Identification of a Novel Class of Membrane-Bound [NiFe]-Hydrogenases in *Thermococcus onnurineus* NA1 by *In Silico* Analysis^{∇†}

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***In silico* analysis of group 4 [NiFe]-hydrogenases from a hyperthermophilic archaeon, *Thermococcus onnurineus* NA1, revealed a novel tripartite gene cluster consisting of dehydrogenase-hydrogenase-cation/proton antiporter subunits, which may be classified as the new subgroup 4b of [NiFe]-hydrogenases-based on sequence motifs.**

Hydrogenases are the key enzymes involved in the metabolism of H₂, catalyzing the following chemical reaction: 2H⁺ + 2e⁻ ↔ H₂. Hydrogenases can be classified into [NiFe]-hydrogenases, [FeFe]-hydrogenases, and [Fe]-hydrogenases, based on their distinctive functional core containing the catalytic metal center (11, 17).

The genomic analysis of *Thermococcus onnurineus* NA1, a hyperthermophilic archaeon isolated from a deep-sea hydrothermal vent area, revealed the presence of several distinct gene clusters encoding seven [NiFe]-hydrogenases and one homolog similar to Mbx (membrane-bound oxidoreductase) from *Pyrococcus furiosus* (1, 6, 8, 12). According to the classification system of hydrogenases by Vignais et al. (17), three hydrogenases (one F₄₂₀-reducing and two NADP-reducing hydrogenases) belong to group 3 [NiFe]-hydrogenases, and four hydrogenases belong to group 4 [NiFe]-hydrogenases. The group 4 hydrogenases are widely distributed among bacteria and archaea (17), with Hyc and Hyf (hydrogenase 3 and 4, respectively) from *Escherichia coli* (19), Coo (CO-induced hydrogenase) from *Rhodospirillum rubrum* (4), Ech (energy-converting hydrogenase) from *Methanosarcina barkeri* (7), and Mbh (membrane-bound hydrogenase) from *P. furiosus* (6, 10, 12) being relatively well-characterized hydrogenases in this group. One of the four group 4 hydrogenases from *T. onnurineus* NA1 was found to be similar in sequence to that of *P. furiosus* Mbh (10).

Gene organization for three distinct hydrogenases. The genes encoding the other three group 4 hydrogenases from *T. onnurineus* NA1 were found to be organized into the following three separate gene clusters: *fdh1-mfh1-mnh1*, *fdh2-mfh2-*

mnh2, and *codH-mch-mnh3* (Fig. 1). The open reading frames in the clusters can be divided into the following three modules: the first encodes an oxidoreductase, such as a formate dehydrogenase or a carbon monoxide dehydrogenase; the second encodes a multimeric membrane-bound hydrogenase with five to seven subunits; and the third module encodes a cation/proton antiporter (Table 1). This kind of tripartite gene cluster has not yet been reported, and only a few bipartite gene clusters have been reported in *Thermococcales* strains, such as the *Thermococcus litoralis* formate hydrogen lyase (formate dehydrogenase-coupled hydrogenase [FDH-MHY]) and the *P. furiosus* Mbh hydrogenase with a cation/proton antiporter (6, 13).

In silico genomic analysis revealed the presence of tripartite gene clusters homologous to *fdh1-mfh1-mnh1* or *fdh2-mfh2-mnh2* in the genomes of *Thermococcus gammatolerans* (GenBank accession number CP001398) and *Thermococcus* sp. AM4 (whole-genome shotgun sequence) (GenBank accession number DS999059) (Fig. 1; see also Table S1A in the supplemental material) (20). The *Pyrococcus abyssi* formate hydrogen lyase gene cluster had been reported to be similar to that of *T. litoralis* (13), but we performed an in-depth genomic analysis and found that the gene cluster was followed by a cation/proton antiporter module, as shown in Fig. 1 (see Table S1A in the supplemental material).

The gene organization of the *codH-mch-mnh3* gene cluster, containing a distinct gene encoding carbon monoxide dehydrogenase (CODH), was found in the genomes of *Thermococcus* sp. AM4 and *Thermococcus barophilus* MP (obtained from the Moore Foundation; unfinished sequence) (GenBank accession number DS990558) (Fig. 1; see also Table S1B in the supplemental material). *Thermococcus* sp. AM4 was isolated as a hydrogenogenic carboxydrotroph, and *T. barophilus* MP was recently found to be capable of growing hydrogenogenically on CO by T. Sokolova (personal communication).

Phylogenetic analysis. To assess the relationship of the above-mentioned distinct hydrogenases with group 4 [NiFe]-

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FIG. 1. Comparison of the gene organizations of the *fdh1-mfh1-mnh1*, *fdh2-mfh2-mnh2* (A), and *codH-mch-mnh3* (B) clusters.

hydrogenases, phylogenetic analysis of the large subunits of all the target hydrogenases was performed using Molecular Evolutionary Genetics Analysis 4.1 (MEGA 4.1) software (14). We found that the target hydrogenases grouped together in a separate cluster in group 4 (Fig. 2). Although only bacteria were thought to possess Coo-type hydrogenases, our research provides the first evidence of Coo-type hydrogenases in archaea (16). According to the above-described grouping, we correctly assigned HycE from *Thermoanaerobacter tengcongensis* as a Coo-type hydrogenase. In support of this designation, HycE- and CODH-encoding genes were found to reside proximate to each other in the genome.

We collected all the sequences of the large subunits belonging to group 4 hydrogenases and assigned them to either sub-

group 4a or 4b by phylogenetic analysis (see Fig. S1 in the supplemental material). We then performed sequence alignment of subgroup 4b hydrogenases using the ClustalW program (15), and a pair of highly conserved motifs (L1 and L2) were revealed, which represent two regions surrounding the two pairs of cysteine ligands of the NiFe center of the large subunits of hydrogenases. The sequence logo was then derived to quantify the sequence conservation at each position within the L1 and L2 motif patterns of group 4b hydrogenases (Fig. 3) (3). In comparison to L1 (C[GS][ILV]C[AGNS]xxH) and L2 ([DE][PL]Cx[AGST]Cx[DE][RL]) motif patterns (“x” denotes any amino acid) characterizing group 4 hydrogenases, those of group 4b hydrogenases presented some invariant residues and additional residues (16).

Distribution of group 4b hydrogenases. The gene distribution for group 4b hydrogenases was then investigated (see Fig. S2 in the supplemental material), and the genes encoding the large subunits of hydrogenases were identified in strains from five bacterial phyla (*Proteobacteria*, *Firmicutes*, *Actinobacteria*, *Planctomycetes*, and *Nitrospirae*) and two archaeal phyla (*Crenarchaeota* and *Euryarchaeota*). Interestingly, all of the above-mentioned archaeal strains and some bacterial strains (*Thermodesulfovibrio yellowstonii* from the *Nitrospirae* phylum, *Moorella thermoacetica* and *T. tengcongensis* from the *Firmicutes* phylum, and *Caminibacter mediatlanticus* from the delta/epsilonproteobacteria subphylum) were thermophiles or hyperthermophiles, isolated mostly from hot springs, deep-sea hydrothermal vents, solfataras, or other thermal environments (2, 5, 18). The habitat similarity could create opportunities to interact with distantly related neighbors, possibly by horizontal gene transfer (9). Investigation into the acquisition routes for

TABLE 1. Functional annotation of the components of the *fdh1-mfh1-mnh1*, *fdh2-mfh2-mnh2*, and *codH-mch-mnh3* gene clusters in *T. onnurineus* NA1

Proposed function	Component(s) of gene cluster ^a :		
	<i>fdh1-mfh1-mnh1</i>	<i>fdh2-mfh2-mnh2</i>	<i>codH-mch-mnh3</i>
Transcription regulator	0282		1016
Dehydrogenase	0281	1563	1018
Electron transfer	0280, 0275	1564, 1570	1017, 1023-1
Nickel insertion			1019
H ⁺ translocation	0279, 0278, 0277	1565, 1566, 1567, 1568	1021, 1022
Hydrogenase	0276, 0274	1569, 1571	1023, 1024
Unknown	0273	1572	1020
Formate transporter		1573	
Na ⁺ /H ⁺ antiporter	0272, 0271, 0270, 0269, 0268, 0267, 0266	1574, 1575, 1576, 1577, 1578, 1579, 1580	1025, 1026, 1027, 1028, 1029, 1030, 1031

^a Locus tags are indicated with the “TON_” initial part omitted.

FIG. 2. Phylogenetic tree of the large subunits of group 4 hydrogenases. The tree was constructed using the MEGA 4.1 program with the neighbor-joining algorithm, using 1,000 bootstrap replicates. Scale bar represents 0.2 substitutions per amino acid position.

group 4b hydrogenases among bacteria would provide insight into the explanation of the gene distribution.

In conclusion, this is the first report on the unique tripartite dehydrogenase-hydrogenase-cation/proton antiporter clusters from the hyperthermophilic archaeon *Thermococcus onnurineus* NA1. We report for the first time that group 4 [NiFe]-hydrogenases can be further subdivided into subgroups 4a and 4b based on their specific motifs. We anticipate that biochemical and physiological experiments can assess the roles of the novel hydrogenases and other components of the tripartite clusters.

FIG. 3. Sequence logo representations of L1 and L2 motif patterns of group 4b hydrogenases generated using the WebLogo program. Residues determined as L1 (A) and L2 (B) motif patterns are boxed in red. Sixty representative group 4b hydrogenases composed of only one gene from several strains of the same species were selected to avoid a biasing effect due to identical residues within hydrogenases that arose recently from a common ancestor.

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