Detection and Quantificaiton of Methanogenic Communities in Anaerobic Processes Using a Real-Time PCR

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Abstract

A method for detection and quantification of aceticlastic methanogens using a real-time PCR with a TaqMan probe was developed. Two sets of primers and probes targeting the family *Methanosarcinaceae* and *Methanosaetaceae* were designed by using the Ribosormal Database Project (RDP) II, and softwares for phylogenetic probe design and sequence analysis. Target-group specificity of each set of primers and probe was verified by testing DNAs isolated from pure cultures of 28 archaeal strains purchased from DSMZ. Cell numbers in the 28 archaeal cultures and in the samples from anaerobic processes were quantified using a real-time PCR with the sets of primers and probe. In conclusion, the real-time PCR assay was very specific for the corresponding target methanogenic family and was proved to be a powerful method for quantification of aceticlastic methanogens in anaerobic processes.

Introduction

Aceticlastic methanogenesis is a very important reaction in anaerobic processes because approximately 70 % of the methane is produced by this metabolic pathway. Furthermore, dominant populations in most of anaerobic digesters are known to aceticlastic methanogens. Two major types of aceticlastic methanogens can be present in anaerobic systems and bioreactor conditions imposed affect the predominant species because their growth kinetics are quite different. *Methanosarcina* can grow rapidly (μ_{max}= 0.336 d^{-1}), but do not have a high affinity for acetic acid (Ks = 300 mg/L). *Methanosaeta*, on the other hand, grow slowly (μ_{max}= 0.072 d^{-1}), but have a higher affinity for acetic acid (Ks=30 mg/L) (3). Consideration of differences of biokinetic coefficients between methanogenic groups and quantification of their cell numbers are essential for better design and control of anaerobic biological processes. In this study, therefore, real-time PCR (5) with a TaqMan probe (4), a highly sensitive method for quantification of target DNAs, was used to enumerate cell numbers of aceticlastic methanogens in samples from archaeal cultures and anaerobic processes.

Materials and Methods

Two sets of primers and probes for each family *Methanosarcinaceae* (Msc) and *Methanosaetaceae* (Mst) were designed. Complete or partial sequences of the 16S ribosomal RNA gene (rDNA) were downloaded from the Ribosomal Database Project (RDP) II (http://rdp.cme.msu.edu/html/), and used to design of primers and probes. These sequences were rearranged and then assigned to corresponding family using PHYDIT (2), a software for phylogenetic analysis. Based on the 16S rDNA sequence alignments, appropriate regions on the16S rDNA sequences were selected for primers or probes using the PHYDIT and PRIMROSE (version 1.1.7) (1), a program for phylogenetic probe design obtained from the RDP II. Specificities of all the primers and probes were examined using PROBE MATCH program, an online analyses tool provided through the RDP II. Each TaqMan probe was labeled with a fluorescent reporter dye (6-carboxyfluorescein) attached to the 5' end, and a fluorescent quencher dye (6-carboxytetramethylrhodamine) attached to the 3' end.

Specificity of each set of primers and TaqMan probe for its target-group was empirically confirmed using a real-time PCR (LightCycler II, Roche Diagnostics) by testing DNAs isolated from pure cultures of 28 archaea purchased from DSMZ. Among the 28 archaea, strains belonging to the family *Methanosarcinaceae* were *Methanosarcina barkeri* str. MS (DSM 800), *Methanosarcina acetivorans* str. C2A (DSM 2834), *Methanosarcina mazei* str. Go1 (DSM 3647), *Methanosarcina thermophila* str. TM-1 (DSM 1825), *Methanococcoides methylutens* str. TMA-10 (DSM 2657), *Methanolobus tindarius* str. Tindari 3 (DSM 2278), and *Methanosalsum zhilinae* str. WeN5 (DSM 4017). Strains belonging to the family *Methanosaetaceae* were *Methanosaeta concilii* str. Opfikon (DSM 2139) and *Methanosaeta thermoacetophila* str. PT (DSM 6194).

For each family, rDNA was prepared by PCR amplification with appropriate archaeal DNAs as templates and corresponding primers. Concentrations of 10-fold serially diluted rDNAs were plotted against their C_T values by real-time PCR reactions. The rDNA concentrations of unknown samples were calculated using the standard curve, which was created by linear regression line through the data points on the plot. All sets of experiments were duplicated. An absolute cell number of an archaea culture was estimated by dividing the rDNA amount by its rDNA copy number, which is available in the Ribosomal RNA Operon Copy Number Database (rrndb) (http://rrndb.cme.msu.edu/rrndb). For strains with unknown rDNA copy number, an average value of rDNA copy numbers for corresponding family was used. For DNA samples from anaerobic reactors, rDNA amounts were quantified using the real-time PCR with each set of primers and a probe, and cell numbers were calculated by using the average of rDNA copy number for the corresponding family. For both families, rDNA copy numbers were assumed to three by referring to the rrndb.

Results and Discussion

The primers and probe sets designed in this study are shown in Table 1. In the specificity test of real-time PCR assays with the Msc set, *M. thermoacetophila* str. PT DSM 6194 was detected as a single non-target strain and all seven target strains were detected. However, only 1.6 % of rDNA

amount of the *M. thermoacetophila* quantified by the Mst set was detected by the Msc set. Real-time PCR assay with the Mst set was specific to the family *Methanosaetaceae* and only rDNA of the two strains belonging to this family were detected. Regression coefficients for each standard curve are presented in Table 2. For each family, standard curve was prepared from each of two DNA sources. The linear ranges of each standard curve(i.e., detection range) were over seven orders of magnitude and the coefficients of determination (R^2) were over 0.998.

For each C_T value by real-time PCR assay, an average value of rDNA amounts calculated by each standard for corresponding family was always used. Cell numbers were calculated by dividing the average rDNA values by their rDNA copy numbers (i.e., three copies for both families (Table 3)). Cell numbers of each family in all anaerobic reactors corresponded with operating conditions of the reactors, such as HRT and acetate concentration. The family *Methanosarcinaceae* was a dominant population at lower HRT and higher acetate content. In this study, quantifications by real-time PCR were rapid, sensitive, and reproducible. Two families of aceticlastic methanogens could be separately quantified by the corresponding set of primers and a probe.

Table 1. The characteristics of the primers and TaqMan probe sets for real-time PCR

Name	Function	Sequence (5'→3')	E.coli No.	T_m(℃)	Length (bp)
Msc380F	F primer	GAAAC CGYGA TAAGG GGA	380-397	56.6	
Msc492F	TaqMan	TTAGC AAGGG CCGGG CAA	492-509	64.3	408
Msc828R	R primer	TAGCG ARCAT CGTTT ACG	811-828	56.4	
Mst702F	F primer	TAATC CTYGA RGGAC CACCA	702-721	58.6	
Mst753F	TaqMan	ACGGC AAGGG ACGAA AGCTA GG	753-774	68.8	164
Mst862R	R primer	CCTAC GGCAC CRACM AC	846-862	59.8	

Table 2. Characteristics of standard curves for the Msc and the Mst sets of primers and robe

Primers	Source strain of template DNA	rDNA[a] (copies/$\mu\ell$)	Standard curve			
			Linear range	Slope	Intercept	R^2
Msc380F	*M. acetivorance* (DSM2834)	5.927×10^{10}	$10^1 - 10^9$	-3.5608	44.27	0.9980
Msc828R	*M. mazei* (DSM3647)	9.893×10^{10}		-3.5461	44.59	0.9994
Mst702F	*M. concilii* (DSM2139)	3.381×10^{10}	$10^2 - 10^9$	-4.0084	46.83	0.9991
Mst862R	*M. thermoacetophila* (DSM6194)	4.490×10^{10}		-4.1633	47.54	0.9989

[a] A copy number-based Concentration was calculated from a mass-based concentration of rDNA amplified with the corresponding template DNA and primers.

Table 3. Quantification of aceticlastic methanogens in archaeal cultures and anaerobic reactors by real-time PCR, and operating conditions of a full-scale anaerobic digester and three lab-scale anaerobic reactors

Archaea strain/Anaerobic reactor sample	*Methanosarcinaceae* (Cell numbers/ml)	*Methanosaetaceae* (Cell numbers/ml)	Substrate	Acetate (mg/L)
M. barkeri str. MS (DSM 800)	4.01 (0.35) $\times 10^9$			
M. acetivorans str. C2A (DSM 2834)	2.13 (0.59) $\times 10^6$			
M. mazei str. Go1 (DSM 3647)	3.83 (0.33) $\times 10^9$			
M. thermophila str. TM-1 (DSM 1825)	2.22 (0.14) $\times 10^7$			
M. methylutens str. TMA-10 (DSM 2657)	1.64 (0.02) $\times 10^{10}$			
M. tindarius str. Tindari 3 (DSM 2278)	4.89 (0.13) $\times 10^8$			
M. zhilinae str. WeN5 (DSM 4017)	5.07 (0.19) $\times 10^9$			
M. concilii str. Opfikon (DSM 2139)		3.24 (0.25) $\times 10^6$		
M. thermoacetophila str. PT (DSM 6194)		1.42 (0.08) $\times 10^7$		
Full-scale digester (HRT:28d, pH 7)	1.03 (0.02) $\times 10^6$	1.66 (0.07) $\times 10^8$	WAS[a]	4.3 (0.2)
Anaerobic SBR (5L, HRT:7d, pH 7)	1.18 (0.05) $\times 10^8$		Whey[b]	1,470 (13)
Anaerobic CSTR (5L, HRT:10d, pH 7)	1.01 (0.07) $\times 10^8$	2.11 (0.34) $\times 10^7$	Synthetic[c]	347 (14)
Acidogenic CSTR (1.5L, HRT:0.5d, pH 6)	1.12 (0.08) $\times 10^4$		Whey[d]	5,794 (36)

( ) : Standard deviation
[a] Waste Activated Sludge; [b] (10g whey powder + 1g yeast extract)/L
[c] [acetate (10) + butyrate (5) + propionate (5) + methanol (10) + ethanol (5)]/L, where, ( ) : grams of COD equivalent
[d] (20g whey powder + 0.3g NH_4Cl)/L

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