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Abstract

Microbial source tracking has historically focused on the origin of traditional enteric indicators including coliforms, enterococci, or *Escherichia coli*. Recently, questions of genetic variability and environmental persistence have encouraged researchers to search for additional animal specific indicators of fecal pollution. To date only eubacteria have been utilized as markers of human and animal-specific pollution. In this study we developed a molecular primer pair specific for *Methanobrevibacter smithii*, a methanogen found only in the human intestine. PCR primers for the *nifH* gene of *M. smithii* were designed, tested, and used to detect the presence or absence of the organism in fecal and environmental samples. Product amplification was observed in 28.6% of all human fecal samples and 93% of sewer samples, and water contaminated with human sewage. No amplification was observed when primers were tested against 43 bacterial stock cultures and fecal samples from 204 animals. Sequencing of PCR products from sewers and *M. smithii* cells demonstrated that the 222bp product amplified was the *nifH* gene of *M. smithii*. Sensitivity assays demonstrated a detection limit of 10ng in human feces, 10ng in fecally contaminated water, and 5 ng in sewer samples. In addition, samples seeded with *M. smithii* established a lower detection limit of 13 cells/ml. The Mnif method for *M. smithii* detection appears to be a rapid, inexpensive, and reliable test for determining the presence or absence of human fecal pollution in recreational waters.

Introduction

To measure fecal pollution in coastal recreational waters, regulators enumerate fecal coliforms and/or enterococci. These bacteria originate in the intestines of animals and are also found in environmental samples. Frequently, the source of bacterial contamination is not known. Researchers have evaluated genotypic and phenotypic protocols to pinpoint the source of fecally contaminated water. Often, these methods are inconsistent in assigning host sources. Furthermore, problems concerning the persistence of *E. coli* and enterococci in sediments, vegetation, and beach water have been shown. For these reasons, researchers have investigated rapid, simple, and reliable tests to identify underutilized organisms found in specific animals. Human-specific indicators such as *Bifidobacterium dentium, Bacteroides spp., Bacteroides thetaiotamicron*, and the *Enterococcus faecium* esp gene have been identified and used for tracking the source of fecal pollution. Animal specific markers such as swine and bovine-specific genes from enterotoxigenic *E. coli* have been identified for specific animal identification. Thus far, all organisms targeted for use as human or animal-specific indicators have been gut-associated eubacteria. However, archaeabacteria, particularly gut-associated methanogens, may serve as possible animal or human markers of fecal pollution.

The genus *Methanobrevibacter* is a member of the order *Methanobacteriales* within the domain archaea of the kingdom *Euryarchaeota*. This genus includes ten known species that inhabit animal intestinal tracts, decaying plant material, and anaerobic sludge of wastewater treatment plants. *Methanobrevibacter smithii* is the only species known to specifically colonize the human large intestine and vaginal tract. Methane production has been found in approximately 33% of the human population in the United States and Great Britain, and enumeration studies have shown that *M. smithii* is the most abundant methanogen in the human gut in concentrations of 10^7- 10^{10} per gram (dry weight). In this study, we designed and tested a molecular primer pair targeting the gene coding for the nitrogenase iron protein (*nifH*) of *Methanobrevibacter smithii* to determine the viability of this organism as a human-specific marker of fecal pollution.

Materials and Methods

Primer Development & DNA Extraction:

Primer sets specific for the *Methanobrevibacter* genus and *M. smithii* were developed using the Seqweb primer development program (Seqweb; Accelerys, San Diego, CA) from published *Methanobrevibacter* 16S rRNA sequences and the *nifH* gene of *M. smithii*. Primer specificity was tested against 12 species of *Methanobrevibacter*, and 12 additional methanogen genera. DNA was extracted using both the (~ 5 x 10^7 bacteria/ml) was extracted using the MO BIO Ultraclean Soil DNA Extraction Kit (MO BIO Labs, Carlsbad California). In addition, a 100µl sample of methanogen culture was applied to a Whatman FTA® Classic Card (Whatman, Florham Park, NJ) and allowed to dry for one hour at room temperature. A 2.0mm punch was removed from the card and placed directly into a PCR tube. The punches were washed 3X (5 minutes each) with 200µl of FTA Purification Reagent, and 2X with 200µl TE^{-1} buffer. The punches were allowed to dry for approximately 2 hours at room temperature and used directly in a 50µl PCR reaction.

DNA from human and animal feces was extracted from fecal samples using the UltraClean Soil DNA Extraction kit and the Powersoil DNA Kit (MO BIO) following the manufacturer instructions. Extracted DNA was quantified using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). Sewer samples (500ml) were prefiltered through a 3µm cellulose acetate filter (Pall Gelman) and concentrated onto a 0.2µm cellulose acetate filter (Pall Gelman). The filter was placed in a sterile 50ml beaker containing 5ml of PBS and agitated with a magnetic stir bar for 2 minutes to dislodge bacteria. The sample was centrifuged for 15 minutes at 1300 x g, the cell pellet reconstituted in 100µl of sterile water, and DNA from pelleted cells extracted using the Powersoil DNA Kit (MO BIO) or BIO 101 FastDNA Spin Kit for Soil (Q-Biogene, Inc.).

To determine the cell detection limit of *M. smithii*, a stock culture was counted using a Petroff-Hausser Counting Chamber (Hausser Scientific, Horsham, PA) and diluted in filtered marine water (filtered through a 0.45µm cellulose acetate filter; Pall Gelman) in ten-fold increments from 1.32 X 10^8 to 13.2 cells/ml. Dilutions were filtered as described above, and the DNA was extracted using the MO BIO Powersoil DNA Kit.

Marine and freshwater samples were collected during (and every week after for 2 months) a documented sewage spill event in which 30,000 gallons per minute (for 24 hours) of raw sewage were pumped into Coffee Creek adjacent to the sewer line break in Gulfport, MS. As Coffee Creek feeds directly into the Mississippi Department of Environmental Quality (MDEQ) beach station 10, samples were collected from three sites in Coffee Creek, the open culvert feeding into station 10, and the beach water at station 10. A clean marine water sample was taken from MDEQ station 9 approximately 0.5mi west of station 10 as a non-contaminated control. For each sample, 500ml of water was collected in a sterile sample bottle, pre-filtered, and concentrated in a similar manner as sewage samples. The DNA was extracted from all samples using the BIO 101 Fast DNA Spin Kit for Soil (Q-biogene, Inc.) and quantified using the Nanodrop ND-1000 spectrophotometer.

DNA was extracted from sediment cores in the Mississippi Sound to determine the presence of *M. smithii*. Sediment cores were obtained from MDEQ stations 8, 9, 10, 11, & 13 (Figure 1) using a sterile conical-shaped 50ml centrifuge tube (Sarstedt, Newton, North Carolina). Further, 12-inch cores were taken from MDEQ stations 8 and 9, as well as a sediment trap west of station 9, to determine the presence or absence of *M. smithii* in deep, anoxic sediments. DNA was extracted from the 0, 6, and 12 inch layers of the cores using the Ultraclean Soil DNA Extraction Kit (MO BIO).

PCR Conditions:

The MET and Mnif primers were tested against 19 different Gram (+) and Gram (-) eubacteria using whole cell PCR. In preparation for whole cell PCR, overnight cultures (approximately 1 x 10^9 bacteria/ml) were centrifuged at 1300 x g for 3 minutes. The resultant pellet was washed 2X with 1X PBS, resuspended in 250µl DNA grade water and frozen at -20ºC.

Each whole cell and DNA extract 10µl PCR reaction contained: 1X PCR Buffer (New England BioLabs), 0.1% BSA, 200µM dNTP (USB Corporation), 0.5U Taq Polymerase (New England BioLabs), 100µM each primer (MET or Mnif), 1µl template cells (approximately 1 x 10^9 cells/ml) or 1µl DNA template, and QS with water to 10µl. FTA Card DNA extracts were amplified in 50µl reactions containing 1X PCR Buffer (New England BioLabs), 0.1% BSA, 200µM dNTP (USB Corporation), 2.5U Taq Polymerase (New England BioLabs), 100µM each primer (MET or Mnif), and the 2.0mm washed punch. The cycling conditions for both MET and Mnif primers were an initial denaturation of 3 minutes at 92ºC and 30 cycles of: denaturation for 2 min at 92ºC, annealing for 30 sec at 55.1ºC, and elongation for 1 min at 72ºC. A final elongation of 6 min at 72ºC was performed to allow duplexing to occur. All PCR cycling procedures were conducted in an Eppendorf Mastercycler PCR instrument (Brinkman Instrument, Inc., Westbury, NY). PCR products were separated on a 1.5% agarose gel, stained in 1µg/ml ethidium bromide (Amresco, Solon, OH) for 15 minutes, and digitally photographed with the Alpha Imager (Alpha Innotech Corporation, San Leandro, CA).

Sequencing of sewer PCR products.

To verify the identity of the PCR product amplified by the Mnif primers, the amplified products from 4 sewage lift stations and a stock culture of *M. smithii* were sequenced. Amplified products were run on a 1.5% agarose gel, the bands excised, and the DNA extracted using the Zymoclean Gel DNA Recovery Kit (Zymo Research, Orange, CA). The purified DNA was quantified using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE), cloned into the pT7Blue Vector (Novagen, San Diego, CA), and transformed into competent *E. coli* cells. The plasmids were isolated using the E.Z.N.A. Plasmid Mini Prep Kit I (Omega Bio-tek, Inc., Doraville, GA) and sequenced by Macrogen, Inc. (Macrogen, Seoul, Korea) using an ABI3730 XL automated DNA sequencer.

Sensitivity Assays

Sensitivity assays were performed for the *M. smithii* stock culture, sewer DNA extracts, human fecal DNA extracts, and cellular DNA extracted from environmental samples. Detection limits for the *M. smithii* pure culture were conducted by adding 10 to 10^{-5}ng of *M. smithii* stock culture DNA extract to the Mnif PCR reaction. Detection limits for sewer and human fecal DNA were determined by adding a range of DNA from 1ng to 50ng in the PCR reaction. Sensitivity of the Mnif PCR assay for sewage-contaminated water was determined by adding a range of DNA concentrations from 1ng to 20ng in the PCR reaction. Detection limits were determined as presence or absence of 222bp PCR products on a 1.5% agarose gel.

Results

Primers specific for the *Methanobrevibacter* genus (MET) amplified a product of 282bp. The Mnif primers amplified a 222bp product. The MET primers were positive for all *Methanobrevibacter* species, and did not amplify a product in any other bacterial or methanogen genus. The Mnif primers targeting the *nifH* gene of *M. smithii* were positive only for *M. smithii* and did not amplify products in any other bacteria tested. Tests of fecal DNA showed that 28.6% of human fecal samples and 25 out of 27 sewers tested were positive for *M. smithii*. All animal fecal DNA tested showed no amplification using the Mnif PCR reaction. Out of a total of 12 plasmids sequenced to confirm the presence of the *M. smithii nifH* gene, 10 were identified by BLAST search as the nitrogenase-Fe (*nifH*) gene of *M. smithii*. Two plasmids showed sequence homology to the pT7Blue vector, indicating that the plasmids did not contain the PCR product. This illustrates that only the *nifH* gene of *M. smithii* was amplified with the primer set.

Sensitivity assays showed that the Mnif PCR amplified the expected product in pure culture to 1pg, and sewer samples showed a detection limit in 5ng of total DNA. Detection limits in human fecal samples were dependent on the individual sample. All samples showed detection to 10ng, although some were more sensitive and could be detected to 1ng. In addition, dilution of a pure culture of *M. smithii* showed detection of the Mnif PCR product to 1.3 X 10^1 cells/ml. Sampling conducted during a known sewer line break showed strong amplification for *M. smithii* at all stations tested except MDEQ station 9. Sensitivity tests showed that all sites amplified products with as little as 10ng of total DNA. The *M. smithii* signature was still detectable at 24 days at 10ng but was not detected on the 31st day.

Previous investigations in our laboratory have indicated that sediments may be a source of coliforms and enterococci in coastal recreational waters. For this reason, seventeen sediment samples were analyzed for the presence of *M. smithii*. Only 2 sediments out of 17 tested positive for *M. smithii* (MDEQ station 8 sediments showed the presence of *M. smithii* at a 12-inch depth, and station 9 sediments showed the presence of *M. smithii* at a 6-inch depth).

Table 1. Species tested for MET and Mnif primer specificity

Species Negative for both MET and Mnif	Species positive for MET and negative for Mnif	Species positive for MET and Mnif
P. aeruginosa, S. aureus ss aureus, S. pneumoniae, E. coli, S. bovis, S. marcescens, P. vulgaris, S. sonnei, S. flexneri, S. choleraesuis, E. faecalis, E. faecium, E. hirae, E. casseliflavus, E.durans, E. gallinarum, L. lactis ss lactis, E. saccharolyticus, E.avium, Methanococcus maripaludis, Methanocalculus pumilus, Methanocorpusculum aggregans, Methanosphaera stadtmaniae, Methanocaldococcus infernus, Methanofollis liminatans, Methanomicrobium mobile, Methanomicrococcus blatticola, Methanosarcina barkeri, Methanolobus oregonensis, Methanosaeta concilii, Methanobacterium bryantii	*Methanobrevibacter oralis, M. ruminantium, M. acididurans, M. woliniі, M. gottschalkii, M. arboriphilus, M. curvatus, M. cuticularis, M. filiformis, M. thaueri,* and *M. woesei*	*Methanobrevibacter smithii*

Table 2. Number of fecal samples positive for *M. smithii*

Environmental Sources Tested	# Samples Tested	Mnif PCR Amplification
Human	70	20/70
Cow	46	0/46
Sheep	2	0/2
Swine	24	0/24
Horse	23	0/23
Deer	20	0/20
Goat	2	0/2
Turkey	20	0/20
Goose	17	0/17
Chicken	23	0/23
Dog	27	0/27
Sewer	27	25/27

Discussion & Conclusions

Methanogens are normally found in marine and terrestrial environments. Only a few are residents of the animal gut, and as such, are good candidates for human and animal specific markers in the environment. To date, *Methanobrevibacter* species, with the exception of *M. arboriphilus*, have been found to inhabit only the intestines and oral cavities of animals and activated sludge. Few species occur in more than one organism, although *M. ruminantium* has been found in ruminants other than cows (deer), and *M. gottschalkii* and *M. thaueri* are found in both horses and pigs. *Methanobrevibacter smithii* has been found to inhabit only the intestinal tract of humans and appears to be a prime candidate for use as a human-specific marker of fecal pollution in the environment. In this study, a primer set specific for *Methanobrevibacter smithii* was developed from the partial sequence of the *nifH* gene of *M. smithii*. To determine the specificity of the primer to *M. smithii* and humans, a total of 70 human fecal samples, 24 methanogens, 19 Gram (+) and Gram (-) eubacteria, 27 sewer samples, and 204 animal fecal samples were tested with the MET and Mnif primers. The only organism out of the 43 tested that gave an amplified product with the Mnif primers was *M. smithii*. The only organisms that gave a product with the MET primers were the *Methanobrevibacter* species. In addition, all animal feces known to harbor *Methanobrevibacter* were negative for the Mnif primers at 10ng or lower concentration, and all but two sewer samples were positive for *M. smithii*. In addition, sequences of the sewer and *M. smithii* PCR products were shown to be the *M. smithii nifH* gene by BLAST search. These results demonstrate that the Mnif primer set is specific for *M. smithii*, and that *M. smithii* is indeed part of the human intestine and sewage environments.

Preliminary studies in our laboratory have shown that indicator organisms residing in sediments may be released into the water column after normal storm events. This release can cause counts to increase dramatically leading to beach closure events. For this reason, sediments were tested to determine the presence of *M. smithii* in both the fluffy, bacterial-rich layer at the sediment surface and the deep anoxic sediments. The results showed that *M. smithii* is present only in deep (6 inches or deeper) cores in the Mississippi Sound. Since the Sound is a low wave energy environment protected from the Gulf of Mexico by a string of barrier islands located 10-20 km from the mainland, the system does not produce sufficient wave action during normal conditions to disturb the sediment. During normal storm conditions (excluding tropical storms and hurricanes) the upper 1-2 inches may be disturbed. Therefore, the *M. smithii* detected in the deep sediments (6-12 inches) should not be released under normal conditions into the surrounding water, and sediments should not be considered a source of these organisms.

Since the sensitivity levels of the sewer and contaminated water Mnif PCR product detected were comparable (5ng and 10ng respectively), this method shows promising detection of human sewage in contaminated water even with dilution of the sewage. In addition, 10ng of DNA consistently showed the presence of *M. smithii* in contaminated water for as long as 24 days after the sewage spill indicating its ability to last in the environment comparably longer than other anaerobic host-specific markers such as *Bifidobacterium.* In addition, the ability of the assay to detect as few as 13 cells/ml indicates the possible future use of the Mnif PCR assay as a quantifiable test for human pollution levels in recreational water.

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