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5′-MGB Probes Allow Rapid Identification of Methanogens and Sulfate Reducers in Cold Marine Sediments by Real-Time PCR and Melting Curve Analysis

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The analysis of microorganism communities in uncultured environmental samples requires laborious and cumbersome techniques such as denaturing gradient gel electrophoresis of amplicons generated with 16S rRNA generic primers with subsequent fragment sequencing. We have developed a simple method for genus identification of methanogen archaea and sulfate-reducing bacteria based on a real-time PCR hybridization probe melting curve analysis. The method takes advantage of a recent explosion of microorganism sequencing data conveniently packaged in the Ribosomal Database Project. Specificity of detection is based on a genus-specific real-time PCR fluorescent 5′-MGB-probe melt. As the probes are designed to have destabilizing mismatches with undesired genera, only samples with a proper melting temperature are called positive.

Introduction

IDENTIFICATION AND MONITORING OF microbial communities in environmental samples is becoming increasingly important. Microbes could both produce and consume greenhouse gases such as methane and have a global effect on a climate change. Modern economics favors renewable energy sources over fossil fuel, and many countries have considered building more hydroelectric power stations including those based on tidal waves. However, artificial isolation of coastal waters by constructions that limit water circulation could lead to an accumulation of organic matter in the upper level of bottom sediments, oxygen deprivation, and creation of anaerobic conditions. As a result, sediment microbial community might shift toward methanogens and sulfate reducers potentially damaging ecosystem and releasing greenhouse gases.

In the past cultivation-independent molecular methods have been applied to investigate microbial diversity in natural and anthropogenic sites. Reported RNA and DNA detection methods are usually PCR-based (Matsuki et al., 2004; Park and Crowley, 2005; Chen et al., 2007; Kendall et al., 2007).

One-step primer-specific amplification of rRNA-based sequences of any microbial group in most cases is not possible due to a lack of conserved regions that would restrict cross-amplification and at the same time amplify all members of the target group. Extra level of specificity is provided by using nested and/or degenerate primers, denaturing gradient gel electrophoresis, terminal restriction fragment length polymorphism, and sequencing (Park and Crowley, 2005; Chen et al., 2007; Kendall et al., 2007). Those multistep methods are quite involved and labor-intensive. At the same time there is an explosion of microbial sequencing occurring right now, especially for rRNA genes. Development of modern bioinformatics software that allows storage and processing of accumulated sequencing information calls for a revision of existing detection techniques for a possible modernization and simplification. Recent advances in modified bases and real-time PCR hybridization probe chemistry make that technically possible (Kutyavin et al., 2002; Afonina et al., 2006; Lukhtanov et al., 2007).

We have developed a fast method for genus identification based on a real-time PCR hybridization probes melting curve analysis. Specificity of detection is based on a genus-specific real-time PCR fluorescent 5′-MGB probe melt. The method was successfully applied to detect specific genera of methanogens and sulfate reducers in environmental samples from marine sediments.

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Materials and Methods

Samples

Sediment samples were collected from Dolgaya fiard, Russia (69°N, 35°E). Dolgaya fiard is located in the south part of Barents Sea in Murmansk region of Russia. Dolgaya fiard is 93 m deep at the sampling site, and the temperature of the sediment is about 4°C year round. Samples were collected from 2 depths: 0–1 cm and 2–6 cm from the sediment–water interface as described (Kendall et al., 2007), immediately sealed excluding air and gas and stored at 4°C.

DNA extraction

DNA extractions from 0.25 g of each of the sediment samples were performed using a PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA) according to the manufacturer's instructions. The kit was chosen because it is intended for use with environmental samples including difficult soil types such as sediment and has a humic substance/brown color removal procedure effective at removing PCR inhibitors. The extracted DNA was resuspended in 100 µL of 10 mM Tris and stored at −20°C until further analyses. Five microliters of that solution or its 50-fold dilution in water was used for each PCR.

Primer and probe development

16S rRNA gene sequences from *Desulfotomaculum, Desulfobacter, Desulfococcus, Desulfuromonas, Desulfomicrobium, Desulfovibrio* bacteria and *Methanolobus, Methanosarcina, Methanothrix, Methanobacterium, Methanococcus, Methanococcoides, Methanogenium* archaea were downloaded from Ribosomal Database Project (http://rdp.cme.msu.edu) databases, aligned using BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) program and checked manually for alignment accuracy. Primrose program (available at http://rdp.cme.msu.edu) was used to do preliminary search for sequences specific to each of the taxons. Each sequence was further examined using BioEdit tools to identify primers and probes for real-time PCR. Special attention was applied to probe design. Probe design criteria were the following: (1) no destabilizing mismatches within taxon; (2) at least 1 destabilizing mismatch with all other taxons. Pleiades (Lukhtanov et al., 2007) technology was chosen for the real-time PCR probe chemistry. Pleiades probes have minor groove binder (MGB) and fluorescent dye linked to the 5′-end of the probe. Quencher ligand attached to the 3′-end of the probe makes sure that the probe fluoresces only upon hybridization to its complement. MGB prevents probe digestion by Taq polymerase during PCR. That allows not only fluorescent signal collection during each PCR cycle but also a post-PCR melting curve analysis to check for probe specificity. If the probe encounters a mismatch in its complement, its melting profile would change (melting temperature will decrease compared to a full match). It means that specific taxon amplification can be easily distinguished from unspecific by checking post-PCR melting curve. Primers were designed around the probe to yield 100% PCR efficiency. Primers were placed in the regions conservative for each taxon to make sure that all known members will be amplified. Sometimes modified bases (Kutyavin et al., 2002; Afonina et al., 2006) were introduced in primers and probes to fine-tune the desired oligonucleotide stability. The 12-mer AT-rich noncomplementary "flaps" were added to each primer to increase fluorescent signal (Afonina et al., 2007). When needed degenerate bases and/or a mixture of several primers and probes were used in each PCR to cover all members of the appropriate taxons. The real-time PCR primers and probes used for this study are listed in Table 1. Primers were synthesized using a DNA synthesizer (PE Applied Biosystems, Forster City, CA); probes were synthesized as described (Lukhtanov et al., 2007). Synthetic amplicons covering primer and probe area for each taxon were made by Integrated DNA Technologies (San Diego, CA). Synthetic amplicons were quantified by spectrophotometry and used as positive controls in real-time PCR.

Real-time PCR

PCR was performed in a 20 µL total reaction volume containing 2 µL of 10× Lightcycler FastStart DNA Master HybProbe PCR Mix (Roche Applied Science, Switzerland; Catalog no. 12239272001), 1 µL each of 20× primers and probes, 3.2 µL of 25 mM $MgCl_2$ (to a final concentration of 5 mM), and 0.2 µL of 1 U/µL AmpErase (Applied Biosciences,

TABLE 1. PRIMERS AND PROBES USED FOR REAL-TIME PCR

Genus	*Forward primer*	*Reverse primer*	*Probe*
Desulfotomaculum	AATAAATCATAA TTGTAGYACGTGTGTAGCCCA	AATAAATCATAAAGGAAGGTGGGGATGACGT	G*GCATGATGATTTGACGTC
Methanolobus	AATAAATCATAACTATCAGGTWGTAGTGGGTGTA	AATAAATCATAACCGGGCTCTTGCTCTCAC	CG*ACGACGGGTACGGG
Desulfobacter	AATAAATCATAATCCCGTTCGCTACCCACG	AATAAATCATAAGGCGAAGGCATCTCTCTGGAC	CTCAGCGTCAATATCGGT
Methanosarcina	AATAAATCATAAGCCGCCGCGGTAACA	AATAAATCATAACTACGGACCCTTTAGACCCAAT	G*CCCGAGTGGTGAG*CGT
Desulfococcus	AATAAATCATAA GAGTAACGCGTGGGTAATCTA	AATAAATCATAA TCCGGTATTAGCACTCCTTTCG	G*GATTCGGGATAACCC
Methanothrix	AATAAATCATAAAAGGCCAGGCTGAAGAC	AATAAATCATAAGACGACGGCCATGCACCA	G*GATTAGCTGAGAGGT
Desulfuromonas	AATAAATCATAAGCTACACACGTGCTACAATGG	AATAAATCATAATGGAGTCGAGTTGCAGACTC	G*GTCTCAGTTCGGATTGG
Desulfomicrobium	AATAAATCATAAGGGAGCAAACAGGATTAGATAC	AATAAATCATAACCCCGACACCTAGTATCCATCG	G*TAGTCCACACCGTAAAC
Methanobacterium	AATAAATCATAATTACCGCGGCGGCTG	AATAAATCATAACCCAAGTGCCACTCTTAACG	CTTGCCCAGCCCTTATTC
Methanococcus	AATAAATCATAAGGGTACTCCAAGGGTAGC	AATAAATCATAACGCCATAGGTGGTCCTCCA	G*AATGTGTTGATCCTTGG
Methanococcoides	AATAAATCATAACGGCCCGAGTGGTGAT	AATAAATCATAAACCGGCTACGGACCCTT	ACT*T*T*T*ATTGG*GTCTAA
Desulfovibrio	AATAAATCATAACGCAGCCGCGGTAATAC	AATAAATCATAAGCGCTTTACGCCCAGT	G*CGTTAATCGGAATYA*CT
Methanogenium	AATAAATCATAAAAGGGCYGGGYAAGAC	AATAAATCATAATCGAGCCGCCGGTRTTAC	CCAGCCGCCGCGGTAA
	AATAAATCATAAAAGGAGAGGGCAAGGCT		

Non-complementary "flap" sequences in primers are underlined; * indicates a modified base; all probes have minor groove binder and fluorescent dye linked to the 5′-end of the probe; quencher ligand is attached to the 3′-end of the probe.

Foster City, CA; Catalog no. N808–0096) to prevent PCR carryover contamination. The rest of the reaction volume was filled with 5 µL sample and water. Water was used for no template control. Final concentration of all forward primers was 250 nM; all reverse primers were at 1 µM, and all probes were at 500 nM. Probes were always complementary to the reverse primer strand.

Thermocycling was performed on Prism 7900 (Applied Biosystems, Foster City, CA), thermocycling fluorimeter. Thermocycling consisted of 2 minutes at 50°C for AmpErase reaction, 10 minutes at 95°C for Taq polymerase, 50 cycles of 95°C denaturation for 5 seconds, 56°C annealing/detection for 20 seconds, 76°C extension for 20 seconds, and dissociation stage of 95°C denaturation for 15 seconds, beginning melt of 35°C for 15 seconds, ramping (10% ramp rate) to an ending melt of 85°C for 15 seconds. Data (cycle threshold, or C_T) was collected at the annealing step and during the temperature ramp in the dissociation stage (melting). Amplification was detected using the FAM detector channel. Samples were considered positive for a taxon if they had the same melting temperature (T_m) as the positive control (within 1°C). Relative taxon template quantities were determined using $\Delta\Delta C_T$ method with modifications (Afonina et al., 2006). Tenfold dilution series of respective positive controls were used to determine dynamic range and linearity of the assays. To determine the limit of detection positive controls were serially diluted 3-fold from 10 to ~0.3 copy per reaction and used as templates in real-time PCR.

TABLE 2. PARTIAL 16S rRNA CONSENSUS GENE SEQUENCES AROUND GENUS-SPECIFIC PROBES FOR *Desulfovibrio, Methanosarcina,* AND *Methanogenium*

Desulfovibrio	GCAGCCGCGGTAATACKGARGGYGCRAGCGT-TAATCGGAATYACTGGGCGTAAAGCGY
Desulfotomaculum	···K·····N···T···KR·YD·R····-·GTC···W·····G··
Desulfomicrobium	···K···H·····S·····T·····S··
Desulfobacter	·····M·····Y····G···S·····T·····T·····G··
Desulfococcus	·····T·····T·····A··
Desulfuromonas	·····-·GT·····T·····M··
Methanobacterium	··C·····C··C·GCA··Y·D··T·G-·RGC·RYTH···T·····C·····
Methanococcus	·····Y··C·····C····TAGG·RRC·ACTY···T····YC·····
Methanococcoides	··C···R·····C··C·GC···C····T·G-·R···AYTW···T····TC·····G··
Methanolobus	··C·····C··C·GC···C····T·G-·RRY·AYTW···T····TC·····G··
Methanosarcina	··C·····C··C·GC···C····T·G-·G·Y··TK····T····TC·····G··
Methanothrix	··C·····C··C·GC···Y····T·G-···C··NTD···T····TC·····G··
Methanogenium	··C·····H··CNGCDS·B·D··T·V-YNDBSRNNW···Y··R·YY····RBR·
Desulfovibrio	··A·····T··K·AR··YG·R··C·T--A····GA··Y·C····CG·····C·YD·····GYB
Desulfotomaculum	··AK·····N··GTAG·KRGYD·RC·T-··T···GAW·Y·C····CG·····CGH·Y··GB·
Desulfomicrobium	··AK···H·····T··S·AG··TG·R··C·T-·AT···GA··Y·C····CG·····S·CR·····G·S
Desulfobacter	··A··M·····Y··G··G··SG·A··C·T-·AT···GA·····CG·····CG··Y··G·R
Desulfococcus	··A·····T··G·AG··TG·A··C·T-·AT···GA·····CG·····A·CG·····G·K
Desulfuromonas	··A·····T··G·AG··TG·R··C·T-··T···GA·····CG·····M·CGT·Y··G··
Methanobacterium	·····A··Y·D·····-·RG··RY·H·····C·····C··Y·····
Methanococcus	··A·····Y·····A····R···A·G·RR··AC·Y·····Y·····C·····
Methanococcoides	·····R·····-·R···AY·W·····
Methanolobus	·····-·RR··AY·W·····K
Methanosarcina	GCCGCCGCGGTAACACCGGCGGCCCGAGTGG-TGAYCGTKATTATTGGGTCTAAAGGGTCCGTAGCCG
Methanothrix	·····Y·····-····N·D·····H·····
Methanogenium	·····H···N··DSYB·D····V-YNDBSRNNW···Y··R·YY····RBR·H·····YK
Desulfovibrio	·A·CR·C···T·AHT·M·····A·····K·AR·····R·
Desulfotomaculum	·A·CYMC···T·ACT·····AK·····N··G·AG·KR·Y··
Desulfomicrobium	·A·CA·C···T·ACTM·K·····AK···H·····S·AG·····R·
Desulfobacter	·ASSR·C···T·ACT·····A·M·····Y··G··G··S····
Desulfococcus	·A·CA·C···T·ACT·····A·····G·AG·····
Desulfuromonas	·A·CA·C···T·ACT·····A·····G·AG·····R·
Methanobacterium	··R·····Y···
Methanococcus	·····T·····A·····Y····A·····R·
Methanococcoides	·····Y·····R·····R·····
Methanolobus	·····
Methanosarcina	·····
Methanothrix	·····Y···
Methanogenium	AGNRSHGGGYAAGDCNVGTGCCAGCCGCCGCGGTAAHACCNGCDSYBCDA

Only nucleotides that differ from the target sequences are shown. Primers are underlined, probes are shaded.

Results and Discussion

Methanogens and sulfate reducers are a diverse group of different genera from archaea and eubacteria superkingdoms. Thirteen genera from that group that live in cold marine sediments (Berge et al. *Manual of Determinative Bacteriology* Ninth Edition J.G. Holt) were chosen for the study: *Desulfotomaculum, Methanolobus, Desulfobacter, Methanosarcina, Desulfococcus, Methanothrix, Desulfuromonas, Desulfomicrobium, Methanobacterium, Methanococcus, Methanococcoides, Desulfovibrio,* and *Methanogenium*. We have used Ribosomal Database Project (http://rdp.cme.msu.edu) maintained by a Center for Microbial Ecology of Michigan State University to align all known 16S rRNA gene sequences and identify regions unique for each genus using Primrose program (http://rdp.cme.msu.edu). Consensus sequences for each genus-specific alignment were created with greater than 98% specificity using BioEdit program (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and aligned. Genus-specific regions identified by Primrose were manually inspected, and real-time PCR hybridization probes were designed as described in Materials and Methods of the manuscript. Since the 5′-MGB probe is not degraded in the course of amplification (Lukhtanov et al., 2007), the probe is available for melt analysis. The melt can confirm a result, and also be used to resolve closely related targets. MGB probes hybridize more strongly to their complementary sequences than regular DNA probes and display increased melting temperature allowing for the use of shorter, yet highly specific, probes. The use of shorter probes can improve mismatch discrimination, when possible primers were placed in the genus-specific regions to enhance amplification specificity. However, in most cases it was not feasible as rRNA genes are by definition conservative. Our goal in primer design was to amplify most members of the genus with close to 100% PCR efficiency. Inevitably some cross-amplification from other groups might occur, so it was necessary to rely on the probe for specificity. Due to a complexity of the alignments, it was impossible to avoid mismatch primer pairing with a small number of isolates. In most cases variable regions are located at the 5′-parts of the primers where destabilizing effect is the least. G:T base pairing was allowed in some cases as it forms a rather stable bond that does not destabilize primer/template binding at permissive temperatures. As PCR conditions are mild with only 56°C annealing temperature, the mismatch templates will still be amplified. When needed modified G* was used in the probes instead of Y because it bonds equally well with T and C. Respective alignment (Table 2) shows that all probes except *Methanogenium* do not have other than nondestabilizing end mismatches within the genus and have at least 2 destabilizing mismatches with other groups. *Methanogenium* genus is on one hand very diverse and on the other too close to *Methanosarcina* for a specific design.

We have used positive controls as real-time PCR templates to determine performance characteristics of the developed reagents. The linear range of detection was over 7 orders of magnitude for all the reagent sets: standard curves derived from 10^7 to 10^1 copies per reaction dilution series had $R^2 \geq 0.99$ for all assays. Threefold serial dilutions of positive controls starting from 10 copies per reaction were used to determine the limit of detection for each genus-specific assay. Six repeats were used for each dilution point. The limit of detection (template amount that amplifies only a portion of the repeats) for all the reagent sets was between 3 and 1 copy per reaction, which is common for all PCR-related detection platforms. As we have used DNA extracted from ~2.5 mg of sediment per each reaction, it means that we can detect 400–1200 copies per gram of sediment assuming extraction efficiency to be close to 100%. Having in mind that ribosomal RNA genes have multiple copies in genome actual number of organisms we can potentially detect could be even lower.

We have used synthetic templates covering primer and probe area to measure match probe T_m for each genus and compare it to the sediment samples T_m. Only *Desulfovibrio* probe had a match T_m with both samples. *Methanosarcina* and *Methanogenium* probes had a specific T_m only with the second sample that was taken from a deeper level of the sediment.

TABLE 3. PROBE T_m MEASURED DIRECTLY AFTER REAL-TIME PCR USING SEDIMENT SAMPLE DNA OR CONTROL SYNTHETIC TEMPLATES

	T_m, °C		
Genus	*Control*	*Sample 1*	*Sample 2*
Desulfotomaculum	68	64	62
Methanolobus	67	61	60
Desulfobacter	64	60	58
Methanosarcina	**69**	—	**69**
Desulfococcus	69	—	—
Methanothrix	67	—	—
Desulfuromonas	69	60	58
Desulfomicrobium	70	64	64
Methanobacterium	68	—	—
Methanococcus	69	—	—
Methanococcoides	72	—	53
Desulfovibrio	**68**	**68**	**68**
Methanogenium	**69**	62	**69**

Bold indicates instances where the control and at least one of the sample values are the same.

TABLE 4. RELATIVE QUANTITIES OF GENUS-SPECIFIC TEMPLATES AMPLIFIED FROM TWO SEDIMENTS

	Relative quantities	
Genus	*Sample 1*	*Sample 2*
Desulfotomaculum	—	—
Methanolobus	—	—
Desulfobacter	—	—
Methanosarcina	—	10
Desulfococcus	—	—
Methanothrix	—	—
Desulfuromonas	—	—
Desulfomicrobium	—	—
Methanobacterium	—	—
Methanococcus	—	—
Methanococcoides	—	—
Desulfovibrio	100	100
Methanogenium	—	1

However, it could be seen on Table 2 that *Methanogenium* probe could also hybridize to several methanogens genera. As we have also detected *Methanosarcina* in the same sample, we cannot exclude a possibility that *Methanogenium* detection is a result of a cross-amplification with that genus.

Typically, in marine sediments sulfate reduction is the dominant microbial process in the upper sediments (Kendall et al., 2007) with very little of methane-producing activity. Methanogens are mainly located in deeper sediments, where sulfate is depleted. Our results (Tables 3 and 4) confirm that sulfate-reducing *Desulfovibrio* was detected in relative abundance in both samples. *Methanosarcina* and possibly *Methanogenium* were detected only in the second sample at ~10 times less quantity compared to *Desulfovibrio*. That finding is quite typical for healthy cold marine sediment (Kendall et al., 2007).

Author Disclosure Statement

The authors declare that there is no conflict of interest and no competing financial interests exist.

References

AFONINA, I., ANKOUDINOVA, I., MILLS, A., LOKHOV, S., HUYNH, P., and MAHONEY, W. (2007). Primers with 5′-flaps improve real-time PCR. Biotechniques. **43,** 770–774.

AFONINA, I., MILLS, A., SANDERS, S., KULCHENKO, A., DEMPCY, R., LOKHOV, S., VERMEULEN, N.M., and MAHONEY, W. (2006). Improved biplex quantitative real-time polymerase chain reaction with modified primers for gene expression analysis. Oligonucleotides. **16,** 401–409.

CHEN, Y., DUMONT, M.G., CEBRON, A., and MURRELL, J.C. (2007). Identification of active methanotrophs in a landfill cover soil through detection of expression of 16S rRNA and functional genes. Environ. Microbiol. **9,** 2855–2869.

KENDALL, M.M., WARDLAW, G.D., TANG, C.F., BONIN, A.S., LIU, Y., and VALENTINE, D.L. (2007). Diversity of Arhaea in marine sediments from Skan Bay, Alaska, including cultivated methanogens, and description of *Methanogenium boonei* sp. nov. Appl. Environ. Microbiol. **73,** 407–414.

KUTYAVIN, I.V., LOKHOV, S.G., AFONINA, I.A., DEMPCY, R., GALL, A.A., GORN, V.V., LUKHTANOV, E., METKALF, M., MILLS, A., REED, M.W., SANDERS, S., SHISHKINA, I., and VERMEULEN, N.M.J. (2002). Reduced aggregation and improved specificity of G-rich oligodeoxyribonucleotides containing pyrazplo[3,4-d] pyrimidine guanine bases. Nucleic Acid Res. **22,** 4952–4959.

LUKHTANOV, E., LOKHOV, S.G., GORN, V.V., PODYMINOGIN, M.A., and MAHONEY, W. (2007). Novel DNA probes with low background and high hybridization-triggered fluorescence. Nucleic Acid Res. **35,** e30.

MATSUKI, T., WATANABE, K., FUJIMOTO, J., TAKADA, T., and TANAKA, R. (2004). Use of 16S rRNA gene-targeted group-specific primers for real-time PCR analysis of predominant bacteria in human feces. Appl. Environ. Microbiol. **70,** 7220–7228.

PARK, J.W., and CROWLEY, D.E. (2005). Normalization of soil DNA extraction for accurate quantification of target genes by real time PCR and DGGE. Biotechniques. **38,** 579–586.

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