Molecular characterization of uptake hydrogenase in *Frankia*

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Abstract
A molecular characterization of uptake hydrogenase in *Frankia* was performed by using two-dimensional gel electrophoresis, matrix-assisted laser-desorption ionization–time-of-flight mass spectrometry, PCR amplification and Southern blotting. A polypeptide of approx. 60 kDa was recognized in *Frankia* UGL011102, AVCI1 and KB5 on the two-dimensional gel by blotting with *Ralstonia eutropha* (Hox G) antibody. Further analysis by MS resulted in a peptide ‘fingerprint’, which was similar to the membrane-bound hydrogenase 2 large subunit (HYD2) in *Escherichia coli*. In addition, a 127 bp PCR fragment could also be amplified from *Frankia* AVCI1, which gave a 76% similarity with the large subunit of hydrogenase in, e.g., *Azotobacter chroococcum*, *Bradyrhizobium japonicum* and *Rhizobium leguminosarum*. Although immunological similarity between the small subunit of *Frankia* hydrogenase and that of other organisms has not yet been found, a PCR product of 500 bp could be amplified from the local source of *Frankia*, the analysis of which gave 69 and 67% identity with the small subunit of hydrogenases in *B. japonicum* and *R. leguminosarum* respectively. A Southern-blot analysis further indicated evidence for the presence of the small hydrogenase subunit in other *Frankia* strains, i.e. KB5, Avci1 and CcI3.

Introduction
Hydrogenases are microbial enzymes that catalyse the reversible oxidation of molecular hydrogen according to the reaction H₂ ↔ 2H⁺ + 2e⁻. Hydrogenase activity has been reported in a large number of anaerobic and aerobic prokaryotes, as well as in some eukaryotes like algae, green plants and protozoa [1–4].

*Frankia* is filamentous, Gram-positive nitrogen-fixing soil actinomycetous bacterium, that in some ways resembles a fungus and which is phylogenetically and morphologically distinct from rhizobia bacteria that are responsible for nitrogen fixation in legumes [5].

The screening of 18 *Frankia* strains isolated from nine different host plants for hydrogenase activity indicated that uptake hydrogenase is common in *Frankia* [6]. The presence of hydrogenase in some *Frankia* strains has also been indicated by immunological studies [7]. Previous immunogold localization studies have shown that hydrogenase is localized in both hyphae and vesicles in *Frankia* [8]. However, the structure of *Frankia* hydrogenase is not known to date.

Therefore, this paper aimed at shedding more light on the molecular characterization of hydrogenase in *Frankia*, by use of different techniques.

Materials and methods
Bacterial strain and growth conditions
*Frankia* strains UGL011102, AVCI1 and KB5, isolated from *Alnus incana*, *A. viridis* and *Casuarina equisetifolia* respectively, were grown with shaking, in PUM + N medium [9] at 27°C [9]. To induce vesicle formation and nitrogen fixation, cultures were transferred to PUM – N medium [9].

Protein preparation and Western-blot analysis
*Frankia* proteins were prepared as described earlier [9]. Proteins for two-dimensional gel electrophoresis were centrifuged (15 000 g, 10 min, 4°C), and the pellet was air-dried and dissolved in sample rehydration buffer for two-dimensional electrophoresis. Immunoblots were performed as described before [6].

One-dimensional gel electrophoresis
ZOOM™ Strips (immobilized pH gradient gels) were rehydrated in the ZOOM™ IPGRunner Cassette (Invitrogen) in 155 µl of the rehydration buffer containing 100 µg of protein for 16 h at room temperature. Then, isoelectric focusing was performed by using the ZOOM™ IPGRunner System by increasing the voltage gradually and maintaining the final focusing voltage for approx. 2 h (175 V for 15 min, 175–2000 V ramp for 45 min and 2000 V for 105 min).

Two-dimensional gel electrophoresis
The electrofocused IPG strip was incubated in a reducing solution containing dithiothreitol at a final concentration of 50 mM equilibration buffer (NuPAGE™ LDS sample buffer) for 15 min. The IPG strip was then incubated for 15 min in an alkylating solution containing 125 mM iodoacetamide and electrophoresed on NuPAGE™ 4–12% Bis-Tris ZOOM™ gel (Invitrogen) at 200 V for 50 min.

Peptide analysis
Proteins were excised from Coomassie stained two-dimensional mini gels and cleaved with trypsin by in-gel
**Results and discussion**

**Immunoblotting**

Two-dimensional gel electrophoresis performed on *Frankia* proteins followed by blotting showed recognition to a polypeptide at approx. 60 kDa. This polypeptide corresponded to the large hydrogenase subunit of *Frankia* strains UGL011102, AVCI1 and KB5 (results not shown), a finding that is supported by earlier studies [7].

**Peptide analysis and partial sequence of the large subunit of hydrogenase**

The analysis of the polypeptide spot at 60 kDa in *Frankia* KB5 by matrix-assisted laser-desorption ionization–time-of-flight mass spectrometry resulted in a peptide ‘fingerprint’, which showed identity (20% matches) with the membrane-bound hydrogenase 2 large subunit (HYD2) in *Escherichia coli* [Acc. P37181]. The matched peptides cover 83% (476/567 amino acids) of the protein.

In addition, a 127 bp PCR fragment (CGAACTGATCAAGTTCTCACCAGGACTACGCTCATACCAGA-TCTCCTCGAAGATCGGTACCTGCGCCTGCGCA-CGGGCGTCATCGACGCAGCCCAGCGGCTGACCGTGGGACGGGTACAAGGCCTGACCGCCTGGGACGGTGACCGTGGGACGGGTCACCG) could also be amplified from *Frankia* AVCI1, using the sense 5′-TAAAGGGCCGCGACCCGCGACGCCTGG-3′ and antisense 5′-CGGTGACGCCGTCCCACGGGTGCAGGCC-3′ primers. The NCBI blast search with the translated query versus protein database (blastx) gave a 76% similarity with the large hydrogenase subunit, e.g. *Azotobacter chroococcum* [gi:2506512], *Bradyrhizobium japonicum* [gi:123744], *Rhizobium leguminosarum* [gi:123745]. We can therefore confirm here that *Frankia* has a great similarity to other Ni-Fe(se) hydrogenases in the protobacteria [4].

**Partial sequence and Southern-blot analysis of the small hydrogenase subunit**

Conserved regions of the structural gene *hupS*, which encodes the small hydrogenase subunit, were identified after aligning sequences from several bacterial strains, and used for the construction of primers. The sense 5′-CGGAA-TTCGTTGCTGTGATCATCTGCCTC-3′ and antisense 5′-CCCAAGCTGAGTGCAGTCCACCTTC-3′ primers were used in PCR with *Frankia* DNA isolated from nodules of the symbiosis between *Frankia* 'local source' and *A. incana* [10]. The PCR product, a fragment of 500 bp, was sequenced (Figure 1) and analysed using the NCBI translated query versus protein database (blastx). A 69 and
67% identity with the small subunit of hydrogenases of *B. japonicum* and *R. leguminosarum* respectively were found. However, the *Frankia* DNA used was extracted from nodules in which several different *Frankia* strains may be present. In addition, using Southern-blot analysis, the *hupS* fragment of *Frankia*, 'local source' hybridized with DNA isolated from *Frankia* KB5, giving a positive result (results not shown). A dendrogram of an unrooted tree comparing hydrogenases from five different organisms revealed that *hupS* showed greatest similarities to *R. leguminosarum* (Figure 2).

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**References**


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