Real-time quantifications of dominant anaerobes in an upflow reactor by polymerase chain reaction using a TaqMan probe

D. W. Liang, T. Zhang and H. H. P. Fang

ABSTRACT

This study was conducted to demonstrate the application of quantitative real-time polymerase chain reaction (qRT-PCR) for the quantification of dominant bacteria in an anaerobic reactor using a designed TaqMan probe. A novel group of uncultured thermophilic bacteria affiliated with *Thermotogales* was first found in a phenol-degrading sludge from a 55°C upflow anaerobic sludge blanket (UASB) reactor, which effectively removed 99% of phenol at loading of 0.51 g-phenol l^{-1} d^{-1} h of hydraulic retention. A TaqMan probe was then designed targeting this group of *Thermotogales* affiliated bacteria (TAB), and used to monitor its concentration in the reactors. Results showed that the TAB population in the 55°C reactor increased proportional to the phenol degrading rate. Results also showed that the TAB population ranged 3.5–9.9% in the 55°C phenol-degrading sludge, but only 0.0044% in the 37°C sludge and 0.000086% in the 26°C sludge.

Key words | anaerobe, PCR, phenol, 16S rDNA, TaqMan, thermophilic

D. W. Liang
T. Zhang
H. H. P. Fang (corresponding author)
Department of Civil Engineering,
The University of Hong Kong,
Pokfulam Road, Hong Kong SAR,
China
E-mail: *hrechef@hkucc.hku.hk*

INTRODUCTION

Microbial population is traditionally quantified by the culture-based methods. The reliability of using these methods for environmental samples is, however, limited since the majority of microbes have not yet been cultured. This problem may be overcome by using new techniques targeting the 16S rDNA or the functional gene of the microbe of interest. These techniques include those rely on DNA–DNA or DNA–RNA hybridization, such as membrane hybridization (Raskin *et al.* 1997) and fluorescence *in situ* hybridization (FISH), and those rely on DNA amplification by polymerase chain reaction (PCR), such as denaturing gradient gel electrophoresis (DGGE) (Zhang & Fang 2001), cloning-sequencing (Fang *et al.* 2006), and quantitative real-time PCR (qRT-PCR) (Zhang & Fang 2006). Among them, the qRT-PCR method has become increasingly popular (Grüntzig *et al.* 2001).

During the qRT-PCR, the concentration of PCR products is monitored throughout the amplification cycles using fluorescent reagents either specific for double stranded DNA or specific to certain fragment of the target denatured DNA. The fluorescent intensity, which reflects the amplicon concentration in real time, increases with the number of PCR cycle. The threshold cycle, C_t, at which the amplification crosses over the detection threshold level, 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. This method has so far been mostly applied to the quantification of pure cultures, such as toxigenic *Escherichia coli* (Oberst *et al.* 1998), and phytopathogenic *Ralstonia solanacearum* (Weller *et al.* 2000). Its application to quantification of uncultured bacteria, including many of those in anaerobic reactors, has still been very limited (Zhang & Fang 2006).

This study was conducted to demonstrate the application of this method for the quantification of a novel group of thermophilic bacteria in the phenol-degrading sludge in an upflow anaerobic sludge blanket (UASB) reactor, using a

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designed TaqMan probe targeting this group of bacteria with high specificity. Attempt was also made to correlate its population in the reactor with operational parameters.

MATERIALS AND METHODS

Phenol-degrading sludge samples

Five thermophilic (55°C) phenol-degrading sludges were sampled from a 2.8 L UASB reactor treating a wastewater containing 630 mg l^{-1} of phenol as the sole substrate (Fang *et al.* 2006) when the reactor was operated under steady state conditions at three respective hydraulic retention times, i.e. 60 h, 40 h and 28 h, corresponding respectively to the loading rates of 0.25, 0.38 and 0.51 g-phenol l^{-1} d^{-1}. Two additional phenol-degrading sludges were also sampled from two mesophilic reactors at 37°C (Fang *et al.* 1996) and 26°C (Fang *et al.* 2004) from previous studies for comparison.

Plasmid with 16S rDNA fragment insert and copy number calculation

The genomic DNA of all phenol-degrading sludge samples were extracted, according to the method described previously (Fang *et al.* 2006). The 16S rDNA of the thermophilic sludge sampled under the optimal HRT of 40h was amplified by PCR using the *Bacteria* specific primer set of EUB8F (5′-AGAGTTTGATCMTGGCTCAG-3′) and UNIV1492R (5′- TACCTTGTTACGACTT-3′) at the annealing temperature of 54°C. The amplified fragments, about 1,500 base pairs, were then used to build a clone library using pCR®2.1 plasmid (TA Cloning Kit, Invitrogen Corporation, Carlsbad, CA) (Zhang & Fang 2001). The plasmids with the 16S rDNA fragment insert were recovered (Sambrook *et al.* 1989). According to sequencing results of the inserted 16S rDNA fragment, 21 operational taxonomy units (OTUs) in *Bacteria* in this sludge were identified. Among the *Bacteria* OTUs, the most abundant OTU, TPD-2 (27.8%), and two others, i.e. TPD-22 (1.9%) and TPD-29 (1.9%), formed a group which was most closely related to species in the order of *Thermotogale* with 91–92% similarity. This novel group of uncultured thermophilic bacteria found in the phenol-degrading sludge is designated as TAB (*Thermotogale* affiliated bacteria) in this study.

The TPD-2 plasmid, which contained the pCR®2.1 plasmid (3929 base pairs) and the 16S rDNA insert of TPD-2 (1,500 bps), had a total molecular weight of 3.39×10^6 assuming average molecular weight of each base pair is 660 (Sambrook *et al.* 1989). The DNA concentration in the TPD-2 plasmid solution was 675 ng μl^{-1} measured by a UV spectrometer (UV-160, Shimadzu, Kyoto, Japan), assuming each A_{260} unit corresponding to 50 ng μl^{-1} of double-stranded DNA (Sambrook *et al.* 1989). The TPD-2 plasmid concentration based on plasmids molecule was, thus, calculated as 1.27×10^{11} molecule μl^{-1}. As each plasmid molecule containing one copy of insert, the TPD-2 16S rDNA concentration in the plasmid solution was 1.27×10^{11} copies μl^{-1}. This solution was ten-fold serially diluted and used as the standards in the subsequent experiment.

TaqMan probe and primers design

The 16S rDNA sequences of TPD-2, -22 and -29 were aligned with the reference sequences from the GenBank using BioEdit (Hall 1999). By manual comparison, a specific region was identified and, based on which, a TaqMan probe and a primer set were designed using the Primer Express software (Applied Biosystems, Foster City, CA). The TaqMan probe (5′-ATCCCTCAGCGTTTCC-3′) was labeled at the 5′-end with the 6-carboxyfluorescein (FAM) reporter dye and at the 3′-end with MGB quencher (Applied Biosystems, Foster City, CA). The primer set (forward primer: 5′-CTT GAC ATG CTG GTA GTA CCT TAG T-3′, and reverse primer: 5′-CCT GTG CTG GCT TCT ACC T-3′) amplified 68 bps DNA fragments.

Quantitative Real-Time PCR

Quantification of this group of TAB was performed by a qRT-PCR assay using an iCycler IQ System (Bio-Rad, Hercules, CA, USA) in triplicate in 20 μl PCR solution. Nine 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, the temperature profile followed a two-step cycle

pattern with a combined annealing and primer extension phase at 60°C for 1 min and a short denaturation at 95°C for 15 s for fifty cycles of amplification.

Accession numbers

The nucleotide sequence data reported in this paper have been assigned by the GenBank, EMBL and DDBJ databases the following accession numbers: AY862519 (TPD-2), AY862524 (TPD-22) and AY862526 (TPD-29).

RESULTS AND DISSCUSSIONS

Design of TaqMan probe and its specificity

Comparison of the nearly full-length 16S rDNA sequences of TPD-2, -22 and -29 by BLAST showed that this group is closely related to an uncultured bacterium TUG22 (similarity 99%) from a thermophilic UASB reactor (Sekiguchi *et al.* 1998) treating synthetic wastewaters comprising sucrose, acetate, propionate, peptone and yeast extract. As shown in Figure 1, the TAB forms a distinct phylogenetic branch with TUG22 in the order of *Thermotogales*, and is remotely (<92% similarity) related to other species, such as uncultured bacteria *Thermotogales* OPB85 and *Thermotogales* sp. SRI-15, and the known species of *Dictyoglomus thermophilum*. *Thermotogales*, which has only one family *Thermotogaceae*, is the only order in the sole subdivision *Thermotogae* of division *Thermotogae*. Only a few species in *Thermotogales* have been ever characterized.

The 16S rDNA sequences of TPD-2, -22 and -29 were used to design a TaqMan probe and a primer set specific to this group of TAB. The TaqMan probe designed in this study had a perfect match with TPD-2, -22 and -29 and TUG22, but a few mismatches with other closely related species, such as the uncultured *Thermotogales* OPB85 and *Thermotogales* sp. SRI-15. The specificity of the TaqMan probe was confirmed using the CHECK_PROBE software provided by the Ribosomal Database Project (Maidak *et al.* 1997). Results showed that species outside the TAB had at least one mismatch with the designed TaqMan probe.

Standard curve and detection limit

Figure 2 is the standard curve of C_t values plotted against the corresponding serial dilutions of the TPD-2 plasmid solution. Over the whole dilution series, ranging from 1.1×10^2 to 1.1×10^{10} TPD-2 16S rDNA copies per PCR sample, the C_t values increased linearly with the $\log_{10}$ value of initial copy number with a slope of -3.37, corresponding to a PCR efficiency of 98% (Grüntzig *et al.* 2001). The relationship between the C_t value and $\log_{10}$ value of the initial copy number became nonlinear below 110 copies per PCR, equaling to 12.7 copies μl^{-1}. Such detection limit was similar to those found in other TaqMan probe applications (Nelson *et al.* 1999; Zhang & Fang 2006).

An additional PCR series was conducted to examine the reproducibility of the standard curve. The slope of this second plot was -3.35, corresponding to an efficiency of 99%. Variation of two independent measurements was 2–10% in concentrations ranging from 10^3 to 10^9 copies μl^{-1}.

Detection of TPD-2 plasmid spiked into samples

Various chemicals, including protein, polysaccharide and humic acids, in the extracted genomic DNA samples may possibly inhibit the PCR, resulting in a decreased PCR efficiency and a doubtful reliability. Thus, in this study, a TPD-2 plasmid was spiked into the above genomic DNA samples to test applicability of the TaqMan method proposed. Spiking 1.27×10^8 copies of TPD-2 plasmid into the genomic DNA sample resulted in a measured value

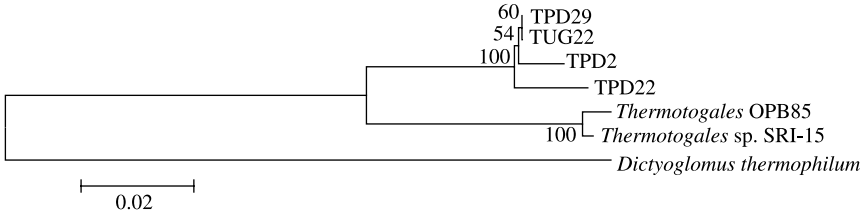

Figure 1 | Phylogenetic tree of TAB and related species with the closest known species *Dictyoglomus thermophilum* as the outgroup.

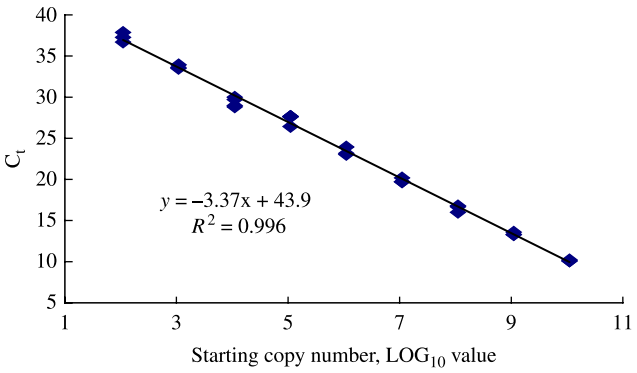

Figure 2 | Standard curve of C_t plot against LOG_{10} value of TPD-2 partial 16S rDNA copy number in 10-fold diluted solutions.

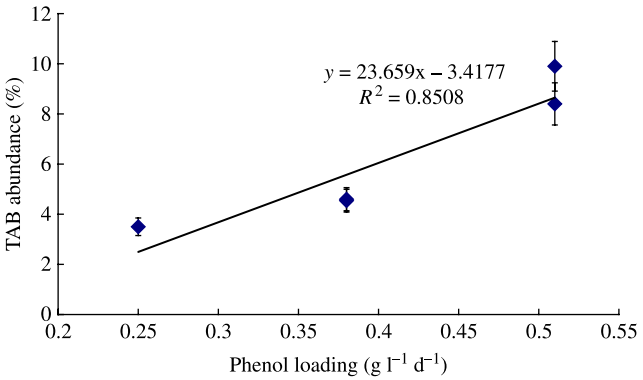

Figure 3 | The linear relationship between TAB percentage and phenol loading.

of 1.43×10^8 copies with a 113% recovery. This indicates that the substances in extracted DNA sample did not significantly affect the quantification, considering the measurement variation of two independent PCR runs (up to 10%).

Relative abundance of TAB in sludge of various temperature and phenol loading rates

Assuming each cell of TAB contains one copy of 16S rDNA as in *Thermotogales* (Nelson *et al.* 1999), the cell number of TAB may be determined by the qRT-PCR. Since each genome in *Bacteria* and *Archaea* contains on the average 2.78×10^6 base pairs (http://www.ncbi.nlm.nih.gov/genomes/MICROBES), 2.85×10^5 cells equal to 1 ng DNA. Assuming DNA extractions of all cells are of the same efficiency, the relative abundance of TAB in all the cells of the sludge may be calculated. Results are shown in Table 1 and Figure 3.

Table 1 shows the abundances of TAB in the total community at sludges sampled from phenol-degrading reactors operated under mesophilic conditions. Populations of TAB were accounted only as 0.0044% of the 37°C sludge and 0.000086% in the 26°C sludge.

Thermophilic reactors were usually started up using mesophilic sludge as seed. As temperature step-increased from mesophilic condition to 55°C, reactors often encounter sludge washout and reduced removal of COD. Full recovery of COD removal efficiency would take 3 to 4 months (Fang & Lau 1996). Result in this study showed that TAB population was very low in mesophilic sludges, and its population may require a long time to build up when the sludge temperature is shifted to 55°C. In this study, the TAB population in the 55°C sludge was 800–40,000 times higher than those in the mesophilic sludge. Assuming a generation time of 7 d (Syutsubo *et al.* 1998), TAB would take 67–107 days to increase their population to 3.5% at 55°C. For comparison, the starting-up time required for a 55°C phenol-degrading reactor using 37°C sludge as seed in a previous study was reported as 75 d (Fang & Lau 1996).

Figure 3 shows that the abundance of TAB in biomass increased from 3.5% to 4.6% and lastly to 9.9%, as the phenol loading increased from 0.25, 0.38 and 0.51 g-phenol l^{-1} d^{-1}. This implies that the rate of phenol degradation was due to specifically the increase of TAB population, instead of the overall biomass in the reactor.

Table 1 | TAB in sludge at various temperatures

Temperature (°C)	C_t	TAB concentration (copies/μl)	Extracted genomic DNA (ng/μl)	TAB abundance (%)
26	32.3	179	727	0.000086
37	29.3	1510	120	0.0044
55	20.5	791000	79	3.5

CONCLUSION

A TaqMan probe was designed targeting a group of *Thermotogales* affiliated bacteria in the anaerobic phenol-degrading sludge, and was successfully used to monitor the

concentration of TAB population in the reactors. Results showed that the TAB population in the 55°C reactor increased proportionally to the phenol degrading rate. Also, the TAB population ranged 3.5–9.9% in the 55°C phenol-degrading sludge, but only 0.0044% in the 37°C sludge and 0.000086% in the 26°C sludge.

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