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Detection and Quantification of Methyl *tert*-Butyl Ether-Degrading Strain PM1 by Real-Time TaqMan PCR

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The fuel oxygenate methyl *tert*-butyl ether (MTBE), a widely distributed groundwater contaminant, shows potential for treatment by in situ bioremediation. The bacterial strain PM1 rapidly mineralizes and grows on MTBE in laboratory cultures and can degrade the contaminant when inoculated into groundwater or soil microcosms. We applied the TaqMan quantitative PCR method to detect and quantify strain PM1 in laboratory and field samples. Specific primers and probes were designed for the 16S ribosomal DNA region, and specificity of the primers was confirmed with DNA from 15 related bacterial strains. A linear relationship was measured between the threshold fluorescence (C_T) value and the quantity of PM1 DNA or PM1 cell density. The detection limit for PM1 TaqMan assay was 2 PM1 cells/ml in pure culture or 180 PM1 cells/ml in a mixture of PM1 with *Escherichia coli* cells. We could measure PM1 densities in solution culture, groundwater, and sediment samples spiked with PM1 as well as in groundwater collected from an MTBE bioaugmentation field study. In a microcosm biodegradation study, increases in the population density of PM1 corresponded to the rate of removal of MTBE.

Methyl *tert*-butyl ether (MTBE) has been used as a gasoline additive since the late 1970s in an effort to increase combustion efficiency and reduce air pollution. A major source of MTBE into the environment is from leaking underground storage tanks at gas stations. MTBE has emerged as an important water pollutant because of its persistence, toxicity, mobility, and widespread use. A report by the U.S. Geological Survey identified MTBE as the second most common volatile organic contaminant of urban aquifers in the United States (1997). Bacterial and fungal cultures isolated from various environmental media are capable of degrading MTBE either as a primary source of carbon and energy or cometabolically following growth on another substrate (9, 14, 15, 28, 34). Evidence of MTBE biodegradation in the field has been reported in oxygen-amended groundwater (29) and surface waters (5, 19).

An MTBE-degrading bacterial strain (strain PM1), isolated from a compost biofilter, has been classified as a member of the *Rubrivivax gelatinosus* subgroup of β-*Proteobacteria* (14). The 16S ribosomal DNA (rDNA) analysis confirmed that the PM1 sequence is most similar to other members of β-*Proteobacteria*, such as *Aquabacterium*, *Leptothrix*, *Rubrivivax*, and *Ideonella* (6). PM1 rapidly mineralizes MTBE at concentrations of up to 500 mg/liter in laboratory cultures (14) and can degrade MTBE when inoculated into groundwater or soil microcosms. In core material from a fuel-contaminated aquifer at Port Hueneme, California, inoculation with PM1 rapidly accelerated the biodegradation of MTBE. A bioaugmentation field pilot test with PM1 is now under way at Port Hueneme.

Biodegradation rate is strongly dependent upon the population size of those organisms carrying out the process of degradation (31). To better understand the factors controlling MTBE biodegradation rate as well as to monitor the survival and movement of PM1 after introduction into the environment, a method to quantify the population density of PM1 was established. A common way of monitoring microbial populations in the environment is to use molecular techniques that detect and quantify specific phylogenetic groups of microorganisms based on 16S rDNA sequences or relevant structural genes (8, 13, 27, 30, 33, 37). Direct hybridization to rRNA extracts or whole cells are preferable to PCR methods for actual quantification of population densities. Many environmental organisms, however, are present at such low densities in mixed microbial communities that PCR-based amplification techniques must be used to detect them. Given that conventional qualitative PCR does not provide reliable quantitative information about densities (30), quantitative PCR methods have been developed to address this deficiency.

One method of competitive quantitative PCR relies on the inclusion of a competitive sequence serving as an internal control in each reaction and requires time- and resource-consuming post-PCR analyses (26). A promising alternative method is real-time quantitative PCR based on 5′ nuclease chemistry (TaqMan assay) (10, 17, 18). The kinetic real-time TaqMan PCR method calculates a precise quantitative measure of a specific sequence from the initial exponential phase of the PCR. This is in contrast to methods that use endpoint detection PCR, in which the final product of the PCR is determined (17). The TaqMan PCR method uses a fluorescent oligonucleotide probe with a 5′ reporter dye and 3′ quencher dye. During the PCR, the 5′-3′ nuclease activity of *Taq* DNA polymerase cleaves nucleotides from an oligonucleotide probe annealed to a target DNA strand. As the amplification reactions proceed, more amplicons become available for probe binding and consequently the fluorescence signal intensity per cycle increases

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TABLE 1. Bacterial strains used to test the specificity of the PM1 TaqMan assay

Bacterial strain[a]	Medium	C_T value[b]
PM1	MSM	23.2
E. coli K-12 ATCC 10798	ATCC medium 3	40
Bacillus subtilis ATCC 6051	ATCC medium 3	40
P. putida ATCC 12633	ATCC medium 3	38.1
S. lactis ATCC 19435	ATCC medium 17	38.68
Staphylococcus aureus ATCC	ATCC medium 3	40
A. facilis ATCC 11228	ATCC medium 81	37.03
H. flava ATCC 33667	ATCC medium 3	40
Micrococcus luteus ATCC 4698	ATCC medium 18	40
Acinetobacter calcoaceticus	ATCC medium 3	40
Azomonas agilis ATCC 7494	ATCC medium 11	40
A. commune DSMZ 11901	DSMZ medium 464	39.55
Mycobacterium sp.	ATCC medium 90	40
Gr(+) bacterial isolate	0.1 × TS	40
Gr(−) bacterial isolate	0.1 × TS	40
Nitrosomonas europaea ATCC 19718	ATCC medium 221	40

[a] Strains were obtained from American Type Culture Collection (ATCC; Manassas, Va.) or Deutsche Sammlung von Mikroorganismen and Zellkulturen GmbH (DSMZ; Braunschweig, Germany).

[b] C_T values obtained when 1,000 pg of DNA was used as a DNA template in the TaqMan PCR.

(17). The initial copy number is estimated from the exponential phase of product accumulation and by comparison to a standard curve. Early applications of the TaqMan PCR method were to detect pathogenic organisms, such as hepatitis C, *Salmonella* spp., *Listeria monocytogenes*, toxigenic *Escherichia coli*, *Neisseria meningitidis*, *Stachybotrys chartarum* conidia, *Borrelia burgdorferi*, *Ehrlichia* spp., and phytopathogenic *Ralstonia solanacearum* (3, 7, 12, 16, 21, 24, 25, 38–40). In the last year, the TaqMan method has been developed for use in studies of microbial ecology. Grüntzig et al. (11) quantified the abundance of nitrate reductase genes in various environmental samples. Suzuki et al. (35) and Takai and Horikoshi (36) quantified 16S rRNA genes at the domain and/or group levels in bacterial communities in marine waters, hot springs, and freshwater sediments.

The objective of this study was to develop a quantitative real-time TaqMan PCR method for detection of rDNA sequences specific to the MTBE-degrading strain PM1. We compared TaqMan PCR estimates of PM1 cell densities to MTBE biodegradation dynamics and quantified PM1 population densities in microcosms of MTBE-spiked groundwater as well as in MTBE-contaminated groundwater and sediment.

MATERIALS AND METHODS

Bacterial strains and growth conditions. For subsequent DNA extractions, bacterial strain PM1 was grown in mineral salt media [MSM; 0.66 g of $(NH_4)_2SO_4$, 1.3 g of KH_2PO_4, 0.123 g of $MgSO_40 \cdot 7H_2O$, 0.017 g of $CaSO_4 \cdot 2H_2O$, and 0.006 g of $FeSO_4$] containing 250 μg of MTBE ml^{-1} to cell densities of 10^9 ml^{-1}. Bacterial strains used for evaluation of primer specificity are listed in Table 1. Two pure lab cultures isolated from soil and designated Gr(+) and Gr(−) were grown on 0.1× TS media (3 g of tryptic soy broth per liter; Difco Laboratories, Detroit, Mich.) and used in the analyses as well. Genomic DNA was extracted with standard methods (2) and quantified using a Lambda 10 UV/Vis spectrometer (PE Applied Biosystems).

Laboratory microcosm experiments. Microcosm experiments were conducted in MSM or groundwater from Port Hueneme MTBE plumes within (Plot B, well B32D) and outside (well CBC61CS) of the bioremediation field plot. At Port Hueneme, the University of California, Davis, field site is located 610 m down a gradient from the source NEX Service Station. In a field trial, oxygen is provided via a pulsed sparging system. The three treatments include oxygen only (Plot A), oxygen and strain PM1 (Plot B), and air only (Plot C) (32). Strain PM1 was injected at plot B in November 1999. The 100-ml microcosms (in six replicates; 5 ppm of MTBE was added in the lab) with MSM (or groundwater from well CBC61CS) were inoculated with strain PM1 (density of 2×10^6/ml as determined by heterotrophic plate counting). Groundwater from well B32D was taken from the field plot at Port Hueneme 7 months after injection of strain PM1 into the ground (32), and no PM1 was added in the lab. The experiment was performed in 250-ml microcosm bottles with Teflon-lined Mini-Nert closures (Alltech, Deerfield, Ill.), and bottles were kept in the dark on an orbital shaker. Duplicate sterile controls using 1% sodium azide were also established for each experiment. Of the six replicate microcosms, three were monitored for MTBE biodegradation and the other three were sampled (5-ml samples) in the time and filtrated for DNA extraction (protocol for groundwater DNA extraction).

MTBE concentrations in the headspace were monitored using a Shimadzu GC-14A gas chromatograph with a 15-m-long, 0.53-mm-diameter DB1 column (J&W Scientific, Folsom, Calif.) and a photoionization detector. Fifty microliters of sample headspace was injected per sample. Flow rates and operating procedures have been described by Hanson et al. (14). The headspace method provides a detection limit of 0.1 mg of MTBE per liter.

DNA extraction from groundwater. DNA was extracted from 5-ml microcosms or 130-ml ground water samples by using the same protocol. Groundwater samples were collected from sampling wells in a field plot at Port Hueneme by using Cole Parmer Masterflex peristaltic pumps and were shipped in ice to the University of California, Davis. Bacterial cells were concentrated from ground water on white polycarbonate filters (diameter, 47-mm; pore size, 0.2 μm; type GTTP 2500; Millipore, Bedford, Mass.), which are placed on nitrocellulose support filters (diameter, 47 mm; pore size, 0.45 μm) by applying a vacuum. After the tubes were frozen in liquid nitrogen, the filters were broken into small pieces and 750 μl of Ground Water Extraction Buffer (10 mM Tris-HCl, 1 mM EDTA [pH 7.8], 0.2% sodium dodecyl sulfate [SDS]) and 0.25 g of glass beads were added to each tube. After a short bead beating (speed, 4.0 m/s; time, 20 s; Savant Instruments, Inc., Holbrook, N.Y.) the tubes were placed in a boiling water bath for 1 min. The samples were cooled on ice and centrifuged for 2 min at 12,000 × *g*. A 0.4 volume of 7.5 M ammonium acetate (to a final concentration of 2.5 M) was added to the supernatant. Crude lysates were extracted two times with chloroform:isoamyl alcohol (24:1). The nucleic acids from the aqueous phase were concentrated and washed with TE (10 mM Tris-HCl, 1 mM EDTA [pH 7.8]) in a microconcentrator (Centricon 100; Amicon), and the preparations were reduced to a final volume of 30 μl.

DNA extraction from sediment samples. One gram of sediment was extracted in a 2-ml-volume screw-cap microcentrifuge tube with 0.5 ml of Na_2HPO_4 buffer (100 mM, pH 8.0) and 0.25 ml of NaCl-Tris-SDS solution (100 mM NaCl, 500 mM Tris-HCl, 10% SDS [pH 8.0]). Sterile glass beads (0.25 g) were added, and physical disruption was performed with a bead beater Savant Instruments for 20 s at a speed of 4.0 followed by centrifugation at 12,000 × *g* for 3 min. Ammonium acetate (7.5 M) was added to the supernatant to give a final concentration of 2.5 M, and samples were incubated on ice for 20 or 10 min at −20°C. The solution was centrifuged at 12,000 × *g* for 5 min, and the supernatant

TABLE 2. Oligonucleotides used as primers and probe

Oligonucleotide	Function	Sequence (5′–3′)	Position[a]
963F	TaqMan PCR forward primer	CCTTGACATGTCTAGAAGTTACCAGAGA	963
1076R	TaqMan PCR reverse primer	GCGGGACTTAACCCAACATCT	1076
1030T	TaqMan PCR probe	ACACGAGCTGACGACGGCCATG	1030

[a] Position according to *E. coli* numbering.

TABLE 3. TaqMan PM1 detection in spiked sediment or groundwater microcosms

Sample[a]	Mean C_T[b]	Standard deviation	CV for C_T (%)	CFU[c]
Sediment	39.7	0.70	1.8	ND
Sediment plus MSM	39.7	0.64	1.6	ND
Sediment plus 10^6 PM1	19.8	0.14	0.7	$8.1 \times 10^6 \pm 0.6 \times 10^6$
Sediment plus 10^7 PM1	17.8	0.32	1.8	$2.0 \times 10^7 \pm 0.4 \times 10^7$
Control groundwater	34.3	0.61	1.8	<DL
Groundwater plus 10^6 PM1	19.7	0.32	1.6	$1.1 \times 10^6 \pm 0.4 \times 10^6$
Groundwater plus 10^8 PM1	15.2	0.35	2.3	$1.1 \times 10^8 \pm 0.5 \times 10^8$

[a] Sediment or groundwater microcosms with different numbers of PM1 cells added.
[b] Mean C_T represents replicate samples from each of three microcosm bottles times two PCR replicates ($n = 12$).
[c] CFU of PM1 per gram of sediment or per milliliter of groundwater; CFU were calculated by using the standard curve (Fig. 1B). ND, not detectable. <DL, below the detection limit of 180 CFU/ml.

was concentrated and washed with TE (10 mM Tris-HCl, 1 mM EDTA [pH 7.8]) in a microconcentrator (Centricon 100; Amicon).

Oligonucleotide probes and primers. TaqMan probe and primer sequences (Table 2) were designed with Primer Express software (Applied Biosystems, Foster City, Calif.) based on alignments of 30 bacterial 16S rDNA sequences. Primers and TaqMan probe were designed using the default parameters of the Primer Express software (22). The fluorogenic probe is 5′ labeled with FAM (6-carboxyfluorescein) and 3′ labeled with TAMRA (6-carboxytetramethylrhodamine), which serves as a quenching dye.

The specificity of all listed primers and probe (Table 2) were checked by using the CHECK_PROBE software provided through the Ribosomal Database Project (23) and the Basic Local Alignment Search Tool (BLAST) network service of GenBank (4).

TaqMan assay and quantitation. PCR was performed in 25-μl volumes using MicroAmp Optical 96-well reaction plates and MicroAmp Optical Caps (Applied Biosystems). A 113-bp product was amplified using primers 963F and 1076R and probe 1030T (Table 2). DNA extraction was performed in duplicate or triplicate, and two PCRs were run for each extraction. Dilution series were tested in triplicate.

Five microliters of diluted template was added to 20 μl of PCR master mix (12.5 μl of TaqMan Universal Master Mix, which is a 2× concentrated mixture of AmpliTaq Gold DNA Polymerase, uracyl-*N*-glycosylase [UNG], deoxynucleoside triphosphates with UTP, passive reference dye, and optimized buffer), a 2.5-μl mixture of 100 nM oligonucleotide primers and 100 nM TaqMan probe and 6 μl of double-distilled H_20. All the reagents were obtained from Applied Biosystems. After an initial incubation at 50°C for 2 min to activate the UNG and a denaturation phase of 10 min 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. Forty cycles of amplification, data acquisition, and data analysis were carried out routinely in an ABI Prism 7700 Sequence Detector (PE Applied Biosystems). Data were analyzed with Sequence Detector Software (version 1.7). Threshold determinations were automatically performed by the instrument for each reaction. The cycle at which a sample crosses the threshold (a PCR cycle where the fluorescence emission exceeds that of nontemplate controls) is called the threshold cycle, or C_T. A high C_T value corresponds to a small amount of template DNA, and a low C_T corresponds to a large amount of template present initially. Holland et al. provide more detailed information on the TaqMan PCR quantification method (18). The C_T values were exported into Microsoft Excel for further statistical analysis.

Sensitivity and detection limit. Various standard curves were generated to determine the detection limit of the assay. Tenfold serial dilutions of PM1 DNA with or without herring sperm DNA as a carrier were prepared. The corresponding CFU per PCR were calculated based on heterotrophic plate counts. Additionally, 10-fold dilutions of strain PM1 between 10^7 to 10^0 CFU were mixed with different concentrations of *E. coli* cells to make a final total cell density of 10^8 CFU/ml. DNA was extracted and used for standard curve generation. All determinations were performed in triplicate.

Detection and recovery of PM1 spiked into groundwater and sediment samples. To examine the accuracy of real-time TaqMan for quantitative measurement of PM1 in MTBE-contaminated sediments, three different setups were conducted. Treatments were established in triplicates as follows: (i) 5 g of sediment plus 2.5 ml of MSM; (ii) 5 g of sediment plus 10^6 CFU of PM1/g of sediment; (iii) 5 g of sediment plus 10^7 CFU of PM1/g of sediment. After the treatments DNA was extracted. The inoculum density was calculated based on a PM1 standard curve representing optical densities versus plate counts. DNA from the sediment without any treatment was extracted as a control.

The accuracy of TaqMan PCR estimations of PM1 in groundwater with low and high cell density was tested in microcosms and groundwater from the MTBE field test at Port Hueneme, where strain PM1 was injected into an oxygen-amended plot.

The C_T standard deviations and corresponding coefficients of variation (CV) were calculated as shown in Tables 3 and 4. The CFU of PM1 were determined from C_T values by using a standard curve (Fig. 1b).

RESULTS

Specificity. Primer specificity was tested against DNA from 15 bacterial strains, including organisms similar and dissimilar

TABLE 4. PM1 detection in groundwater DNA samples collected from an MTBE-contaminated plume at Port Hueneme, California

Sample[a]	Mean C_T[b]	Standard deviation	CV for C_T (%)	CFU/ml of groundwater[c]
BIBD	23.5	0.12	0.5	$2.0 \times 10^4 \pm 0.3 \times 10^4$
B1-D	27.6	0.27	1.0	$1.4 \times 10^3 \pm 0.4 \times 10^3$
B2-D	24.5	0.08	0.3	$1.4 \times 10^4 \pm 0.3 \times 10^4$
B1-S	23.1	0.09	0.4	$2.5 \times 10^4 \pm 0.2 \times 10^4$
B4-S	23.5	0.25	1.1	$1.9 \times 10^4 \pm 0.4 \times 10^4$
B4-D	24.6	0.13	0.5	$9.4 \times 10^3 \pm 0.4 \times 10^3$
B5-S	28.0	0.25	0.7	$9.9 \times 10^3 \pm 0.3 \times 10^3$
CBC61	35.5	0.33	0.9	<DL
CBC10	39.0	0.60	1.6	<DL
PHB4	32.9	0.39	1.2	<DL

[a] B1-D, B2-D, and B4-D correspond to deep wells and B1-S, B4-S, and B5-S, correspond to shallow wells in the field test plot where strain PM1 was injected. BIBD, injection bed. CBC10 is (649 m) upstream and PHB4 is (623 m) downstream of the test plot. CBC61 is close (40 m) to the test plot.
[b] Mean C_T represents two PCR replicates of two replicate groundwater samples ($n = 4$).
[c] CFU were calculated by using a standard curve, shown in Fig. 1B. <DL, below the detection limit of 180 CFU/ml.

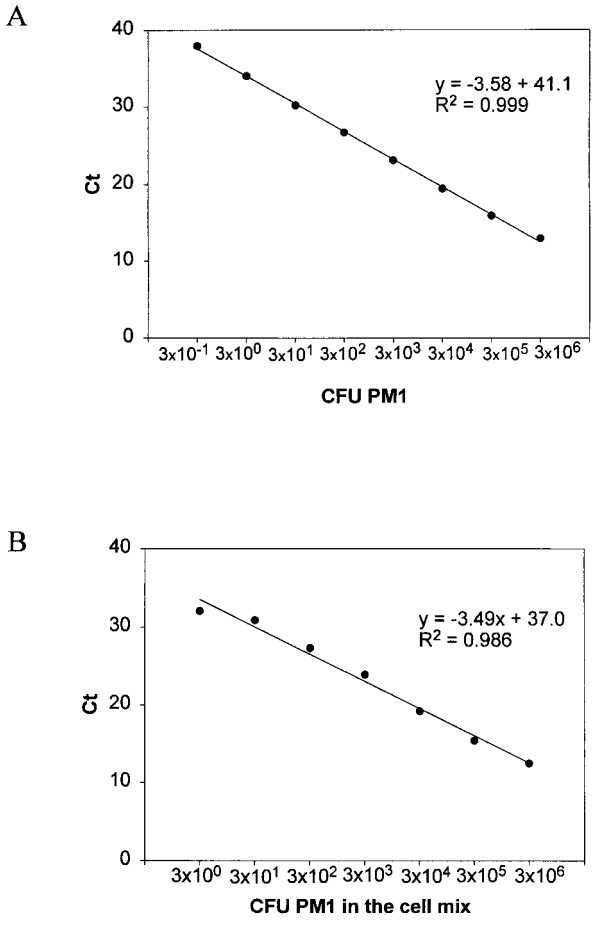

FIG. 1. PM1 standard curve. Real-time analysis of serial 10-fold dilutions of PM1 DNA (A) or PM1 cells (B). The C_T values are plotted against the corresponding PM1 cell numbers in the PCR. Template DNA was extracted from 1.8×10^7 CFU of PM1 corresponding to 3×10^6 CFU/PCR and was diluted 10-fold (A). For the second curve (B) DNA was extracted from serial dilutions of PM1 cells mixed with *E. coli* cells until 10^8 total CFU/ml was reached.

to PM1, based on the phylogenetic relationship to strain PM1. BLAST comparisons of the nearly full-length 16S rDNA sequences between PM1 and strains *Aquabacterium commune*, *Acidovorax facilis*, and *Hydrogenofaga flava* showed 95, 93, and 93% similarity, respectively. The rest of the tested bacterial strains were <83% similar to strain PM1. We relied on the specificity of the forward primer to discriminate between rDNAs of strain PM1 and closely related strains. Two different concentrations of DNA extracted from the bacterial pure cultures were tested in the TaqMan PCR assay to determine PM1 primers and probe specificity. Of the 15 bacterial strains (Table 1), *Aquabacterium commune*, *Acidovorax facilis*, *Streptococcus lactis*, and *Pseudomonas putida* had C_T mean values of 39.55, 37.03, 38.68, and 38.1, respectively, when 1,000 pg of DNA was used as a DNA template. The same concentration of PM1 DNA gave a C_T value of 23.2. The unspecific signal associated with non-target bacterial cultures was equivalent to or below approximately 0.3 CFU of PM1 per PCR (Fig. 1). Decreasing the target DNA concentration by twofold resulted in no signal for all bacterial strains, with the exception of PM1. Based on the specificity test results, we determined that environmental samples spiked with 10^6 to 10^7 cells of PM1 per ml needed to be diluted to 100 to 500 pg of total DNA per PCR to overcome unspecific signal yet provide good quantification of PM1.

Standard curve and detection limit. To generate a standard curve, the TaqMan C_T values were plotted relative to the corresponding serial dilutions of template DNA extracted from a culture of PM1 (Fig. 1A) or to DNA extracted from different cell densities of PM1 (data not shown). Target DNA was detectable when the PCR mixture contained 0.3 cells (~2 CFU/ml) by using a primer concentration of 100 nM. The C_T values increased with each 10-fold dilution of the target PM1 DNA. Linearity between the TaqMan C_T values and target concentration was observed over the entire 8 orders-of-magnitude dilution series, demonstrating that quantification of the target DNA was possible. The slope of the curve was −3.58, and the linear square regression coefficient was 0.999. To test whether the higher dilutions of target DNA were influenced by adsorption to tubes walls or pipetting error, a second standard curve was generated using DNA from the same extraction described above but in the presence of 200 ng of carrier DNA (calf thymus DNA; Sigma). No TaqMan signal could be detected in samples of carrier DNA only. In contrast to previously reported results (20), the carrier DNA had no effect on the results as evidenced by no substantial difference in the slopes (−3.62 plus carrier DNA versus −3.58 without carrier DNA; data not shown). To account for possible differences in DNA content in PM1 at different growth stages, we compared standard curves with DNA extracted from cells harvested during stationary phase versus logarithmic growth phase. The difference in the slopes of curves was very small (0.08), and the linear regression coefficient (R^2) remained the same (data not shown).

The TaqMan assay was able to detect less than 1 cell per PCR (2 cells per ml), as calculated from viable plate counts of CFU of PM1 at each dilution (Fig. 1). All non-template controls and all dilutions with DNA corresponding to less than 0.3 cells per reaction gave negative results.

Since environmental samples contain diverse bacterial communities, the quantification of PM1 was tested in the presence of non-target DNA. The TaqMan assay was able to detect as few as 30 PM1 cells per PCR (180 CFU/ml), below which the relationship between the TaqMan signal and cell density became nonlinear (Fig. 1B). The slope difference is −0.7 (data not shown) for PM1 only versus PM1 plus *E. coli* cells. The higher difference is due to limitations of DNA extraction when only a few cells are present in the highest dilutions of PM1 cells only, and this problem is overcome with the presence of non-target DNA in the mixtures.

Detection and recovery of PM1 spiked into groundwater and sediment samples. Strain PM1 was added to and recovered from environmental samples to test the reliability of the TaqMan method under field conditions. Strain PM1 could be detected in samples of inoculated groundwater or aquifer sediments (Table 3), with small mean C_T standard deviations ranging from 0.14 to 0.35 (and CVs for C_Ts ranging from 0.72 to 2.33%). The low CV values confirmed the reproducibility of the DNA extraction method and were associated with a small amount of error during the PCR setup. Three out of the 10 sediment control samples showed a very low fluorescence, be-

low the detection limit. This was likely due to cross-contamination during the PCR setup rather than cross-contamination during DNA extraction, because the replicate PCRs for the same samples were negative. A small increase in fluorescence with DNA extracted from the control groundwater microcosm bottles (containing water from well CBC61 only) was observed at the end of the experiment, but this increase was below the detection limit (C_T of 32.01, or 180 CFU/ml; Table 3).

Reproducibility and detection of PM1 in groundwater samples from bioaugmentation field trial. In November 1999, strain PM1 was inoculated into oxygen-amended groundwater in an MTBE-contaminated plume at Port Hueneme Naval Base (32). Groundwater samples were collected from locations within the injection bed and outside the treatment area and then were analyzed by TaqMan for PM1 population density. PM1 was detectable in the samples from the treatment zone but not in the adjacent samples (Table 4). The replicability of the assay was high, with the standard deviation of C_T values for the four PCRs (2 DNA extractions $\times$ 2 PCR repetitions) per environmental sample ranging from 0.009 to 0.25 for the PM1-positive samples. The differences in CV values between two runs of the same DNA dilutions were less than 3.2% (data not shown).

With respect to the standard curve, there was a higher degree of error associated with samples with low versus high cell densities. For example, the standard deviation for the C_T values was 0.5 for cell densities ranging from 3×10^1 to 3×10^{-1}, whereas the standard deviation for C_T values ranged from 0.04 to 0.08 for densities ranging from 3×10^2 to 3×10^6.

MTBE degradation by strain PM1 in microcosms. Microcosm studies were conducted to determine the relationship between PM1 cell density and aerobic MTBE biodegradation in mineral media, inoculated groundwater, and in groundwater collected from the bioaugmentation study at Port Hueneme. MTBE (10 ppm) was biodegraded by $\sim 1 \times 10^6$ CFU/ml of PM1 in mineral media to undetectable levels within 250 h after a short lag period (Fig. 2A). Concentrations of MTBE in the sodium azide controls did not change during the experiment (data not shown). The MTBE degradation rate was highest in groundwater collected upstream of the bioaugmentation treatment area at Port Hueneme (Fig. 2B). Two- and 1.5-log increases in PM1 cell density was observed in groundwater and mineral media microcosms, respectively (Fig. 2A and B). Increases in cell density were concomitant with declines in MTBE concentration. This relationship supported the previous observation (based on cell protein; 14) that MTBE can support growth of PM1. Total heterotrophic plate counts performed at the beginning and end of the experiment also showed a 2- and 1.5-log increase in cell densities in the inoculated groundwater and mineral media, respectively.

In groundwater collected from the field test plot at Port Hueneme (well B32D) 7 months after injection of strain PM1 (32), the initial PM1 concentration was $3.3 \times 10^4 \pm 2.4 \times 10^4$ per ml, as detected by TaqMan assay. Following incubation with added MTBE, a 1-log-order increase in PM1 density was measured by both the TaqMan assay (Fig. 2C) and plate counts (data not shown). The rate of MTBE biodegradation was lower (8.5 ppm removed in 500 h) than what was observed in the other microcosms, probably due to large differences in the initial PM1 cell densities in the inoculated versus field samples.

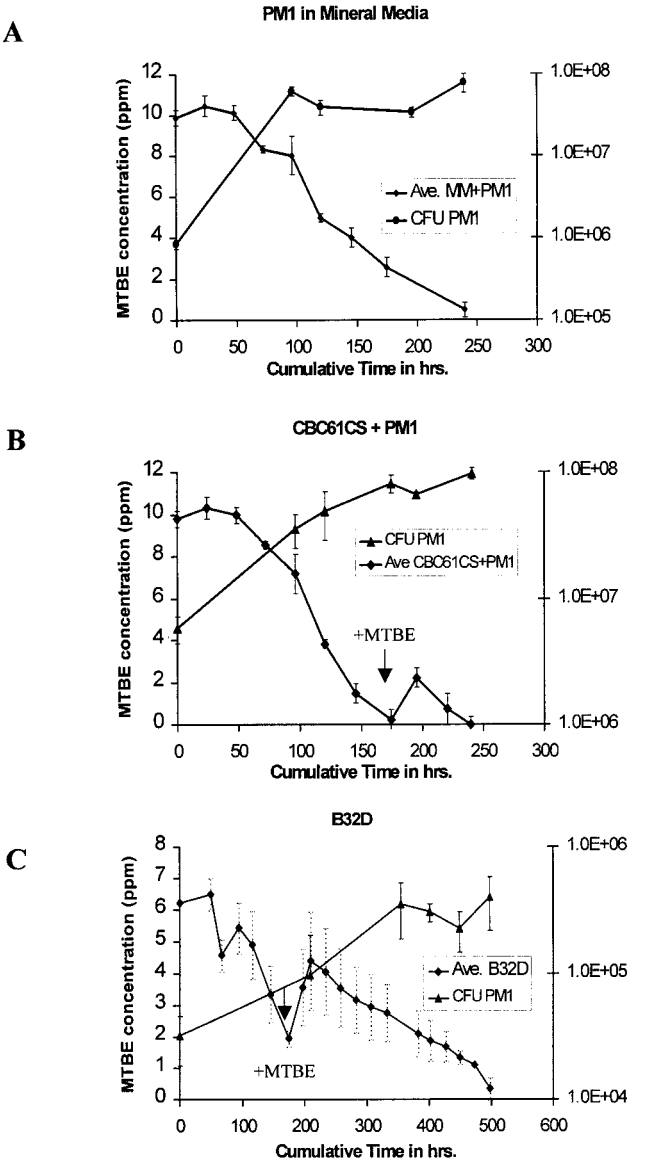

FIG. 2. TaqMan-estimated PM1 cell density and aerobic MTBE biodegradation in mineral media, inoculated groundwater, and groundwater collected from the bioaugmentation study at Port Hueneme. Biodegradation of MTBE in microcosms of mineral media (A) or groundwater from Port Hueneme (well CBC61CS located upstream from the field plots [B] or well B32D from the field test plot [C]), with or without addition of 10^6 CFU of PM1 per ml. Ave, average.

DISCUSSION

We succeeded in developing a quantitative real-time TaqMan PCR assay targeting the 16S rDNA for detection and quantification of the MTBE-degrading strain PM1. The method was successful in detecting PM1 in laboratory cultures and inoculated environmental samples (groundwater and sediment) as well as in groundwater samples collected from a contaminated MTBE site where PM1 had been added months previously. In addition, MTBE removal was related to increases in PM1 cell density, as estimated by the TaqMan method in laboratory incubation studies.

The quantitative real-time TaqMan PCR has several advantages over conventional PCR or TaqMan endpoint analysis. Without any post-PCR manipulation of the samples, cross-contamination between samples is greatly reduced. The real-time TaqMan PCR method has an increased dynamic range for quantification of target sequences (at least 5 orders of magnitude). Determining the initial target copy number from the early exponential phase by kinetic PCR avoids the potential error associated with endpoint analysis (where the rate-limiting conditions during the PCR plateau phase may confound the estimate of the target concentration). Standardized commercial reagents together with the 96-well format and PE-ABI 7700 thermocycler allow a reproducible assay within 2 h. Real-time quantitative TaqMan PCR is precise and less labor-intensive than present quantitative PCR methods (17) but requires expensive equipment.

The specificity of the PM1 PCR primers and TaqMan probe was confirmed both by homology searches in nucleotide databases and by testing 15 bacterial strains with different degrees of relatedness to PM1 based on 16S rRNA comparative analyses. We targeted the 16S rDNA for designing probes and primers for PM1 because the large 16S rDNA database available allows the identification of sequences exclusive to PM1. The major disadvantage of this approach is that variable regions within the 16S rDNA can be almost identical for closely related bacteria. To overcome this problem, stringent conditions such as hot-start PCR technique and high annealing temperatures can be defined to exclude amplification of closely related organisms.

The PM1 TaqMan PCR assay showed high analytical sensitivity and precision. DNA standard curves showed a detection limit for PM1 below 1 CFU per PCR and, in the presence of non-target DNA, 30 PM1 cells per PCR. Our detection limit was similar to levels found in other TaqMan applications. Reported detection limits using TaqMan endpoint analysis were 2 CFU for a pure culture of *Salmonella enterica* serovar Typhimurium (7) and 50 CFU for *Listeria monocytogenes* (3). Recently Nogva et al. (24) reported a detection limit of 1 CFU per PCR for *Campylobacter jejuni* based on a DNA standard curve and 10 CFU per PCR based on a cell standard curve. The precision of the PM1 TaqMan assay was good, with the CV values for C_Ts between replicate PCRs of the same environmental samples ranging from 0.33 to 2.33% and between independent PCR runs of the same sample ranging from 0.8 to 3.2%.

A challenge in using the TaqMan PCR method is to convert measurements of fluorescent signal into target cell densities. Our approach was to directly relate the TaqMan signal to measured cell densities in groundwater using standard curves for PM1. This approach was possible because our target DNA was associated with a specific bacterial isolate that we could culture. This is in contrast to other environmental studies, where TaqMan PCR has been used to estimate population sizes of uncultured organisms (35, 36) and where only relative quantification was possible. The indirect methods used in these studies (based on comparison to estimates from probing, cloned rRNA genes, or universal bacterial TaqMan primers) require a priori knowledge of rDNA copy number and genome size.

We compared standard curves of DNA extracted from cells (grown on MTBE) harvested during stationary phase to curves of DNA extracted from cells harvested during logarithmic growth phase and, by finding no significant difference in the slopes of the curves, concluded there were no major differences in DNA (rDNA, respectively) content in PM1 at different growth stages. The linearity of the standard curves and the observed constant amplification efficiency confirm that the PM1 TaqMan PCR assay is valid and well suited for quantitative measurements.

Presently a field trial is under way at Port Hueneme Naval Base, where PM1 has been added to MTBE-contaminated groundwater (32). Preliminary analysis of samples from this trial suggests that PM1 has survived up to 7 months after injection into the plots. Our next step is to adapt the PM1 TaqMan assay for quantification of rRNA so that we can determine which portion of the PM1 population is metabolically active at the site. Quantification of specific microorganisms in the environment has been a very labor-intensive and often inaccurate process. Being able to reliably and sensitively detect and enumerate MTBE-degrading bacteria in environmental samples should substantially improve our understanding of why bioremediation of contaminated aquifers succeeds or fails.

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