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Localized gene expression in *Pseudomonas aeruginosa* Biofilms

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Abstract

Gene expression in biofilms is dependent on bacterial responses to the local environmental conditions. Most techniques for studying bacterial gene expression in biofilms characterize average values over the entire population. Here, we describe the use of laser capture microdissection microscopy (LCMM) combined with multiplex quantitative real time reverse transcriptase polymerase chain reaction (qRT-PCR) to isolate and quantify RNA transcripts from small groups of cells at spatially resolved sites within biofilms. The approach was first tested and analytical parameters determined for *Pseudomonas aeruginosa* containing an IPTG-inducible gene for the green fluorescent protein (*gfp*). The results show that amounts of *gfp* mRNA were greatest in the top zones of the biofilms, and that *gfp* mRNA levels correlated with the zone of active GFP-fluorescence. The method was then used to quantify transcripts from wild-type *P. aeruginosa* biofilms for a housekeeping gene, *acpP*; the 16S rRNA; and two genes regulated by quorum-sensing, *phzA1* and *aprA*. The results demonstrated that the amount of *acpP* mRNA was greatest in the top 30 μm of the biofilm, with little or no mRNA for this gene at the base of the biofilms. In contrast, 16S rRNA amounts were relatively uniform throughout biofilm strata. Using this strategy, the RNA amounts of individual genes are determined, and therefore results are dependent on both gene expression and the half-life of transcripts. Therefore, the uniform amount of rRNA throughout the biofilms is likely due to the stability of the rRNA within ribosomes. Levels of *aprA* mRNA showed stratification, with the greatest amounts in the upper 30 μm zone of these biofilms. The results demonstrate that mRNA levels for individual genes are not uniformly distributed throughout biofilms, but may vary by orders of magnitude over small distances. The LCMM/qRT-PCR technique can be used to resolve and quantify this RNA variability at high spatial resolution.

Introduction

Bacteria thrive in spatially defined microenvironments, and therefore are exposed to local environmental conditions that may vary at the micrometer scale (53). When growing on surfaces in biofilms, bacteria are typically distributed in a heterogeneous manner. The cell distribution reflects heterogeneities in the properties of the surface and the concentration gradients of chemicals dissolved in the interstitial fluid within the biofilm matrix (22, 27, 38, 54). These physical and chemical heterogeneities in the biofilms promote differences in bacterial enzymatic activities at different regions (25, 61, 64) and influence gene expression in different zones within biofilms (4, 9, 13, 14, 52, 55).

Most studies of gene expression by bacteria in biofilms report levels that reflect the average gene expression over the entire population (2). Techniques are often insensitive to the diverse range of activities that occur among cells at different locations within the biofilm, and therefore do not account for the contribution of specific parameters, such as the role of changing environmental conditions on the regulation of gene expression (2). However, due to the importance of understanding bacterial activities *in situ*, several approaches have been developed to evaluate local gene expression within biofilms. One approach uses fluorescent reporter genes fused to promoter regions of interest and epifluorescence microscopy (14, 33, 55, 65). Although useful, this technique requires prior genetic manipulation of the bacteria and reintroduction of the constructed strains back into the environment. Expression of the reporter gene may induce physiological changes in the cells of the constructed strains that do not occur in the wild-type cell. Thus, this approach precludes the ability to study gene expression in the wild-type strain in its native state. Fluorescent *in situ* hybridization (FISH) has been used to detect specific RNA sequences in bacterial cells in their native assemblages (1, 15, 32). This approach is most useful for studying high abundance RNAs, such as rRNAs, but less useful for detecting mRNA transcripts in low abundance. Measurements of cellular activity may be obtained by combining FISH with microautoradiography or Raman spectroscopy following uptake of labeled substrates into cellular material (26, 56). To measure gene expression of cells in biofilms, *in situ* reverse transcription polymerase chain reaction (IS-RT-PCR) has been developed to amplify mRNA present in low copy number (29). The approach uses fluorescently labeled probes to detect individual cells expressing a gene of interest, but is limited by lack of mRNA quantification.

Here, we developed a strategy based on advances in laser capture microdissection microscopy (LCMM) (7, 16-18, 39, 48, 49) and multiplex quantitative real time reverse transcriptase PCR (qRT-PCR) (10, 23, 24, 34) for quantitative assessment of any gene expressed by bacteria in their native environment. The approach allows quantification of low abundance mRNA transcripts without the need to genetically manipulate the cells or expose cells to labeled substrates. We used this strategy to study the vertical distribution of RNA transcripts in *Pseudomonas aeruginosa* biofilms. In particular, we characterized the distribution of housekeeping gene, *acpP*, the 16S rRNA, and two genes regulated by quorum sensing, *phzA1* and *aprA*. The results demonstrate that RNA abundances for individual genes are not uniformly distributed throughout biofilms, and that cells in relatively close proximity to each other may have vast differences in abundances of individual RNA transcripts.

Materials and Methods:

Bacterial Strains and Growth Conditions. *P. aeruginosa* PAO1 and *P. aeruginosa* PAO1 (pAB1) were used for these studies. Plasmid pAB1 allows IPTG-induction of the green fluorescent protein (GFP) (3). Inocula of *P. aeruginosa* containing pAB1 were supplemented with 150 µg/ml of carbenicillin. Two biofilm growth conditions were used: colony biofilms and biofilms cultivation in drip-flow reactors (61, 64). For colony biofilms, planktonic cultures of *P. aeruginosa* were incubated overnight at 37^0C in Luria-Bertani broth (LB) (6). Cultures were diluted in sterile LB to an optical density of 0.4 (600 nm, 1-cm path length) and 25 µl was used to inoculate sterilized black, polycarbonate membrane filters (13 mm diameter, 0.2 µm pore size; GE Water & Process Technologies). The filters were placed on LB agar (Difco Laboratories) and incubated for a total of 52 h at 37^0C. The membranes containing the colonies were transferred to fresh LB agar every 12 h. For IPTG induction of the GFP in strains containing pAB1, membranes were transferred to LB agar containing 1.0 mM IPTG, and incubated for an additional 4 h.

Biofilms were also cultivated on stainless steel coupons in continuous flow by using drip flow reactors (64). The reactor consisted of a once flow-through system containing a medium reservoir, pump, silicon tubing, drip flow chamber, and a waste container. Biofilm minimal medium (BMM) (42) contained (per liter): 9.0 mM sodium glutamate, 50 mM glycerol, 0.02 mM $MgSO_4$, 0.15 mM NaH_2PO_4, 0.34 mM K_2HPO_4, 145 mM NaCl, 20 µl trace metals, and 1 ml

vitamin solution. Trace metal solution contained (per liter of 0.83 M HCl); 5.0 g $CuSO_4.5H_2O$, 5.0 g $ZnSO_4.7H_2O$, 5.0 g $FeSO_4.7H_2O$, 2.0 g $MnCl_2.4H_2O$. Vitamins solution contained (per liter): 0.5 g thiamine, 1 mg biotin. The pH of the medium was adjusted to 7.0. Prior to inoculation into the system, each strain was incubated in BMM for 18 h at 37°C. The cultures were transferred to fresh BMM for 4 h to reach an $O.D._{600}$ of 0.2. The inoculum was diluted 20-fold in 0.85% NaCl, and 5 ml was used to inoculate steel coupons for 25 min. BMM was then pumped through the reactors at 1.2 ml/min for 72 h. Reactors were maintained at 37°C throughout the incubation. For biofilms where induction of the GFP was necessary, BMM was supplemented with 1.0 mM IPTG for the final 4 h of incubation.

Cryoprocessing of biofilms. Following incubation, biofilms were cryoembedded by flash freezing the stainless steel slides containing drip-flow cultivated or colony biofilms on dry ice. Biofilms were immersed in Tissue-Tek O.C.T. compound (Sakura Finetechnical Co.). Vertical transects of the biofilms were obtained by sectioning the solidified O.C.T. containing biofilms with a cryomicrotome. Thin sections (5 μm) vertical transects of the biofilms were placed onto membrane-coated microscope slides (P.A.L.M. microlaser technologies). The microscope slides were maintained on dry ice until examination and sampling.

Laser capture microdissection microscopy (LCMM). LCMM (Zeiss/P.A.L.M. Laser-MicroBeam System) was used to dissect and capture sections from different regions within the biofilms. Microscope slides containing the O.C.T.-embedded biofilms were thawed on the microscope stage for 5 sec. The biofilms were then examined using 10x to 40x objective lenses. Areas of the biofilms ranging from 500 to 100,000 μm^2 were obtained using the laser catapult parameter (LCP), allowing dissected samples to be catapulted into 40 μl of lysis buffer (0.3 M sucrose and 0.02 M sodium acetate at pH 4.5) contained in the caps of 0.5 ml microcentrifuge tubes.

RNA extraction. RNA extraction followed the phenol-chloroform-based protocol of Chomczynski and Sacchi (11) as modified by Barton et al. (5). The samples, obtained by laser catapulting in 40 μl lysis buffer, were mixed by inversion with additional buffer (160 μl of lysis buffer and 200 μl of 2% sodium dodecyl sulfate). An aliquot of luciferase (*lucI*) RNA ($1.7x10^4$ transcript copies) (Promega) was added as a spike-in control to assay for RNA loss during sample preparation. Samples were centrifuged for 10 sec, then transferred to a 1.5 ml microcentrifuge tube containing water-saturated phenol (400 μl), then heated in a water bath at

65^0C for 5 min with frequent vortexing. The aqueous layer, formed after 25 min of centrifugation was extracted with an equal volume of phenol:chloroform:isoamyl-alcohol (25:24:1) in a heavy Phase-Lock gel tube (Eppendorf). RNA was precipitated by adding 3 µl of PolyAcryl Carrier (Molecular Research Center, Inc.), 32 µl of 0.25 µM sodium acetate and 1200 µl of absolute ethanol. Resuspended RNA was treated with DNase using the Turbo DNA-free kit (Ambion, Inc.) in 10 µl reactions for 20 min. RNA was diluted to a final volume of 25 µl to reduce interference from possible PCR inhibitors. Total RNA was quantified using a NanoDrop fluorescence spectrophotometer (NanoDrop Technologies, Inc) with Molecular Probes Ribogreen (Invitrogen). All samples were stored at -80^0C until analysis by qRT-PCR.

Primers and dual-labeled probes. PCR primer and dual-labeled probe sequences are shown in Table 1. The primers were designed using the Primer3 Program (41) or with Primer Express software v 2.0 (Applied Biosystems)**.** Primers were assayed for melting temperature, primer dimers, and secondary structure using Mfold (30, 67) and Oligo Analyzer version 3.0 (Integrated DNA Technologies, Inc.). Primers were purchased from Integrated DNA Technologies.

In vitro transcription. RNA standards were generated by in vitro transcription (IVT) (19). The gene of interest was amplified from *P. aeruginosa* PAO1 genomic DNA by using gene specific primers containing a T7 promoter (Table 1). The PCR products were purified and used for IVT with the MEGAscript T7 kit (Ambion); which included a DNase treatment (TURBO DNase for 15 min at 37^0C). IVT RNA products were purified using the RNeasy mini kit (QIAGEN), and analyzed by electrophoresis on a RNA Nanochip using the Bioanalyzer 2100 (Agilent Technologies). RNA standards were quantified using UV absorption at 260 nm, aliquoted, and stored at -80^0C.

The copy number of RNA standards was calculated using the following formula: molecules/µL = (Xg/µL RNA/M.W.)*Avogadro's constant. Copy numbers per reaction were assigned to each standard using Rotor Gene software version 6.0 (Corbett Research). For each standard, the concentration was plotted against the cycle number at which the fluorescence exceeded the background (C_T- threshold cycle). The slope of the calibration curve was used to determine the reaction efficiency (E) of the C_T according to the equation $E = 10^{[-1/\text{slope}]} - 1$, where an E of 1 indicates an exponential amplification of the product. The efficiency of serially diluted

samples was evaluated to determine assay sensitivity and to ensure assay compatibility with different probes in the multiplex reactions.

Quantitative real time reverse transcriptase PCR (qRT-PCR). One step qRT-PCR was used for all mRNA quantifications. qRT-PCR reactions (25 µl) containing 2-3 µl of template RNA were used according to the manufacturer's instructions. Three kits were used depending on the type of reaction. QuantiTect Probe RT-PCR and QuantiTect Multiplex RT-PCR NR kits (Qiagen) were used for dual-labeled probe analysis and multiplex dual-labeled probe analysis. QuantiTect SYBR Green RT-PCR kit was used for SYBR green labeling analysis.

Primer and probe concentrations were determined by performing the optimization protocol recommended by the Rotor Gene system (Real time summary, Version 1.7). Every assay contained negative controls (samples lacking reverse transcriptase or template), and positive controls (samples with appropriate standards added). Standard RNA, derived from IVT, and the external spike-in control RNA, *lucI*, were serially diluted in 8 µL/mL of PolyAcryl Carrier (Molecular Research Center, Inc.) to accommodate 10^1 - 10^6 molecules per reaction. Cycling parameters were established according to kit instructions for single probe assays or for multiplex assays (Corbett Research). Three replicates were used to generate standard curves. Each sample was assayed in duplicate. Primer specificity (single PCR product) was confirmed by electrophoresis using a Bioanalyzer 2100 and a DNA 500 chip (Agilent Technologies).

Transcript amounts for *acpP, gfp, aprA, phzA1* and *lucI* were calculated from calibration curves, with a normalization factor of $x/1.7x10^4$, since $1.7x10^4$ *lucI* RNA transcripts were added to each sample immediately after laser capture as a spike-in control. To quantify relative amounts of 16S rRNA, C_T values were log transformed (LT) using the following equation; LT = $(\text{amplification}^{-C_t})*10^{10}$, where C_T and amplification values were derived from the Rotor Gene software comparative quantitation analysis (40).

Validation of qRT-PCR efficiency, linearity and reproducibility. qRT-PCR efficiencies were calculated from the slope of each calibration curve run in multiplex reactions. A qRT-PCR efficiency (E) of 1 indicates an exponential amplification of the product. We observed high efficiency for *acpP* (0.91), *gfp* (0.93) and *lucI* (1.02) when run in multiplex reactions using dual-labeled probes. Standard curves for these transcripts exhibited linear responses from 50 to 500,000 transcripts ($r^2 > 0.98$ for each). The efficiencies for multiplex

reactions were: *aprA* (1.04)*, phzA1* (1.04) and *acpP* (0.86), *lucI* (0.94). These transcripts exhibited linear responses from approximately 300 to 30,000 transcript copies ($r^2 > 0.96$ for each).

SYBR Green based qRT-PCR was used to measure 16S rRNA. This assay had an efficiency of 0.95 and was linear from 10 and 100,000 transcripts ($r^2 > 0.99$). Inter-experimental precision was approximately 16%, and intra-sample precision was 11%. These calculations were based on calculated copies per reaction and their variation from the mean. We chose to obtain the reproducible measure from calculated copies per reaction and not from C_T values to avoid underestimating the true variability (28).

Statistical analysis. Since data sets obtained from qRT-PCR do not have uniform distributions, difference between means were calculated using the two-tailed Mann-Whitney test.

Results:

GFP fluorescence correlates with *gfp* mRNA amounts. To quantify RNA levels from small groups of cells from within a larger surrounding biofilm population, we first developed a system where mRNA transcript numbers could be determined and compared to a spatially defined phenotypic trait. For this we used a strain of *P. aeruginosa* with an IPTG-inducible gene for the green fluorescent protein (*gfp*). GFP fluorescence was assayed by microscopy and *gfp* mRNA abundance was assayed by LCMM/qRT-PCR. When biofilms of this strain are incubated for 52 h in the absence of IPTG, then induced with IPTG for the final 4 h of incubation, a band of green fluorescence is observed at the top 30 μm zone of the biofilm (Fig. 1A,B) (8, 61). This fluorescent band is observed whether the IPTG is applied from the bottom of the biofilm in the colony biofilm format (Fig 1B), or from the liquid medium in drip flow-cultivated biofilms (Fig 1A). The LCMM was used to dissect and capture cells from the top fluorescent layer in the IPTG-induced biofilms, and from the equivalent top zone of control biofilms not amended with IPTG. The captured dissects ranged from 500 to 48,000 μm^2 (Fig. 1B**)**. Figure 1C shows the *gfp* mRNA copy abundances for 26 samples after normalizing to 60,000 um^3 biofilm volume. On average, in IPTG-induced biofilms, the copy number of *gfp* mRNA was 250-fold greater than from the biofilm samples not induced with IPTG ($P < 0.001$), where no fluorescent zone was observed. Of the 12 samples taken from biofilms where no IPTG was added, nine had *gfp* mRNA levels below the detection limit, indicating little or no expression of this gene in the

absence of inducing agent. The other three samples had transcript levels above the detection limits, but approximately 100-fold less than the IPTG-induced biofilms. Of the 26 samples from the IPTG-induced biofilms, one sample showed no *gfp* mRNA transcripts. The lack of *gfp* mRNA in this sample was not due to sampling error or mRNA degradation, since the *lucI* spike-in control was not anomalous and since the sample contained 16S rRNA (described below).

Stratified *gfp* expression in IPTG-induced biofilms. Since GFP requires oxygen for activity, there is the possibility that it is expressed throughout the biofilm, but only shows fluorescence in the oxygen-exposed top layer. To determine if *gfp* expression, as opposed to GFP activity, is greatest at the top of the biofilms, LCMM was used to sample the non-fluorescent zones of the IPTG-induced biofilms. The results show little *gfp* mRNA amounts in the underlying portions of the biofilm compared to the top 30 μm fluorescent zone ($P < 0.001$ top vs. middle or bottom layers) (Fig. 1C). No *gfp* expression was observed for any of the samples in the middle zone, and six samples in the bottom zone had no *gfp* expression. Some of the samples in the bottom zones had low amounts of *gfp* expression, that were approximately 1000-fold less than in the top fluorescent zone. Therefore, in this system, the deeper biofilm zones are not fluorescent due to low levels of *gfp* expression, rather than to lack of GFP activation with oxygen. These results demonstrate that it is possible to use LCMM and qRT-PCR to quantify RNA amounts of a regulated gene that is not homogenously distributed throughout a stratified biofilm.

Localized expression of the housekeeping gene *acpP*. By using multiplex qRT-PCR, it is possible to assay RNA amounts of several genes simultaneously. In addition to *gfp* and the *lucI* spike-in control, we chose to assay the gene for the acyl carrier protein, *acpP*, as an internal housekeeping gene control. AcpP is required for fatty acid biosynthesis, and therefore required for production of new cell membrane material. Interestingly, *acpP* mRNA amounts are not uniform throughout these biofilms. In the colony biofilms (Fig 2A), *acpP* mRNA abundances reflected *gfp* amounts, with the greatest amounts in the top 30 μm zone of the biofilm ($P < 0.001$ top vs. middle or bottom layers). None of the samples isolated from the middle of the colony biofilms had *acpP* mRNA amounts that were above the detection limit by qRT-PCR. Similarly, most samples from the bottom of the biofilm had *acpP* mRNA that were below dection. Several of the samples from the bottom of the biofilm had *acpP* above detection, but each of these was approximately 100-fold less than the average amounts in the top 30 μm of the biofilm. The

results indicate that in these biofilms, which averaged 200 μm thick, the top 30 μm contain cells with high abundance of *acpP* mRNA, suggesting that these cells are likely synthesizing new membrane material, whereas the deeper portions of the biofilms had much less expression of this gene.

The drip-flow cultivated biofilms also showed greatest *acpP* abundance in the top 30 μm zone of the biofilm. However, these biofilms had greater variability in mRNA amounts than the colony biofilms (Fig 2B). Most samples from the bottom of the drip-flow biofilm had *acpP* mRNA levels that were below detection (14 of 18 samples), and the average abundance of *acpP* was 100-fold less at the base of the biofilm compared to the top zone. The middle of the biofilm showed a large range of variability in *acpP* mRNA amounts, with an average that was approximately tenfold less than at the top of the biofilm. The drip flow biofilms have more structural features than the colony biofilms (as shown in Fig1A,B), with increased amounts of water channels and mushroom-like features. The increased variability observed in Fig 2B for the middle of the biofilms likely reflects the proximaty of the dissected sample to adjacent water channels, which affects nutrient and oxygen availability. The results for *acpP* for both the colony biofilms and for the drip-flow biofilms indicate that there is signficant variability in expression of this housekeeping gene, that is dependent on location of the cells within a biofilm. The results also demonstrate that *acpP* may not provide a good internal housekeeping gene control for normalizing the activity of other genes, since its expression is not uniform throughout these biofilms.

Uniform amounts of 16S rRNA throughout biofilms. In contrast to the heterogeneous pattern observed for *acpP* and *gfp*, the 16S rRNA amounts were relatively constant in the different biofilm layers (Fig 3). No statistically significant difference was observed for the 16S rRNA gene product at the top versus the middle or bottom of the biofilms, cultivated in either the colony biofilm or the drip-flow biofilm format ($P > 0.1$ for each analysis). In addition, the average amounts of 16S rRNA was fairly consistent among all of the biofilms and biofilm zones. Although the expression of rRNA varies with cell growth (35, 43), the homogenous distribution of 16S rRNA is likely due to the higher stability of rRNA contained within ribosomes. The abundance of individual RNAs measured here by qRT-PCR is dependent on both expression and half-life of the transcript. Whereas the half-life of the *acpP* and *gfp* mRNAs is short, the rRNA contained within ribosomes is more stable. These results are consistent with visual observations

of the 16S rRNA using FISH probing, which showed relatively constant amounts of 16S rRNA throughout a biofilm (63). The results also suggest that the 16S rRNA may be used as an internal control for comparison of induction of genes that are differentially regulated through the biofilms. However, rRNA must be used with caution as an internal control, since the abundance of rRNA is often much higher than of any mRNA species.

Stratified expression of quorum sensing-regulated genes. Bacterial cells within biofilms communicate through small diffusible chemical signals by quorum sensing (QS) (20, 21, 36, 37). We assayed the localized expression of two genes, *aprA* (encoding alkaline protease) and *phzA1* (necessary for pyocyanin biosynthesis) known to be regulated by QS (46, 57), and that have been shown by proteomics to be upregulated in *P. aeruginosa* biofilms (42). *P. aeruginosa* PAO1 biofilms, cultivated in drip flow reactors were cryoembedded and thin-sectioned for LCMM, then assayed for *aprA* and *phzA1* expression along vertical transects of the biofilms. Amounts of *aprA* mRNA showed a similar trend to that observed for *gfp* and *acpP*, with the greatest expression in the outermost 30 μm portion of the biofilm($P < 0.001$) (Fig. 4A). Expression of *aprA* averaged 1000-fold higher than in the bottom of the biofilms, where most samples had expression that was below detection limit. The middle of the biofilms had intermediate *aprA* expression, that averaged approximately 100-fold less than the top of the biofilms. In contrast, *phzA1* amounts, although greater at the top of the biofilm, were more uniformly distributed at the different levels ($P > 0.1$) (Fig. 4b), possibly due to the lower overall expression of *phzA1* compared to *aprA*. The 16S rRNA gene product was used as an internal control and showed uniform amounts throughout these biofilms ($P > 0.5$). The differences observed between the expression patterns of *aprA* and *phzA1* demonstrate that factors other than QS, such as the physiological status of the cells , may also influence expression of genes that are regulated by QS.

Limits of detection for localized mRNA and rRNA amounts in biofilms. To determine the range of detection limits for LCMM/qRT-PCR, biofilm samples were dissected from the top zone of IPTG-induced colony biofilms. The amounts of *gfp*, *acpP*, and 16S rRNA were quantified and plotted against sample volume. Since samples were taken from cryoembedded biofilms, viable cell counts could not be determined. However, assuming a cell volume of approximately 1 μm^3 (51), a 500 μm^2 area of a 5 μm thick section contains approximately 2,500 cells. For *acpP* and *gfp*, a linear relationship was observed between the

sample volume and the number of mRNA transcripts, ranging from $1x10^3$ to $1x10^5$ μm^3 (r^2 = 0.94 for *acpP*, r^2 = 0.90 for *gfp*) (Fig. 5A,B). Therefore, this technique is useful for quantifying mRNA transcripts from approximately 2,000 cells. Similarly, log transformed C_T values of 16S rRNA also correlated with sample size (r^2 = 0.90) (Fig. 5C). Due to the higher cellular amounts of 16S rRNA, the detection limit for rRNA is much less than 1,000 cells.

The detection limits of qRT-PCR for *gfp*, *acpP*, and 16S rRNA were also determined for exponentially growing planktonic cells, allowing comparison of the mRNA transcripts with viable cell counts. RNA was extracted from serially diluted planktonic *P. aeruginosa* (pAB1) cultures containing from 1 to 10^9 cells, determined as colony forming units (CFU) (Fig. 5A,B,C). Linear responses were observed for cell numbers and mRNA transcripts, *gfp* (r^2 = 0.97) and *acpP* (r^2 = 0.99). A linear response was also observed for the 16S rRNA (r^2 = 0.99). According to the regression analysis, there are approximately 40 *gfp* and 900 *acpP* mRNA transcripts per cell growing in planktonic culture.

These results allowed comparison of the gene expression levels of exponentially growing planktonic cells and of cells growing in the top zone of the IPTG-induced colony biofilms (Fig. 5). Planktonic cells had mRNA levels of *gfp* and *acpP* per cell that were higher than the expression in the equivalent volume of the biofilm. RNA amounts were approximately 40-fold, 250-fold, and 33-fold greater in planktonic cells for *gfp*, *acpP* and 16S rRNA, respectively, than in the top biofilm zone. Therefore, although mRNA amounts are highest at the top zone of these biofilms, the amounts in biofilms are less than for exponentially growing planktonic cultures.

Discussion:

The goals of this research were twofold; (i) to develop methodology to quantify bacterial gene expression from small groups of bacterial cells isolated from biofilms, without prior genetic manipulation of the cells, and (ii) to use the method to determine if local conditions found within biofilms result in spatial variation of bacterial gene expression. By using cryosectioning of biofilms and LCMM, we were able to isolate and extract RNA from small groups of bacterial cells isolated from different regions within biofilms. Multiplex qRT-PCR allowed quantification of gene expression of up to four genes simultaneously from these small groups of cells.

The approach was validated using an artificially induced tracer gene (*gfp*), a housekeeping gene (*acpP*), an RNA spike-in control (*lucI*), an internal control (16S rRNA), and two genes regulated by quorum sensing. For *gfp* and *acpP*, the qRT-PCR approach using dual-labeled probes was sensitive for detecting less than 1,000 mRNA transcripts. For exponentially growing planktonic cells, where transcripts ranged from 100 to 400 copies per cell (Fig 5), the qRT-PCR approach may be useful for estimating mRNA abundances from less than 10 cells. At the oxygen interface of these biofilms, we estimate that the number of transcripts for *gfp* and *acpP* is approximately 40 to 250-fold less per cell than for exponentially growing planktonic cells (Fig 5). We measured expression of these genes for approximately 2,000 biofilm-associated cells. However, based on the detection limits obtained for planktonic cultures, it should be possible to investigate gene expression from even smaller numbers of biofilm cells by using this approach.

The GFP-fluorescent zone of the biofilm correlated with *gfp* mRNA amounts (Fig. 1). GFP amounts were significantly greater when comparing IPTG-induced biofilm cultures with non-induced samples, and when comparing the GFP-fluorescent zone with the non-fluorescent deeper areas of the biofilms. Variation in *gfp* mRNA at different regions of the biofilms was expected given the localized nature of the fluorescent zone. Interestingly, a similar active zone of *acpP* mRNA amounts was also observed (Fig. 2). Housekeeping genes, such as *acpP*, often serve as internal controls in qRT-PCR experiments to normalize expression of regulated genes (45). In biofilms, gradients of pH, oxygen, nutrients, and waste products are established at different depths in biofilms (53, 54). These variable environmental factors contribute to varying metabolic activity of the bacteria. Since the AcpP analyzed in this study is required for the synthesis of fatty acids and new membrane material, the results suggest that the cells in the upper 30 μm of these biofilms are likely the most active in membrane synthesis. The cells in the deeper portions of the biofilm are likely in slow-growth state. These results are consistent with other studies that indicate that *P. aeruginosa* cells in deeper portions of the biofilm are less metabolically active (60). In the prior study, transmission electron microscopy was used to demonstrate that *P. aeruginosa* cells located in the top layer of a biofilm, at the air/biofilm interface, are more susceptible to antibiotic killing than those in the deeper portions (60), even though the antibiotics penetrate through biofilms (3, 60, 66). The prior study suggested that the cells in the deeper portions of the biofilm are more tolerant to the antibiotics, due primarily to

their slow growth rate. The results here, showing localized expression of *acpP*, further supports that cells in deeper portions of these *P. aeruginosa* biofilms are not as metabolically active as those at the top of the biofilm.

Due to the differential expression of *acpP* throughout the biofilms, *acpP* may not be used as an internal control for normalizing activity of genes differentially expressed in biofilms. Using FISH probing, 16S rRNA showed a fairly uniform distribution in *P. aeruginosa* biofilms (63). The qRT-PCR results here confirm those results by demonstrating little variability in 16S rRNA amounts along vertical layers of the biofilm (Fig 3). These results were similar in all conditions tested including, colony biofilms, drip-flow-cultivated biofilms, as well as biofilms supplemented with IPTG. Expression of ribosomal RNAs is highly regulated in bacteria (35). 16S rRNA has been shown to vary according to cellular activity, with slower metabolisms having lower levels of 16S rRNA (43). Although the top portion of these biofilms may be most active in membrane biogenesis, the cells at the top of the biofilm are not equivalent in growth to exponentially growing planktonic cells (Fig 5). The final abundance of rRNA per cell, as measured here, was approximately 33-fold less for cells at the top of the biofilm compared to exponentially growing planktonic cells. Reduced growth of these cells (as indicated by the lower *acpP* and rRNA amounts) combined with the stability of rRNA within ribosomes of cells in the deeper protion of the biofilms may explain the relative uniformity in rRNA abundance throughout the biofilm layers observed here and in the previous study (63).

The results indicate rRNA may be used as a technical control, for determining if cell material is captured by the laser catapult method and retained during RNA sample preparation. However, the abundance of 16s rRNA (which is much higher than any of the mRNA species tested) is a drawback to using it as an internal control for quantify relative expression of other genes. In order to identify an appropriate alternative transcript that combines an average level of abundance with a uniform distribution along the biofilm layers it may be necessary to develop genome wide approaches that look at global distributions of gene expression throughout subpopulations within the biofilm.

Genes regulated by quorum sensing are responsive to small diffusible molecules, such as acyl homoserine lactones, whose concentration increase with increasing cell density (20, 21, 36, 37). Since cells in biofilms are at high density, quorum sensing is one factor that plays a role during biofilm development (12, 47). Here, we assayed expression of two genes regulated by

QS, and found differences in their expression patterns. Amounts of *aprA* mRNA showed stratification and were from 100 to 1000-fold greater at the top of the drip-flow biofilms, compared to the middle or bottom layers (Fig 4). In contrast, *phzA1* amounts, although slightly higher at the top of the biofilm, had more equivalent average amounts throughout the biofilms compared to *aprA*. The results demonstrate genes that are regulated by high cell density may show stratification and heterogeneity of expression in response to growth in biofilm microenvironments. The results for *aprA* are in contrast to a previous study using reporter genes to characterize local expression of other QS-regulated gene, *lasI* and *rhlI* (13). The prior studies demonstrated that the greatest percentage of cells expressing *lasI* and *rhlI* were near the biofilm substratum. Difference between the QS-regulated genes shown here and those in the prior study are likely due to the difference in biofilm cultivation systems. Whereas the *lasI/rhlI* study used flow-through biofilms, we used drip-flow biofilms that resulted in much thicker biofilms. The thicker biofilms likely resulted in steeper chemical gradients of oxygen and nutrients (53, 54), thereby possibly reducing the metabolic activity of bacteria in the deeper portions of the biofilms. QS may be affected by cell density, as well as other factors such as the metabolic status of the cells.

Global proteomic and transcriptomic studies have been performed to characterize physiological differences of *P. aeruginosa* growing in biofilms versus cells growing in planktonic cultures (44, 50, 58, 59, 62). Studies have also compared changes in protein profiles and gene expression during biofilm developmental processes (44). Those studies have been useful for identifying genes uniquely expressed in biofilms. Here, we demonstrate that gene expression differences occur not only between planktonic and biofilm-associated cells, but also for cells at different locations within biofilms. The results may help explain some of the differences observed between global transcriptional and proteomic data. Since proteins in general have a longer half-life than mRNA, proteomic data would represent the accumulation of protein and cell material in biofilms up to the time of sampling (i.e. from all layers of the biofilms). However, due to the short half-life of the mRNA, the transcriptional studies would only assay genes that are actively expressed at the time of sampling. The results here suggest transcription of individual genes may vary dramatically in different regions of the biofilms. Combining LCMM and qRT-PCR will be useful for characterizing the spatial and temporal aspects of these localized gene expression events.

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Figure Legends

Figure 1. Localized GFP fluorescence of (A) biofilm cultivated in drip-flow reactor, (B) colony biofilm. Biofilms were incubated without inducing agent for 72 and 52 h, respectively, then induced with 1 mM IPTG for the final 4 h. (B) The LCMM was used to obtain microdissected samples from regions within the biofilm ranging from 500 to 60,000 μm^2. (C) qRT-PCR of *gfp* mRNA obtained from the top fluorescent zone of the IPTG-induced biofilms (n=26) and from the equivalent top zone of the non-induced biofilms (n=12). Sections were normalized to 60,000 μm^3 for comparison. Each point represents an individual measurement. *The points plotted at 10^1 are below detection limits (+IPTG - 1 sample, -IPTG - 9 samples). A two-tailed Mann-Whitney test between IPTG-induced and non-induced biofilm was used ($P<0.01$). The LCMM was also used to obtain biofilm sections from the middle (n=7) and bottom (n=13) of the biofilm. $^{\#}10^1$ indicates below detection (middle - 7 samples, bottom – 6 samples).

Figure 2. Stratified expression of *acpP* mRNA in biofilms. qRT-PCR was performed on *acpP* obtained from the top (n=26 and 21), middle (n=8 and 11) or bottom (n=15 and 18) of colony biofilms and drip flow biofilms. Two-tailed Mann-Whitney test results demonstrated significant differences in *acpP* expression ($P<0.01$ for top vs. middle or top vs. bottom). *Number of samples below detection, shown as 10^1 for colony biofilms (top-1, middle-8, bottom-11) and drip flow biofilms (top-0, middle-1, bottom-14).

Figure 3. Box plot of 16S rRNA in biofilms. Sections from the top (n=10 and 12), middle (n=11 and 8) or bottom (n=12 and 17) biofilm layers from colony biofilms or drip flow biofilms respectively, were analyzed by SYBR Green-based qRT-PCR. C_T values were log transformed and adjusted to 60,000 μm^3. Line indicates the median; 50% of observations are within the box; bars indicate samples with the minimum and maximum value. Two-tailed Mann-Whitney test for comparisons between top and bottom levels of 16S rRNA in colony biofilms showed no significant differences ($P > 0.5$). In addition, no significant difference was observed for the different layers of the drip flow biofilms.

Figure 4. Expression of quorum-sensing regulated genes *aprA* and *phzA1* in *P. aeruginosa* PAO1 biofilms, cultivated in drip-flow reactors. (A) *aprA* mRNA obtained from the top (n=20), middle

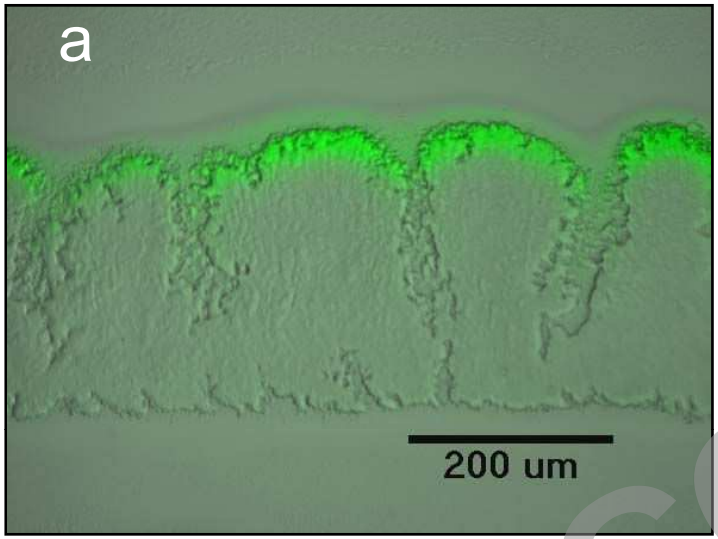
(11) and bottom (n=17) of biofilms. * Samples Below detection (top-1, middle-4, bottom-13). (B) *phzA1* mRNA from the same samples. * Samples Below detection (top-8, middle-4, bottom-11). Two-tailed Mann-Whitney test results demonstrated significant differences in *aprA* expression ($P<0.01$ for top vs middle or top vs. bottom). Two-tailed Mann-Whitney test results demonstrated no significant differences in *phzA* expression ($P>0.1$ for top vs middle or top vs. bottom).

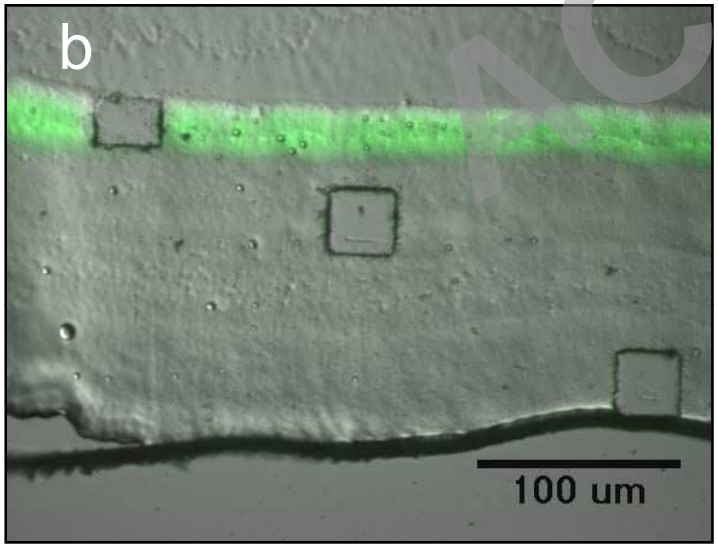
Figure 5. Limits of detection for microdissected biofilm samples and for planktonic cultures for (a) *gfp*, (b) *acpP*, and (c) 16S *rRNA*. Detection limits were based on biofilm volume using 5 μm thick sections of the top zones of the biofilms. Detection limits for planktonic cells were based on colony forming units (CFU) of serially diluted cultures. Dual-labeled probes were used in multiplex reactions for *gfp* ($r^2=0.89$ biofilm and $r^2=0.99$ for planktonic), and for *acpP* ($r^2=0.94$ biofilm and $r^2=0.99$ for planktonic). Levels of 16S rRNA were measured using the SYBR Green based chemistry of qRT-PCR ($r^2=0.87$ for biofilm and $r^2=0.99$ for planktonic).

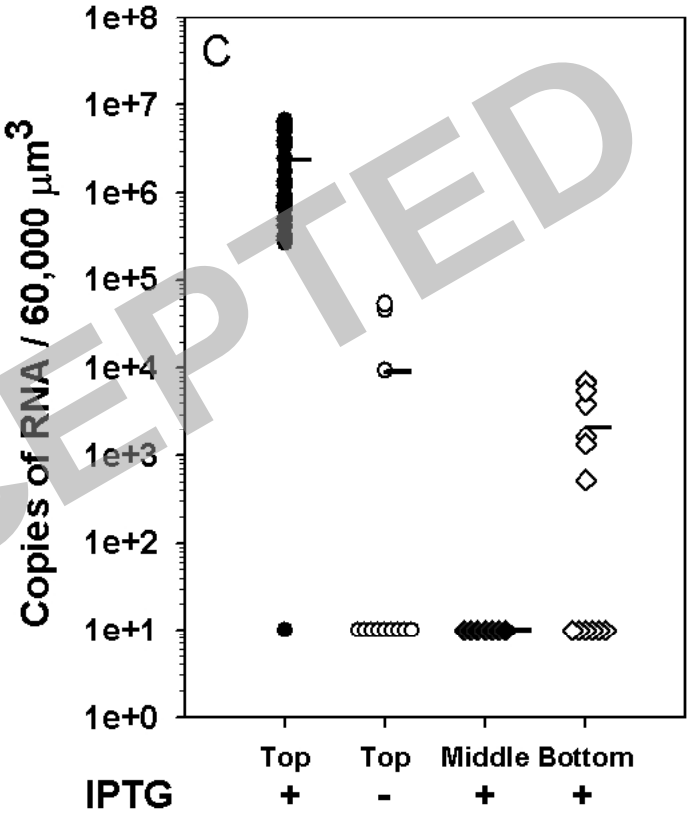
Table 1. qRT-PCR Primer and Probe sequences and labels.

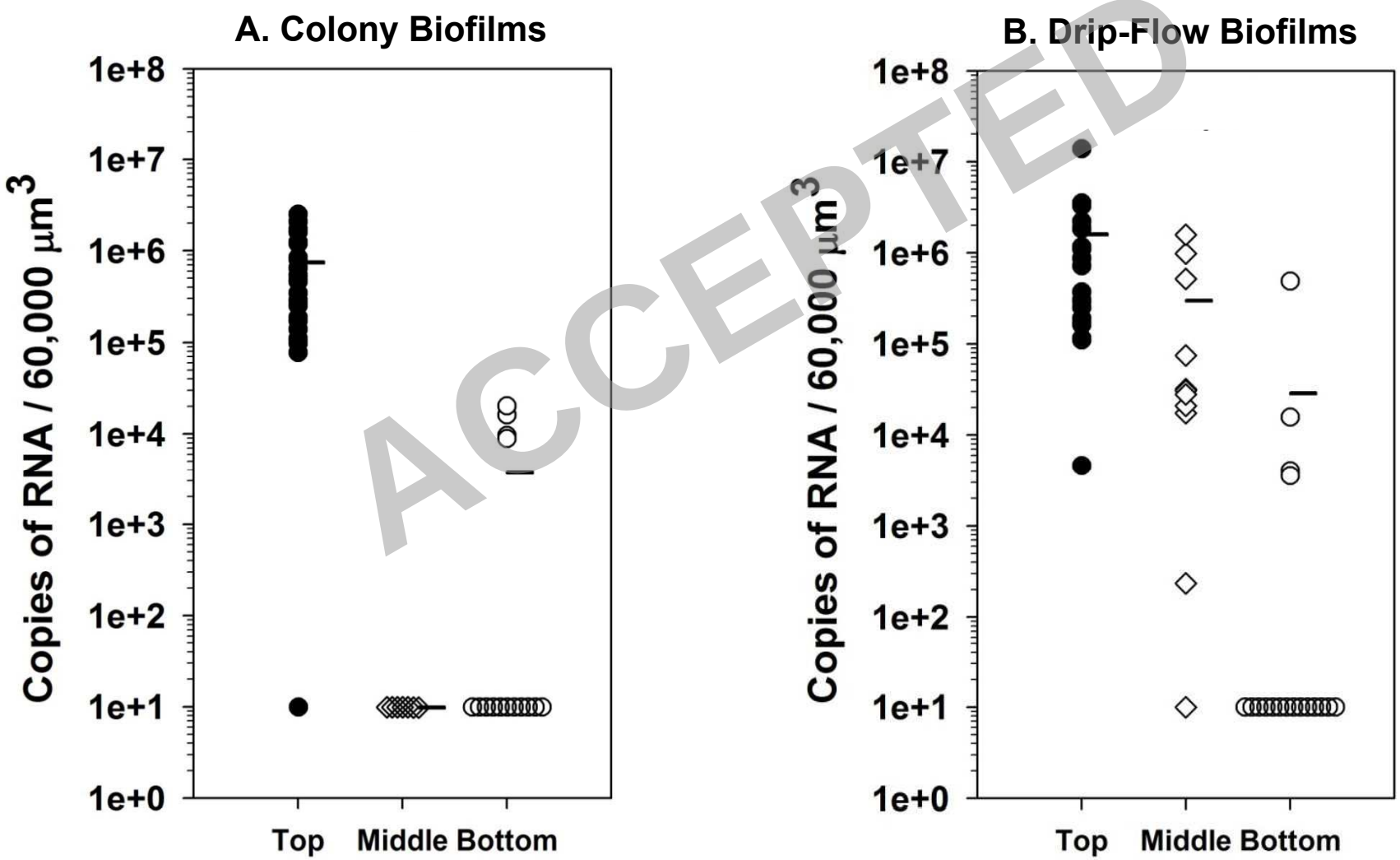
Primer	Sequence	Label	Conc.[a]	Temp[b]	Product[c]
gfp-For	TTTCACTGGAGTTGTCCCAATTC		400	60	80
gfp-Rev	CACCCTCTCCACTGACAGAAAAT		400		
gfp-Probe	TGTGCCCATTAACATCACCATCTAATTCAACA	5' FAM, 3' BHQ-1	300		
acpP-For	ACTCGGCGTGAAGGAAGAAG		400[d]/50[e]	60	80
acpP-Rev	CGACGGTGTCAAGGGAGT		400[d]/900[e]		
acpP-Probe	AAGTCACCAACAGCGCTTC	5' JOE, 3' BHQ-1	200		
lucI-For	GTGTTGGGCGCGTTATTTATC		200	60	78
lucI-Rev	ACTGTTGAGCAATTCACGTTCA		200		
lucI-Probe	CGCCCGCGAACGACATTTAT	5' Cy5, 3' BHQ-2	200		
aprA-For	GCTTCAGCCAGAACCAGAAGAT		200	60	78
aprA-Rev	TCGACACATTGCCCTTCAAC		200		
aprA-Probe	ACATCGGACAGCGCCTTCTCGTTG	5' FAM, 3' BHQ-1	100		
phzA1-For	TAAAACGTAATCGCGAGTTCATG		900	60	74
phzA1-Rev	TTTTATTTGCGGAACGGCTATT		900		
phzA1-Probe	CCAATGCACGCAGTTTCTGTATCGGGT	5' ROX, 3' BHQ-2	150		
16S rRNA-For[f]	CAAAACTACTGAGCTAGAGTACG		300	59	215
16S rRNA-Rev[f]	TAAGATCTCAAGGATCCCAACGGCT		300		
Primers used for in vitro transcription					
gfp-T7-for	TAATACGACTCACTATAGGGGGAGAAGAACTTTTCACTGG				
gfp-T7-rev	GAAAGGGCAGATTGTGTGGAC				
acpP-T7-for	TAATACGACTCACTATAGGGCCATCGAAGAACGCGTTAAG				
acpP-T7-rev	CCTGAACGGTGGTGATCTTT				
aprA-T7-for	TAATACGACTCACTATAGGGCTTGCATTGAAAGGTCGTAGC				
aprA-T7-rev	GATATCGCCGTAGACGAAGGT				
phzA1-T7-for	TAATACGACTCACTATAGGGGTCAGCGGTACAGGGAAACAC				
phzA1-T7-rev	GTGGGAATACCGTCACGTTT				

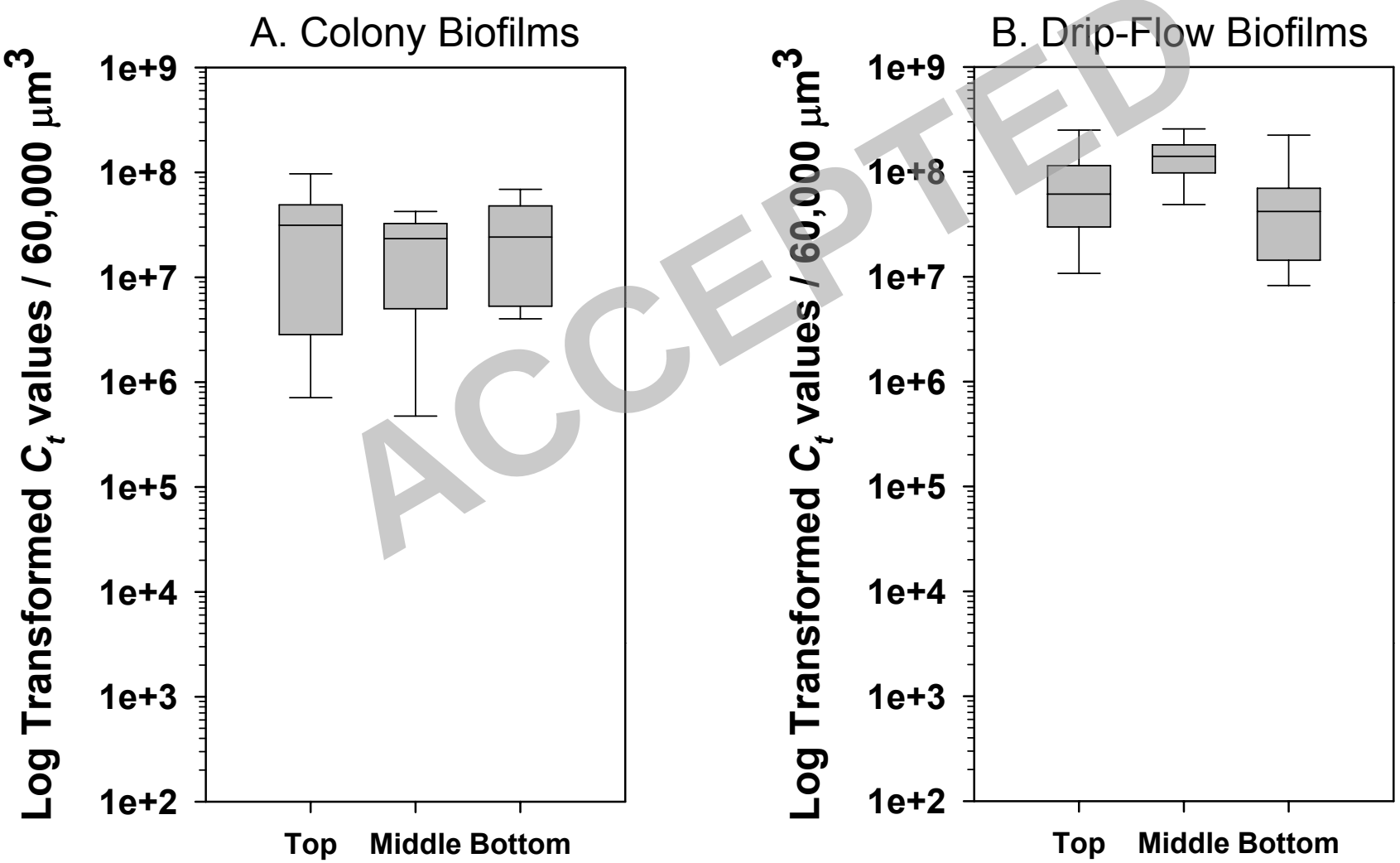
[a]Primer and probe concentrations used in RT-PCR reactions (nM).
[b]Annealing temperature for PCR reaction.
[c]Size of PCR product in base pairs.
[d]Primer concentrations used in *acpP, gfp,* and *lucI* triplex reactions (nM).
[e]Primer concentrations used in *acpP, aprA,* and *phzA1* triplex reactions (nM).
[f]Primers sequences used by Matsuda, et al. (31)The acquisition step of the 16S rRNA assay was performed at 72^{0}C.

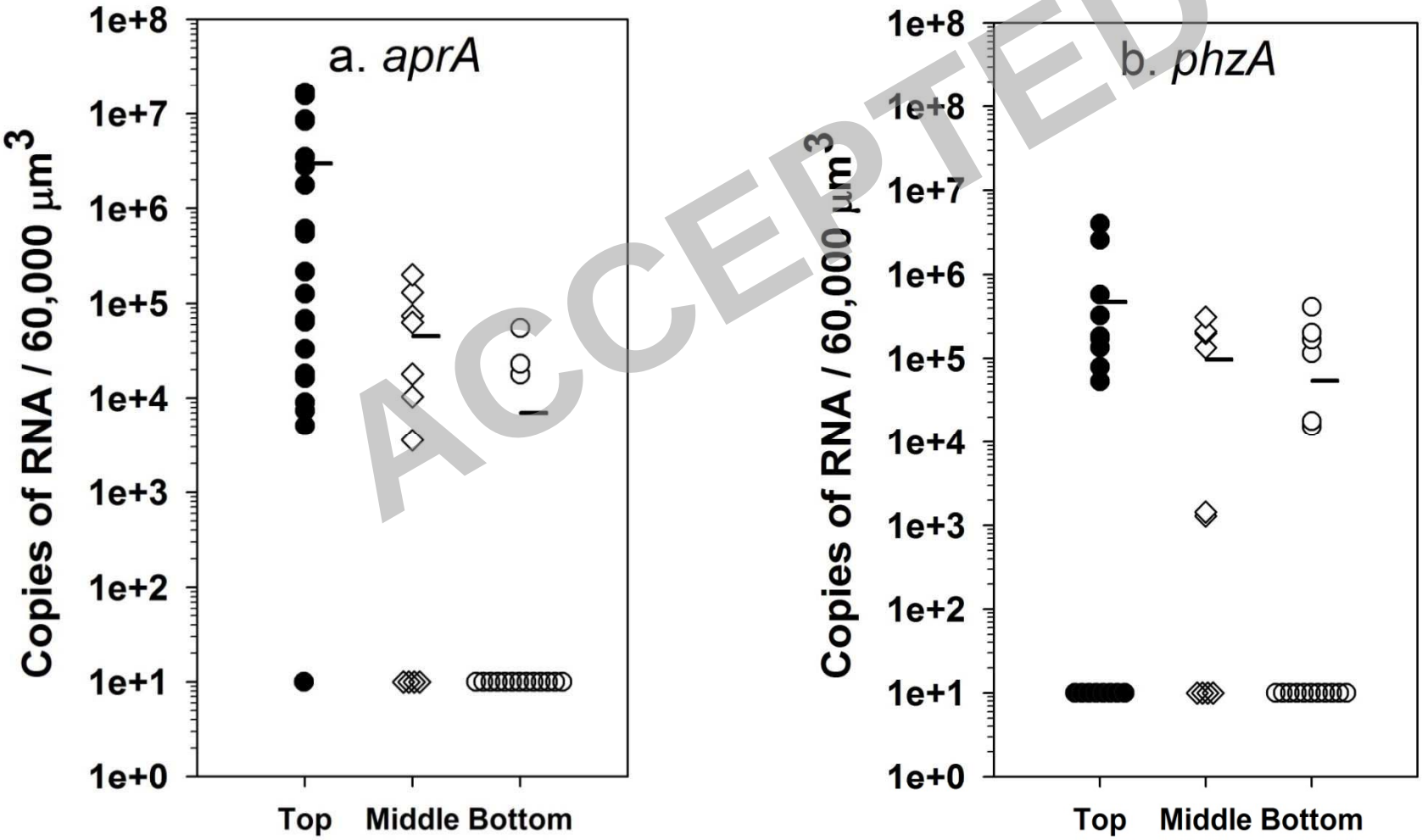

a
200 um

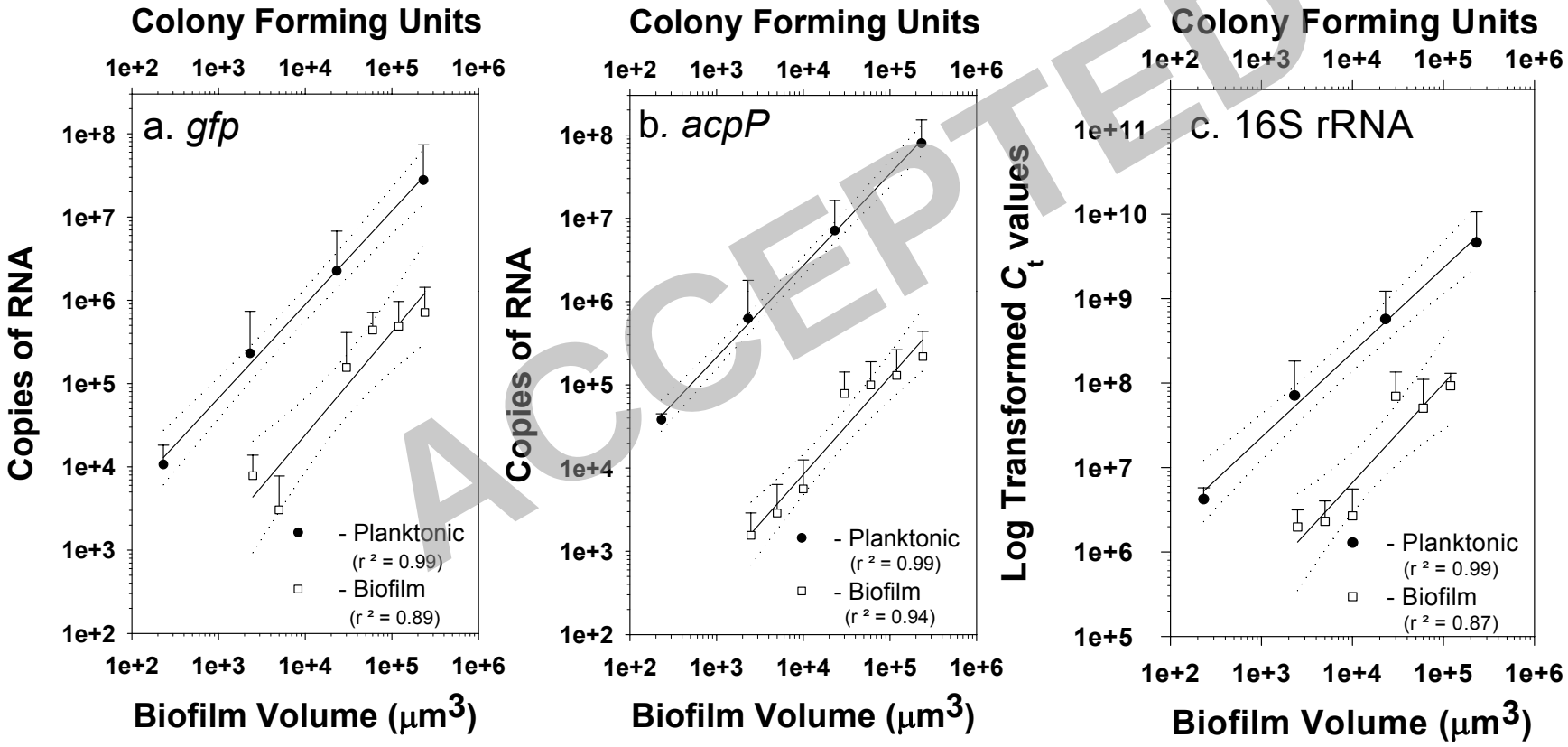

b
100 um


C
Copies of RNA / 60,000 µm3
1e+8
1e+7
1e+6
1e+5
1e+4
1e+3
1e+2
1e+1
1e+0
Top
Top
Middle
Bottom
IPTG
+
-
+
+


A. Colony Biofilms
B. Drip-Flow Biofilms
Copies of RNA / 60,000 µm3
1e+8
1e+7
1e+6
1e+5
1e+4
1e+3
1e+2
1e+1
1e+0
Top
Middle
Bottom


A. Colony Biofilms
B. Drip-Flow Biofilms
Log Transformed C_t values / 60,000 μm^3
1e+9
1e+8
1e+7
1e+6
1e+5
1e+4
1e+3
1e+2
Top
Middle
Bottom


a. aprA
b. phzA
Copies of RNA / 60,000 µm3
1e+8
1e+7
1e+6
1e+5
1e+4
1e+3
1e+2
1e+1
1e+0
Top
Middle
Bottom


Colony Forming Units
Copies of RNA
Biofilm Volume (μm3)
a. gfp
- Planktonic
(r ² = 0.99)
- Biofilm
(r ² = 0.89)
b. acpP
- Planktonic
(r ² = 0.99)
- Biofilm
(r ² = 0.94)
Log Transformed Ct values
c. 16S rRNA
- Planktonic
(r ² = 0.99)
- Biofilm
(r ² = 0.87)