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Enrichment and specific quantification of *Methanocalculus* in anaerobic digestion

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Members of the genus *Methanocalculus* are characterized as hydrogenotrophic methanogens and present in diverse natural and engineered environments. *Methanocalculus* populations were enriched from anaerobic digesters treating dairy waste using formate as the substrate. *Methanocalculus* sequences retrieved from the enrichment cultures were subsequently used to develop a *Methanocalculus*-specific TaqMan qPCR assay to determine the abundance of *Methanocalculus* populations in the environment, representing the first quantitative tool specifically targeting *Methanocalculus*. The *Methanocalculus*-specific primer/probe set was shown to have high coverage with perfect match to >80% of all *Methanocalculus* 16S rRNA gene sequences in the Ribosomal Database Project (RDP). High specificity of the qPCR assay was also validated by both *in silico* and experimental analyses. Amplification efficiency of the qPCR assay was determined to be 91.9%, which is satisfactory for quantitative applications. Results from the *Methanocalculus*-specific qPCR analysis of formate-enriched methanogenic cultures were consistent with those from clone library analysis of the same cultures, validating the accuracy of the qPCR assay. Subsequent field application of the qPCR assay found low relative abundance of *Methanocalculus* in anaerobic digesters treating dairy waste, accounting for 0.01% of the archaeal populations. The qPCR results were consistent with the lack of detection of *Methanocalculus* in previous studies of the same anaerobic digesters with clone library analyses, which are less sensitive than qPCR. Thus, the *Methanocalculus*-specific qPCR assay developed in this study is a highly sensitive tool for the rapid and efficient quantification of *Methanocalculus* populations in methanogenic environments and understanding of the ecological functions of these methanogens.

[**Key words:** Anaerobic digestion; Formate; *Methanocalculus*; Methanogen; qPCR]

Methanogenesis is of great significance in global carbon cycling in diverse ecosystems (1). Methanogenesis has also been exploited in anaerobic digestion processes for the treatment of various organic waste streams, including animal manure, food waste, and sludge from wastewater treatment (2). Methanogenic anaerobic digestion of these waste materials has the benefit of producing biomethane as a renewable energy source and reducing greenhouse gas emission, leading to increased effort to enhancing the efficiency and stability of anaerobic digestion processes.

Methanogens as a group of archaeal populations are key members of the microbial communities exclusively involved in the terminal steps of the anaerobic transformation of organic materials into methane (3). Given the importance of methanogens in both natural environments and engineered systems, monitoring the abundance and dynamics of methanogen populations is of great importance to understanding how these microorganisms respond to changing environmental conditions and subsequently affect the performance of the anaerobic food web. While methanogens are characterized by the common trait of methanogenic metabolism, it is well established that methanogen populations differ in substrate spectrum, substrate affinity, and growth kinetics, among others (4). Therefore, a number of quantitative tools, such as 16S rRNA gene-based quantitative PCR assays (qPCR), have been developed for the monitoring of specific methanogen populations at fine phylogenetic resolutions (5).

Methanocalculus represents a group of hydrogenotrophic mesophilic methanogen populations found in diverse natural habitats in marine, estuarine, terrestrial environments as well as solid waste disposal sites and anaerobic digestion processes (6–12), suggesting its potential significance in anaerobic environments. Understanding of the ecological functions of these methanogens, however, is incomplete, in particular hindered by the lack of quantitative tools specifically targeting *Methanocalculus*. Therefore, the objectives of this study were to identify cultivation conditions for the enrichment of *Methanocalculus* and subsequently develop a *Methanocalculus*-specific qPCR assay with high sensitivity for the monitoring of these archaeal populations in the environment. Results from this study show that enrichment of *Methanocalculus* was specific to formate as the substrate and the *Methanocalculus*-specific qPCR assay developed with the sequences of *Methanocalculus* enriched by formate had excellent specificity, coverage, and amplification efficiency, providing a much needed tool for the quantitative monitoring of *Methanocalculus* populations in methanogenic environments.

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MATERIALS AND METHODS

Anaerobic digester set-up Triplicate anaerobic digesters were established as model systems to study methanogen communities and as sources to enrich for specific methanogen populations. These mesophilic continuous bench-scale anaerobic digesters used dairy waste as the feedstock at an organic loading rate of 1.0 gVS/L/day as described previously (13). The temperature of the anaerobic digestion process was maintained at 35°C and the hydraulic retention time was controlled at 20 days.

Enrichment for methanogens To enrich for hydrogenotrophic methanogen populations including *Methanocalculus*, an anaerobic basal medium was prepared according to the following recipe (per liter) as previously described (14): NaCl, 1.0 g; $MgCl_2 \cdot 6\,H_2O$, 0.5 g; KH_2PO_4, 0.2 g; NH_4Cl, 0.3 g; KCl, 0.3 g; $CaCl_2 \cdot 2H_2O$, 0.015 g; trace element solution, 1.0 mL; Se/Wo solution, 1.0 mL; and resazurin, 1.0 mg. L-cysteine (0.031 g/L) and $Na_2S \cdot 9\,H_2O$ (0.048 g/L) were added as reductants into the basal medium after it had been boiled and cooled to room temperature under an oxygen-free N_2 atmosphere. $NaHCO_3$ (2.52 g/L) was added to the medium as the buffer. The medium was subsequently dispensed into 160-mL serum bottles flushed with pure N_2 and sealed with butyl rubber stoppers and aluminum caps. A sterile vitamin solution (1%) was added after autoclaving (15).

Initial enrichments for methanogens were seeded with a 10% (vol/vol) inoculum of digestate from the continuous bench-scale anaerobic digesters fed with dairy waste along with 50 mM formate as the sole substrate, which was replenished when depleted. At the completion of 10 feedings of formate, secondary enrichments were established by transferring a 10% (vol/vol) inoculum from the initial enrichment cultures into fresh medium followed by repeated feedings of 50 mM formate.

To determine whether *Methanocalculus* could be enriched with other substrates, enrichment cultures were set up and handled following the same protocols described above, with the only exception being the substrate used. The following organic compounds were used as the sole methanogenic substrate in individual enrichment cultures: methanol, acetate, propionate, and butyrate.

All enrichment cultures were set up in triplicates and maintained in a shaking incubator at 80 rpm and 35°C. Biogas production was measured using a previously described water displacement method (16). Methane content in biogas was analyzed by a Hewlett Packard 5890 Series II gas chromatograph (GC) equipped with a thermal conductivity detector (TCD) and a Supelco packing column (60/80 Carbonxen-1000; Sigma–Aldrich, St Louis, MO, USA). Argon was used as the carrier gas with a flow rate of 5 mL/min. GC analysis of methane used the following temperature scheme: oven 125°C, injection port 150°C and TCD detector 170°C.

Clone library analysis of enrichment cultures Clone library analysis was performed to examine the composition of methanogen communities in the enrichment cultures and to obtain 16S rRNA gene templates for the design of qPCR assays specific to enriched methanogen populations. Biomass from secondary enrichments was pelleted by centrifugation and stored at −80°C. Whole community DNA was extracted and purified as previously described (17). DNA extracts from triplicate enrichments were pooled for PCR amplification of the 16S rRNA genes using the following *Archaea*-specific primers: Arch21F (5′-TTCCGGTTGATCCYG CCGGA-3′) and Arch958R (5′-YCCGGCGTTGAMTCCAATT-3′) following previously described PCR protocols (18). Amplicons of 16S rRNA gene sequences were subsequently purified and cloned into plasmid vectors following procedures described previously (19). Twenty cloned plasmid inserts were randomly selected for sequencing with the ABI Prism Big Dye chemistry (Applied Biosystems, Foster City, CA, USA) using M13 forward and reverse primers. The obtained sequences were checked for chimeric artifacts using the Chimera Check program in the Ribosomal Database Project II (20). High quality 16S rRNA gene sequences were deposited at GenBank under the following accession numbers: KJ435044—KJ435059.

Subsequently, phylogenetic analysis was conducted on the 16S rRNA gene sequences retrieved from the formate enrichment cultures by searching the NCBI GenBank database for closely related sequences. The phylogenetic positions of these 16S rRNA gene sequences were assessed by phylogenetic trees constructed with the neighbor-joining algorithm (1000 bootstrap re-samplings) using MEGA 4.0 (21).

Primer/probe design for *Methanocalculus*-specific qPCR assay Since 16S rRNA gene sequences belonging to the genus *Methanocalculus* dominated the methanogen community in the formate enrichments (see Results), *Methanocalculus*-specific qPCR assay for the quantification of this group of methanogens was developed based on all of the 376 16S rRNA gene sequences of the genus *Methanocalculus* in RDP Release 11 as well as *Methanocalculus* sequences retrieved from the formate enrichment cultures in this study using primer/probe design procedures previously described for TaqMan chemistry (22). The primer/probe sequences were determined following guidelines provided by Applied Biosystems on parameters including amplicon size, nucleotide position, melting temperature (T_m), GC content, self-annealing, complementarity, and hairpin formation, according to the Oligo Calculator program (23). *In silico* evaluation of the specificity and coverage of the primer/probe set was performed using the ProbeMatch program of RDP II (24).

Experimental evaluation and field application of *Methanocalculus*-specific qPCR assay Primer/probe specificity was further assessed using partial 16S rRNA genes of representative methanogen populations cloned from the continuous bench-scale anaerobic digesters in a previous study (19), including *Methanoculleus* (GenBank accession no. JN052755), *Methanosaeta* (GenBank accession no. JN052761), and *Methanosarcina* (GenBank accession no. JN052757), in addition to *Methanocalculus* (GenBank accession no. KJ435045) derived from the formate enrichment cultures in this study. Moreover, one non-methanogen archaeal 16S rRNA gene clone (Genbank accession no. JN052741) phylogenetically related to *Crenarchaeota* was also included in the assessment because of the potential significance of *Crenarchaeota* populations in anaerobic digestion (13,25). These 16S rRNA gene clones were used as DNA templates for qPCR assays to test the specificity of the *Methanocalculus*-specific primer/probe set designed in this study. Amplification efficiency (E) of the qPCR assay was determined with the C_T-Log [Template] plot derived from the quantification of 10-fold dilution series of *Methanocalculus* 16S rRNA gene templates (18−1.8 × 10^9 copies/reaction) as previously described (26).

To further validate the accuracy of the *Methanocalculus*-specific qPCR assay, quantification of *Methanocalculus* populations was conducted for the secondary formate enrichment cultures. Results from qPCR were compared with those from clone library analysis performed on the same samples. Moreover, the *Methanocalculus*-specific qPCR assay was applied to quantify *Methanocalculus* populations in the archaeal communities in the continuous anaerobic digesters treating dairy waste.

TaqMan qPCR protocol The *Methanocalculus*-specific primers and dual-labeled TaqMan probe, 5'-end labeled with 6-carboxyfluorescein (FAM) and 3'-end labeled with the Black Hole Quencher (BHQ), were obtained from Biosearch Technologies (Novato, CA, USA). All qPCR assays were performed in 25 μL reaction volume with 15 pmol of the primers, 5 pmol of the probe, and Brilliant II QPCR Master Mix (Agilent, Santa Clara, CA, USA). Thermal cycling consisted of a starting incubation at 50°C for 2 min and an initial denaturation at 95°C for 10 min, followed by up to 45 cycles at 95°C for 30 s and 60°C for 45 s. To determine the relative abundance of *Methanocalculus* populations in the archaeal community, total *Archaea* was quantified with the *Archaea* domain-specific TaqMan primer/probe set as previously described (5): forward primer Arc787F-ATTAGATACCCSBGTAGTCC, probe Arc915P-AGGAATTGGCGGGGGAGCAC, and reverse primer Arc1059R-GCCATGCACCWCCTCT. Thermal cycling and fluorescence detection were performed with a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Fluorescence response data were processed with the software provided by the manufacturer (Bio-Rad). For all qPCR assays, standards, controls, and samples were run in triplicates. Gene copy numbers were determined from standard curves based on the log transformation of known concentrations versus the threshold cycle (C_T).

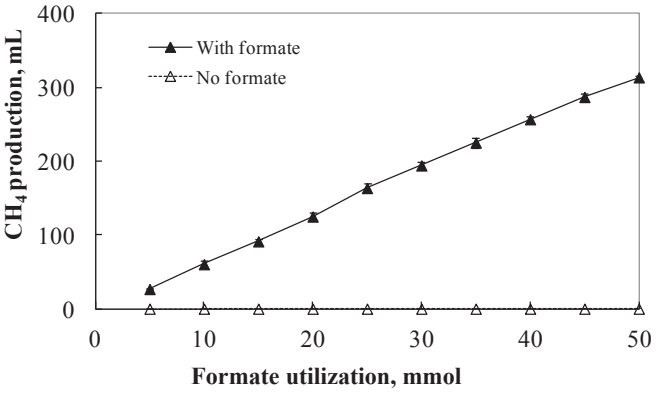

FIG. 1. Methane production and substrate utilization in secondary enrichment cultures. Controls lacked formate as the substrate. Data points were means of triplicate cultures with the error bars showing standard deviation.

RESULTS

Enrichment of methanogen populations with formate Formate was used as the sole substrate to enrich hydrogenotrophic methanogen populations from anaerobic digesters treating diary waste. The consumption of formate was accompanied with the production of methane gas in the enrichment cultures (Fig. 1), which could be attributed solely to formate since no methane production was observed in the controls without formate amendment. Methane production averaged 31 mL from each feeding of 50 mM formate, corresponding to a