enzyme (Ni-A State). *J. Mol. Biol*. **402** 428–444

Ouellet H, Ouellet Y, Richard C, Labarre M, Wittenberg B, Wittenberg J and Guertin M 2002 Truncated hemoglobin HbN protects Mycobacterium bovis from nitric oxide. *Proc. Nat. Acad. Sci. USA* **99** 5902–5907

Parsons R, Silvester WB, Harris S, Gruijters WTM and Bullivant S 1987 *Frankia* vesicles provide inducible and absolute oxygen protection for nitrogenase. *Plant Physiol*. **83** 728–731

Pathania R, Navani NK, Rajamohan G. and Dikshit KL 2002 Mycobacterium tuberculosis hemoglobin HbO associates with membranes and stimulates cellular respiration of recombinant *Escherichia coli*. *J. Biol. Chem*. **277** 15293–15302

Pawlowski K, Jacobsen KR, Alloisio N, Denison RF, Klein M, Tjepkema JD, Winzer T, Sirrenberg A, Guan C and Berry AM 2007 Truncated hemoglobins in Actinorhizal nodules of *Datisca glomerata. Plant Biol*. **9** 776–785

Pfaffl MW 2001 A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res*. **29** e45

Pierik AJ, Roseboom W, Happe RP, Bagley KA and Albracht SPJ 1999 Carbon monoxide and cyanide as intrinsic ligands to iron in the active site of [NiFe]-hydrogenases - NiFe(CN)(2)CO, biology's way to activate H-2. *J. Biol. Chem*. **274** 3331–3337

Pruitt KD, Tatusova T and Maglott DR 2007 NCBI reference sequences (RefSeq): a curated non-redundant sequence database of genomes, transcripts and proteins. *Nucleic Acids Res*. **35** D61–D65

Robson R 2001 Biodiversity of hydrogenases; in *Hydrogen as fuel: Learning from nature* (eds) R Cammack and KY Florence (London: Taylor & Francis) pp 73–92

Rozen S and Skaletsky H 2000 Primer3 on the WWW for general users and for biologist programmers; in *Bioinformatics methods and protocols: Methods in molecular biology* (ed) S Krawetz and S Misener (Totowa: Humana Press) pp 365–386

Schrödinger L 2010 The PyMOL Molecular Graphics System, Version 1.5.0.4.

Sellstedt A and Mattsson U 1994 Hydrogen metabolism in Casuarina-Frankia - immunolocalization of nitrogenase and hydrogenase. *Soil Biol. Biochem*. **26** 583–592

Sellstedt A, Reddell P and Rosbrook P 1991a The occurrence of hemoglobin and hydrogenase in nodules of 12 Casuarina-Frankia symbiotic associations. *Physiol. Plant*. **82** 458–464

Sellstedt A, Reddell P, Rosbrook PA and Ziehr A 1991b The relations of hemoglobin and lignin-like compounds to acetylene-reduction in symbiotic Casuarina. *J. Expt. Bot*. **42** 1331–1337

Siedenburg G and Jendrossek D 2011 Squalene-Hopene Cyclases. *Appl. Environ. Microbiol*. **77** 3905–3915

Suharjo UKJ and Tjepkema JD 1995 Occurrence of hemoglobin in the nitrogen-fixing root-nodules of Alnus-Glutinosa. *Physiol. Plant*. **95** 247–252

Tjepkema JD 1983 Hemoglobins in the nitrogen-fixing root-nodules of actinorhizal plants. *Can. J. Bot.* **61** 2924–2929

Tjepkema JD and Asa DJ 1987 Total and co-reactive heme content of actinorhizal nodules and the roots of some nonnodulated plants. *Plant Soil* **100** 225–236

Tjepkema JD, Cashon RE, Beckwith J and Schwintzer CR 2002 Hemoglobin in *Frankia*, a nitrogen-fixing actinomycete. *Appl. Environ. Microbiol*. **68** 2629–2631

Vallenet D, Belda E, Calteau A, Cruveiller S, Engelen S, Lajus A, Le Fevre F, Longin C, *et al.* 2013 MicroScope-an integrated microbial resource for the curation and comparative analysis of genomic and metabolic data. *Nucleic Acids Res*. **41** E636–E647

Vallenet D, Labarre L, Rouy Z, Barbe V, Bocs S, Cruveiller S, Lajus A, Pascal G, *et al.* 2006 MaGe: a microbial genome annotation system supported by synteny results. *Nucleic Acids Res.* **34** 53–65

Vignais PM 2007 Hydrogenases and H+ readuction in primary energy conservation. *Results Probl. Cell Differ.* **45** 1223–1252

Vignais PM and Billoud B 2007 Occurrence, classification, and biological function of hydrogenase: an overview. *Chem. Rev.* **107** 4206–4272

Volbeda A, Charon MH, Piras C, Hatchikian EC, Frey M and Fontecillacamps JC 1995 Crystal-structure of the nickel-iron hydrogenase from Desulfovibrio-Gigas. *Nature* **373** 580–587

Volbeda A and Fontecilla-Camps JC 2003 The active site and catalytic mechanism of NiFe hydrogenases. *Dalton Trans*. 4030–4038

Wittenberg JB, Bolognesi M, Wittenberg BA and Guertin M 2002 Truncated hemoglobins: a new family of hemoglobins widely distributed in bacteria, unicellular eukaryotes, and plants. *J. Biol. Chem*. **277** 871–874