Genome Data Mining and Soil Survey for the Novel Group 5 [NiFe]-Hydrogenase To Explore the Diversity and Ecological Importance of Presumptive High-Affinity H$_2$-Oxidizing Bacteria[▽][†]

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Streptomyces soil isolates exhibiting the unique ability to oxidize atmospheric H$_2$ possess genes specifying a putative high-affinity [NiFe]-hydrogenase. This study was undertaken to explore the taxonomic diversity and the ecological importance of this novel functional group. We propose to designate the genes encoding the small and large subunits of the putative high-affinity hydrogenase *hhyS* and *hhyL*, respectively. Genome data mining revealed that the *hhyL* gene is unevenly distributed in the phyla *Actinobacteria*, *Proteobacteria*, *Chloroflexi*, and *Acidobacteria*. The *hhyL* gene sequences comprised a phylogenetically distinct group, namely, the group 5 [NiFe]-hydrogenase genes. The presumptive high-affinity H$_2$-oxidizing bacteria constituting group 5 were shown to possess a hydrogenase gene cluster, including the genes encoding auxiliary and structural components of the enzyme and four additional open reading frames (ORFs) of unknown function. A soil survey confirmed that both high-affinity H$_2$ oxidation activity and the *hhyL* gene are ubiquitous. A quantitative PCR assay revealed that soil contained 10^6 to 10^8 *hhyL* gene copies g (dry weight)$^{-1}$. Assuming one *hhyL* gene copy per genome, the abundance of presumptive high-affinity H$_2$-oxidizing bacteria was higher than the maximal population size for which maintenance energy requirements would be fully supplied through the H$_2$ oxidation activity measured in soil. Our data indicate that the abundance of the *hhyL* gene should not be taken as a reliable proxy for the uptake of atmospheric H$_2$ by soil, because high-affinity H$_2$ oxidation is a facultatively mixotrophic metabolism, and microorganisms harboring a nonfunctional group 5 [NiFe]-hydrogenase may occur.

Hydrogenases catalyze the interconversion of H$_2$ to protons and electrons (H$_2$ $\leftrightarrow$ 2H$^+$ + 2e$^-$). Under physiological conditions, these metalloenzymes couple H$_2$ oxidation to respiration or reduce protons as a way to disperse accumulated reducing equivalents (21). Hydrogenases are divided into three different classes on the basis of the metal content of their catalytic sites: [NiFe]-, [FeFe]-, and [Fe]-hydrogenases (55, 56). Among these, [NiFe]-hydrogenases from the domain *Bacteria* are the most numerous and the most extensively studied. These hydrogenases usually are O$_2$ tolerant or are transiently inactivated following O$_2$ exposure (20), a feature that argues for their integration into biofuel cells (58) and biohydrogen production (27).

Phylogenetic analysis of the gene sequences encoding the structural subunits of [NiFe]-hydrogenases has revealed the occurrence of 4 distinct groups (55). Group 1 comprises membrane-bound hydrogenases coupling H$_2$ oxidation to aerobic or anaerobic respiration in bacteria and archaea. Group 2 includes two subclasses of cytoplasmic hydrogenases: the cyanobacterial uptake hydrogenases (group 2a), which recycle H$_2$ evolved from nitrogenase, and the H$_2$ sensor hydrogenases (group 2b), which control the transcription of hydrogenase genes in aerobic H$_2$-oxidizing bacteria (35). Hydrogenases belonging to group 3 are physiologically bidirectional and include four subclasses defined on the basis of additional subunits that are able to bind cofactors such as NAD, F$_{420}$, and NADP. These bidirectional hydrogenases either are found in methanogenic archaea (51) or act as electron valves in the photosynthetic electron transport of cyanobacteria (2). The function of H$_2$-evolving hydrogenases in group 4 is to dissipate reducing equivalents generated through the fermentation of low-potential carbon, such as carbon monoxide and formate (47).

Recent evidence for a fifth group of [NiFe]-hydrogenases was obtained by analyzing the H$_2$ oxidation activity of *Streptomyces* spp. isolated from soil (13). Gene sequences specifying a putative high-affinity [NiFe]-hydrogenase were detected in *Streptomyces avermitilis* and several *Streptomyces* isolates displaying an unusually high affinity H$_2$ oxidation activity (apparent K_m [$K_{m(app)}$], <100 parts per million by volume [ppmv]) and the unique ability to oxidize atmospheric H$_2$ (13). These microorganisms contribute to the uptake of H$_2$ by soil, which represents the main sink for atmospheric H$_2$ (15, 18). Inter-

estingly, inactivation of the putative high-affinity [NiFe]-hydrogenase caused a significant reduction in the growth yield of *Mycobacterium smegmatis* (5). Expression of the genes encoding auxiliary and structural components of this hydrogenase increased under starvation conditions without an exogenous H_2 supply, suggesting that the genes encoding the putative high-affinity [NiFe]-hydrogenase conferred on *M. smegmatis* the ability to scavenge atmospheric H_2 (5). Based on the Gibbs free energy of atmospheric H_2 oxidation activity and bacterial maintenance energy requirements, Conrad (10) estimated that the H_2 uptake activity of soil typically observed in the environment would sustain a maximal population of 6×10^7 H_2-oxidizing bacteria per gram of soil. Virtually nothing is known about the taxonomic diversity, distribution, and abundance of high-affinity H_2-oxidizing bacteria in the environment, impeding evaluation of the extent of their contribution to the global budget of atmospheric H_2.

The objective of this study was to explore the ecological importance of high-affinity H_2-oxidizing bacteria by using the genes specifying the putative high-affinity [NiFe]-hydrogenase in *S. avermitilis* as functional biomarkers. Although gene sequences homologous to those encoding putative high-affinity hydrogenases have been observed in the genomes of *Ralstonia eutropha* (50), *Frankia* spp. (36), and *Thermomicrobium roseum* (60), the taxonomic diversity of high-affinity H_2-oxidizing bacteria in publicly available genome databases has not been thoroughly analyzed.

MATERIALS AND METHODS

Gene sequence data mining and phylogenetic analyses. Sequences similar to those of genes encoding the auxiliary and structural components of the putative high-affinity [NiFe]-hydrogenase in *S. avermitilis* were retrieved from the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/) using the Basic Local Alignment Search Tool (1). Nucleic acid sequences were assembled into 14 gene-specific databases and were imported into the ARB software package (37). Imported sequences were translated *in silico*, and the amino acid sequences were aligned using ClustalW (52). The alignments were optimized manually, and functional amino acid sequence motifs in the structural (HhySL) and auxiliary (HypABCDEFX) components of [NiFe]-hydrogenases were examined in order to validate the sequences (7). Conserved sequence motifs in the accessory proteins of [NiFe]-hydrogenase are described in Table S1 in the supplemental material. The accession numbers of the sequences retrieved from the NCBI database are provided in Table S2 in the supplemental material. The amino acid sequences of open reading frames (ORFs) 1 to 4 and HhyS were imported into SignalP, version 3.0, in order to search for N-terminal signal peptides (3, 19). The amino acid sequence of ORFs 1 to 4 was searched for putative transmembrane helices using the TMHMM (transmembrane protein topology based on the hidden Markov model) package (31).

Phylogenetic trees of amino acid sequences translated from the gene specifying the large subunit of [NiFe]-hydrogenases were constructed with neighbor-joining and maximum-parsimony algorithms by using the ARB software package (37).

16S rRNA gene sequences were retrieved from the genomes of 23 presumptive high-affinity H_2-oxidizing bacteria. The sequences were imported into the SILVA database, containing aligned 16S rRNA sequences with a minimum length of 1,200 bp for bacteria (44). Pairwise distance matrices (23 by 23) were computed (37) to obtain the similarity scores of all possible combinations of 16S rRNA gene, *hhyL* gene, and HhyL amino acid sequences. Comparisons between the percentages of similarity of all *hhyL* and HhyL pairs and the sequence similarities of the 16S rRNA genes of the same bacteria were performed by regression analysis ($n = 252$). This pairwise similarity score analysis has recently been utilized to establish a similarity score threshold value for particulate methane monooxygenase gene (*pmoA*) sequences in methanotrophic bacteria at the species level (17).

Soil samples. Soil samples were collected in ecosystems encompassing a broad range of climate and soil properties (see Table S3 in the supplemental material).

Soil samples collected in a deciduous forest (Mainz, Germany), an agricultural area (Heidelberg, Germany), a paddy field (Fuyang, China), a hyperarid desert (Arava, Israel), an arid desert (Avdat, Israel), and peatland (Loppi, Finland) were recently used to study the thermal deactivation of high-affinity hydrogenase activity (9). Other soil samples were collected in Siberian grassland and in experimental afforestation plots maintained for 30 years under the influence of five tree species: Arolla pine (*Pinus sibirica*), Scots pine (*Pinus silvestris*), Norway spruce (*Picea abies*), birch (*Betula fruticosa*), and aspen (*Populus tremula*) (40, 41). The soil samples were sieved (mesh size, <2 mm) and stored at 4°C for more than 2 years. For the measurement of H_2 oxidation activity, 1 to 3 g of soil was suspended in 5 ml Tris buffer (100 mM Tris-HCl [pH 7.0]).

H_2 oxidation activity. Before H_2 uptake activity was measured, 60-ml vials containing soil suspensions were sealed with rubber stoppers and were flushed for 30 min with synthetic air (80% N_2 and 20% O_2). Standard H_2 gas (1,000 ppmv $\pm$ 2%; Messer Schweiz AG, Switzerland) was then added to the headspace of the bottles to obtain an initial mixing ratio of 1.5 to 1.8 ppmv H_2. The decrease in the H_2 mixing ratio was monitored as a function of time by analyzing aliquots (0.5 ml) of the headspace air in a gas chromatograph with a reduction gas detector as described previously (49). Vials were continuously agitated at 100 rpm on an orbital shaker kept at 25°C to enhance the transfer of H_2 from the gas to the liquid phase. Apparent first-order H_2 uptake rate constants were obtained by integrating the logarithmic decrease in the headspace H_2 mixing ratio. H_2 concentrations were typically measured over 3 h, and the H_2 oxidation activity was proportional to the amount of soil. The reproducibility of the H_2 analyses was assessed before and over the course of each set of experiments by repeated analysis of certified standard H_2 gas (2.0 ppmv $\pm$ 5% H_2; Messer Schweiz AG, Switzerland), and standard deviations were typically <5%. No significant H_2 uptake was observed for blank experiments with sterile Tris buffer. H_2 oxidation activity measurements were performed in triplicate.

DNA extraction, PCR amplification, and cloning of *hhyL* genes. Genomic DNA was extracted from 500-mg soil samples using the SL Buffer(I) and Enhancer solution provided in the NucleoSpin soil kit (Macherey-Nagel GmbH & Co. KG, Germany) according to the manufacturer's instructions. Cells were disrupted using a FastPrep system (Thermo Scientific) with 1 burst at 5.0 m s^{-1} for 30 s. DNA was eluted in 50 μl SE buffer (Macherey-Nagel GmbH & Co. KG, Germany) and was serially diluted (1:5, 1:10, and 1:100). PCRs were performed in 50-μl reaction volumes containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate (dNTP), 0.2 μM primers NiFe-244f and NiFe-1640r (13), 20 μg of bovine serum albumin (Roche Diagnostics GmbH, Germany), 2.5 U DNA polymerase (AccuPrime *Taq* DNA polymerase system; Invitrogen GmbH, Germany), and 2 μl diluted DNA. The reactions were performed using a Primus thermal cycler (MWG-Biotech AG, Germany) as follows: 94°C for 5 min; 10 cycles of 94°C for 1 min, 65°C for 1 min, with a decrease in steps of 0.5°C/cycle, and 68°C for 1 min; 20 cycles at 94°C for 1 min, 57°C for 1 min, and 68°C for 1 min; and a final extension for 10 min at 68°C. PCR-amplified *hhyL* genes from soil samples were cloned into the pGEM-T Easy vector system (Promega GmbH, Germany) according to the manufacturer's instructions. Recombinant colonies were manually picked and shipped to GATC Biotech AG (Germany) for plasmid extraction; PCR amplification using primers T7f and M13r, covering regions flanking the insert; and double-strand DNA sequencing. A total of 107 *hhyL* gene sequences were edited and assembled using the DNAStar software package and were compared with the GenBank database using a standard nucleotide-nucleotide BLAST search (1). Operational taxonomic units (OTUs) were defined at an arbitrary evolutionary distance of ≤10%, resulting in 54 OTUs.

Quantitative PCR of *hhyL* genes. The copy number of *hhyL* genes in soil was determined by quantitative PCR using primers NiFe-244f and NiFe-568r (12). PCR-amplified *hhySL* genes (~3 kb) from *Streptomyces avermitilis* DSMZ 46492 were used to generate a quantitative PCR standard. A dilution series of the PCR-amplified DNA was made to generate a standard curve of *hhySL* covering 30 to 3.0×10^9 copies of the template per assay, quantified by using PicoGreen (Quant-iT PicoGreen; Invitrogen GmbH, Germany). Quantitative PCR was performed using the iCycler (Bio-Rad Laboratories Inc.) with a soil DNA extract dilution series (1:5, 1:10, and 1:100) as the template. The reaction mixture (25 μl) contained 12.5 μl SYBR green Jump-Start *Taq* ReadyMix, 0.5 μM each primer, 3.0 mM $MgCl_2$, and 5.0 μl template DNA. The thermal protocol used was 94°C for 6 min, followed by 45 cycles at 94°C for 35 s, 57°C for 30 s, and 72°C for 45 s, with plate reading at 86.5°C for 10 s. The threshold cycles (C_T) of all reactions were determined by analysis of PCR kinetics with iCycler software (version 2.3.1370). Specific amplification of the *hhyL* gene was confirmed by melting curve analysis. The data reported are the averages of quantifications performed using triplicate DNA extracts.
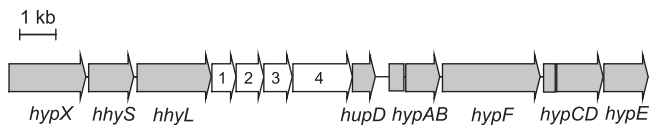
FIG. 1. Arrangement of auxiliary and structural genes of the putative high-affinity [NiFe]-hydrogenase in *S. avermitilis*.

Theoretical population size of high-affinity H$_2$-oxidizing bacteria in soil. The theoretical population size of high-affinity H$_2$-oxidizing bacteria in soil was estimated based on the theoretical maintenance energy consumption rate and the free energy of atmospheric H$_2$ oxidation, using the following model formulated by Conrad (10): $N = 1.4 \times 10^{14} (-\Delta G)d/m_E$, where N is the theoretical population size of H$_2$-oxidizing bacteria in soil (expressed as the number of cells g of soil [dry weight]$^{-1}$), 1.4×10^{14} is a constant expressing the density of bacterial cells containing 1 mol carbon (number of cells [mol of C biomass]$^{-1}$), ΔG is the Gibbs free energy of atmospheric H$_2$ oxidation (-199 kJ mol of H$_2^{-1}$), d is the measured H$_2$ oxidation rate (mol of H$_2$ g of soil [dry weight]$^{-1}$), and m_E is the energy maintenance requirement of the population (kJ [mol of C biomass]$^{-1}$). This model provides an estimate of the maximal population for which the H$_2$ oxidation reaction fully supplies m_E. The parameter m_E is dependent primarily on temperature and was estimated using the following model derived by Tijhuis et al. (54):

$$m_E = 4.5 \exp\left[\frac{-6.94 \times 10^4}{R}\left(\frac{1}{T}\right) - \left(\frac{1}{298}\right) \right]$$

where R is the universal gas constant (8.314 J mol^{-1} K^{-1}) and T is the temperature (K).

Nucleotide sequence accession numbers. All *hhyL* gene sequences determined in this study have been deposited in GenBank (4) with accession numbers HM029252 to HM029358.

RESULTS

Genome data mining for the putative high-affinity [NiFe]-hydrogenase. The genome of *S. avermitilis*, which exhibits the unusual ability to oxidize atmospheric H$_2$, includes a unique gene cluster specifying a putative high-affinity [NiFe]-hydrogenase (13). This gene cluster comprises 14 genes encoding the structural and auxiliary components of the putative metalloenzyme (Fig. 1). We designated the ORFs specifying putative proteins with similarity to the [NiFe]-hydrogenase small and large subunits *hhyS* and *hhyL*. These gene designations were chosen according to the recommended hydrogenase gene nomenclature (56), where *hhyS* or *hhyL* stands for <u>h</u>igh-affinity <u>h</u>ydrogenase <u>s</u>mall or <u>l</u>arge subunit. No N-terminal signal peptide recognized by the twin-arginine translocation apparatus was detected in the small subunit, indicating that the [NiFe]-hydrogenase either is soluble or is anchored to an internal membrane-bound electron acceptor. The eight ORFs *hypABCDEFX* and *hupD* encode putative proteins with similarity to hydrogenase pleiotropic maturation proteins and endopeptidase, respectively. The HypABCDEF proteins are responsible for the assembly of active-site constituents and their insertion into the large subunit of the hydrogenase (7, 34, 57). ORFs 1 to 4 specify hypothetical proteins of unknown function in which neither a consensus N-terminal signal peptide cleavage site nor a putative transmembrane helix was detected.

The *S. avermitilis hhyL* gene, encoding the large subunit of the putative high-affinity [NiFe]-hydrogenase, was used to search public gene sequence data. The sequences were retrieved from a genome sequencing project for microorganisms isolated from diverse ecological niches, including contaminated soils and humans (Table 1). The HhyL-like translated amino acid sequences were added to the extensive [NiFe]-hydrogenase large-subunit sequence database of Vignais and Billoud (55), and they clustered with sequences specifying the putative high-affinity hydrogenases in H$_2$-oxidizing streptomycetes isolated from soil (Fig. 2). This analysis substantiated the definition of the phylogenetically distinct group 5 [NiFe]-hydrogenases (13) and revealed that homologous genes for *hhyL* are unevenly distributed across the phyla *Actinobacteria*, *Proteobacteria*, *Acidobacteria*, and *Chloroflexi*. According to phylogenetic analysis, the conserved L1 and L2 motifs, including the four cysteine residues of HhyL, differed from the conserved motif in the large subunit of [NiFe]-hydrogenases belonging to groups 1 to 4 (Fig. 3). The pairwise sequence similarity scores of 16S rRNA genes and HhyL or *hhyL* were not correlated (Fig. 4), leading to incongruence between classifications in the *hhyL* and 16S rRNA gene-based phylogeny. For instance, *hhyL* gene sequences showed 75 to 97% and 72 to 87% pairwise similarity scores in *Streptomyces* spp. and *Frankia* spp., respectively, and did not make up species-specific monophyletic clusters (Fig. 2). The *hhyL* gene sequences of *Streptomyces* spp. formed two clusters. Cluster 1 comprises *S. avermitilis* and other soil isolates displaying high-affinity H$_2$ oxidation activity, while cluster 2 encompasses *S. ghanaensis*, *S. viridochromogenes*, and *Streptomyces* sp. AP1 (Fig. 2). Strain AP1 was isolated from arid desert soil and showed high-affinity H$_2$ oxidation activity, with a $K_{m(\text{app})}$ of 30 ppmv H$_2$ (see Fig. S1 in the supplemental material). Owing to the unusual high-affinity H$_2$ oxidation activity of *Streptomyces* isolates possessing the *hhyL* gene, the microorganisms possessing group 5 [NiFe]-hydrogenases are defined as "presumptive high-affinity H$_2$-oxidizing bacteria." Genes encoding additional [NiFe]-hydrogenases belonging to groups 1 to 3 were also detected in a number of representatives (Table 1).

We searched the genome sequences of presumptive high-affinity H$_2$-oxidizing bacteria for the [NiFe]-hydrogenase auxiliary genes of *S. avermitilis*. The sequences retrieved were assembled in gene-specific databases and were examined for functional amino acid sequence motifs after alignment (see Table S1 in the supplemental material). Hydrogenase gene clusters comprising sequences homologous to the *hypABCDEF*-like genes and the four ORFs encoding hypothetical proteins of *S. avermitilis* were detected in 69% of the presumptive high-affinity H$_2$-oxidizing bacteria (Table 1). However, auxiliary genes encoding critical components of the hydrogenase maturation apparatus were missing in *Mycobacterium avium* and *Sphaerobacter thermophilus* (Table 1). The *hypX*-like gene was detected only in *Streptomyces* spp. and in the soluble [NiFe]-hydrogenase (group 3) operon of *Ralstonia eutropha*. Except for the fact that *hhySL* and ORFs 1 to 3 always flanked each other, no consensus was observed in the arrangement of the genes encoding the auxiliary components of group 5 [NiFe]-hydrogenase. The genes encoding structural and auxiliary components of the hydrogenase are arranged within a single cluster in all the presumptive high-affinity H$_2$-oxidizing bacteria except *Thermonospora bispora* and *Methylosinus trichosporium* OB3b (see Table S2 in the supplemental material).

Environmental soil survey for the putative high-affinity [NiFe]-hydrogenase. High-affinity H$_2$ oxidation activity was

TABLE 1. Identification and sources of microorganisms possessing genes specifying auxiliary and structural components of the group 5 [NiFe]-hydrogenase

Phylum and species[a]	Source[b]	Auxiliary gene(s) not detected[c]	Other [NiFe]-hydrogenase(s)
Actinobacteria			
Streptomyces avermitilis (**hypX+**)	Soil		
Streptomyces scabiei (**hypX+**)	Soil		
Streptomyces sviceus (**hypX+**)	Soil		
Streptomyces griseoflavus	Soil		Group 3b
Streptomyces sp. Mg1 (**hypX+**)	Soil		Group 3b
Streptomyces sp. C (**hypX+**)	Soil		
Streptomyces clavuligerus	Soil		
Streptomyces viridochromogenes (**hypX+**)	Soil	ORF3	Group 1
Streptomyces ghanaensis (**hypX+**)	Soil	ORF3	
Mycobacterium sp. KMS	PAH-contaminated soil		Group 3b
Mycobacterium sp. MCS	PAH-contaminated soil		Group 3b
Mycobacterium sp. JLS	PAH-contaminated soil		Group 3b
Mycobacterium smegmatis	Human smegma		Groups 2a, 3b
Mycobacterium avium	Clinical isolate	*hypA, hypB, hypC, hypD, hypE, hypF*	
Mycobacterium kansasii	Clinical isolate		Group 3b
Mycobacterium marinum M	Human isolate		Group 3b
Mycobacterium parascrofulaceum	Clinical isolate		Group 3b
Rhodococcus jostii RHA1	Lindane-contaminated soil		Group 3b
Rhodococcus erythropolis PR4	Seawater		Group 3b
Rhodococcus erythropolis SK121	Human skin	*hypA*	Group 3b
Rhodococcus equi	Human abscess		Group 3b
Rhodococcus opacus B4	Gasoline-contaminated soil		Group 3d
Frankia sp. EAN1pec	Root nodule		Group 1
Frankia sp. CcI3	Root nodule	ORF3	Groups 2a, 3b
Frankia alni ACN14a	Root nodule	ORF3	Group 2a
Saccharopolyspora erythraea	Soil		
Saccharomonospora viridis	Peat		
Thermobispora bispora	Decaying manure	ORF4	Groups 2a, 3b
Thermomonospora curvata	Straw	ORF3, ORF4, *hypB*	
Amycolatopsis mediterranei U32	Soil		
Streptosporangium roseum	Soil	*hypB*	Group 3b
Conexibacter woesei	Soil		
Proteobacteria			
Ralstonia eutropha H16 (**hypX+**)	Sludge		Groups 1, 2b, 3d
Methylosinus trichosporium OB3b	Sediment, soil, and water		Groups 1, 2b, 3b
Chloroflexi			
Thermomicrobium roseum	Hot spring	ORF1	
Sphaerobacter thermophilus	Thermophilic sewage sludge	ORF3, *hypD, hypE, hypF*	
Ktedonobacter racemifer	Soil		
Acidobacteria			
Acidobacterium sp. MP5ACTX8	Acidic soil		
Solibacter usitatus Ellin6076	Grazed pasture	*hypA*	Groups 1, 3d

[a] Microorganisms possessing the *hypX*-like gene are identified (**hypX+**).
[b] PAH, polycyclic aromatic hydrocarbon.
[c] A list of the accession numbers of the auxiliary genes detected is provided in Table S1 in the supplemental material.

analyzed in soil samples collected in ecosystems encompassing a broad range of climate and soil properties (Fig. 5A). The soil physicochemical parameters analyzed, including pH, nitrogen content, and carbon content, were not significantly correlated with H_2 oxidation rates. Indeed, the soil samples displaying the highest H_2 oxidation activity were collected in peatland and desert ecosystems encompassing extreme ranges in climate and physicochemical properties of soil (see Table S3 in the supplemental material). Six clone libraries were generated on the basis of *hhyL* gene sequences amplified from soil samples collected in a deciduous forest, an agricultural area, a hyperarid desert, an arid desert, a paddy field, and peatland. Clone sequences were incorporated into the hydrogenase phylogenetic analysis (Fig. 2).

Measured soil H_2 oxidation rates (Fig. 5A) were used to estimate the maximal population of high-affinity H_2-oxidizing bacteria that could survive using H_2 as an energy source (10). Based on the model, H_2 oxidation would fully supply the maintenance energy requirements of 10^4 to 10^7 H_2-oxidizing bacteria g of soil (dry weight)$^{-1}$ (Fig. 5B). The abundance of presumptive high-affinity H_2-oxidizing bacteria in soil was determined using a quantitative PCR assay targeting the *hhyL* gene. Soil samples contained 10^6 to 10^8 *hhyL* gene copies g of soil (dry weight)$^{-1}$ (Fig. 5B). Assuming one *hhyL* gene copy per genome (55), the abundances of H_2-oxidizing bacteria determined by quantitative PCR were thus generally larger than the theoretical maximal population size estimates. H_2 oxida-
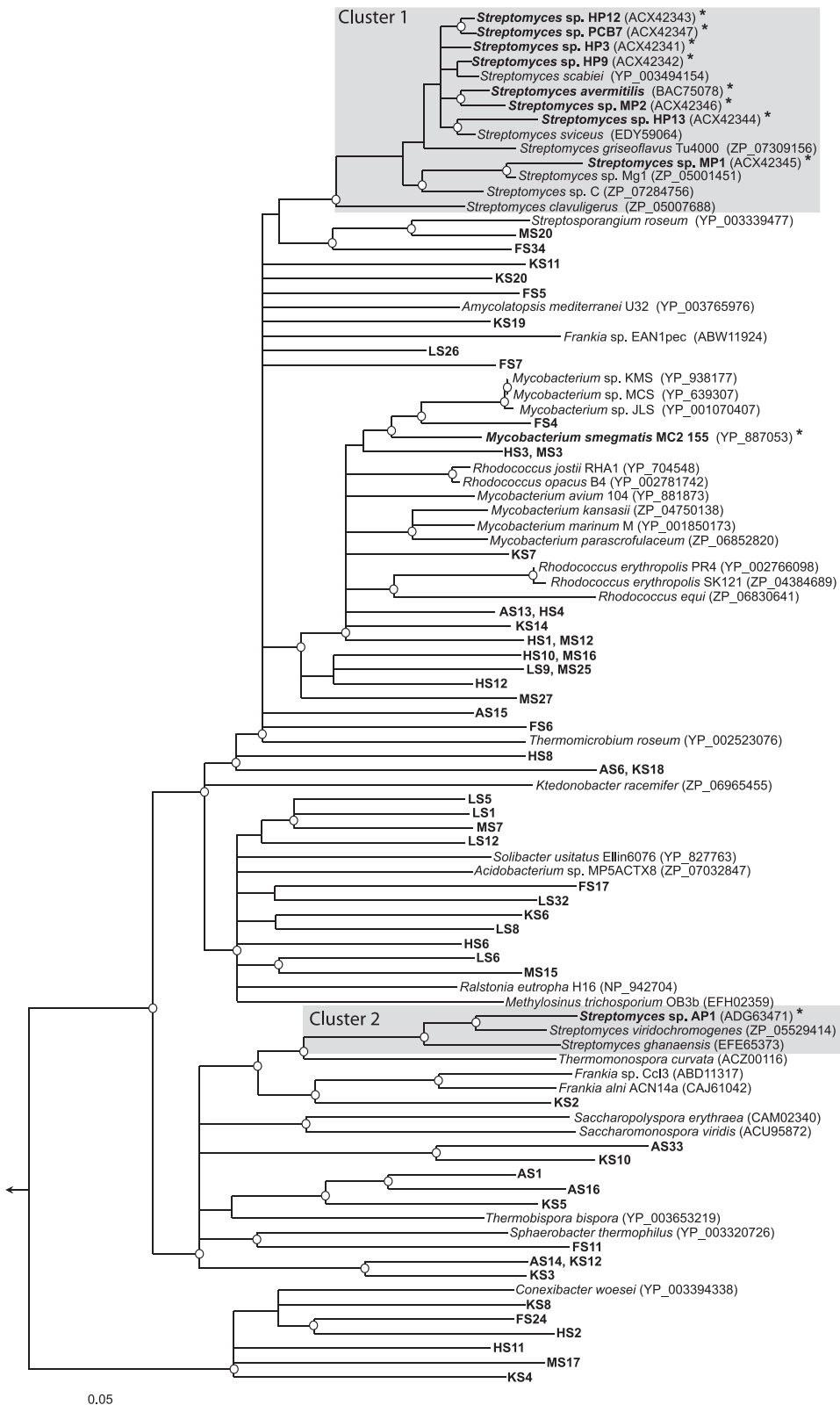
FIG. 2. Neighbor-joining tree of translated amino acid sequences from *hhyL* gene sequences retrieved from public databases, soil isolates, and soil clone libraries. Prefixes of *hhyL* clone sequences indicate the soil type, as follows: AS, arid desert; FS, paddy field; HS, agricultural area; KS, hyperarid desert; LS, peatland; MS, deciduous forest. Microorganisms in which high-affinity H₂ oxidation activity was demonstrated are asterisked. *Streptomyces* clusters 1 and 2 are shaded. Bar, 0.05 change per amino acid position. Only the nodes supported by ≥50% of the bootstrap replicates are shown. Circles mark the nodes validated with the maximum-likelihood algorithm.
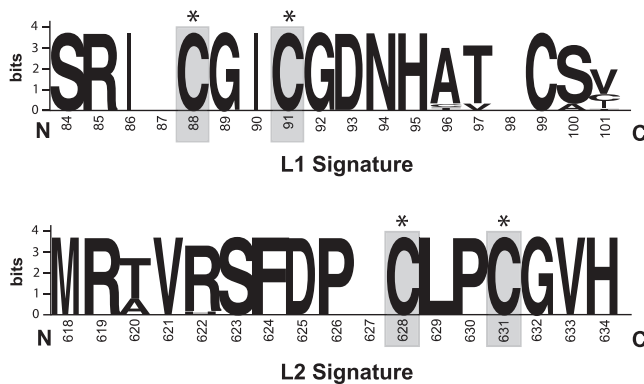
FIG. 3. Consensus L1 and L2 signatures in the large subunit of the hydrogenase generated from the alignment of sequences comprising the group 5 [NiFe]-hydrogenase cluster using the Weblogo program (16). Amino acid residue positions in the HhyL sequence alignment are given below the letters. The overall height of the symbols indicates the relative frequency of each amino acid and the sequence conservation at each position. Asterisked, shaded C's represent the cysteine residues coordinating the NiFe cofactor in the large subunit of the hydrogenase. An alignment of the predicted amino acid sequences of selected group 1 to 5 [NiFe]-hydrogenase large subunits is provided in File S1 in the supplemental material.
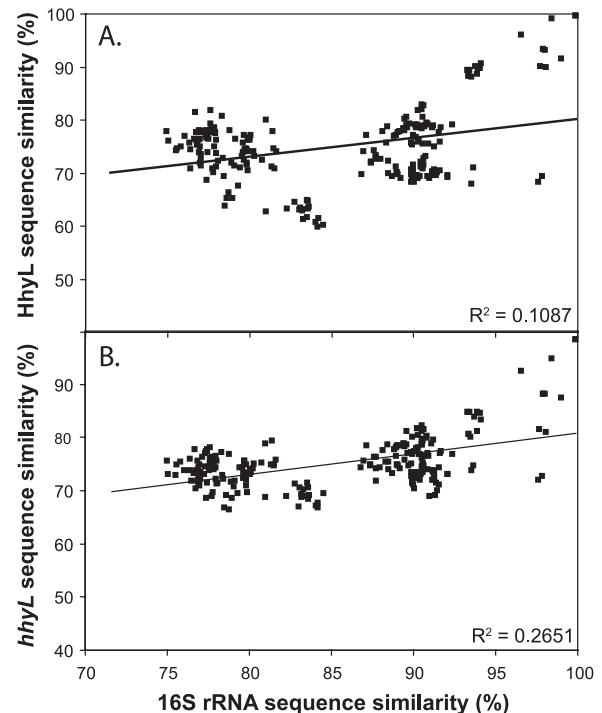

FIG. 4. Correlation between the pairwise sequence similarity scores of HhyL and the 16S rRNA gene (A) and the *hhyL* gene and the 16S rRNA gene (B) in presumptive high-affinity H$_2$-oxidizing bacteria with group 5 [NiFe]-hydrogenases.

tion activity was not proportional to the *hhyL* gene copy number detected in soil.

DISCUSSION

Compelling experimental evidence suggests that high-affinity H$_2$-oxidizing *Streptomyces* spp. possessing the genes encoding a putative high-affinity [NiFe]-hydrogenase are involved in the uptake of atmospheric H$_2$ (13, 14). Quantitative assessment of the contribution of high-affinity H$_2$-oxidizing bacteria to the budget of atmospheric H$_2$ remains elusive, however, since virtually nothing is known about their abundance, diversity, and distribution in the environment. This study constitutes a first attempt to bridge this knowledge gap.

The putative high-affinity [NiFe]-hydrogenase is distributed across different phyla and constitutes a phylogenetically distinct group, namely, the group 5 [NiFe]-hydrogenases. The definition of this novel group was further supported by the conserved L1 and L2 signatures of the large subunit (Fig. 3), distinguishing the group 5 [NiFe]-hydrogenases from those belonging to groups 1 to 4 (55). The presence of conserved ORFs 1 to 4 within the hydrogenase coding region and the unusual high-affinity H$_2$ oxidation activity displayed by *Streptomyces* isolates possessing the genes encoding the putative metalloenzyme (13) are other unique features of group 5 [NiFe]-hydrogenases. The novel hydrogenase was not detected in publicly available archaeal genome sequences. We propose the designations *hhyS* and *hhyL* for the gene sequences specifying the small and large subunits of [NiFe]-hydrogenases belonging to group 5. Consistent with the recommended hydrogenase gene nomenclature based on phylogenetic relationships (56), these gene designations are proposed to eliminate inconsistencies in the designation of the genes homologous to *hhySL* in current genome annotations.

The incongruence between 16S rRNA and *hhyL* gene se-

quence phylogenies (Fig. 4) implies a lateral inheritance of group 5 [NiFe]-hydrogenases. Indeed, the potential lateral acquisition of group 5 [NiFe]-hydrogenases was observed in analyses of the genome sequence data of *R. eutropha* (50), *Frankia* spp. (36), and *Thermomicrobium roseum* (60). According to the selfish-operon hypothesis, the arrangement of the auxiliary and structural genes of the hydrogenase within a single cluster constitutes an important factor promoting the diversification of presumptive high-affinity H$_2$-oxidizing bacteria (33). Genes encoding factors involved in genetic mobility, a tRNA gene, and a phage motif typical of genomic islands (25) were observed in the chromosome region of a number of group 5 [NiFe]-hydrogenase gene clusters (see Table S4 in the supplemental material). The origin and functionality of a laterally inherited hydrogenase gene complex would require special attention in future investigations. With the exception of *Streptomyces* soil isolates (13, 14) and *M. smegmatis* (28), there has been no report on high-affinity H$_2$ oxidation activity in microorganisms possessing group 5 [NiFe]-hydrogenases. *Frankia* spp. have been shown to express the *hhyL* gene in soil, suggesting that these actinobacteria possess a functional group 5 [NiFe]-hydrogenase (36, 39). In contrast, *R. eutropha* H16 harbors a megaplasmid carrying the genes encoding group 1, 2, 3, and 5 [NiFe]-hydrogenase isoenzymes and has been shown to be unable to oxidize atmospheric H$_2$ (11, 50). Whether the genes specifying the putative high-affinity hydrogenase are functional in *R. eutropha* or not remains elusive, since strain HF500, defective in all but the group 5 [NiFe]-hydrogenase, showed no H$_2$ oxidation activity (30). The putative high-affinity hydrogenase of *R. eutropha* might be cryptic, constituting a genetic
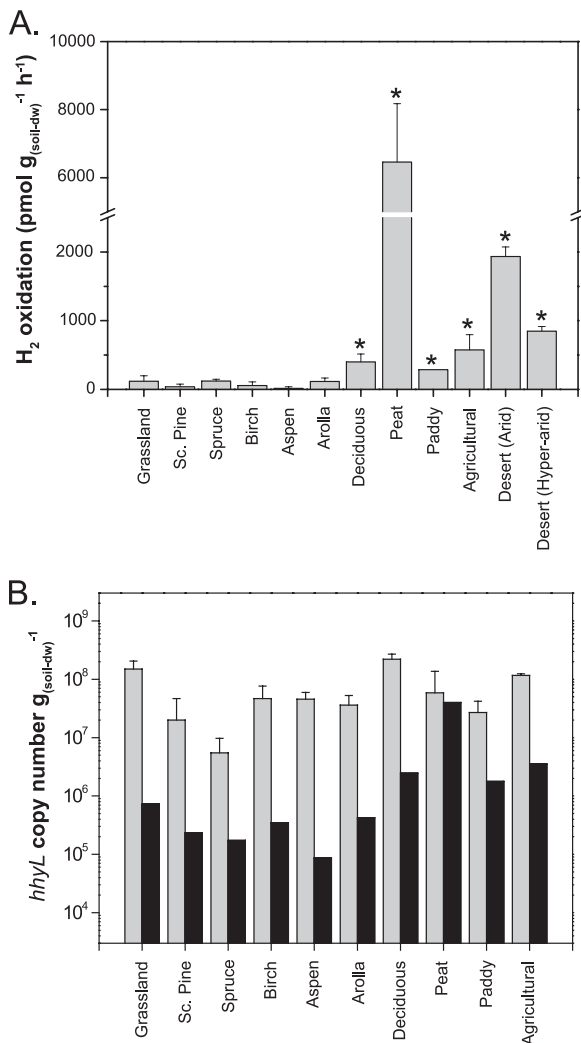
FIG. 5. (A) H$_2$ oxidation activities of soil samples collected in contrasting terrestrial ecosystems exposed to 1.5 ppmv H$_2$. Asterisked soil samples were utilized for *hhyL* gene clone libraries. Sc. pine, Scots pine. (B) Copy number of the *hhyL* gene (shaded bars) and maximal population of high-affinity H$_2$-oxidizing bacteria for which maintenance energy requirements would be fully supplied through the H$_2$ oxidation activity measured in the soil (filled bars). dw, dry weight.

reservoir that could potentially be activated following genetic modifications or could be expressed under particular growth conditions.

The function of the auxiliary genes of group 5 [NiFe]-hydrogenase was predicted based on the current stage of knowledge about the hydrogenase maturation apparatus in *Escherichia coli* and *R. eutropha* (7, 34, 57). Auxiliary proteins designated HypABCDEF are involved in the assembly of active-site components into the large subunit. The endopeptidase HupD cleaves the C-terminal extension of the large subunit after nickel insertion, triggering the final folding and assembly of the polypeptide with the small subunit. However, it must be mentioned that conserved amino acid motifs shown to be essential for the activity of [NiFe]-hydrogenase maturation proteins in *Escherichia coli* (7) were absent from some auxiliary gene sequences retrieved from the genomes of *Streptomyces* spp. (see

Table S1 in the supplemental material). Whether the whole set of genes identified in *S. avermitilis* (Fig. 1) is essential for group 5 [NiFe]-hydrogenase activity or not remains to be established, especially for *hypX* and ORFs 1 to 4, whose physiological function is unknown. The *hypX*-like gene encodes a putative protein, HypX, that has been shown to confer oxygen tolerance on the soluble [NiFe]-hydrogenase in *R. eutropha* H16 through the attachment of an additional cyanide ligand to the active site (6) and to participate in the maturation of the large subunit of the H$_2$ uptake hydrogenase in *Rhizobium leguminosarum* (45). The *hypX* gene was detected only in the hydrogenase gene clusters of *Streptomyces* representatives (Table 1), suggesting that they synthesize hydrogenases with unusual biochemical properties. The conservation of ORFs 1 to 4 in the hydrogenase gene clusters of presumptive H$_2$-oxidizing bacteria suggests that their function could be linked to the activity of group 5 [NiFe]-hydrogenase (61). Since ORFs 1 to 4 are not found in group 1 to 4 [NiFe]-hydrogenase gene clusters, biochemical characterization of these hypothetical proteins would provide valuable information on the biological functions of [NiFe]-hydrogenases belonging to group 5. The deduced amino acid sequence of ORF1 comprises two conserved redox-active CxxC motifs, designated "rheostat in the active site" due to the influence of the residues separating the two cysteines on the redox function of the enzymes (8). Coupling of ORFs 1 to 4 with the structural subunits of group 5 [NiFe]-hydrogenase to mediate electron flow is thus conceivable. The *hypABCDEF* genes coordinate the NiFe cofactor and the assembly of the hydrogenase. The HypC, HypD, HypE, and HypF proteins participate in the transfer of the Fe-(CN$^-$)$_2$-(CO) moiety to the precursor of the large subunit of [NiFe]-hydrogenase, while HypA and HypB are involved in nickel storage and insertion (7, 34, 57). Only HypCDEF have been shown to be essential for a functional hydrogenase. Indeed, *hypA* and *hypB* mutants are readily complemented by the addition of exogenous nickel (>0.5 mg liter^{-1}) in cultivation media, while microorganisms deficient in *hypCDEF* have been shown to lack hydrogenase activity (23, 24, 38, 43). Bioavailable nickel concentrations of 0.5 to 6 mg kg^{-1} have been detected in sewage sludge-amended soils (53), while low- and high-affinity nickel transport systems allow microorganisms to satisfy their demands for this metal (42). Thus, the concentration of bioavailable nickel in soil should be sufficient to complement a *hypAB* gene deficiency in H$_2$-oxidizing bacteria. In contrast, the occurrence of a functional hydrogenase is questionable in *M. avium* and *S. thermophilus*, which possess only the group 5 [NiFe]-hydrogenase and lack the *hypABCDEF* and *hypDEF*-like genes, respectively (Table 1).

Soil samples collected in different ecosystems were analyzed for H$_2$ oxidation activity and the occurrence of presumptive high-affinity H$_2$-oxidizing bacteria using the *hhyL* gene as a functional biomarker. Among the sampling sites selected, peatland and agricultural ecosystems were recently investigated for annual H$_2$ fluxes. These ecosystems are natural sinks for atmospheric H$_2$, with annual average deposition velocities of 0.057 and 0.031 cm s^{-1}, respectively (32, 48). Accordingly, peat displayed a higher H$_2$ oxidation activity than agricultural soil (Fig. 5A). Differences in H$_2$ oxidation activity may reflect H$_2$-oxidizing bacterial populations that are divergent in terms of abundance and diversity. This concept was already proposed in

an extensive soil survey revealing that the H$_2$ oxidation potential of soil was proportional to substrate-induced respiration and potential nitrification rates (22). The incongruence between the phylogenies of the 16S rRNA and *hhyL* gene sequences observed in newly identified presumptive high-affinity H$_2$-oxidizing bacteria (Fig. 4) impaired the identification of sequences retrieved from soil at the taxonomic level. Nevertheless, the detection of the *hhyL* gene in every soil sample indicated that high-affinity H$_2$-oxidizing bacteria are ubiquitous in terrestrial ecosystems.

A first attempt to quantify presumptive high-affinity H$_2$-oxidizing bacteria in soil, using quantitative PCR targeting *hhyL* genes, was made. The number of *hhyL* gene copies was compared to the maximal population theoretically sustained with the H$_2$ oxidation activity measured. The fact that the abundance of *hhyL* gene copies was higher than the theoretical population estimates (Fig. 5B) reflects the lack of discrimination between active and inactive H$_2$-oxidizing bacteria inherent in the *hhyL* gene detection approach. Indeed, high-affinity H$_2$ oxidation is a facultative metabolism induced under definite growth conditions (5, 13, 14). Atmospheric H$_2$ is likely an alternative substrate providing complementary energy sources (13), and therefore, presumptive high-affinity H$_2$-oxidizing bacteria may grow heterotrophically without expressing hydrogenase genes. Moreover, our *in silico* analysis revealed that the expression of a functional high-affinity [NiFe]-hydrogenase remains questionable in several bacteria possessing the *hhyL* gene (Table 1). The copy number of the *hhyL* gene in soil is therefore not a reliable proxy for assessing high-affinity H$_2$ oxidation activity in soil. Notwithstanding these limitations, application of the quantitative PCR approach showed that presumptive high-affinity H$_2$-oxidizing bacteria are abundant and ubiquitous in soil, supporting further investigations aimed at defining the environmental control of high-affinity hydrogenase gene expression and activity.

Conclusion. Presumptive high-affinity H$_2$-oxidizing bacteria are taxonomically diverse and encompass representatives isolated from various ecological niches. A reason for the wide distribution of the *hhyL* gene may be its specific function in mixotrophic metabolism—the ability to acquire energy from organic and inorganic substrates—providing microorganisms with metabolic versatility for long-term survival or growth (13, 26, 46). Interestingly, several presumptive high-affinity H$_2$-oxidizing bacteria do possess a high-affinity carbon monoxide dehydrogenase conferring the ability to oxidize atmospheric carbon monoxide mixotrophically (29). The utilization of H$_2$ and CO as complementary energy sources should provide a metabolic benefit, enabling microorganisms to colonize oligotrophic environments as well as thriving and making up an important fraction of microbial assemblages in nutrient-rich environments, as has been observed across a succession gradient of volcanic deposits (59). Such a potentially wide distribution of high-affinity H$_2$-oxidizing bacteria was supported by the presence of *hhyL* genes in soil collected in contrasting terrestrial ecosystems. The abundance of *hhyL* gene copies in soil supports the notion that high-affinity H$_2$-oxidizing bacteria contribute to the uptake of atmospheric H$_2$ by soil. However, the lack of discrimination between active and inactive components of high-affinity H$_2$-oxidizing bacterial assemblages impairs quantitative assessment of the extent to which these mi-

croorganisms contribute to the uptake of atmospheric H$_2$ by soil. There are significant gaps in our current knowledge about the physiological and mechanistic aspects of the uptake of atmospheric H$_2$ by soil. We anticipate that the availability of high-affinity H$_2$-oxidizing isolates and the genetic information presented in this article will stimulate future investigations to elucidate the regulation and biochemical properties of group 5 [NiFe]-hydrogenases, the ecophysiology of high-affinity H$_2$-oxidizing bacteria, and ultimately environmental control of the uptake of atmospheric H$_2$ by soil. A gene-driven mutagenesis approach will be crucial to confirming the functions of the genes specifying the auxiliary and structural components of group 5 [NiFe]-hydrogenases.

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