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The phylogeny of uptake hydrogenases in *Frankia*

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Summary. Uptake hydrogenase is an enzyme that is beneficial for nitrogen fixation in bacteria. Recent studies have shown that *Frankia* sp. has two sets of uptake hydrogenase genes, organized in synton 1 and synton 2. In the present study, phylogenetic analysis of the structural subunits of hydrogenase syntons 1 and 2 showed a distinct clustering pattern between the proteins of *Frankia* strains that were isolated from different host plants and non-*Frankia* organisms. The structural subunits of hydrogenase synton 1 of *Frankia* sp. CpI1, *Frankia alni* ACN14a, and *F. alni* AvCI1 were grouped together while those of *Frankia* spp. CcI3, KB5, UGL140104, and UGL011102 formed another group. The structural subunits of hydrogenase synton 2 of *F. alni* ACN14a and *Frankia* spp. CcI3 and BCU110501 grouped together, but those of *Frankia* spp. KB5 and CpI1, *F. alni* ArI3, and *F. alni* AvCI1 comprised a separate group. The structural subunits of hydrogenase syntons 1 and 2 of *Frankia* sp. EAN1pec were more closely related to those of non-*Frankia* bacteria, i.e., *Streptomyces avermitilis* and *Anaeromyxobacter* sp., respectively, than to those of other *Frankia* strains, suggesting the occurrence of lateral gene transfer between these organisms. In addition, the accessory Hyp proteins of hydrogenase syntons 1 and 2 of *F. alni* ACN14a and *Frankia* sp. CcI3 were shown to be phylogenetically more related to each other than to those of *Frankia* EAN1pec. [**Int Microbiol** 2009; 12(1):23-28]

Key words: *Frankia* cluster · lateral gene transfer · hydrogenase syntons

Introduction

Uptake hydrogenases catalyze the oxidation of hydrogen to protons and electrons, with the latter fed to the respiratory chain in the production of energy through oxidative phosphorylation. Hydrogenase activity has been reported in many anaerobic and aerobic prokaryotes, as well as in some eukaryotes, including various algae, green plants (such as barley), and protozoa [1,22]. Uptake hydrogenases are beneficial to nitrogen-fixing organisms in both free-living and symbiotic states [6] since hydrogen produced during nitrogen fixation can be consumed, and the reductant generated in the process used by the cells in various ways. To our knowledge, all strains of *Frankia* [10,19] investigated to date have uptake hydrogenases, except one. Hydrogenases require the concerted action of many proteins in addition to the enzymes' large and small structural subunits. Thus, hydrogenase genes encode regulatory elements, post-translational-processing enzymes, nickel-sensing and nickel-metabolism proteins, and electron-transport components, as well as proteins required for maturation (*hyp* genes) [12].

The three available genome sequences of *Frankia* have revealed the presence of two distinctly separated hydrogenase syntons in each one [11]. The genes encoding *Frankia* hydrogenases are arranged tightly together in each synton.

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Hydrogenase synton 1 and synton 2 are uptake hydrogenases and have many features in common with the hydrogenases of other *Frankia* strains and of other organisms. For example, organization of the uptake hydrogenase synton 1 (or synton 2) of *F. alni* ACN14a is very similar to that of *Frankia* sp. CcI3. However, hydrogenase syntons also have differences. For example, sequence conservation between the structural subunits of hydrogenase syntons 1 and 2 in *F. alni* ACN14a (e.g., HupL1 of synton 1 vs. HupL2 of synton 2) was found to be as low as 27%. Phylogenetic studies of the structural subunits of *F. alni* ACN14a and *Frankia* spp. CcI3 and EAN1pec have shown that hydrogenase syntons 1 and 2 of *Frankia* are divergent and that hydrogenase synton 1 is probably ancestral among the actinobacteria [11]. Moreover, the uptake hydrogenase synton 1 of *Frankia* ACN14a is highly expressed under free-living conditions whereas hydrogenase synton 2 is mainly involved in symbiotic interactions [11].

Three well-defined *Frankia* phylogenetic clusters have been identified: (i) *F. alni* and *Casuarina* infective strains; (ii) unisolated symbionts of Rosaceae, Datiscaceae, Coriariaceae, and Rhamnaceae; and (iii) *Frankia* species infective on Elaeagnaceae [16]. In this work, the term "hydrogenase synton" is used instead of "hydrogenase cluster" to avoid confusion with the "*Frankia* clusters" described herein.

In the present work, several *Frankia* strains isolated from different host plants originating from different geographical areas were screened to study the occurrence of two hydrogenase syntons in a wide range of *Frankia* strains. In addition, the phylogenetic relationships of the genes encoding the structural subunits and Hyp proteins of each hydrogenase synton across different *Frankia* strains are discussed, also in relation to the hydrogenases of other non-*Frankia* bacteria and archaea.

Materials and methods

Frankia strains and growth conditions. *Frankia* strains isolated from various different actinorhizal host plants (Table 1) were grown at 27°C, with shaking, in liquid propionate uniquely modified (PUM) medium [13]. Cells were successively transferred on a weekly basis to fresh medium containing 0.1 g NH4Cl/l. The cells were inoculated at a total protein concentration of 5 µg/ml in 50 ml PUM medium.

Extraction of _Frankia_ DNA. Genomic DNA was extracted from 7-day old *Frankia* cultures according to the bacterial protocol in the Blood and Tissue Genomic DNA Extraction Kit (Viogene).

Amplification and sequencing of _Frankia_ hydrogenase genes. Gene fragments about 800 bases long and corresponding to the small and large structural subunits of hydrogenase syntons 1 and 2 were amplified by touchdown PCR using different primers (Table 2). The PCR was carried out with 25 ng of DNA in a 20-µl assay (0.6 µM of each primer, 3 mM $MgCl_2$ or Q solution, and 1 U Taq, from Qiagen, with temperature programs of 3 min at 95°C followed by 35 cycles of 30 s at 95°C, 15 s at 58°C, 15 s at 54°C, and 1 min at 72°C, followed by a final elongation step of 10 min at 72°C. PCR products were electrophoretically separated on agarose gels, purified using a QIAquick Gel Extraction Kit (Qiagen GmbH, Hilden, Germany) and sequenced in an ABI377 sequencer (Applied Biosystems, Foster City, CA, USA) using 300 fmol of PCR product in a volume of 15 µl.

Sequence alignments and phylogenetic analysis. Multiple alignments of the hydrogenase sequences were constructed with ClustalX [21]. Matrix pair-wise comparisons were corrected for multiple-base substitutions according to the method of Kimura [8], followed by a phylogenetic analysis using the neighbor-joining clustering method [18] with the standard parameters. A bootstrap confidence analysis was performed with 1000 replicates to determine the reliability of the distance tree topologies obtained [7]. Tree representations were constructed with the Tree-View program [17].

Phylogenetic analysis was carried out using ~800-base nucleotide sequences of the hydrogenase structural subunits of *F. alni* strain ACN14a, *Frankia* spp. CcI3 and EAN1pec (obtained from *Frankia* database), and sequences amplified from *F. alni* UGL011102, AvCI1, ArI3, and *Frankia* spp. KB5, BCU110501, and UGL140104 (Table 1). The nucleotide sequences of non-*Frankia* bacteria and of archaea were used for comparative study. The complete amino acid sequences of the Hyp proteins of *F. alni*

Table 1. *Frankia* sp. strains used in the study, their source or reference, locations, and host plant origins

Frankia strains	Location	Host plant	Source or reference
F. alni ACN14a	Canada	*A. viridis* subsp. *crispa*	[14]
F. alni AvCI1	USA	*A. viridis* subsp. *crispa*	[2]
Frankia sp. KB5	Australia	*C. equisetifolia*	[20]
F. alni UGL011102	Sweden	*A. incana*	C. Wheeler (pers. commun)
F. alni ArI3	USA	*A. rubra*	[3]
Frankia sp. HFPCcI3	USA	*C. cunninghamiana*	|24]
Frankia sp. EAN1pec	USA	*E. angustifolia*	[9]
Frankia sp. BCU110501	Argentina	*D. trinervis*	[5]
Frankia sp. UGL140104	Scotland	*H. rhamnoides*	C. Wheeler (pers. commun)
Frankia sp. CpI1	USA	*C. peregrina*	[4]

Table 2. List of HupL primers used in the study

Product	Designation	Forward (5′–3′)	Reverse (5′–3′)
HupL1	HupL 20/HupL 21	CCTCGTTGACCCAGTCCTTG	CGCATCATCGGCAACCTC
HupL1	HupL 20/HupL-13	BCCTCGTTGACCCAGTCCTTG	AAGGGGAAGGATCCACGCGACGCC
HupS1	HupS-6F/HupS-6	BGTTGTGCCGCCACCTCGGCTC	TGCGACGGCGACACGGTCTCG
HupS1	HupS-6F/HupL 24	GTTGTGCCGCCACCTCGGCTC	ACGGTCCACCTGCACAACAA
HupL2	HupL-F1/HupL33-2-(b)	GACGTCACCCACTCGTTCTAC	CGTTGATGACGAACCTGCT
HupL2	HupL32-2-(f)/HupL33-2-(b)	TCACCCACTCGTTCTACGC	CGTTGATGACGAACCTGCT
HupS2	HupS34-2-(f)/HupS-B2	GATGTCATCCGTGCTCTGG	AGCCGAACTCGTAGAACAGG
HupS2	HupS-F1/HupS35-2-(b)	TCATCCGTGCTCTGGTTTC	GTGGGTGAACGTGGTGAAG

ACN14a and *Frankia* spp. CcI3 and EAN1pec and the Hyp proteins of non-*Frankia* bacteria and archaea were used in phylogenetic studies.

Results and Discussion

Phylogenetic analysis of the structural subunits of hydrogenase. Recently, Vignais and Billoud [23] published data on the phylogeny of hydrogenases based on sequence alignments of the enzymes' catalytic subunits. The results led to the identification of three distinct classes of hydrogenases: (i) [NiFe]-hydrogenases, (ii) [FeFe]-hydrogenases, and (iii) [Fe]-hydrogenases. The largest and most important group of these is the [NiFe]-hydrogenases, which also includes the uptake hydrogenase of *Frankia*.

Our phylogenetic analysis of hydrogenase synton 1 showed a distinct clustering pattern between hydrogenases of various *Frankia* strains and non-*Frankia* organisms (Fig. 1). The phylogeny of the small subunit of hydrogenase synton 1 showed essentially the same topology as that of the large subunit (data not shown).

The occurrence of two different hydrogenase syntons was reported by Leul et al. [11] and confirmed by Vignais and Billoud [23]. Figure 2 shows the results of our analysis, in which the structural subunits of the hydrogenases of *Frankia* strains isolated from different genera of host plants were found to group together. Of the *Frankia* strains isolated from the same genera, hydrogenase synton 2 of *F. alni* ArI3 grouped together with that of *F. alni* AvCI1 whereas hydrogenase synton 2 of *F. alni* ACN14a was distinct (Fig. 2). Note that the structural subunits of hydrogenase synton 2 of both *Frankia* sp. EAN1pec and phylogenetic *Frankia* cluster 3 [16] grouped more closely with the subunits of non-*Frankia* *Anaeromyxobacter* sp. than with those of the other *Frankia* strains. The structural subunits of the hydrogenases of the archaea *Methanosarcina mazei* Go1 and *M. acetivorans* C2A grouped together but were distant from those of *Frankia* and

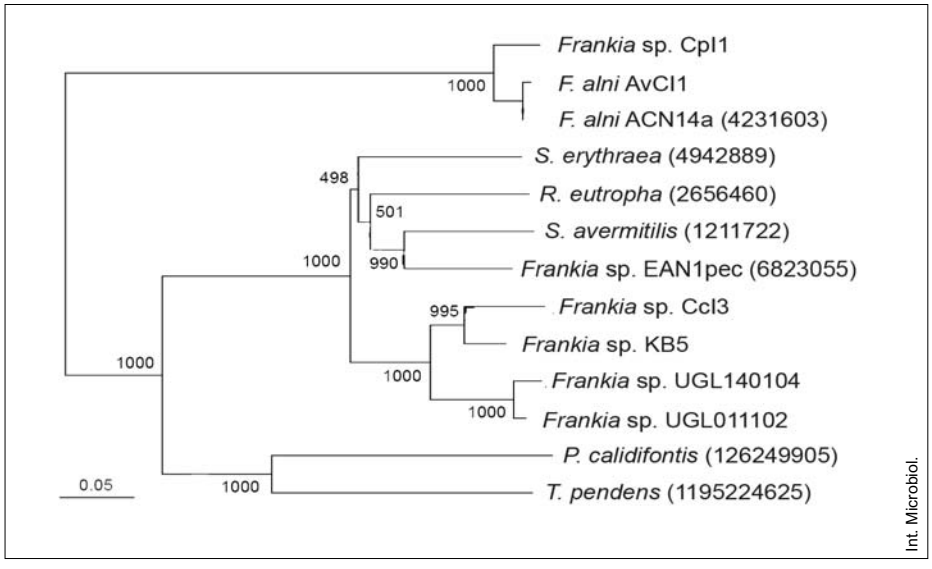

Fig. 1. Phylogenetic analysis of the large subunit of hydrogenase synton 1 of various *Frankia* strains, non-*Frankia* bacteria, and archaea. GeneIDs of the hydrogenases are given in parentheses. Numbers at nodes indicate bootstrap values, which were obtained from 1000 replicates [7].

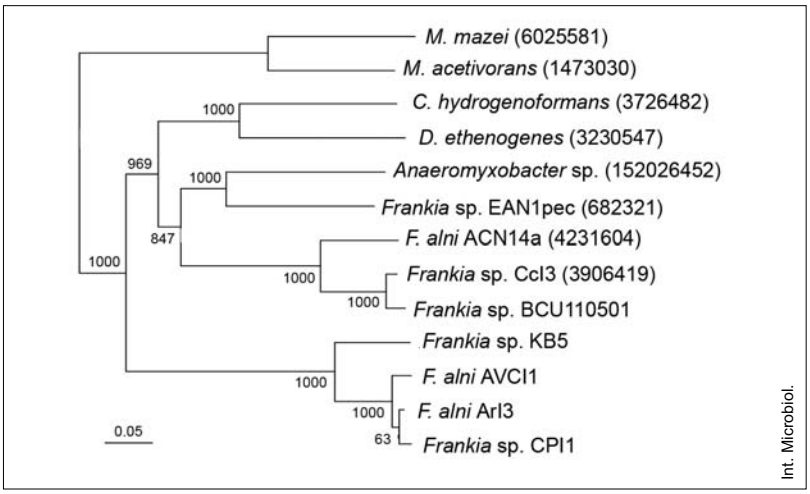

Fig. 2. Phylogenetic analysis of the large subunit of hydrogenase synton 2 of various *Frankia* strains, non-*Frankia* bacteria, and archaea. GeneIDs of the hydrogenases are given in parentheses. Numbers at nodes indicate bootstrap values, which were obtained from 1000 replicates [7].

non-*Frankia* bacteria. The phylogeny of the small subunit of hydrogenase synton 2 likewise showed a topology similar to that of the large subunit (data not shown).

Phylogenetic analysis of the Hyp proteins of hydrogenase. HypB1, HypD1, HypE1, and HypF1 (hydrogenase synton 1) of *F. alni* ACN14a and *Frankia* spp. CcI3 and EAN1pec grouped relatively closely together but were closer to the corresponding Hyp proteins of non-*Frankia* bacteria than to those of archaea. However, the Hyp proteins of *F. alni* ACN14a and *Frankia* sp. CcI3 were consistently more related to each other than to those of *Frankia* sp. EAN1pec (Fig 3A–D). HypE1 of *Frankia* sp. EAN1pec was more related to the corresponding protein in *R. eutropha* than to the proteins of *F. alni* ACN14a and *Frankia* sp. CcI3. HypD1 and HypF1 of *F. alni* ACN14a and *Frankia* sp. CcI3,

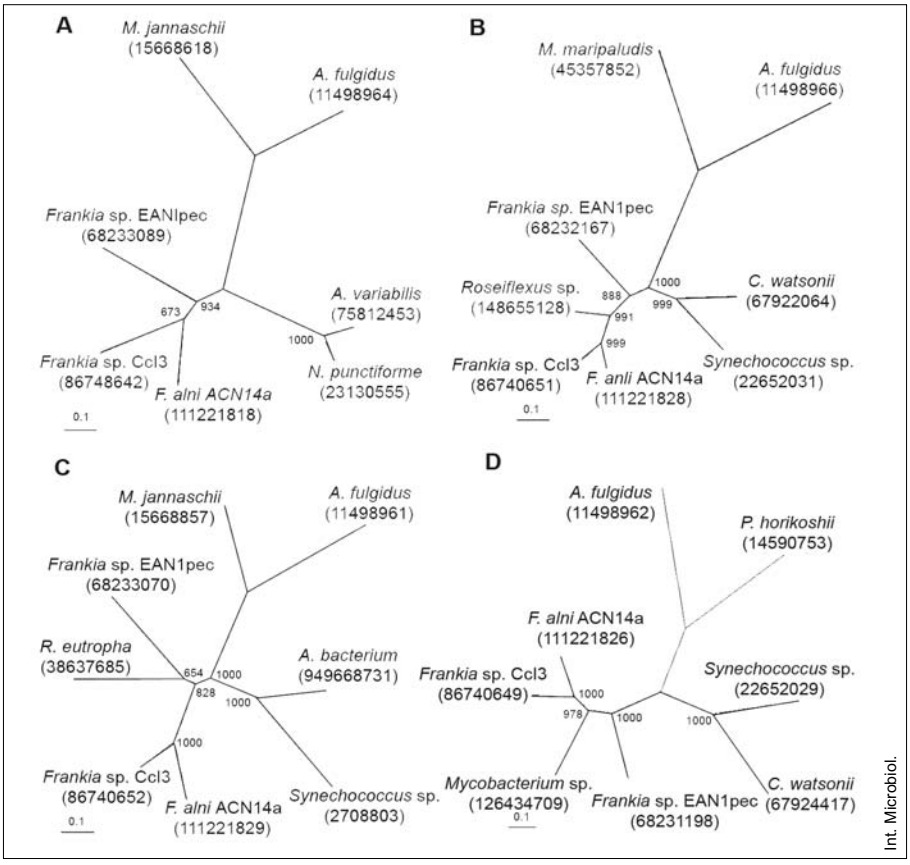

Fig. 3. Phylogenetic analysis of the complete proteins of HypB (**A**), HypD (**B**), HypE (**C**), and HypF (**D**) of *F. alni* ACN14a, *Frankia* sp. CcI3, and *Frankia* sp. EAN1pec (hydrogenase synton 1) compared with the hydrogenases of non-*Frankia* bacteria and archaea. Numbers in parentheses indicate the ProteinID of the hydrogenases used. Numbers at nodes indicate bootstrap values, which were obtained from 1000 replicates [7].

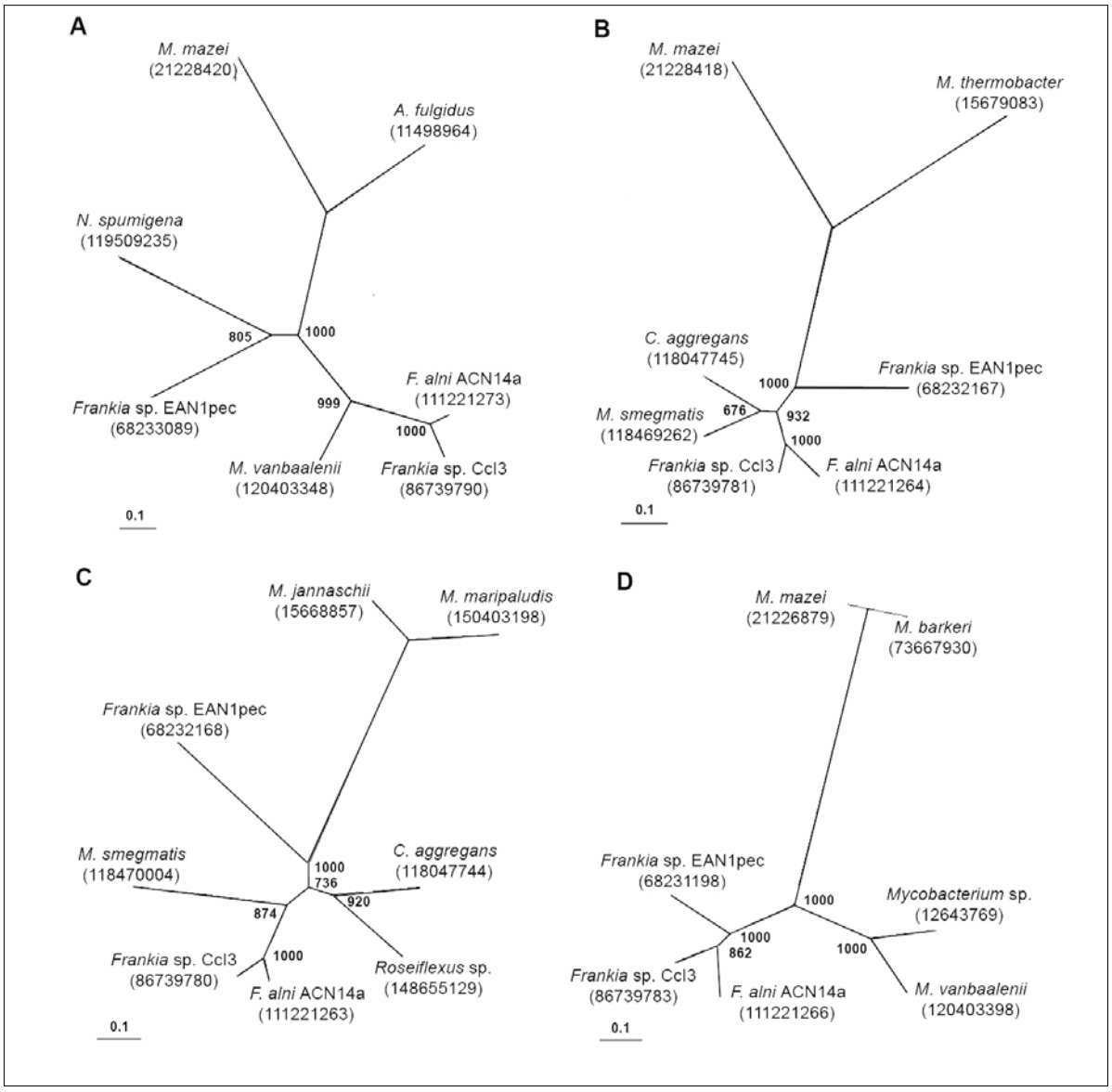

Fig. 4. Phylogenetic analysis of the complete proteins of HypB (**A**), HypD (**B**), HypE (**C**), and HypF (**D**) of *F. alni* ACN14a, *Frankia* sp. CcI3, and *Frankia* sp. EAN1pec (hydrogenase synton 2) compared with the hydrogenases of non-*Frankia* bacteria and archaea. Numbers in parentheses indicate the ProteinID of the hydrogenases used. Numbers at nodes indicate bootstrap values, which were obtained from 1000 replicates [7].

however, were more related to the corresponding proteins of *Mycobacterium* sp. and *Roseiflexus* sp., respectively, than to those of *Frankia* sp. EAN1pec.

The proteins encoded by the *hyp* genes are genes required for maturation of the hydrogenase enzyme of several [NiFe]-hydrogenases in *Escherichia coli* [23]. In our study, HypB, HypD, HypE, and HypF of *M. mazei* and *A. fulgidus* (Fig. 4A), *M. mazei* and *M. thermobacter* (Fig. 4B), *M. jannaschii* and *M. maripaludis* (Fig. 4C), and *M. mazei* and *M. barkeri* (Fig. 4D), respectively, grouped closely together but were distant from the same proteins in *Frankia* and non-*Frankia* bacteria. HypB2, HypD2, HypE2, and HypF2 (hydrogenase synton 2) of *Frankia* sp. CcI3 and *F. alni* ACN14a grouped consistently together (Fig. 4A–D). HypB2 and HypE2 of *Frankia* sp. CcI3 and *F. alni* ACN14a were more closely related to the same proteins of *M. vanbaalenii* and *M. smegmatis*, respectively, than to those of *Frankia* sp. EAN1pec.

Hydrogenase syntons 1 and 2 of *Frankia* sp. CcI3 and *F. alni* ACN14a have about the same genetic content and in a similar physical position and orientation, while in *Frankia* sp. EAN1pec the genes are both reduced and rearranged. *Frankia* sp. EAN1pec has the largest genome in terms of size

(9.0 Mb), followed by *F. alni* ACN14a (7.5 Mb), and *Frankia* sp. CcI3 (5.4 Mb) [15]. *Frankia* EAN1pec might have acquired its hydrogenase by lateral gene transfer (LGT) from non-*Frankia* bacteria, in contrast to *Frankia* sp. CcI3 and *F. alni* ACN14a.

The structural subunits of hydrogenase syntons 1 and 2 of *Frankia* sp. EAN1pec were found to be more closely related to those of non-*Frankia* bacteria *S. avermitilis* and *Anaeromyxobacter* sp., respectively, than to those of other *Frankia* strains, suggesting the occurrence of LGT involving these organisms. According to the results of our study, the Hyp proteins of hydrogenase syntons 1 and 2 of *F. alni* ACN14a, and *Frankia* sp. CcI3 (phylogenetic *Frankia* cluster 1) are phylogenetically more related to each other than they are to *Frankia* EAN1pec (phylogenetic *Frankia* cluster 3). A previous study reported that the structural subunits of hydrogenase synton 1 (and 2) of *F. alni* ACN14a and *Frankia* sp. CcI3, which are closely related to each other, are relatively closer to those of non-*Frankia* bacteria than to those of *Frankia* sp. EAN1pec and vice versa [11]. The Hyp proteins of archaea grouped closely together but were distant from the Hyp proteins of *Frankia* and non-*Frankia* bacteria, suggesting LGT between the latter. The events by which *Frankia* participated in hydrogenase synton 1 gene transfer with bacteria must have been different from and independent of those that involved hydrogenase synton 2.

In our study, the structural subunits of hydrogenases of several *Frankia* strains isolated from the same genera of host plants grouped separately, while those of *Frankia* strains isolated from different genera of host plants often grouped together. It was not possible to establish a connection between the phylogenetic relationships of *Frankia* uptake hydrogenases and the geographical distribution of *Frankia* strains or their hosts. The occurrence of two hydrogenase syntons is a common phenomenon in *Frankia*.

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