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Identification of the truncated hemoglobin gene in *Frankia*

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Abstract
A PCR approach was used to identify the truncated hemoglobin (trHb) gene in *Frankia*. Primers were designed from the conserved regions of the actinomycete TrHb sequences. With these primers, a 202 bp amplicon was detected for several *Frankia* strains. Amplicons for *Frankia* strains EAN1pec, EUN1f, CN3, and EuI1c were sequenced and found to have 69–76% DNA homology with the trHbO gene from *Streptomyces coelicolor*. Several important amino acid residues including residues B9F, B10Y, CD1Y, E7A, and E14F that are involved in heme coordination were identified and were unique to the actinomycete cluster of trHbO genes. Phylogenetic analysis suggested that the *Frankia* trHb genes were grouped based on their respective genotype and clustered closest to *Mycobacterium* trHb genes.

Keywords: Actinorhizal symbiosis, hemoglobin, truncated hemoglobin, *Frankia*

1. Introduction

Frankia, a member of the Actinomycetales, forms a symbiotic nitrogen-fixing association with over 200 species of woody dicotyledonous plants that comprise eight families of angiosperms (Benson and Silvester, 1993). Hemoglobin has been identified and extracted from several *Frankia* isolates suggesting that its presence is widespread among all of the isolates (Tjepkema et al., 2002; Beckwith et al., 2002). The function(s) of hemoglobin in *Frankia* remains to be determined. Hemoglobin may play a vital role in nitrogen fixation, oxygen transfer, or oxidative stress.

Truncated hemoglobins (trHb) are the newest branch of the hemoglobin superfamily. Three distinct groups of trHbs (trHbN, trHbO, and trHbP) are distributed among eubacteria, cyanobacteria, protozoans, and plants (Wittenberg et al., 2002). Although trHbs are 20–40 amino acid residues shorter than (non-) vertebrate hemoglobins, they still maintain the conserved classical globin fold as a 2-over-2 α-helical sandwich (Pesce et al., 2000; Milani et al., 2001). The functions of these trHbs are currently being elucidated.

For example, *Mycobacterium tuberculosis* TrHbN has been hypothesized to provide protection from nitric oxide produced by macrophages in tubercles (Ouellet et al., 2002). Under the hypoxic conditions found in these granulomas, *M. tuberculosis* TrHbO has been proposed to act as an oxygen delivery protein for terminal oxidases (Pathania et al., 2002; Liu et al., 2004).

Biochemical evidence supports the hypothesis that *Frankia* hemoglobin is a trHb. The molecular weight of the *Frankia* hemoglobin is consistent with known trHbs. The presence or absence of a combined nitrogen source does not affect hemoglobin production. However, hemoglobin levels are greater when cells are grown at 2% oxygen as opposed to 20% oxygen (Beckwith et al., 2002). The purpose of this study was to identify the truncated hemoglobin gene in several diverse *Frankia* isolates.

2. Materials and Methods

Growth conditions

Frankia strains ACN1^{AG} (Lalonde et al., 1981), Cc1.17 (Meesters et al., 1985), CN3 (Mirza et al., 1994), CpI1 propionate variant (Callaham et al., 1978; Tisa et al.,

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1983), EAN1pec (Lalonde et al., 1981), EuI1c (Baker et al., 1980), and EUN1f (Lalonde et al., 1981) were grown and maintained in basal medium with NH_4Cl as a nitrogen source as described previously (Tisa et al., 1999). *Streptomyces coelicolor* NRRL B-16638 and *Escherichia coli* HB101 were grown in YEME (Hopwood et al., 1985) and Luria-Bertani (LB) (DIFCO), respectively.

Gene sequences used

The following trHb sequences (with their respective accession numbers) were used in this study: *Streptomyces coelicolor* trHbO (CAB71209); *Streptomyces avermitilis* trHbO (BAC73082); *Mycobacterium bovis* trHbN (CAD96236), trHbO (CAD97358); *Mycobacterium tuberculosis* trHbN (CAA98320), trHbO (CAA16047); *Mycobacterium avium* subsp. *paratuberculosis* trHbN (AAS03570), trHbO (AAS04608), trHbP (AAS05726); *Mycobacterium leprae* trHbO (CAC31634); *Corynebacterium glutamicum* trHbO (CAF21110); *Corynebacterium efficiens* trHbO (BAC19155); *Corynebacterium diptherae* trHbO (CAE50330); Sperm whale (*Physeter catodon*) Mb (P02185). TrHbO sequences from *Arthrobacter* sp., *Brevibacterium linens*, *Kinecoccus radiotolerans*, *Leifsonia xyli*, *Nocardia farcinica*, and *Thermobifia fusca* were all obtained using the Integrated Microbial Genomes System from the Joint Genome Institute (http://img.jgi.doe.gov/v1.0/main.cgi).

Preliminary sequence data for *Frankia* strains EAN1pec and CcI3 trHbO and trHbN were obtained from D. Benson and L. Tisa. Preliminary sequence data for ACN14a trHbO and trHbN were obtained from P. Normand.

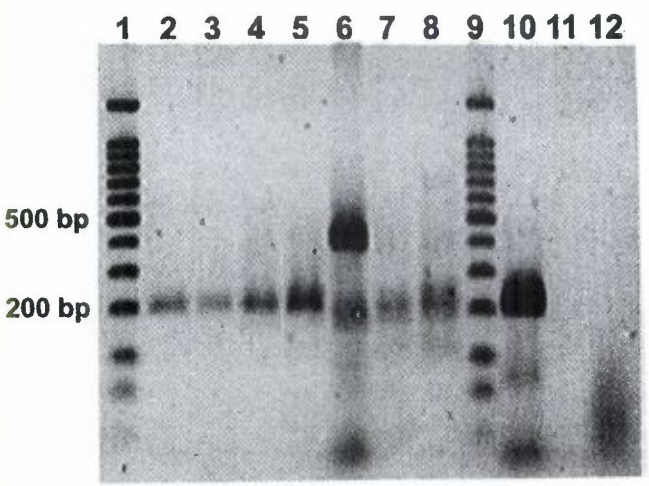

Figure 1. Agarose gel electrophoresis of TrHb PCR products (202 bp) amplified using TrHb-F and TrHb-R and DNA template from *Frankia* strains and controls. Lanes: (1 and 9) 100 bp DNA ladder, (2) EAN1pec, (3) EUN1f, (4) EuI1c, (5) CN3, (6) CcI.17, (7) ACN1ag, (8) CpI1-P, (10) *S. coelicolor* (+) control, (11) no template (–) control and (12) *E. coli* (–) control.

Polymerase chain reaction

Genomic DNA (gDNA) was isolated using the CTAB method (Wilson, 1989). A 202 bp trHb amplicon was amplified by PCR with the primer set TrHb-F (5'-GTCGGCGGGGAGGAGACCTTC-3') and TrHb-R (5'-CGTGCCGCATCCGCAGCCGCGG-3') using 250 ng of template DNA. The PCR was performed in 50 µl reaction volumes with 0.5 µM of each primer using the Failsafe™ PCR System (Epicentre Tech.) according to the manufacturer's recommendations. The primers were designed from *S. coelicolor* (bp positions 61 to 81 and positions 241 to 262) within conserved regions of the trHbO gene between *S. coelicolor*, *M. tuberculosis*, *M. leprae*, and *C. glutamicum*. Thermocycling parameters were as follows: i) initial denaturing at 95°C for 2 min; ii) 35 cycles of denaturization at 95°C for 1 min, primer annealing at 61°C for 45 sec, and primer extension at 72°C for 1 min; iii) and a final extension step at 72°C for 5 min. The amplicons were resolved by gel electrophoresis in a 2% agarose matrix containing 1X TBE according to Sambrook et al. (1989).

DNA sequencing and phylogenetic analyses

PCR products were purified with a Qiaquick® PCR Purification Kit, according to the manufacturer's recommendations, and used as a template for DNA sequencing reactions with a DYEnamic ET terminator cycle sequencing Kit (Amershan Pharmacia Biotech) in a ABI PRISM 377 sequencer (Perkin Elmer). Both the TrHb-F and TrHb-R primers were used for these sequencing reactions. Sequence analyses were performed using the SeqEd program version 1.0.3 (Applied Biosystems). The sequences were compared to those available from the GenBank and EMBL database using the BLAST program (Altschul et al., 1997). Partial sequences of the *Frankia* trHb amplicons have been deposited in GenBank under the following accession numbers: AY768545 (EUN1f), AY768546 (EAN1pec), AY768547 (EuI1c), and AY768548 (CN3). Frameplot 2.3.2 (Ishikawa and Hotta, 1999) was used to establish an open reading frame for each sequence. Hemoglobin sequences were aligned using ClustalX (Thompson et al., 1997). For phylogenetic analysis, a neighbor-joining tree was constructed from 1000 bootstrap replicates using PAUP 4.0b10 (Swofford, 2003).

3. Results and Discussion

Identification of Frankia *trHb amplicons*

An analysis of trHb amino acid sequences indicates that the actinomycetes form a single clade within the trHbO subgroup of truncated hemoglobins (Wittenberg et al., 2002). These gene sequences were aligned. and conserved regions were used to design the primers TrHb-F and

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                                                  * ** ***:*** **:***:
      EAN1pec -VAQNGPVNQPSPARPRADFYEAVGGEATFRALVARFYEGVASDPVLRPL    49
        EUN1f ----------------------------RALVARFYEGVASDPVLRPL    20
         CcI3 VPQNGRVNQSPPRTLPISSFYDAAGGEPTFRKLVARFYQGVANDPVLRPL    50
       ACN14a -----VSQPPTPAQPTTTTFFDAVGGEPTFRRLVARFYQGVANDPVLRPL    45
          CN3 ----------------------------RRLVARFYEGVAADPVLRPL    20
        EuI1c ----------------------------RRLVARFYEGVATDPVLRPL    20
S.coelicolor ---MDGVNEIRRGTLQEQTFYEQVGGEETFRRLVHRFYEGVAEDPILRPM    47

             **:::*  **:*: :**:******: * . ******* **
      EAN1pec YPDEDLAAAEERLRLFLIQYWGGPTTYSEQRGHPRLRMRHVPFAIGPAER    99
        EUN1f YPDEELAEAEERLRMFLIQYWGGPSTYSELRGHPRLRMRH----------    60
         CcI3 YPEEDLTGAEERLRMFLIQYWGGPTDYQEQRGHPRLRRRHAPFAIGPTQR   100
       ACN14a YPEDDLAGAEDRLRLFLIQYWGGPSDYQELRGHPRLRMRHVPFAIGPAQR    95
          CN3 YPEEDLGPAEERLRLFLIQYWGGPTTYHERRGHPRLRMRH----------    60
        EuI1c YPEEDLGPAEERLRLFLIQYWGGPATYHKKRGHPRLRMRH----------    60
S.coelicolor YPEEDLGPAEDRFALFLMQYWGGPTTYSDNRGHPRLRMRHAPFAVDRAAH    97

      EAN1pec DAWLRIMESAVDSLGLAPEHRAQLWDYLLMAANSLQNRPG   139
        EUN1f ----------------------------------------    60
         CcI3 DAWLKIMRAAVDSLDLPPDLDRQLWDYLSMAANSLQNRPD   140
       ACN14a DAWLVVMRAAVDSLGLPPDQYKTLWDYLQMAANSLQNRAD   135
          CN3 ----------------------------------------    60
        EuI1c ----------------------------------------    60
S.coelicolor DAWLKHMRVALDELGLSEEHEQTLWKYLTYAAASMINTPG   137
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Figure 2. Alignment of the full and partial trHb amino acid sequences from six *Frankia* strains, with the *S. coelicolor* trHbO sequence. Predicted amino acid sequences were obtained using Frameplot 2.3.2. The alignment was established using ClustalX program.

TrHb-R. The primer set was tested on 7 *Frankia* strains and yielded the expected 200 bp amplicon (Fig. 1). A larger amplicon of about 400 bp was also observed with strain Cc1.17. As expected, *S. coelicolor* yielded a 200 bp amplicon while *E. coli*, which is not known to produce a trHb, failed to yield any PCR product (Fig. 1).

From these samples, four (CN3, EAN1pec, EuI1c, and EUN1f) were sequenced and compared to the trHbO gene sequence of *Streptomyces coelicolor*. During the course of this study, three genome sequencing projects were initiated for *Frankia* strains EAN1pec, CcI3, and ACN14a, and the full trHbO genes for these isolates were identified from the draft sequences. Overall DNA similarities between the *Frankia* trHbO partial sequences ranged from 75% (ACN14a and CN3) to 87% (CN3 and EuI1c). Similarities observed between *S. coelicolor* and the *Frankia* isolates ranged from 69% (EUN1f, CcI3) to 76% (EuI1c). A database search using BLAST (Altschul et al., 1997) also showed high DNA similarities between these sequences and trHbO genes of other actinomycetes (data not shown).

Wittenberg et al. (2002) identified several important amino acid residues of trHbs in relation to heme coordination. Frameplot analysis (Ishikawa and Hotta, 1999) of the *Frankia* trHbO partial sequences determined their predicted amino acid sequences. The alignment of six predicted full and partial amino acid sequences is shown in Fig. 2. All six *Frankia* sequences possessed residues (B9F, B10Y, CD1Y, E7A, and E14F) that were unique to the actinomycete trHbO subgroup. These data including their high DNA sequence similarity to other trHbO genes, supports the presence of a trHbO in *Frankia*.

Phylogenetic analysis of truncated Hb sequences

An analysis of the draft genome sequences for the three *Frankia* strains revealed the presence of a gene that was homologous to the *Mycobacterium* spp. trHbN gene. The complete trHb gene sequences encompassing all 3 subgroups of trHbs (trHbN, trHbO, and trHbP) from *Frankia* and other actinomycetes were aligned to create a neighbor-joining distance tree (Fig. 3). Although our sample size was small, three distinct clades were evident that corresponded to the respective trHb subgroups. For HbO and HbNgenes, all of the *Frankia* strains were closest to the *Mycobacterium* sp. A tree that incorporated the partial *Frankia* trHbO sequences was also constructed and yielded a similar topology (data not shown).

Three distinct phylogenetic groups (I, II, III) of *Frankia* have been identified based on 16S rRNA sequence studies (Benson and Clawson, 2000; Normand et al., 1996). For the Group II representatives (CcI3 and ACN14a), the HbO and HbN genes formed a distinct group that was separate from the other *Frankia* strains.

The three representatives of Group III (EAN1pec, EUN1f and EuI1c) and a representative (CN3) from a fourth group of related "*Frankia*-like" nodulation and fixation defective (Nod^-/Fix^-) actinomycetes formed a separate subgroup of the *Frankia* strains. This clade branched out into the two groups: effective strains and defective strain. Strain EuI1c forms nodules on its host plant, but generates ineffective nodules (Baker et al., 1980). Present studies are focused on determining the level of expression for these two *Frankia* TrHb genes.

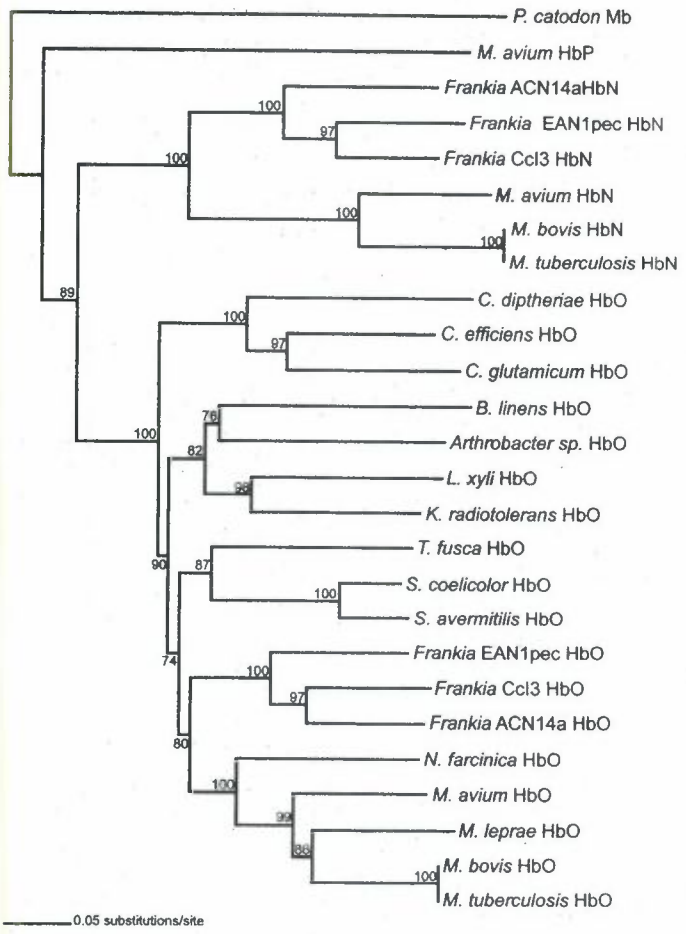

Figure 3. Phylogenetic dendogram representing neighbor-joining analysis of the trHb gene sequences (HbP, HbO, HbN subgroups) from *Frankia* and other actinomycetes. Sperm whale (*Physeter catodon*) Mb was included as an outgroup. The scale bar indicates 0.05 substitutions per site. Bootstrap values (above 50%) are shown as a percentage of 1000 replicates. Maximum Parsimony and Maximum Likelihood analyses produced a similar dendogram results (data not shown).

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