APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 2005, p. 3866–3871 Vol. 71, No. 7
0099-2240/05/$08.00+0 doi:10.1128/AEM.71.7.3866–3871.2005

An Internal Reference Technique for Accurately Quantifying Specific mRNAs by Real-Time PCR with Application to the *tceA* Reductive Dehalogenase Gene

David R. Johnson, Patrick K. H. Lee, Victor F. Holmes, and Lisa Alvarez-Cohen*

Department of Civil and Environmental Engineering, University of California, Berkeley, California

Received 18 September 2004/Accepted 4 February 2005

The accuracy of mRNA quantification by reverse transcription (RT) in conjunction with real-time PCR (qPCR) is limited by mRNA losses during sample preparation (cell lysis, RNA isolation, and DNA removal) and by inefficiencies in reverse transcription. To control for these losses and inefficiencies, a technique was developed that utilizes an exogenous internal reference mRNA (*ref* mRNA) along with mRNA absolute standard curves. The technique was applied to quantify mRNA of the trichloroethene (TCE) reductive dehalogenase-encoding *tceA* gene in an anaerobic TCE-to-ethene dechlorinating microbial enrichment. Compared to RT-qPCR protocols that utilize DNA absolute standard curves, application of the new technique increased measured quantities of *tceA* mRNA by threefold, demonstrating a substantial improvement in quantification. The technique was also effective for quantifying the loss of mRNA during specific steps of the sample processing protocol. Analysis revealed that the efficiency of the RNA isolation (56%) step was significantly less than that of the cell lysis (84%), DNA removal (93%), and RT (88%) steps. The technique was applied to compare the effects of cellular exposure to different chlorinated ethenes on *tceA* expression. Results show that exposure to TCE or *cis*-1,2-dichloroethene resulted in 25-fold-higher quantities of *tceA* mRNA than exposure to vinyl chloride or chlorinated ethene starvation.

The application of reverse transcription (RT) in conjunction with real-time PCR (qPCR) is rapidly becoming the preferred approach for quantifying specific mRNAs from environmentally relevant microbial samples (2, 3, 6–13, 22, 31, 36, 38, 39, 41) (for technical reviews of RT-qPCR, see references 4, 5, 15, and 32). RT-qPCR is distinguished from alternative RNA quantification methods, such as Northern blotting, in situ hybridization, RNase protection assays, RT-PCR, and microarray analyses, by its ability to rapidly analyze large numbers of samples while maintaining high degrees of both sensitivity and specificity (4, 15). The accuracy of RT-qPCR measurements, however, is limited by mRNA losses during sample preparation and by inefficiencies in reverse transcription (4, 5, 15). Loss of mRNA during sample preparation may result from the incomplete lysis of cells, enzymatic and abiotic degradation, incomplete volume transfers and phase separations, and, when mRNA is isolated using glass fiber binding columns, incomplete adsorption to filters. Inefficient reverse transcription may result from inhibition of the reverse transcriptase by residual alcohol, phenol, and salts.

To control for some or all of these losses and inefficiencies, mRNA quantities are frequently normalized to a reference measurement. One common approach is to normalize mRNA quantities to the mass of total RNA analyzed (4). This approach has two principal drawbacks. First, total RNA is composed of mRNA, rRNA, tRNA, and small noncoding RNAs. Since the stabilities of these RNAs may significantly differ, normalizing to the mass of total RNA cannot specifically control for the degradation and loss of mRNA (5). Second, to accurately compare mRNA quantities from different samples, the mass of total RNA per cell must not significantly differ between the samples of interest. The mass of total RNA per cell, however, can change dramatically throughout cellular growth cycles and with different environmental conditions (17, 26).

As an alternative, mRNA quantities can be normalized to the quantity of mRNA of an endogenous housekeeping gene (4, 5). Assuming that target and housekeeping mRNAs behave similarly, the processing and amplification of both mRNAs in parallel can control for mRNA losses during sample preparation, including enzymatic and abiotic degradation, and for inefficiencies in reverse transcription. Accurate comparison of mRNA quantities between different samples, however, requires the expression levels of the selected housekeeping genes to remain relatively stable. As with the mass of total RNA, dramatic changes in the expression levels of housekeeping genes have been observed in both eukaryotic (35, 42, 43) and prokaryotic (36) cells.

In this study, a third normalization approach is developed that utilizes an exogenous internal reference mRNA (*ref* mRNA). The *ref* mRNA is an in vitro-transcribed mRNA described as "exogenous" because it is not found in the sample of interest, "internal" because it is added to culture samples prior to sample processing, and "reference" because the initial amount is known. After sample processing, the recovered quantities of *ref* and target mRNAs are independently measured by multiplex real-time PCR using independent standard curves and the absolute standard curve method (19, 27). The sample-specific fractional recovery of *ref* mRNA is then used as a normalization factor to control for the loss of mRNA during the cell lysis, RNA isolation, and DNA removal steps. The primary advantage of this approach is that measurements are

* Corresponding author. Mailing address: Department of Civil and Environmental Engineering, University of California, Berkeley, CA 94720-1710. Phone: (510) 643-5969. Fax: (510) 642-7483. E-mail: alvarez@ce.berkeley.edu.

independent of cell state, growth stage, and gene expression level, consequently allowing for accurate comparison of mRNA quantities across different experimental conditions. One limitation of this approach, however, is that it cannot control for mRNA losses resulting from the incomplete lysis of cells. Therefore, the cell lysis procedure must be carefully optimized for the specific organism of interest.

The *ref* mRNA technique developed here was applied to quantify mRNA of the trichloroethene (TCE) reductive dehalogenase-encoding *tceA* gene in an anaerobic TCE-to-ethene dechlorinating microbial enrichment. Although *ref* mRNA techniques have been applied to mammalian (1, 18, 34) and plant (25) cells, to our knowledge this is the first application to prokaryotic cells or to complex microbial communities.

MATERIALS AND METHODS

Microbial culture. The anaerobic TCE-to-ethene dechlorinating microbial enrichment, designated ANAS, and procedures for culture maintenance have been previously described (29). Briefly, cells were grown in a continuously stirred semibatch reactor at 25 to 28°C. The total reactor volume was 1.5 liters, and the liquid volume was 400 ml. To prevent oxygen intrusion, the reactor was pressurized to 1.8 atm with N_2/CO_2 (90:10). The reactor was periodically amended with a total of 111 μmol TCE (approximate aqueous concentration of 0.1 mM) and with sodium lactate to an aqueous concentration of 25 mM. After complete dechlorination of all the TCE to ethene, which typically required 7 days, the reactor was purged with N_2 and 60 to 100 ml of culture was withdrawn and replaced with an equal volume of fresh anaerobic medium.

For chlorinated ethene exposure experiments, 100 ml of culture was transferred to a 160-ml anaerobic bottle and maintained in the absence of chlorinated ethenes for 72 h. Thereafter, 25-ml aliquots were transferred to four parallel 70-ml anaerobic bottles. The subcultures were purged with N_2/CO_2 (90:10) for 15 min and amended with sodium lactate to an aqueous concentration of 4 mM. Three of the subcultures were further amended with 6 μmol of TCE, *cis*-1,2-dichloroethene (cDCE) (added as chlorinated ethene-saturated water), or vinyl chloride (VC) (added as a pure gas). The fourth subculture was starved of chlorinated ethenes to serve as a control. Subcultures were incubated at 30°C for 8 h with shaking while chlorinated ethene concentrations were monitored by gas chromatography. Cells for mRNA and protein analyses were collected from 1-ml culture samples by centrifugation (5 min at 10,000 × *g*) in RNase-free 2-ml screw-cap microcentrifuge tubes. The supernatants were discarded, and the cell pellets were stored at −80°C until further processing.

Cell lysis and RNA isolation (acid phenol method). Cells were lysed and RNA was isolated using a modified method of Siering and Ghiorse (33). Frozen cell pellets were resuspended in 250 μl lysis buffer (50 mM sodium acetate, 10 mM EDTA; pH 5.1), 100 μl 10% sodium dodecyl sulfate, 1.0 ml pH 4.3 buffer-equilibrated phenol (Sigma Chemical Company, St. Louis, MO), and 2 μl of 10^8 transcripts μl^{-1} luciferase control RNA (Promega, Madison, WI). Cells were lysed by amending samples with 1 g 100-μm-diameter zirconia-silica beads (Biospec Products, Bartlesville, OK), heating them to 65°C for 2 min, bead beating them with a Mini Bead Beater (Biospec Products) for 2 min, incubating them for 10 min at 65°C, and bead beating them for an additional 2 min. Cellular debris was collected by centrifugation (5 min at 15,000 × *g*), and the aqueous lysate was transferred to a new RNase-free microcentrifuge tube.

RNA was isolated by being extracted twice with 1 volume of pH 4.3 buffer-equilibrated phenol:chloroform:isoamyl alcohol (125:24:1) and once with 1 volume of chloroform:isoamyl alcohol (24:1) (Sigma Chemical Company). Isolated RNA was precipitated overnight at −20°C with 0.5 volume of 7.5 M ammonium acetate, 2 volumes of absolute ethanol, and 2 μl of 10 μg μl^{-1} glycogen (Fermentas, Hanover, MD). The precipitate was collected by centrifugation, washed once with 80% ethanol, and resuspended in 40 μl of diethyl pyrocarbonate-treated deionized water (ISC BioExpress, Kaysville, UT).

Alternative cell lysis and RNA isolation methods. In addition to the acid phenol method described above, cells were lysed and RNA was isolated using two different commercial kits, the UltraClean Microbial RNA kit (Mo Bio Laboratories, Carlsbad, CA) and the RiboPure Bacteria kit (Ambion, Austin, TX). For both kits, total RNA was isolated from 1-ml ANAS culture samples according to the manufacturers' instructions.

DNA removal. Contaminating DNA was removed by DNase I treatment using the DNA-*free* kit (Ambion). To stringently remove contaminating DNA, two serial 50-μl DNase I treatment reactions were performed: the first reaction mixture contained 4 U DNase I, and the second contained 2 U DNase I. Treated RNA was stored at −80°C prior to further use.

qPCR standards. DNA standards for *tceA* were synthesized by cloning a 3.6-kb region containing the entire *tceA* open reading frame into the pCR 2.1-TOPO plasmid (Invitrogen, Carlsbad, CA). Recombinant plasmid DNA was purified from a 100-ml *Escherichia coli* culture using a standard alkali lysis ethanol precipitation protocol (30). Mass of DNA per volume was quantified using the PicoGreen double-stranded DNA quantitation kit (Molecular Probes, Eugene, OR) according to the manufacturer's instructions. Copy number of *tceA* DNA per volume was calculated using a recombinant plasmid size of 7.5 kb and an average molecular mass of 660 Da nucleotide $pair^{-1}$. When single-stranded cDNA samples produced by RT were quantified, the theoretical copy number of the *tceA* double-stranded DNA standard was multiplied by two.

Standards for *tceA* mRNA were synthesized by linearizing *tceA*-containing plasmid DNA with XmnI restriction endonuclease (New England Biolabs, Beverly, MA), followed by in vitro transcription using T7 RNA polymerase (Roche Diagnostics, Indianapolis, IN). Plasmid template was removed by DNase I treatment using the DNA-*free* kit (Ambion) according to the manufacturer's instructions. Effective plasmid digestion and synthesis of transcripts of the expected size were confirmed by DNA and RNA analyses on a 2100 Bioanalyzer (Agilent, Palo Alto, CA). The standard for *ref* mRNA was purchased from Promega (luciferase control RNA [1 mg ml^{-1}]). Masses of both *tceA* and *ref* mRNA per volume were quantified with the RiboGreen RNA quantification kit (Molecular Probes) according to the manufacturer's instructions. Copy numbers of *tceA* and *ref* mRNA per volume were calculated using transcript sizes of 1.6 kb and 1.8 kb, respectively, and an average molecular mass of 330 Da $nucleotide^{-1}$.

Primers and probes. *Taq*Man primer-probe sets were purchased from PE Applied Biosystems, Foster City, CA. One set was designed to target all the *tceA* gene sequences currently deposited in the NCBI GenBank database (www.ncbi.nlm.nih.gov) (accession numbers AF228507, AY165309, AY165310, AY165311, and AY165312) (*tceA* forward primer ATCCAGATTATGACCCT GGTGAA, *tceA* reverse primer GCGGCATATATTAGGGCATCTT, *tceA* probe 6-carboxyfluorescein–TGGGCTATGGCGACCGCAGG–6-carboxytetramethylrhodamine). A second primer-probe set was designed to target *ref* mRNA (luciferase, accession number X65316) (*ref* forward primer TACAACACCCCA ACATCTTCGA, *ref* reverse primer GGAAGTTCACCGGCGTCAT, *ref* probe VIC-CGGGCGTGGCAGGTCTTCCC–6-carboxytetramethylrhodamine). The *tceA* and *ref* reverse primers were used for both reverse transcription and qPCR amplification.

Quantification of reverse transcription efficiency. Serially diluted *tceA* mRNA standards were reverse transcribed using the RT core reagent kit (PE Applied Biosystems). Each 10-μl reaction volume contained 2 μl of standard mRNA and 0.5 μM of the *tceA* reverse primer. The reaction mixture was incubated for 30 min at 55°C followed by 5 min at 95°C. Reactions were performed at 55°C to increase the stringency of primer-template binding and therefore improve RT specificity (14, 16).

The reverse-transcribed *tceA* mRNA standards and serially diluted *tceA* DNA standards were amplified in parallel on an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Each 25-μl qPCR volume contained 2 μl of *tceA* DNA standard or reverse-transcribed *tceA* mRNA standard, 12.5 μl of 2× *Taq*Man Universal PCR Master Mix (Applied Biosystems), 0.2 μM of *tceA* probe, and 0.7 μM of each *tceA* primer (forward and reverse). Thermocycling conditions were as follows: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C and 1 min at 60°C. A single fluorescence value (*R*) was identified that corresponded with exponential amplification in every sample. The threshold cycle [$C_T(R)$] values associated with this fluorescence were then plotted against the initial number of DNA or mRNA copies in each reaction. The efficiency of RT was calculated as the ratio of initial *tceA* mRNA copies to initial DNA copies for a fixed value of $C_T(R)$.

Quantification of *tceA* mRNA in culture samples. Multiplex RT-qPCR was applied to independently quantify *tceA* and *ref* mRNA in each culture sample. Sample RNA, 10-fold serially diluted *tceA* mRNA standards, and 10-fold serially diluted *ref* mRNA standards were reverse transcribed in parallel 10-μl reaction mixtures using the RT core reagent kit (PE Applied Biosystems), 2 μl of sample or standard mRNA, and 0.5 μM of each reverse primer (*tceA* and *ref*). RT incubation conditions were identical to those described above. Multiplex qPCR was performed in 25-μl reaction volumes containing 2 μl of RT product, 12.5 μl of 2× *Taq*Man Universal PCR Master Mix (Applied Biosystems), 0.7 μM of each forward and reverse primer (*tceA* and *ref*), and 0.2 μM of each probe (*tceA* and *ref*). Thermocycling conditions were identical to those above. The quantities of *ref* and *tceA* mRNA were independently measured using the absolute standard curve method (19, 27).

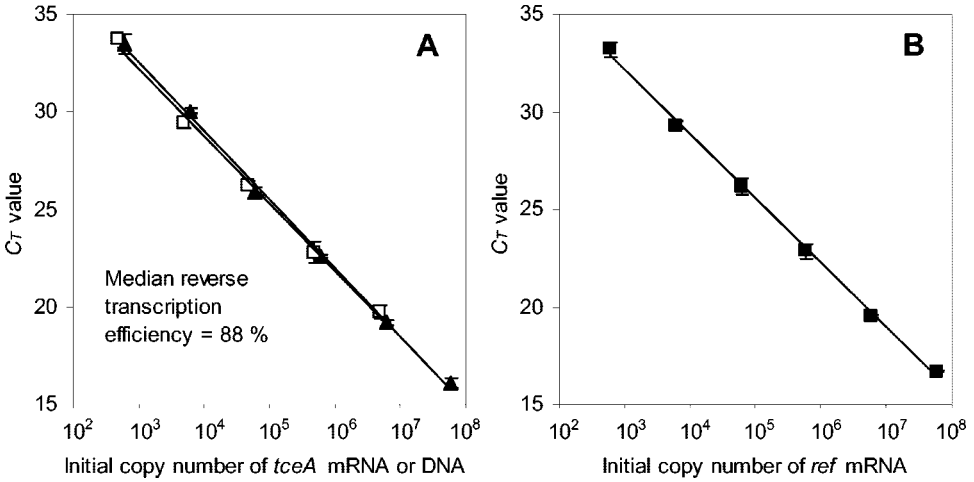

FIG. 1. Standard curves were generated by plotting C_T values against the known initial mRNA or DNA copy number per 25-μl qPCR mixture volume. A: C_T values were obtained using plasmid DNA containing the entire *tceA* gene (□) or in vitro-transcribed *tceA* mRNA (▲). B: C_T values were obtained using in vitro-transcribed *ref* mRNA (■). Data points are averages of three independent dilution series, and error bars are 1 standard deviation.

Biomass quantification. Biomass was quantified as mass of cellular protein. Frozen cell pellets from 1-ml culture samples were resuspended in 100 μl of 48 mM NaOH. The mixture was boiled at 100°C for 20 min followed by centrifugation (10 min at 10,000 × *g*) to remove cellular debris from the supernatant. Protein mass per volume was quantified from 50 μl of the supernatant using the Coomassie Plus protein assay reagent kit with bovine serum albumin as the standard (Pierce Biotechnology, Rockford, IL).

RESULTS

Analysis and control of RT inefficiencies. To assess the efficiency of the RT protocol described here, *tceA* DNA standards and reverse-transcribed *tceA* mRNA standards were amplified and analyzed in parallel. Both sets of standards exhibited similar linear slopes over 5 orders of magnitude (DNA, −3.47; 95% confidence interval [CI], −3.06 to −3.87; mRNA, −3.50; 95% CI, −3.29 to −3.72) (Fig. 1A). In addition, the two sets of standards returned nearly identical C_T values for a given initial copy number, which is indicative of a high RT efficiency. The median separation distance between the *tceA* DNA and mRNA standard curves was 0.27 C_T, corresponding to a median RT efficiency of 88% (evaluated at C_T = 26). This RT efficiency is consistent with RT efficiencies reported elsewhere (15).

Optimization of the cell lysis and RNA isolation protocol. The cell lysis and RNA isolation protocol described here (acid phenol method) was optimized to maximize the lysis efficiency of the *tceA*-containing organism in the ANAS enrichment. Bead beating for 2 min increased yields of *tceA* mRNA by 3.5-fold over those for lysis without bead beating, while bead beating for longer than 2 min (maximum of 8 min) did not affect *tceA* mRNA quantification. All results presented here used 4 min of bead beating.

The optimized cell lysis and RNA isolation protocol (acid phenol method) was compared to two commercial RNA isolation methods, the UltraClean Microbial RNA kit (Mo Bio Laboratories), and the RiboPure Bacteria kit (Ambion). Of the three methods, the acid phenol method yielded the most RNA from the ANAS culture. All results presented here used the acid phenol method for cell lysis and RNA isolation.

***ref* mRNA selection and quantification.** Firefly (*Coleoptera*) luciferase mRNA was selected as the exogenous internal reference mRNA (*ref* mRNA) because a primer-probe set could be designed that does not target any bacterial or archaeal genes submitted to the NCBI GenBank database (www.ncbi.nlm.nih.gov). The specificity of the primer-probe set for only *ref* mRNA was verified by performing RT-qPCR with total RNA from the ANAS culture. After RT and 40 cycles of qPCR, no detectable amplification was observed. To quantify *ref* mRNA, a set of *ref* mRNA external standards were developed. As with the *tceA* mRNA standards, amplification of reverse-transcribed *ref* mRNA standards exhibited low variability over 5 orders of magnitude (Fig. 1B).

Dynamic range of the *ref* mRNA protocol. Because *tceA* and *ref* mRNAs are quantified simultaneously by multiplex RT-qPCR, the relative initial quantities of each mRNA may affect quantification through competition for RT-qPCR reagents. To test for possible complications with the protocol described here, a range of *ref* mRNA quantities (2.5×10^6 to 7.5×10^8 copies) were added to homogenized sets of 1-ml ANAS culture samples (three replicates for each *ref* mRNA quantity) and the recovered quantities of *ref* and *tceA* mRNA were measured by multiplex RT-qPCR. As shown in Fig. 2, a linear relationship was observed between the added and recovered amounts of *ref* mRNA (slope, 0.94; 95% CI, 0.91 to 0.97). The mean fractional recovery of *ref* mRNA was 29% (standard deviation, 7.2%), and normalization to the sample-specific fractional recovery of *ref* mRNA increased *tceA* quantities by more than threefold. While the quantity of unnormalized *tceA* mRNA was slightly reduced as the added amount of *ref* mRNA was increased (slope, −0.06; 95% CI, −0.05 to −0.07), sample-specific normalization to the fractional recovery of *ref* mRNA (slope, 0.004; 95% CI, −0.02 to 0.02) resulted in more consistent reporting of the uniform *tceA* mRNA quantity (Fig. 2).

To examine the effective dynamic range of the cell lysis and RNA isolation protocol (acid phenol method), cells were harvested from homogenized sets of 1-ml ANAS culture samples that differed in cellular protein concentration (3.3 to 330 ng

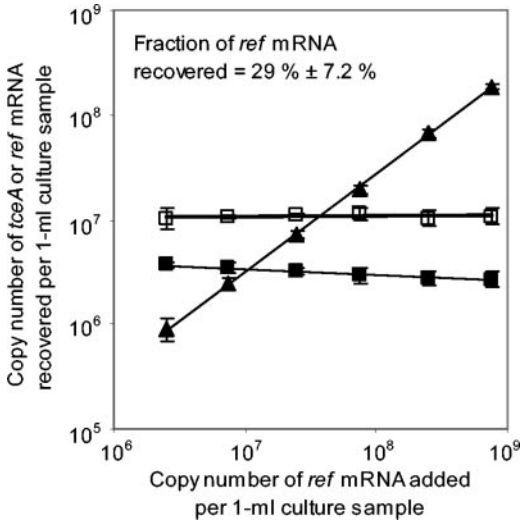

FIG. 2. Effect of the initial *ref* mRNA copy number per ml of ANAS culture on the quantification of *tceA* mRNA (■), *ref* mRNA (▲), and *tceA* mRNA normalized by the sample-specific fractional recovery of *ref* mRNA (□). Data points are averages of triplicate culture samples, and error bars are 1 standard deviation.

cellular protein ml^{-1}, three replicates for each protein concentration) and amended with a fixed amount of *ref* mRNA (2.5×10^8 copies). The recovered quantities of *ref* and *tceA* mRNA were then measured by multiplex RT-qPCR. As shown in Fig. 3, the quantity of *tceA* mRNA increased linearly with increasing mass of cellular protein (slope, 1.0; 95% CI, 0.86 to 1.2) while the fractional recovery of *ref* mRNA was not significantly affected (average, 33%; standard deviation, 8.3%). Normalization to the sample-specific fractional recovery of *ref* mRNA increased quantities of *tceA* mRNA approximately 3.0-fold while the slope of the linear relationship and datum variability

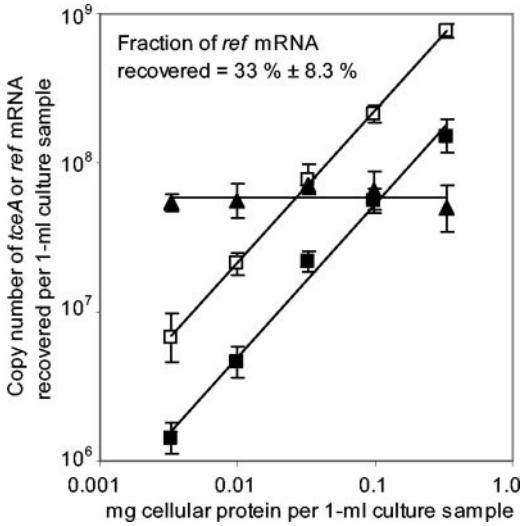

FIG. 3. Effect of mass of cellular protein per ml of ANAS culture sample on the quantification of *tceA* mRNA (■), *ref* mRNA (▲), and *tceA* mRNA normalized by the sample-specific fractional recovery of *ref* mRNA (□). All data points are averages of triplicate culture samples, and error bars are 1 standard deviation.

were not substantially affected (unnormalized slope, 1.0; 95% CI, 0.86 to 1.2; *ref* mRNA-normalized slope, 1.0; 95% CI, 0.98 to 1.1).

Loss of mRNA during specific protocol steps. The *ref* mRNA technique was applied to quantify the loss of mRNA during specific protocol steps. Four parallel sets of 1-ml ANAS culture samples (five replicates for each set) were amended with 2×10^8 copies of *ref* mRNA immediately prior to the cell lysis, RNA isolation, DNA removal, or RT steps, and the recovered quantities of *tceA* and *ref* mRNA were measured by multiplex RT-qPCR (Table 1). As expected, measured quantities of *tceA* were consistent across all sample sets and hence independent of when *ref* mRNA was added to samples. By calculating differences in the mean fractional recoveries of *ref* mRNA when *ref* mRNA was added prior to each step, the contribution of each step towards the overall loss of mRNA was estimated to be 16% for cell lysis (Table 1, [B] − [A]), 44% for RNA isolation (Table 1, [C] − [B]), and 7% for DNA removal (Table 1, [D] − [C]). These fractional losses of mRNA correspond to mRNA recovery efficiencies of 84% for the cell lysis step, 56% for the RNA isolation step, and 93% for the DNA removal step.

Effect of chlorinated ethene exposure. The *ref* mRNA technique was applied to examine the effect of chlorinated ethene exposure on the quantity of *tceA* mRNA in the ANAS enrichment. Parallel subcultures were exposed to TCE, cDCE, or VC or starved of chlorinated ethenes. To control for any variability in cellular density between the subcultures, *tceA* mRNA measurements were normalized to the mass of cellular protein per sample. Without *ref* mRNA normalization, the quantities of *tceA* mRNA in the TCE-, cDCE-, and VC-exposed subcultures were 89-, 57-, and 3-fold greater, respectively, than in the chlorinated ethene-starved subculture (Fig. 4). Normalization to the sample-specific fractional recovery of *ref* mRNA changed the results in two ways. First, the quantity of *tceA* mRNA increased more than threefold in all the subcultures. Second, as a result of differing sample-specific fractional recoveries of *ref* mRNA, the ratio of *tceA* mRNA quantities in the VC-exposed and chlorinated ethene-starved subcultures decreased from 3-fold to 1.4-fold, which substantially reduced the apparent effect of VC exposure on the quantity of *tceA* mRNA.

DISCUSSION

The primary objective of this work was to develop an RT-qPCR technique that provides accurate quantification of specific mRNAs from prokaryotic samples. Limitations to the accuracy of mRNA quantification result from mRNA losses during sample processing (cell lysis, RNA isolation, and DNA removal) and from inefficiencies in RT (4, 5, 15). Traditional methods for controlling some or all of these losses, such as normalizing to the mass of total RNA or to quantities of housekeeping mRNA, suffer from dependencies on cell state, growth stage, and/or environmental conditions (17, 26, 35, 36, 42, 43). The novel approach developed here overcomes this limitation by normalizing to an internal reference mRNA (*ref* mRNA) that is not present in the experimental culture. Although *ref* mRNA techniques have been applied to eukaryotic cells (1, 18, 25, 34), to our knowledge this work represents the first application to prokaryotic cells.

TABLE 1. Summary of mRNA quantities and *ref* mRNA recoveries

Step immediately following *ref* mRNA addition	Step(s) contributing towards *ref* mRNA losses	Mean copy no. of *tceA* mRNA/sample[a]	SD[a]	Mean copy no. of *ref* mRNA/sample[a]	SD[a]	Mean fraction of *ref* mRNA recovered (%)[a,b,d]	SD[a,b]
Cell lysis[c]	Cell lysis,[c] RNA isolation,[c] DNA removal	9.7×10^6	8.6×10^5	5.6×10^7	4.2×10^6	(A) 28	2.1
RNA isolation[c]	RNA isolation,[c] DNA removal	1.2×10^7	1.7×10^6	8.7×10^7	1.5×10^7	(B) 44	7.6
DNA removal	DNA removal	1.0×10^7	7.2×10^5	1.8×10^8	1.5×10^7	(C) 88	7.5
RT		1.0×10^7	1.9×10^6	1.9×10^8	1.6×10^7	(D) 95	7.7

[a] All means and standard deviations were calculated from results for five replicate samples.
[b] 2×10^8 copies of *ref* mRNA were added to each sample.
[c] Cells were lysed and RNA was isolated using the acid phenol method.
[d] Capital letters A to D refer to adjacent values.

One limitation of the *ref* mRNA technique developed here is that it cannot control for the underestimation of mRNA quantities resulting from the incomplete lysis of cells. In this work, this limitation has been addressed by specifically optimizing the cell lysis protocol for the *tceA*-containing organism in the ANAS enrichment.

The *ref* mRNA technique coupled with the absolute standard curve method (19, 27) dramatically improved the quantification of *tceA* mRNA in the ANAS enrichment. Repeated experiments revealed that only 30% of the *ref* mRNA was recovered after the cell lysis, RNA isolation, and DNA removal steps (Fig. 2 and 3; Table 1). This overall mRNA recovery efficiency is consistent with RNA recovery efficiencies reported for other microbial cells, which range from 20 to 87% (28, 37, 40). Of the 30% of mRNA remaining after RNA isolation and purification, 88% was effectively synthesized to cDNA by RT (Fig. 1). Taking these mRNA losses and process inefficiencies together, the amount of cDNA amplified by qPCR corresponds to only 26% of the original quantity of mRNA [(30% of mRNA remaining after the cell lysis, RNA isolation, and DNA removal steps) × (88% RT efficiency)].

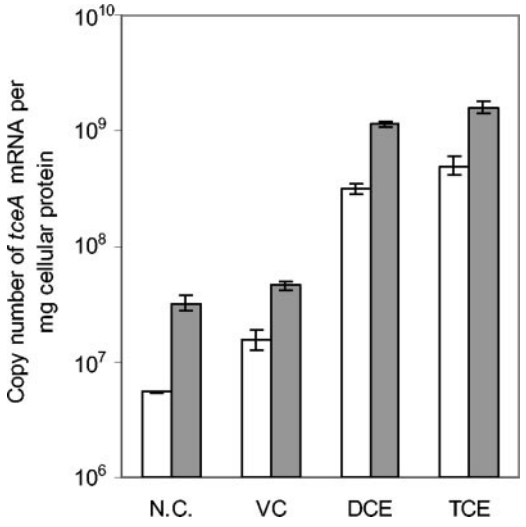

FIG. 4. Comparison of *tceA* mRNA copy numbers in the ANAS culture after 8 h of exposure to VC, cDCE, TCE, or chlorinated ethene starvation (N.C.). Filled bars are normalized by the sample-specific fractional recovery of *ref* mRNA while outlined bars are not. All measurements are averages of triplicate RT-qPCRs, and error bars are 1 standard deviation.

The *ref* mRNA technique proved to be a valuable tool for assessing the performance of different RNA processing steps. The mRNA recovery efficiency of the RNA isolation step (56%) was significantly less than that of the cell lysis (84%) and DNA removal (93%) steps. The RNA isolation step as employed here involves three aqueous-organic phase separations. During these phase separations, 10 to 15% of the aqueous phase was consistently left behind to prevent carryover of genomic DNA to downstream processes (DNA accumulates near the interface of acid phenol). Over three serial phase separations, the inefficient transfer of aqueous volumes likely accounts for the 56% efficiency of the RNA isolation step. Similarly, the cell lysis step involves one organic-aqueous phase separation (bead beating is performed in a two-phase phenol-aqueous lysis buffer), and the incomplete transfer of the aqueous layer could account for the 84% efficiency of this step. Taken together, this analysis suggests that physical losses contributed significantly more towards the overall loss of mRNA than mRNA degradation.

As a proof of concept, the *ref* mRNA technique was applied to investigate the effects of chlorinated ethene exposure on the quantity of *tceA* mRNA in the ANAS enrichment. Results show that the quantities of *tceA* mRNA in the TCE- and cDCE-exposed subcultures were 25-fold higher than in the VC-exposed and chlorinated ethene-starved subcultures. This effect was likely not due to cell growth, as the reported 19-hour doubling time of the *tceA* host organism, *Dehalococcoides ethenogenes* 195 (24), is much longer than the 8-hour time period of the chlorinated ethene exposure experiment. Instead, these differences likely represent chlorinated ethene-dependent differences in the expression level of the *tceA* gene.

The observation of increased *tceA* gene expression after exposure to TCE or cDCE but not VC is consistent with a regulatory scheme where *tceA* expression increases only in response to growth-supporting substrates of the TCE reductive dehalogenase. Physiological studies of *D. ethenogenes* 195 have shown that this strain can couple growth with the dechlorination of TCE and cDCE but not with VC (23, 24). In addition, biochemical studies of the purified TCE reductive dehalogenase have shown that dechlorination rates for TCE and cDCE

were more than 2 orders of magnitude greater than the dechlorination rate for VC (20, 21). Interestingly, prior to *ref* mRNA normalization, *tceA* mRNA quantities in the VC-exposed subculture were substantially higher than in the starvation subculture (3-fold). After *ref* mRNA normalization, however, this difference was reduced to 1.4-fold, demonstrating the substantial effect that application of the *ref* mRNA technique can have on the interpretation of RT-qPCR data.

ACKNOWLEDGMENTS

This work was supported by the Lawrence Berkeley National Laboratory Earth Science Division LDRD, National Science Foundation grant no. 0104740, and the NIEHS Superfund Basic Research Project ES04705.

REFERENCES

1. **Baker, P. J., and P. J. O'Shaughnessy.** 2001. Expression of prostaglandin D synthetase during development in the mouse testis. Reproduction **122:**553–559.
2. **Barloy-Hubler, F., A. Chéron, A. Hellégouarch, and F. Galibert.** 2004. Smc01944, a secreted peroxidase induced by oxidative stresses in *Sinorhizobium meliloti* 1021. Microbiology **150:**657–664.
3. **Bordi, C., M. Ansaldi, S. Gon, C. Jourlin-Castelli, C. Iobbi-Nivol, and V. Mejéan.** 2004. Genes regulated by TorR, the trimethylamine oxide response regulator of *Shewanella oneidensis*. J. Bacteriol. **186:**4502–4509.
4. **Bustin, S. A.** 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J. Mol. Endocrinol. **25:**169–193.
5. **Bustin, S. A.** 2002. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. J. Mol. Endocrinol. **29:**23–39.
6. **Cabrol, S., A. Olliver, G. B. Pier, A. Andremont, and R. Ruimy.** 2003. Transcription of quorum-sensing system genes in clinical and environmental isolates of *Pseudomonas aeruginosa*. J. Bacteriol. **185:**7222–7230.
7. **Chin, K.-J., A. Esteve-Núñez, C. Leang, and D. R. Lovley.** 2004. Direct correlation between rates of anaerobic respiration and levels of mRNA for key respiratory genes in *Geobacter sulfurreducens*. Appl. Environ. Microbiol. **70:**5183–5189.
8. **Corbella, M. E., and A. Puyet.** 2003. Real-time reverse transcription-PCR analysis of expression of halobenzoate and salicylate catabolism-associated operons in two strains of *Pseudomonas aeruginosa*. Appl. Environ. Microbiol. **69:**2269–2275.
9. **Denef, V. J., J. Park, T. B. Tsoi, J.-M. Rouillard, H. Zhang, J. A. Wibbenmeyer, W. Verstraete, E. Gulari, S. A. Hashsham, and J. M. Tiedje.** 2004. Biphenyl and benzoate metabolism in a genomic context: outlining genome-wide metabolic networks in *Burkholderia xenovorans* LB400. Appl. Environ. Microbiol. **70:**4961–4970.
10. **Devers, M., G. Soulas, and F. Martin-Laurent.** 2004. Real-time reverse transcription PCR analysis of expression of atrazine catabolism genes in two bacterial strains isolated from soil. J. Microbiol. Methods **56:**3–15.
11. **Dinamarca, M. A., I. Aranda-Olmedo, A. Puyet, and F. Rojo.** 2003. Expression of the *Pseudomonas putida* OCT plasmid alkane degradation pathway is modulated by two different global control signals: evidence from continuous cultures. J. Bacteriol. **185:**4772–4778.
12. **El-Shehawy, R., C. Lugomela, A. Ernst, and B. Bergman.** 2003. Diurnal expression of *hetR* and diazocyte development in the filamentous non-heterocystous cyanobacterium *Trichodesminum erythraeum*. Microbiology **149:**1139–1146.
13. **Fey, A., S. Eichler, S. Flavier, R. Christen, M. Hofle, and C. A. Guzmán.** 2004. Establishment of a real-time PCR-based approach for accurate quantification of bacterial RNA targets in water, using *Salmonella* as a model organism. Appl. Environ. Microbiol. **70:**3618–3623.
14. **Freeman, W. M., S. L. Vrana, and K. E. Vrana.** 1996. Use of elevated reverse transcription reaction temperatures in RT-PCR. BioTechniques **20:**782–783.
15. **Freeman, W. M., S. J. Walker, and K. E. Vrana.** 1999. Quantitative RT-PCR: pitfalls and potential. BioTechniques **26:**112–125.
16. **Fuchs, B., K. Zhang, M. G. Rock, M. E. Bolander, and G. Sarkar.** 1999. High temperature cDNA synthesis by AMV reverse transcriptase improves the specificity of PCR. Mol. Biotechnol. **12:**237–240.
17. **Gourse, R. L., T. Gall, M. S. Bartlett, J. A. Appleman, and W. Ross.** 1996. rRNA transcription and growth rate-dependent regulation of ribosome synthesis in *Escherichia coli*. Annu. Rev. Microbiol. **50:**645–677.
18. **Jelaso, A. M., E. Lehigh-Shirey, J. Means, and C. F. Ide.** 2003. Gene expression patterns predict exposure to PCBs in developing *Xenopus laevis* tadpoles. Environ. Mol. Mutagen. **42:**1–10.
19. **Liu, W., and D. A. Saint.** 2002. A new quantitative method of real time reverse transcription polymerase chain reaction assay based on simulation of polymerase chain reaction kinetics. Anal. Biochem. **302:**52–59.
20. **Magnuson, J. K., M. F. Romine, D. R. Burris, and M. T. Kingsley.** 2000. Trichloroethene reductive dehalogenase from *Dehalococcoides ethenogenes*: sequence of *tceA* and substrate range characterization. Appl. Environ. Microbiol. **66:**5141–5147.
21. **Magnuson, J. K., R. V. Stern, J. M. Gossett, S. H. Zinder, and D. R. Burris.** 1998. Reductive dechlorination of tetrachloroethene to ethene by a two-component enzyme pathway. Appl. Environ. Microbiol. **64:**1270–1275.
22. **Mary, I., C.-J. Tu, A. Grossman, and D. Vaulot.** 2004. Effects of high light on transcripts of stress-associated genes for the cyanobacteria *Synechocystis sp*. PCC 6803 and *Prochlorococcus* MED4 and MIT9313. Microbiology **150:**1271–1281.
23. **Maymó-Gatell, X., T. Anguish, and S. H. Zinder.** 1999. Reductive dechlorination of chlorinated ethenes and 1,2-dichloroethene by "*Dehalococcoides ethenogenes*" 195. Appl. Environ. Microbiol. **65:**3108–3113.
24. **Maymó-Gatell, X., Y.-T. Chien, J. M. Gossett, and S. H. Zinder.** 1997. Isolation of a bacterium that reductively dechlorinates tetrachloroethene to ethene. Science **276:**1568–1571.
25. **McMaugh, S. J., and B. R. Lyon.** 2003. Real-time quantitative RT-PCR assay of gene expression in plant roots during fungal pathogenesis. BioTechniques **34:**982–986.
26. **Nomura, M., R. Gourse, and G. Baughman.** 1984. Regulation of the synthesis of ribosomes and ribosomal components. Annu. Rev. Biochem. **53:**75–117.
27. **Peirson, S. N., J. N. Butler, and R. G. Foster.** 2003. Experimental validation of novel and conventional approaches to quantitative real-time PCR data and analysis. Nucleic Acids Res. **31:**e73.
28. **Pichard, S. L., and J. H. Paul.** 1991. Detection of gene expression in genetically engineered microorganisms and natural phytoplankton populations in the marine environment by mRNA analysis. Appl. Environ. Microbiol. **57:**1721–1727.
29. **Richardson, R. E., V. K. Bhupathiraju, D. L. Song, T. A. Goulet, and L. Alvarez-Cohen.** 2002. Phylogenetic characterization of microbial communities that reductively dechlorinate TCE based upon a combination of molecular techniques. Environ. Sci. Technol. **36:**2652–2662.
30. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
31. **Semighini, C. P., M. Marins, M. H. S. Goldman, and G. H. Goldman.** 2002. Quantitative analysis of the relative transcript levels of ABC transporter *Atr* genes in *Aspergillus nidulans* by real-time reverse transcription-PCR assay. Appl. Environ. Microbiol. **68:**1351–1357.
32. **Sharkey, F. H., I. M. Banat, and R. Marchant.** 2004. Detection and quantification of gene expression in environmental bacteriology. Appl. Environ. Microbiol. **70:**3795–3806.
33. **Siering, P. L., and W. C. Ghiorse.** 1997. Development and application of 16S rRNA-targeted probes for detection of iron- and manganese-oxidizing sheathed bacteria in environmental samples. Appl. Environ. Microbiol. **63:**644–651.
34. **Smith, R. D., B. Brown, P. Ikonomi, and A. N. Schechter.** 2003. Exogenous reference RNA for normalization of real-time quantitative PCR. BioTechniques **34:**88–91.
35. **Thellin, O., W. Zorzi, B. Lakaye, B. De Borman, B. Coumans, G. Hennen, T. Grisar, A. Igout, and E. Heinen.** 1999. Housekeeping genes as internal standards: use and limits. J. Biotechnol. **75:**291–295.
36. **Vandecasteele, S. J., W. E. Peetermans, R. Merckx, and J. Van Eldere.** 2001. Quantification of expression of *Staphylococcus epidermidis* housekeeping genes with Taqman quantitative PCR during in vitro growth under different conditions. J. Bacteriol. **183:**7094–7101.
37. **Ward, D. M., A. L. Ruff-Roberts, and R. Weller.** 1995. Methods for extracting RNA or ribosomes from microbial mats and cultivated microorganisms, p. 1–14. *In* A. Akkermans, J. van Elsas, and F. de Bruijn (ed.), Molecular microbial ecology manual. Kluwer Academic Publishers, Dordrecht, The Netherlands.
38. **Watanabe, T., H. Fujihara, and K. Furukawa.** 2003. Characterization of the second LysR-type regulator in the biphenyl-catabolic gene cluster of *Pseudomonas pseudoalcaligenes* KF707. J. Bacteriol. **185:**3575–3582.
39. **Wawrik, B., J. H. Paul, and F. R. Tabita.** 2002. Real-time PCR quantification of *rbcL* (ribulose-1,5-bisphosphate carboxylase/oxygenase) mRNA in diatoms and pelagophytes. Appl. Environ. Microbiol. **68:**3771–3779.
40. **Weinbauer, M. G., I. Fritz, D. F. Wenderoth, and M. G. Höfle.** 2002. Simultaneous extraction from bacterioplankton of total RNA and DNA suitable for quantitative structure and function analyses. Appl. Environ. Microbiol. **68:**1082–1087.
41. **Yarzábal, A., and C. Appia-Ayme.** 2004. Regulation of the expression of the *Acidithiobacillus ferrooxidans* rus operon encoding two cytochromes c, a cytochrome oxidase and rusticyanin. Microbiology **150:**2113–2123.
42. **Zhong, H., and J. W. Simons.** 1999. Direct comparison of GAPDH, β-actin, cyclophilin, and 28S rRNA as internal standards for quantifying RNA levels under hypoxia. Biochem. Biophys. Res. Commun. **259:**523–526.
43. **Zhou, G., Y. Chang, J. Zuo, X. Dong, M. Zhang, G. Hu, and F. Fang.** 2001. Fudenine, a C-terminal truncated rat homologue of mouse prominin, is blood glucose-regulated and can up-regulate the expression of GAPDH. Biochem. Biophys. Res. Commun. **281:**951–956.