Unification of [FeFe]-hydrogenases into Three Structural and Functional Groups

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ABSTRACT

Background. [FeFe]-hydrogenases (Hyd) are structurally diverse enzymes that catalyze the reversible oxidation of hydrogen (H_2). Recent biochemical data demonstrate new functional roles for these enzymes, including those that function in electron bifurcation where an exergonic reaction is coupled with an endergonic reaction to drive the reversible oxidation/production of H_2 .

Methods. To identify the structural determinants that underpin differences in enzyme functionality, a total of 714 homologous sequences of the catalytic subunit, HydA, were compiled. Bioinformatics approaches informed by biochemical data were then used to characterize differences in inferred quaternary structure, HydA active site protein environment, accessory iron-sulfur clusters in HydA, and regulatory proteins encoded in HydA gene neighborhoods.

Results. HydA homologs were clustered into one of three classification groups, Group 1 (G1), Group 2 (G2), and Group 3 (G3). G1 enzymes were predicted to be monomeric while those in G2 and G3 were predicted to be multimeric and include HydB, HydC (G2/G3) and HydD (G3) subunits. Variation in the HydA active site and accessory iron-sulfur clusters did not vary by group type. Group-specific regulatory genes were identified in the gene neighborhoods of both G2 and G3 Hyd. Analyses of purified G2 and G3 enzymes by mass spectrometry strongly suggests that they are post-translationally modified by phosphorylation.

Conclusions. These results suggest that bifurcation capability is dictated primarily by the presence of both HydB and HydC in Hyd complexes, rather than by variation in HydA.

General Significance. This classification scheme provides a framework for future biochemical and mutagenesis studies to elucidate the functional role of Hyd enzymes.

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1. INTRODUCTION

Hydrogenases catalyze the reversible reduction of protons to hydrogen (H₂) gas using a variety of electron donors and acceptors. The three primary variants of hydrogenase, termed [NiFe]-, [FeFe]-, and [Fe]-hydrogenases, are evolutionarily distinct and differ in the composition of their active site metal clusters [1-10]. Of the three variants, [FeFe]-hydrogenases (Hyd) are of substantial interest for biotechnology and bioenergy purposes due to their high rate of proton reduction [11-13]. Hyds are widely distributed in Bacteria and also have been detected in a number of Eukarya but have yet to be identified in Archaea [14-17]. Hyds vary in their cellular localization which corresponds with differing functionalities. For instance, Hyds can be localized in the periplasm or the cytoplasm and can be either membrane-bound or soluble [18-24]. Periplasmic Hyds are primarily involved in H₂ uptake while membrane-bound or soluble cytoplasmic Hyds are involved in reversible oxidation of H₂ [18-22, 24].

At a minimum Hyds comprise a catalytic subunit, HydA, which contains a conserved ~350 residue H-cluster domain that harbors the ligands for the novel active site iron-sulfur cluster. The active site iron-sulfur cluster consists of a [4Fe-4S] cluster bridged to a 2Fe subcluster via a cysteine thiol [1, 11, 17, 25]. In addition to the H-cluster domain, HydA often contains N-terminal (F-cluster) and C-terminal (C-cluster) domains that add flexibility to the enzyme and that modulate electron flow to and/or from the active site H-cluster [11, 14, 16, 17, 25, 26]. The simplest form of HydA includes those identified in green algae, such as *Chlamydomonas reinhardtii*, which generally only contains the H-cluster domain [27-32]. More complex forms of HydA are more common and possess several additional iron-sulfur clusters, including those with both the F- and C-clusters [2, 11, 33]. In addition to F- and C-cluster mediated functional flexibility, HydA can exist in multiple isoforms with different quaternary structures, including those that are monomeric and those that are multimeric [14, 16, 17, 26, 34]. Multiple HydAs of variable quaternary structure can be present in a single genome [22, 35-37] where they form complexes with paralogs of the large and small subunits of NADH dehydrogenase (referred to as HydB and HydC, respectively) [21, 35-39].

Recent biochemical studies indicate that several soluble, cytoplasmic and, multimeric Hyds are capable of electron bifurcation, a novel mechanism of energy conservation that involves the simultaneous reduction or oxidation of two electron acceptors or donors in an enzyme complex, whereby a thermodynamically favorable exergonic reaction drives a thermodynamically unfavorable endergonic reaction [21, 35, 37-42]. Soluble cytoplasmic Hyds identified from *Acetobacterium woodii* [21], *Clostridium autoethanogenum* [40], *Moorella thermoacetica* [36] and *Thermotoga maritima* [35] are all capable of electron bifurcation [38,

39]. The trimeric Hyd from *T. maritima* (i.e. HydABC complex) couples the simultaneous oxidation of ferredoxin (Fd) and NADH to the production of H₂ [35]. In contrast, the tetrameric Hyd from *A. woodii* (i.e. HydABCD complex) couples the oxidation of H₂ to the simultaneous reduction of Fd and NAD⁺ [21]. Interestingly, a trimeric Hyd from the acetogen *M. thermoacetica* functions *in vivo* to either oxidize or produce H₂, contingent on cultivation conditions, by coupling Fd and NAD⁺/NADH reduction/oxidation [43]. Autotrophically-grown *M. thermoacetica* cells oxidize H₂ by simultaneously reducing both Fd and NAD⁺ while glucose-grown cells used the same enzyme complex to reduce protons to produce H₂ by coupling with the oxidation of Fd and NADH. Conversely, the Hyd from *C. pasteurianum* (cytoplasmic and soluble) has been shown to not bifurcate electrons, which is consistent with its lack of a full length HydB [2, 11, 36]. Likewise, the ‘dimeric’ Hyd from *Desulfovibrio desulfuricans*, which has a truncated HydB that functions in part to target the enzyme to the periplasm, is unlikely to bifurcate [23, 44].

The genomes of many organisms encode for monomeric and multimeric Hyds, for example, *A. woodii* [21], *Ruminococcus albus* [37], and *T. maritima* [35], among others [14, 16]. The presence of multiple copies of HydA including those that bifurcate and those that do not suggests a need to regulate one or more of the enzymes in order to maximize energy conservation. Indeed, a recent study identified several genes that encode for proteins putatively involved in sensing H₂ and in the post-translational modification (PTM) in the gene neighborhood of seven different HydAs (including both monomeric and trimeric proteins) [37]. One of those proteins, HydS, was proposed as a H₂ sensor based on the presence of a Per-ARNT-Sim (PAS) domain, which are widely used to promote protein-protein interactions or signal

transduction [37, 45]. Moreover, proteins hypothesized to be involved in signal transduction (e.g. serine-threonine kinases/phosphatases and histidine kinases) have been identified in the flanking gene environment of *hydA* in *C. thermocellum* and *T. maritima* [37]. Whether these enzymes are involved in PTM of Hyd proteins and the extent to which they are distributed in the gene neighborhoods of *hydA* among other microorganisms is not yet known.

In order to develop a robust classification scheme for use in predicting bifurcating and non-bifurcating HydA, we compiled all the available HydAs in completed genomes. Our goal was to build upon and at the same time reconcile past classification schemes [14, 16]. A comprehensive characterization of the proteins encoded in the neighboring genomic regions of *hydA* was also conducted using bioinformatics approaches. This information was then used to develop classifications of Hyd that unify patterns in enzyme diversity at the level of primary and predicted quaternary structure. These groups were then analyzed in the context of biochemically-characterized bifurcating and non-bifurcating enzymes to demarcate their diverse functionalities. Finally, we used this classification scheme to identify proteins that might be involved in the PTM of Hyds and to characterize their distribution in the Hyd classification groupings. The regulatory proteins identified include a histidine kinase-like (Hkl) protein (sometimes annotated as HydE [21]), serine/threonine kinase (Stk), and serine phosphatase (Sp) that may be involved in PTM of putatively multimeric bifurcating Hyds. Herein we report a new classification scheme that assigns HydA into one of these three groups, based on the composition of proteins encoded in adjacent gene neighborhoods and structural features associated with these groups. Finally, we provide evidence from biochemical and mass spectrometry assays for PTM in multimeric Hyd complexes.

2. MATERIALS AND METHODS

2.1 Identification and compilation of HydA homologs

The catalytic subunit of [FeFe]-hydrogenase (HydA) from *C. pasteurianum* (WP_004455619.1) was used in a BLASTp query to identify homologs present in publically available genomes in the Department of Energy (DOE) Integrated Microbial Genomes (IMG) database in June 2014 using default settings and an e-value of 10^{-5} . The extracted homologs were aligned using Clustal Omega [46] specifying default alignment parameters. Aligned homologs were screened manually for the presence of three H-cluster binding motifs, previously defined as L1 (TSCCPxW), L2 (MPCxxKxxE), and L3 (ExMACxxGCxxGGGxP) [34]. A total of 714 sequences were identified that contained the signature L1, L2, and L3 motifs. Despite previously reported conservation of the three motifs [34], our analyses indicated slight variations in each of them. Therefore, we analyzed the extent of variation in these three H-cluster binding motifs using the weblogo server [47]. The taxonomy associated with each HydA sequence was compiled using the DOE-IMG database.

2.2 Construction of an extended sequence dataset

A custom python (ver. 2.7.6) script was used to extract gene sequences (10 upstream and 10 downstream) that flanked each *hydA*. The twenty inferred protein sequences were clustered by pairwise BLASTp using the NCBI blastclust program [48] specifying a threshold of 30% sequence identities and a coverage of 60% for each pair of sequences. Clusters that contained a minimum of 12 sequences were considered for further analysis, resulting in a total of 123

clusters. The protein sequences within each of the 123 clusters were blasted against the CDD: a public database that contains all known protein motifs and domains [49] using an e-value of 0.01 to confirm bin assignments that were made by cluster analysis. Protein sequences were pruned from the clusters based on the absence of key domains (as identified by CDD blast) associated with each protein cluster. In rare cases the proteins were manually reassigned to other clusters. The filtered protein clusters were then annotated using the UniProt database [50]. Proteins encoded in the gene neighborhood of *hydA* are reported in **Supp. Table 1**.

2.3 Frequent pattern mining using the extended dataset

The protein clusters (n=123) encoded in the gene neighborhoods of the 714 HydA homologs were converted into binary matrix for use in classifying the HydA homologs based on the presence/absence of proteins. To objectively identify the most frequent patterns in the composition of proteins encoded by genes flanking *hydA*, the Apriori algorithm was used as implemented with the arules package in R [51]. Apriori is an algorithm that identifies frequent items (i.e., protein clusters) within a database and characterizes associations among the distribution of those items to objectively identify patterns. We applied the Apriori algorithm to our protein cluster database while specifying the following parameters: a confidence threshold of 0.95 and a support threshold of 0.1 [51, 52]. The confidence threshold is the measure of the statistical significance of an identified pattern within a dataset while the support threshold is indicative of the relative abundance of a given pattern in a dataset. The Apriori algorithm works in two phases with the first phase consisting of a scan of the entire database of proteins encoded in flanking regions of *hydA* in order to objectively identify and extract frequently observed proteins. In the second phase, the algorithm uses those frequently occurring proteins to identify

the dominant patterns in the distribution of these proteins or their combinations. In doing so, the algorithm identifies correlations (within the user defined confidence and support threshold) between and among proteins in the database [52]. The 32 most frequent patterns and their confidence intervals are reported in **Supp. Table 2**.

2.4 Statistical analysis

To further evaluate the extent to which Hyd group designations correspond to differences in the composition of proteins encoded in the flanking regions of *hydA*, the binary presence/absence table of the protein encoding genes flanking *hydA* (+/- 10 genes) were subjected to Principal Component Analysis (PCA). Only proteins that were present in flanking genes that were represented in >2% of all HydA encoding genomes were considered in the generation of the dissimilarity matrix. The dissimilarity matrix was subjected to PCA (using variance-covariance matrix) to visualize variation in the composition of genes that flank *hydA* using PAST (version 3) [53].

2.5 Network analysis

The abundant patterns that were (i.e. $\geq 95\%$ confidence value and $\geq 10\%$ support value) identified by the Apriori algorithm were used to create a pattern correlating network with Cytoscape specifying the force directed organic layout [54]. Each unique pattern was denoted as a node and the edges between nodes represent the degree of correlation between the nodes. The network was further analyzed using the Network Analyzer plugin in Cytoscape [55] to calculate the relatedness (i.e., betweenness centrality) of each node in the network where the edges

represent the degree of correlation between the nodes. Proteins with the highest betweenness centrality were overlaid on the network.

Proteins encoded in the flanking regions of *hydA* that had a relative frequency of $\geq 10\%$ were extracted and used to create a binary matrix to identify the presence/absence of the protein in the gene context of each *hydA*. This matrix was then used to generate a network map to identify the protein clusters most commonly identified in each Hyd group. Cytoscape was used to visualize the network using a force directed organic layout and the network was further analyzed using the Network Analyzer plugin.

2.6 Determining the F- and C-cluster composition of HydA homologs

The N- and C-terminal regions that flank the H-cluster (i.e., F- and C-cluster domains, respectively) of HydA were trimmed from alignment blocks and were subjected to BLASTp against CDD using the CDSEARCH/cdd v3.13 algorithm [49] (version 3.13) using an e-value of 0.01 as previously described [14]. The identified F- and C-cluster domains were compiled for each sequence using a custom python (ver. 2.7.6) script and were used to classify the putative domains of HydA and to determine if these structures varied according to group designation.

2.7 Analysis of Post-Translational Modification

The Hyd (i.e. HydABC) from *T. maritima* was purified as described previously [22] while purification and properties of the HydABCD enzyme from *C. bescii* will be reported elsewhere. *Pyrococcus furiosus* transhydrogenase (also known as NfnI or sulfide dehydrogenase) was purified as previously described [56].

PTM identification (phosphorylation) was conducted on intact protein complexes. Samples were generated by spiking 4 µg of protein complexes with Calf Intestinal Alkaline Phosphatase (CIP, New England BioLabs) (1 CIP unit/1 µg protein) and incubation for 3 h. at 37 °C in 50 mM ammonium bicarbonate, pH 8.5. Casein (Affymetrix Life Science) was chosen as a positive control for phosphorylation. After treatment samples were immediately mixed with gel-loading buffer, boiled for 5 min., centrifuged, and loaded onto 4-20% mini-protean TGX gel (Bio-Rad). Controls were prepared using the same protocol, however no CIP was added. Proteins were visualized with Pro-Q Diamond Phosphoprotein Stain (Invitrogen/Molecular Probes) and GelCode Blue Stain (ThermoFisher) according to manufacturer instructions.

Concurrently, intact protein samples were analyzed using a 1290 ultrahigh pressure (UPLC) series chromatography stack (Agilent Technologies) coupled directly to an electrospray-time of flight (ESI-TOF) mass spectrometer (Bruker Micro-TOF). Before infusion to ESI source, samples were separated on a reverse-phase (RP) column PLRP-S column (50 x 2.1 mm, 3 µm, 100 Å, Agilent Technologies) or a size exclusion (SEC) Polyhydroxyethyl A column (100 x 4.6 mm, 5 µm, 500 Å, PolyLC Inc.) with flow rates of 600 µL/min. (RP column) and 300 µL/min. (SEC column). Fast gradient conditions (1 min., 10% B; 1.0-6.0 min., 10-70% B; 6.0-7.0 min., 10% B) were applied to elute proteins from the RP column (temperature of 50°C), while isocratic conditions (25% B) were used to elute proteins for 4 min. from the SEC column (temperature of 25°C). Solvent A comprised 0.1% formic acid (FA, Sigma) in water (ThermoFisher) and solvent B comprised 0.1% FA in acetonitrile (ThermoFisher).

Electrospray conditions for RP and SEC chromatography were as follow: nebulizer 3.0 or 3.5 bar (RP and SEC columns, respectively), drying gas at a flow rate of 7.0 or 6.0 L/min. (RP and SEC columns, respectively), drying temperature at 200°C, capillary voltage at 4.5 kV, and capillary exit voltage at 100 V. Data was collected in positive mode only at 2 Hz rate over the 200-3000 m/z scan range. Data processing and analysis were performed using the Bruker Data Analysis package 4.0. Charge deconvolution was performed using a maximum entropy algorithm for H⁺ adducts only and 0.1 m/z data point spacing. The low mass end was defined by the mass of lightest component while the high mass end was defined as 3.3x of the heaviest component within the complex. Measured m/z errors for all proteins were less than 0.8 m/z from calculated values.

3. RESULTS AND DISCUSSION

3.1 Taxonomic distribution and classification of HydA homologs.

The taxonomic distribution of *hydA* was examined in 30 bacterial, 5 archaeal, and 9 eukaryotic phyla with available and complete genome sequences, as determined in June 2014 (**Table 1**). None of the archaeal phyla (167 total genomes) contained organisms whose genomes encoded for HydA while 17 of the 30 bacterial phyla (287 total genomes) contained organisms whose genomes encoded for HydA. Three of the 9 eukaryotic phyla (5 total genomes) contained organisms whose genomes encoded for HydA. Ninety percent of the 714 *hydA* homologs identified were distributed among the bacterial phyla Firmicutes, Proteobacteria, Spirochaetes, Thermotogae and which encoded on average ~ 2 HydA isoforms per HydA encoding genome.

The genes flanking (+/- 10 genes) the 714 *hydA* homologs were compiled and the encoded proteins clustered into homologous bins. The Apriori algorithm was then applied to this dataset to identify genes present at a high frequency in genomic regions flanking *hydA* and to calculate their co-occurrence patterns for use in predicting potential interactions. HydB and HydC were the most prevalent proteins encoded in genomic regions flanking *hydA* and these were identified in ~40% of the 714 *hydA* flanking gene neighborhoods (**Fig. 1**). An ABC transporter protein, HydD, and Hkl were the next most frequently identified proteins which were encoded in ~28%, 15%, and 12% of the *hydA* flanking gene neighborhoods, respectively (**Fig. 1**).

The categorized proteins from the first phase of the Apriori algorithm were then used to generate association rules for classifying HydA based on the distribution of proteins or combinations therein encoded in the flanking regions of *hydA*. A total of 32 different flanking protein combinations were identified that were within the confidence value of 0.95 or higher (**Supplementary Table 1**). Of the 32 statistically supported flanking protein combinations, 98% of them contained either HydB, HydC, HydD, or combinations of these proteins (**Supplementary Table 1**). The identified flanking protein combinations (n=32) were subjected to network analysis to identify the most prevalent co-occurrence patterns.

HydB, HydC, HydD, Hkl, a protein containing a polymerase and histidinol phosphatase (PHP) domain, and a protein with a DRTGG domain also had high betweenness centrality (e.g., connectivity among nodes) (**Fig. 2**). This indicates that these proteins were shared with a number of the 32 patterns and therefore behave as ‘bridges’ that connect the entire protein cluster

network. Of these proteins, HydB, HydC, and HydD had the highest betweenness centrality. This indicates that these three proteins are highly represented in flanking regions of genomes that encode *hydA* and presumably, given the available biochemical evidence discussed below, interact with HydA.

To further characterize the proteins in the flanking regions of *hydA*, a network map was generated using the proteins that were identified within 10 genes upstream and downstream of >2% of the 714 *hydA* homologs (**Fig. 3**). This network, like that presented in **Fig. 2**, again indicates that the HydB and HydC are highly prevalent in *hydA* gene neighborhoods and their distribution is strongly co-correlated. Likewise, the distribution of HydD and the Hkl protein also strongly co-vary. Rex (redox sensing repressor protein which is involved in transcriptional redox regulation [57-59]) was commonly found in the gene neighborhood of all three groups of Hyd (thus giving it a high ‘betweenness centrality’ value) and may represent a general transcriptional level mechanism to regulate Hyd as a function of cellular redox status. Based on these results we categorized each HydA into 3 groups: Group 1 (G1), Group 2 (G2), and Group 3 (G3), which are differentiated primarily on the basis of the presence of HydBCD (**Fig. 4 and Supplementary Table 2**). The Hyds identified in each group were then compared to those of biochemically characterized Hyds [21, 35-37, 40, 60] in order to putatively assign functionality and quaternary structure.

On the basis of sequence alignments and BLASTp analyses against the Conserved Domain Database (CDD), HydB is predicted to bind a flavin and ligate several [4Fe-4S] clusters while HydC is predicted to ligate a [2Fe-2S] cluster. Electron paramagnetic resonance (EPR) on

HydB from *T. maritima* also indicated the presence of multiple iron-sulfur clusters and sequence analysis of HydB predicted the presence of a FMN binding site and a variable number of iron-sulfur clusters [22]. Similarly, bioinformatics analysis of HydB from *A. woodii*, *C. autoethanogenum*, *M. thermoacetica*, and *R. albus* also indicated the presence of variable number of iron sulfur clusters and a FMN binding site [21, 35-37, 40]. Sequence analysis of HydC from *T. maritima* predicted the presence of one [2Fe-2S] cluster [22, 35]. In addition, based on our sequence analysis, HydD exhibits homology to a thioredoxin-like Fd and, based on the presence of two vicinal Cys residues, is predicted to bind a [2Fe-2S] cluster.

Hyd gene clusters that did not encode for or encoded incomplete HydB (e.g., truncated HydB lacking a flavin binding site) and HydC proteins in the *hydA* gene neighborhood (i.e. +/- 10 genes) were classified as G1 Hyds (n=483 HydA). Representatives of these monomeric enzymes are from the bacterium *C. pasteurianum* [2, 11, 14, 16, 34, 61] and the alga *C. reinhardtii* [14, 16, 27, 29, 30]. These have been subjected to extensive biochemical analysis and are prototypical examples of this group. Biochemical data of HydA from *C. pasteurianum* [2, 11, 36] and *C. reinhardtii* [27, 29, 30] indicate that they are unable to bifurcate electrons. These findings suggest that the other G1-group Hyds also likely do not bifurcate, including the previously characterized 'dimeric' Hyd from *D. desulfuricans* (note the presence of truncated HydB which lacks a flavin binding site [23, 44]). Of the 483 G1 HydAs, the gene neighborhood of seven homologs encoded for HydB, HydC, or HydD or a combination of these, but they did not meet the classification scheme for complete HydBC in their gene neighborhoods. For example, of these seven Hyds, one encoded for only HydB, three encoded for only HydC, one encoded for only HydCD, and two encoded for only HydBD. Based on available biochemical

data, it is not clear how or if these proteins interact with HydA. At this point, it is not known whether these Hyds can bifurcate since all biochemically characterized bifurcating enzymes encode for either HydABC or HydABCD complexes [21, 35-37, 40, 41].

Hyds that encode HydBC in the flanking regions of *hydA* were categorized as G2 enzymes (n=155 HydA). Of these 155 Hyds, 92% shared a common pattern of HydABC encoded in the ABC orientation (**Fig. 4**). Representatives of this Hyd group have been purified from *M. thermoacetica* and *T. maritima*, which were biochemically characterized as trimeric complexes (i.e. HydABC) [35, 36]. In these examples, HydB is the subunit that has been predicted to contain a unique flavin binding site along with variable numbers of iron-sulfur clusters while HydC contains a single [2Fe-2S] cluster [35, 36]. Both of these trimeric Hyds have been shown to bifurcate electrons, indicating that other G2 Hyds are likely capable of bifurcation [35, 36]. Both Fd and NADH are necessary for bifurcating Hyd activity and it has been proposed that HydC, which contains a Fd-like [2Fe-2S] cluster, accepts electrons from Fd while HydB accepts electrons from NADH [35]. If verified, this suggests the importance of both subunits HydBC for Hyd and bifurcating capability. Furthermore, the biochemical study conducted on the trimeric G2 Hyd from the thermophile *T. maritima* indicated that when HydBC were separated from HydA, the HydA enzyme lost its stability at elevated temperature and also lost the ability to reduce quinones [22]. This indicates that these two HydBC subunits not only play a role in electron transfer but also function to stabilize HydA.

In addition to HydBC, genes flanking certain G2 *hydA* were found to encode electron transfer proteins (*etp*) (n=2), formate dehydrogenase D (*fdhD*) (n=2), a homolog of HydA termed

HydS (n=17), and Sp (n=28). HydS contains an additional sensory domain (i.e. PAS [45]) at its C-terminus that is not present in canonical HydA. These results are consistent with previous informatics studies which revealed the genes flanking *hydA* included *hydS* and *sp* in *C. thermocellum* DSM 1313 [37, 62] and *T. maritima* [35, 37]. In addition, ETP and FdhD, the latter of which has a NADP⁺ binding site, have been shown to form a complex with G2 Hyds in *C. autoethanogenum* [40]. This multimeric complex is involved in the reversible reduction of CO₂ in the presence of H₂ to formate and also in the reversible reduction of Fd and NADP⁺ using formate. Interestingly, six of the 155 G2 Hyds were found to have fused HydB and HydC subunits and one G2 Hyd (*Nyctotherus ovalis*; AAU14235) was found to have fused HydABC subunits. These observations provide additional evidence that these two proteins function synergistically, as has been observed in numerous other co-occurring enzyme systems, including Hyd maturases [1, 63-67].

Lastly, *hydA*-containing gene clusters that encode for HydBCD were classified into G3 enzymes (n=76). Interestingly, 71% of the G3 Hyds (58 of 76) also encoded an Hkl protein that was present within the *hyd* gene cluster. The identity of Hkl proteins was confirmed based on the presence of a HATPase_c motif (PF02518) which is the ATP binding site of the ATPase domain [68, 69]. This motif composition differs from canonical histidine kinase proteins. Canonical histidine kinase typically has an additional two motifs including the HAMP motif [histidine kinase, adenylyl cyclase, methyl-accepting protein and phosphatase (PF00672)] which is a phosphoacceptor domain that acts as a cytoplasmic intermediate linker connecting the phosphoryl transfer (HisKA) motif [68, 70-74] with the sensor domains outside of cytoplasm [70, 75, 76]. Biochemical characterization of the G3 Hyd in *A. woodii* indicates that it forms a

tetrameric complex that includes HydBC and a Fd-like HydD protein that contains a [2Fe-2S] cluster [21].

Like representatives of G2 Hyds, the tetrameric HydABCD enzyme of *A. woodii* has been shown to bifurcate and also to require two electron carriers, Fd and NADH, for activity [21]. However, unlike the trimeric G2 Hyd identified in *T. maritima* [35], which is involved in fermentative H₂ production, the tetrameric G3 Hyd identified in *A. woodii* is involved in oxidation of H₂ [35]. It has been proposed that electrons are transferred from HydA to HydC and then to HydB where the electrons are bifurcated simultaneously to reduce Fd and NAD⁺ [21]. This mechanism, like that proposed for trimeric G2 Hyd [35], implies an important role for HydBC in electron transfer. The function of HydD in G3 Hyd and its role in bifurcation and in reaction directionality is still unknown. Importantly, in several instances, HydBCD were not present in this arrangement, but rather, were interspersed among other genes in the 20 protein encoding genes flanking G3 *hydA*. Examples include Hyd from *Desulfarculus baarsii* DSM 2075 (Deba_1523), *Desulfobacterium autotrophicum* HRM2 (HRM2_16550), and *Kosmotoga olearia* TBF 19.5.1 (Kole_0172). Thus, it is unclear if these Hyds have the capability to bifurcate. In addition to HydBCD and Hkl, genes flanking a number of G3 Hyds encoded various regulatory genes including Stk proteins (n = 39 HydA), and/or transpeptidase proteins (n = 20 HydA).

In support of the group designations identified by Apriori algorithm classification, PCA ordination of a matrix describing the dissimilarity in the composition of proteins encoded by genes in flanking regions of *hydA* formed clusters that largely corresponded to the same group designations (**Supplementary Figure. 1**). A network map was also used to confirm the extent to

which proteins encoded by genes in flanking regions of *hydA* correlated with group designation (**Fig. 5**). Based on the Apriori analysis, regulatory genes including Sp and HydS were enriched in G2 Hyds while Hkl, DRTGG, a PHP domain-containing protein and Stk were enriched in flanking regions of G3 Hyds. The difference in regulatory gene association between the two groups indicates that G2 and G3 Hyds may be subject to different modes of PTM and/or that these PTM enzymes exhibit specificity for target Hyd proteins allowing for their differential regulation if both types of protein are present in the same organism.

The HydA copy number per genome varied as a function of taxonomy (**Table 1**). Moreover, the HydA copy number per genome, when broken out by group designation, also varied (**Supplementary Figure 2A**). Multiple copies (isoforms) of G1 Hyd in the same genome was the most common pattern identified (n=117 of 287 genomes). Several genomes contained three or more copies of HydA, with a maximum copy number/genome of seven HydAs (*Desulfotomaculum carboxydivorans* CO-1-SRB, DSM 14880). Fifty-two percent of the HydA-encoding genomes encoded for at least two different types of Hyd (**Supplementary Figure 2B**). For example, 77 genomes encoded for G1 and G2 Hyds, 44 encoded for G2 and G3 Hyds, and 17 genomes encoded for all three groups of Hyds. The co-existence of G2 and G3 Hyds with G1 Hyds potentially suggests they have different functional roles within the organisms and again is consistent with data presented above indicating that they may be regulated at the post-translational level by different PTM enzymes.

Hyd variants were distributed unequally among bacterial phylum-level lineages. G1 Hyds were prevalent in the Bacteroidetes, Firmicutes, Proteobacteria, Spirochaetes and Thermotogae

phyla while G2 Hyds were enriched in Chloroflexi and Thermotoga phyla. G3 Hyds were enriched in Firmicutes and Spirochaetes phyla (**Table 1**). Two of the three eukaryotic phyla that contained homologs of Hyd encoded G1 Hyds: heterokonts with one genome (i.e. *Thalassiosira pseudonana*) and chlorophytes with 3 genomes. One of the three eukaryotic phyla encoded a G2 Hyd: Ciliophora with one genome (i.e. *Nyctotherus ovalis*). Multiple copies of HydA were often encoded in chlorophyte genomes and these were all classified as G1 Hyd. Interestingly, HydA identified in the ciliate *Nyctotherus ovalis* [77-79] forms a fused complex with HydB-like and HydC-like subunits, which begs the question as to whether this enzyme can also bifurcate electrons as it meets the minimal requirement of gene complements (i.e., encodes HydABC) hypothesized to afford bifurcation activity.

3.2 Structural composition of HydA (H-, F- and C- cluster).

Various biochemical [2, 11, 29, 30] and bioinformatics studies [14, 16, 32, 34, 61] indicate substantial variation in the iron-sulfur cluster content of the F- and C- clusters of HydA, which are hypothesized to act as electron transfer conduits for the various Hyds. Thus, the composition of iron-sulfur cluster binding domains in the F- and C-clusters of HydA were characterized in order to determine if variations in these clusters correlated with Hyd group designations. Based on the presence or absence of cluster binding motifs and other identifiable motifs defined in the CDD, the 714 HydA homologs were categorized into modular structures according to the scheme of Calusinska *et al.*, 2010 [14]. A total of 12 modular structures were identified and these were nearly equally represented across all groups of Hyd (**Fig. 6**). Out of 12 modular structures, 10 were predicted to ligate two [4Fe-4S] clusters in the F-cluster domain. More than 50% of HydA in G2 and G3 exhibited the M2i modular structure which were

predicted to ligate two [4Fe-4S] clusters or the M3a modular structure which comprised multiple iron-sulfur clusters (i.e. [2Fe-2S], histidine substituted [4Fe-4S] and 2[4Fe-4S]) in the F-cluster domain. Likewise, more than 50% of the HydA lacked C-clusters and of those that did have C-clusters, most were classified as the non-bifurcating G1 Hyds.

We also examined the variation in the three amino acid sequence motifs (i.e. L1, L2 and L3 [14, 16]) that are involved in ligating the H-cluster in relation to group designations (**Fig. 7**). The majority of the alignment positions were comprised of residues that did not vary substantially in amino acid usage for any single group. However, two positions in each of the three motifs contained residues that differed among groups. For example, in the L1 motif region, the 4th residue, cysteine, is more common in G1 and G2 Hyds while serine is more common in G3 Hyds. Similarly, glycine is commonly observed in the 6th position of the L1 motif in G2 and G3 Hyds, whereas alanine is more commonly observed at this position in G1 Hyds. Within the L2 motif region, methionine is more common in the 1st position in G2 and G3 Hyds, whereas glycine is more common in G1 Hyds. Within the L3 motif region, glycine and valine in the 2nd position are more common in G1 Hyds while valine and isoleucine are more common in G2 and G3 Hyds. Finally, proline and glycine are more common in the 13th position of the L3 motif in G1 Hyds whereas glycine is more common in G2 and G3 Hyds.

The lack of consistency between F-, C-, and H-cluster and Hyd group designations indicates that bifurcation capability is more likely to be predicated by the presence/absence of accessory subunits, in particular the flavin binding subunit HydB. Moreover, these results may suggest that variations in F-, C-, and H-cluster domains are refinements to enzymes that are

primarily under selection for bifurcating versus non-bifurcating abilities. Biochemical characterization of Hyds from *A. woodii*, *C. autoethanogenum*, *M. thermoacetica* and *T. maritima* all showed the ability to bifurcate and all the Hyds identified in these species were either trimeric or tetrameric [21, 35, 36, 40]. Conversely, the monomeric Hyd from *C. pasteurianum* [2, 11, 36] and *C. reinhardtii* [27, 29, 30] indicate that they are unable to bifurcate electrons. These findings suggest that the other G1-group Hyds also likely do not bifurcate, including the ‘dimeric’ Hyd from *D. desulfuricans* which forms a complex with a truncated HydB that lacks a flavin binding site [23, 44]. A few additional putative dimeric forms of Hyd were identified that contained full length HydB with a flavin binding motif, but due to lack of biochemical characterization of an enzyme with this configuration, they were classified as G1 enzymes. For example, the HydA encoded in the *Coprococcus catus* GD/7 genome (locus tag: CC1_10640) also encoded for HydB in its flanking region but lacked genes encoding for HydC. Hence, based on available biochemical data, we hypothesize that the enzymes that lack either of the subunits i.e. HydB or HydC do not have the capability to bifurcate.

3.3 Post translational modification of bifurcating G2 and G3 Hyds

Among the more intriguing and novel findings from our bioinformatics work was the identification of genes encoding proteins that could be involved in PTM in the gene neighborhoods of G2 and G3 Hyds. Identified proteins included Hkl and Stk in G3 Hyds while Sp was commonly observed in G2 Hyds. Despite both having an ATPase domain and exhibiting ~25% homology with ~40% pairwise sequence coverage, an alignment of our identified Hkl and Stk proteins indicated they are distinct (**Supplementary Figure 3**). Protein kinases have been a subject of extensive study which have demonstrated their involvement in various regulatory

processes. Among those most widely studied are Hkl and Stk which have been biochemically shown to target histidine (H) and serine/threonine (S/T) for phosphorylation, respectively [70, 80-83]. The presence of Hkl and Stk in the gene neighborhood of G3 *hydA* indicate that these kinases and phosphatases may be involved in reversible phosphorylation of H, S and T residues. By extension, this may suggest conservation of these residues in Hyd subunits.

Multiple sequence alignments (MSA) of all the sequences for the Hyd subunits, i.e. HydA, HydB, and HydC from G1, G2, and G3, revealed few or no conserved (i.e. $\geq 90\%$ conservation) H, S, and T residues. We therefore screened alignments of only HydA and its subunits in gene clusters that also encode Sp (G2), Stk (G3), and Hkl (G3) in the flanking region, which resulted in a total of 18 and 46 sequences, respectively. When HydABC that were not flanked by Hkl or Stk were removed from MSA, several conserved H, S, and T were identified (**Supplementary Figure 4, 5, and 6**). HydB subunits also harbored conserved (i.e. $\geq 90\%$ conservation) H and S/T residues regardless of whether they were classified as G2 or G3 Hyds (**Supplementary Figure 5**). HydC in G2 harbored multiple conserved (i.e. $\geq 90\%$ conservation) H and T residues while G3 harbored multiple conserved T residues only at the same position (**Supplementary Figure 6**). Lastly, HydD in G3 also harbored conserved (i.e. $\geq 90\%$) H and T residues (**Supplemental Figures 7**). However, the extent of conservation of H, S, and T residues in HydD did not vary substantially in enzymes that were not flanked by Hkl or Stk versus those that were flanked by these proteins. While the presence of conserved H and S/T residues does not, by itself, indicate that those conserved sites are post-translationally modified, they do provide potential targets for *in vitro* studies. To provide further evidence for PTM of Hyd, we

undertook a biochemical assay to probe PTM using the G2 Hyd from *T. maritima* and the G3 Hyd from *Caldicellulosiruptor bescii*.

Sequence analysis of the G2 HydA from *T. maritima* indicates that it comprises multiple iron-sulfur cluster (i.e. 2[Fe-2S], histidine substituted [4Fe-4S] and 2[4Fe-4S]) binding motifs in the F-cluster and a [2Fe-2S] cluster binding motif in the C-cluster and thus is classified within the 3c modular structure (**Fig. 6**). The structural proteins identified in *T. maritima* G2 Hyd (i.e. HydABC) and a homolog of Sp (TM1423) have been identified in the flanking region of the trimeric Hyd complex (TM1424-TM1426). Similarly, sequence analysis of HydA from *C. bescii* indicates that it also contains multiple iron-sulfur cluster (i.e. 2[Fe-2S], histidine substituted [4Fe-4S] and 2[4Fe-4S]) binding motifs in its F-cluster and is thus classified as having the M3a modular structure, which is a common structure associated with G3 Hyds (**Fig. 6**). The structural proteins identified in *C. bescii* G3 Hyd (i.e. HydABCD) are encoded in an apparent operon (YP_002573168 to YP_002573172). Homologs of Hkl (YP_002573169) and Stk (YP_002573164) were also identified in the gene neighborhood of *hydA* in *C. bescii*. The presence of Hkl and Stk in the flanking gene neighborhood of *hydA* in *C. bescii* and Sp in the flanking region of *hydA* in *T. maritima* suggests that phosphorylation of these Hyd proteins may occur.

To test the possibility for PTM, purified Hyd protein complexes from *T. maritima* and *C. bescii* were analyzed by SDS-PAGE followed by phospho-specific protein staining. Image analysis showed strong signals for HydA/B proteins (68/72 kDa for *T. maritima* and 63/64 kDa for *C. bescii*) as well as for HydD (14 kDa) from *C. bescii* (**Fig. 8**, left panel). Positive (casein,

Cas from *Bos taurus*) and negative (transhydrogenase, Nfn1 from *Pyrococcus furiosus*) controls for phospho-staining were included on the gel for reference. To further establish the presence of phosphorylation, protein samples were treated with calf intestinal phosphatase (CIP). A reduction in phosphorylation intensity was observed for high molecular weight Hyd subunits from *T. maritima* and casein. Incubation of *C. bescii* Hyd complex with CIP did not lead to a significant change in fluorescence. The same gel was then stained with Coomassie Blue to verify protein loading (**Fig. 8**, right panel). *T. maritima* HydC (18 kDa) was poorly visualized, as observed by the less than stoichiometric band intensity. Coomassie stain showed that HydC (17 kDa) and HydD (14 kDa) from *C. bescii* were present at an equal ratio, as expected, in contrast to their signal when visualized for phosphorylation.

Mass spectrometry was used to further investigate Hyd subunit modification. High-resolution mass analysis of intact proteins is a sensitive method for observing heterogeneity. Purified Hyd complexes were subjected to size-exclusion and reverse-phase chromatography to remove salts and buffers prior to electrospray time of flight mass analysis. All Hyd subunits, except for HydC from *C. bescii*, were observed as a mixture of several predominant forms. HydB from *T. maritima* showed significant heterogeneity, with all of the forms being 300-400 Da greater than the expected molecular weight of 68,551 Da, based on the amino acid sequence (**Fig. 9**, top panel). HydC from *T. maritima* had three distinct forms separated by 98 Da (**Fig. 9**, middle panel). Analysis of *C. bescii* proteins showed that HydD was present as two dominant forms (1:3 ratio), separated by 80 Da (**Fig. 9**, bottom panel).

Phosphorylation is often difficult to confirm by mass spectrometry due to sub-stoichiometric modification and loss of the attached phosphate during sample processing and electrospray ionization. Attachment of a phosphate group (HPO_3) adds 80 Da to the protein for each phosphorylated amino acid [84-87]) as we observed for HydD from *C. bescii*. In contrast, a difference 98 Da (H_3PO_4) is indicative of an ionization induced loss of phosphorylation specific to serine and threonine residues [86, 88, 89]. This result suggests modification of serine and threonine residues of *T. maritima* HydC, although phosphorylation at the peptide level will be necessary to confirm the specific sites of phosphorylation. Together, the SDS-PAGE and LCMS analysis strongly suggest that HydAB and HydC of *T. maritima* G2 Hyd are phosphorylated. Along with our bioinformatics-based analysis, this provides strong evidence that Hyd proteins are likely to be post-translationally modified. This is an attractive mechanism for regulating the activity of bifurcating G2 Hyd complexes. Similarly, SDS-PAGE and LCMS analysis strongly suggest that the HydAB and HydD subunits of *C. bescii* G3 Hyd are phosphorylated. Interestingly, HydC in *C. bescii* G3 Hyd was not modified. Since G3 HydC lacked conserved H residues but G3 HydABD all contained a number of conserved H residues, this result suggests that Hkl is potentially responsible for phosphorylating the H residues in G3 HydA. Future biochemical studies are needed to identify the specific sites that are putatively involved in phosphorylation activity, their effects on modulating hydrogenase activities, and the specific enzymes involved in PTM activities.

4. CONCLUSIONS

Our bioinformatics-based analysis of the gene neighborhood of *hydA* revealed three major groups of Hyd (G1, G2, and G3) that are differentiated on the basis of the presence of HydBC (G2) or HydBCD (G3). The G1 enzymes appear to be monomeric and based on biochemical data are predicted to not bifurcate while G2 and G3 Hyds are in trimeric and tetrameric forms, respectively. Based on bioinformatics analysis and albeit limited biochemical data, G2 and G3 enzymes are predicted to bifurcate. Variation in the F- and C-clusters and the residues that ligate the H-cluster of HydA do not vary in a systematic manner with respect to Hyd group designations, indicating that they are under different selection and unlikely to delineate the ability of enzymes to bifurcate electrons.

Our analysis also identified regulatory genes in the flanking regions of *hydA*, including Sp (in G2 Hyds), Stk (in G3 Hyds), and an Hkl (in G3 Hyds), all of which we hypothesize to play a role in PTM regulation of Hyds. Indeed, our MS and biochemical data indicate that examples of G2 and G3 Hyds are phosphorylated alluding to the role of the aforementioned proteins in potentially regulating Hyd. That PTM proteins associate with G2/G3 Hyd but not G1 Hyd is consistent with the prevalence of G1 Hyd in genomes that encode for G2/G3 Hyd, necessitating mechanisms to differentially regulate the activities of these enzymes. Moreover, the presence of different PTM proteins in the gene neighborhoods of G2 and G3 Hyds and differences in the protein subunits that were apparently phosphorylated is consistent with the co-occurrence of G2 and G3 Hyd in the same organisms and their need to differentially regulate these enzyme complexes. Collectively, these results show that the vast inferred structural diversity of Hyds can be classified into only three fundamental groups, and variations within them allude to their various functions in microbial metabolism. The classification scheme

reported herein provides the first framework for prioritizing biochemical and mutagenesis studies of Hyds aimed at furthering our understanding of the functional role of this surprisingly cohesive group of enzymes.

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6. FIGURE LEGENDS

Figure 1. Relative frequency of encoded proteins that were identified among genes flanking 714 *hydA* homologs (+/-10 genes). For simplicity, only those proteins that were encoded in $\geq 6\%$ of the 714 gene neighborhoods are shown. The identity of all proteins that flank each individual HydA can be found in Supplementary Table 2. (Single column).

Figure 2. Network map depicting the dominant patterns in proteins encoded in the genes that flank *hydA* (+/- 10 genes), as identified by the Apriori algorithm (18). Each unique pattern is given as a node and the edges between nodes represent significant correlations between those nodes (confidence value of ≥ 0.95). Node color represents the betweenness centrality (a measure of the ‘connectedness’ of each gene to the total network) that connects multiple groups (i.e., are shared in multiple groups). Node size represents the number of patterns/genes significantly associated with other patterns/genes. The force directed organic layout within Cytoscape was

used to visualize the correlation in the network. Patterns in the distribution of genes flanking *hydA*, as revealed by this network, were used to classify the 714 HydA homologs into 3 categories (described in text). For simplicity, only nodes with betweenness centrality of 0.06 or greater are labeled. (Single column)

Figure 3. Network map depicting the correlation of proteins encoded by genes flanking (± 10 genes) *hydA*. Each unique protein is given as a node and the edges between nodes represent significantly correlated proteins ($p < 0.05$). Node color represents the betweenness centrality that connects proteins (a measure of the ‘connectedness’ of each gene to the total network) while edge color represents the Pearson correlation describing the co-distribution of proteins encoded in flanking regions of *hydA*. Node size represents the number of proteins encoded by genes flanking *hydA* that exhibit significant associations with other proteins in these flanking regions. (Double column)

Figure 4. Classification of HydA (n=714) homologs based on the composition of proteins encoded in flanking gene neighborhoods (± 10 genes). The number of HydA homologs that fall within each group is indicated in parentheses. The percent of HydA homologs within a specified group that exhibit the exact gene neighborhood as depicted is given as a percentage. An organism and the locus tag for a representative gene cluster for each specified group is indicated. Asterisks denote Hyd homologs that have been biochemically characterized (see results and discussion for details). Those with a red asterisk have been biochemically shown to bifurcate while those with a black asterisk have been biochemically shown to not bifurcate. (Double Column)

666

667 **Figure 5.** Network analysis of proteins encoded by genes flanking (± 10 genes) *hydA*. Only
668 proteins ($n = 22$) encoded in the flanking regions of $>10\%$ of the each HydA group (i.e. relative
669 frequency of $>10\%$) were considered in this analysis. Here, node color represents the
670 betweenness centrality (a measure of the ‘connectedness’ of each gene to the total network)
671 while edge color represents the relative frequency of each protein in each group. The force
672 directed organic layout was used to visualize the correlation in the network. The average pattern
673 associated with each of the 3 defined Hyd groups were included in this analysis [indicated by
674 Group 1 (G1), Group 2 (G2), and Group 3 (G3)]. (Single column)

675

676 **Figure 6.** Inferred domain composition of the F- and C-clusters associated with HydA homologs.
677 Homologs and modular structures are organized by Hyd group designations given on the left side
678 of the figure. The number in the parenthesis next to the modular structure designation represents
679 the number of HydA homologs out of 714 that have the specified modular structure. An example
680 of a HydA in an organism and its associated locus tag with each modular structure is indicated
681 on the right side of the figure. Hyd homologs that have been biochemically characterized are
682 denoted with an asterisk. Hyd homologs with a red asterisk have been biochemically shown to
683 bifurcate while those with a black asterisk have been biochemically shown to not bifurcate.
684 (Double column)

685

686 **Figure 7.** Schematic diagram indicating the extent of conservation in residues comprising the H-
687 cluster motifs (L1, L2, and L3, as outlined by [11]) in HydA as organized by Hyd group

designations. Residues with higher bit scores (i.e., larger font sizes) suggest a greater degree of conservation at a given aligned position within a given group. (Single column)

Figure 8. Phosphoprotein-specific staining. Purified Hyd were separated using SDS-PAGE and stained with a fluorescent dye that is specific for phosphoproteins (left panel) followed by Coomassie staining (right panel) to observe protein abundance. Samples were run with and without treatment with calf intestinal phosphatase (CIP). The G2 Hyd from *Thermotoga maritima* (Tm) and the G3 Hyd from *Caldicellulosiruptor bescii* (Cb) were positive for phosphorylation. Casein (Cas) from *Bos taurus* and transhydrogenase (Nfn1) from *Pyrococcus furiosus* served as positive and negative controls for phosphorylation, respectively. (Single column)

Figure 9. LCMS analysis of Hyd subunits from *T. maritima* (Tm) and *C. bescii* (Cb). Intact protein analysis of G2 and G3 Hyd separated by SEC-ESI-Q-TOF shows heterogeneity of proteins. The HydB (G2) and HydC (G2) subunits have three prominent forms, expected MW = 68,551 Da (top panel) and expected MW = 18,193 Da (middle panel), respectively. The HydD (G3) subunit (expected MW = 14,688 Da, bottom panel) was found in two major forms spaced by 80 Da. (Single column)

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TABLE 1. Taxonomic distribution of HydA homologs in 2921 complete archaeal, bacterial, and eukaryal genomes. The total number of genomes for each phylum is indicated along with the number of genomes within that phylum that encode for HydA. The average number of HydA in genomes that encode HydA is indicated along with the number of HydA in each of the 3 defined Hyd groups (see results and discussion section for a description of the group designations). (Double column)

Domain	Phylum	Total number of genomes	Total number of genomes with HydA	Average # of HydA in the genomes	Total number of Hyd in each group		
					Group 1	Group 2	Group 3
Archaea	Crenarchaeota	50	0	0	0	0	0
	Euryarchaeota	110	0	0	0	0	0
	Korarchaeota	1	0	0	0	0	0
	Nanoarchaeota	1	0	0	0	0	0
	Thaumarchaeota	5	0	0	0	0	0
Bacteria	Acidobacteria	7	0	0	0	0	0
	Actinobacteria	301	6	1.5	9	0	0
	Aquificae	14	1	1	1	0	0
	Armatimonadetes	2	0	0	0	0	0
	Bacteroidetes	94	10	1.5	11	2	2
	Caldiserica	1	0	0	0	0	0
	Chlamydia	109	0	0	0	0	0
	Chlorobi	11	0	0	0	0	0
	Chloroflexi	24	8	1	0	8	0
	Chrysiogenetes	1	0	0	0	0	0
	Cloacimonetes	1	1	1	0	0	1
	Cyanobacteria	79	0	0	0	0	0
	Deferribacteres	4	0	0	0	0	0
	Deinococcus-Thermus	21	0	0	0	0	0
	Dictyoglomi	2	2	2	2	2	0
	Elusimicrobia	1	1	1	0	1	0
	Fibrobacteres	2	0	0	0	0	0
	Firmicutes	622	165	2.9	347	92	52
	Fusobacteria	11	2	4.5	4	5	0
	Gemmatimonadetes	1	0	0	0	0	0
	Ignavibacteriae	2	2	3.5	2	5	0
	Nitrospirae	4	1	2	2	0	0
	Planctomycetes	8	0	0	0	0	0
	Proteobacteria	1240	39	1.5	44	7	6
	Spirochaetes	65	22	2.5	31	14	11
	Synergistetes	5	2	1	0	1	1
	Tenericutes	89	0	0	0	0	0
	Thermodesulfobacteria	2	1	4	4	0	0
	Thermotogae	18	17	2.3	20	16	3
	Verrucomicrobia	5	1	1	0	1	0
Eukaryota	Heterokonts	1	1	1	1	0	0
	Chlorophyta	3	3	1.7	5	0	0
	Ciliophora	1	1	1	0	1	0
	Ascomycota	14	0	0	0	0	0
	Microsporidia	1	0	0	0	0	0
	Euglenozoa	1	0	0	0	0	0
	Magnoliophyta	1	0	0	0	0	0
	Chordata	2	0	0	0	0	0
	Nematoda	1	0	0	0	0	0

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

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Figure 8

Figure 9