Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 568549, 8 pages
http://dx.doi.org/10.1155/2014/568549

Research Article

Contrasted Reactivity to Oxygen Tensions in *Frankia* sp. Strain CcI3 throughout Nitrogen Fixation and Assimilation

Faten Ghodhbane-Gtari,[1,2] Karima Hezbri,[1] Amir Ktari,[1] Imed Sbissi,[1] Nicholas Beauchemin,[2] Maher Gtari,[1,2] and Louis S. Tisa[2]

[1] *Laboratoire Microorganismes et Biomolécules Actives, Université Tunis El Manar (FST) and Université Carthage (INSAT), Campus Universitaire, 2092 Tunis, Tunisia*
[2] *Department of Molecular, Cellular & Biomedical Sciences, University of New Hampshire, 46 College Road, Durham, NH 03824-2617, USA*

Correspondence should be addressed to Louis S. Tisa; louis.tisa@unh.edu

Received 18 April 2014; Revised 28 April 2014; Accepted 15 May 2014; Published 28 May 2014

Academic Editor: Ameur Cherif

Copyright © 2014 Faten Ghodhbane-Gtari et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Reconciling the irreconcilable is a primary struggle in aerobic nitrogen-fixing bacteria. Although nitrogenase is oxygen and reactive oxygen species-labile, oxygen tension is required to sustain respiration. In the nitrogen-fixing *Frankia*, various strategies have been developed through evolution to control the respiration and nitrogen-fixation balance. Here, we assessed the effect of different oxygen tensions on *Frankia* sp. strain CcI3 growth, vesicle production, and gene expression under different oxygen tensions. Both biomass and vesicle production were correlated with elevated oxygen levels under both nitrogen-replete and nitrogen-deficient conditions. The mRNA levels for the nitrogenase structural genes (*nif*HDK) were high under hypoxic and hyperoxic conditions compared to oxic conditions. The mRNA level for the hopanoid biosynthesis genes (*sqh*C and *hpn*C) was also elevated under hyperoxic conditions suggesting an increase in the vesicle envelope. Under nitrogen-deficient conditions, the *hup*2 mRNA levels increased with hyperoxic environment, while *hup*1 mRNA levels remained relatively constant. Taken together, these results indicate that *Frankia* protects nitrogenase by the use of multiple mechanisms including the vesicle-hopanoid barrier and increased respiratory protection.

1. Introduction

The genus *Frankia* is comprised of nitrogen-fixing actinobacteria that are able to establish a mutualistic symbiosis with a variety of dicotyledonous host plants that results in the establishment of a root nodule structure [1–6]. The bacteria nourish their host plant with combined nitrogen and the plants provide in return carbon and energy. This symbiosis allows actinorhizal host plants to colonize nutrient-poor soils. Besides its life style within the host plant, these bacteria are members of soil community although less information is known about this life style [7]. Under arid tropic and subtropic conditions of North Africa, actinorhizal plants are essentially represented by fast growing and highly tolerant trees from the family Casuarinaceae [8].

Under atmospheric oxygen conditions, *Frankia* actively fixes dinitrogen to ammonium within the root nodules of the host plants and aerobically in culture [9–15]. The oxygen-labile nitrogenase enzyme is localized within specialized thick-walled structures, termed vesicles that are formed *in planta* and *in vitro* [2, 16–18]. Their shape is strain dependent and host-plant-influenced. Vesicles act as specialized structures for the nitrogen fixation process and are formed terminally on short side branches of hyphae that have a septum near their base. The mature vesicle is surrounded by an envelope that extends down the stalk of the vesicle past the basal septum, which separates the vesicle from the hypha. The envelope surrounding the vesicle is composed of multilaminated lipid layers containing primarily bacteriohopanetetrol and its derivatives [19–22]. It is believed that this lipid envelope acts as an oxygen diffusion barrier to protect the nitrogenase enzyme from oxygen inactivation [19].

Unlike other actinorhizal plants, *Frankia* found within the root nodules of *Casuarina* and *Allocasuarina* plants are

devoid of symbiotic vesicle structures [23, 24]. A positive correlation was observed between the differentiation of intracellular hyphae and the lignifications of the host-infected cell walls [23]. In several actinorhizal nodules, a low oxygen tension was shown to be consistent with the high concentrations of hemoglobin [2]. *Frankia* are known to produce truncated hemoglobins [25–27]. Besides hemoglobins, *Frankia* possess hydrogenases that may act as oxygen-scavenging enzymes [28]. Sequencing of several *Frankia* genomes [29–34] has provided insight on the physiology and opened up new genomics tools for these microbes. These databases have been used in transcriptomics [35–37] and proteomics studies [38–40] on these bacteria. The aim of the present study was to investigate the expression levels for several selected genes involved under different oxygen concentration for the *Casuarina* compatible *Frankia* sp. strain CcI3. These genes were involved in the following functions: nitrogen fixation and assimilation, hopanoid biosynthesis, hydrogen uptake, and oxidative stress.

2. Materials and Methods

2.1. Culture Conditions and Experimental Design. *Frankia* sp. strain CcI3 [41] was grown and maintained at 28°C in basal MP growth medium with 5.0 mM propionate and 5.0 mM NH_4Cl as carbon and nitrogen sources, respectively, as described previously [42].

In all experimental procedures, *Frankia* cells were grown for 7 days in 250 mL cylindrical bottles with a working MP medium volume of 50 mL with and without NH_4Cl for nitrogen-deficient and nitrogen-replete conditions, respectively. Three sets of oxygen tensions were considered: oxic (atmospheric condition), hypoxic (reduced partial pressure of oxygen), and hyperoxic (elevated oxygen levels). Hypoxic conditions were generated by placing the cultures in Brewer's jar that contained reduced partial pressures of oxygen by the use of gas packets (BBL GasPak BBL CampyPak System). For this system, water interacts with catalyst in the packet generating a reduced partial pressure of oxygen within the chamber. Hyperoxic conditions were generated by continuously air-sparging the cultures via an aquarium pump.

2.2. Growth Assessment and Vesicle Count. For dry weight determinations, cell cultures were collected on tarred membrane filters (type HA, 0.45 um pore size; Millipore Corp.). The filters were placed in a Petri dish over desiccant and dried at 90°C to constant weight [43]. In parallel, protein content was measured. Briefly, cell samples were solubilized by heating for 15 min at 90°C in 1.0 N NaOH and total proteins were measured using BCA method [44].

Vesicle numbers were determined as previously described [45, 46]. Briefly, cells were sonicated for 30 s with a Braun model 350 sonifier under power setting of 3 using microtip probe. This treatment disrupted the mycelia and released vesicles. The numbers of vesicles were counted by using a Petroff-Hausser counting chamber with a phase-contrast microscope at magnification of 400x.

2.3. Determination of Ammonia. Ammonium concentration was determined in cell-free media using modified protocol of Berthelot's reagent [47].

2.4. RNA Extraction, RT-PCRs, and Q-PCR. For these experiments, all solutions and materials were DEPC-treated to prevent RNA degradation. RNA extractions were performed by the Triton X100 method as previously described [48]. RNA samples were treated with DNase I (New England Biolabs) according to the manufacturer's recommendations. RNA samples were quantified with a Nanodrop 2000c spectrophotometer (Thermo Scientific) and stored at −80°C until use. The cDNA synthesis was performed using hexamer primers, 400 ng RNA and SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer's recommendations. The cDNA was quantified by a Nanodrop 2000c spectrophotometer, diluted to 10 ng/μL working stocks in DNAse-free, RNAse-free H_2O, and stored at −20°C until use.

Frankia gene expression analyses were performed by qRT-PCR using specific primers (Table 1) and SYBR Green PCR Master Mix (Applied Biosystems) as described previously [49]. Briefly, each 25 μL reaction contained 50 ng template cDNA, 300 nM of the forward and reverse primer mix, and SYBR Green PCR Master Mix. Parameters for the Agilent MP3000 were as follows: (1) 95°C for 15 min, (2) 40 cycles of 95°C for 15 s and 60°C for 30 s, and (3) thermal disassociation cycle of 95°C for 60 s, 55°C for 30 s, and incremental increases in temperature to 95°C for 30 s. Reactions were performed in triplicates and the comparative threshold-cycle method was used to quantify gene expression. The results were standardized with *rps*A expression levels. Relative expression (fold changes) was determined by the Pfaffl method [50] with the control as the calibrator. Two biological replicates of the triplicate samples were averaged.

3. Results

3.1. Growth and Vesicle Production under Different Oxygen Pressures. Figure 1 shows the effect of oxygen on the growth yield of *Frankia* sp. strain CcI3. Under nitrogen-replete conditions (NH_4), the biomass of cells grown under hyperoxic conditions was greater than both cultures grown under oxic and hypoxic conditions. Under nitrogen-deficient (N_2) conditions, the biomass correlated with the oxygen level with the hyperoxic conditions generating the greatest biomass. Furthermore, vesicle production under nitrogen-deficient (N_2) conditions positively correlated with oxygen tension. Cells under hyperoxic (air-sparged) conditions produced 2.6- and 5.4-fold more vesicles ($6.50 \pm 0.41 \times 10^6$/mg) than oxic ($2.45 \pm 0.29 \times 10^6$/mg) and hypoxic ($1.20 \pm 0.36 \times 10^6$/mg) conditions, respectively. Analysis of ammonia metabolism by *Frankia* CcI3 indicates that it was correlated with oxygen tension. With nitrogen-replete conditions, hyperoxic conditions resulted in the highest ammonia consumption, followed by oxic condition and lastly hypoxic condition (Figure 1(c)). Under nitrogen-deficient conditions the level of ammonium ions increased under lower oxygen tension. This level decreased with corresponding increases in oxygen tension.

Table 1: Primers used in this study.

Locus tag	Gene	Gene identity	Sequence
francci3_4488	*nif*H	Nitrogenase reductase iron-sulfur protein	5′-CGACAACGACATGAAGACC-3′ 5′-CTTGCCGATGATGCTCTC-3′
francci3_4487	*nif*D	Nitrogenase molybdenum-iron protein alpha chain	5′-AAGGACATCGTCAACATCAGCCAC-3′ 5′-AACTGCATCGCGGCGAAGTTATTC-3′
francci3_4486	*nif*K	Nitrogenase molybdenum-iron protein beta chain	5′-TGACGACGACTCCGGAAACAAACA-3′ 5′-TGTGGTAGACCTCGTCCTTGAACA-3′
francci3_4496	*hup*1	Nickel-dependant hydrogenase, large subunit	5′-AACAAATCTGCGACGTCACGGTCA-3′ 5′-ACTCTCGATCCATTCACCGCAGTA-3′
francci3_1076	*hup*2	Uptake hydrogenase, large subunit	5′-TGGAAGGTCAACTGGCTGGAGAA-3′ 5′-ATGTCTAGGCAGTACCGGAGGAAGAA-3′
francci3_1149	*hbo*O	Truncated hemoglobin	5′-GGGACGCCTGGCTGAAGA-3′ 5′-CCAGAGCTGCCTGTCGAGATC-3′
francci3_2581	*hbo*N	Truncated hemoglobin	5′-CACCCCTCTTTGCCAACCG-3′ 5′-GGTGGTTTCCGTCGGGAC-3′
francci3_0823	*sqh*C	Squalene hopene cyclase	5′-TGCAATGGCTGCTGGACAA-3′ 5′-TGCCGTAGACGTGGTTGAT-3′
francci3_0819	*hpn*C	Squalene synthase	5′-AACTTCCCGGTCTCGCCGTT-3′ 5′-AACGCGTTGAAGTGGAAACGAACC-3′
francci3_2949	*kat*A	Catalase	5′-ACATGCCGGTGTTCTTCATTCAGG-3′ 5′-ACATCATCATGTGGCATCGACTCGG-3′
francci3_2817	*sod*A	Superoxide dismutase	5′-GTGCCAATGACACCCTTGAGAAGA-3′ 5′-AGTGGAGAATATGCCCGGAAAGGT-3′
francci3_3012	*glt*D	Glutamate synthase, small subunit	5′-TGCATGCGACGAACAACTTCCC-3′ 5′-ATGATGCTGACCTCGATCTGCTTG-3′
francci3_3013	*glt*B	Glutamate synthase, large subunit	5′-CGTGCTGAAGGTGATGTCCAAGAT-3′ 5′-AAATAGGCGTCGATCAGTTCCTGG-3′
francci3_3142	*gln*A	Glutamine synthetase, type I	5′-ATGACCCGATCACCAAGGAACAGT-3′ 5′-GGGTTGTAGTCATAACGGACATCG-3′
francci3_3143	*gln*A	Glutamine synthetase, type II	5′-AACTTCTCCACCAGGCAGACGAT-3′ 5′-AGAACTTGTTCCACGGAGCTGTCT-3′
francci3_4059	*gln*A	Glutamine synthetase, catalytic region	5′-TACAACATCGACTACGCGCTTTCC-3′ 5′-ATACCGGAACACGATCTCGAACTG-3′
francci3_1057	*rps*A	30S ribosomal protein S1	5′-CGAAGTCCGTTCCGAGTTC-3′ 5′-CGCCGAAGTTGACGATGG-3′

Locus tag and gene designation were determined from the Integrated Microbial Genomes System (IMG) at the Joint Genome Institute (https://img.jgi.doe.gov/) [51].

3.2. Expression of Nitrogen Fixation and Assimilation Genes under Different Oxygen Pressures. The effect of oxygen on the expression of several genes involved in nitrogen fixation and assimilation was measured by detecting changes in mRNA levels via qRT-PCR (Figure 2). For nitrogen-deficient conditions, the level of structural nitrogenase genes (*nif*HDK) mRNA increased >10-fold under hyperoxic and hypoxic conditions compared to oxic condition (Figure 2(a)). Under nitrogen-replete conditions, the expression levels for these genes were very low and there was no change with different oxygen tensions.

The *Frankia* genome contains two glutamate synthase genes (*glt*B and *glt*D) encoding the large and small subunits of the enzyme. These two glutamate synthase genes were studied for their expression levels under three oxygen tensions. The mRNA levels of the *glt*B gene were reduced except under hyperoxic and nitrogen-replete conditions (Figure 2(b)). The *glt*D mRNA levels increased slightly (1.3–2.5-fold) under the different nitrogen and oxygen conditions. There were four glutamine synthetase orthologs found within the *Frankia* sp. strain CcI3 genome. We were able to follow the expression of three of these *gln*A genes (Figure 2(c)). The level of *francci3_3143* mRNA was controlled by nitrogen. Under all oxygen conditions, *francci3_3143* mRNA levels increased 10–15-fold under nitrogen-deficient (N_2) conditions. Both high and low oxygen tensions increased the level of *francci3_3143* mRNA. The level of *francci3_3142* mRNA was decreased under nitrogen-deficient (N_2) conditions and showed 7-fold increase under hyperoxic under nitrogen-replete conditions. The levels of *francci3_4059* mRNA remained constant except under hyperoxic conditions, in which levels increased 15-fold. Under hyperoxic conditions, the levels of *francci3_4059* mRNA were controlled by nitrogen status and increased approximately 2-3-fold from nitrogen-replete (NH_4) conditions.

3.3. Expression of Genes Known to Protect Nitrogenase from Oxygen and Reactive Oxygen Species. The biosynthesis of

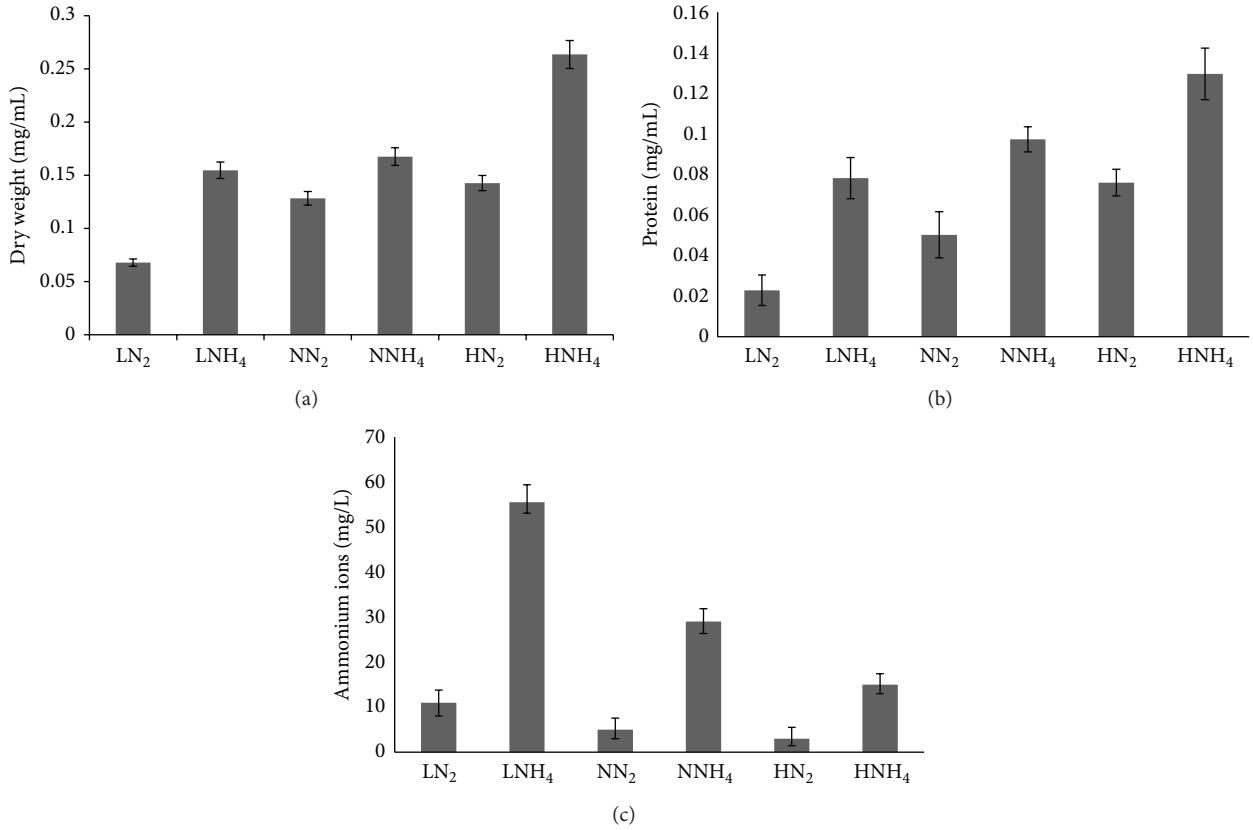

Figure 1: Biomass yields of *Frankia* sp. strain CcI3 grown under nitrogen fixation (N_2) and nitrogen-replete (NH_4) at hypoxic (L), oxic (N), and hyperoxic (H) conditions as estimation by (a) dry weight and (b) total protein and determination of (c) ammonium ion concentrations.

hopanoids has been correlated with vesicle development [19]. The effect of oxygen tension on the expression of the squalene synthase (*hpn*C) and squalene/phytoene cyclase (*sqh*C) genes was examined (Figure 2(d)). Under nitrogen-replete conditions (NH_4), the level of mRNA for *sqh*C showed a 2-fold increase for hyperoxic conditions. A smaller increase was observed for *hpn*C mRNA levels. In general, *sqh*C and *hpn*C were expressed constitutively with comparable mRNA levels for hypoxic and oxic levels. Under nitrogen-deficient (N_2) conditions, the mRNA levels of both genes (*sqh*C and *hpn*C) increased 2- and 1.5-fold, respectively.

The *Frankia* CcI3 genome contains two hydrogenase operons [30, 52, 53]. We tested the effects of oxygen tension and nitrogen status of their gene expression levels (Figure 2(e)). Under nitrogen-replete (NH_4) conditions, the level of mRNA for *hup*2 increased proportionally with the level of oxygen present, while the level of mRNA for *hup*1 only increased under hyperoxic conditions. The expression of *hup*2 was influenced by the nitrogen status of the cells and by the oxygen levels. Under both conditions, *hup*2 mRNA levels increased, but *hup*1 expression remained constant.

The effect of oxygen tension and nitrogen status was investigated on the expression of two truncated hemoglobins (*hbo*O and *hbo*N). The level of mRNA of *hbo*O and *hbo*N increased under hyperoxic condition for both nitrogen conditions (Figure 2(f)). Under nitrogen-replete (NH_4) conditions, mRNA levels for *hbo*O increased proportionally to the oxygen tension levels. Under hypoxic nitrogen-deficient conditions, mRNA levels for *hbo*N increased about 1.5-fold.

The effects of oxygen tension and nitrogen status on the expression levels of two oxygen defense enzymes, catalase (*kat*A) and superoxide dismutase (*sod*A), were also tested (Figure 2(g)). Under hyperoxic conditions, the mRNA levels of *kat*A increased 6.5- and 8-fold under nitrogen-deficient (N_2) and nitrogen-replete (NH_4) conditions, respectively. The expression of the *sod*A gene appeared to be constitutive under all oxygen tensions and both nitrogen statuses.

4. Discussion

Without a doubt, the vesicle is the most characteristic morphogenetic structure produced by *Frankia* [1]. Vesicles are functionally analogous to cyanobacterial heterocysts providing unique specialized cells that allow nitrogen fixation under aerobic condition [54, 55]. In this study, the growth of *Frankia* strain CcI3 was evaluated under three oxygen tensions. The results indicate that growth increased with elevated oxygen tensions (Figure 1) confirming the aerobic nature of the microbe. Although the dry weight measurement increased, the total protein values were reduced under hyperoxic nitrogen-deficient (N_2) conditions. This result would imply that the cells were producing other metabolic products under this condition and a similar level of protein compared to hypoxic nitrogen-deficient (N_2) condition. Thus, this result

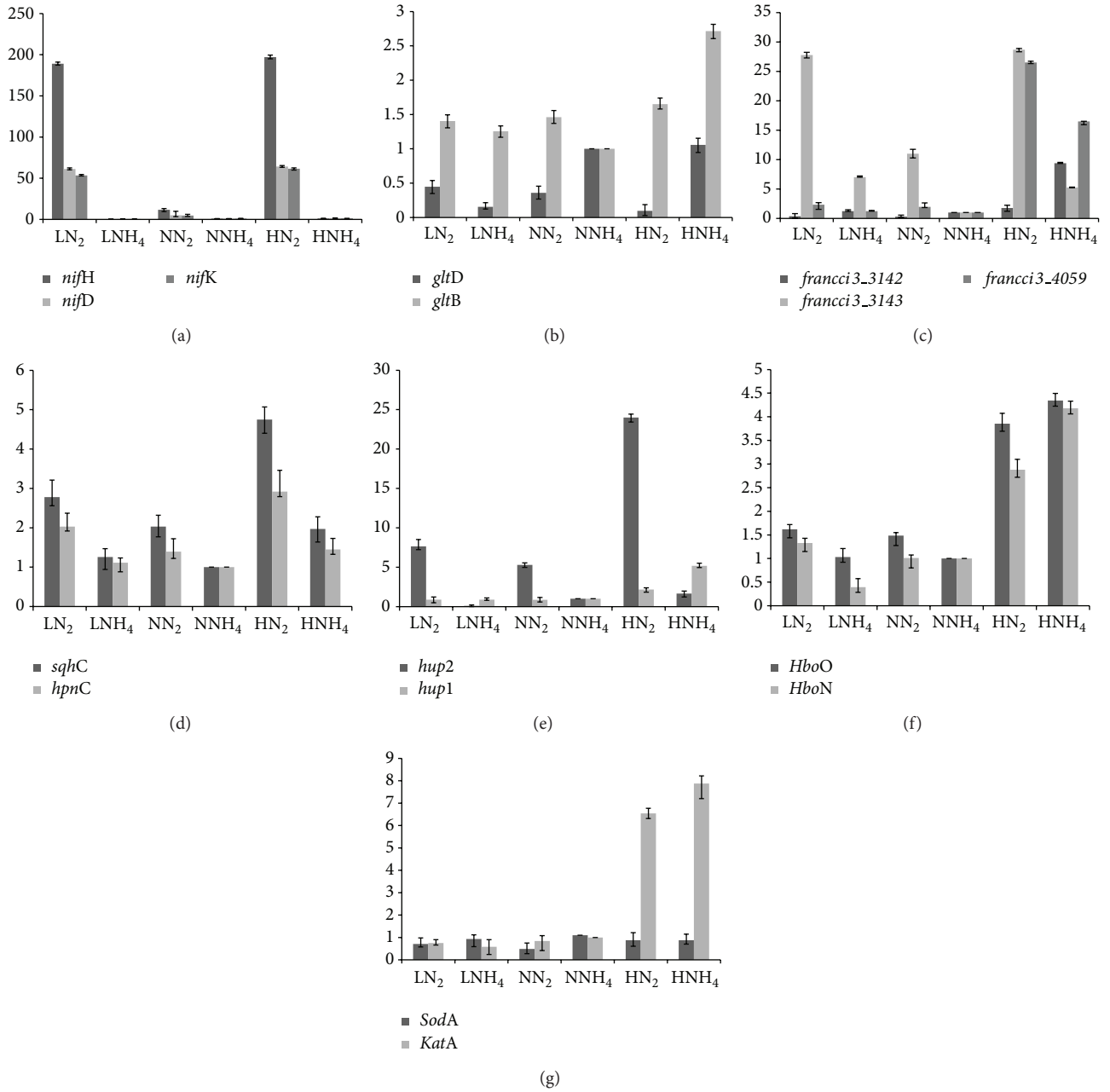

Figure 2: Relative gene expression (fold change) in response to hyperoxic and hypoxic conditions. *Frankia* cultures were grown under nitrogen-replete (NH_4) or nitrogen-deficient (N_2) conditions. These cultures were exposed to oxic (N), hyperoxic (H), and hypoxic (L) conditions as described in Section 2. Experimental gene expression was normalized to the rpsA housekeeping gene and compared to the calibrator (NH_4 oxic conditions). The following genes were analyzed: (a) *nif* HDK (b) *glt*B and *glt*D, (c) *gln*A genes, (d) *hpn*C and *sqh*C, (e) *hup*1 and *hup*2, (f) *hbo*N and *hbo*O, and (g) *sod*A and *kat*A.

suggests that part of the respiration was uncoupled providing some oxygen protection. *Frankia* contains two respiratory systems and a cyanide-insensitive system was proposed to help protect nitrogenase from oxygen inactivation [46]. With other aerobic nitrogen-fixing bacteria, increased respiratory rates in response to elevated oxygen tensions help maintain low levels of intracellular oxygen protecting nitrogenase from inactivation [56, 57]. Under nitrogen-deficient (N_2) conditions, vesicles were produced and correlated with oxygen tensions. The numbers of vesicles produced per mg dry weight increased with elevated oxygen levels. These results confirm those obtained previously [58, 59].

In our study, we investigated the effects of oxygen on gene expression for a variety of functional genes involved in nitrogen fixation, nitrogen assimilation, and protection from oxygen and other reactive oxygen species [60]. The levels of expression for the structural nitrogenase genes (*nif* HDK) indicate a concordant profile with clear induction under

nitrogen-deficient (N_2) conditions. Transcriptome studies on *Frankia* sp. strain CcI3 under nitrogen-deficient and nitrogen-replete conditions also show an increase in *nif* HDK gene expression [35, 36]. The levels of *nif* HDK mRNA showed an increase under hypoxic and hyperoxic conditions indicating that nitrogenase induction was influenced by oxygen levels.

The hopanoid envelope has been postulated to be involved in the protection of nitrogenase from oxygen inactivation [19]. We found that mRNA levels of squalene synthase (*hpn*C) and squalene-hopene cyclase (*sqh*C) genes increased in response to oxygen tension under nitrogen-deficient conditions, but remained constant under nitrogen-replete conditions (Figure 2(d)). The results correlate with the increase in vesicle envelope observed under high oxygen levels [61]. Nalin et al. [62] found only a slightly higher hopanoid content under nitrogen-deficient conditions suggesting remobilization rather than nascent biosynthesis. Furthermore, the *Frankia* sp. strain CcI3 transcriptome profiles under nitrogen-deficient and nitrogen-replete conditions did not show any significant differences in hopanoid biosynthetic genes [35, 36]. However, these studies were performed under one oxygen tension while our study has investigated three different oxygen tensions.

Analysis of the nitrogen assimilation genes (*glt*B, *glt*D, and *gln*A) is a bit more complex. The *Frankia* CcI3 genome contained several homologues of *gln*A. The mRNA level of *francci3_3143* correlated the best with nitrogen regulation, being increased under nitrogen-deficient conditions. Transcriptome studies have shown that *francci3_3143* expression increased significantly under nitrogen-fixing conditions [35, 36], while all of the other homologues remained consistent. This result would suggest that this gene encoded primary nitrogen scavenging enzyme. The levels of expression were also influenced by elevated oxygen tensions during increased nitrogenase activity. The expression levels of the *glt*B and *glt*D appear to be less influenced by oxygen tension. These effects seemed in agreement with the ammonia metabolism results that showed an increase in consumption under hyperoxic conditions.

Our results on hemoglobin gene expression correlate with previous results [48] that showed no increase in *hbo*N and *hbo*O expression in response to nitrogen status increased under low oxygen tension. However, our results conflict in response to oxygen. We found that both *hbo*N and *hbo*O mRNA levels increased under hyperoxic conditions. The use of the more sensitive qRT-PCR in our study compared to RT-PCR is the best explanation for these differences.

Frankia possesses two uptake hydrogenase systems [52, 53]. One of them has been correlated with symbiotic growth and the other to free-living conditions [53]. Our results show that *hup*2 gene expression was influenced by nitrogen status suggesting that it was associated with vesicle production, while *hup*1 gene expression was relatively constant. The levels of *hup*2 mRNA increased proportionally with oxygen tensions suggesting potential oxygen protection mechanism. Anoxic conditions have no effect on hydrogenase gene expression by *Frankia* CcI3 but increased by 30% for *Frankia alni* ACN14a [60]. We did not test anoxic conditions in our study.

Increased oxygen tension can lead to elevated oxidative stress conditions. We investigated the influence of oxygen tensions on reactive oxidative stress genes. While *sod*A expression levels were constitutive, *kat*A gene expression increased under hyperoxic conditions. In general, our results confirm those of Steele and Stowers [63], which examined enzymatic activity levels. They reported an increase in catalase activity in cultures derepressed for nitrogen fixation compared to ammonium-grown cultures.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

Louis S. Tisa was supported in part by Agriculture and Food Research Initiative Grant 2010-65108-20581 from the USDA National Institute of Food and Agriculture, Hatch Grant NH530, and the College of Life Sciences and Agriculture at the University of New Hampshire, Durham, NH, USA. This is scientific contribution number 2556 from the NH Agricultural Experimental Station. Maher Gtari and Faten Ghodhbane-Gtari were supported in part by a Visiting Scientist and Postdoctoral Scientist Program administered by the NH AES at the University of New Hampshire.

References

[1] D. R. Benson and W. B. Silvester, "Biology of *Frankia* strains, actinomycete symbionts of actinorhizal plants," *Microbiological Reviews*, vol. 57, no. 2, pp. 293–319, 1993.

[2] K. Huss-Danell, "Actinorhizal symbioses and their N_2 fixation," *New Phytologist*, vol. 136, no. 3, pp. 375–405, 1997.

[3] L. G. Wall, "The actinorhizal symbiosis," *Journal of Plant Growth Regulation*, vol. 19, no. 2, pp. 167–182, 2000.

[4] C. Santi, D. Bogusz, and C. Franche, "Biological nitrogen fixation in non-legume plants," *Annals of Botany*, vol. 111, no. 5, pp. 743–767, 2013.

[5] J. Schwencke and M. Carú, "Advances in actinorhizal symbiosis: host plant-*Frankia* interactions, biology, and applications in arid land reclamation. A review," *Arid Land Research and Management*, vol. 15, no. 4, pp. 285–327, 2001.

[6] A. Sellstedt and K. H. Richau, "Aspects of nitrogen-fixing actinobacteria, in particular free-living and symbiotic *Frankia*," *FEMS Microbiology Letters*, vol. 342, no. 2, pp. 179–186, 2013.

[7] E. E. Chaia, L. G. Wall, and K. Huss-Danell, "Life in soil by the actinorhizal root nodule endophyte *Frankia*. A review," *Symbiosis*, vol. 51, no. 3, pp. 201–226, 2010.

[8] M. Gtari and J. O. Dawson, "An overview of actinorhizal plants in Africa," *Functional Plant Biology*, vol. 38, no. 8-9, pp. 653–661, 2011.

[9] J. D. Tjepkema, W. Ormerod, and J. G. Torrey, "Vesicle formation and acetylene reduction activity in *Frankia* sp. CPI1 cultured in defined nutrient media," *Nature*, vol. 287, no. 5783, pp. 633–635, 1980.

[10] J. D. Tjepkema, W. Ormerod, and J. G. Torrey, "Factors affecting vesicle formation and acetylene reduction (nitrogenase activity) in *Frankia* sp. CpI1," *Canadian Journal of Microbiology*, vol. 27, no. 8, pp. 815–823, 1981.

[11] D. Gauthier, H. G. Diem, and Y. Dommergues, "In vitro nitrogen fixation by 2 actinomycete strains isolated from *Casuarina* nodules," *Applied and Environmental Microbiology*, vol. 41, pp. 306–308, 1981.

[12] M. A. Murry, Z. Zhongze, and J. G. Torrey, "Effect of O_2 on vesicle formation, acetylene reduction, and O_2-uptake kinetics in *Frankia* sp. HFPCcI3 isolated from *Casuarina cunninghamiana*," *Canadian Journal of Microbiology*, vol. 31, no. 9, pp. 804–809, 1985.

[13] Z. Zhongze and J. G. Torrey, "Biological and cultural characteristics of the effective *Frankia* strain HFPCcl3 (Actinomycetales) from *Casuarina cunninghamiana* (Casuarinaceae)," *Annals of Botany*, vol. 56, no. 3, pp. 367–378, 1985.

[14] M. S. Fontaine, S. A. Lancelle, and J. G. Torrey, "Initiation and ontogeny of vesicles in cultured *Frankia* sp. strain HFPArI3," *Journal of Bacteriology*, vol. 160, no. 3, pp. 921–927, 1984.

[15] A. J. P. Burggraaf and W. A. Shipton, "Studies on the growth of *Frankia* isolates in relation to infectivity and nitrogen fixation (acetylene reduction)," *Canadian Journal of Botany*, vol. 61, no. 11, pp. 2774–2782, 1983.

[16] T. M. Meesters, "Localization of nitrogenase in vesicles of *Frankia* sp. Cc1.17 by immunogoldlabelling on ultrathin cryosections," *Archives of Microbiology*, vol. 146, no. 4, pp. 327–331, 1987.

[17] T. M. Meesters, S. T. Van Genesen, and A. D. L. Akkermans, "Growth, acetylene reduction activity and localization of nitrogenase in relation to vesicle formation in *Frankia* strains Cc1.17 and Cp1.2," *Archives of Microbiology*, vol. 143, no. 2, pp. 137–142, 1985.

[18] T. M. Meesters, W. M. Vanvliet, and A. D. L. Akkermans, "Nitrogenase is restricted to the vesicles in *Frankia* strain EAN1pec," *Physiologia Plantarum*, vol. 70, no. 2, pp. 267–271, 1987.

[19] A. M. Berry, O. T. Harriott, R. A. Moreau, S. F. Osman, D. R. Benson, and A. D. Jones, "Hopanoid lipids compose the *Frankia* vesicle envelope, presumptive barrier of oxygen diffusion to nitrogenase," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 90, no. 13, pp. 6091–6094, 1993.

[20] A. M. Berry, R. A. Moreaua, and A. D. Jones, "Bacteriohopanetetrol: abundant lipid in *Frankia* cells and in nitrogen-fixing nodule tissue," *Plant Physiology*, vol. 95, no. 1, pp. 111–115, 1991.

[21] O. T. Harriott, L. Khairallah, and D. R. Benson, "Isolation and structure of the lipid envelopes from the nitrogen-fixing vesicles of *Frankia* sp. strain CpI1," *Journal of Bacteriology*, vol. 173, no. 6, pp. 2061–2067, 1991.

[22] H. C. Lamont, W. B. Silvester, and J. G. Torrey, "Nile red fluorescence demonstrates lipid in the envelope of vesicles from N_2-fixing cultures of *Frankia*," *Canadian Journal of Microbiology*, vol. 34, no. 5, pp. 656–660, 1988.

[23] R. H. Berg and L. McDowell, "Endophyte differentiation in *Casuarina* actinorhizae," *Protoplasma*, vol. 136, no. 2-3, pp. 104–117, 1987.

[24] R. H. Berg and L. Mcdowell, "Cytochemistry of the wall of infected-cells in *Casuarina* actinorhizae," *Canadian Journal of Botany*, vol. 66, no. 10, pp. 2038–2047, 1988.

[25] V. Coats, C. R. Schwintzer, and J. D. Tjepkema, "Truncated hemoglobins in *Frankia* CcI3: effects of nitrogen source, oxygen concentration, and nitric oxide," *Canadian Journal of Microbiology*, vol. 55, no. 7, pp. 867–873, 2009.

[26] J. D. Tjepkema, "Hemoglobins in the nitrogen-fixing root nodules of actinorhizal plants," *Canadian Journal of Botany*, vol. 61, no. 11, pp. 2924–2929, 1983.

[27] J. D. Tjepkema, R. E. Cashon, J. Beckwith, and C. R. Schwintzer, "Hemoglobin in *Frankia*, a nitrogen-fixing actinomycete," *Applied and Environmental Microbiology*, vol. 68, no. 5, pp. 2629–2631, 2002.

[28] A. Sellstedt, P. Reddell, and P. Rosbrook, "The occurrence of hemoglobin and hydrogenase in nodules of 12 *Casuarina-Frankia* symbiotic associations," *Physiologia Plantarum*, vol. 82, no. 3, pp. 458–464, 1991.

[29] F. Ghodbhane-Gtari, N. Beauchemin, D. Bruce et al., "Draft genome sequence of *Frankia* sp. strain CN3, an atypical, non-infective (Nod-) ineffective (Fix-) isolate from *Coriaria nepalensis*," *Genome Announcements*, vol. 1, no. 2, Article ID e0008513, 2013.

[30] P. Normand, P. Lapierre, L. S. Tisa et al., "Genome characteristics of facultatively symbiotic *Frankia* sp. strains reflect host range and host plant biogeography," *Genome Research*, vol. 17, no. 1, pp. 7–15, 2007.

[31] A. Sen, N. Beauchemin, D. Bruce et al., "Draft genome sequence of *Frankia* sp. strain QA3, a nitrogen-fixing actinobacterium isolated from the root nodule of *Alnus nitida*," *Genome Announcements*, vol. 1, no. 2, Article ID e0010313, 2013.

[32] S. R. Mansour, R. Oshone, S. G. Hurst, K. Morris, W. K. Thomas, and L. S. Tisa, "Draft genome sequence of *Frankia* sp. strain CcI6, a salt-tolerant nitrogen-fixing actinobacterium isolated from the root nodule of *Casuarina cunninghamiana*," *Genome Announcements*, vol. 2, no. 1, Article ID e01205-13, 2014.

[33] I. Nouioui, N. Beauchemin, M. N. Cantor et al., "Draft genome sequence of *Frankia* sp. strain BMG5.12, a nitrogen-fixing actinobacterium isolated from Tunisian soils," *Genome Announcements*, vol. 1, no. 4, Article ID e00468-13, 2013.

[34] L. G. Wall, N. Beauchemin, M. N. Cantor et al., "Draft genome sequence of *Frankia* sp. strain BCU110501, a nitrogen-fixing actinobacterium isolated from nodules of *Discaria trinevis*," *Genome Announcements*, vol. 1, no. 4, Article ID e00503-13, 2013.

[35] D. M. Bickhart and D. R. Benson, "Transcriptomes of *Frankia* sp. strain CcI3 in growth transitions," *BMC Microbiology*, vol. 11, article 192, 2011.

[36] H.-I. Lee, A. J. Donati, D. Hahn, L. S. Tisa, and W.-S. Chang, "Alteration of the exopolysaccharide production and the transcriptional profile of free-living *Frankia* strain CcI3 under nitrogen-fixing conditions," *Applied Microbiology and Biotechnology*, vol. 97, no. 24, pp. 10499–10509, 2013.

[37] N. Alloisio, C. Queiroux, P. Fournier et al., "The *Frankia* alni symbiotic transcriptome," *Molecular Plant-Microbe Interactions*, vol. 23, no. 5, pp. 593–607, 2010.

[38] N. Alloisio, S. Félix, J. Maréchal et al., "*Frankia alni* proteome under nitrogen-fixing and nitrogen-replete conditions," *Physiologia Plantarum*, vol. 130, no. 3, pp. 440–453, 2007.

[39] J. E. Mastronunzio and D. R. Benson, "Wild nodules can be broken: proteomics of *Frankia* in field-collected root nodules," *Symbiosis*, vol. 50, no. 1-2, pp. 13–26, 2010.

[40] J. E. Mastronunzio, Y. Huang, and D. R. Benson, "Diminished exoproteome of *Frankia* spp. in culture and symbiosis," *Applied and Environmental Microbiology*, vol. 75, no. 21, pp. 6721–6728, 2009.

[41] Z. Zhang, M. F. Lopez, and J. G. Torrey, "A comparison of cultural characteristics and infectivity of *Frankia* isolates from root nodules of *Casuarina* species," *Plant and Soil*, vol. 78, no. 1-2, pp. 79–90, 1984.

[42] N. J. Beauchemin, T. Furnholm, J. Lavenus et al., "*Casuarina* root exudates alter the physiology, surface properties, and plant infectivity of *Frankia* sp. strain CcI3," *Applied and Environmental Microbiology*, vol. 78, no. 2, pp. 575–580, 2012.

[43] L. Tisa, M. Mcbride, and J. C. Ensign, "Studies of growth and morphology of *Frankia*strains $EAN1_{pec}$, $EuI1_c$, CpI1, and $ACN1^{AG}$," *Canadian Journal of Botany*, vol. 61, no. 11, pp. 2768–2773, 1983.

[44] P. K. Smith, R. I. Krohn, G. T. Hermanson et al., "Measurement of protein using bicinchoninic acid," *Analytical Biochemistry*, vol. 150, no. 1, pp. 76–85, 1985.

[45] L. S. Tisa and J. C. Ensign, "Comparative physiology of nitrogenase activity and vesicle development for *Frankia* strains CpI1, $ACN1^{AG}$, $EAN1_{pec}$ and $EUN1_f$," *Archives of Microbiology*, vol. 147, no. 4, pp. 383–388, 1987.

[46] L. S. Tisa and J. C. Ensign, "The calcium requirement for functional vesicle development and nitrogen fixation by *Frankia* strains $EAN1_{pec}$ and CpI1," *Archives of Microbiology*, vol. 149, no. 1, pp. 24–29, 1987.

[47] E. D. Rhine, G. K. Sims, R. L. Mulvaney, and E. J. Pratt, "Improving the Berthelot reaction for determining ammonium in soil extracts and water," *Soil Science Society of America Journal*, vol. 62, no. 2, pp. 473–480, 1998.

[48] J. Niemann and L. S. Tisa, "Nitric oxide and oxygen regulate truncated hemoglobin gene expression in *Frankia* strain CcI3," *Journal of Bacteriology*, vol. 190, no. 23, pp. 7864–7867, 2008.

[49] F. Perrine-Walker, P. Doumas, M. Lucas et al., "Auxin carriers localization drives auxin accumulation in plant cells infected by *Frankia* in *Casuarina glauca* actinorhizal nodules," *Plant Physiology*, vol. 154, no. 3, pp. 1372–1380, 2010.

[50] M. W. Pfaffl, "A new mathematical model for relative quantification in real-time RT-PCR," *Nucleic Acids Research*, vol. 29, no. 9, article e45, 2001.

[51] V. M. Markowitz, F. Korzeniewski, K. Palaniappan et al., "The integrated microbial genomes (IMG) system," *Nucleic Acids Research*, vol. 34, pp. D344–D348, 2006.

[52] P. Normand, C. Queiroux, L. S. Tisa et al., "Exploring the genomes of *Frankia*," *Physiologia Plantarum*, vol. 130, no. 3, pp. 331–343, 2007.

[53] M. Leul, P. Normand, and A. Sellstedt, "The organization, regulation and phylogeny of uptake hydrogenase genes in *Frankia*," *Physiologia Plantarum*, vol. 130, no. 3, pp. 464–470, 2007.

[54] N. A. Noridge and D. R. Benson, "Isolation and nitrogen-fixing activity of *Frankia* sp. strain CpI1 vesicles," *Journal of Bacteriology*, vol. 166, no. 1, pp. 301–305, 1986.

[55] L. S. Tisa and J. C. Ensign, "Isolation and nitrogenase activity of vesicles from *Frankia* sp. strain $EAN1_{pec}$," *Journal of Bacteriology*, vol. 169, no. 11, pp. 5054–5059, 1987.

[56] A. Hochman and R. H. Burris, "Effect of oxygen on acetylene reduction by photosynthetic bacteria," *Journal of Bacteriology*, vol. 147, no. 2, pp. 492–499, 1981.

[57] H. Dalton and J. R. Postgate, "Effect of oxygen on growth of *Azotobacter chroococcum* in batch and continuous cultures," *Journal of General Microbiology*, vol. 54, no. 3, pp. 463–473, 1968.

[58] M. A. Murry, Z. Zhongze, and J. G. Torrey, "Effect of O_2 on vesicle formation, acetylene reduction, and O_2-uptake kinetics in *Frankia* sp. *HFPCc*I3 isolated from *Casuarina cunninghamiana*," *Canadian Journal of Microbiology*, vol. 31, no. 9, pp. 804–809, 1985.

[59] Z. Zhongze, M. A. Murry, and J. G. Torrey, "Culture conditions influencing growth and nitrogen fixation in *Frankia* sp. *HFPCc*I3 isolated from Casuarina," *Plant and Soil*, vol. 91, no. 1, pp. 3–15, 1986.

[60] K. H. Richau, R. L. Kudahettige, P. Pujic, N. P. Kudahettige, and A. Sellstedt, "Structural and gene expression analyses of uptake hydrogenases and other proteins involved in nitrogenase protection in *Frankia*," *Journal of Biosciences*, vol. 38, no. 4, pp. 703–712, 2013.

[61] R. Parsons, W. B. Silvester, S. Harris, W. T. Gruijters, and S. Bullivant, "*Frankia* vesicles provide inducible and absolute oxygen protection for nitrogenase," *Plant Physiology*, vol. 83, no. 4, pp. 728–731, 1987.

[62] R. Nalin, S. R. Putra, A.-M. Domenach, M. Rohmer, F. Gourbiere, and A. M. Berry, "High hopanoid/total lipids ratio in *Frankia* mycelia is not related to the nitrogen status," *Microbiology*, vol. 146, no. 11, pp. 3013–3019, 2000.

[63] D. B. Steele and M. D. Stowers, "Superoxide dismutase and catalase in *Frankia*," *Canadian Journal of Microbiology*, vol. 32, no. 5, pp. 409–413, 1986.