Microb Ecol (2017) 74:771–775
DOI 10.1007/s00248-017-1011-1

NOTE

Survey of High-Affinity H_2-Oxidizing Bacteria in Soil Reveals Their Vast Diversity Yet Underrepresentation in Genomic Databases

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Received: 20 April 2017 / Accepted: 5 June 2017 / Published online: 17 June 2017

Abstract While high-affinity H_2-oxidizing bacteria (HA-HOB) serve as the main sink of atmospheric H_2, the ecology of this specialist functional group is rather unknown due to its recent discovery. The main purpose of our study is to provide the first extensive survey of HA-HOB in farmland, larch, and poplar soils exposed to 0.5 and 10,000 ppmv H_2. Using qPCR and qRT-PCR assays along with PCR amplicon high-throughput sequencing of *hhyL* gene encoding for the large subunit of high-affinity [NiFe]-hydrogenases (HAH), we found that *hhyL* gene expression ratio explained better variation in measured H_2 oxidation rates than HA-HOB species richness. Carbon, nitrogen, pH, and bacterial species richness appeared as the most important drivers of HA-HOB community structure. Our study also highlights the need to cultivate HA-HOB due to the huge gap in current genomic databases.

Keywords Biogeochemistry · H_2-oxidizing bacteria · High-throughput sequencing · Trace gas

State-of-the-art atmospheric chemistry-transport models indicate that nearly 70% of atmospheric H_2 losses are caused by microbial H_2-oxidizing metabolism in upland soils [1]. Microbes accountable for this crucial ecosystem service remained unknown until *Streptomyces* spp. harboring high-affinity [NiFe]-hydrogenases (HAH) were discovered [2, 3]. Genome database mining and measurement of H_2 uptake in pure cultures demonstrated that other Actinobacteria such as *Mycobacterium* spp. [4] and *Rhodococcus* spp. [5], and Acidobacteria [4, 6], have the ability to scavenge trace levels of H_2. The ecology of this functional group is fairly unknown, yet soil moisture, carbon content, and pH were identified as key drivers of high-affinity H_2 oxidation in soils [7]. While soil moisture limits H_2 diffusion [8], the stimulating effect of carbon and pH on H_2 uptake is not fully understood. They both act as ecological niche filters selecting or activating high-affinity H_2-oxidizing bacteria (HA-HOB), resulting in higher uptake rates in forests than in grassland and farmland ecosystems [9]. Our study aims to provide the first extensive survey of HA-HOB in soil and relate their diversity profile to soil H_2 uptake rate and physicochemical properties. We used total genomic DNA and RNA extracts from a previous study in which soil samples encompassing three land use types were exposed to either ambient H_2 (aH_2; 0.5 ppmv H_2) or elevated H_2 (eH_2; 10,000 ppmv H_2) treatments during 15 days in a dynamic microcosm chamber unit [10]. Briefly, soil samples were collected in a 12-year-old larch plantation, a 10-year-old poplar plantation, and a farmland consisting of potato and maize rotation. The dynamic microcosm chamber unit consisted of a gas mixer and a gas distribution network that continually supplied soil microcosms with synthetic air mixtures, containing precise H_2 stoichiometric ratios, and vented them to the atmosphere. Distribution of HA-HOB was examined through qPCR/qRT-PCR and PCR amplicon sequencing of *hhyL* gene, encoding for the large structural subunit of HAH. We hypothesized that bacterial species richness varies in tandem with HA-HOB species richness and that land use defines HA-HOB abundance and diversity.

The abundance of *hhyL* gene estimated by qPCR ranged from 10^8 to 10^9 gene copies $g^{-1}_{(dw)}$ (Table 1), akin to previous soil surveys [11]. Single linear regression analysis showed that

Electronic supplementary material The online version of this article (doi:10.1007/s00248-017-1011-1) contains supplementary material, which is available to authorized users.

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Table 1 HA-HOB species richness and quantification of *hhyL* gene and transcript across H_2 treatments and land uses

Variables	Farmland		Larch monoculture		Poplar monoculture	
	aH_2	eH_2	aH_2	eH_2	aH_2	eH_2
Number of observed OTUs	261	273	240	242	270	238
ACE richness estimator	266 (7)	278 (7)	249 (7)	245 (7)	279 (7)	240 (7)
Chao1 richness estimator	267 (5)	279 (4)	255 (10)	248 (5)	282 (8)	241 (3)
Shannon richness index	3.14 (0.01)	3.06 (0.27)	3.20 (0.05)	3.11 (0.11)	3.13 (0.04)	3.09 (0.08)
Pielou evenness index	0.62 (0.01)	0.60 (0.05)	0.65 (0.02)	0.63 (0.02)	0.62 (0.02)	0.63 (0.01)
hhyL gene (copies $g^{-1}_{(dw)}$)	1.49×10^8 (2.43×10^7)	7.07×10^8 (1.43×10^8)	9.30×10^8 (2.29×10^8)	5.39×10^8 (2.56×10^8)	1.48×10^8 (1.10×10^8)	1.09×10^9 (1.26×10^8)
hhyL transcript (copies $g^{-1}_{(dw)}$)	2.69×10^{11} (8.24×10^{10})^{bc}	5.10×10^8 (2.10×10^8)^{bc}	1.16×10^{10} (8.56×10^8)^{bc}	3.67×10^5 (1.35×10^5)^{bc}	6.98×10^{11} (2.79×10^{11})^{bc}	4.09×10^9 (1.48×10^9)^{bc}
hhyL gene expression ratio[d]	3.45×10^2 (1.70×10^2)^{bc}	2.59 (2.38×10^{-1})^{bc}	1.44×10^1 (1.28)^{bc}	4.71×10^{-4} (2.73×10^{-4})^{bc}	1.66×10^4 (1.61×10^2)^{bc}	3.42 (2.30)^{bc}
High-affinity H_2 oxidation rate (pmol $g^{-1}_{(dw)}$ h^{-1})[a]	361 (87)^{bc}	22 (2)^{bc}	1045 (250)^{bc}	47 (18)^{bc}	1003 (45)^{bc}	59 (0)^{bc}

Mean and standard deviation (in parentheses) values computed from three independent replicates are presented

[a] Published data [10]

[b] Variable showing significant difference between land use types (ANOVA, $p < 0.05$)

[c] Variable showing significant difference between H_2 exposure treatments (ANOVA, $p < 0.05$)

[d] The expression ratio of *hhyL* gene was computed by dividing the absolute number of transcripts by the absolute gene count as detected by qRT-PCR and qPCR, respectively

hhyL expression ratios (number of transcripts divided by gene count detected by qRT-PCR and qPCR) explained variations in high-affinity H_2 oxidation rates ($R^2 = 0.45$, $p = 0.04$, $n = 18$), which is comparable to the resolution of a previous approach relying on the relative abundance of HA-HOB estimated by *hhyL* and 16S ribosomal RNA (rRNA) gene qPCR analysis [12]. On the other hand, neither *hhyL* gene transcripts ($R^2 = 0.21$, $p > 0.05$) nor *hhyL* gene counts ($R^2 = 0.00$, $p = 0.98$) alone reflected measured activities. Sequences of PCR-amplified *hhyL* gene obtained from Illumina MiSeq (2× 250-bp configuration) sequencing platform were clustered into 664 OTUs using a 99% identity cutoff (Supplemental Method 1). This stringent threshold was selected as a tradeoff to delineate HA-HOB species, considering both unavoidable technical limitations (i.e., sequencing error rate [13] and potential errors introduced by paired-end assembly [14]) and frequent lateral transfers resulting in considerable underestimations of HA-HOB richness [11]. An extensive database of [NiFe]-hydrogenase large subunit genes was used for phylogenetic analysis of *hhyL* OTUs (Supplemental Method 1). Consensus phylogenetic tree of reference sequences displays the expected clusters outlining [NiFe]-hydrogenases encompassing group 1 to 5 (Fig. 1a). All OTUs were accurately assigned to group 5 cluster, establishing the specificity of the oligonucleotides targeting *hhyL* gene. However, only nine OTUs were closely associated with reference sequences (Fig. S1). The most abundant OTU genotype from our dataset (OTU_1, 20–40% abundance) has an uncultivated clone retrieved from deciduous forest soil in Germany as its closest neighbor (clone MS12, 100% similarity). Similarly, OTU_858 was classified in a cluster comprising environmental clones LS15 and LS27 (97% similarity) detected in Finnish peatlands, although seven other OTUs were affiliated to clusters comprising known bacterial species. OTU_294 was related to *Rhodococcus* sp. (89% similarity) and OTU_237 encompassed a cluster comprising *Streptosporangium roseum* and the environmental clone MS20 (92% similarity) originating from deciduous forest soil in Germany. OTU_81 and OTU_423 were affiliated to *Streptomyces* spp. (97–99% similarity), while OTU_869, OTU_1305, and OTU_1312 were related to *Conexibacter woesei* (76–81% similarity). Considering this, all HA-HOB harboring *hhyL* genes within this survey remain undiscovered.

The number of observed OTUs was comparable to species richness estimators (Table 1), indicating sufficient sequencing effort to cover HA-HOB diversity. Neither pairwise correlation between 16S rRNA and *hhyL* genes richness (Pearson, $P = 0.4788$) nor evenness (Pearson, $P = 0.0708$) showed significant covariation, suggesting that HA-HOB diversity is independent of bacterial species richness in soil. Furthermore, H_2 treatments caused no significant alteration in HA-HOB richness or evenness (Table 1). Similarly, the impact of eH_2 exposure on *hhyL* OTU distribution profiles was

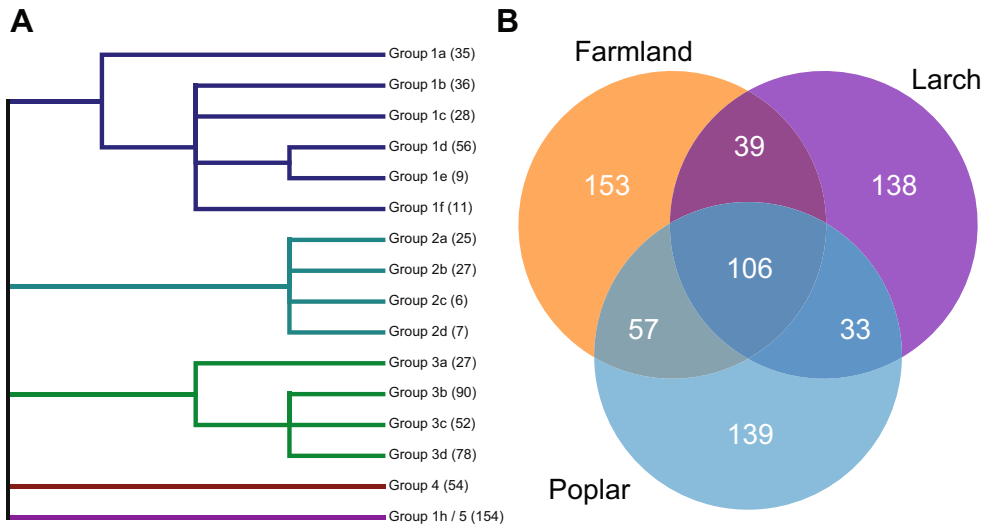

Fig. 1 Classification of *hhyL* OTUs across **a** a consensus phylogeny analysis and **b** the three land use types. Consensus phylogenetic tree of translated amino acid sequences was computed from near-complete genes encoding the large subunit of [NiFe]-hydrogenases of groups 1 through 5 retrieved from public databases. The consensus tree was generated from the combination of neighbor-joining, maximum likelihood, and maximum parsimony phylogenetic trees using the "ape" R package [15]. Only nodes supported by over 75% of bootstrap replications (out of 500 bootstraps) from all three initial trees are shown. Sequences with 100% identity at the amino acid level were removed until only one identical representative remained. *Numbers between parentheses* represent the number of sequences encompassing each genotype. The Venn diagram represents the number of OTUs present in each combination of land use types

undiscernible at the community level (PERMANOVA, $p = 0.254$). Nevertheless, differential analysis of OTU count data (Supplemental Method 1) indicated that 27 OTUs were affected by eH_2 exposure, mostly with higher relative abundance in eH_2-treated soils, with contradicting patterns between land uses (Supplemental Table 2). This agrees with previous observations in which microbial response to eH_2 exposure was idiosyncratic due to the most influential role of abiotic factors such as carbon, nitrogen, and pH on microbial communities [10, 16]. Genotypes showing higher abundance under eH_2 could be representatives of HOB possessing both HAH (i.e., group 5 hydrogenase) and low-affinity [NiFe]-

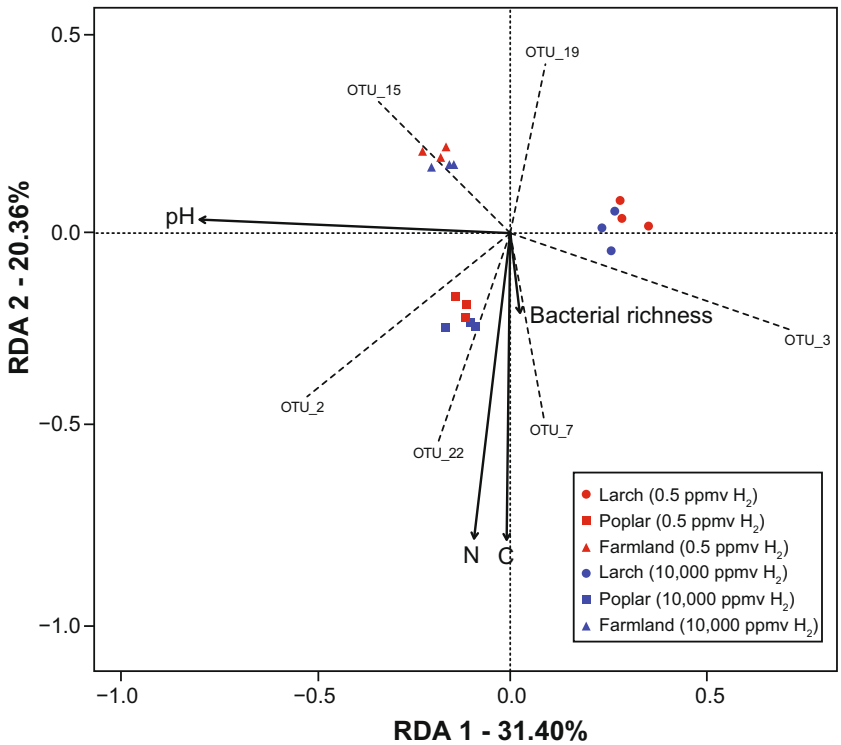

Fig. 2 Parsimonious RDA representing the distribution of soil microcosms in a reduced space according to their *hhyL* OTU distribution profiles constrained with environmental variables. Land uses are distinguished by three different symbols (*circle* larch, *square* poplar, *triangle* farmland). *Red symbols* indicate soil microcosms exposed to aH_2 (0.5 ppmv), and *blue symbols* indicate soil microcosms exposed to eH_2 (10,000 ppmv). Only OTUs whose relative abundance exert more weight than the average to separate land use types in terms of *hhyL* genes profiles are depicted

hydrogenases (e.g., group 3 hydrogenase), like *Mycobacterium smegmatis* [17]. Indeed, multiple hydrogenases provide greater flexibility to switch between survival mixotrophy supported by HAH, at low H_2 mixing ratios, and lithoautotophic growth under elevated H_2 mixing ratios. Bacteria possessing only HAH are expected to be hindered by eH_2 exposure [18]. Although the exact biochemical mechanism is unknown, such potential substrate inhibition on HA-HOB metabolism was supported by lower *hhyL* gene expression ratio in eH_2-exposed soils (Table 1).

Neither HA-HOB species richness nor evenness was distinguishable across the three land use types (Table 1). In contrast to H_2 exposure treatments, land use was a significant driver of HA-HOB community structure, explaining 62% of the variance in *hhyL* gene distribution (PERMANOVA, $p < 0.001$). Most OTUs were land use-specific (Fig. 1b), yet 106 OTUs common to the three land uses were most abundant, since they represented up to 88% of *hhyL* genes in any given sample. A parsimonious redundancy analysis (RDA) was then computed to pinpoint which biotic and abiotic parameters measured in soil microcosms [10] acted as environmental filters for *hhyL* gene distribution profiles. Nearly 50% variance in HA-HOB community structure was explained by soil carbon and nitrogen contents, pH, and bacterial species richness (Fig. 2). Several OTUs also discriminated land uses in the reduced space, including OTU_15 which was more abundant in farmland soils. The important contribution of carbon and pH in explaining HA-HOB community structure complies with previous studies investigating environmental drivers of H_2 oxidation activity in soil [7, 12, 19].

In short, this study displayed the diversity of soil HA-HOB and the crucial need to cultivate more HA-HOB in order to fill the gap in genomic databases, which are currently not suited to depict soil microbiomes [20].

Acknowledgements This work was supported by a Natural Sciences and Engineering Research Council of Canada Discovery grant to P.C. The authors acknowledge use of McGill University and Génome Québec Innovation Centre for the preparation of *hhyL* gene libraries and sequencing services. S.P.-C. and M.K. are grateful to the Fondation Universitaire Armand-Frappier INRS for their Ph.D. scholarship.

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Accession numbers

Raw sequence reads of *hhyL* genes from PCR amplicon sequencing were deposited in the Sequence Read Archive of the National Center for Biotechnology Information under BioProject PRJNA382186.