Distribution of Hydrogenases in Cyanobacteria: A Phylum-Wide Genomic Survey

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Microbial Molecular hydrogen ($H_2$) cycling plays an important role in several ecological niches. Hydrogenases ($H_2$ases), enzymes involved in $H_2$ metabolism, are of great interest for investigating microbial communities, and producing Bio$H_2$. To obtain an overall picture of the genetic ability of Cyanobacteria to produce $H_2$ases, we conducted a phylum wide analysis of the distribution of the genes encoding these enzymes in 130 cyanobacterial genomes. The concomitant presence of the $H_2$ase and genes involved in the maturation process, and that of well-conserved catalytic sites in the enzymes were the three minimal criteria used to classify a strain as being able to produce a functional $H_2$ase. The [NiFe] $H_2$ases were found to be the only enzymes present in this phylum. Fifty-five strains were found to be potentially able produce the bidirectional Hox enzyme and 33 to produce the uptake (Hup) enzyme. $H_2$ metabolism in Cyanobacteria has a broad ecological distribution, since only the genomes of strains collected from the open ocean do not possess $hox$ genes. In addition, the presence of $H_2$ase was found to increase in the late branching clades of the phylogenetic tree of the species. Surprisingly, five cyanobacterial genomes were found to possess homologs of oxygen tolerant $H_2$ases belonging to groups 1, 3b, and 3d. Overall, these data show that $H_2$ases are widely distributed, and are therefore probably of great functional importance in Cyanobacteria. The present finding that homologs to oxygen-tolerant $H_2$ases are present in this phylum opens new perspectives for applying the process of photosynthesis in the field of $H_2$ production.

Keywords: cyanobacteria, genomes, hydrogenase, oxygen tolerance

INTRODUCTION

Microbial hydrogen ($H_2$) metabolism is a process that occurs in many different environments. In addition to being a key metabolic factor in several biological communities, $H_2$ has attracted considerable interest as a candidate environmentally friendly energy carrier. The use of photosynthetic organisms such as microalgae and cyanobacteria has been tested worldwide for this purpose. In cyanobacteria, the main enzymes involved in $H_2$ metabolism are nitrogenases and hydrogenases ($H_2$ases) (Reviewed in Bothe et al., 2010). Nitrogenases fix molecular nitrogen ($N_2$) and produce $H_2$ as a byproduct (D’Eustachio and Hardy, 1964). $H_2$ases are metalloprotein enzymes which catalyze in several microorganisms the reversible reaction:

$$2H^+ + 2e^- \leftrightarrow H_2$$ (for a recent Review see Peters et al., 2015).

They are usually classified into three phylogenetically independent classes: [Fe] $H_2$ases, [FeFe] $H_2$ases, and [NiFe] $H_2$ases (Vignais and Billoud, 2007). Since [Fe] $H_2$ases are light-sensitive...
enzymes (Chen et al., 2002), they can be considered as directed limited interest in the context of H₂ photoproduction. The [FeFe] H₂-ases present in anaerobic bacteria and some phototrophic eukaryotes preferentially catalyze the evolution of H₂ at high frequencies; these enzymes are also characterized by their high sensitivity to oxygen (O₂) (Melis et al., 2000; Florin et al., 2001; Winkler et al., 2002; Peters et al., 2015). The [NiFe] H₂-ases, which have been found to exist in Archaea and in several aerobic and anaerobic bacterial phyla, are mainly involved in H₂ oxidation but can also catalyze the reduction of protons to H₂ (Vignais and Billoud, 2007). They consist of a large subunit containing the bimetallic center [NiFe] and a small subunit containing [FeS] clusters (Volbeda et al., 1995, 1996; Peters et al., 2015). Based on a phylogenetic analysis of the large subunit, and more specifically, on two highly conserved regions located in this subunit near the [NiFe] center (the L1 and L2 regions), the [NiFe] H₂-ases have been classified into the eight different groups presented in Table 1 (Vignais et al., 2001; Vignais and Billoud, 2007). The maturation of [NiFe] H₂-ases involves six proteins (HypFCDEAB), which synthesize the non-protein ligands (CO and CN) and assemble the active site (Dernedde et al., 1996; Hansel et al., 2001; Hoffmann et al., 2006). In the last step in the process of biosynthesis, the C terminal part of the large subunit is cleaved by a specific peptidase (Thiemermann et al., 1996; Devine et al., 2009).

Although the activity of most of the [NiFe] H₂-ases tends to be inhibited by O₂, some members of this class remain active in the presence of O₂ and have therefore been called O₂-tolerant. The O₂-tolerant H₂-ases described for the first time in the anoxicogenic bacterium Rubrivivax gelatinosus (Maness et al., 2002) occur in the Group 1 membrane-bound H₂-ases (MBH), the H₂-signaling group (RH, Group 2b) (Buhrke et al., 2005; Duchê et al., 2005), the tetrameric bifunctional H₂-ases (group 3b) (Jenney and Adams, 2008; Kwan et al., 2015), and the recently identified Group 5 Actinobacterial-[NiFe]-H₂-ases (Table 2) (Constant et al., 2010; Lubitz et al., 2014). In the case of the MBH enzymes, the main difference between the standard and tolerant members focuses on the [FeS] cluster located near the [NiFe] site. Instead of the canonical [4Fe4S]⁻ present in the standard enzymes, a [4Fe3S]⁻ cluster coordinated by six cysteine residues occurs in the tolerant enzymes (Pandelia et al., 2011; Shomura et al., 2011). This proximal [4Fe3S]⁻ is the most striking feature thought to be linked to O₂-tolerance (Goris et al., 2011; Lukey et al., 2011). The O₂-insensitivity of the RH-H₂-ases ofRalstonia eutropha H16 depends on the size and shape of the intramolecular hydrophobic cavity giving access to the active [NiFe] site (Buhrke et al., 2005). The molecular mechanism underlying the O₂-tolerance of the Group 3 SH enzymes and that of the actinobacterial H₂-ases still remains to be elucidated.

Cyanobacteria, the only prokaryotes capable of oxygenic photosynthesis, form a large and morphologically diverse bacterial group consisting of five morphological subsections. The unicellular organisms that undergo binary fission belong to subsection I (Chroococcales). The unicellular strains that divide through multiple fission processes form subsection II (Pleurocapsales), and subsection III consists of filamentous strains which are unable to perform cell differentiation (Oscillatoriaceae). The strains in subsections IV and V are filamentous and able to differentiate specific cells called heterocysts, which are dedicated to N₂ fixation (Rippka et al., 1979). Cyanobacteria are widely distributed in various environments (from oceans to desert crusts), where they contribute importantly to primary production and N₂ fixation processes (Garcia-Pichel et al., 2003). N₂-fixation in these organisms is mainly achieved by a molybdenum-iron ([MoFe]) nitrogenase which consists of two subunits, a Fe-protein encoded by nifH, and a Mo-Fe protein encoded by nifDK genes (Smith and Eady, 1992). The maturation process requires three essential processes (H202 evolution, energy-conserving, membrane-associated H2-ases)

| Group | Name | Function | H₂ | O₂ sensitive/resistant | References |
|-------|------|----------|----|------------------------|------------|
| 1     | Membrane bound H₂ uptake H₂-ases | H₂ uptake under aerobic and/or anaerobic conditions. | Oxidation | Sensitive and Resistant | Higuchi et al., 1999; Marques et al., 2010; Dementin et al., 2011 |
| 2a    | Cyanobacterial uptake H₂-ases | Uptake of H₂ produced by nitraginase. | Oxidation | Sensitive | Oxelfelt et al., 1998; Tamagnini et al., 2007; Zhang et al., 2014 |
| 2b    | H₂-signaling H₂-ases | H₂ perception and signaling. | Oxidation | Resistant | Buhrke et al., 2004, 2005; Roncaroli et al., 2015 |
| 3a    | F₄200-reducing H₂-ases | H₂ utilization during methanogenesis. | Oxidation evolution | Sensitive | Hendrickson and Leigh, 2008; Vitt et al., 2014 |
| 3b    | Tetrameric bifunctional H₂-ases | Regulation and redox balance. | Oxidation evolution | Sensitive and resistant | Bryant and Adams, 1989; Jenney and Adams, 2008; Berney et al., 2014 |
| 3c    | Methyl-viologen-reducing H₂-ases | H₂ uptake during methanogenesis. | Oxidation | Sensitive | Kaster et al., 2011 |
| 3d    | Soluble bidirectional H₂-ases | Regulation and redox balance. | Oxidation evolution | Sensitive and resistant | McIntosh et al., 2011; Lauterbach and Lenz, 2013 |
| 4     | H₂-evolving, energy-conserving, membrane-associated H₂-ases | Coupling of formate or carbon monoxide to H₂ evolution. | Evolution | Sensitive | Bagramyan et al., 2002; McDowall et al., 2014 |
| 5     | Actinobacteria [NiFe]-H₂-ases | H₂ uptake during starvation. | Oxidation | Resistant | Schäfer et al., 2013 |
TABLE 2 | Overview of the main features of O$_2$-tolerant H$_2$ases in several organisms.

| Group          | Name             | Cluster Fe-S small subunit | Structural basis of O$_2$-tolerance                                                                 | Example                                              | References                                      | Homolog in cyanobacteria                  |
|---------------|------------------|---------------------------|----------------------------------------------------------------------------------------------------|------------------------------------------------------|------------------------------------------------|-------------------------------------------|
| 1             | Membrane bound H$_2$ uptake H$_2$ases (MBH) | p [4Fe3S] m [3Fe4S] q[4Fe4S] | Transfer electron from the proximal cluster to active site to reduce O$_2$ to water.             | Rubrivivax gelatinosus, Hyd-1 Escherichia coli       | Maness et al., 2002; Evans et al., 2013       | Lyngbya confervoides                        |
| 2b            | H$_2$-signaling H$_2$ases (RH)                    | p [4Fe4S] m [4Fe4S] q[4Fe4S] | The gas channel is narrower than standard H$_2$ases and the O$_2$ cannot interact with the active site. | Rhodobacter capsulatus, Ralstonia eutropha          | Buhrie et al., 2005; Duché et al., 2005     | None                                      |
| 3b            | Tetrameric bifunctional H$_2$ases (PISH)         | p [4Fe4S] m [2Fe2S] q[4Fe4S] | No formation of the slowly reactivating state Ni-A                                               | Pyrococcus furiosus                                 | Jenney and Adams, 2008; Kwan et al., 2015   | Cyanobacteria sp. PCC 7425, Leptolyngbya boryana PCC 6306, Mastigocoleus testarum BC0008 |
| 3d            | Soluble bidirectional H$_2$ases (FeSH)           | [4Fe4S]                   | Reduction of O$_2$ in water. Cys39 and Thr42 are demonstrated important for O$_2$ tolerance      | Ralstonia eutropha                                  | Horch et al., 2013; Karstens et al., 2015   | Aphanocapsa montana BDH-KU210001       |
| 5             | Actinobacteria [NiFe] H$_2$ases (AH)             | p [4Fe4S] m [4Fe4S] q[4Fe4S] | Unknown                                                                                           | Streptomyces avermitilis, Ralstonia eutropha        | Constant et al., 2010; Lubitz et al., 2014   | None                                      |

(nifBEN) and three no essential genes (nifUSV) (Reviewed in: Rubio and Ludden, 2008). The reduction of N$_2$ is accompanied by the formation of H$_2$ (Berman-Frank et al., 2003). Cyanobacteria contain two different [NiFe] H$_2$ases: the bidirectional [NiFe] H$_2$ase (Hox, Group 3d) and the uptake H$_2$ase (Hup, Group 2a) (Tamagnini et al., 2007). The Hup H$_2$ase is a heterodimeric enzyme encoded by the hupSL genes, which consumes the H$_2$ produced by the nitrogenase (Houchins and Burris, 1981; Lindblad and Sellstedt, 1990). The bidirectional Hox H$_2$ase, which can oxidize H$_2$ and reduce H$^+$, can exist in both diazotrophic and non-diazo-trophic strains, and is thought to be a heteropentameric enzyme encoded by hoxEFUYH genes (Schmitz et al., 1995). In the unicellular cyanobacterium Synechocystis PCC 6803, the bidirectional H$_2$ase has been shown to be essential under mixotrophic and nitrate limiting conditions, which suggests that this enzyme functions as electron sink for reduced flavodoxin/ferredoxin (Gutkunst et al., 2014). The ability of the Hox enzymes to be quickly reactivated after being inhibited by O$_2$ has made them the most frequently used H$_2$ase in studies on H$_2$ production in cyanobacteria (Serebryakova et al., 1996; Germer et al., 2009; McIntosh et al., 2011). The main limitations of using the cyanobacterial Hox enzymes in large scale H$_2$ production processes are the low levels of H$_2$ produced and the fast reversal of the enzymatic reaction into oxidation (Tamagnini et al., 2007; Rögnar, 2013). During the last decade, genetic engineering approaches were used in several studies in order to overcome these technological barriers with a relative success (Masukawa et al., 2002; McNeely et al., 2010; Baebprasert et al., 2011; Ortega-Ramos et al., 2014; Nyberg et al., 2015). Cyanobacterial strains and/or genomes have also been widely explored in order to unravel the complex picture of H$_2$ases (Ludwig et al., 2006; Barz et al., 2010; Kothari et al., 2012, 2013). These studies have opened new perspectives, since they have shed light on the H$_2$ production potential of strains other than those previously used as laboratory models. Since the publication of these studies, larger numbers of cyanobacterial genomes have been sequenced, which has greatly improved the genomic coverage of all the phylum (Shih et al., 2013). In order to investigate cyanobacterial H$_2$ metabolism more closely, we performed a large-scale analysis of H$_2$ases genes distribution in cyanobacteria, which consisted in searching for the genes encoding H$_2$ases and the proteins required for their maturation in 130 cyanobacterial genomes. The distribution of H$_2$ases in the cyanobacterial phylum inhabiting various environments is discussed.

RESULTS
Distribution of H$_2$ase Encoding Genes and of Genes Involved in Their Maturation Process
Our genomic search for genes encoding H$_2$ase and the proteins involved in their maturation helped to complete the picture of which strains may possibly synthesize functional H$_2$ase.
A phylum-wide analysis of the genomic distribution of H$_2$ase genes among the cyanobacterial genomes in the CyanoGEBA dataset (Shih et al., 2013) showed that only [NiFe] H$_2$ases are present in these organisms. No obvious homologs of [FeFe] or [Fe] H$_2$ases were identified. We assumed that only genomes possessing all the hox and hup genes carry a complete set of H$_2$ase-encoding genes. A complete set of
H$_2$ase-encoding genes was deciphered in 52% of the genomes studied (Figure 1A). Among the 130 genomes analyzed, 49 did not show any H$_2$ase-encoding genes (Figure 1A), and 13 genomes did not present the complete set of genes required to encode a functional H$_2$ase (Supplementary Table 1). The lack of H$_2$ase genes may be attributable to the bacterial habitat, since the proportions of H$_2$ase-free genomes differ from one ecological niche to another: the highest proportion of H$_2$ase-free strains was detected in the open ocean (89%), and the remaining 11% carried only hup genes, which suggests that the cyanobacterial contribution to H$_2$ production in the open ocean is negligible (Figure 1B). The distribution of H$_2$ase genes and of genes required for their maturation was found to vary in the cyanobacterial phylum, but all the organisms belonging to subsections IV and V have a complete set of genes encoding H$_2$ases. H$_2$ oxidation and H$^+$ reduction activities seem to be generally conserved in these species (Figure 1C). Since the uptake H$_2$ase is involved in functional nitrogenase processes, the co-occurrence of H$_2$ases, and nitrogenase in various environments was investigated by studying the distribution of [FeMo] nitrogenase structural genes (nifH and nifDK), the nifBEN, and the nifUSV genes involved in the synthesis of the [FeMo]-cofactor synthesis. The nifH, nifDK, and nifBEN genes were found in all the cyanobacteria genomes (Supplementary Table 2). The nifBEN genes were found in co-occurrence with nifUSV genes except in six genomes (Supplementary Table 2). Since the nifSU genes have been reported to be dispensable in Anabaena variabilis (Lyons and Thiel, 1995), one might conclude that their absence does not necessarily mean that the strain is not able to fix nitrogen. It is therefore concluded that all the strains listed in Supplementary Table 2, and whose genomes contain nifH, nifDK, and nifBEN genes are potentially nitrogen-fixing.

The data obtained here, indicate that nif genes are present in genomes harboring hup (10%) or hox (8%), or both (15%). Eleven percent of the genomes possess nifH and nifDK without harboring the hox and hup genes (Figure 1A). The co-occurrence of nif and hup genes seems to be significantly more frequent in the genomes of strains belonging to subsection V (Figure 1C).

To further assess the distribution of genes encoding H$_2$ases among the cyanobacterial phylum, a phylogenetic tree was constructed using 21 concatenated sequences corresponding to the 130 cyanobacterial proteins listed in Supplementary Table 3 (see Methods Section). The hox and hup genes were found to occur more frequently in the late branches of the tree, although the distribution of hox is patchier (Figures 2, 3). The presence of hup genes was always associated with that of at least one of the Hox or Hup sequences, and these genes therefore occur less frequently in the early branching clades, which is clearly illustrated in the case of clade g (Figures 2, 3). The distribution of the nitrogenase-encoding genes (nifH and nifDK) is in agreement with the phylogenetic tree previously presented (Bandyopadhyay et al., 2010). These genes are present in four genomes in the early branches of cyanobacterial evolution: clade a, [Synechococcus sp. JA-2-3B′(2–13), Synechococcus sp. JA-3-3Ab], clade b (Pseudanabaena sp. PCC 6802), clade c (Cyanothece sp. PCC 7425), and six genomes of clade d. No hup genes were ever detected in these early clades, which suggests that the nitrogenase may function naturally in the absence of uptake H$_2$ase. This was previously found to occur in Synechococcus sp. and Cyanothece PCC 7425, which fix N$_2$ under anaerobic conditions (Bandyopadhyay et al., 2011). In the genomes of the

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**FIGURE 1** | Co-occurrence of H$_2$ases and nitrogenase in cyanobacteria. The number of genomes analyzed in each graph is shown in parentheses. The various combinations of hox, hup, nif genes are presented in different colors. The symbols (+) and (−) indicate that the genes are present or absent respectively. (A) Co-occurrence of H$_2$ase and nitrogenase encoding genes in the 130 genomes analyzed in this study. (B) Distribution of H$_2$ase and nitrogenase-encoding genes depending on the habitat of the strains. (C) Distribution of H$_2$ase and nitrogenase-encoding genes depending on the morphological classification of the strains.
FIGURE 2 | Phylogenetic distribution of H$_2$ases, and nitrogenase in Cyanobacteria. The species tree used in this study is shown in the left panel. The tree was rooted using the sequences of four outgroup organisms (See Section Methods). The genomes are shown in different colors depending on the habitat of the strains. The presence or absence of selected genes is indicated by green and red squares, respectively. The blue square indicates genomes where the set of hyp, hup, or hox genes is incomplete (See Supplementary Table 1 for details). The green barred square indicates genetic polymorphism in catalytic residues. The cluster arrangement of hup, hox, and hyp genes shown in Figure 4 is summarized in the right panel of this picture.
FIGURE 3 | Phylogenetic distribution of H_2ases, and nitrogenase in Cyanobacteria. The species tree used in this study is shown in the left panel. The tree was rooted using the sequences of four outgroup organisms (See Section Methods). The genomes are shown in different colors depending on the habitat of the strains. The presence or absence of selected genes is indicated by green and red squares, respectively. The blue square indicates genomes where the set of hyp, hup, or hox genes is incomplete (See Supplementary Table 1 for details). The green barred square indicates genetic polymorphism in catalytic residues. The cluster arrangement of hup, hox, and hyp genes shown in Figure 4 is summarized in the right panel of this picture.
strains Nostocales and Stigmatales (subsections V and VI), which belong to clade h, the nif and hup genes were always found to co-occur (Figures 2, 3). It is also worth noting that the co-occurrence of hox, hup and nif genes was observed only in the late branches of the tree.

Distribution, Conservation, and Physical Organization of the hox Genes

The genes encoding the bidirectional H$_2$ases (hoxY, hoxH) and the hoxU, hoxE, and hoxF genes encoding the diaphorase part are widely distributed among the cyanobacterial phylum and are particularly abundant in the genomes of organisms belonging to subsections II, III, and IV (Figures 1B, C and Supplementary Table 4). All the hox genes listed in Supplementary Table 4 potentially encode soluble H$_2$ases belonging to subgroup 3d (Vignais et al., 2001). In the large subunit (HoxH), the sequences of the L1 and L2 motifs typical of each [NiFe] group show a high level of conservation. Only a few amino-acid substitutions were observed in the L1 motif in seven genomes of strains from various habitats (terrestrial, coastal, and freshwater strains) (Supplementary Table 5). The Cysteine residues involved in the coordination of metal ions are strictly conserved in all the HoxH and HoxY sequences. The three subunits in the diaphorase of the bidirectional H$_2$ase part (HoxE, HoxF, and HoxU) also contain the conserved cysteine residues potentially required for the coordination of [2Fe2S] and [4Fe4S] clusters. These cysteine residues are largely conserved, since the only few exceptions observed were HoxF and HoxU proteins in Synechococcus sp. CB0205, P. hollandica PCC 9006, and Cyanobium sp. PCC 7001 (Supplementary Table 1). These genomes also lack some of the genes involved in the maturation process (Supplementary Table 1). The bidirectional H$_2$ase in these strains may therefore not be active. The last step in the maturation of the bidirectional H$_2$ases involves the HoxW endopeptidase. The co-occurrence of the hoxW gene and the hox structural genes (HYUEF) was observed in all the genomes analyzed (Supplementary Table 4). Based on the difference between the patterns of expression of the structural hox genes and hoxW, it has been suggested that the endopeptidase HoxW might have multiple functions in cyanobacteria (Wünschers et al., 2003). The results of the present study confirm this assumption, since hoxW homologs were found to exist in four genomes containing no hoxYHUEF genes (Supplementary Table 4). In addition, the presence of multicopies of the hoxW gene observed in three genomes provides a further argument supporting this hypothesis (Supplementary Table 4).

Seven different patterns of organization were observed among the structural hox genes (Figure 4A, Supplementary Figure 1). In Group 1, the hoxE, hoxF, hoxU, hoxY, and hoxH genes are clustered together and show the same orientation, whereas the hoxW gene occupies another position in the genome. Group 1 includes 26 genomes belonging to all the subsections except subsection V. Group 2 includes 20 genomes belonging to subsections I, II and IV, and all the structural hox genes (EFUYHW) are clustered together in the same orientation (Figure 4A). Group 3 contains two genomes belonging to subsections I and V: the hoxE, hoxF, hoxU, hoxY are clustered together and in the same orientation, whereas the hoxW and hoxH are located in another part of the genomes. The hox genes are more widely scattered in Groups 4-6: hoxE and hoxF are clustered together and the other hox genes are either clustered or scattered in various combinations. All the hox genes hoxF, hoxU, hoxY, hoxH, and hoxW are clustered together in Fiscarella sp. PCC 9605 (Group 7), whereas hoxE is located in another part of the genome. The organization of the hox genes is generally not conserved throughout the tree of species, where the seven groups are randomly distributed among the eight clades (Figures 2, 3).

Distribution, Conservation, and Physical Organization of the hup Genes

HupL and HupS homologs encoding the large and small subunits of the uptake H$_2$ase, respectively, were identified only in genomes of diazotrophic strains belonging to subsections I, III, IV, and V (Figure 1C and Supplementary Table 6). The strains carrying uptake H$_2$ase genes are widely distributed in various habitats. They are absent only in the genomes of strains collected from salt lakes (Figure 1B). The amino acid sequences of HupS and HupL show a high degree of conservation: the L1 and L2 motifs typical of H$_2$ase belonging to group 2a (Vignais and Billoud, 2007) were found to be conserved in all the Hup sequences analyzed. These motifs include the cysteine residues involved in the coordination of the [NiFe] in the case of HupL and [FeS] in that of HupS (Vignais and Billoud, 2007). In the genomes of Calothrix sp. PCC 7103, Tolypothrix sp. PCC 9009, Rivularia sp. PCC 7116, Cyanobacterium sp. UCYN A2, and Calothrix sp. PCC 6303, since the HupS sequence shows deletions and substitutions of the residues involved in the binding of [FeS] cluster, these enzymes may be inactive or might have different enzymatic characteristics (Supplementary Figure 2). The specific peptidase HupW was identified in all the genomes carrying hupSL genes (Supplementary Table 6). The HupW sequences consistently showed well-conserved residues thought to contribute importantly to the specific interactions between the peptidase and its cognate H$_2$ase subunit (Devine et al., 2009).

In all the genomes analyzed, the hupS and hupL genes form clusters. The organization of the five groups of hup genes depends on the location of the hupW gene and the disruption (or otherwise) of hupS or hupL genes by the xisc gene (Figure 4B). The distribution of these clustering groups varies in the tree of species (Figures 2, 3). Groups 1 or 2 are mostly present throughout the late branches of the tree (clades f, e, g, and h), whereas groups 3, 4, and 5 occur only in clade h (Figure 4B).

Distribution, Conservation, and Physical Organization of the hyp Genes

Almost all the cyanobacterial genomes harboring structural H$_2$ase genes (hox, hup, or both) also harbor the hypABCDEF genes known to encode proteins involved in the maturation of the H$_2$ase (Supplementary Table 7), apart from the genomes of Chroococcidiopsis thermalis PCC 7203, Synechococcus elongatus PCC 7942, Synechococcus sp. CB0101, and Synechococcus sp. PCC 7336, from which some hyp genes are missing. (Supplementary Table 1). Whether the maturation of the H$_2$ase
in these strains involves different mechanisms, or whether the maturation process is not efficient in these cases is still an open question.

Since little is known about the process of H₂ase maturation in cyanobacteria, we analyzed the amino acid composition of the Hyp proteins in the light of the data available in the...
literature on other organisms. All the information based on the resolution of the crystal structure of the HypF protein of *Caldanaerobacter subterraneus* (Shomura and Higuchi, 2012), that of the HypECDA of *Thermococcus kodakarenensis* (Watanabe et al., 2009, 2012; Tominaga et al., 2013) and that of the HypB of *Archaeoglobus fulgidus*, *Bradyrhizobium japonicum*, and *Escherichia coli* (Olson and Maier, 2000; Chan et al., 2012; Douglas et al., 2013) are summarized in Supplementary Table 7. The result and that of the HypECDA of *E. coli* are analyzed in different environments. The maturation process of the H2ase enzyme in cyanobacteria might be similar to that described in other organisms (Hansel et al., 2001; Shomura and Higuchi, 2012; Watanabe et al., 2012; Douglas et al., 2013; Tominaga et al., 2013). The hyp genes are either clustered together in various combinations or scattered throughout the genome without any correlations being detected with the diazotrophic ability of the strains or their habitat or their classification (Figure 4C, Supplementary Figure 1). The hyp genes can be classified into 11 main classes depending on their patterns of organization. The genomes in class 1 carry all the hyp genes in a single cluster, while those in class 2 carry five clustered hyp genes and one gene located in another part of the genome, for example. Many rearrangements of the hyp clusters have occurred during the evolution of cyanobacteria, and the number of clusters increases in the late branches of the tree. In clade h, the hyp genes are all clustered together and show a similar pattern of organization (Figures 2, 3, Supplementary Figure 1).

**O2-Tolerant H2ases**

A search for homologs of O2-tolerant H2ase encoding genes in all the cyanobacterial genomes available in the NCBI database yielded positive findings in five genomes (Table 2, Supplementary Table 8). A blast analysis using the MBH H2ase HydI from *E. coli* (Group 1, accession number: 3UQY PDB) showed a match with a protein from *Lyngbya confervoides* BDU141951 (Chandrababunaidu et al., 2015). Multiple sequence alignments indicated that the six cysteine residues (C17, C19, C20, C115, C120, and C149 in *E. coli* HydI) involved in the coordination of the proximal [4Fe3S] as well as the proline residue (residue 242 in HydI), both of which are typical of this class of O2-tolerant enzymes, are conserved in the protein of *L. confervoides* BDU141951 (Figure 5).

The Hox enzyme of *Aphanocapsa montana* BDHKU210001 (Bhattacharyya et al., 2015) showed similarities with the SH H2ase of *R. eutropha* (Group 3d, accession number: AAP85843.1). The HoxH, HoxY, and HoxU proteins showed 51, 50, and 45% identity, respectively, with their respective homologs in the *R. eutropha* enzyme. Homologs of the *Pyrococcus furiosus* H2ase SH (Group 3b) were identified in *Leptolyngbya boryana* PCC 6306, *Cyanothece* sp. PCC 7425 and *Mastigocoleus testarum* BC008. The sequences encoding the four subunits α (pf0894), β (pf0894), γ (pf0892), and δ (pf0893) showed an average rate of identity of 33% with those of *P. furiosus*. In the small subunit, the four cysteine residues serving as ligands in the coordination of the [4Fe-4S] cluster in the small subunit are conserved. In conclusion, three of the four O2-tolerant enzymes described so far are present in the cyanobacterial phylum. Three of the strains potentially producing these enzymes are marine (*Aphanocapsa montana* BDHKU210001, *Lyngbya confervoides* BDU141951 and *Mastigocoleus testarum* BC008), and the other two originate from freshwater environments (*Cyanothece* sp. PCC 7425, and *Leptolyngbya boryana* PCC 6306).

The maturation process of the MBH-O2 tolerant H2ase of *Ralonasia eutropha* has been shown to involve some hox specific genes in addition to the hyp genes (Bernhard et al., 1996; Schubert et al., 2007; Ludwig et al., 2009; Fritsch et al., 2011a). The peptidase specific for this enzyme is encoded by the hoxM gene. hoxO and hoxQ genes encode for specific chaperones and hoxZ for a b-type cytochrome (Bernhard et al., 1996; Schubert et al., 2007). The maturation process of the MBH-O2 tolerant H2ase of *R. eutropha* has been shown to also involve the Hox LRTV proteins (Fritsch et al., 2011b). Homologs of the hoxZMLOQRTV genes were searched in the genome of the cyanobacterium *Lyngbya confervoides* BDU14195, and as a control in genomes of other organisms known to harbor the MBH-O2 tolerant enzyme (*E. coli* (Evans et al., 2013), *Alteromonas macleodii* (Vargas et al., 2011), *Hydrogenovibrio marinus* DSM 11271 (Shomura et al., 2011), *Rubrivivax gelatinosus* (Maness et al., 2002), and *Salmonella enterica* (Bowman et al., 2014). The result of this analysis showed that while the hoxZ, hoxM, hoxL, hoxO, and hoxQ were conserved in all non-cyanobacterial genomes analyzed, only the hoxZ, and hoxM genes were identified in...
Lyngbya confervoides BDU14195 (Supplementary Table 9). The ability of this cyanobacterium to produce an active MBH-O₂ tolerant enzyme is therefore questionable. Since the maturation process of the other O₂-tolerant H₂ases found in cyanobacteria has not been reported to require any specific proteins other than the Hyp, it is possible that Aphanocapsa montana BDHKU210001, Cyanothecae sp. PCC 7425 and Mastigocoleus testarum BC008 might produce active O₂-tolerant H₂ases. The genome of Leptolyngbya boryana PCC 6306 was found to contain only the hypAB genes, this strains can therefore regarded as unable to build an active O₂-tolerant H₂ase (Supplementary Table 1).

**DISCUSSION**

The present analyses of the distribution of genes encoding H₂ases in cyanobacterial genomes suggest that H₂ metabolism is widely distributed among the various ecological niches that have been colonized by these organisms. H₂ase genes and the genes encoding proteins necessary to the maturation process feature prominently in the late branching clades of the cyanobacterial tree of species, which suggests that the need for H₂ production and/or uptake has followed the phylogenetic evolution of this phylum. The fact that all the structural genes in these enzymes and their maturation process genes have been largely conserved in many cyanobacterial genomes indicates, if these genes are really expressed, that they might play an important physiological role in the bacterial strains inhabiting various environments. Considerable rates of H₂ production by cyanobacteria have been reported to occur in microbial mats (Marshall et al., 2012), and Microcoleus spp has been found to be a predominant H₂ producer in the microbial mats formed in the Elkhorn Slough estuary, Monterey Bay (Burow et al., 2012). These data further indicate that functional studies on H₂ases in environmental strains in addition to laboratory models would greatly improve our understanding of H₂ metabolism in this bacterial phylum. No bidirectional H₂ase genes were detected in the genomes of open ocean strains (Prochlorococcus and Synechococcus in particular), in agreement with previous results (Barz et al., 2010). The latter study also showed that heterotrophic bacteria inhabiting this environment also lacked bidirectional H₂ase encoding genes. The O₂ concentration of open ocean waters measured during a period of several months was found to be above 200 μM (Emerson et al., 2002) which may not favor the contribution of the Hox enzyme to the process of H₂ metabolism under anaerobic conditions (Khanna and Lindblad, 2015). The distribution of hup, hox and nif genes is highly variable in freshwater, hot spring and terrestrial environments (Figure 1), possibly because of the various conditions that organisms may encounter in these ecological niches.

Nineteen genomes of strains belonging to subsections I, II, III and IV contain nif genes but no hup genes (Figures 1–3 and Supplementary Table 2). In this background, one might expect the H₂ production rate of nitrogenase to play an important role in the absence of uptake H₂ase. The deletion of the hupL gene in the filamentous diazotrophic strains Nostoc PCC 7120 and Nostoc PCC 7422 has indeed been found to improve the H₂ production (Masukawa et al., 2002; Yoshino et al., 2007). In the unicellular cyanobacterium Cyanothecae PCC 7822, which fixes nitrogen under aerobicosis, HupL has been shown to be essential to activity of the nitrogenase in the presence of O₂. The authors concluded that the main function of the HupL complex in this bacterium is the protection of the nitrogenase from O₂ (Zhang et al., 2014). The present data show that most of the strains possessing nif genes and lacking the uptake H₂ase are unicellular [Aphanocapsa montana BDHKU210001, Chroococcidiopsis sp. PCC 6712, Nodosilinea nodulosa PCC 7104, Synechococcus sp. JA-2-3'B'a(2–13), Synechococcus sp. JA-3-3Ab]. All these strains are known to undergo N₂ fixation under anaerobic conditions (Supplementary Table 2). In future studies, it would be interesting to investigate whether the absence of the uptake H₂ase in these strains results in high H₂ production.

The finding that genes potentially encoding O₂-tolerant H₂ases are present in five cyanobacterial genomes is of great interest. Since Lyngbya confervoides BDU141951 genome does not contain all the accessories hox genes important for the maturation process of the MBH-O₂ tolerant enzyme, and since the genome of Leptolyngbya boryana PCC 6306 contains only the hypAB genes, it is likely that these two strains are not able to produce an active O₂-tolerant enzyme. Whether the other three cyanobacterial strains found here to possess genes encoding for O₂-tolerant enzyme actually produce these enzymes needs to be analyzed. The possible input of these enzymes to the physiology of these organisms in both marine and freshwater environments is an intriguing question. These enzymes are probably involved in the oxidation of H₂, like most of their homologs in other organisms. However, in the aerobic soil bacterium Mycobacterium smegmatis, an O₂-tolerant H₂ase has been found to produce H₂, thus enabling this organism to cope with the hypoxia occurring in its ecological niche (Berney et al., 2014). The possibility that O₂-tolerant H₂ase may play a similar role in cyanobacteria is a tempting hypothesis. Whether the cyanobacterial strains found to possess genes encoding for O₂-tolerant H₂ases could be of interest in the context of photosynthetic H₂ production is a perspective worth exploring in the future.

**METHODS**

**Datasets**

The genome set analyzed in this study includes 126 cyanobacterial genomes of the Cyanobacteria dataset (Shih et al., 2013; Calteau et al., 2014), and genomes of Aphanocapsa montana BDHKU210001, Cyanobacterium sp. UCYN-A2, Lyngbya confervoides BDU141951, Mastigocoleus testarum BC008 which are present in the JGI database (https://img.jgi.doe.gov/cgi-bin/m/main.cgi). In the case of H₂ases not generally found to occur in cyanobacteria (the [FeFe] H₂ases, and [NiFe] H₂ases other than Hox and Hup), the analysis also included cyanobacterial genomes present in the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The complete list of the genomes analyzed and their accession numbers is given in Supplementary Table 3.
Phylogenetic Analysis

The species tree was generated by concatenating 21 conserved proteins selected from the phylogenetic markers proposed for use with bacterial genome trees (Wu and Eisen, 2008). The 21 selected proteins are: DnaG, Pgk, PyrG, RplB, RplC, RplD, RplE, RplF, RplL, RpmM, RplN, RplP, RpsD, RpsE, RpsF, RpsK, RpsS, SmbP, and Tsf. The sequences of these proteins from Anabaena variabilis ATCC 29413 were used as queries in BlastP analyses. The genomes of Chloroflexus auranticus J-10, Rhodobacter sphaeroides 2.4.1, Helio bacterium modestialdum Ice1, and Chlorobium tepidum TLS were used as outgroups to root the tree as previously used (Calteau et al., 2014). Multiple sequence alignments of the proteins were performed using MUSCLE 3.8.31 (Edgar, 2004). The alignments were concatenated and the phylogenetic tree was generated with PhyML 3.3.2 (BioNJalgorithm/default parameters) (Guindon et al., 2009).

AUTHOR CONTRIBUTIONS

AL designed the study and wrote the paper, VP conducted the work, and ST participated in the analysis.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fgene.2016.00223/full#supplementary-material

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