APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 2010, p. 3387–3390 Vol. 76, No. 10
0099-2240/10/$12.00 doi:10.1128/AEM.02444-09

Succession of the Bacterial Community and Dynamics of Hydrogen Producers in a Hydrogen-Producing Bioreactor▽†

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Received 8 October 2009/Accepted 10 March 2010

Variation in the hydrogen production rate was consistent with the succession of dominant bacteria during the batch fermentation process. Denaturing gradient gel electrophoresis (DGGE) of 16S rRNA genes and quantitative analysis of the *hydA* genes at both the DNA and mRNA levels confirmed that *Clostridium perfringens* was the most dominant hydrogen producer in the bioreactor.

Biological hydrogen production is expected to be an important strategy in the development of clean and sustainable alternative energy sources (6). Anaerobic mixed cultures are frequently used as inocula in hydrogen production (3, 4, 10). Compared with pure cultures, mixed cultures have the advantage of more technical feasibility and the potential for using complex carbohydrates as substrates (14), possibly because different members of the bacterial community play complementary or mutually beneficial roles in utilizing substrates, providing growth factors, eliminating feedback inhibition, etc. (12, 17). Many sources of natural microflora, including compost (20), different sludges (4, 10, 24), and soils (21), have been used as inocula for hydrogen production after inactivation of hydrogenotrophic methanogens. However, whether pretreated natural microflora is the most efficient mixed culture for hydrogen production is still unknown. Understanding the relationships between variation in microbial composition and its hydrogen production efficiency is the first step in reconstructing more-efficient hydrogen-producing consortia.

The 16S rRNA gene has been widely used as a universal molecular marker (7, 15). The Fe-hydrogenase gene (*hydA*), which is usually involved in proton reduction (H_2 production) to dispose of excess reducing equivalents (1, 2, 22) in *Clostridium* spp. and sulfate reducers, has recently been used as a molecular marker to distinguish potential hydrogen-producing bacteria in mixed cultures (3, 8, 23). Therefore, in this study, the V3 regions of the 16S rRNA gene and the *hydA* gene were used as biomarkers to investigate the succession of the bacterial community during hydrogen production in a batch culture.

Hydrogen production. Hydrogen production was conducted in a 10-liter continuously stirred tank reactor (CSTR) with a 7-liter working volume and no specific requirement to remove oxygen from the culture and headspace. Cattle dung compost was pretreated by boiling for 5 min and then used as an inoculum. The fermentation reaction mixture was kept at pH 5.8, with stirring at 100 rpm. Other fermentation conditions for batch hydrogen production from 18 g liter^{-1} sucrose were the same as in a previous study (27). The evolved biogas was collected by the water displacement method. The concentrations of hydrogen in the biogas were quantified as previously described (18). A total of 25.6 liters of hydrogen was evolved in the batch fermentation, corresponding to a hydrogen yield of 3.11 mol H_2 mol sucrose^{-1} or 200 ml H_2 g sucrose^{-1}. The reported high hydrogen yields from sucrose in dark fermentation were 3.8 mol H_2 mol sucrose^{-1} by a *Clostridium pasteurianum* strain and 2.73 mol H_2 mol sucrose^{-1} by microflora (5, 25).

Over 72 h, the hydrogen-producing process was divided into five phases according to the hydrogen production rate: the lag phase (0 to 12 h after inoculation), exponential phase (12 to 16 h), stationary phase (16 to 24 h), early decline phase (24 to 36 h), and late decline phase (36 to 72 h) (Fig. 1). The oxidation reduction potential (ORP) in the medium was traced with a built-in ORP probe (Mettler-Toledo, Columbus, OH). The ORP value was above zero during the hydrogen-producing lag phase, dropped dramatically from 148 mV to −246 mV when hydrogen production was started, and then remained below −156 mV in the hydrogen-producing exponential and stationary phases (Fig. 1).

Succession in the bacterial community as revealed by 16S rRNA genes. Genomic DNA was extracted, using the sodium dodecyl sulfate (SDS)-based DNA extraction procedure (26). The V3 regions of the bacterial 16S rRNA genes were PCR amplified and analyzed by denaturing gradient gel electrophoresis (DGGE) as previously described (16). During the fermentation process, the bacterial compositions of all DNA samples clustered into three groups, based on similarities among the DGGE profiles as assessed with Quantity One software, version 4.4 (Bio-Rad) (Fig. 2B). The three groups were consistent with the variations in hydrogen production rates and ORP values during the hydrogen-producing process.

All of the sequenced fragments recovered from clear DGGE

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† Supplemental material for this article may be found at http://aem.asm.org/.

▽ Published ahead of print on 19 March 2010.

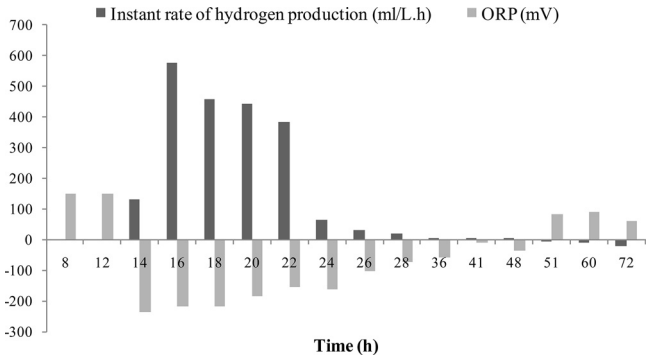

FIG. 1. Changes in the hydrogen production rate and ORP during the fermentation process (8 to 72 h).

bands were most closely affiliated with the 16S rRNA genes of species from the genera *Bacillus* and *Clostridium* (Fig. 2A and Table 1). It appeared that boiling the cattle dung compost for 5 min had killed almost all non-spore-forming microorganisms. As the bioreactor was not sparged with N_2 to remove oxygen in the headspace and in the culture, the ORP was above 100 mV at the initial stage of fermentation, which is why *Bacillus* spp.,

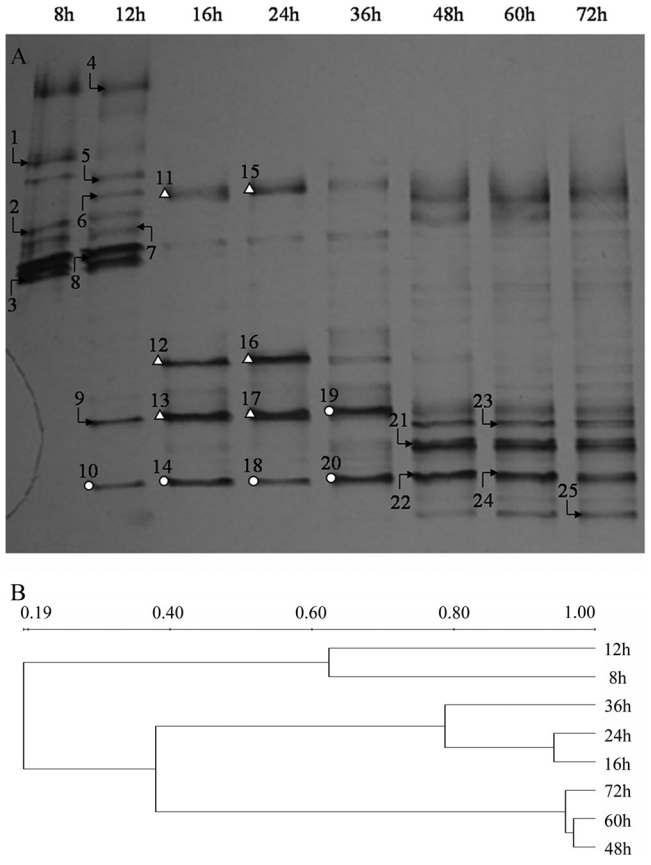

FIG. 2. 16S rRNA gene DGGE profiles of samples collected at different fermentation stages (8 to 72 h). (A) Bands marked with triangles (△) were identified as *C. perfringens*. Bands marked with circles (○) were identified as *C. beijerinckii*. Other bands marked with numbers were identified as shown in Table 1. (B) Clustering of DGGE profiles as determined by using Quantity One software.

TABLE 1. Sequence analyses of 16S rRNA genes recovered from single DGGE bands

Band ID[a]	Most closely affiliated organism in GenBank database	% Similarity
8h-1	*Bacillus* sp. TTV-7	100
8h-2	*Bacillus licheniformis*	100
8h-3	*Bacillus mojavensis*	100
12h-4	*Bacillus megaterium* strain A2P4	100
12h-5	*Bacillus subtilis* strain B1-33	100
12h-6	*Bacillus circulans*	100
12h-7	*Bacillus* sp. MC6B22	98
12h-8	*Bacillus cereus* strain TpP-2	98
12h-9	*Bacillus* sp. BBDP1032	99
12h-10	*Clostridium beijerinckii*	100
16h-11	*Clostridium perfringens*	100
16h-12	*Clostridium perfringens*	100
16h-13	*Clostridium perfringens*	100
16h-14	*Clostridium beijerinckii*	100
24h-15	*Clostridium perfringens*	100
24h-16	*Clostridium perfringens*	100
24h-17	*Clostridium perfringens*	100
24h-18	*Clostridium beijerinckii*	100
36h-19	*Clostridium beijerinckii*	100
36h-20	*Clostridium beijerinckii*	100
48h-21	*Clostridium lundense* strain DSM 17049	100
48h-22	*Clostridium peptidivorans*	100
60h-23	*Clostridium lundense* strain DSM 17049	100
60h-24	*Clostridium lundense* strain DSM 17049	100
72h-25	*Clostridium vincentii*	100

[a] The number before the hyphen represents the lane of the DGGE gel shown in Fig. 2A. The number after the hyphen represents the band mark in Fig. 2A. ID, identity.

facultative anaerobes, dominated in the lag phase. With the growth of *Bacillus* spp., oxygen was exhausted, the ORP began to decrease rapidly, and an anaerobic environment suitable for the growth of anaerobic and hydrogen-producing bacteria began to appear (9). *Clostridium beijerinckii* and *Clostridium perfringens* were found to be the dominant species in the exponential and stationary phases. *Clostridium lundense* and other *Clostridium* spp. began to appear in the decline phases, when hydrogen production had stopped and hydrogen uptake was observed (Table 1 and Fig. 1).

Variation in copy numbers of *hydA* genes at the DNA or cDNA level as revealed by quantitative real-time PCR. At least five *Clostridium* species were detected by DGGE analysis during the hydrogen-producing process in our bioreactor, suggesting that the *Clostridium* species were the major hydrogen producers in this study, as in other anaerobic bioreactors (13, 19). Based on the conserved regions of the H domains in the *hydA* genes from nine *Clostridium* species (see Table S1 in the supplemental material), a primer set for fragments with an average length of 200 bp (see Table S2 and Fig. S1A in the supplemental material) was designed and used in quantitative PCR to monitor the quantity of potential hydrogen producers and the expression levels of their *hydA* mRNAs. Total RNA was extracted, using an RNeasy kit (Qiagen, Hilden, Germany) (11). Reverse transcription of isolated RNA was carried out with random primers, using an iScript cDNA synthesis kit (Toyobo, Osaka, Japan). The calibration curves against the cycle threshold (C_T) for the *hydA* gene showed a good linear relationship over six orders of concentration, from 1.0×10^1 to 1.0×10^6 copies μl^{-1}, with a R^2 value of 0.9999.

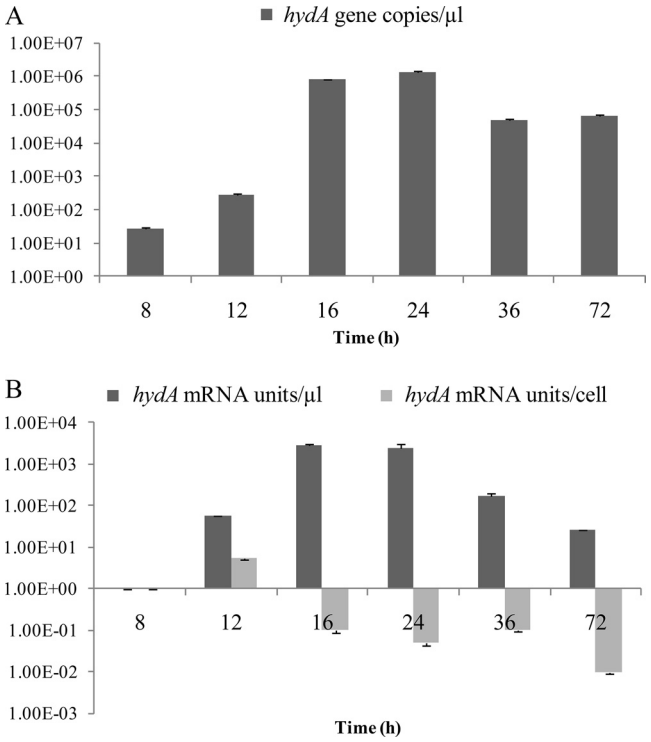

FIG. 3. Numbers of *hydA* gene copies (A) and *hydA* mRNA units (B) at different fermentation phases, based on quantitative PCR. One unit is defined as the number of *hydA* mRNA copies in 1 μl of a sample at 8 h or the number of *hydA* mRNA copies expressed by one hydrogen producer at 8 h.

The copy number of the *hydA* genes at DNA level, which represents the abundance of the potential hydrogen producers, was very low in the lag phase and then increased to approximately 1.0×10^6 copies μl^{-1} in the exponential phase. Several hours after the bioreactor stopped evolving hydrogen, the copy number of the *hydA* genes decreased dramatically, to less than 1.0×10^5 copies μl^{-1} (Fig. 3A).

The copy number of the *hydA* genes per reaction volume at the cDNA level, which reflects the total expression level of *hydA* mRNA per reaction volume (*hydA* mRNA units μl^{-1}), peaked in the exponential and stationary phases (Fig. 3B), consistent with the highest instant hydrogen production rate and the dramatic decrease in the ORP (Fig. 1). By contrast, the relative *hydA* mRNA expression level per cell was highest in the early exponential phase (12 h), while no hydrogen production was observed (Fig. 3B). The rapid increase in the abundance of hydrogen producers in the late exponential phase might be related to the highest total expression level of *hydA* mRNA.

Succession of potential hydrogen producers and their gene expression levels based on *hydA* DNA/cDNA clone libraries. Another primer set for fragments with an average length of 695 bp (see Table S2 and Fig. S1B in the supplemental material) was designed based on the H domains in the *hydA* genes and used to investigate the diversity and composition of the *hydA* genes in the bioreactor by sequence analysis. In total, 13 different *hydA* gene types were identified from the libraries (Table 2). No potential hydrogen producers and no *hydA* mRNA expression were detected in the early lag phase. Hydrogen production was still not detected 12 h after inoculation; however, the *hydA* genes of *C. perfringens*, *C. beijerinckii*, and two *Paenibacillus* D14-like species were detected in the *hydA* DNA library from this phase, but only the *hydA* mRNAs from *C. perfringens* and *C. beijerinckii* were detected in the corresponding *hydA* cDNA library. In the exponential and stationary phases, *C. perfringens* became the dominant hydrogen producer and showed active expression of the *hydA* mRNA. In the early decline phase, three *hydA* gene types closely matched to that of a *Clostridium* 7_2_43 FAA-like species appeared, and the relative abundance of *C. perfringens* decreased significantly, but its *hydA* mRNAs were still dominant in the *hydA* cDNA library. In the late decline phase, six new *Clostridium*-like species were detected at either the DNA or cDNA level. The *hydA* mRNAs of *C. perfringens* decreased to the lowest level, and the

TABLE 2. Distribution of *hydA* DNA and cDNA clones in different fermentation phases

Type no.	GenBank accession no.	Most closely affiliated organism (% similarity)[a]	Relative abundance of clones at indicated fermentation phase[b] (%)				
			12 h	16 h	24 h	36 h	72 h
1	GQ915195	*Clostridium perfringens* SM101 (99%)	29[c]/90[d]	93/90	100/100	25/90	7/7
2	GQ915139	*Clostridium beijerinckii* (99%)	21/10	7/10	ND	ND	ND
3	GQ915142	*Paenibacillus* sp. D14 (96%)	43/ND	ND	ND	ND	ND
4	GQ915151	*Paenibacillus* sp. D14 (82%)	7/ND	ND	ND	ND	ND
5	GQ915188	*Clostridium* sp. 7_2_43FAA (82%)	ND	ND	ND	8/ND	ND
6	GQ915191	*Clostridium* sp. 7_2_43FAA (97%)	ND	ND	ND	8/ND	ND
7	GQ915185	*Clostridium* sp. 7_2_43FAA (89%)	ND	ND	ND	59/10	58/ND
8	GQ915201	*Clostridium* sp. 7_2_43FAA (68%)	ND	ND	ND	ND	21/ND
9	GQ915203	*Clostridium butyricum* (100%)	ND	ND	ND	ND	7/ND
10	GQ915205	*Clostridium carboxidivorans* P7 (72%)	ND	ND	ND	ND	7/13
11	GQ915255	*Clostridium botulinum* Ba4 (80%)	ND	ND	ND	ND	ND/13
12	GQ915259	*Alkaliphilus oremlandii* OhILAs (72%)	ND	ND	ND	ND	ND/40
13	GQ915260	*Paenibacillus* sp. D14 (71%)	ND	ND	ND	ND	ND/27

[a] Similarities are based on BLASTp results for HydA amino acid sequences.
[b] No obvious band was detected on agarose gel after 30 cycles of PCR amplification from the DNA and cDNA samples at 8 h. Therefore, corresponding clone libraries were not constructed. ND, not detected.
[c] Percentage of the corresponding clone in the *hydA* DNA clone library.
[d] Percentage of the corresponding clone in the *hydA* cDNA clone library.

hydA cDNA library became dominated by the *hydA* mRNAs from these newly emerging species.

Clostridium peptidivorans, *C. lundense*, and *Clostridium vincentii* were identified in the decline phase by sequencing the obvious bands on 16S rRNA gene DGGE profiles. However, sequencing of the *hydA* DNA and cDNA libraries from the early and late decline phases indicated nine newly emerging types of *hydA*, namely, types 5 to 13. As there are no reported sequences for the *hydA* genes from *C. peptidivorans*, *C. lundense*, and *C. vincentii*, it is difficult to determine which of the three possible *Clostridium* species are represented by the above newly emerging *hydA* genes. In the decline phases, nearly no hydrogen was produced, hydrogen uptake was observed when the ORP rose to about 100 mV, and a low level of expression of *hydA* mRNA was detected. This phenomenon might be because the Fe-hydrogenases are bidirectional hydrogenases (1) that catalyze hydrogen uptake when the metabolic system is more likely to accept electrons.

Conclusions. Both *C. perfringens* and *C. beijerinckii* contributed to hydrogen production in the bioreactor, but *C. perfringens* was the most dominant hydrogen producer there. The hydrogen production rate and ORP values were consistent with the succession of dominant bacterial species during the different phases of the hydrogen-producing process. It appears that rapid and complex bacterial succession occurs even within a batch culture during hydrogen production.

Nucleotide sequence accession numbers. Nucleotide sequences of the *hydA* and 16S rRNA genes from clone libraries were deposited in GenBank under accession numbers GQ915138 to GQ915262 and GU735571 to GU735638, respectively.

This work was funded by the National Natural Science Foundation of China (30670042), the Knowledge Innovation Key Program of the Chinese Academy of Sciences Project (grant no. KSCX2-YW-G-022), and the High-Tech Research and Development Program of China (863: 2007AA021302).

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