Distribution Analysis of Hydrogenases in Surface Waters of Marine and Freshwater Environments

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Abstract

**Background:** Surface waters of aquatic environments have been shown to both evolve and consume hydrogen and the ocean is estimated to be the principal natural source. In some marine habitats, H2 evolution and uptake are clearly due to biological activity, while contributions of abiotic sources must be considered in others. Until now the only known biological process involved in H2 metabolism in marine environments is nitrogen fixation.

**Principal Findings:** We analyzed marine and freshwater environments for the presence and distribution of genes of all known hydrogenases, the enzymes involved in biological hydrogen turnover. The total genomes and the available marine metagenome datasets were searched for hydrogenase sequences. Furthermore, we isolated DNA from samples from the North Atlantic, Mediterranean Sea, North Sea, Baltic Sea, and two freshwater lakes and amplified and sequenced part of the gene encoding the bidirectional NAD(P)-linked hydrogenase. In 21% of all marine heterotrophic bacterial genomes from surface waters, one or several hydrogenase genes were found, with the membrane-bound H2 uptake hydrogenase being the most widespread. A clear bias of hydrogenases to environments with terrestrial influence was found. This is exemplified by the cyanobacterial bidirectional NAD(P)-linked hydrogenase that was found in freshwater and coastal areas but not in the open ocean.

**Significance:** This study shows that hydrogenases are surprisingly abundant in marine environments. Due to its ecological distribution the primary function of the bidirectional NAD(P)-linked hydrogenase seems to be fermentative hydrogen evolution. Moreover, our data suggests that marine surface waters could be an interesting source of oxygen-resistant uptake hydrogenases. The respective genes occur in coastal as well as open ocean habitats and we presume that they are used as additional energy scavenging devices in otherwise nutrient limited environments. The membrane-bound H2-evolving hydrogenases might be useful as marker for bacteria living inside of marine snow particles.

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Introduction

The composition of earth’s atmosphere is the result of a number of concurrent processes and a matter of continuous change. Especially the amount of trace gases governs important aspects of the gas cover of our planet, such as its retention capacity of heat or the amount of ozone present. After methane, hydrogen is the second most abundant trace gas in the atmosphere, making up around 0.5 ppm to 0.6 ppm [1,2].

Approximately 90% of hydrogen evolution is due to photochemical oxidation of hydrocarbons such as methane in the atmosphere, the combustion of fossil fuels and biomass burning. Natural evolution results from volcanic activity, the nitrogen fixation process in legumes and an uncharacterized source in the oceans. The latter comprises the majority with around 6% (6 Tg per year [3]).

The removal of hydrogen is either due to its reaction with hydroxyl radicals in the atmosphere or by its reaction with hydrogenases in the soil. In particular, hydrogen uptake into the soil is responsible for the largest term with an estimated 75% to 77% globally [1–5]. This is further corroborated by the lower average concentration of hydrogen found on the northern hemisphere, with its larger landmass [1]. Hydrogen uptake was attributed to aerobic hydrogen-oxidizing bacteria and extracellular enzymatic activity. Abiotic removal has been previously considered since hydrogen concentrations are below the threshold level found for cultures of aerobic hydrogen oxidizing bacteria that still maintains growth [6].

In contrast to soil, supersaturating concentrations of hydrogen have been measured in aquatic environments. In all cases, concentrations were highest at the surface and steeply decreased...
down to the thermocline while the deep ocean is undersaturated. Although a systematic analysis is not available it appears that surface waters of tropical and subtropical oceans are generally hydrogen sources [7–9]. In contrast, concentrations lower than the expected atmospheric equilibrium have been observed in higher latitudes and both hydrogen uptake and production vary depending on the season [10,11]. In some fresh water lakes supersaturation has also been found [12], with a maximum at dawn [13]. The highest hydrogen concentrations were in the upper water column, which correlated with the maximum of primary production [13,14].

Marine hydrogen uptake has been attributed to particulate fractions of 0.2 μm to 5 μm in size [11] and, like in freshwater lakes, most probably correlates with aerobic hydrogen-oxidizing bacteria [13]. Hydrogen production in the oceans was found to depend on solar radiation and clearly shows a diurnal variation with a maximum around noon [8,9]. Since the nitrogenase inevitably produces at least one molecule of hydrogen per dinitrogen reduced to ammonia, cyanobacterial nitrogen fixation is thought to be the major source of hydrogen in these oceanic regions. Studies on heterocystous cyanobacteria demonstrated that hydrogen cycling by these strains is highly effective, although regions. Studies on heterocystous cyanobacteria demonstrated that hydrogen cycling by these strains is highly effective, although

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In the microbial world hydrogen is a valuable energy source that is exchanged efficiently between different prokaryotes and anaerobic eukaryotes. Some produce hydrogen while fermenting whereas others capture it to drive anaerobic or aerobic respiration and make use of its energy. A wealth of different enzymes called hydrogenases have been found in microorganisms that are able to split or form hydrogen [18,19].

Hydrogenases are classified according to their metal content into the Fe-, FeFe-, and NiFe-varieties. Fe-hydrogenases are confined to the methanogenic archaea and FeFe-hydrogenases occur in bacteria and anaerobic eukaryotes. NiFe-hydrogenases are separated into 4 different groups and are widespread in archaea and bacteria [19,20]. Most purified hydrogenases are only active under anoxic conditions, but there are some NiFe-hydrogenases from aerobic H2-oxidizing bacteria that are able to oxidize hydrogen at ambient oxygen concentrations [21].

Although hydrogenases have been investigated for a long time in a variety of different microorganisms it is rather difficult to deduce their physiological function on the basis of their classification alone. In Table 1 a tentative assignment of their metabolic roles is given. However, this assignment needs to be treated cautiously since several studies found surprising variations.

### Table 1. Overview of all the known hydrogenase enzymes.

| Group       | Name                        | Tentative function                                | O2 resistance |
|-------------|-----------------------------|---------------------------------------------------|---------------|
| Fe-hydrogenase |                             |                                                   |               |
| One Group  | Hmd hydrogenase             | Occurs only in methanogens and is used for H2 uptake during methanogenesis | Its cofactor is sensitive against oxygen |
| FeFe-hydrogenases | Periplasmic and cytoplasmic enzymes | Periplasmic enzymes are probably H2-oxidizing whereas cytoplasmic enzymes are H2-evolving | No resistant enzymes known, rapid inactivation by O2 |
| NiFe-hydrogenases | Membrane-bound H2-uptake hydrogenases | H2 uptake under anaerobic and aerobic conditions | Some resistant enzymes known |
|             | Cytoplasmic hydrogenases    | H2 uptake under N2-fixing conditions              | No resistant enzymes known |
|             | H2-sensing hydrogenases     | H2 receptor that activates the expression of hydrogenase structural genes | Resistant |
|             | F420-reducing hydrogenases  | H2 uptake during methanogenesis                   | No resistant enzymes known |
|             | Bifunctional NAD(P) hydrogenases | Function unknown                               | No resistant enzymes known |
|             | Methyl-viochlorin-reducing hydrogenases | H2 uptake during methanogenesis | No resistant enzymes known |
|             | Bidirectional NAD(P)H hydrogenases | H2 uptake for the generation of NAD(P)H or H2 evolution | Some resistant enzymes known |
|             | Membrane-bound H2-evolving hydrogenases | H2 evolution under fermentative conditions in some bacteria and H2 uptake for the reduction of ferredoxin in others, both processes are either accompanied by a proton gradient formation or the use of a proton gradient for reverse electron transfer | No resistant enzymes known |

For all the different classes [19,20] a tentative function is given.

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Systematic studies concerning the distribution of hydrogenases in different habitats to unravel their ecophysiological role are not yet available. Apart from the investigation of some specific soil hydrogenases [27,28] only two studies attempted the amplification of FeFe-hydrogenase sequences from microbial mats [29,30]. Although these works showed a surprising variety of these hydrogenases the short sequences amplified preclude any assignment of their function.

The hydrogen concentrations found in a variety of surface waters prompted us to investigate the presence and distribution of all known hydrogenases in marine and freshwater environments. Moreover, the ecological distribution of their genes was analyzed to collect valuable hints for their physiological functions and their oxygen tolerance.

To this end we analyzed the distribution of hydrogenases in cyanobacteria since they are one of the largest prokaryotic groups that occur in aquatic surface waters. The search was then expanded to the complete genomes of bacteria isolated from marine surface waters (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi [31]) and the global ocean sampling metagenomic database [http://camera.calit2.net/][32–34] for all the families of hydrogenases as classified by Vignais et al. [20] and Vignais and Billoud [19]. In parallel, we investigated DNA isolated from samples taken from the North Atlantic, Mediterranean Sea, North Sea, the Baltic Sea and the fresh water lakes Westensee and Selenter See in Northern Germany for the presence of the genes of the bidirectional NAD(P)-linked hydrogenase. Our results reveal that these enzymes are surprisingly widespread in surface waters and a clear bias toward waters with terrestrial influence is obvious.

**Results**

**Distribution of hydrogenases in cyanobacterial genomes**

Cyanobacteria are known to harbor two different NiFe-hydrogenases. One is called bidirectional (group 3d) since it can produce or take up hydrogen, depending on the physiological conditions and the other is an uptake hydrogenase (group 2a) that is linked to the nitrogen fixation process and seems to be confined to diazotrophic strains [35,36]. A phylogenetic analysis revealed a close ancestry of both hydrogenases to the filamentous anoxygenic photosynthetic bacteria (the former green non-sulfur bacteria)[37].

A search of genebank (http://www.ncbi.nlm.nih.gov/) and cyanobase (http://bacteria.kazusa.or.jp/cyanobase/) for all available cyanobacterial sequences revealed the presence of the bidirectional NAD(P)-linked hydrogenase (the large subunit HoxH was used in the BLAST search [38]) in all the freshwater strains and all the strains isolated from microbial mats, salt marshes, and the intertidal zone (Table 2). In contrast, only four out of the seven available coastal genomes harbor the gene for the bidirectional enzyme and it was completely absent in oceanic strains. Genomestreamlining and iron limitation [39] in the open ocean could be used as arguments for the absence of the bidirectional hydrogenase genes in the picoplanktonic Prochlorococcus and Synechococcus strains. But even the typical open ocean strains Crocosphaera watsonii and Thrichodesmium erythraeum with genome sizes above 6 Mbp do not harbor this hydrogenase, although both have the uptake hydrogenase, which has an iron requirement similar to the bidirectional enzyme (Fig. 1 and Table 2). In addition the unicellular marine strain UCYN-A that lacks photosystem II shows an extremely reduced genome and still contains the hyp genes [40].

All the completely sequenced cyanobacterial strains that harbor the bidirectional hydrogenase genes also harbor the gene of a pyruvate:ferredoxin oxidoreductase (PFOR), nifJ. In two genomes (Synechococcus WH 5701 and Arthrospira maxima), this gene is either part of the hyp-gene cluster or in close proximity to the hox-genes, suggesting that the bidirectional hydrogenase is used to dispose of electrons during fermentation via a PFOR-like enzyme (Table 2).

The occurrence of the uptake hydrogenase (HupL, group 2a) in cyanobacteria does not correlate with a specific habitat but with the diazotrophy of the respective strains, as indicated by the presence of the nitrogenase genes (e.g. NifD)(Table 2). Of the completely sequenced genomes two Synechococcus strains isolated from a hot spring and Cyanothoe sp. PCC 7425 harbor the nitrogenase genes but no uptake hydrogenase. This confirms the previous finding of a marine nitrogen-fixing Synechococcus strain without an uptake hydrogenase [37].

*Cyanothoe* sp. PCC 7425 is the only strain containing the genes of the bifunctional NAD(P) linked hydrogenase (group 3b)(Table 2) but expression and metabolic activity of this enzyme have not yet been demonstrated.

**Distribution of hydrogenases in genomes of heterotrophic bacteria isolated from marine surface waters**

Representatives of each of the hydrogenase classes were used to search the completely sequenced prokaryotic genomes in the genebank (Table 3). Of the approximately 1210 prokaryotic genomes (as of March 2010) 149 were isolated from marine surface waters and in 33 of these genomes, one or several hydrogenases occur, making up 22% of the total (Table 4, Table S1 supporting information). Since a number of the analyzed genomes is still not complete, this proportion is a minimum estimate. If divided into coastal and open ocean isolates, 25% of the coastal and 14% of the open ocean strains have hydrogenase genes.

The genomes of two *Shewanella* strains (ANA-3 and MR-4) all have the genes necessary for the expression of a FeFe-hydrogenase. Since this type of hydrogenase is extremely sensitive against and irreversibly inactivated by oxygen [41], this is a surprising finding. However, it should be noted that one strain (ANA-3) has been isolated from a wooden pier that might have been occupied by biofilms that could become anaerobic and the other strain (MR-4) was isolated from the Black Sea, which is the world largest anoxic basin [42]. Therefore, both are considered exceptions and will not be discussed any further.

Concerning the NiFe-hydrogenases, there are 24 genomes with a membrane-bound H₂-uptake hydrogenase (group 1), two genomes with a cyanobacterial-type uptake hydrogenase (group 2a)(*Sphingopyxis alaskensis* RB2256 and *Neptunomonas caesariensis*), six genomes with a sensor hydrogenase (group 2b), seven genomes with a bifunctional hydrogenase (group 3b), four genomes with a bidirectional NAD(P)-linked hydrogenase (group 3d), and three genomes with a membrane-bound H₂-evolving hydrogenase (group 4) similar to hydrogenase 3 of *E. coli*.

The genomes of the *Roseovarius* group contain large gene clusters with the membrane-bound hydrogenase in conjunction with a sensor hydrogenase and the whole complement of the two-component system (Fig. S1, supporting information). The sensor enzyme is a receptor that enables the cells to detect hydrogen in the environment and to activate transcription of the hydrogenase structural genes [43–46]. The same gene clusters also contain a number of additional genes that encode for proteins such as HupK that have been shown to be necessary for the production of an oxygen tolerant hydrogenase in *R. eutropha* [47,48].
Table 2. Occurrence of the bidirectional NAD(P)-linked hydrogenase (HoxH) and the membrane-bound uptake hydrogenase (HupL) in cyanobacteria.

| Strain | Environment | size | HoxH* | NifJ  | HupL   | NifD   |
|--------|--------------|------|-------|-------|--------|--------|
| *Anabaena variabilis* ATCC 29413 | freshwater | 6.36 | YP_325153 | YP_323551 | YP_325087 | YP_324742 |
| *Arthrospira maxima* CS-328 | freshwater | 6.0  | ZP_03273562 | ZP_03273569 |        |        |
| *Cylindrospermopsis raciborskii* CS-505 | freshwater | ZP_06307638 | ZP_06307770 | ZP_06309351 | ZP_06309411 |        |
| *Gloeocapsa alpicola* str. CALU 743 | freshwater |      |        |        | AAO85440 |        |
| *Microcystis aeruginosa* NIES-843 | freshwater | 5.84 | YP_001656435 | YP_001658828 |        |        |
| *Microcystis aeruginosa* PCC 7806 | freshwater | 5.17 | CAO89286 | CAO88863 |        |        |
| *Nostoc* sp. PCC 7120 | freshwater | 6.41 | NP_484809 | NP_485951 | NP_484742 | NP_485484 |
| *Prochlorothrix hollandica* | freshwater |      |        |        | U88400 |        |
| *Raphidiopsis brookii* D9 | freshwater |      |        |        |        |        |
| *Synechococcus elongatus* PCC 6301 | freshwater | 2.69 | YP_172265 | YP_172431 |        |        |
| *Synechococcus elongatus* PCC 7942 | freshwater | 2.69 | YP_401572 | YP_401401 |        |        |
| *Synechocystis* sp. PCC 6803 | freshwater | 3.57 | NP_441411 | NP_442703 |        |        |
| *Anabaena* siamensis TISTR 8012 | rice field |      |        |        | AAN65267 | AAN65266 |
| *Cyanothece* sp. PCC 7424 | rice field | 5.94 | ZP_02972728 | YP_002376576 | ZP_02973433 | YP_002377414 |
| *Cyanothece* sp. PCC 7425* | rice field | 5.37 | YP_002484718 | YP_002485040 | ZP_03139427 |        |
| *Cyanothece* sp. PCC 8801 | rice field | 4.68 | ZP_02942892 | YP_002374020 | ZP_02941033 | ZP_02943179 |
| *Cyanothece* sp. PCC 8802 | rice field | 4.8  | ZP_03143669 | ZP_03141892 | ZP_03142797 | ZP_03144923 |
| *Arthrospira maxima* FACHB5M | saline marsh |      |        |        | AAQ63961 |        |
| *Arthrospira platensis* FACHB341 | saline marsh |      |        |        | AAQ63964 |        |
| *Arthrospira platensis* FACHB349 | saline marsh |      |        |        | AAQ63960 |        |
| *Arthrospira platensis* FACHB440 | saline marsh |      |        |        | AAQ63963 |        |
| *Arthrospira platensis* FACHB791 | saline marsh |      |        |        | AAQ91344 |        |
| *Arthrospira platensis* FACHBOUQDS6 | saline marsh |      |        |        | AAQ63959 |        |
| *Microcoleus chthonoplastes* PCC 7420 | saline marsh | 8.67 | YP_002619903 | YP_002620835 |        |        |
| *Lyngbya aestuarii* CCY 9616 | marine microbial mat | 7.0 | ABD34839 | ABD34838 | ABD34836* |        |
| *Lyngbya majuscula* CCAP 1446/4 | marine microbial mat | 7.0 | AY536043 | AAO66476 | AAY78884 |        |
| *Lyngbya sp.* PCC 8106 | marine microbial mat | 7.0 | ZP_01622077 | ZP_01622083 | ZP_01619041 | ZP_01620767 |
| *Gymnobium* sp. PCC 7001 | intertidal zone | 2.8 | YP_002597857 | YP_002597848 |        |        |
| *Cyanothece* sp. ATCC 51142 | intertidal zone | 4.93 | YP_001803731 | YP_001803270 | YP_001801977 |        |
| *Synechococcus* sp. PCC 7002 | intertidal zone | 3.00 | YP_001733469 | YP_001734690 |        |        |
| *Synechococcus* sp. PCC 7335 | intertidal zone | 6.0 | YP_002710310 | YP_002711016 | YP_002711054 |        |
| *Cyanothece* sp. CCY 0110 | coastal | 5.9  | ZP_01727423 | ZP_01730229 | ZP_01728928 | ZP_01727766 |
| *Nodularia spumigena* CCY 9414 | coastal | 5.3  | ZP_01629499 | ZP_01630855 | ZP_01628406 | ZP_01628430 |
| *Spirulina subsalsa* FACHB351 | coastal |      |        |        | AY345592 |        |
| *Synechococcus* sp. CC9605 | coastal | 2.51 |        |        |        |        |
| *Synechococcus* sp. CC9902 | coastal | 2.23 |        |        |        |        |
| *Synechococcus* sp. BL107 | coastal | 2.3  |        |        |        |        |
| *Synechococcus* sp. WH 5701 | coastal | 3.0  | ZP_01085930 | ZP_01085923 |        |        |
| *Crocospheara watsonii* WH 8501 | open ocean | 6.24 | YP_00518015 | YP_00519188 | ZP_00516387 |        |
| *Prochlorococcus marinus* str. AS9601 | open ocean | 1.67 |        |        |        |        |
| *Prochlorococcus marinus* str. MIT 9211 | open ocean | 1.69 |        |        |        |        |
| *Prochlorococcus marinus* str. MIT 9215 | open ocean | 1.74 |        |        |        |        |
| *Prochlorococcus marinus* str. MIT 9301 | open ocean | 1.64 |        |        |        |        |
The genomes of the *Vibrionaceae* harbor a membrane-bound H₂-evolving hydrogenase (Fig. S2, supporting information) and a second membrane-bound hydrogenase. This is the necessary combination that can be used under anaerobic conditions to establish a proton gradient by hydrogen cycling in a single cell [49].

Additionally, the genome of *N. caesariensis* (former *Oceanospirillum* [50]) is worth mentioning. It contains a membrane-bound enzyme, a cyanobacterial like uptake hydrogenase, a sensor, and a bifunctional hydrogenase. A phylogenetic analysis confirmed that the HypX encoded in its genome belongs to the group of hydrogenase maturation factors (Fig. S3, supporting information). HypX was shown to render the soluble hydrogenase of the Knallgas bacterium *Ralstonia eutropha* oxygen insensitive [51]. The membrane-bound hydrogenase of *N. caesariensis* is a close relative of the same hydrogenase of *R. eutropha* (Fig. S4, supporting information), which is evidence that this bacterium and the *Roseovarius* strains are able to perform aerobic hydrogen oxidation in marine environments.

The genomes have been searched by using the respective protein sequences.

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### Distribution of hydrogenases in metagenomic databases

Single bacterial strains allow a detailed analysis of part of the genomes that occur in the specific environment they have been isolated from. However, isolated strains provide only a glimpse on the genetic diversity that might be present in the habitat from which they originate, given that most microbial strains are unculturable [52–54]. Therefore, we searched the global ocean sampling database (GOS) [32–34] with the same representative hydrogenases as given in Table 3 and the representatives of the small hydrogenase subunits (Fig. S5 to S7, supporting information).

This database contains millions of sequence reads that have been obtained mostly from biological samples with a particle size of 0.2 to 0.8 μm. Due to this size fractionation, the major proportion of the sequences belongs to *Pelagibacter ubique* and the *Prochlorococcus*/*Synechococcus* group of cyanobacteria [33]. Since the large number of sequences in the Sargasso Sea metagenome belonging to the *Shewanellaceae* and the *Burkholderiaceae* was discussed to be a contamination [55] Station 11 was not included in the analysis.

| Strain | Environment | size | HoxH* | NifJ | HupL | NifD |
|--------|-------------|------|------|------|------|------|
| *Prochlorococcus marinus* str. MIT 9303 | open ocean | 2.68 | | | | |
| *Prochlorococcus marinus* str. MIT 9312 | open ocean | 1.70 | | | | |
| *Prochlorococcus marinus* str. MIT 9313 | open ocean | 2.41 | | | | |
| *Prochlorococcus marinus* str. MIT 9515 | open ocean | 1.70 | | | | |
| *Prochlorococcus marinus* str. NATL1A | open ocean | 1.86 | | | | |
| *Prochlorococcus marinus* str. NATL2A | open ocean | 1.84 | | | | |
| *Prochlorococcus marinus* subsp. marinus str. CCMP1375 | open ocean | 1.75 | | | | |
| *Prochlorococcus marinus* subsp. pastoris str. CCMP1986 | open ocean | 1.75 | | | | |
| *Synechococcus* sp. CC9311 | open ocean | 2.61 | | | | |
| *Synechococcus* sp. WH 7803 | open ocean | 2.37 | | | | |
| *Synechococcus* sp. WH 7805 | open ocean | 2.6 | | | | |
| *Synechococcus* sp. WH 8102 | open ocean | 2.43 | | | | |
| *Trichodesmium erythraeum* MS101 | open ocean | 7.75 | YP_722943 | YP_723618 | | |
| Cyanobacterium UCYN-A | open ocean | 1.44 | YP_003421184 | YP_003421697 | | |
| *Synechococcus* sp. RCC307 | Mediterranean Sea | 2.22 | | | | |
| *Synechococcus* sp. RS9916 | Red Sea | 2.7 | | | | |
| *Synechococcus* sp. RS9917 | Red Sea | 2.6 | | | | |
| *Synechococcus* sp. JA-2-3'B'a(2-13) | hot spring | 3.04 | YP_476681 | | | |
| *Synechococcus* sp. JA-3-3Ab | hot spring | 2.93 | YP_475237 | | | |
| *Thermosynechococcus elongatus* BP-1 | hot spring | 2.59 | | | | |
| *Acaryochloris marina* | ascidian | 6.50 | YP_001521996 | YP_001522063 | | |
| *Arthrospira platensis* str. Paraca | Salt lake | ZP_06307638 | ZP_06381891 | | | |
| *Gloebacter violaceus* sp. PCC 7421 | rock | 4.66 | | | | |
| *Nostoc azollae* | Symbiont with water fern | ZP_03765204 | ZP_03768004 | ZP_03768758 | | |
| *Nostoc punctiforme* sp. PCC 73102 | symbiont with cycad | 8.23 | YP_001867453 | ZP_00112356 | ZP_00112319 | |
| *Nostoc* sp. PCC 7422 | symbiont with cycad | ~10 | BAE46796 | BAE46791 | | |

*The genomes have been searched by using the respective protein sequences.

*Cyanobacterium sp. PCC 7425 is the only cyanobacterium with the gene of a bifunctional (NADP) hydrogenase (YP_002483374).

The 69 strains have been separated according to the habitat they have been isolated from. *Leptolyngbya valderiana* BDU 20041 has been omitted from the analysis although it is provided in the genebank (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi) since only 89 kbp of its genome has been sequenced. The presence of NifD is given as a marker for the nitrogenase. Completely sequenced strains are given in bold.

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Table 2. Cont.
We could not detect any cyanobacterial bidirectional hydrogenase in the samples taken from the open ocean. All the cyanobacterial HoxH sequences that could be found in the database are from a single sample taken at Punta Comorant, a hypersaline pond with low oxygen levels [56] on the Galapagos Islands (Fig. 2). These sequences were most similar to the available bidirectional hydrogenases of Synechococcus strains (Fig. S8, supporting information). Thus, the GOS sampling and sequencing effort should have been able to capture any HoxH sequence present in the Prochlorococcus/Synechococcus group. Although it has to be taken into account that environmental sequencing does not capture 100% of the present DNA sequences it seems highly probable that this cyanobacterial hydrogenase is absent in these strains in these environments as already deduced from the whole genomes (Table 2, Table S1, supporting information).

These findings are also corroborated when looking at the hoxH sequences of the Burkholdericeae. Although these bacteria make up a major fraction of all the oceanic metagenome sequences, there are only representatives from Punta Cormorant with this hydrogenase (Fig. 2), whereas no sequences of this group have been retrieved from the open ocean. Altogether 48 hoxH sequences could be found but apart from three coastal stations (Mangrove on Isabella Island, Cape May and Dirty Rock), which accounted for 4 sequences all of the other 44 were exclusively from Punta Cormorant. This confirms the presence of hoxH in marine, coastal environments.
| Coastal/open ocean | strain | FeFe | group 1 | group 2a | group 2b | group 3a | group 3b | group 3c | group 3d | group 4 | HypX |
|--------------------|--------|------|---------|----------|----------|----------|----------|----------|----------|---------|-------|
| Actinobacteria     |        |      |         |          |          |          |          |          |          |         |       |
| C                  | Mycobacterium marinum M | C    | YP_001850173 |         |          |          |          |          |          |         |       |
| O                  | Rhodococcus erythropolis PR4 | O    | YP_002766098 |         |          |          |          |          |          |         |       |
| Bacteroidetes/Chlorobi |      |      |         |          |          |          |          |          |          |         |       |
| C                  | Flavobacteria bacterium M524-2A | C    | ZP_03702421 |         |          |          |          |          |          |         |       |
| C                  | Prosthecochloris aestuarii DSM 271 | C    | YP_002015547 |         |          |          |          |          |          |         |       |
| O                  | Rhodinilaeana bififorma HTCC2501 | O    | ZP_01119574 |         |          |          |          |          |          |         |       |
| Mollicutes/others  |        |      |         |          |          |          |          |          |          |         |       |
| C                  | Candidatus Koribacter versatilis Ellin345 | C    | YP_593314 |         |          |          |          |          |          |         |       |
| C                  | Planctomycyes maris DSM 8797 | C    | ZP_01852867 |         |          |          |          |          |          |         |       |
| C                  | Verrucomicrobiace bacterium DG1235 | C    | YP_002715357 |         |          |          |          |          |          |         |       |
| Proteobacteria     |        |      |         |          |          |          |          |          |          |         |       |
| C                  | Magnetococcus sp. MC-1 | C    | YP_866409 | YP_866399 |          |          |          |          |          |         |       |
| α-Proteobacteria   |        |      |         |          |          |          |          |          |          |         |       |
| C                  | Labrenzia aggregata IAM 12614 | C    | ZP_01550392 | ZP_01550270 |          |          |          |          |          |         | ZP_01545563 |
| C                  | Labrenzia alexandrii DFL-11 | C    | ZP_01550392 | ZP_01550270 |          |          |          |          |          |         | ZP_01545563 |
| O                  | Roseovarius sp. HTCC2601 | O    | ZP_01443057 | ZP_01443054 |          |          |          |          |          |         |       |
| C                  | Roseovarius sp. TM1035 | C    | ZP_01881109 | ZP_01881113 |          |          |          |          |          |         |       |
| C                  | Sagittula stellata E-37 | C    | ZP_01748533 | ZP_01748530 |          |          |          |          |          |         |       |
| O                  | Sphingopyxis alaskensis RB2256 | O    | YP_611130 |     |          |          |          |          |          |         |       |
| δ-Proteobacteria   |        |      |         |          |          |          |          |          |          |         |       |
| C                  | Hahella chejuensis KCTC 2396 | C    | YP_431451 |     |          |          |          |          |          |         |       |
| O                  | Neptunibacter caesaris | O    | ZP_01166042 | ZP_01166042 |          |          |          |          |          |         |       |
| C                  | Psychromonas ingrahamii 37 | C    | YP_942646 | YP_942646 |          |          |          |          |          |         |       |
| C                  | Shewanella baltica OS155 | C    | YP_001050263 |     |          |          |          |          |          |         |       |
| C                  | Shewanella baltica OS185 | C    | YP_00364620 |     |          |          |          |          |          |         |       |
| C                  | Shewanella baltica OS195 | C    | YP_00155432 |     |          |          |          |          |          |         |       |
| C                  | Shewanella baltica OS223 | C    | YP_00235832 |     |          |          |          |          |          |         |       |
| C                  | Shewanella frigidimarina NCIMB 400 | C    | YP_750788 |     |          |          |          |          |          |         |       |
| C                  | Shewanella putrefaciens CN-32 | C    | YP_001183609 |     |          |          |          |          |          |         |       |
| C                  | Shewanella sp. ANA-3 | C    | YP_868355 | YP_869516 |          |          |          |          |          |         |       |
| C                  | Shewanella sp. MR-4 | C    | YP_735375 | YP_739552 |          |          |          |          |          |         |       |
| C                  | Shewanella sp. MR-7 | C    | YP_738201 |     |          |          |          |          |          |         |       |
| C                  | Shewanella sp. W3-18-1 | C    | YP_963312 |     |          |          |          |          |          |         |       |
shallow coastal environments and ponds in a variety of different bacterial groups.

The largest group of sequences in the metagenome database were those of the membrane-bound NiFe-hydrogenases. Again most of the 51 sequences were found at Punta Cormorant, although 11 sequences were detected in the datasets of coastal stations (New Harbor, Dirty Rock, Yucatan Channel, Nags Head, a Mangrove on Isabella Island) and two were found in the open ocean (outside Seychelles and 250 miles of Panama) (Fig. 3).

Cyanobacterial-like uptake hydrogenases could also be found in the metagenomic dataset (Fig. 4). Because of the size fractionation (0.2–0.8 μm) most of the larger diazotrophic cyanobacteria have been excluded from this analysis. Therefore, although many of the samples have been taken in regions known to be inhabited by this cyanobacterial group only two sequences could be retrieved from the whole dataset. A total of 33 sequences could be found. Most of these sequences originate from coastal sites (20) but four sequences are from the open ocean (Sargasso Sea, Reunion Island and 250 miles off Panama City).

Searches for the small hydrogenase subunit genes retrieved 23 sequences of the bidirectional NAD(P)+-linked hydrogenases, 37 of the membrane bound H2 uptake hydrogenases and 18 of the cyanobacterial-like uptake hydrogenases. In all these cases the numbers are close to the expected number when comparing the gene sizes of the respective large and small hydrogenase genes (Fig. S5 to S7, supporting information).

Sequences of the oxygen sensitive FeFe-hydrogenases retrieved from the GOS database were from a Mangrove (Isabella Island) and the hypersaline pond at Punta Cormorant. In all other samples no FeFe-hydrogenase was found (Fig. 5) and none of the archaeabacterial hydrogenases were found in the metagenome sequences.

Recently large amounts of metatranscriptomics data became available (e.g. [57]). A search of the respective dataset revealed the presence of three transcripts of membrane-bound H2-uptake hydrogenases. One transcript was most similar to a cyanobacterial uptake hydrogenase, one to the Flavobacteriaceae and one to the Bradyrhizobiaceae. In this dataset only samples from the open ocean are available.

Detection of sequences of the bidirectional NAD(P)-linked NiFe-hydrogenase in the North Atlantic, Mediterranean Sea, North Sea, Baltic Sea, and two freshwater lakes

Although all NiFe-hydrogenases share two characteristic motifs with altogether four cysteins at the N- and C-terminus for the binding of the NiFe active site, it is impossible to design degenerated primers that bind to the genes of all different classes of these enzymes. Therefore, we limited our effort to a single class and constructed degenerated primers specific for the bidirectional NAD(P)-linked hydrogenases of cyanobacteria, the Chloroflexaceae and some proteobacteria. In cyanobacteria this enzyme is known as the bidirectional hydrogenase. It is closely related to the soluble hydrogenase of Ralstonia eutropha [58,59].

We collected surface water from Stollergrundrinne outside the Kielfjord (Baltic Sea), in the Norderpiep west of Büsum (North Sea) and two freshwater lakes in northern Germany, Westensee and Selenter See. These samples were sequentially filtered on 10 μm and 0.2 μm filters and DNA isolated from the retained material. In samples from all these locations we could detect hoxH. In Fig. 6 the distribution of sequences on the different bacterial groups is shown for the different stations.

From the Baltic Sea as well as the fresh water lakes we could amplify a large number of cyanobacterial hoxH that are most similar to the Chroococcales (most closely related to Cyanothrix, Microcystis and
Synechocystis) or the filamentous, heterocystous Nostocaceae. In the North Sea the α-proteobacterial group Rhodobacteraceae made up the same proportion as all the cyanobacterial sequences taken together. From the freshwater mesotrophic lakes Westensee and Selenter See we could only amplify cyanobacterial hoxH (Chlorococcales, Nostocaceae and Oscillatoriales) and in each case some sequences of methylotrophic bacteria and Dictyoglomaceae.

In contrast to this, all attempts to amplify sequences of the bidirectional NAD(P)H hydrogenases from the samples taken in the North Atlantic off the west African coast and the Ionian Sea (Mediterranean Sea) were negative. This corroborates that the open ocean and marine oligotrophic waters are devoid of this hydrogenase type.

**Discussion**

Any conclusion concerning the activity of a gene from its environmental distribution is hampered by the fact that it is not necessarily expressed in a specific environment. Genomes might have genes in store that are not necessary to survive under the present-day conditions, but can be used to invade other niches or to prepare the organism for a drastic change. In the case of the

**Figure 2. Distribution of bidirectional NAD(P) linked hydrogenases found in the GOS database of the different prokaryotic groups.**

The hoxH sequence of Synechocystis sp. PCC 6803 (Table 3) was used for the search and a total of 48 sequences has been found. On the right the number of sequences from the different sampling stations is shown.

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**Figure 3. Distribution of membrane-bound hydrogenases found in the GOS database of the different prokaryotic groups.** The hupL sequence of Desulfovibrio vulgaris (Table 3) was used for the search and a total of 51 sequences has been retrieved. On the right the number of sequences from the different sampling stations is shown.

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distribution of hydrogenases found in this work, this scenario seems highly unlikely. For several reasons described in detail below, we think that biological hydrogen production and consumption, as depicted in Fig. 6, might be common in a large number of marine and freshwater habitats.

All strains from the open ocean were free of the bidirectional NAD(P) linked hydrogenase. Neither the cyanobacterial genomes nor all of the heterotrophic bacteria (Table 2 and Table S1, supporting information) or the metagenomic sequences harbor this hydrogenase. In addition, our efforts to amplify these hydrogenase genes from the North Atlantic or the Mediterranean Sea were unsuccessful. Since the diazotrophic cyanobacterial strains and the heterotrophic bacteria from the open ocean have other types of hydrogenases, there is no selection pressure against these enzymes per se. However, there is a clear bias of the bidirectional type to environments such as coastal marine waters, ponds, freshwater lakes and microbial mats (Table 2, Fig. 2, Fig. 6 and Table S1, supporting information), where cyanobacteria and heterotrophic bacteria might encounter micro-oxic or anaerobic conditions. In cyanobacteria this type of enzyme was shown to be activated under anaerobiosis and to be responsible for fermentative hydrogen production [60]. This is corroborated by the distribution of the PFOR gene, nifH, in the same cyanobacteria (Table 2).

Starting from anaerobiosis, the bidirectional hydrogenase is known to be used as an electron valve, when cells switch from fermentation to photosynthesis [61–64]. These findings might explain the high hydrogen concentration found in the morning hours in a eutrophic lake that coincided with the phytoplankton maximum [13]. Oxygen depletion due to high respiratory activity during the night could have activated the hydrogenase in this zone and elicited a fermentative hydrogen production in the dark that continued at dawn until the next morning when photosynthesis resumed, thus causing supersaturating H2 concentrations. A
similar diel variation of hydrogen concentrations has also been described for cyanobacterial mats (see e.g. [65]).

In both cases, hydrogen production is certainly not confined to the resident cyanobacteria but can also result from the activity of algae and other heterotrophic bacteria living in the same community.

The large number of genomes of marine bacteria from surface seawaters containing the membrane-bound H₂-uptake hydrogenase is remarkable. A search of the current marine metatranscriptomics data [57] revealed the expression of these hydrogenases in cyanobacteria as well as other bacteria in the open ocean.

The membrane-bound hydrogenase gene clusters found in the Rhodobacteraceae (Fig. S1 supporting information) include all the accessory genes that are known from the membrane-bound hydrogenase of R. eutropha. One of the four hydrogenases of N. caerariensis and the hydrogenases of the Roseovarius strains are closely related to this hydrogenase as revealed by phylogenetic analysis (Fig. S1 and S4, supporting information). This type of enzyme is known to be oxygen insensitive and was shown to be active at ambient oxygen concentrations [66,67]. Electrochemical investigations of this hydrogenase found measurable hydrogen uptake down to levels of 1 to 10 nM [67], which is well in the range of H₂ concentrations in surface waters. One of these strains (Roseovarius sp. HTCC 2601) was isolated from the Sargasso Sea, but all of the others were from coastal areas. In these regions, this α-proteobacterial subclass makes up as much as 24% of the bacterioplankton [68] and therefore, their hydrogenases might be widespread in these environments.

Mycobacteria, known to colonize aquatic ecosystems, take up hydrogen in the same concentration range under aerobic conditions [69], supporting the notion that hydrogen consumption in these environments is a common microbial feature. Even though the supersaturating concentrations found in surface waters are below the threshold necessary to support growth exclusively on H₂, hydrogen uptake could add to the ability to survive in a variety of these habitats. Similar suggestions have already been made for hydrogen uptake for long-term survival of bacteria [70] and for the ability to oxidize carbon monoxide in the coastal ocean [71,72]. These suggestions coincide with the aerobic hydrogen uptake demonstrated for particle sizes between 0.2 and 5 μm in coastal waters [11]. This trait is especially important for litho- and heterotrophic bacteria that have to capitalize on as much of the

Figure 6. Distribution of bidirectional NAD(P) linked hydrogenases in samples taken from Norderpiep (North Sea), Stollergrundrinne (Baltic Sea) and the freshwater lakes Westensee and Selenter See.
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available energy supply as possible, but can be disregarded by photoautotrophs like cyanobacteria.

Bacterial activity was found to be capable of depleting oxygen in marine organic aggregates. In particles as small as 1.5 mm, anoxic conditions emerged. In the same aggregates no methanogenic or sulfate-reducing bacteria could be detected [73]. Our results suggest that these anaerobic microniches might be specifically occupied by bacteria of the Flabonulaceae (Fig. S2, supporting information). Since their membrane-bound H2-evolving NiFe-hydrogenases are encoded in conjunction with subunits of the formate dehydrogenase it seems highly likely that it performs the formate–hydrogen lyase reaction. This reaction is well known from E. coli, where it detoxifies formate produced during fermentation, evolves hydrogen and might be involved in an additional energy-generating step [24]. The membrane-bound hydrogen uptake hydrogenase encoded in the same genomes would allow hydrogen cycling and might be used for additional net transport of protons across the cell membrane [49].

The Alteromonadaceae are widespread in marine waters. Two different ecotypes have been sequenced, one is predominant in surface waters whereas the other is known from the deep Mediterranean Sea. The deep ecotype was originally found to harbor the genes of the membrane-bound H2 uptake hydrogenase but our analysis and that of others [74] also found the same sequences at the surface of the Sargasso Sea. It was speculated that these two strains are separated by either being associated with small aggregates (surface type) or large aggregates (deep ecotype) [75]. This might be further support for the use of hydrogenases in transiently anoxic microniches in the ocean.

The diel variation of the H2 concentration in marine surface waters [8, 9] that parallels solar radiation is still awaiting conclusive explanation. Nitrogen fixation is a major source of hydrogen in terrestrial ecosystems [4]. In situ measurements of the diazotrophic cyanobacterium T. thiebautii suggest that it is a negligible source of hydrogen in the Sargasso Sea [15]. Therefore, nitrogen fixation by filamentous cyanobacteria is an insignificant source of H2 in aquatic ecosystems. Interestingly, a unicellular marine diazotrophic cyanobacterium has been shown to be devoid of the uptake hydrogenase [37] and to produce hydrogen while fixing nitrogen [76]. In general unicellular cyanobacteria perform a temporal separation of the oxygen sensitive energy consuming nitrogen fixation process and oxygenic energy generating photosynthesis between night and day, but some strains also fix nitrogen during the light phase [76, 77]. Unicellular strains are known to provide a considerable part of fixed nitrogen in marine waters [78, 79] and might therefore be responsible for part of the evolved H2. The newly discovered unicellular cyanobacteria without photosystem II [40, 80] harbor the genes of the cyanobacterial uptake hydrogenase (Table 2), which is most similar to those of the Cyanobacteria group (Fig. S4, supporting information) as expected. Therefore, these strains should be able to recycle the H2 evolved by the nitrogenase.

The distribution of cyanobacterial nitrogen fixers in the ocean and their seasonal abundance are poorly characterized although qPCR data has shown that all groups are widely distributed [81, 82]. One investigation suggests that their distribution is patchy and their rate of nitrogen fixation highly variable [79] and might therefore result in hydrogen evolution in some parts and very low or no evolution in other parts.

Although unicellular nitrogen-fixing cyanobacteria might be responsible for hydrogen evolution in some regions, part of the H2 produced during the day might be of photochemical origin, such as dissociation of organic matter by UV light [17].

Coastal waters are rich in hydrogenase sequences, as suggested by our analysis of complete genomes (Table 2, 4, Table S1, supporting information), and the number of sequences we could amplify of a single class of NiFe-hydrogenases from the North Sea and the Baltic Sea (Fig. 5). The apparent scarcity of sequences from coastal samples in the GOS database can be explained by the filtration procedure. Since mainly particle sizes between 0.2 and 0.8 µm have been used for DNA isolation many of the coastal bacteria and particle associated bacteria have been excluded from the analysis. We hypothesize that the membrane-bound H2 evolving hydrogenase in the genomes of the Flabonulaceae might be used as indicator for bacteria that colonize the inner parts of organic aggregates and thus, have not been sequenced yet in the GOS database.

Our analysis shows that the genetic repertoire of bacteria from surface waters of different environments enables them to produce hydrogen either by their nitrogenase, by hydrogenases linked to fermentative pathways (such as the bidirectional NAD(P) linked hydrogenase), or the membrane-bound H2-evolving hydrogenase. A number of bacteria could oxidize hydrogen as an energy source probably down to the lower nM range and might be responsible for biological hydrogen consumption in freshwater and marine systems.

This study intends to deliver a first key to the elucidation of the underlying biological processes of hydrogen turnover in aquatic ecosystems. Whether a specific body of water is a hydrogen sink or source will depend on a number of factors such as primary production, nitrogen fixation, the concentration of photodegradable organic compounds and organic particles, and the availability of electron acceptors. This is the first evidence that microorganisms can be an integral part of hydrogen turnover in marine waters, but much more remains to be learned. This is especially true when considering oxygen minimum zones [83] that have not been investigated for the presence of hydrogen or hydrogenases until now.

Materials and Methods

Sample collection

Samples were collected from the surface. In the North Sea water was sampled in the Norderpier (54°13′N/8°27′E), in the Baltic Sea it was collected in the Stollergrundrine (54°29′N/10°13′E) and from the freshwater lakes Selenter See (54°18′25″N/10°28′53″E) and Westensee (54°17′53″N/9°57′09″E) at least four times a year from every season. These samples were sequentially filtered on 10 µm and 0.2 µm filters with a peristaltic pump 620 S (Watson-Marlow Bredel).

Samples from the Mediterranean Sea were taken from the Ionian Sea at station 2 (36°41′N/21°39′E), station 3 (36°50′N/21°31′E), station 5.2 (36°37′N/21°17′E) and station 6 (36°42′N/21°04′E). In this case 5 l water from a depth of 5 m was filtered on 5 µm and then on 0.2 µm.

The samples from the North Atlantic were taken during the Poseidon 284 cruise at 18°N/30°W, 25°N/30°W and 29°30′W in April 2002.

For DNA isolation the UltraClean™ Soil DNA Kit (Mo Bio, Carlsbad CA, USA) was used.

DNA amplification and sequence analysis

Sequences of the bidirectional NAD(P)-linked hydrogenase were amplified with the primers HoxH-f GTATYTGYGGYATT-TGTCCTGT and HoxH-r GGCATTTGTCCTRCTGTGATG-TGT were used. Prior to 40 cycles of the program the DNA was denatured for 5 min at 95°C. The temperature program was as
follows: 30 sec at 95°C, 40 sec at 50°C, 2 min at 72°C. In a final step the temperature was kept at 72°C for 10 min. The reaction contained 0.5 μM of the two primers, 0.2 mM of dNTPs, 2.5 mM MgCl₂, 0.025 U/μl Taq polymerase (MBI Fermentas, St. Leon-Roth, Germany) and 10x buffer as recommended by the manufacturer in a total volume of 50 μl. Of each sample different amounts of DNA between 2 and 100 ng were tested as template. If no PCR product was detected DNA concentrations were increased at least 10 times. Positive controls were run in parallel to prove the efficiency of the PCR. The approximate size of the product is around 1190 bp and covers close to 84% of the hoxH gene.

The resulting PCR products were ligated into the pCRII-topo (Invitrogen), sequenced with the Big-Dye Kit, and applied on a 96 capillary sequencer (3730 DNA Analyzer, Applied Biosystems).

If possible contigs were assembled from the obtained sequence data and the respective sequences deposited in the genebank (Accession numbers GQ454441 to GQ454443 and GU238237 to GU238258) including two additional cyanobacterial hoxI sequences of Aphanothece halophilica and Mastigocladus laminosus SAG 4.84 (Accession numbers GQ454444 and GQ454445).

Database searches

The genebank, cyanobase, and the GOS database were searched for hydrogenase specific sequences by using the hydrogenase sequences given in Table 3. Retrieved sequences were either run against the genebank by using the BLAST algorithm [38] to deduce the closest homolog or searched for the signature sequences as given by Vignais and Bilou [19] to unambiguously classify the respective hydrogenase. In case of the GOS database, the sequences found were aligned, and, if possible, larger contigs were formed from the same sampling station and used for all further analysis.

Phylogenetic analysis

In the case of critical candidates or unclear phylogenetic affiliation phylogenetic trees were used. Sequence alignments were made with ClustalW [34]. After manual optimization and removal of gaps from the alignments, parsimony, maximum likelihood, and distances were calculated with the 3.63 release of the PHYLIP package [85], using the Jones-Taylor-Thornton matrix and the algorithm of Fitch and Margoliash [86]. Maximum parsimony and distances were calculated for 1000 bootstraps and maximum likelihood for 100 bootstraps. The Unix-cluster at the computer center of the University of Kiel was used for most of the calculations. The resulting trees are given in Fig. S3 to S5 (supporting information).

Supporting Information

Table S1 Complete list of all marine bacteria searched for hydrogenase genes

Found at: doi:10.1371/journal.pone.0013846.s001 (0.05 MB XLS)

Figure S1 Structure of the gene cluster of the membrane bound hydrogen uptake NiFe-hydrogenase of marine Rhodobacteraeac and the delta-proto bacterium Neptunibacter caesaris. The structural genes of the hydrogenase (hupS, hupL, and hupZ the membrane bound cytochrome) are shown in blue. Red genes (hoxAJBC) are involved in the regulation of the hydrogenase. HoxJ encodes a histidine kinase that is known to interact with a hydrogen sensor encoded by hoxB and hoxC and regulates the activity of the response regulator encoded by hoxA. HupK might encode a protein necessary to express an oxygen-tolerant hydrogenase. Accessory genes known to be necessary for this type of membrane hydrogenase are shown in grey, whereas grey patterned genes are general accessory genes for all NiFe-hydrogenases. Genes depicted in green are putative proteases that cleave the C-terminus of the hydrogenase. HypX of Ralstonia eutropha is known to render its soluble hydrogenase oxygen tolerant.

Found at: doi:10.1371/journal.pone.0013846.s002 (0.06 MB DOC)

Figure S2 Structure of three hydrogenase gene clusters of Vibrionaceae isolated from marine environments that are most similar to the energy converting H₂-evolving NiFe-hydrogenases. The color code is the same as in Figure S1. Genes shown in plaid are part of the formate dehydrogenase. FluA is the transcriptional activator of the formate-hydrogen lyase. Those in black and grey-blue are additional subunits of the whole complex.

Found at: doi:10.1371/journal.pone.0013846.s003 (0.05 MB DOC)

Figure S3 Phylogenetic tree of HypX. Representatives of enoyl-CoA hydratase/crotonase have been used as outgroup. The abbreviations and the respective accession numbers are as follows: Aaeoli, Aquifex aeolicus VF5 NP_213788; Achrl, Alkalilimnicola chrlchei MLHE-1 YP_742845; Amarin, Acaryochloris marina MBIC11017 YP_001529046; BjaUSDA, Bradyrhizobium japonicum USDA 110 NP_773566; Cviola, Chromobacterium violaceum ATCC 12472 NP_903812; Daroma, Dechloromonas aromatica RCB YP_287160; Frankia Cc Frankia sp. Cc13 YP_482743; Frankia EA Frankia sp. EAN1pcc YP_001505433; MmmagAMB, Magnetospirillum magnetocitricum AMB-1 YP_420998; MmmagMS-1, Magnetospirillum magnetotacticum MS-1 ZP_00055441; Mmarr, Marinoplastis marina ATCC 23134 ZP_01691937; Mpetro, Methylobium petroleophillum PM1 YP_001021998; Ncaesar, Neptuniibacter caesaris ZP_01166042; Naritri, Nitratiruptor sp. SB155-2 YP_001358952; Pedobac Pedobacter sp. BAL39 ZP_01833535; Phiapht, Polaromanas naphthalenivorans CJ2 YP_982187, PflufP-5, Pseudomonas fluorescens Pf-3 YP_260772; Pfluoere, Pseudomonas fluorescens PFO-1 YP_348556; Reutro, Ralstonia eutropha H16 NP_942660; Rferri, Rhodoferax ferriferdencs T118 YP_525349; Rmetalli, Ralstonia metallidurans CH34 YP_583693; Savermi, Streptomyces avermitilis MA-4680 NP_828541; Savermi, Streptomyces avermitilis MA-4680 NP_828541; Savermi, Streptomyces avermitilis MA-4680 NP_829362; Scoel, Scoeli, Stenotrophomonas maltophilia R551-3 YP_002219307; Sdegra, Saccharophagus degradans 2-40 YP_85-10 YP_363011.

Found at: doi:10.1371/journal.pone.0013846.s004 (0.50 MB DOC)

Figure S4 Phylogenetic tree of HupL sequences. Representatives of the 49 kDa subunit of the complex I have been used as outgroup. The abbreviated and their respective accession numbers are as follows: Abac345 Candidatus Korbacter versatilis Elinn345 YP_593314; Abut4018 Arcobacter butzleri RM4018 YP_001490338; Afer3393 Acidithiobacillus ferrooxidans ATCC 53993 YP_00219307; Ahyd7966 Aeromonas hydrophila subsp. hydrophila ATCC 7966 YP_857036; AmacDE Alteromonas macroeloides ‘Deep ecology’ YP_00214659; Aplf4074 Actinobacillus pleurophomense serovar 1 str. 4074 ZP_00134404; AsalaA494 Alcaligenes salmonicida subsp. salmonicida A494 YP_00114167; Asiam Anaabaena siensis TISTR 8012 AAN65266; Avari Anaabaena variabilis ATCC 29413 YP_325087; Bae Elin bacterium Elin514 ZP_03626632; BTAI1-2 Bradyrhizobium sp. BTAI YP_001220511; BTAI-3 Bradyrhizobium sp. BTAI YP_
Distribution of small subunits of the cyanobacterial hydrogenases in Surface Waters

Figure S5

Distribution of small subunits of the bi-directional NAD(P)+ linked hydrogenase found in the GOS database of the different prokaryotic groups. The small subunit gene, hoxY, of Synechocystis has been used for the search. All genes have been retrieved from the GOS database of the different prokaryotic groups. The hupS sequence of Desulfovibrio vulgaris was used for the search. On the right the number of different prokaryotic groups. The hupS sequence of Desulfovibrio vulgaris was used for the search.

Figure S6

Distribution of small subunits of the membrane bound H2 uptake hydrogenases found in the GOS database of the different prokaryotic groups. The hupS sequence of Desulfovibrio vulgaris was used for the search. On the right the number of different prokaryotic groups. The hupS sequence of Desulfovibrio vulgaris was used for the search.
Found at: doi:10.1371/journal.pone.0013846.s008 (0.05 MB DOC)

**Figure S8** Phylogenetic tree of HoxH sequences. Representatives of the 49 kDa subunit of the complex I have been used as outgroup. The used abbreviations and their respective accession numbers are as follows: Afla Actinobacterium flavidum CAAC4646; Ahalo Aphanothec halophylica GQ544444; Amar Acaryochloris marina MBIC11107 VP_001521996; Amax Archaeorhithrix maxima FACHBMS AAQ3961; Apl1 Arthroporpa platensis FACHB341 AAQ53964; Apl2 Arthroporpa platensis FACHB2036 AAQ39595; Apl3 Arthroporpa platensis FACHB439 AAQ53960; Apl4 Arthroporpa platensis FACHB911 AAQ14344; Avar Anaebana variabilis ATCC 29413 VP_3251535; Bxn Burkholderia xenovorans LB400 YP_555781; Cagg Chloroflexus aggregans DSM 9485 VP_002463784; CaggL Chlororibium chlororomutatii CaD3 YP_378564; Caur Caurorolaxus aurantia J-10-fl VP_001634807; Ccay10 Cynoothec sp. Ccay 0110 ZP_01724237; Clm1 Chlororibium limicola DSM 245 VP_001944410; Cncc Ranstaon europa H16 NF_942730; CplhL Chlororibium phaeobacteroides DSM 266 VP_912598; CtepL Chlororibium tepidum TLS NP_662771; Daro Dechloromacet ba Ruser from FTZ (Bu¨sum) and Christoph Keller and Conndy Schmidt and Fischerei Reese while taking samples on Seleran Se. Special thanks to Jonathan Zehr and Jim Tripp for providing sequence reads of the unicellular Nd-ﬁxing cyanobacteria in "group A".

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**Author Contributions**

Conceived and designed the experiments: RS JL RS JA. Performed the experiments: MB CB TS FG FO CM. Analyzed the data: MB CB TS FG CM CS KG JA. Contributed reagents/materials/analysis tools: KHV RS JL RS. Wrote the paper: JA.

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