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Development of a dual-internal-reference technique to improve accuracy when determining bacterial 16S rRNA:16S rRNA gene ratio with application to *Escherichia coli* liquid and aerosol samples

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ABSTRACT

Accurate enumeration of rRNA content in microbial cells, e.g. by using the 16S rRNA:16S rRNA gene ratio, is critical to properly understand its relationship to microbial activities. However, few studies have considered possible methodological artifacts that may contribute to the variability of rRNA analysis results. In this study, a technique utilizing genomic DNA and 16S rRNA from an exogenous species (*Pseudomonas fluorescens*) as dual internal references was developed to improve accuracy when determining the 16S rRNA:16S rRNA gene ratio of a target organism, *Escherichia coli*. This technique was able to adequately control the variability in sample processing and analysis procedures due to nucleic acid (DNA and RNA) losses, inefficient reverse transcription of RNA, and inefficient PCR amplification. The measured 16S rRNA:16S rRNA gene ratio of *E. coli* increased by 2–3 fold when *E. coli* 16S rRNA gene and 16S rRNA quantities were normalized to the sample-specific fractional recoveries of reference (*P. fluorescens*) 16S rRNA gene and 16S rRNA, respectively. In addition, the intra-sample variation of this ratio, represented by coefficients of variation from replicate samples, decreased significantly after normalization. This technique was applied to investigate the temporal variation of 16S rRNA:16S rRNA gene ratio of *E. coli* during its non-steady-state growth in a complex liquid medium, and to *E. coli* aerosols when exposed to particle-free air after their collection on a filter. The 16S rRNA:16S rRNA gene ratio of *E. coli* increased significantly during its early exponential phase of growth; when *E. coli* aerosols were exposed to extended filtration stress after sample collection, the ratio also increased. In contrast, no significant temporal trend in *E. coli* 16S rRNA:16S rRNA gene ratio was observed when the determined ratios were not normalized based on the recoveries of dual references. The developed technique could be widely applied in studies of relationship between cellular rRNA abundance and bacterial activity.

1. Introduction

The small subunit ribosomal RNA (ssu rRNA) of bacteria (in particular, 16S rRNA or 16S rRNA gene) has been commonly used to assess the phylogenetic structure of microbial communities in various ecosystems, because it is ubiquitous and has low mutation rates throughout prokaryotic evolution (Woese, 1987). Unlike the rapid turnover of messenger RNA (mRNA) within the cell, rRNA is perceived as a relatively stable RNA type (Deutscher, 2003). As a result, the 16S rRNA has been frequently used as an endogenous reference to normalize the mRNA quantities when studying the expression of a specific functional gene (Takle et al., 2007; Arunasri et al., 2014; Fey et al., 2004). However, the rRNA content within a cell is also known to change depending on the physiological state of the cell. For example, a number of studies have shown a positive correlation between rRNA content and growth rates of specific bacterial species in pure cultures (Kerkhof and Ward, 1993; Muttray et al., 2001; Dortch et al., 1983; Poulsen et al., 1993). A more recent study also reported elevated rRNA content for *Sphingomonas aerolata* aerosols supplied with gaseous growth substrates in a rotating bioreactor (Krumins et al., 2014). Blazewicz et al. indicated in a review paper that little information is available regarding the relationship between non-growth activities and bacterial rRNA content (Blazewicz et al., 2013). Thus, the relationship between the microbial rRNA content and change of environmental conditions or environmental stressors is still inconclusive and needs further exploration.

The relative abundance of 16S rRNA with respect to DNA, often presented as the 16S rRNA:16S rRNA gene ratio, is considered to be an indicator of microbial activity or potential of being active (Campbell et al., 2011; Perez-Osorio et al., 2010; Pitkanen et al., 2013; Kim and Wang, 2009; Campbell et al., 2009; Hawkins et al., 2012). To determine the rRNA abundance in a microbial sample, the 16S rRNA and 16S rRNA gene must be quantified separately. A number of studies have applied both quantitative polymerase chain reaction (qPCR) and reverse

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transcription-qPCR (RT-qPCR) to examine the 16S rRNA:16S rRNA gene ratio for microbes from a variety of environmental systems, including plant leaves (Kim and Wang, 2009), marine surface waters (Campbell et al., 2011, 2009), biofilms (Perez-Osorio et al., 2010), nitrification reactor (Hawkins et al., 2012) and water from sewage treatment plants (Pitkanen et al., 2013). Most studies do not specifically report the extent of DNA or RNA losses during sample processing when determining the rRNA abundance, thus the accuracies of those reported ratios, which spanned almost 5 orders of magnitude from ~0.1 (Hawkins et al., 2012) to ~10^4 (Perez-Osorio et al., 2010), are largely unknown. In addition, high intra-sample variation, as indicated by large coefficients of variation (CV) within sample replicates, has also been observed in these studies (Perez-Osorio et al., 2010; Pitkanen et al., 2013; Kim and Wang, 2009; Campbell et al., 2009; Hawkins et al., 2012). The large intra-sample variation could reduce the statistical power to detect true differences among multiple samples. Therefore, a more robust analytical method is needed to determine the 16S rRNA:16S rRNA gene ratio with improved accuracy and reduced intra-sample variation so that the relationship between the rRNA abundance and bacterial activity could be examined.

In most gene expression studies, the variation among individual samples comes from two different sources: the biological variability and technical variability (Cheng et al., 2012; Oberg et al., 2012; Whitehead and Crawford, 2006). The biological variability is the inherent difference in RNA transcription level between individual samples such as sample replicates (Cheng et al., 2012; Oberg et al., 2012). On the other hand, the technical variability is mainly influenced by experimental artifacts (Cheng et al., 2012; Whitehead and Crawford, 2006), and thus could be controlled and reduced by modification of experimental procedures and parameters. For studies of 16S rRNA:16S rRNA gene ratio, the technical variability can stem from multiple sources, e.g. sample losses during DNA/RNA isolation and DNase treatment of RNA samples, inefficient reverse transcription of 16S rRNA, and biased quantification of 16S rRNA genes or reverse transcribed 16S rRNA during qPCR. To control this variability, an exogenous internal reference technique has been applied in a number of previous studies of RNA quantification by RT-qPCR (Smith et al., 2003; Johnson et al., 2005; McMaugh and Lyon, 2003; Wagner et al., 2009). It is assumed that the reference RNA, a known amount of which is amended to the sample of interest prior to RNA extraction, behaves similarly to the target RNA analyte through the entire analysis process. The quantity of the target RNA analyte is then obtained by normalizing the recovered target RNA quantity to the fractional recovery of the reference RNA. In addition to its application in RNA quantification, the exogenous internal reference technique has also been used to enumerate DNA in environmental samples (Hatt et al., 2013; Sen et al., 2007; Luciano et al., 2007).

To further improve accuracy and reduce variability when analyzing 16S rRNA:16S rRNA gene ratio in bacterial samples, including bioaerosol samples, we developed an exogenous dual-internal-reference technique. Specifically, known amounts of 16S rRNA and genomic DNA from a reference bacterial species (*Pseudomonas fluorescens*) were spiked into the target *Escherichia coli* samples prior to DNA and RNA co-extraction from both species. This technique was then applied to *E. coli* from both liquid culture and bioaerosol samples to investigate the variation of 16S rRNA:16S rRNA gene ratio as a function of bacterial growth and potential activity in response to environmental stressors. To the best of our knowledge, this is the first study to apply dual internal references simultaneously to accurately quantify RNA and DNA from a microbial sample.

2. Materials and methods

2.1. Extraction of nucleic acids

Total nucleic acids including genomic DNA and RNA were simultaneously extracted from *E. coli* (ATCC 15597, Manassas, VA) cell pellets or *E. coli* aerosols collected on filters (sampling described in section "Aerosolization of *E. coli*") using a phenol/chloroform-based method developed by Kerkhof et al. (Kerkhof and Ward, 1993; Kerkhof and Kemp, 1999). Briefly, bacterial cells were suspended in 50 μl buffer A (50 mM glucose, 10 mM EDTA and 25 mM pH = 8.0 Tris) and treated with five quick freeze/thaw cycles of freezing with liquid nitrogen and thawing in a water bath at 55 °C, respectively. Thereafter, 200 μl of buffer A, 100 μl of 4 mg/ml lysozyme in buffer A and 50 μl of 500 mM EDTA were added in sequence into the sample. After incubation at room temperature for 5–10 min, a 50 μl 10% sodium dodecyl sulfate (SDS) solution was added into the sample solution. The cell lysate was then extracted twice with 800 μl phenol–chloroform–isoamyl alcohol mixture (25:24:1, pH = 6.7, Fisher Scientific, Pittsburgh, PA). Next, the aqueous phase was mixed with 50 μl of 3.0 M sodium acetate, 2 μl of glycogen (20 mg/ml), and 1 ml 100% ice-cold ethanol. The nucleic acids were pelleted by centrifugation at 16,100 ×*g* at 4 °C for 15 min and then washed once with 400 μl 70% ethanol solution. The pellets were dried under a laminar flow hood for 10 min and stored at −80 °C.

2.2. Preparation of dual internal references

The 16S rRNA reference was prepared from the total nucleic acid extracts of 8 ml of pure *P. fluorescens* (ATCC 13525) culture ($7.6 \times 10^8 \pm 2.3 \times 10^8$ cells/ml as determined by microscopy) that was initially obtained using aforementioned phenol/chloroform protocol (described in section "Extraction of nucleic acids"). Particularly, the total nucleic acids were dissolved in 600 μl DEPC-water and loaded in small quantities into individual wells on a 1.2% agarose gel. The volume of each loaded sample after mixing with loading dye was 10 μl (<5 μg nucleic acids) per well, and thus a total of ~70 wells on three individual agarose gels were used. Electrophoresis was performed at 110 V for 45 min in cold 1× TAE buffer and the 16S rRNA was separated from other nucleic acids. The 16S rRNA bands on each gel were excised using a sterile stainless-steel blade and collected into 10 microcentrifuge tubes, each of which contained ~400 mg gel material. The agarose gel pieces containing 16S rRNA bands were then extracted and purified with a Zymoclean™ Gel RNA Recovery Kit (Zymo Research Corp., Irvine, CA) according to the manufacturer's protocol. The quality of total nucleic acid extracts and purified 16S rRNA reference were initially checked by agarose gel electrophoresis (1.2%, 110 V for 45 min) with a Lambda DNA/HindIII marker (Thermo Scientific, Waltham, MA), and examined further with the BioRad Experion™ automated electrophoresis system (Hercules, CA).

To prepare the genomic DNA reference from *P. fluorescens*, 4 ml of freshly grown pure culture ($7.6 \times 10^8 \pm 2.3 \times 10^8$ cells/ml) were harvested and subjected to nucleic acid extraction using aforementioned phenol/chloroform protocol (described in section "Extraction of nucleic acids") with the following modifications: once SDS solution was added, 1 μl of 10 unit RNase ONE™ ribonuclease (Promega, Madison, WI) was applied to each crude extract for 10 min at 37 °C; the cell lysate was extracted by phenol–chloroform–isoamyl alcohol mixture three times instead of twice; the total nucleic acids dissolved in DEPC-water after ethanol precipitation were further treated with 2.5 units of RNase ONE™ ribonuclease for 30 min at 37 °C, and then RNase was inactivated by adding 5 mM Dithiothreitol (DTT) and incubating at 70 °C for 15 min. The complete degradation of RNA in DNA/RNA mixture was verified by gel electrophoresis on a 1.2% agarose gel (110 V, 45 min) with 1000-bp DNA ladders (Thermo Scientific). In the final step, the genomic DNA extracts were purified using a DNeasy blood & tissue kit (Qiagen, Valencia, CA) according to the manufacturer's protocol.

The genomic DNA and 16S rRNA standards for *E. coli* were prepared in the same way as for *P. fluorescens*. Two separate 2-ml aliquots of *E. coli* pure culture ($1.6 \times 10^9 \pm 3.3 \times 10^8$ cells/ml, as determined by microscopy) were used for the preparation of each standard.

2.3. Quantification of genomic DNA and 16S rRNA standards

The purified genomic DNA and 16S rRNA extracts from both bacteria were quantified individually by a Qubit dsDNA HS assay kit and RNA HS assay kit (Life Technologies, Grand Island, NY) with a Qubit 2.0 fluorometer (Life Technologies). After quantification, the DNA and RNA extracts were divided into several microcentrifuge tubes and stored at −80 °C prior to use.

The concentrations of genomic DNA were converted from a mass basis to a 16S rRNA gene copy number basis using Eq. (1):

$$C_{16S\ rRNA\ gene} = \frac{C_{mass} \times f \times n}{N} \quad (1)$$

where $C_{16S\ rRNA\ gene}$ is the number concentration of 16S rRNA gene in a sample (copies/ml); C_{mass} is the mass concentration of genomic DNA in the same sample (ng/ml); f is the average number of base pairs per unit mass of DNA, which is equal to 0.978×10^{12} bp/ng (Dolezel et al., 2003); n is the average number of 16S rRNA gene copies in the bacterial genome, which are 7 and 5 for *E. coli* (Blattner et al., 1997) and *P. fluorescens* (Redondo-Nieto et al., 2012), respectively; and N is the number of base pairs in the bacterial genome: 4,639,221 and 6,845,832 bp for *E. coli* (Blattner et al., 1997) and *P. fluorescens* (Redondo-Nieto et al., 2012), respectively. The number concentration of 16S rRNA was determined by Eq. (2):

$$C_{16S\ rRNA} = \frac{C_{mass} \times K}{M} \times 10^{-9} \quad (2)$$

where $C_{16S\ rRNA}$ is the number concentration of 16S rRNA in a sample (copies/ml); C_{mass} is the mass concentration of 16S rRNA in the same sample (ng/ml); K is the Avogadro's Constant ($6.02214129 \times 10^{23}$/mol); and M is the molecular weight of target 16S rRNA (g/mol), which was determined using Eq. (3):

$$M = 320.5 \times N + 159 \quad (3)$$

where M is the molecular weight of 16S rRNA (g/mol); and N is the number of nucleotides for a complete 16S rRNA sequence, which are 1542 and 1529 for *E. coli* (Blattner et al., 1997) and *P. fluorescens* (Redondo-Nieto et al., 2012), respectively.

2.4. Primers and probes

Taqman primer–probe sets were purchased from Sigma-Aldrich (Woodlands, TX). The primer–probe sets were designed using Beacon Designer's program (Premier Biosoft, Palo Alto, CA) to target the 16S rRNA gene sequence of *E. coli* and *P. fluorescens*. For *E. coli* 16S rRNA gene, the set included a forward primer: GGGAGTAAAGTTAATACCTTTG, a reverse primer: CCAGTATCAGATGCAGTTC, and a FAM-labeled probe: TCACATCTGACTTAACAAACCGCCT-FAM. The primer–probe set for *P. fluorescens* 16S rRNA gene included a forward primer: CCTTGTCCTTAGTTACCAG, a reverse primer: CTCTGTACCGACCATTGTA, and a HEX-labeled probe: CACTCTAAGGAGACTGCCGGTGAC-HEX.

2.5. Quantitative PCR

Multiplex quantitative PCR was performed using the iCycler iQ5 RT-PCR detection system (Bio-Rad Laboratories, Hercules, CA) to quantify *E. coli* and *P. fluorescens* 16S rRNA genes or reverse transcribed 16S rRNA, simultaneously. The determined optimal reaction conditions in a 20 μl mixture included 10 μl of 2× TaqMan® universal PCR master mix (Life Technologies), 650 nM of each forward and reverse primer, 200 nM of each probe, 0.04 mg/ml of bovine serum albumin (Sigma, St. Louis, MO) and 4 μl of sample DNA or cDNA. The amplification reaction was performed using the following temperature program: 10 min of denaturation at 95 °C and 40 cycles of 15 s of denaturation at 95 °C and 1 min of annealing/extension at 58 °C and 30 s further extension at 72 °C. Data analysis was performed using the iCycler iQ real-time detection system software. A 10-fold serial dilution of mixture containing *E. coli* and *P. fluorescens* genomic DNA standards [with similar 16S rRNA gene copy numbers for each organism] was performed in multiplex qPCR along with samples in each plate. Standard curves for individual amplicons (*E. coli* or *P. fluorescens* 16S rRNA gene sequence) were prepared by plotting each cycle threshold (C_T) value against the log of target gene copy number contained in the mixture. Standard curves for single-stranded cDNA (reverse transcribed 16S rRNA) were produced by plotting each C_T value against the log of double the theoretical copy number of the double-stranded 16S rRNA gene standard (genomic DNA extracts). Each sample and standard was measured by multiplex qPCR in triplicate.

2.6. Determination of 16S rRNA:16S rRNA gene ratio of E. coli samples

Prior to nucleic acid extraction, *E. coli* samples were spiked with dual internal references: 5 μl of genomic DNA from *P. fluorescens* (1.1×10^6 copies of 16S rRNA gene/μl) and 5 μl 16S rRNA extracts from *P. fluorescens* (8.6×10^9 copies/μl). After extraction, the pellets, including genomic DNA and RNA of both bacterial species, were resuspended in 100 μl diethylpyrocarbonate (DEPC)-treated water and divided into two aliquots. The first aliquot was used to quantify 16S rRNA genes of both bacterial species using the multiplex qPCR protocol described in section "Quantitative PCR", while the other aliquot was saved at −80 °C for subsequent RNA analysis. The quantity of *E. coli* 16S rRNA gene was obtained by normalizing the recovered quantity to the fractional recovery of *P. fluorescens* 16S rRNA gene.

The nucleic acid extracts aliquoted for RNA quantification were subjected to DNA removal and reverse transcription prior to qPCR. DNA was removed using the Ambion TURBO DNA-free DNase kit (Life Technologies, Grand Island, NY) following the manufacturer's instructions. No amplicons were obtained in PCR reaction with DNase-treated RNA sample and primer sets targeting general bacterial 16S rRNA gene sequences. The DNA-free RNA sample was reverse transcribed into cDNA using SuperScript® VILO cDNA synthesis kit (Life Technologies), and then cDNA was diluted and analyzed for 16S rRNA from both bacterial species in a multiplex qPCR reaction. The quantity of *E. coli* 16S rRNA was obtained by normalizing the recovered quantity to the fractional recovery of *P. fluorescens* 16S rRNA.

2.7. Change in 16S rRNA:16S rRNA gene ratio for E. coli growing in liquid culture

The dual-internal-reference technique was applied to investigate the temporal variation of 16S rRNA:16S rRNA gene ratio for *E. coli* during non-steady-state growth in a complex liquid medium. Briefly, a few *E. coli* colonies from a TSA agar plate were randomly picked and inoculated in a 50 ml freshly made TSB medium under strict aseptic conditions. The inoculated medium was incubated at 37 °C on a mechanical shaker at 120 rpm for over 16 h. 100 μl homogenized culture samples were taken 1, 2, 3, 4, 5, 6, 7, 9, 12 and 16 h after inoculation and stored at −80 °C prior to analyzing their 16S rRNA:16S rRNA gene ratio. The optical density at 600 nm (O.D. 600) of *E. coli* culture was measured using a spectrophotometer (PerkinElmer, Shelton, CT) hourly over 16 h after inoculation. The relative growth rate, V_t, of *E. coli* in the liquid culture each hour was determined using Eq. (4):

$$V_t = (Abs_{t+1} - Abs_t)/Abs_t \quad (4)$$

where Abs_t is the O.D. 600 of culture at time t; and Abs_{t+1} is the O.D. 600 of culture at $t + 1$ h.

2.8. Aerosolization of E. coli

The dual-internal-reference technique was also applied to investigate the temporal variation of 16S rRNA:16S rRNA gene ratio of *E. coli* aerosols that were collected on a filter and then exposed to particle-free air for different time periods. Briefly, the precultured *E. coli* cells were first pelleted and then washed twice with 1× PBS solution by centrifugation at 6000 ×*g* for 5 min at 25 °C (BR4; Jouan, Winchester, VA) and refilled with 50 ml 1× PBS solution after the second wash. A 100 μl of liquid *E. coli* culture was saved at −80 °C to enable the determination of the 16S rRNA:16S rRNA gene ratio of *E. coli* before aerosolization. The bacterial suspensions were aerosolized using a Single-Pass Aerosolizer (CH Technologies Inc., Westwood, NJ) which was previously shown to induce lower aerosolization stress to bacteria compared to other commonly used bioaerosol generators (Zhen et al., 2014). The culture was supplied by a syringe pump (Kent Scientific Corp., Torrington, CT) at a rate of 0.1 ml/min, and the aerosolization air flow rate was 1.2 l/min. The aerosolized *E. coli* was diluted with a particle-free airflow at 80 l/min and introduced into an open cylinder-shaped chamber (36 cm in length × 10 cm in diameter). The aerosolization time was 1 min. Five Button aerosol samplers (SKC Inc., Eighty Four, PA) were placed inside the chamber and simultaneously operated for 1 min to sample *E. coli* bioaerosol on 0.8 μm-pore-size and 22 mm-diameter polyethersulfone (PES) membrane filter (SUPOR filter, Pall Corp., Port Washington, NY). Once *E. coli* aerosolization stopped, all Button samplers continued to sample particle-free air (relative humidity at 25–30%) for another 0, 2, 4, 6 and 10 h (one Button sampler for each extra time period). After that, filters from each sampler were removed and cut into 1 cm × 1 cm pieces with sterile scissors and stored at −80 °C. The entire experiment was conducted inside a Class II Biosafety cabinet (NUAIRE Inc., Plymouth, MN) to prevent the release of bioaerosols into the laboratory environment.

2.9. Statistical analysis

Statistical analysis was performed using SPSS v.18.0 (IBM Corp., Armonk, NY). The specific statistical tests and investigated variables are described in more detail in Table S1 in Supplementary Information. Overall, a statistically significant difference was assumed for $p < 0.05$.

3. Results

3.1. Quality of genomic DNA and 16S rRNA extracted from E. coli and P. fluorescens

The quality of genomic DNA and 16S RNA was initially assessed by agarose gel electrophoresis as shown in Fig. S1 in Supplementary Information. Tight bands of 16S rRNA (Fig. S1a) and genomic DNA (Fig. S1b) for both bacterial species indicated integrity of both nucleic acids. A BioRad Experion™ automated electrophoresis system was employed to examine further the integrity of total RNA extracts used for 16S rRNA isolation and the isolated 16S rRNA for both bacterial species (Fig. 1). The ratios of 23S rRNA to 16S rRNA in total RNA extracts are closed to 1.5 (1.43 for *P. fluorescens*) or higher than 1.5 (1.80 for *E. coli*), a sign of high RNA quality (Bhagwat et al., 2013). In addition, the flat baseline and low background in electrophorogram of 16S rRNA isolated from both species indicated minimal degradation of RNA. It was estimated that 6 μg and 3 μg of genomic DNA and 16S rRNA, respectively, were obtained per ml of *E. coli* ($1.6 \pm 0.3 \times 10^9$ cells/ml), and 3 μg and 2 μg of genomic DNA and 16S rRNA, respectively, were obtained per ml of *P. fluorescens* ($7.6 \pm 2.3 \times 10^8$ cells/ml).

3.2. Development of multiplex qPCR assay for the determination of 16S rRNA genes of E. coli and P. fluorescens

Prior to multiplex qPCR assay, the validity of the designed primer and probe sets for each bacterial species was evaluated by singleplex qPCR assays using *E. coli* and *P. fluorescens* genomic DNA (Fig. 2). Linear amplifications with quantification ranging from 10^2 to 10^7 copies of 16S rRNA gene per reaction were obtained for both bacteria. The amplification efficiencies for *E. coli* and *P. fluorescens* singleplex assays were 93.2% and 89.4%, respectively, when the corresponding genomic DNA was applied for each assay. No amplification was observed in simplex assays using *E. coli* primer and probe set with *P. fluorescens* genomic DNA or *P. fluorescens* primer and probe set with *E. coli* genomic DNA (data not shown). In multiplex assay, when approximately equal quantities of DNA from the two species were mixed and then serially diluted, linear amplification was observed in the range from 10^2 to 10^7 copies of 16S rRNA gene per assay. The slopes of the amplification curves did not exhibit a significant difference between two target genes in the multiplex assay ($p = 0.659$), indicating similar amplification efficiencies for both targets (85.7% for *E. coli* and 85.4% for *P. fluorescens*). Similarly, the difference in amplification efficiencies between the multiplex and singleplex assay for *E. coli* and *P. fluorescens* (reference) 16S rRNA genes was not statistically significant: $p = 0.08$ in both cases.

One limitation of the multiplex qPCR technique is that when two target amplicons are present at greatly different quantities, the amplification signals often become distorted, especially for the less abundant amplicon (Mutter and Boyton, 1995). This effect could result from reagent limitation and/or amplification inhibition (Mutter and Boyton, 1995). Thus, to determine the conditions when such experimental errors could be avoided, a suite of 10-fold dilutions (10^2–10^7 copies per sample) of *E. coli* 16S rRNA gene was mixed with similar dilutions of *P. fluorescens* 16S rRNA gene (10^2–10^7 copies per sample) but in reverse order, e.g. ~10^7 copies of *E. coli* amplicon mixed with ~10^2 copies of *P. fluorescens* 16S rRNA gene and ~10^6 copies of *E. coli* amplicon mixed with ~10^3 copies of *P. fluorescens* 16S rRNA gene. These results are presented in Fig. S2. When the 16S rRNA gene quantity of *E. coli* was greater than that of *P. fluorescens* by a factor of 1000:1 or higher, the C_T of *P. fluorescens* increased compared to the singleplex assay (Fig. S2a). In contrast, when *P. fluorescens* 16S rRNA gene quantities exceeded *E. coli* 16S rRNA gene quantities by a factor of 1000:1 or higher (Fig. S2b), the C_T of *E. coli* decreased dramatically compared with the singleplex assay. Thus, to achieve reliable quantification of both target genes when performing multiplex assay, the difference in relative initial quantities of *E. coli* and *P. fluorescens* 16S rRNA genes should not exceed a factor of 1000. As a result, an initial estimate of the target *E. coli* 16S rRNA gene quantity relative to that of the reference *P. fluorescens* in each sample is needed prior to multiplex qPCR reaction.

3.3. Effect of initial cell quantities on the determination of 16S rRNA:16S rRNA gene ratio with dual-internal-reference technique

The data in Fig. S2 show that a substantial difference in initial quantities of 16S rRNA gene from *E. coli* and *P. fluorescens* affects the quantification of both analytes when performing multiplex qPCR. Likewise, when different amounts of *E. coli* 16S rRNA and rRNA gene are analyzed with a fixed quantity of reference *P. fluorescens* 16S rRNA and rRNA gene, the initial quantity of *E. coli* cells could have an effect on the amplification efficiency of both species and thus on the accuracy of the results. To investigate this possibility, aliquots with a fixed quantity of reference *P. fluorescens* genomic DNA and RNA (4.3×10^{10} and 5.5×10^6 copies of 16S rRNA gene and rRNA, respectively) were mixed with different numbers of *E. coli* cells (2.0×10^4 to 2.0×10^6) prior to DNA/RNA co-extraction. The *E. coli* cells were prepared by making serial dilutions from the same batch of pure culture, and three separate batches were evaluated.

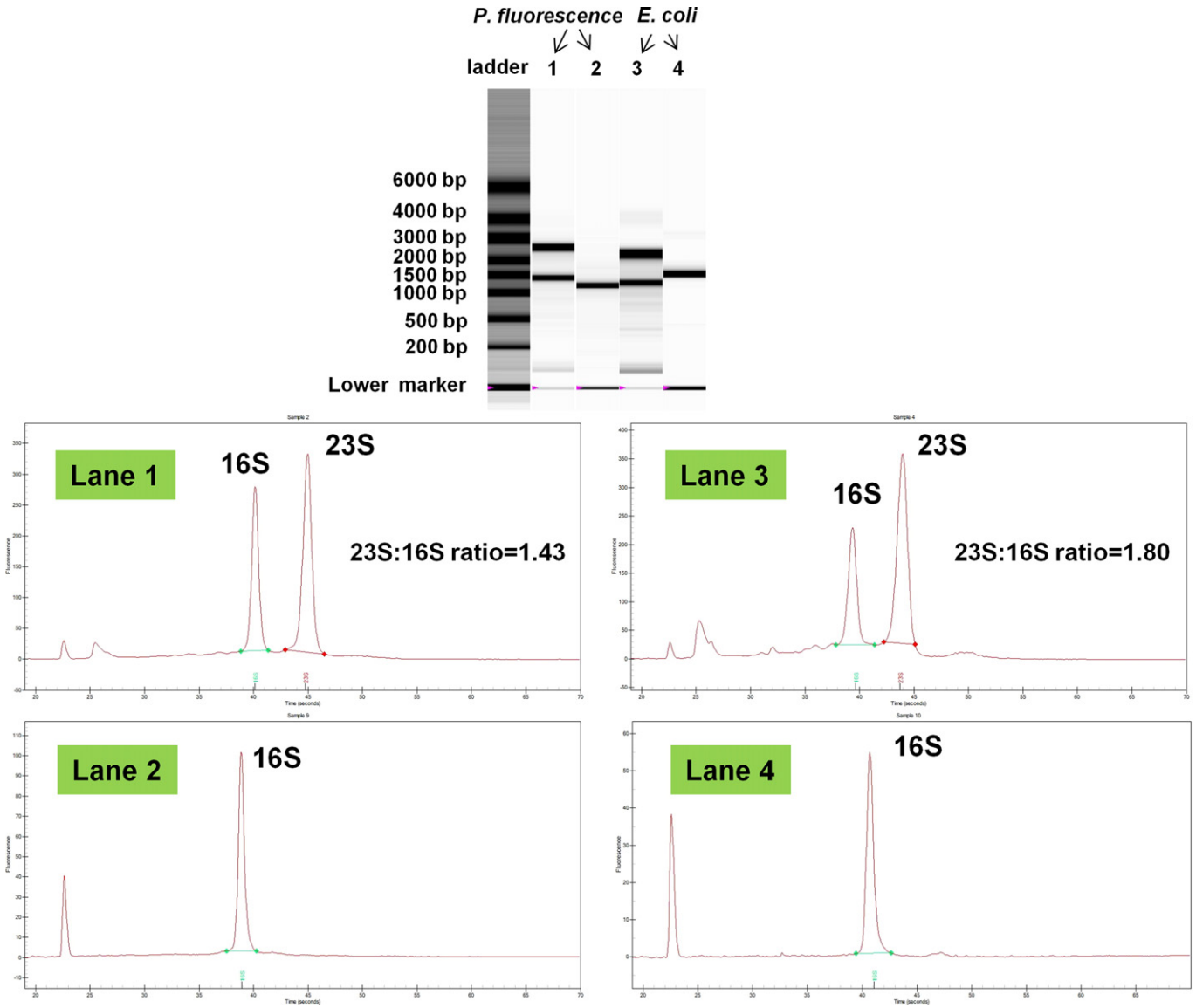

Fig. 1. Electrophorogram of total RNA and 16S rRNA isolated from *P. fluorescens* (lane 1: total RNA and lane 2: 16S rRNA) and *E. coli* (lane 3: total RNA and lane 4: 16S rRNA) on the BioRad Experion™ automated electrophoresis system. The 23S/16S ratios for *P. fluorescens* and *E. coli* are 1.43 and 1.80, respectively.

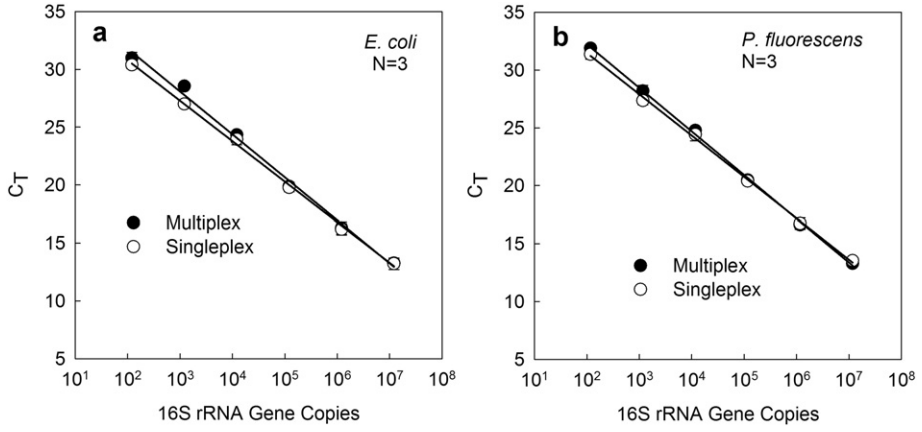

Fig. 2. Standard curves for *E. coli* (a) and *P. fluorescens* (b) 16S rRNA gene in multiplex (closed circle) and singleplex (open circle) qPCR reactions. All data points are averages of triplicate samples, and error bars are 1 standard deviation. Where not visible, error bars are smaller than the symbols.

The results presented in Fig. 3a show that the recovered copy number of *E. coli* 16S rRNA gene increased linearly with increasing cell quantity. The recovery of reference (*P. fluorescens*) 16S rRNA gene was on average 52 ± 28% across different initial *E. coli* cell quantities. When *E. coli* 16S rRNA gene quantities were normalized using the *P. fluorescens* recovery as a reference, they increased by nearly a factor of two, but the difference between the slopes of the regression lines with (0.93) and without (1.06) normalization was not statistically significant ($p = 0.143$). A similar normalization approach was performed with *E. coli* 16S rRNA (Fig. 3b). The average recovery for *P. fluorescens* 16S rRNA (a reference) was 19 ± 6% across all test sample groups with different initial *E. coli* cell quantities; the quantities of *E. coli* 16S rRNA increased by approximately a factor of five once normalized based on the recovery of the reference *P. fluorescens* 16S rRNA. The difference between slopes of regression lines with (0.97) and without (0.94) normalization was not statistically significant ($p = 0.150$).

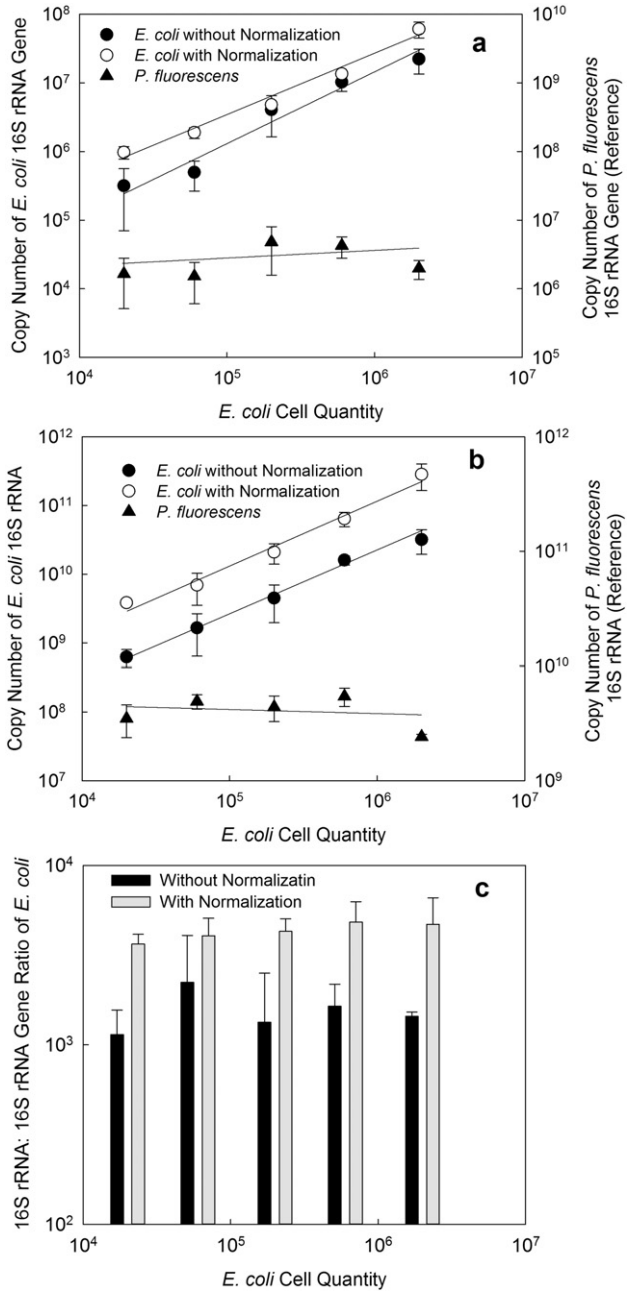

Fig. 3. Effect of initial *E. coli* cell quantity on the determination of 16S rRNA:16S rRNA gene ratio with the dual-internal-reference technique: a) the quantities of *E. coli* 16S rRNA gene with (open circle) and without (closed circle) normalization by the recovered quantity of *P. fluorescens* 16S rRNA gene (triangle) for different initial cell quantities; b) the quantities of *E. coli* 16S rRNA with (open circle) and without (closed circle) normalization by the recovered quantity of *P. fluorescens* 16S rRNA (triangle) for different initial cell quantities; c) the determined 16S rRNA:16S rRNA gene ratio for *E. coli* with different initial cell quantities with and without normalization by dual internal references. All data points are averages of triplicate samples, and error bars are 1 standard deviation.

Fig. 3a and b clearly indicates that accounting for the recoveries of dual references (*P. fluorescens* 16S rRNA gene and 16S rRNA) substantially affects the amount of reported *E. coli* 16S rRNA gene and 16S rRNA. When recoveries of dual references are taken into account, one can calculate 16S rRNA:16S rRNA gene ratios for *E. coli* samples with different initial cell quantities (Fig. 3c). When normalization based on the recoveries of dual references (*P. fluorescens* 16S rRNA and 16S rRNA gene) was not applied, the average 16S rRNA:16S rRNA gene ratio was $1.6 \times 10^3 \pm 9 \times 10^2$ (n = 15). When the recoveries of the dual references were taken into account, the average *E. coli* 16S rRNA:16S rRNA gene ratio increased to $4.3 \times 10^3 \pm 1.1 \times 10^3$ (n = 15) by almost a factor of 3, and the difference was statistically significant ($p < 0.001$). More importantly, once normalization was applied, the *E. coli* 16S rRNA:16S rRNA gene ratio was no longer dependent on the initial cell quantity ($p = 0.76$).

3.4. Application of dual-internal-reference technique for E. coli liquid culture at different growth stages

The dual-internal-reference technique was applied to explore the temporal variation of 16S rRNA:16S rRNA gene ratio for *E. coli* growing in TSB at 37 °C. The bacteria grew rapidly during the exponential growth stage at approximately 2–6 h after inoculation, as measured by optical density of the liquid at 600 nm (Fig. 4a). The 16S rRNA:16S rRNA gene ratio (normalized to the recoveries of dual references) increased rapidly from 2.4×10^3 to 1.1×10^4 during the initial 3 h after inoculation, then decreased to ~5.0×10^3 during 3–6 h, and finally remained stable during 6–16 h after inoculation (Fig. 4b). The ratio at 3 h was significantly higher ($p < 0.05$) than the ratios at all other times except for the 4-hour sample ($p = 0.372$). The ratio of the 4-hour sample was higher than the rest of ratios ($p < 0.05$) except for the 5-hour sample ($p = 0.074$). However, the 16S rRNA:16S rRNA gene ratio calculated without normalization of DNA and RNA quantities to recoveries of dual references did not exhibit any distinct temporal trend, and the only statistical difference was found for the ratios determined at 7 h and 1 h ($p = 0.026$). The CVs for ratios at each time point with normalization were also lower compared to those without normalization ($p = 0.003$).

3.5. Application of dual-internal-reference technique for E. coli aerosol samples

Fig. 5 illustrates the application of the dual-internal-reference technique to investigate the potential change in 16S rRNA:16S rRNA gene ratio for non-growth activities of bacteria in response to environmental stress, e.g. stress to *E. coli* aerosols when collected on a filter and then subjected to particle-free air for prolonged periods of time. In Fig. 5, the ratio for *E. coli* collected on a filter and not subjected to additional stress (t = 0 h) was 4.0×10^3, nearly identical to the ratio of *E. coli* ($p = 0.772$) in liquid suspension (3.8×10^3). Interestingly, when exposure to air flow was extended to 2 h, the ratio increased slightly, but not significantly ($p = 0.305$) to 4.4×10^3. As the exposure to particle-free air continued for another 2 h (4 h total), this ratio reached ~6.5×10^3 and then leveled off for the remaining exposure time. The ratios for 4, 6 and 10 h were all significantly higher ($p < 0.05$) than those ratios at 0 and 2 h and the ratio of *E. coli* in liquid suspension. In contrast, if the ratios were determined without normalization with dual references, they would not show any significant or substantial temporal trend ($p > 0.05$). Likewise, the CVs for ratios determined with normalization of DNA and RNA quantities by recoveries of dual references were significantly lower compared to those without normalization ($p = 0.02$).

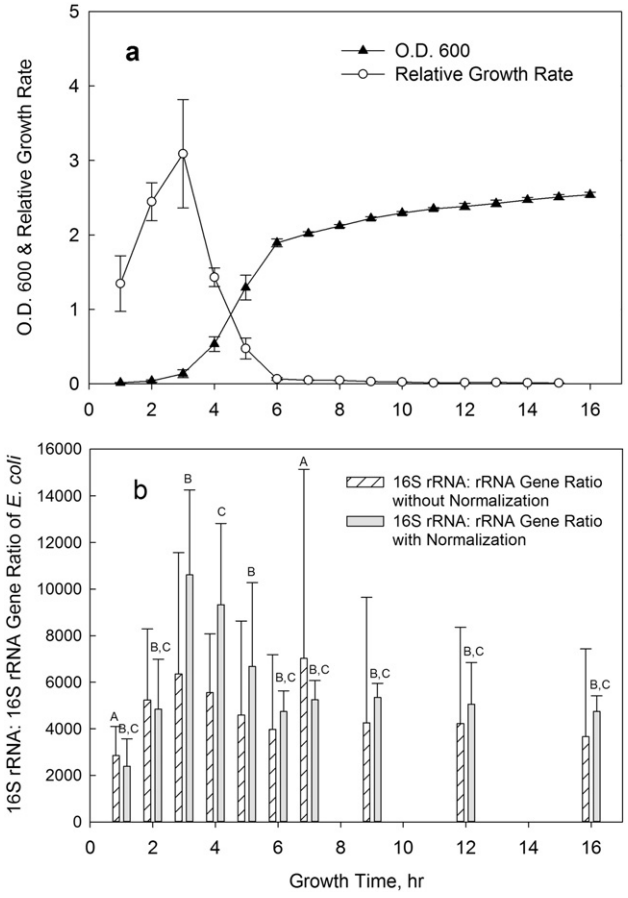

Fig. 4. a) Optical density of *E. coli* culture at 600 nm (O.D. 600) and its relative growth rates; b) temporal changes of 16S rRNA:16S rRNA gene ratio (with and without normalization by dual internal references) for *E. coli* growing in tryptic soy broth at 37 °C. All data points are averages of triplicate samples, and error bars are 1 standard deviation. Capitalized symbol pairs (A, B or C) on bars indicate statistical difference ($p < 0.05$) of 16S rRNA:16S rRNA gene ratios at different growth times.

4. Discussion

In this study, a dual-internal-reference technique was developed to improve the accuracy and reduce the experimental variability when determining 16S rRNA:16S rRNA gene ratio of bacterial samples by taking into account DNA and RNA losses due to sample processing and analysis procedures. Specifically, two exogenous references (*P. fluorescens* 16S rRNA gene and *P. fluorescens* 16S rRNA) were added into bacterial samples prior to their DNA/RNA co-extraction to act as surrogates for the target (*E. coli*) DNA and rRNA, respectively. The absolute quantities of recovered target DNA and rRNA were then normalized to the recoveries of respective internal reference, and then the 16S rRNA:16S rRNA gene ratio was calculated.

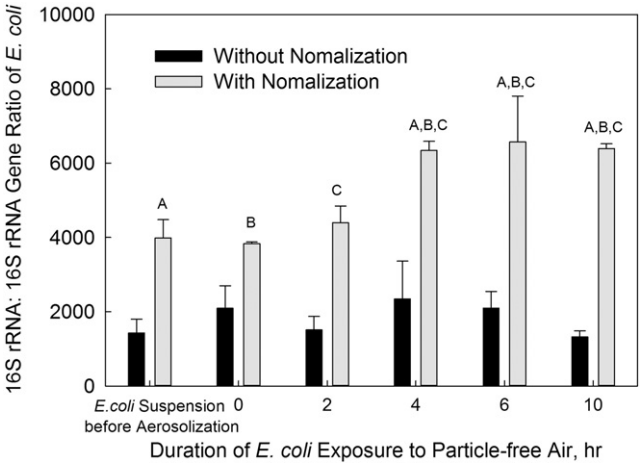

Fig. 5. Effect of exposure to particle-free air on the 16S rRNA:16S rRNA gene ratio for *E. coli* aerosols collected on filter. All data points are averages of triplicate samples, and error bars are 1 standard deviation. Capitalized symbol pairs (A, B or C) on bars indicate statistical difference ($p < 0.05$) of 16S rRNA:16S rRNA gene ratios of different samples.

Analysis of 16S rRNA:16S rRNA gene ratio using qPCR and RT-qPCR often involves multiple procedures in DNA and RNA co-extraction from a microbial sample, e.g. cell lysis, DNA/RNA isolation, and DNA removal and reverse transcription for RNA. In these steps, sample losses could result from incomplete cell lysis, incomplete volume transfer and phase separation, enzymatic and abiotic degradation of RNA, and inefficient reverse transcription of RNA (Johnson et al., 2005). Presumably, the exogenous references behave similarly to the target analyte due to their similar physical and chemical properties. Thus, the percentage loss of the target analyte in each step should be the same as the percentage loss of a corresponding reference. For example, in this study, a similar RT efficiency of RNA for both reference (*P. fluorescens* 16S rRNA) and target analyte (*E. coli* 16S rRNA) was verified (Fig. S3). One limitation of this technique is that the loss of target DNA and RNA due to incomplete cell lysis cannot be controlled by the dual references (Johnson et al., 2005). Thus, depending on a specific cell lysis method, e.g. mechanical disruption, enzyme digestion, freeze-thaw cycles, or a combination of these, the determined 16S rRNA:16S rRNA gene ratios from the same sample could display some variation. Nonetheless, the sample loss during extraction steps other than cell lysis should be corrected by this technique (see Fig. S4 in Supplementary Information).

In order to quantify two gene sequences (*E. coli* and *P. fluorescens* 16S rRNA gene), singleplex qPCR could be performed for each gene on two separate reaction plates. Alternatively, a multiplex qPCR targeting both gene sequences could be performed on a single plate to avoid the variability caused by inconsistent pipetting and amplification when using two plates, which should further improve the accuracy of results. However, it is critical to determine the optimal conditions for multiplex qPCR in order to avoid any potential amplification bias for each amplicon (Hatt et al., 2013). In our case, when *E. coli* 16S rRNA gene was present at ratios of 1000:1 or higher to the *P. fluorescens* 16S rRNA gene quantity, the *P. fluorescens* sequence showed distorted amplification and its C_T was delayed (higher) compared to a singleplex reaction. This could be due to limited availability of PCR reagents for *P. fluorescens* amplification when competing with more abundant *E. coli*. In contrast, when *P. fluorescens* 16S rRNA gene is at ratios of 1000:1 or higher to the *E. coli* 16S rRNA gene quantity, the C_T for the *E. coli* sequence decreased dramatically compared to the C_T obtained in a singleplex reaction. This was mainly caused by the cross-talk effect of HEX fluorophore (*P. fluorescens* probe) on FAM fluorophore (*E. coli* probe) (Data not shown). In addition, the effect of initial cell quantities on the accuracy of this technique was determined by mixing different amounts of cells with a fixed quantity of DNA and rRNA reference. The experiment allowed determining an optimal range of initial cell quantities where the determined 16S rRNA:16S rRNA gene ratios were independent of the initial cell quantity (Fig. 3): another important factor to consider for internal-reference-based analytical method.

Normalization of *E. coli* DNA and rRNA quantities to the recoveries of *P. fluorescens* DNA and rRNA (references), respectively, changed the 16S rRNA:16S rRNA gene ratios in two ways. First, the numerical value of 16S rRNA:16S rRNA gene ratio exhibited an approximately 2–3 fold increase with normalization. This increase was mainly due to lower recovery of the 16S rRNA reference (~20%) than that of DNA reference (~50%) during DNA/RNA co-extraction. Thus, the accuracy of the determined ratio improves when the discrepant recoveries between two analytes are taken into account. For instance, the 16S rRNA:16S rRNA gene ratio of *E. coli* grown in liquid culture at 37 °C for 16 h (stationary phase) was approximately 4.0×10^3 once normalization was applied.

Since seven copies of 16S rRNA gene are present per *E. coli* genome (Blattner et al., 1997), the 16S rRNA content of *E. coli* in the stationary phase was estimated to be approximately 3×10^4 copies/cell. This number is in a good agreement with the previously reported average number of ribosomes ($1–7 \times 10^4$) per *E. coli* cell (Bremer and Dennis, 1996). Second, the intra-sample variability, represented by the CV of triplicate samples, decreased significantly in all investigated *E. coli* samples from the liquid culture (Fig. 4) and aerosol samples (Fig. 5) once the data were normalized based on the recoveries of dual references, thus showing a positive effect of the method on precision of the results.

In our experiments, *P. fluorescens* 16S rRNA and rRNA gene were selected as dual references to investigate the 16S rRNA:16S rRNA gene ratios of pure *E. coli* samples. However, the applicability of dual references from *P. fluorescens* for environmental samples may be limited because *P. fluorescens* has been detected in samples from multiple different environments (Vanderkooij et al., 1982; Delille et al., 2007; Wong et al., 2011). Therefore, a new set of exogenous internal references will most likely be needed to investigate the 16S rRNA:16S rRNA gene ratios for specific bacterial taxa in complex microbial communities. For example, the firefly luciferase *luc* mRNA and DNA might be good candidates for dual internal references (Johnson et al., 2005; Hatt et al., 2013) because, 1) they are not expected in environmental microbial samples; 2) a primer–probe set which does not target any bacterial or archaeal genes can be designed; and 3) luciferase mRNA and a *luc* gene-containing DNA plasmid vector are commercially available. The use of luciferase mRNA to control for the loss of target RNA in the application of RT-qPCR has been reported in earlier studies (Perez-Osorio et al., 2010; Johnson et al., 2005; Wagner et al., 2009; Hatt et al., 2013). However, when applying luciferase mRNA and DNA with the dual-internal-reference technique, one has to consider the following factors: first, discrepancies may exist between the luciferase and target bacteria, e.g. *E. coli* in this study, including the DNA size (5 kbp for *luc* gene-containing plasmid v.s. 4 Mbp for *E. coli* genomic DNA) and RNA stability (mRNA v.s. rRNA), thus the use of *luc* mRNA and DNA plasmid as dual references should be evaluated in detail. Second, a multiplex qPCR assay should be carefully designed to ensure amplification of both target and reference genes with similar and acceptably high efficiencies. If there are conditions where multiplex qPCR fails to produce unbiased amplification, one could apply singleplex qPCR for the two amplicons separately.

When the dual-internal-reference technique was applied for liquid *E. coli* culture, the 16S rRNA:16S rRNA gene ratio of *E. coli* was found to peak at the same time when its relative growth rate reached maximum in a complex liquid medium, which confirmed the direct association between the rRNA content and bacterial growth as indicated by previous studies (Kerkhof and Ward, 1993; Dortch et al., 1983; Poulsen et al., 1993). In addition, the results from these studies, including ours, also strongly suggest that the cellular rRNA abundance and bacterial growth rate are not always simply correlated (Blazewicz et al., 2013).

The developed technique was also applied to investigate the potential change of *E. coli* 16S rRNA:16S rRNA gene ratio due to a non-growth activity, e.g. cellular activities in response to air sampling stress. It was found that the 16S rRNA:16S rRNA gene ratio of *E. coli* bioaerosols collected on a filter increased by more than 50% once exposed to particle-free air (relative humidity at 25-30%) for additional 4 h. This increase in 16S rRNA:16S rRNA gene ratio was apparently due to the production of 16S rRNA by *E. coli* when the cells remained on filters: we observed the increased quantities of 16S rRNA but no change in 16S rRNA gene quantities in 4-hour samples compared to 0-hour samples (data not shown). The production of rRNA for *E. coli* in reaction to the stress associated with air sampling, e.g. osmotic and desiccation stress, suggests the potential cellular activities during continuous exposure to desiccating conditions. In addition, the results also show that to analyze rRNA content and 16S rRNA sequences from airborne bacteria, one has to carefully evaluate the sampling protocols and their potential to bias the results.

No significant temporal trend in *E. coli* 16S rRNA:16S rRNA gene ratio was observed when the determined ratios were not normalized based on the recoveries of dual references. Under such conditions, the variation of the ratio between different samples was likely obscured by large intra-sample variation as represented by the CVs of individual samples. This intra-sample variation was greatly reduced with normalization of results to the recoveries of dual references. Thus, the dual-internal-reference technique could enhance our ability to detect the change in rRNA abundance of microbial samples.

5. Conclusions

A dual-internal-reference technique was successfully developed to examine the 16S rRNA:16S rRNA gene ratio of *E. coli* samples by using multiplex qPCR. The developed technique improved the accuracy of determined 16S rRNA:16S rRNA gene ratios and also substantially reduced intra-sample variability. Application of this technique for *E. coli* cells in liquid culture and *E. coli* aerosol demonstrated its ability to successfully identify the change in 16S rRNA:16S rRNA gene ratios in bacterial samples at different physiological states. It is hoped that this technique will facilitate investigations of the relationship between rRNA abundance and microbial activities.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mimet.2015.07.023.

References

Arunasri, K., Adil, M., Khan, P.A.A., Shivaji, S., 2014. Global gene expression analysis of long-term stationary phase effects in *E. coli* K12 MG1655. PloS One 9.

Bhagwat, A.A., Ying, Z.I., Karns, J., Smith, A., 2013. Determining RNA quality for NextGen sequencing: some exceptions to the gold standard rule of 23S to 16S rRNA ratio. Microbiol. Discov. 1, 10.

Blattner, F.R., Plunkett, G., Bloch, C.A., Perna, N.T., Burland, V., Riley, M., ColladoVides, J., Glasner, J.D., Rode, C.K., Mayhew, G.F., Gregor, J., Davis, N.W., Kirkpatrick, H.A., Goeden, M.A., Rose, D.J., Mau, B., Shao, Y., 1997. The complete genome sequence of *Escherichia coli* K-12. Science 277, 1453–1462.

Blazewicz, S.J., Barnard, R.L., Daly, R.A., Firestone, M.K., 2013. Evaluating rRNA as an indicator of microbial activity in environmental communities: limitations and uses. ISME J. 7, 2061–2068.

Bremer, H., Dennis, P.P., 1996. Modulation of Chemical Composition and Other Parameters of the Cell by Growth Rate. ASM Press, Washington D.C.

Campbell, B.J., Yu, L., Straza, T.R.A., Kirchman, D.L., 2009. Temporal changes in bacterial rRNA and rRNA genies in Delaware (USA) coastal waters. Aquat. Microb. Ecol. 57, 123–135.

Campbell, B.J., Yu, L.Y., Heidelberg, J.F., Kirchman, D.L., 2011. Activity of abundant and rare bacteria in a coastal ocean. Proc. Natl. Acad. Sci. U. S. A. 108, 12776–12781.

Cheng, W.C., Shu, W.Y., Li, C.Y., Tsai, M.L., Chang, C.W., Chen, C.R., Cheng, H.T., Wang, T.H., Hsu, I.C., 2012. Intra- and inter-individual variance of gene expression in clinical studies. PLoS One 7.

Delille, A., Quiles, F., Humbert, F., 2007. *In situ* monitoring of the nascent *Pseudomonas fluorescens* biofilm response to variations in the dissolved organic carbon level in low-nutrient water by attenuated total reflectance-fourier transform infrared spectroscopy. Appl. Environ. Microbiol. 73, 5782–5788.

Deutscher, M.P., 2003. Degradation of stable RNA in bacteria. J. Biol. Chem. 278, 45041–45044.

Dolezel, J., Bartos, J., Voglmayr, H., Greilhuber, J., 2003. Nuclear DNA content and genome size of trout and human. Cytometry A 51A, 127–128.

Dortch, Q., Roberts, T.L., Clayton, J.R., Ahmed, S.I., 1983. RNA/DNA ratios and DNA concentrations as indicators of growth-rate and biomass in planktonic marine organisms. Mar. Ecol. Prog. Ser. 13, 61–71.

Fey, A., Eichler, S., Flavier, S., Christen, R., Hofle, M.G., Guzman, C.A., 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.

Hatt, J.K., Ritalahti, K.M., Ogles, D.M., Lebron, C.A., Loffler, F.E., 2013. Design and application of an internal amplification control to improve *Dehalococcoides mccartyi* 16S rRNA gene enumeration by qPCR. Environ. Sci. Technol. 47, 11131–11138.

Hawkins, S., Robinson, K., Layton, A., Sayler, G., 2012. Molecular indicators of *Nitrobacter spp*. population and growth activity during an induced inhibition event in a bench scale nitrification reactor. Water Res. 46, 1793–1802.

Johnson, D.R., Lee, P.K.H., Holmes, V.F., Alvarez-Cohen, L., 2005. An internal reference technique for accurately quantifying specific mRNAs by real-time PCR with a application to the tceA reductive dehalogenase gene. Appl. Environ. Microbiol. 71, 3866–3871.

Kerkhof, L., Kemp, P., 1999. Small ribosomal RNA content in marine Proteobacteria during non-steady-state growth. FEMS Microbiol. Ecol. 30, 253–260.

Kerkhof, L., Ward, B.B., 1993. Comparison of nucleic-acid hybridization and fluorometry for measurement of the relationship between RNA/DNA ratio and growth-rate in a marine bacterium. Appl. Environ. Microbiol. 59, 1303–1309.

Kim, J.S., Wang, N., 2009. Characterization of copy numbers of 16S rDNA and 16S rRNA of *Candidatus Liberibacter* asiaticus and the implication in detection *in planta* using quantitative PCR. BMC Res. Notes 2, 37.

Krumins, V., Mainelis, G., Kerkhof, L.J., Fennell, D.E., 2014. Substrate-dependent rRNA production in an airborne bacterium. Environ. Sci. Technol. Lett. 1, 376–381.

Luciano, P., Bertea, C.M., Temporale, G., Maffei, M.E., 2007. DNA internal standard for the quantitative determination of hallucinogenic plants in plant mixtures. Forensic Sci. Int. Genet. 1, 262–266.

McMaugh, S.J., Lyon, B.R., 2003. Real-time quantitative RT-PCR assay of gene expression in plant roots during fungal pathogenesis. Biotechniques 34, 982–986.

Mutter, G.L., Boyton, K.A., 1995. PCR bias in amplification of androgen receptor alleles, a trinucleotide repeat marker used in clonality studies. Nucleic Acids Res. 23, 1411–1418.

Muttray, A.F., Yu, Z.T., Mohn, W.W., 2001. Population dynamics and metabolic activity of *Pseudomonas abietaniphila* BKME-9 within pulp mill wastewater microbial communities assayed by competitive PCR and RT-PCR. FEMS Microbiol. Ecol. 38, 21–31.

Oberg, A.L., Bot, B.M., Grill, D.E., Poland, G.A., Therneau, T.M., 2012. Technical and biological variance structure in mRNA-Seq data: life in the real world. BMC Genomics 13, 2–14.

Perez-Osorio, A.C., Williamson, K.S., Franklin, M.J., 2010. Heterogeneous *rpoS* and *rhlR* mRNA Levels and 16S rRNA/rDNA (rRNA gene) ratios within *Pseudomonas aeruginosa* biofilms, sampled by laser capture microdissection. J. Bacteriol. 192, 2991–3000.

Pitkanen, T., Ryu, H., Elk, M., Hokajarvi, A.M., Siponen, S., Vepsalainen, A., Rasanen, P., Domingo, J.W.S., 2013. Detection of fecal bacteria and source tracking identifiers in environmental waters using rRNA-based RT-qPCR and rDNA-based qPCR assays. Environ. Sci. Technol. 47, 13611–13620.

Poulsen, L.K., ballard, G., Stahl, D.A., 1993. Use of rRNA fluorescence in situ hybridization for measuring the activity of single cells in young and established biofilms. Appl. Environ. Microbiol. 59, 1354–1360.

Redondo-Nieto, M., Barret, M., Morrisey, J.P., Germaine, K., Martinez-Granero, F., Barahona, E., Navazo, A., Sanchez-Contreras, M., Moynihan, J.A., Giddens, S.R., Coppoolse, E.R., Muriel, C., Stiekema, W.J., Rainey, P.B., Dowling, D., O'Gara, F., Martin, M., Rivilla, R., 2012. Genome sequence of the biocontrol strain *Pseudomonas fluorescens* F113. J. Bacteriol. 194, 1273–1274.

Sen, K., Schable, N.A., Lye, D.J., 2007. Development of an internal control for evaluation and standardization of a quantitative PCR assay for detection of *Helicobacter pylori* in drinking water. Appl. Environ. Microbiol. 73, 7380–7387.

Smith, R.D., Brown, B., Ikonomi, P., Schechter, A.N., 2003. Exogenous reference RNA for normalization of real-time quantitative PCR. Biotechniques 34, 88–91.

Takle, G.W., Toth, I.K., Brurberg, M.B., 2007. Evaluation of reference genes for real-time RT-PCR expression studies in the plant pathogen *Pectobacterium atrosepticum*. BMC Plant Biol. 7.

Vanderkooij, D., Visser, A., Oranje, J.P., 1982. Multiplication of *Fluorescent Pseudomonads* at low substrate concentrations in tap water. Anton. Leeuw. J. Microbiol. 48, 229–243.

Wagner, A., Adrian, L., Kleinsteuber, S., Andreesen, J.R., Lechner, U., 2009. Transcription analysis of genes encoding homologues of reductive dehalogenases in "*Dehalococcoides*" sp strain CBDB1 by using terminal restriction fragment length polymorphism and quantitative PCR. Appl. Environ. Microbiol. 75, 1876–1884.

Whitehead, A., Crawford, D.L., 2006. Variation within and among species in gene expression: raw material for evolution. Mol. Ecol. 15, 1197–1211.

Woese, C.R., 1987. Bacterial evolution. Microbiol. Rev. 51, 221–271.

Wong, V., Levi, K., Baddal, B., Turton, J., Boswell, T.C., 2011. Spread of *Pseudomonas fluorescens* due to contaminated drinking water in a bone marrow transplant unit. J. Clin. Microbiol. 49, 2093–2096.

Zhen, H.J., Han, T.W., Fennell, D.E., Mainelis, G., 2014. A systematic comparison of four bioaerosol generators: affect on culturability and cell membrane integrity when aerosolizing *Escherichia coli* bacteria. J. Aerosol Sci. 70, 67–79.