Transcription of [FeFe]-Hydrogenase Genes during H$_2$ Production in Clostridium spp. Isolated from a Paddy Field Soil

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Changes in the relative abundances of the transcripts of hydA gene paralogs for [FeFe]-hydrogenase in Clostridium sp. strain H2 and Desulfovibrio sp. strain A1 isolated from paddy field soil were analyzed during H$_2$ production. Strains H2 and A1 had at least five and two phylogenetically different hydA genes, respectively. The relative abundances of their hydA transcripts differed among the paralogs and H$_2$ production activity changed in a manner that depended on the growth phase and conditions. Increases or decreases in the relative abundances of the transcripts of two out of five hydA genes in strain H2 correlated with changes in H$_2$ production rates, whereas those of the others remained unchanged or decreased. In strain A1, the relative abundances of the transcripts of two hydA genes differed between monoculture, sulfate-reducing, and syntrophic, methanogenic conditions. The relative abundance of the transcripts of one hydA gene, predicted to encode a cytosolic [FeFe]-hydrogenase, was higher under syntrophic, methanogenic conditions than sulfate-reducing conditions, while that of the transcripts of the other hydA gene decreased with time under both conditions. This study showed that the transcription of the hydA gene during growth with active H$_2$ production was differently regulated among the paralogs in H$_2$ producers isolated from paddy field soil.

Key words: paddy field soil, [FeFe]-hydrogenase, H$_2$-producing microorganism, hydA-paralog, transcriptional analysis

Molecular H$_2$ produced during the anaerobic decomposition of organic matter is one of the important intermediates in anoxic paddy field soil (7, 12). H$_2$ is produced by various fermenters using protons as the electron acceptor, and is consumed by H$_2$ scavengers such as sulfate reducers and methanogens (32). Although apparent H$_2$ production in paddy field soil is very low because of concomitant H$_2$ consumption (12), the balance between the production and consumption of H$_2$ regulates the decomposition processes of organic matter (7). Thus, elucidating the ecophysiology of key H$_2$ producers is crucial for obtaining a more complete understanding of the biogeochemical cycle in paddy field soil.

However, clarification of the diversity, activities, and roles of H$_2$ producers in the environment is challenging because H$_2$ producers are physiologically and phylogenetically diverse microorganisms. Previous studies estimated the contribution of acetate and CO$_2$/H$_2$ to methane production and emission from paddy field soils using a stable carbon isotopic signature and tracer experiments (9, 28, 34). The findings obtained showed the importance of H$_2$ in methanogenesis in paddy field soil. However, H$_2$ producers in paddy field soil have not been examined in detail. A few studies have investigated members that produce H$_2$ as secondary fermenters using protons as the electron acceptor, and is known that Clostridium spp. are common H$_2$-producing fermenters that often possess more than one paralog for [FeFe]-hydrogenases (21, 40), the relationship between H$_2$ production rate and the relative abundance of the transcripts of each hydA gene remains unknown in paddy field soil because actual H$_2$ production activity cannot be evaluated. Moreover, H$_2$ producers often possess more than one paralog for [FeFe]-hydrogenases (4, 20, 26), and their functions may differ. For example, Clostridium spp. are common H$_2$-producing fermenters that have various types of [FeFe]-hydrogenases (4). The transcriptional regulation of the respective hydA genes was found to differ during H$_2$ production in some Clostridium strains isolated from a digested sludge enrichment (22). Many species of Desulfovibrio have both periplasmic and cytosolic [FeFe]-hydrogenases (25). Desulfovibrio spp., which are representative sulfate-reducing bacteria in anoxic environments, establish a syntrophic relationship with hydrogenotrophic methanogens as secondary fermenters under sulfate-limited conditions (32). Therefore, the two types of [FeFe]-hydrogenases are predicted to play different roles under sulfate-reducing and syntrophic conditions, and their transcriptional patterns may also differ depending on the conditions present. However, information on how H$_2$ producers regulate the transcription of...
hydA paralogs during H₂ production is limited to some defined species (22).

In the present study, we attempted to reveal the transcriptional patterns of hydA paralogs during H₂ production for *Clostridium* sp. strain H2 and *Desulfovibrio* sp. strain A1, which belong to *Firmicutes* and *Deltaproteobacteria*, respectively, isolated from paddy field soil. Both isolates had multiple hydA genes in their genomes, and their transcriptional patterns and H₂-producing activities were analyzed.

Materials and Methods

**Microorganisms**

*Clostridium* sp. strain H2, *Desulfovibrio* sp. strain A1 (NBRC 101757), and *Methanobacterium* sp. strain AH1 (NBRC 103406), which were isolated from paddy field soil in the Aichi-ken Anjo Research and Extension Center, Anjo, Aichi, Japan (Anjo field; latitude 34°58′21″N, longitude 137°04′35″E), were used. The procedures used for isolating and clarifying the physiology and phylogeny of the isolates were described in Supporting information. The sequences of the 16S rRNA genes of strain H2 (LC194786), A1 (AB252583), and AH1 (AB302950 and AB302951) were almost identical (100%, 99%, and 99%) to *C. bifermentans* ATCC 6387 (AVNC01000016), *D. vulgaris* strain Hildenborough (AE017285), and *M. palustre* DSM 3108 (AF093061), respectively.

**Sequencing of hydA paralogs in Clostridium sp. H2 and Desulfovibrio sp. A1**

The sequences of the hydA genes in the genomes of strains H2 and A1 were elucidated by a PCR-based analysis from the genome information of reference bacteria, *C. bifermentans* ATCC 6387 (AVNC00000000), ATCC 19299 (AVNB00000000), and *D. vulgaris* Hildenborough (AE017285). The primer sets targeting each hydA gene were designed using Primer3Plus (36) (Table S1). Each hydA gene in the genomes of strains H2 and A1 was retrieved by PCR. Each reaction premix (25 μL) contained 2.5 μL of 10×PCR Buffer for KOD-Plus- (Toyobo, Osaka, Japan), 2.5 μL of dNTPs (Toyobo), 1 μL of 25 mM MgSO₄, 0.5 μL of KOD-Plus- (Toyobo), 0.15 μL of 50 μM forward and reverse primers, and 2.5 μL of template DNA. PCR was performed under the following conditions: 94°C for 2 min, 30 (H2hydA1, H2hydA2, H2hydA5, A1hydA1, and A1hydA2) or 40 (H2hydA3 and H2hydA4) cycles of 94°C for 15 s, 46°C (H2hydA3 and H2hydA4), 56°C (H2hydA1, H2hydA2 and H2hydA5), or 65°C (A1hydA1 and A1hydA2) for 30 s, and 68°C for 2 min. Amplicons were checked by agarose gel electrophoresis followed by ethidium bromide staining. PCR products were purified with the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany). A sequencing analysis of each amplicon was performed as described in a previous study (2) after direct cycle-sequencing for amplicons. Sequencing analysis of each amplicon was performed as described in a previous study (2) after direct cycle-sequencing for amplicons.

**HydA transcripts and RT-qPCR**

Cells were periodically harvested by centrifugation (15,000 × g, 4°C, 2 min) from 2 mL of cultures and stored at −80°C until used. RNA extraction from the harvested cells was performed using Nucleospin® RNA (Macherey-Nagel), according to the manufacturer’s procedure. DNase I (Promega, Madison, WI, USA) was used for additional DNA digestion. The complete digestion of DNA in RNA preparations was confirmed by PCR using the bacterial universal primer set 357/517r (23) in the absence of the reverse transcriptase. cDNA was synthesized from RNA preparations using the PrimeScript RT reagent Kit (Perfect Real Time) (Takara, Otsu, Japan) with random 6-mer primers according to the manufacturer’s instructions. The synthesized cDNAs were subjected to a qPCR analysis of hydA transcripts and 16S rRNAs. Each reaction premix (25 μL) contained 12.5 μL of SYBR® Premix EX Taq (Perfect Real Time) (Takara), 0.1 μL of 50 μM forward and reverse primers (Table S2), and 2 μL of template cDNA standard DNA (duplicate; 10⁻⁴ to 10⁻¹ copies μL⁻¹ and 10⁻⁴ to 10⁻¹ copies μL⁻¹ of hydA and 16S rRNA gene fragments, respectively, obtained from the genomic DNAs of strains H2 and A1 by PCR using the primer sets in Table S1). qPCR was performed using a Thermal Cycler Dice Real Time System (Takara).
under the following conditions: 95°C for 30 s, and 40 cycles of 95°C for 5 s and 65°C for 45 s. Standard curves showed good reaction efficiencies (76–98%) and $R^2$ values (>0.98). The numbers of 16S rRNA and hydA transcripts were calculated by absolute quantification based on standard curves. Ct values were obtained by the second derivative maximum method. The relative abundance of hydA was calculated with the following formula: the number of hydA transcripts/the number of 16S rRNAs at each sampling time. 16S rRNA was used as the normalization reference according to previous studies that quantified the relative abundances of the hydA transcripts of microorganisms (22, 40). Bartlett’s test and the Tukey-Kramer test were performed based on the relative abundances of hydA, using R (version 3.1.1; R Foundation for Statistical Computing [http://www.R-project.org/]). When the homoscedasticity of data was not confirmed, Dunnett’s T3 test was performed using R package ‘DTK’.

**Accession numbers of nucleotide sequences**

The nucleotide sequences of hydA obtained in this study have been deposited to the DDBJ database under accession numbers LC194779 to LC194785.

**Results**

**hydA paralogs in strains H2 and A1**

Clostridium sp. strain H2 had at least 5 phylogenetically different hydA genes (designated as H2hydA1–H2hydA5) in its genome, and the similarity of these hydA genes to the corresponding hydA genes of C. bifermentans ATCC 638 (AVNC0000000) and ATCC 19299 (AVNB00000000) was very high (99–100%) (Fig. 1A). Desulfovibrio sp. strain A1

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**Fig. 1.** Phylogenetic tree of hydA genes possessed by (A) strain H2 and (B) strain A1 (shown in bold). The neighbor-joining method was used to make the tree. Bootstrap values (500 resampling, ≥50%) are shown at the nodes. A1–A8 and the names of modular structures (M2, M4, M2d, TR[M2], M3, DM3, and TR[M3]) are based on the classification of clostridial hydA proposed by Calusinska et al. (4).
had at least 2 hydA genes (designated as A1hydA1 and A1hydA2, Fig. 1B), and the similarity of these hydA genes to the corresponding hydA genes of D. vulgaris Hildenborough (AE017285) was higher than 99%.

**Growth, metabolites, and H₂ production**

Strain H2 actively grew from 6 to 24 h after the short lag phase (Fig. 2A), and the turbidity of the culture decreased after the stationary phase. Active H₂ production occurred between 8 and 12 h after the inoculation, and approximately 1.9 μmol mL⁻¹ of H₂ was produced during the 96-h incubation (Fig. 2A). During the active growth phase, the concentration of glucose decreased from 40 μmol mL⁻¹ to 36 μmol mL⁻¹ and the concentrations of acetate and formate increased to 4.1 and 3.8 μmol mL⁻¹ respectively (Fig. 3A).

Strain A1 initiated active proliferation without a lag phase under sulfate-reducing conditions, and growth reached the stationary phase after 30 h (Fig. 2B). Lactate was linearly consumed with the concomitant reduction of sulfate. During growth, the concentration of acetate increased to 39 μmol mL⁻¹, while formate was not produced (Fig. 3B). Although H₂ production was very low during growth, its concentration slightly increased during the stationary phase (Fig. 2B).

In the co-culture of strains A1 and AH1, the turbidity of the culture exponentially increased until 138 h after the short lag phase, and CH₄ was produced linearly from 70 h to 240 h after the inoculation (Fig. 2C). Lactate was almost consumed during growth, and acetate and formate concentrations increased to 87 and 44 μmol mL⁻¹, respectively (Fig. 3C). H₂ was produced up to 0.50 μmol mL⁻¹; however, its concentration decreased with the initiation of CH₄ production. A total of 0.058 mmol mL⁻¹ of CH₄ was produced at the end of the incubation. Regardless of the assumption that CH₄ was produced from only H₂/CO₂ or both H₂/CO₂ and formate (29), H₂ production continuously occurred during growth (Fig. 2C).

**Fig. 2.** Turbidity and hydrogen and methane production in monocultures of (A) strain H2 and (B) strain A1, and (C) a co-culture of strains A1 and AH1. (bars=S.D., n=3). Deemed H₂ is calculated as the sum of the amount of H₂ and four-fold of methane. Deemed H₂ (1/2 formate) is estimated under the assumption when 50% of methane was produced from formate.

**Fig. 3.** Amounts of substrates (glucose, lactate, and sulfate) and major metabolites in monocultures of (A) strain H2 and (B) strain A1, and (C) a co-culture of strains A1 and AH1. (bars=S.D., n=3)
Relative abundance of transcripts of hydA and 16S rRNA genes

Based on $H_2$ production activity (Fig. 2), RT-qPCR analyses targeting 16S rRNA and hydA transcripts were performed on samples collected at 8, 12, 18, and 24 h for strain H2, 12, 24, 35, and 54 h for strain A1 under sulfate-reducing conditions, and 16, 69, 117, and 233 h after the inoculation for A1 and AH1 under syntrophic, methanogenic conditions.

In strain H2, the copy numbers of 16S rRNA and hydA transcripts were $10^9–10^{10}$ copies mL$^{-1}$ and $10^3–10^6$ copies mL$^{-1}$, respectively, during the incubation. The relative abundances of the transcripts of $H2hydA3$ and $H2hydA5$ increased 12 h after the inoculation along with increases in the $H_2$ production rate; however, the increase observed in the abundance of $H2hydA5$ was not significant ($p=0.12$) (Fig. 4A and S7A). The other paralogs ($H2hydA1$, $H2hydA2$, and $H2hydA4$) were also transcribed, but their relative abundances were low, unchanged, or decreased (18 h and 24 h versus 8 h in $H2hydA1$) during $H_2$ production (Fig. 4A and S7A).

In strain A1 under sulfate-reducing conditions, the copy numbers of 16S rRNA and hydA transcripts were $10^{10–10^{11}}$ copies mL$^{-1}$ and $10^5–10^7$ copies mL$^{-1}$, respectively. The relative abundances of the transcripts of $A1hydA1$ were markedly lower than those at 12 h, while that of $A1hydA2$ was always low during growth (Fig. 4B and S7B).

In the co-culture of strains A1 and AH1 under syntrophic, methanogenic conditions, the copy numbers of the 16S rRNA and hydA transcripts of strain A1 were $10^8–10^9$ copies mL$^{-1}$ and $10^3–10^6$ copies mL$^{-1}$, respectively. Similar to sulfate-reducing conditions, the relative abundances of the transcripts of $A1hydA1$ linearly decreased during proliferation ($p<0.05$), whereas those of $A1hydA2$ increased until 117 h and then decreased at 233 h after the inoculation, which corresponded to the increase in the deemed $H_2$ production rate (Fig. 4C and S7C).

Discussion

We herein examined the transcriptional regulation of the hydA paralogs of two $H_2$ producers during $H_2$ production and their predicted functions. We also discussed further prospects for elucidating the ecology of $H_2$ producers in paddy field soil by a molecular biological analysis targeting hydA paralogs.

Strains H2 and A1 had at least 5 and 2 hydA paralogs in their genomes by a PCR-based analysis based on the genomic information of their close relatives. The number of paralogs was within a predictable range of hydA paralogs in Clostridium spp. (2–7 hydA paralogs) (4) and Desulfovibrio spp. (1–5 hydA paralogs) (25). The closest relatives of each hydA paralog in strains H2 and A1 were those in C. bifermentans ATCC 636 and D. vulgaris strain Hildenborough with high similarities (99–100%), which have 5 and 2 hydA paralogs in their genomes, respectively. The phylogeny of these hydA paralogs was diverse (Fig. 1A), suggesting that strain H2 has multiple [FeFe]-hydrogenases with different modular structures because [FeFe]-hydrogenases with different modular structures are predicted to contain different HydA subunits (4). According to the classification of clostridial hydA genes by Calusinska et al. (4), the sequence information of the hydA paralogs in strain H2 suggested that $H2hydA1$, $H2hydA2$, and $H2hydA4$ each encodes the monomeric [FeFe]-hydrogenase, and $H2hydA3$ and $H2hydA5$ each encodes a catalytic subunit of the trimeric [FeFe]-hydrogenase. The variety of [FeFe]-hydrogenases in strain H2 indicates an interaction with various electron donors because of different numbers of FeS clusters and modules in their structures (4, 38), suggesting the versatile ability of strain H2.

Positive relationships between hydA transcription and $H_2$ production have previously been reported for Clostridium spp. (6, 40). However, the present study showed that the transcriptional regulation of hydA in strain H2 differed...
among the paralogs. Morra et al. (22) and Calusinska et al. (5) also showed different regulation patterns for hydA paralogs in three Clostridium species (C. beijerinckii, C. butyricum, and C. perfringens) and C. butyricum CWBI 1009. These findings suggested that each hydA has different roles in H₂ metabolism. However, even if a hydA paralog has a similar domain structure among different microorganisms, regulating the transcription of the hydA paralog may differ depending on the microorganisms. In this study, the relative abundances of the transcripts of H₂hydA3 and H₂hydA5 increased during active H₂ production (Fig. 4). The transcription of hydA paralogs Cbei_4110 (C. beijerinckii SM10; [22]) and CBY 2047 (C. butyricum SM32; [22]), which had a TR(M3) structure (22), was constant during H₂-producing growth. However, the relative abundances of the transcripts of H₂hydA3 and H₂hydA5 of strain H₂, which were phylogenetically grouped into A6-TR(M3) [FeFe]-hydrogenases (Fig. 1A), changed during H₂-producing growth. Therefore, the regulation of hydA transcription during H₂ production may depend not only on the types of hydA, but also on microorganisms and growth (environmental) conditions.

H₂hydA3 in strain H₂ was phylogenetically close to hydA genes encoding a subunit of bifurcating [FeFe]-hydrogenases, which produce H₂ by receiving electrons from not only reduced ferredoxin, but also NADH (31). Bifurcating [FeFe]-hydrogenases may catalyze H₂ production under low H₂ pressure (31, 33, 43) possibly when the amount of ATP synthesized increases in cells (43). Therefore, this bifurcating [FeFe]-hydrogenase partly encoded in H₂hydA3 appears to contribute to H₂ production when substrates are rich and H₂ consumers co-exist.

Two hydA paralogs possessed by strain A1 also differed with each other in terms of their phylogeny, although both were closely related to the hydA genes of D. vulgaris strain Hildenborough. A1hydA1 and A1hydA2 were predicted to encode the periplasmic [FeFe]-hydrogenase and cytosolic bifurcating [FeFe]-hydrogenase, respectively, according to the genomic analysis of sulfate reducers (25, 37).

A1hydA1 and A1hydA2 were both transcribed under sulfate-reducing and syntrophic conditions (Fig. 4B and C). Active H₂ production was not observed under sulfate-reducing conditions; however, the relative abundance of the transcripts of A1hydA1 was high at the initial growth phase (Fig. 4B). Since periplasmic [FeFe]-hydrogenases in Desulfovibrio species are known to catalyze H₂ consumption (25, 27, 38), H₂ produced by strain A1 may be consumed in the sulfate-reducing process in parallel, as indicated by Odom and Peck (24). However, since the relative abundance of the transcripts of the other hydA paralog (A1hydA2) did not change under sulfate-reducing conditions, the role of A1hydA1 in H₂ metabolism currently remains unknown.

Under syntrophic conditions, active H₂ production occurred as CH₄ was actively produced by the hydrogenotrophic methanogenic archaeon strain AH1; however, formate also appears to be utilized in part for CH₄ production. Under these conditions, the transcriptional pattern of A1hydA1 was similar to that under sulfate-reducing conditions, namely, the relative abundance of the transcripts decreased with time. On the other hand, the relative abundance of the transcripts of A1hydA2 was 6–31-fold higher than that under sulfate-reducing conditions, particularly at the time point of active H₂ production (Fig. 4C), suggesting that A1hydA2 of strain A1 is related to H₂ production in syntrophic methanogenesis. As described above, A1hydA2 is predicted to encode a cytosolic bifurcating [FeFe]-hydrogenase (25, 37). However, the role of bifurcating [FeFe]-hydrogenases in sulfate reducers including Desulfovibrio species in H₂ metabolism remains unclear (25), and, thus, further studies are needed.

The periplasmic [FeFe]-hydrogenases of sulfate reducers have been reported to play an important role in interspecies H₂ transfer. For example, D. vulgaris strain Hildenborough, which has a mutation in the gene of periplasmic [FeFe]-hydrogenase, showed a low growth rate under sulfate-deficient syntropic conditions co-cultured with a hydrogenotrophic methanogen (39). D. alaskensis G20 increased the relative abundances of the transcripts of the gene of periplasmic [FeFe]-hydrogenase under syntrophic conditions (15). On the other hand, the relative abundance of the transcripts of the gene of D. alaskensis G20 did not differ under sulfate-reducing and syntrophic conditions, and periplasmic [NiFe]-hydrogenases and formate dehydrogenases may contribute to interspecies electron transfer (18). Syntrophic partners (H₂ scavengers) also influenced the relative abundance of the transcripts of the hydrogenase genes of H₂ producers (19). Discrepancies between these findings and the present results suggest that the roles of periplasmic [FeFe]-hydrogenases in H₂ metabolism differ depending on not only the strains, but also growth conditions. Strain A1 may have independently developed a unique H₂ metabolism system to adapt to the environment of paddy field soil, thereby influencing the transcriptional patterns of hydA genes.

Many bacteria have multiple [FeFe]-hydrogenase genes in their genomes (4, 25, 30, 37, 38). This study showed that the relative abundances of the transcripts of some hydA did not have positive relationships with H₂ production irrespective of high or low relative abundances. These findings indicate that analyses of hydA in the environment are associated with the risk of overestimating the diversity and activity of potential H₂ producers, as already discussed in other studies (1, 30). Meanwhile, this study showed the up-regulated transcription of some hydA (H₂hydA3, H₂hydA5, and A1hydA2) during H₂ production and differences in the relative abundances of the transcripts between sulfate-reducing and syntrophic conditions. Some hydA transcripts (14R348 [LC041901] and 1dR151 [LC041552] in Fig. S8) closely related to H₂hydA3 and H₂hydA5 of strain H₂ and A1hydA2 of strain A1 were actually detected during anaerobic rice straw decomposition in paddy field soil (2). These findings indicate that the transcription of some hydA certainly reflects active H₂ production.

Paddy fields, in which apparent H₂ production is very low (12), are flooded during rice cultivation, and the field is drained after rice harvest, resulting in soil conditions that markedly change between oxic and anoxic conditions. These changes affect the metabolic activities and growth/survival strategies of microorganisms to adapt to these changes; however, the community structures of bacteria and methanogenic archaea are known to be stable irrespective of dynamic changes in soil conditions (11, 41). The roles and active members of H₂ producers must also change and shift dynamically during H₂ production depending on soil conditions. However, limited
transcription of $H_2$ producers

Paddy field soil harbors numerous microorganisms including diverse $H_2$ producers (1, 2). The structure and function of [FeFe]-hydrogenases and the transcriptional regulation of the hydA paralogs of soil $H_2$ producers need to be diverse. Therefore, further studies on the regulation of the hydA paralogs of various soil isolates are needed and will provide new perspectives for understanding active $H_2$ producers and $H_2$-dependent microbial interactions in paddy field soil.

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