Comparative Genomics: Understanding Regulation of Hydrogenases in the Nitrogen-Fixing *Frankia*

Chatchai Kosawang

Umeå Plant Science Centre
Department of Forest Genetics and Plant Physiology, SLU, Umeå

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Faculty of Forest Sciences, Umeå Plant Science Centre, Department of Forest Genetics and Plant Physiology, Umeå

Author: Chatchai Kosawang

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Supervisor: Prof Anita Sellstedt, Umeå Plant Science Centre, Department of Plant Physiology, Umeå University, SE-901 87 Sweden

Examiner: Dr Karin Ljung, Umeå Plant Science Centre, Department of Forest Genetics and Plant Physiology, SLU, SE-901 83 Sweden

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Abstract

Frankia is a facultative symbiont actinomycete living with the dicotyledonous plant worldwide. Two hydrogenase functions- uptake hydrogenase and hydrogen-evolving hydrogenase- have been so far reported in Frankia species, while the presence of [Fe] hydrogenase function was surmised. Investigation of three Frankia strains, ACN14a, CcI3 and R43 disclosed non-existence of the [Fe] hydrogenase function, and bidirectional hydrogenase function in two of the Frankia strains, ACN14a and CcI3. The blast of the three available Frankia genomes with the conserved region of [Fe] hydrogenase, subunits of cyanobacterial bidirectional hydrogenase, hyd and hyn genes resulted in no relevant information, but uptake hydrogenase. Regarding the gene expression studies, it is supposed that [Fe] hydrogenase is unlikely to presence in the Frankia strains. On the other hand, bidirectional hydrogenase function is unlikely to occur in the strains ACN14a and CcI3 since hydrogen evolution was not detected in these strains. The hydrogen production in the strain R43 was found both under aerobic and anaerobic condition regardless of nickel availability. Application of hox-specific primers to all the strains evaluated did not provide an informative dataset. It is possible that hydrogen evolution in the strain R43 was retrieved from uptake hydrogenase function acting in reverse direction. In addition, it was shown that uptake hydrogenase was consecutively expressed. However, knowledge about hydrogenase in Frankia is now like the tip of the iceberg. It is not possible to show the presence of bidirectional hydrogenase gene in the strain R43 until its genome is sequenced.

I. Introduction

Frankia is a gram positive actinomycete, living either as a free living or as a symbiont with a variety of dicotyledonous plants. In return, it provides ammonium fixed from nitrogen in the air back to its host as a part of symbiotic relationship. Frankia has been reported to be distributed all over the world with respect to its globally widespread host, the so-called actinorhizal plants that dwell diversely from arctic tundra to alpine forest (Benson and Silvester, 1993). Due to this reason, it could be speculated that Frankia could offer a great variation in genome size and enzymatic activities by virtue of adaptation to host plants. A genome sequence of three Frankia species isolated from different host plants, ACN14a isolated from Alnus sp., CCI3 isolated from Casuarina sp. and recently EAN1pec isolated from Elaeagnus sp., illustrates a concrete dataset depicting that EAN1pec has the largest genome size and CCI3, in contrast, comes up with the smallest genome size among the three strains sequenced (Normand et al., 2007a,b). The 'genus-specific' gene of the three genomes was possessing in a range of 35-51% of the genomes, approximately 3,000 genes, depending on such strains. These were including genes responsible for nutrient exchanges and symbiosis interaction. Analysis of these genomes has been cautiously done for the sake of evolutionary sight. It is certain that host range and host plant biogeography determine the Frankia genome sizing through major evolutionary processes, gene deletion, duplication and acquisition. The genome contraction is resulted from host plant isolation, while host diversity, on the contrary, surges geome expansion (Normand et al., 2007a).

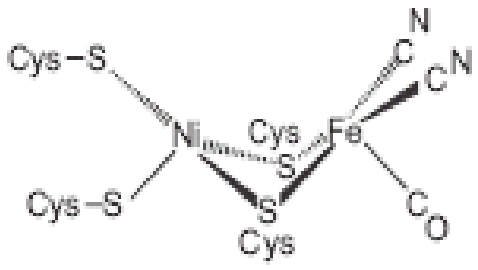

Figure 1 Structure of the cyanobacterial [NiFe] hydrogenase. (Ghirardi et al., 2007)

In microorganisms living solely in an anaerobic condition, hydrogenase is an enzyme that plays a major role in energy conservation by catalysing both hydrogen production and hydrogen oxidation. Hydrogenase can be categorised into 13 classes according to its function (Robson, 2001; table 1). However, with respect to phylogenetic distinct,

Number	Hydrogenase family	Occurrence	Function
1	Fe-only hydrogenases	Obligately anaerobic bacteria and Eukaryotes	Fermentation/ energy conservation?
2	NiFe(Se) membrane-bound or periplasmic/respiratory hydrogenases	Aerobes, facultative anaerobes, obligate anaerobes of the Proteobacteria	Energy conservation
3	NiFe-(thylakoid)uptake hydrogenases	Cyanobacteria	Energy conservation?
4	Bidirectional NAD(P)-reactive hydrogenases	Cyanobacteria	Energy conservation, Redox poising?
5	NAD(P)-reactive hydrogenases	Facultative and obligately anaerobic Eubacteria	Energy conservation
6	NAD(P)-reactive hydrogenases	Obligately anaerobic Archaea	Fermentation
7	F420-non-reactive hydrogenases	Methanogens	Energy conservation
8	F420-reactive hydrogenases	Methanogens	Energy conservation
9	NiFe-sensor hydrogenases	Chemolithotrophic/ phototrophic Proteobacteria	Hydrogen sensing components in genetic regulation of hydrogenase expression
10	NiFe-hydrogenases associated with the formate hydrogen lyases complex	Facultative and obligate anaerobes, Archaea	Fermentation
11	Ech hydrogenases	Methanogens	Methanogenesis pathway
12	Non-metal hydrogenases	Methanogens	Energy conservation
13	Soluble hydrogenase	*Anabaena cylindrica*	Unknown

Table 1 Functional classes of hydrogenases (Robson, 2001).

three types of hydrogenases have been designated regarding to metal content of active site. These hydrogenase classes are (i) Hmd or [Fe]-hydrogenase, (ii) [NiFe]-Hydrogenase and (iii) [FeFe]-Hydrogenase (Vignais and Billoud, 2007; Baffert et al., 2008). To begin with the first type, Hmd hydrogenase is present in ranges of methanogenic species in which current studies is directed toward Hmd from the bacterial *Methanothermobacter marburgensis*, where Hmd has been recognised. Hmd is made up of two subunits of homodimer. The enzyme contains two iron molecules per homodimer, but surprisingly holds no iron-sulphur cluster. The delivery of methylene-H4MPT as the end reversible product acclaims it as the only hydrogenase not catalysing reversible reaction of H_2 (Vignais and Billoud, 2007). In contrast to the homodimer skeleton of Hmd, [NiFe] hydrogenase is a heterodimer enzyme. The α (large, 60 kDa) and β (small, 30 kDa) subunit accommodate bimetallic site and Fe-S cluster of the enzyme, respectively (Figure 1). [NiFe] hydrogenase is the biggest group wtih 13 subgroups (Vignais and Billoud, 2007) and the most extensive studied among three types of hydrogenase, especially cyanobacterial uptake hydrogenase and bidirectional hydrogenase. Cyanobacterial uptake hydrogenase is composed o two subunits: a small subunit (HupS) of 35 kDa and a large subunit (HupL) of 58 kDa, where the Ni core is buried within. However, the crystal structure of the uptake

hydrogenase is still unclear since no active cyanobacterial uptake hydrogenase has been isolated yet (Ghirardi et al., 2007). On the other hand, cyanobacterial bidirectional hydrogenase is unique with hydrogenase structural complex (HoxHY) and diaphorase component (HoxEFU) as shown in figure 2. The last group, [FeFe] hydrogenase, illustrates diverse structures. Apart from dimeric, trimeric and tetrameric, monomeric with the catalytic subunit only is ubiquitously predominant. Its conserved domain is approximately 350 residues containing active site or H-cluster and, often, housing Fe-S clusters as additional domains (Vignais and Billoud, 2007). Not only posing as the highest potential in producing hydrogen among hydrogenases, this kind of hydrogenase is also observed in a wide range of organisms from eukaryote to anaerobic prokaryote.

The attention to hydrogenase and biological hydrogen production has been increasing in recent years due to energy crisis plus a deep concern over global pollution from the consumption of carbon-based fuels. As a house of at least three types of hydrogenase functions- [NiFe] uptake hydrogenase, hydrogen-evolving hydrogenase and [Fe] hydrogenase (Leul et al., 2005b), Frankia has been researched as an alternative hydrogen manufacturer in past years. Hydrogen-evolving hydrogenase function was firstly proposed by Mattson and collaborators in 2001 from the strain R43, where hydrogen evolution was observed. The existence of the hydrogen-evolving hydrogenase function in some other strains was later confirmed by Mohapatra et al. (2004, 2006) and Leul et al. (2005b). Immuno-gold labelling with antibody raised against bidirectional hydrogenase hoxH of *Disulfovibrio vulgaris* revealed that the hydrogenase is accumulated both in vesicle and hyphae, while amino acid sequencing disclosed a close homology with bidirectional hydrogeanase of the cyanobacteria *Anabaena siamensis* strain TISTR8012 (Mohapatra et al., 2006).

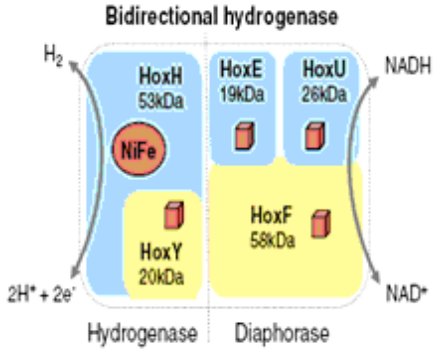

Figure 2 Structural and enzymatic processes of cyanobacterial bidirectional hydrogenase (Ghirardi et al., 2007)

Moreover, a capability to generate hydrogen evolution whether in deprivation of nitrogen source suggested that it is expressed sequentially (Mohapatra et al., 2004). A cross-reaction with antibodies raised against Hox U and Hox H of *Ralstonia eutropha* rises a speculation that hydrogen-evolving hydrogenase in Frankia has a NiFe centre. The increase in hydrogen evolution after $NiCl_2$ added supports the hypothesis that the hydrogen-evolving enzyme belongs to the [NiFe] class of hydrogenase (Leul et al., 2007). However, the clear structural picture of the enzyme has not been depicted so far in respect of difficulty in isolation of the enzyme. Regarding to the recent knowledge, hydrogen-evolving hydrogenase could be distinguished from cyanobacteria bidirectional hydrogenase by NAD-reduction activity (Mohapatra et al., 2004; Leul, 2007).

Frankia is also found to harbour an uptake hydrogenase function and, plausibly, [Fe] hydrogenase function apart from hydrogen-evolving hydrogenase. In the nitrogen-fixing organisms, uptake hydrogenase poses as a key enzyme in energy conservation as it catalyses the consumption of hydrogen produced by nitrogenase. Uptake hydrogenase is also capable of producing hydrogen from its reversible direction as reported in Frankia (Leul et al., 2005b). The uptake hydrogenase as of cyanobacteria and Frankia to date is presumed to be related and is built up of large and small subunits as mention above (Leul et al., 2005a), while the large subunit is more conserved across species than the small subunit. Undoubtedly, the Frankia uptake hydrogenase function also shares common features with uptake hydrogenase from other organisms, while its gene encoded the small subunit lays itself upstream of the large one (Leul et al., 2007). Immunological studies revealed previously that uptake hydrogenase is localised in vesicles and hyphae (Sellstedt and Lindblad, 1990). Similar to *Rhizobium leguminosarum*, the expression of uptake hydrogenase tends substantially concomitant with nitrogenase (Mattson and Sellstedt, 2000). Also, biodiversity of uptake hydrogenase in Frankia has been researched by the employment of different antibodies raised against both small (Hup S) and large (Hox G) subunits. [Fe] hydrogenase is the latest reported type of hydrogenase function in Frankia. No fundamental knowledge is much discovered- besides, the use of antibody raised against [Fe] hydrogenase from Desulfovibrio desulfuricans ATCC 7757 recognised a polypeptide of about 64 kDa in two frankia strains, UGL011102 and KB5 (Leul et al., 2005b). Regarding a green algae [Fe] hydrogenase, it's encoded in the nucleus but delivered to localise in chloroplast. The enzyme is merely irreversible oxygenic-

sensitive as well as vulnerably inhibited by CO_2 (Ghirardi et al., 2007). However, expression of Frankia [Fe] hydrogenase, protein structure, and diversity of the gene remain unknown and need to be studied.

The aims of this study

(I) To study the regulations of hydrogen-evolving hydrogenase function in *Frankia* sp.

(II) To authenticate the [Fe] hydrogenase function in *Frankia* sp.

(III) To study the correlation between hydrogen-evolving hydrogenase and [Fe] hydrogenase in *Frankia* sp if present.

II. Materials and Methods

In silico acquisition of [Fe] hydrogenase, bidirectional hydrogenase, and hydrogenase gene clusters in Frankia sp.

To search for such enzymes, amino acid and nucleotide sequences specific to each enzyme were searched from three databanks: NCBI, the genome database for cyanobacteria and the genome database for Rhizobia. Conserved regions of such enzymes were later identified by aid of web-based ClustalW2 (Largin et al., 2007). Verification for the existence of these enzymes in Frankia was achieved by high-throughput blasting the conserved specific amino acid and nucleotide sequence generated from three Frankia genomes- ACN14a, EAN1pac, and CcI3- available on the website 'Frankiascope'.

Frankia strains culture and growth measurement

Three *Frankia* stains- ACN14a, CCI3 and R43- were investigated regarding the capability of hydrogen production in this experiment. All strains were cultured according to Mohapatra et al. (2004). In brief, the bacteria were firstly grown in approximately 100 millilitres of PUM medium before incubated on a shaker at 27^{o}C for 8 days. The Frankia strains were then transferred to PUM medium without ammonium chloride but supported with 15 μM $NiCl_2$ (as later called PUM –N/+Ni for another 7 days. To avoid hydrogen evolution from nitrogenase activity, the media PUM –N/+Ni was replaced by PUM medium supported with 10 μM NH_4Cl for 24 hours before hydrogen evolution was analysed.

Growth rate of the *Frankia* strains was assessed by mean of total protein concentration. Total protein content was extracted from the *Frankia* strains after measurement of hydrogen evolution by PEB protein extraction buffer kit (Agrisera, Sweden) with sonication (Branson cell disruptor B15, 2 cycles with 30% duty). Quantification of the protein was carried out with a modified Lowry assay (Bio-Rad DC protein assay, Bio-Rad) using a spectrophotometer at absorbance of 750 nm. The amount of proteins revealed in three replicates was correlated to hydrogen evolution expensed as $nmolH_2\,(mg\ protein)^{-1}.h^{-1}$.

Hydrogen evolution

An artificial electron donor system of methyl viologen and sodium dithionite was introduced to assessment of H_2-evolving enzymatic activities according to Mohapatra et al. (2004). A total reaction of 2 ml was made up of 1.8 ml of the *Frankia* cell suspension and a fresh mixture of 0.2 ml of sodium dithionite-reduced methyl viologen in Tris HCl pH 7.0. An anaerobic condition was generated by argonising the vials immediately after the cell suspensions were transferred. Hydrogen evolution was investigated 90 minutes after incubation at 27^{o}C using a GC-8AIT gas chromatrograph (Schimadzu Scientific Instruments, Columbia, USA). Re-measurement of hydrogen evolution was performed 24 hours after the first measurement in the case that no hydrogen production observed.

The effect of nickel on activity of hydrogenase in the Frankia strain R43 were evaluated by growing cell in the PUM –N culture with/without $NiCl_2$ and oxygen. Bacteria cultivation was performed as mentioned above. Upon measurement of hydrogen evolution, methyl viologen was added as the artificial electron donor under anaerobic condition as previously stated. Hydrogen evolution was investigated as above at 90 mins and repeated 24 hours if no hydrogen evolution was detected.

RNA extraction, cDNA synthesis and Polymerase Chain Reaction

Bacterial RNA from cells grown under both aerobic and anaerobic conditions were isolated using Rneast mini kit (Qiagen, CA, USA) with some modifications. cDNA was later synthesized using an iScriptTM cDNA Synthesis Kit (Bio-Rad Laboratories, USA) following the manufacturer's protocols. Five pairs of DNA primers were employed to assess the existence of [Fe] hydrogenase, uptake hydrogenase and bidirectional hydrogenase in *Frankia* sp as shown in Table 2. To the [Fe] hydrogenase primers, PCR conditions were adopted from Hatchikian et al. (1999) with minor modifications. The 20 µl PCR mixtures comprised 200 ng of cDNA, 0.6 mM of each primer, 2mM of dNTP (Fermentus, Germany), 15 mM of MgCl2, and 1 U of Tag polymerase (Invitrogen, USA). PCR condition was set to 3 mins at 95^{o}c for initial denaturation followed by 35 cycles of 30s at 95^{o}c, 30 s at 58^{o}c, 30 s at 54^{o}c and 1 min at 72^{o}c. A final extension was conducted for 3 min at 72^{o}c. To the primers for subunits of bidireactional hydrogenase, the PCR reactions were performed accordingly to Leul et al. (2007) with minor modifications. The 20 µl PCR mixtures was made up of 200 ng of cDNA, 0.6 mM of each primer, 2mM of dNTP (Fermentus,

Germany) 3 mM of MgCl2, and 1 U of Tag polymerase (Quigen, USA). PCR condition was carried out the same for all bidirectional hydrogenase primers, except annealing temperature was adjusted to each primer. In brief, initial denaturation was set to 3 mins at 95^{o}c, 35 cycles of 30s at 95^{o}c for denaturation, 15 s at 54^{o}c for extension, and 1 min at 72^{o}c for extension before ending up with final extension, 3 min at 72^{o}c. The PCR products were electrophorised with 1% agarose gel before visualised under UV illuminator.

DNA sequencing and data manipulation

DNA bands of interests were selected, purified using a QIAquick Gel Extraction Kit (Qiagen, USA) following its protocol, and sequenced by ABI377 sequencer (Applied Biosystems). The retrieved sequences were manipulated using the computer programme Bioedit version 7.0.9 (Ibis Biosciences, California) before blasted against the database of NCBI.

Table 2 List of primers employed for addressing the presence of [Fe] hydrogenase, uptake hydrogenase and bidirectional hydrogenase

Primer	Sequence	Target
Fe1/Fe2	GGGGGTGACAGGATGGTGCAA	[Fe] hydrogenase
	GATCGTGGACAGGTGCTGAC	
HupL2	GACGTCACCCACTCGTTCTAC	LSU, Uptake hydrogenase
	CGTTGATGACGAACCTGCT	
HoxY	GGGGTCAATCGTACTGATTG	hoxY,
	CACTCCGAGAAGAACGAAAT	Bidirectional hydrogenase
HoxY2	TGTGAATTCTCGACATGGACGAGCGGC	
	ATGAAAGCTTCCATCTTGACCAC	
HoxH	GGAGGTCTTGGTATGAGCAC	hoxH,
	GGGTCGAAAGCGAATTTGTG	Bidirectional hydrogenase
HoxF	GATCGTATATGAGCGGAGAC	hoxF,
	TCCACTACACCATCGACATA	Bidirectional hydrogenase
HoxF2/B2	TTCCTCGGCTACGACAGC	[NiFe] Large Subunit
	CGACAAGCTCGGTCAGGT	

III. Results and Discussion

Alignments, identification of conserved regions and blasting of [Fe] hydrogenase, Hydrogen-evolving hydrogenase, Bidirectional hydrogenase, Hyd and Hyn genes

The search across the 3 major databases resulted in 33 amino acid sequences of [Fe] hydrogenase, 42 amino acid sequences of 5 subunits of bidirectional hydrogenase, 23 amino acid sequences of uptake hydrogenase, 71 amino acid sequences of hyd gene cluster (hydA, hydB, hydC, hydD and hydE) and 12 amino acid sequences of hyn gene cluster (hynL and hynS) as listed in appendix I. The multiple sequence alignment with CLUSTALW2 revealed a short-specific amino acid sequence conserved among [Fe] hydrogenases, assigned as FIEVMACPGGCIGGGGQP (figure 3), which was further used to blast against the *Frankia* genomes. Interestingly, only Fe-hydrogenase posed the conserved region while no conserved regions were identified among subunits of cyanobacterial bidirectional hydrogenase. As a matter of fact, bidirectional hydrogenase is found to be structurally diverse even among cyanobacteria (Tamagnini et al., 2007). The constitution of the functionally bidirectional hydrogenase is not limited to the five principal subunits, HoxYHEFU, but accessory subunits are participated in some cases. On the other hand, not all of cyanobacteria own the enzyme; an absence of the enzyme has been so far reported in *Nostoc punctiforme* (Tamagnini et al., 1997). It could be surmised that evolution plays a major role in a selection process while loss of the gene happens during horizontal gene transfer.

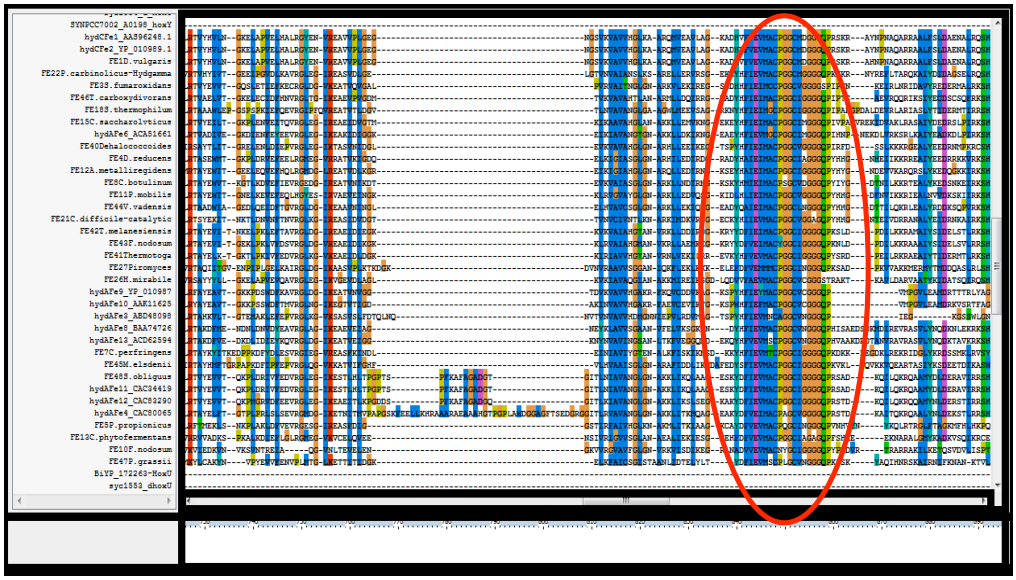

Figure 3 The conserved region of [Fe] hydrogenase (circled in red).

Protein blast of the conserved regions was brought in to FrankiaScope with a minimised identity value of 10% and 0.1 minLrap. No homology sequence was even shown from the search, suggesting two possibilities:

(I) *Frankia* might not possess [Fe] hydrogenase.

(II) The *Frankia* [Fe] hydrogenase is structurally unique and not shared amino acid similarity to those [Fe] hydrogenases.

This information perplexes the hypothesis somehow for the previous experiment evinced the recognition elicited from the antibody raised against [Fe] hydrogenase of *Desulfovibrio vulgaris* (Leul et al., 2005). However, it might be that the [Fe]-hydrogenase of *D. vulgaris* shares similar epitope with hydrogen uptake of *Frankia* sp. Also, amino acid sequences of prokaryotic and eukaryotic [Fe] hydrogenases, Narf-like putative protein in human, brought forth the certainty of high similarity among [Fe] hydrogenase (Nicolet et al., 2002). On the other hand, protein blasts were performed individually in the case of bidirectional subunits, hyd and hyn gene clusters with the identical searching parameters to [Fe] hydrogenase. It was revealed that no such genes searched were substantially defined in the *Frankia* genomes. Nevertheless, the exploration of Hox subunits from cyanobacteria returned hupL and hupS (uptake hydrogenase large and small subunits, respectively) while hydC was returned from *Rhodopsuedomonas palustris* after challenged with the [Fe] hydrogenase sequence. Taking into account all these findings, it seems that the [Fe] hydrogenase antibody recognised inseparable epitope leading to recognition in the case of [Fe] hydrogenase. In other words, it might recognise an epitope of uptake hydrogenase. However, since incomplete protein translation might be hindrance the in silico process, it is of importance to perform further biochemical experiments to prove the existent of the enzymes.

Hydrogen Evolution

The hydrogen-producing capacity of *Frankia* species was examined using the artificial electron donor system. It is shown that, in this experiment, hydrogen evolution was found in all replications of the strain R43 under aerobic condition at the rate of 8.72 nmolH2 (mg protein)$^{-1}$.h^{-1} (Figure 4), whereas no hydrogen production was observed from the strain ACN14a and CcI3. No hydrogen evolution detected in the strains ACN41a and CcI3 was logically predictable by the previous results of data mining that reflected no similarity sequences from the three genomes. This crucial information would convince the prior report of hydrogen-evolving enzyme in the strain CcI3 (Leul et al., 2005; Mohapatra et al., 2006). It is likely that the cross reaction generated by the antibodies raised against Hox F of *R. eutropha* might be due to a certain similarity between bidirectional hydrogenase Hox H of R.eutropha and *Frankia* uptake hydrogenase. On the other hand, it is possible that the microoxygenic condition, or even anaerobic condition, was generated itself in this experiment since loads of the cells were used for measuring hydrogen evolution. If such, the bidirectional function of uptake hydrogenase or reverse function of uptake hydrogenase was able to be triggered and started producing hydrogen.

Hydrogen evolution in *Frankia* is principally emanated from either nitrogenase or hydrogenase. In the case of hydrogenase, it can either be uptake hydrogenase acting in the reverse direction or bidirectional hydrogenase (Mohapatra et al., 2004). In this experiment, the detection of hydrogen was not recorded under anaerobic condition, but aerobic condition. Nitrogenase is known to be oxygenic sensitive in cyanobacteria (Tamagnini et al., 2002; Tamagnini et al., 2007). In addition, nitrogenase-produced hydrogen is hindered by supplementing ammonium chloride (Leul at al., 2005) as it had been added 24 hours before measurement of hydrogen evolution in this experiment. The conclusion could therefore be logically drawn that hydrogen evolution observed in the stain R43 is not from nitrogenase. Alternatively, it is possible that the hydrogen evolution in the strain R43 was from the uptake hydrogenase acting in the reverse direction.

In addition, uptake hydrogenase, like nitrogenase, functions under microoxygenic condition in nitrogen-fixing cyanobacteria (Ghirardi et al., 2007; Tamagnini et al., 2007). These ambivalent results puzzles whether the *Frankia* strain R43 possesses hydrogen-evolving hydrogenase or the hydrogen evolution was of uptake hydrogenase acting in the reverse direction. Although this finding strengthens uptake hydrogenase-generated hydrogen evolution in *Frankia*, it might be too early to determine the absence of bidirectional hydrogenase in *Frankia* since diversity among *Frankia* strains should be counted on. Particularly a genome sequence of the strain R43 has not been released yet. Therefore, further experiment is indubitably required to cut the Gordian knot, whether the hydrogen evolution is from a unique hydrogen-evolving enzyme or the reverse action of uptake hydrogenase.

Effects of nickel on hydrogen evolution

The effect of $NiCl_2$ on hydrogen evolution was carried out in the *Frankia* strain R43, which hitherto demonstrated the hydrogen evolution. As surmised that hydrogen-evolving hydrogenase falls into the [NiFe] class (Mohapatra et al., 2004), the production of hydrogen would be urged correspondingly to the increased amount of nickel fed. The strain R43 astonishingly developed hydrogen evolution in all treatments- irrespective of $NiCl_2$ and oxygenic condition (figure 5). In agreement with previous observation, the capacity for hydrogen production was undoubtedly pronounced under anaerobic condition as compared to aerobic condition. However, $NiCl_2$ did not affect hydrogen evolution as advanced hydrogen evolution was even more pronounced in *Frankia* grown without nickel. Based on available information, it could be speculated that the hydrogen evolutions observed were not brought forth by a bidirectional hydrogenase, but by an uptake hydrogenase acting in reverse direction. Anaerobic condition might be built up during the experiments seducing the reverse function of uptake hydrogenase and hydrogen evolution, eventually. Intriguingly, production of hydrogen was not obtained from the strain R43 cultured under anaerobic condition with nickel in the first experiment; in contrast, hydrogen evolution was found in all conditions in the second experiment as described earlier. The questions concerning this matter were raised and the only explanation falls into the diversity of the *Frankia*. It is reasonable that gene involving with hydrogen

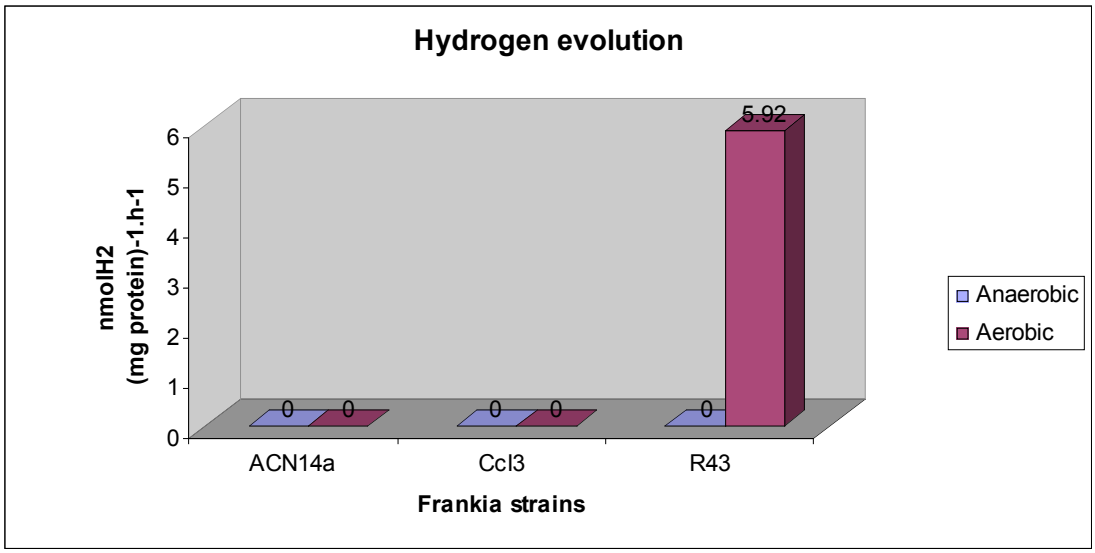

Figure 4 Hydrogen evolution from the *Frankia* strain ACN14a, CcI3 and R43 grown under anaerobic and aerobic conditions.

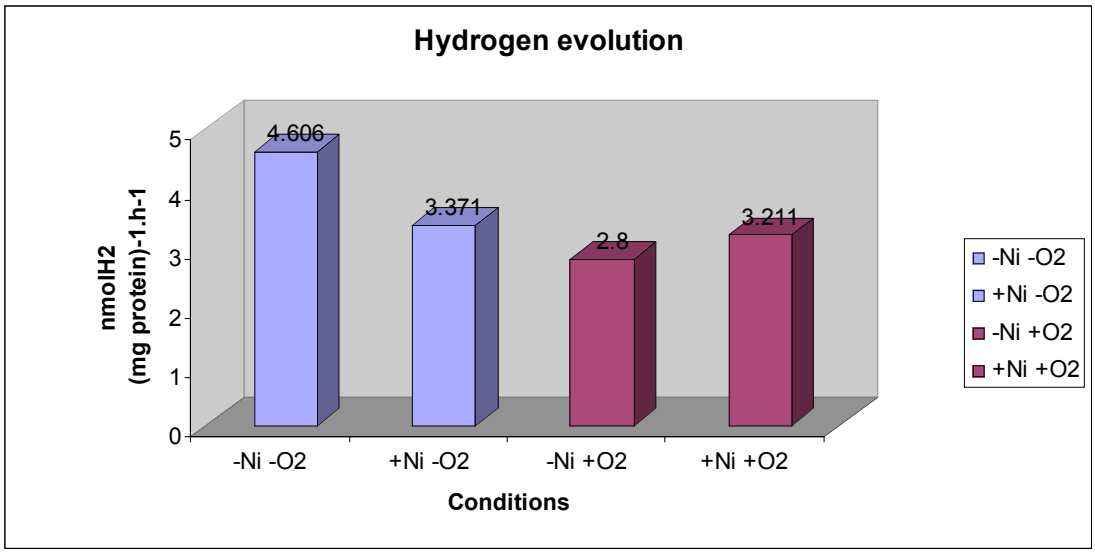

Figure 5 Hydrogen evolution from the *Frankia* strain R43 grown in PUM –N with/without nickel under both anaerobic and aerobic conditions.

evolution were malfunctioned, or deactivated, since the strain R43 used in the first experiments was not actively growing, but it was starved due to long-term preservation. To circumvent this trouble, actively growing *Frankia* strains would be employed in further experiments.

Expression study of Iron-only hydrogenase and hydrogen-evolving hydrogenase

PCR of the *Frankia* strains grown both under anaerobic and aerobic conditions using different primers was done to observe expression of the three enzymes, [Fe] hydrogenase, uptake hydrogenase and hydrogen-evolving hydrogenase. The use of the primer Fe1/Fe2, designed from *D. desulfuricans* ATCC 7757, acquired the product of approximately 1500 bp only from the strain ACN14a while the product size of 2000 bp is expected (Hatchikian et al., 1997). The unspecific amplification was abundantly found although the enzyme was designed specifically to the [Fe] hydrogenase. It is suggested that the primers are not stringent only to [Fe] hydrogenase; in the other words, it amplified other hydrogenases or housekeeping genes sharing high sequence similarity. To tackle the doubt, sequencings of the band were attempted. However, it was difficult to inspect the sequence due to the typical character of high GC content shared among *Frankia* genome. In this case, more endeavours and cloning might be a crucial key to the success.

Five primer pairs specific to hoxH, hoxH, hoxY and [NiFe] hydrogenase were also tried in PCR in this experiment apart from the hupL and [Fe] hydrogenase primers. Only [NiFe]-target, hoxY-targeted and hoxF-targeted primers could generate DNA amplification among the primers used despite non-specific amplification. In exception of hoxY-specific primer, approximately 100 bp of DNA product was amplified as showed in figure 6. The generated DNA profiles with [NiFe] primer ascertains the availability of the [NiFe]-ligand hydrogenase in Frankia. In addition, several sequencing attempts were made to decode the PCR product although it has not been achieved so far. The DNA profile derived from hoxF-targeted primer was, on the contrary, unspecific and redundant DNA bands were developed. One should bear in mind; none of the hox subunit was mined in silico from the ACN14a and CcI3 genomes. None of DNA bands amplified by hoxH and hoxF were as a plus, accentuating the question about presence of bidirectional hydrogenase in *Frankia*. The

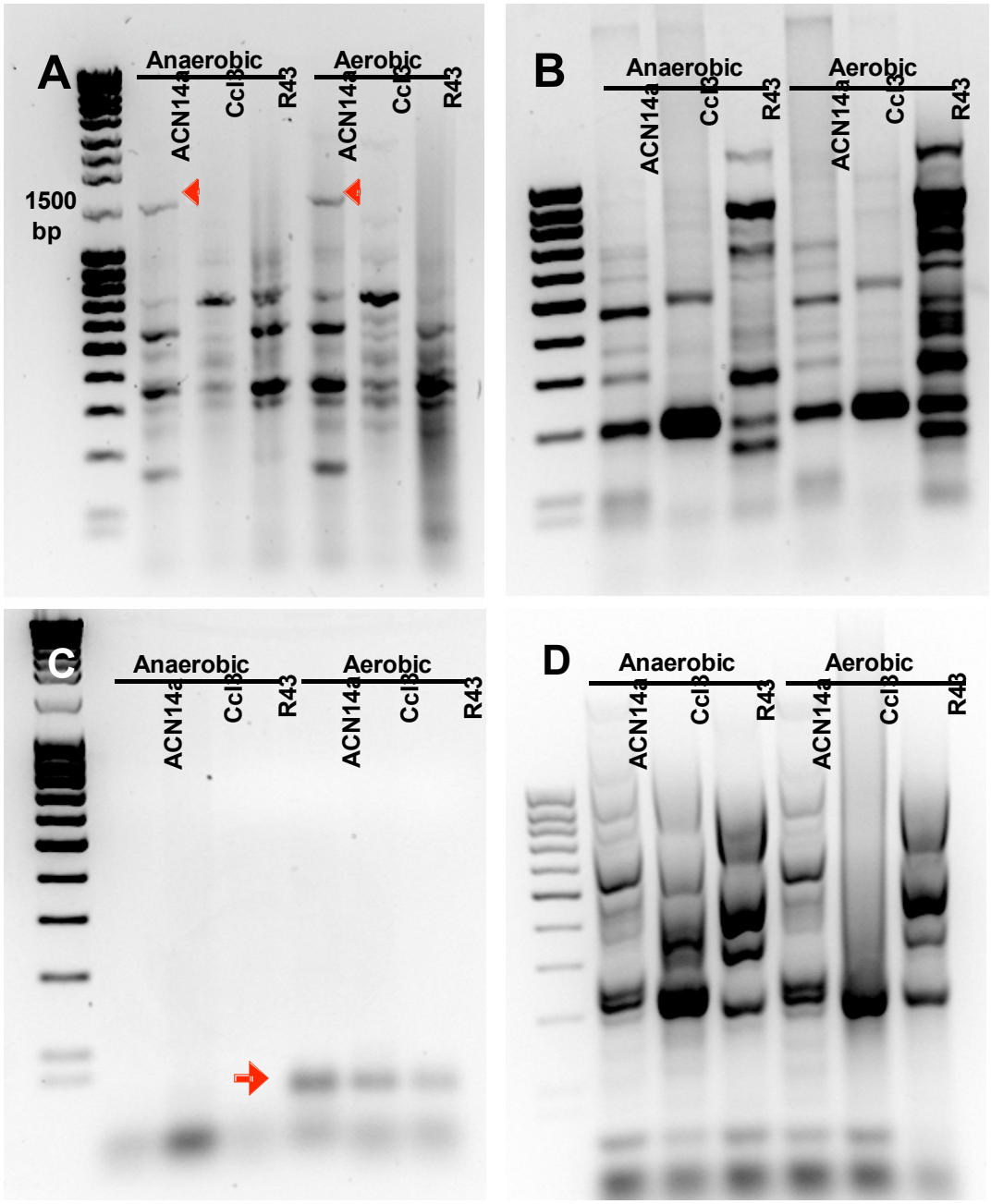

Figure 6 PCR amplification of *Frankia* DNA with primers designed for (A) [Fe] hydrogenase, (B) [NiFe] hydrogenase and (C) hoxY and (D) hoxF subunits of bidirectional hydrogenase, respectively. The first lane from the left is ladder following by ACN14a, CcI3 and R43 grown in anaerobic and aerobic condition, respectively. The anaerobic condition were initiated by argonisation and cultured for 24 hours. The red arrows (4a and 4c) pointed against the bands of interest.

lessons from cyanobacteria we have learnt so far illustrate that bidirectional hydrogenase is much like 'accessory enzyme' to 'principal enzyme'. Not all cyanobacteria have the enzyme and the presence of multiple bidirectional hydrogenase in a single strain is highly unlikely (Ghirardi et al., 2007). Interestingly, Mohapatra et al. (2004, 2006) together with Leul et al. (2005) declared hydrogen evolution from *Frankia* strain R43 and CcI3; nonetheless, all the cases reported hydrogen evolution under anaerobic condition. Amino acid sequencing of the 47 kD protein interacted with Hox F of *R. eutropha* revealed a high homology to the hox H subunit of bidirectional hydrogenase from *Anabaeba siamensis* (Mohapatra et al., 2004). Nonetheless, the search for the amino acid sequence did not put a trace on the three available *Frankia* genomes, but only molecular chaperone. Taken all together, it is possible that the bidirectional hydrogenase night not exist in the *Frankia* strain ACN14a and CcI3.

Frankia and its uptake hydrogenases

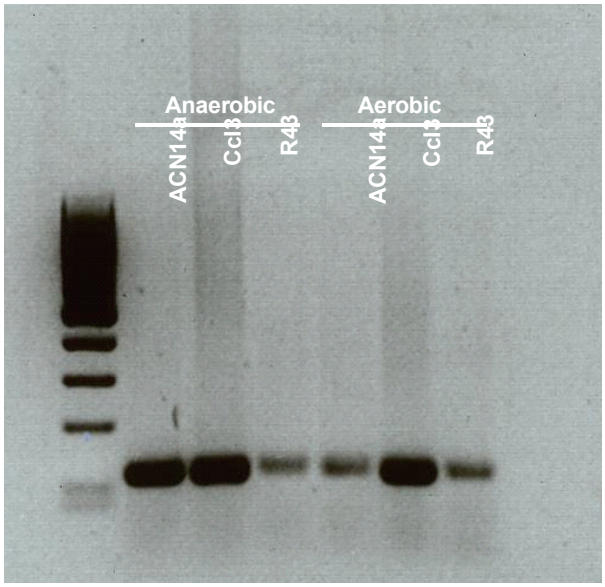

Figure 7 Expression of uptake hydrogenase in the *Frankia* ACN14a, CcI3 and R43 as revealed by the primer hupL2.

A previous report of ubiquitously expressing nature of uptake hydrogenase in *Frankia* species was attested in the strain KB5, where the unprocessed and processed subunit of approximately 62 and 60 kDa were characterised (Mattson and Sellstedt, 2002). The use of hupL2 primers specific to large subunit in this experiment accentuated the consecutive expression of the enzyme in the strains ACN14a, CcI3 and R43 regardless of oxygen as show in figure 7. The previous knowledge

postulates that uptake hydrogenase is less active under aerobic condition as it recycles electron back to nitrogenase in the nitrogen fixation process (Leul et al., 2005a); in contrast, expression pattern of the uptake hydrogenase in the strain R43 seems aberrant. The stronger expression was observed under oxygenic condition to anaerobic one, which possibly describes the reported hydrogen evolution in the strain R43 under aerobic condition if the enzyme posed in the reverse direction. However, one should be reminded, the huge amount of cells was used for measuring hydrogen evolution in this experiment. It is feasible that microoxygenic condition was generated per se from high density of the cells, and this activated the uptake hydrogenase when hydrogen was built up. Nonetheless, the detailed maturation process of the enzyme is still uninformed, and it, of course, needs to be further dissected for more understanding in the uptake hydrogenase of *Frankia*.

IV. Conclusions

(1.) The *Frankia* strains ACN14a, CcI3 and R43 do not possess the [Fe] hydrogenase as obviously depicted by both in silico and in vitro tests in this experiment.

(2.) The uptake hydrogenase function is expressed consecutively in all the strains tested. Also, it is likely that uptake hydrogenase acting in reverse direction, in some extent, involves with hydrogen evolution in the strain R43.

(3.) The bidirectional hydrogenase function is absent in the *Frankia* strains ACN14a and CcI3. However, this conclusion will be only applied to the strain R43 providing that its genome sequence is thoroughly examined.

V. Future Directions

(1.) The [Fe] hydrogenase and its isoforms are found in a range of organisms, from prokaryote to eukaryote. This information introduces questions regarding the possibility that *Frankia* [Fe] hydrogenase has structurally distinct from those found in other bacteria and fungi. If such, how many classes of [Fe] hydronease that *Frankia* possesses?

(2.) Once the probing for hoxH and hoxF were unsuccessful in the three researched strains in coupling with relatedness between hydrogen-involving hydrogenase in the strain R43 and bidirectional hydrogenase of *A. Siamensis* were coined (Mohapatra et al., 2006), these following questions should be addressed.

(I) Are there an enzymatic diversity of hydrogen-evolving hydrogenase among the *Frankia* strains?

(II) How *Frankia*'s hydrogen-evolving hydrogenase and cyanobacterial bidirectional hydrogenase are structurally different and evolutionary related?

(3.) The uptake hydrogenase is one of the enzymes involving with nitrogen fixation. From our current knowledge, uptake hydrogenases has a niche under microoxygenic condition describing the situation where it functions synchronously with nitrogenase inside the vesicle. It is interesting to enhance the function of uptake hydrogenase, principally feed more electrons into the system, which might increase nitrogen fixation.

(4.) Several *Frankia* strains have been isolated and collected worldwide. Among hundreds of isolates, basic knowledge of genetic diversity and evolutionary prospects among them is far from exclusive. Attempts to sequence more *Frankia* strains are taken worldwide. As clearly seen in this experiment, such strains of *Frankia* show certain uniqueness regarding to its origin and adaptation to host. It is neither possible to draw final conclusion concerning number of hydrogenases in *Frankia* nor to conclude that [Fe] hydrogenase nor bidirectional hydrogenase are not present in *Frankia*.

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