

Use of TaqMan gene probe for real-time monitoring of acidophilic hydrogen-producing bacteria

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Abstract Using a TaqMan gene probe targeting the 16S rDNA of acidophilic hydrogen-producing bacteria (HPB), the abundance of this HPB in the biomass was found to increase from 0.02 to 72% in a single batch treating rice slurry waste at pH 4.5 over 130 h. The corresponding abundances were 4.4% in the batch operated at pH 5.0 and 0.01–0.02% at pH 5.5–6.5. During the growth phase, the generation time for the acidophilic HPB at pH 4.5 averaged 3.5 h.

Keywords 16S rDNA · Acidophilic · Hydrogen · Probe · Primer · TaqMan

Introduction

Bio-hydrogen production from wastewater has attracted much attention recently (Adams and Stiefel 1998). The population of hydrogen-producing bacteria (HPB) in a reactor is conventionally quantified by the culture-based methods (Ueno et al. 2001), the reliability of which is limited since the majority of HPB may be unculturable (Ueno et al. 2001). In the past 2 decades, analytic techniques targeting 16S rDNA or functional genes have been widely used for

microbial quantification in the mixed cultures. These include hybridization-based techniques, such as fluorescence in situ hybridization (FISH) (Fang et al. 2005), as well as PCR-based techniques, such as denaturing gradient gel electrophoresis (DGGE) and cloning-sequencing (Ueno et al. 2001). More recently, a new quantitative real time PCR technique based on TaqMan probe has emerged as an effective means for the detection and quantification of microorganisms at very low concentrations (Ginzinger 2002).

TaqMan probe is a fluorescent oligonucleotide with a 5'-end fluorophore (called "reporter") and a 3'-end quencher. As PCR proceeds, the fluorescent intensity increases proportionally with the amount of the amplicons. TaqMan probes have been used for the quantification of pathogens (Oberst et al. 1998) as well as microorganisms in various environmental samples (Takai and Horikoshi 2000). However, applications of TaqMan probe for the study of microbial reactions in still limited. This study was thus conducted first to design a TaqMan gene probe targeting the 16S rDNA of a new group of acidophilic HPB (Fang et al. 2006a), and then applied it to monitor the change of HPB population in a batch reaction in real time.

Materials and methods

DNA extraction and amplification

Genomic DNA were extracted from five acidophilic H₂-producing sludges in batch reactors treating rice

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slurry waste containing 5.5 g carbohydrate/l at 37°C at pH levels varied from pH 4.5 to 6.5 (Fang et al. 2006a). All batch reactions ended after 130 h as the gas production ceased by then. The optimal pH for H₂ production was found as pH 4.5, at which the maximum specific H₂ production rate was 2.1 l/g-VSS-d and the H₂ yield was 346 ml/g carbohydrate, corresponding to a yield of 62.6%. Thus, sludge samples collected throughout the batch reaction in the pH 4.5 reactor was examined.

The procedures of genomic DNA extraction were as follows: a 1 ml mixed liquor sample was homogenized in a Mini-Beadbeater (Biospec Products, Bartlesville, OK) with 0.2 g 0.1 mm glass beads at 5,000 rpm for 10–20 s, after centrifugation and re-suspension in a pH 8.0 lysis buffer (0.75 M sucrose, 100 mM EDTA, 100 mM Tris/HCl). It was then incubated three times sequentially in a sterilized Falcon tube: (1) at 37°C for 30 min after adding 4 ml lysis buffer containing 100 µl each of lysozyme solution (50 mg/ml) and achromopeptidase solution (1 mg/ml); (2) at 37°C for 2 h after adding 0.5 ml SDS solution (10%) and 50 µl proteinase K solution (20 µg/ml); and (3) at 65°C for 20 min after adding 0.8 ml 5 M NaCl and 0.6 ml of a mixed solution containing 10% (v/v) CTAB and 4.1% NaCl. Afterwards, the solution was mixed with a 6.6 ml of phenol/chloroform/isoamylalcohol (25:24:1, by vol.) and centrifuged (3,000g for 20 min). The supernatant (5.0 ml) was mixed with an equal volume of chloroform/isoamylalcohol (24:1 v/v) solution and centrifuged again (3,000g for 20 min). The supernatant (4 ml) was lastly mixed with 2.4 ml isopropanol and kept at 4°C overnight. The DNA precipitate was then centrifuged (1,000g for 10 min) and rinsed with 0.8 ml 70% (v/v) ethanol. After another centrifugation (10,000g for 10 min), the DNA precipitate was air-dried (5 min) before dissolving in 100 µl water.

The DNA of the sludge sampled at 130 h was amplified by PCR using the primer set of EUB8F (5'-AGAGTTTGATCMTGGCTCAG-3') and UNIV1392R (5'-ACGGGCGGTGTGTRC-3') at the annealing temperature of 54°C. The PCR-amplified product was then used to build a clone library with the TA Cloning Kit (Invitrogen Corporation, Carlsbad, CA) as described previously (Fang et al. 2006a). According to sequencing results of the inserted partial 16S rDNA, there were nine operational taxonomy units (OTUs) in the acidophilic hydrogen-producing

sludge. Among them, the most abundant OTU, HPB-R-2, and five others, i.e. HPB-R-1, -4, -16, -25, and -26, formed a group which was most closely related to one known species, *Clostridium acetobutylicum* and one uncultured clone *Clostridium* sp. 44a-T5zd (AY082483) with the similarities less than 97%. These six OTUs were presumably the acidophilic HPB.

Quantitative real-time PCR

A TaqMan gene probe and a primer set were designed based on the common conserved 16S rDNA regions of these acidophilic HPB and the reference microorganisms retrieved from the GenBank using BioEdit (Hall 1999). Quantification of the acidophilic HPB 16S rDNA in each extracted DNA samples was performed by a qRT-PCR assay using an iCycler IQ System (Bio-Rad, Hercules, CA, USA). Most of such quantifications were conducted in triplicate with a standard deviation ranging 10–18%. About 9 µl of template was added to 10 µl of 2X iQ Supermix, plus 1 µl mixture of 100 nM oligonucleotide primers and 100 nM TaqMan probe. After 10 min of denaturation at 95°C, PCR was conducted for 50 cycles, each of which consisted of two steps: a denaturation at 95°C for 15 s plus a step combining annealing and primer extension at 60°C for 1 min. The C_t value, i.e. the number of cycles at which the amplified DNA fragment exceeded the threshold concentration, was analyzed using the iCycler Software (version 3.1) (Zhang and Fang 2006).

The clone containing the 16S rDNA fragment of HPB-R-2 (1400 bps) was selected to recover the DNA insert in the plasmid using the primer set of M13F (5'-CAGGAAACAGCTATGAC-3') and M13R (5'-GTTTGATCCTGGCTCAG-3') at the annealing temperature of 54°C. The obtained PCR product containing the inserted 16S rDNA and the plasmid fragment between M13F and M13R binding sites (200 bps) had a total molecular weight of 1.06×10^6 . DNA concentration of the purified PCR product was 15 ng/µl (assuming that each A₂₆₀ unit corresponded to 50 ng double-stranded DNA/µl; Sambrook et al. 1989). Thus the concentration of PCR product was calculated as 8.55×10^9 molecule/µl. As each molecule contained one copy of inserted DNA, the HPB-R-2 16S rDNA concentration in the PCR product solution was estimated as 8.55×10^9 copies/µl.

This solution was 10-fold serially diluted and used to construct the standard curve of C_t vs LOG_{10} value of the starting copy number. The PCR efficiency may be calculated from the slope of the standard curve (Abell and Bowman 2005) as follows:

$$\text{Efficiency} = [10^{(-1/\text{slope})}] - 1 \tag{1}$$

Accession numbers

The nucleotide sequence data reported in this paper have been assigned by the GenBank, EMBL and DDBJ databases the following accession numbers: AY862515 (HPB-R-1), AY862510 (HPB-R-2), AY862513 (HPB-R-4), AY862512 (HPB-R-16), AY862510 (HPB-R-25) and AY862511 (HPB-R-26).

Results and discussion

Phylogeny of the acidophilic HPB

As shown in Fig. 1, BLAST comparison of the nearly full-length 16S rDNA sequences of HPB-R-1, -2, -4, -16, -25, and -26 showed that the HPB was closely related to two known H_2 -producing species, i.e. *C. acetobutylicum* and *C. butyricum*, plus one uncul-

tured clone *Clostridium* sp. 44a-T5zd (AY082483), which was found in an acid drainage system. The similarity within the HPB was over 97%. However, this group was distantly related to other species, such as *Clostridium* sp. 44a-T5zd (similarity >97%), *C. acetobutylicum* (>95%) and *C. butyricum* (>90%). This indicates that the acidophilic HPB is likely an unknown hydrogen-producing species.

Acidophilic HPB specific TaqMan gene probe

The 16S rDNA sequences of HPB and their closest related species were retrieved from the GenBank. The TaqMan probe including a primer set were consequently designed based on the common reserved regions found in the HPB, using the Primer Express software (Applied Biosystems, Foster City, CA). The TaqMan gene probe (5'-TTTTGCCAAAGAATTC-3', $T_m = 56^\circ\text{C}$) was labeled at the 5'-end with the 6-carboxyfluorescein (FAM) reporter dye and at the 3'-end with the MGB quencher (Applied Biosystems, Foster City, CA). The primer set, which included the forward primer (5'-CCATAACACAAAGGAAACGCATGT-3', $T_m = 58.5^\circ\text{C}$) and reverse primer (5'-GCGGGCCCATCTCACAA-3', $T_m = 60.6^\circ\text{C}$), amplified fragments of 62 bps.

Figure 2 shows that the designed TaqMan probe has a perfect match with all the acidophilic species

Fig. 1 Phylogenetic tree of acidophilic HPB group and its related species with the closest known species *Clostridium butyricum* as the outgroup

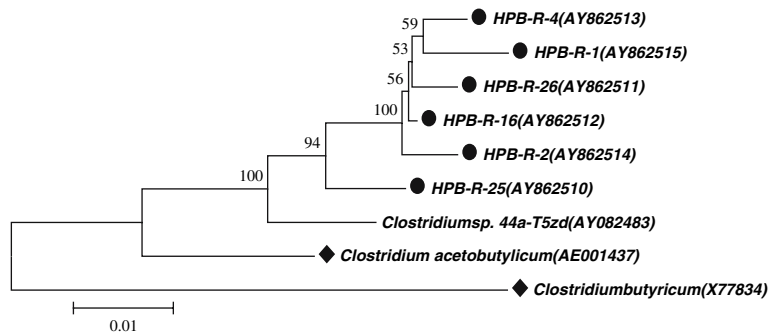


Fig. 2 Specificity of the TaqMan probe and primer set



but a few mismatches with the closely-related uncultured bacteria *Clostridium* sp. 44a-T5zd and the known H₂-hydrogen-producing *C. acetobutylicum* and *C. butyricum*. The specificity of the TaqMan probe was confirmed using the CHECK_PROBE software provided through the Ribosomal Database Project.

Standard curve and detection limit

During the PCR amplification, C_t is inversely proportional to the log value of the initial concentration of the target. Based on a pre-calibrated standard curve, the initial concentration of the target DNA can be estimated from its C_t measurement.

Figure 3 is the standard curve of C_t plotted against the serial dilutions of PCR product of the HPB-R-2 16S rDNA fragment. Over the whole dilutions series, ranging from 7.7×10^2 to 7.7×10^8 DNA copies per PCR sample, C_t increased linearly with the LOG₁₀ value of starting copy number with a slope of -3.49 , corresponding to a PCR efficiency of 93% based on Eq. (1). The correlation between C_t and the LOG₁₀ value of the starting copy number became nonlinear below 770 copies per PCR sample, which corresponds to 85.5 copies/ μ l. Such detection limit was similar to those found in other TaqMan probe applications (Nelson et al. 1999; Zhang and Fang 2006).

HPB quantifications

Table 1 summarizes the concentration of HPB 16S rDNA extracted from the pH 4.5 hydrogen-producing

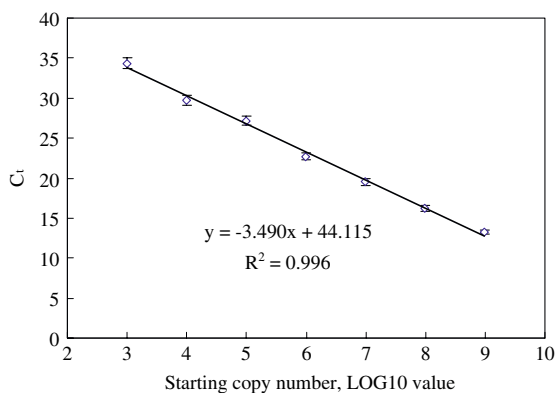


Fig. 3 Standard curve of C_t and LOG₁₀ value of copy number of PCR product containing HPB-R-2 16S rDNA fragment in 10-fold diluted solution

Table 1 Increase of acidophilic HPB 16S rDNA concentration with time

Time (h)	C_t	HPB 16S rDNA (copies/ μ l)
0	34.6	169
24	34.5	146
48	23.7	1.12×10^5
72	18.1	2.11×10^6
96	18.7	1.88×10^6
130	16.4	4.3×10^6

sludge sampled at various time intervals. Results indicate that the HPB 16S rDNA concentration (which reflected the cell population assuming identical DNA extraction efficiency for all samples) increased significantly from the initial 169 to 4.3×10^6 copies/ μ l after 130 h. Figure 4 is a semi-log plot of HPB population over time in the batch. It illustrates that, the HPB population increased exponentially, after a lag phase, before leveling off due to the depletion of substrate. Based on the slope of the plot in Fig. 4 between 24 and 72 h, the generation time for the acidophilic HPB is estimated as 3.5 h, which is comparable to the 4.2 h of uncultured hydrogen-producers estimated based on Fe-hydrogenase (Fang et al. 2006b) and the 4.5 h of *C. cellulolyticum* (Gelhaye et al. 1993) but substantially longer than the 0.58 h of *C. botulinum* (Prescott et al. 2002).

Table 2 shows that the HPB 16S rDNA in biomass decreased significantly with the increase of pH. At pH 4.5, the HPB 16S rDNA concentration was 4.30×10^6 copies/ μ l, 35 times higher than that at pH 5.0 and 2.24–3.14 $\times 10^4$ times higher than those at pH 5.5–6.5.

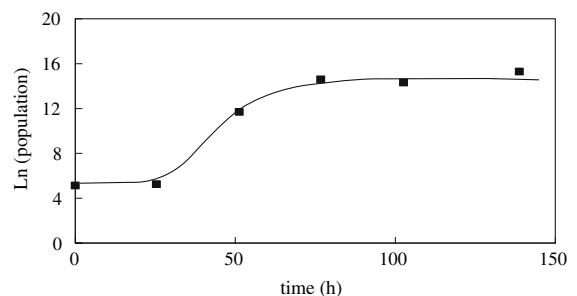


Fig. 4 Population increase of acidophilic HPB in the batch reactor

Table 2 Acidophilic HPB 16S rDNA concentration at various pH

pH	C _t	HPB 16S rDNA (copies/μl)
	16.4	4.3 × 10 ⁶
5.0	23	1.2 × 10 ⁵
5.5	33.7	137
6.0	33.9	158
6.5	34	192

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