Localized Gene Expression in *Pseudomonas aeruginosa* Biofilms[▽]

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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 across the entire population. Here, we describe the use of laser capture microdissection microscopy (LCMM) combined with multiplex quantitative real-time reverse transcriptase PCR (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 were determined for *Pseudomonas aeruginosa* containing an isopropyl-β-D-thiogalactopyranoside-inducible gene for the green fluorescent protein (*gfp*). The results show that the amounts of *gfp* mRNA were greatest in the top zones of the biofilms, and that *gfp* mRNA levels correlated with the zone of active green fluorescent protein fluorescence. The method then was 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 were determined, and therefore the results are dependent on both gene expression and the half-life of the transcripts. Therefore, the uniform amount of rRNA throughout the biofilms likely is due to the stability of the rRNA within ribosomes. The levels of *aprA* mRNA showed stratification, with the largest 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.

Bacteria thrive in spatially defined microenvironments, and therefore they are exposed to local environmental conditions that may vary on the micrometer scale (53). When growing on surfaces in biofilms, bacteria typically are 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 across the entire population (2). Techniques often are 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 as well as epifluorescence microscopy (14, 33, 55, 65). Although useful, this technique requires the prior genetic manipulation of the bacteria and the reintroduc-

tion of the constructed strains back into the environment. The 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 is less useful for detecting mRNA transcripts in low abundance. Measurements of cellular activity may be obtained by combining FISH with microautoradiography or Raman spectroscopy after the uptake of labeled substrates into cellular material (26, 56). To measure the gene expression of cells in biofilms, in situ reverse transcription-PCR has been developed to amplify mRNA present in low copy numbers (29). This approach uses fluorescently labeled probes to detect individual cells expressing a gene of interest, but it is limited by a 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 the quantitative assessment of any gene expressed by bacteria in their native environment. The approach allows the 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 a housekeeping gene, *acpP*; the 16S rRNA; and two genes regulated by quorum

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sensing (QS), *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 the isopropyl-β-D-thiogalactopyranoside (IPTG) induction of 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°C in Luria-Bertani (LB) broth (6). Cultures were diluted in sterile LB broth to an optical density at 600 nm of 0.4 (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°C. The membranes containing the colonies were transferred to fresh LB agar every 12 h. For the IPTG induction of GFP in strains containing pAB1, membranes were transferred to LB agar containing 1.0 mM IPTG and were incubated for an additional 4 h.

Biofilms also were cultivated on stainless steel coupons in continuous flow by using drip-flow reactors (64). The reactor consisted of a one-time flowthrough system containing a medium reservoir, pump, silicon tubing, drip-flow chamber, and 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$_2$PO$_4$, 0.34 mM K$_2$HPO$_4$, 145 mM NaCl, 20 μl trace metals, and 1 ml vitamin solution. The trace metal solution contained (per liter of 0.83 M HCl) 5.0 g CuSO$_4$ · 5H$_2$O, 5.0 g ZnSO$_4$ · 7H$_2$O, 5.0 g FeSO$_4$ · 7H$_2$O, and 2.0 g MnCl$_2$ · 4H$_2$O. The vitamin solution contained (per liter) 0.5 g thiamine and 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 optical density at 600 nm 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 then was pumped through the reactors at 1.2 ml/min for 72 h. Reactors were maintained at 37°C throughout the incubation. For biofilms for which the 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) of 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.

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 s. The biofilms then were examined using lenses with objectives of ×10 to ×40 magnification. Areas of the biofilms ranging from 500 to 100,000 μm^2 were obtained using the laser catapult parameter, which allows 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 being laser catapulted into 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 (1.7 × 10^4 transcript copies) (Promega) was added as a spike-in control to assay for RNA loss during sample preparation. Samples were centrifuged for 10 s and then transferred to a 1.5-ml microcentrifuge tube containing water-saturated phenol (400 μl), and then they were heated in a water bath at 65°C 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 1,200 μl of absolute ethanol. Resuspended RNA was treated with DNase by

using the Turbo DNA-free kit (Ambion, Inc.) in 10-μl reaction mixtures 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°C 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 v2.0 software (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.

IVT. 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°C). IVT RNA products were purified using the RNeasy mini kit (Qiagen) and analyzed by electrophoresis on an RNA nanochip using a Bioanalyzer 2100 (Agilent Technologies). RNA standards were quantified using UV absorption at 260 nm, aliquoted, and stored at −80°C.

The copy number of RNA standards was calculated using the following equation: number of molecules per microliters = (number of grams of RNA per microliter/molecular weight) × 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 (i.e., the threshold cycle [C_T]). 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 the assay's sensitivity and to ensure the assay's compatibility with different probes in the multiplex reactions.

qRT-PCR. One-step qRT-PCR was used for all mRNA quantifications. qRT-PCR mixtures (25 μl) containing 2 to 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. A 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 for 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, which was derived from IVT, and the external spike-in control RNA of *lucI* were serially diluted in 8 μl/ml of PolyAcryl carrier (Molecular Research Center, Inc.) to accommodate 10^1 to 10^6 molecules per reaction. Cycling parameters were established according to the kit's 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 (for the 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 calculated *lucI* per sample/1.7 × 10^4, since 1.7 × 10^4 *lucI* RNA transcripts were added to each sample immediately after laser capture as a spike-in control. To quantify the relative amounts of 16S rRNA, C_T values were log transformed with the formula amplification^{-CT} × 10^{10}, where C_T and amplification values were derived from the comparative quantitation analysis that used Rotor Gene software (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 the following: *aprA*, 1.04; *phzA1*, 1.04; *acpP*, 0.86; and *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 to 100,000 transcripts ($r^2 > 0.99$). The interexperimental precision was approximately 16%, and the intrasample precision was 11%. These calculations were based on the calculated number of copies

TABLE 1. qRT-PCR primer and probe sequences and labels

Primer	Sequence	Label[a]	Concn (nM)	Temp[b] (°C)	Product size (bp)
Primers and probes for qRT-PCR					
gfp-For	TTTCACTGGAGTTGTCCCAATTC		400	60	80
gfp-Rev	CACCCTCTCCACTGACAGAAAT		400		
gfp-Probe	TGTGCCCATTAACATCACCATCTAATTCAACA	5′ FAM, 3′ BHQ-1	300		
acpP-For	ACTCGGCGTGAAGGAAGAAG		400[c]/50[d]	60	80
acpP-Rev	CGACGGTGTCAAGGGAGT		400[c]/900[d]		
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[e]	CAAAACTACTGAGCTAGAGTACG		300	59	215
16S rRNA-Rev[e]	TAAGATCTCAAGGATCCCAACGGCT		300		
Primers used for IVT					
gfp-T7-for	TAATACGACTCACTATAGGGGGAGAAGAACT TTTCACTGG				
gfp-T7-rev	GAAAGGGCAGATTGTGTGGAC				
acpP-T7-for	TAATACGACTCACTATAGGGCCATCGAAGAA CGCGTTAAG				
acpP-T7-rev	CCTGAACGGTGGTGATCTTT				
aprA-T7-for	TAATACGACTCACTATAGGGCTTGCATTGAA AGGTCGTAGC				
aprA-T7-rev	GATATCGCCGTAGACGAAGGT				
phzA1-T7-for	TAATACGACTCACTATAGGGGTCAGCGGTAC AGGGAAACAC				
phzA1-T7-rev	GTGGGAATACCGTCACGTTT				

[a] FAM, 6-carboxyfluorescein; BHQ-1, black hole quencher 1; JOE, carboxy-4′,5′-dichloro-2′,7′-dimethoxyfluorescein; and ROX, carboxy-X-rhodamine.
[b] Annealing temperature for PCR.
[c] Primer concentrations used in *acpP*, *gfp*, and *lucI* triplex reactions.
[d] Primer concentrations used in *acpP*, *aprA*, and *phzA1* triplex reactions.
[e] Primers sequences used by Matsuda et al. (31). The acquisition step of the 16S rRNA assay was performed at 72°C.

per reaction and their variations from the means. We chose to obtain the reproducible measure from the calculated number of 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, differences 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 within a larger surrounding biofilm population, we first developed a system by which mRNA transcript numbers could be determined and compared to those of a spatially defined phenotypic trait. For this we used a strain of *P. aeruginosa* with an IPTG-inducible gene for GFP (*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 and then induced with IPTG for the final 4 h of incubation, a band of green fluorescence is observed in the top 30-μm zone of the biofilm (Fig. 1A and B) (8, 61). This fluorescent band is observed whether 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 μm² (Fig. 1B). Figure 1c shows the *gfp* mRNA copy abundances for 26 samples after normalizing the results to the 60,000-μm³ biofilm volume. On average, in IPTG-induced biofilms the copy number of *gfp* mRNA was 250-fold greater than that from the biofilm samples not induced with IPTG ($P < 0.001$), for which no fluorescent zone was observed. Of the 12 samples taken from biofilms to which no IPTG was added, 9 had *gfp* mRNA levels below the detection limit, indicating little or no expression of this gene in the absence of the inducing agent. The other three samples had transcript levels above the detection limits but approximately 100-fold less than that of 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 fluores-
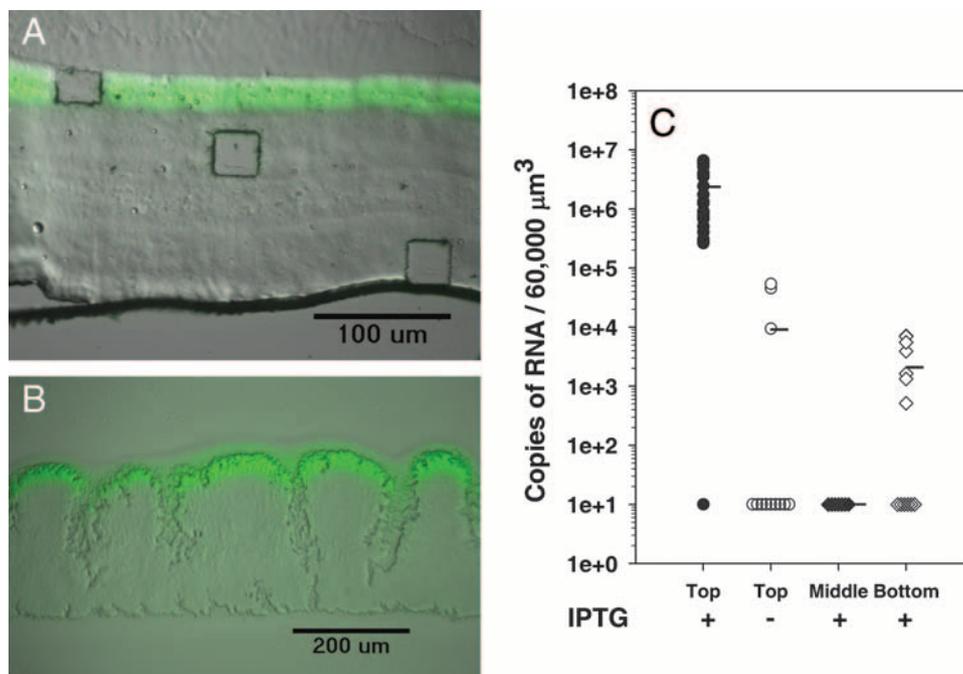
FIG. 1. Localized GFP fluorescence of (A) biofilm cultivated in a drip-flow reactor and (B) colony biofilm. Biofilms were incubated without an inducing agent for 72 and 52 h, respectively, and then were induced with 1 mM IPTG for the final 4 h. (B) LCMM was used to obtain microdissected samples from 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 noninduced 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 to calculate differences in means between IPTG-induced and noninduced biofilms was used ($P < 0.01$). The LCMM also was used to obtain biofilm sections from the middle ($n = 7$) and bottom ($n = 13$) of the biofilm. A value of 10^1 indicates amounts below the level of detection (middle, 7 samples; bottom, 6 samples).

cence 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 nonfluorescent zones of the IPTG-induced biofilms. The results show little gfp mRNA in the underlying portions of the biofilm compared to that in the top 30-μm fluorescent zone ($P < 0.001$ for values for the top layer compared to those for the middle or bottom layer) (Fig. 1C). No gfp expression was observed for any of the samples in the middle zone, and six samples from the bottom zone had no gfp expression. Some of the samples from the bottom zones had small amounts of gfp expression that were approximately 1,000-fold lower than those 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 a lack of GFP activation with oxygen. These results demonstrate that it is possible to use LCMM and qRT-PCR to quantify the 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 the 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 is required for the 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 ($P < 0.001$ for

values for the top layer compared to those for the middle or bottom layer). 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 sam-
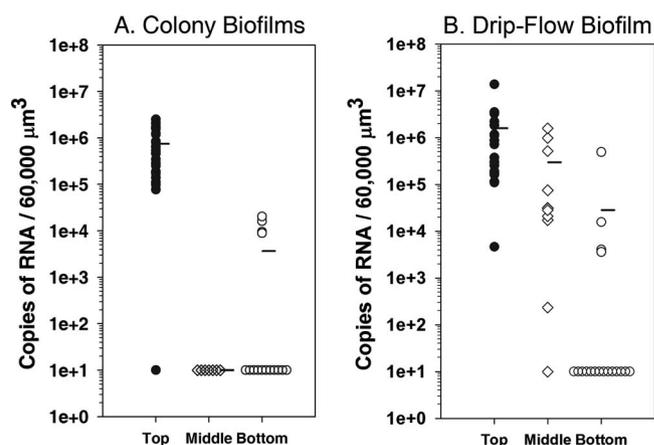

FIG. 2. Stratified expression of $acpP$ mRNA in biofilms. qRT-PCR was performed on $acpP$ obtained from the top ($n = 26$ and 21), middle ($n = 8$ and 11), or bottom ($n = 15$ and 18) of colony biofilms and drip-flow biofilms, respectively. Two-tailed Mann-Whitney test results demonstrated significant differences in $acpP$ expression ($P < 0.01$ for values for the top layer compared to those for the middle or bottom layer). The number of samples below the level of detection are shown as 10^1 for colony biofilms (top, 1 sample; middle, 8 samples; bottom, 11 samples) and drip-flow biofilms (top, 0 samples; middle, 1 sample; bottom, 14 samples).

ples from the bottom of the biofilm had *acpP* mRNA levels that were below the level of detection. Several of the samples from the bottom of the biofilm had *acpP* levels that were above the detection level, but each of these amounts was approximately 100-fold less than the average amounts from samples from the top 30 μm of the biofilm. The results indicate that in these biofilms, which averaged 200 μm thick, the top 30 μm contains cells with a high abundance of *acpP* mRNA, suggesting that these cells are synthesizing new membrane material; the deeper portions of the biofilms had much less expression of this gene.

The drip-flow-cultivated biofilms also showed the 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 than at the top. The middle of the biofilm showed a large range of variability in *acpP* mRNA amounts, with an average that was approximately 10-fold less than that at the top of the biofilm. The drip-flow biofilms have more structural features than the colony biofilms (Fig. 1A and 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 proximity 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 significant variability in the expression of this housekeeping gene that is dependent on the location of the cells within a biofilm. The results also demonstrate that *acpP* may not be 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, middle, or bottom of the biofilms that had been 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 were 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 likely is due to the higher stability of rRNA contained within ribosomes. The abundance of individual RNAs measured here by qRT-PCR is dependent on both the 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 the 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 is used as an internal control for the comparison of the 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 often is much higher than that of any mRNA species.
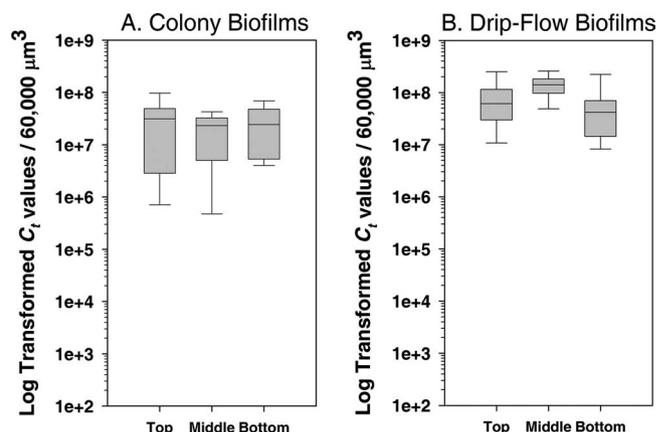

FIG. 3. Box plot of 16S rRNA in biofilms. Sections from the top (n = 10 and 12), middle (n = 11 and 8), and bottom (n = 12 and 17) biofilm layers from colony biofilms or drip-flow biofilms, respectively, were analyzed by SYBR green-based qRT-PCR. C_Ts were log transformed and adjusted to 60,000 μm³. The line indicates the median; 50% of observations are within the box. Bars indicate samples with the minimum and maximum values. A 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.

Stratified expression of QS-regulated genes. Bacterial cells within biofilms communicate through small diffusible chemical signals by QS (20, 21, 36, 37). We assayed the localized expression of two genes, *aprA* (encoding alkaline protease) and *phzA1* (necessary for pyocyanin biosynthesis), that are 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 that had been cultivated in drip-flow reactors were cryoembedded and thinly sectioned for LCMM and then assayed for *aprA* and *phzA1* expression along vertical transects of the biofilms. The amounts of *aprA* mRNA showed a trend similar 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). The expression of *aprA* averaged 1,000-fold higher than that of samples from the bottom of the biofilms, where most samples had expression that was below the detection limit. The middle of the biofilms had intermediate *aprA* expression that averaged approximately 100-fold less than that of samples from 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 that of *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, also may influence the 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 the sample volume. Since samples were taken from cryoembedded
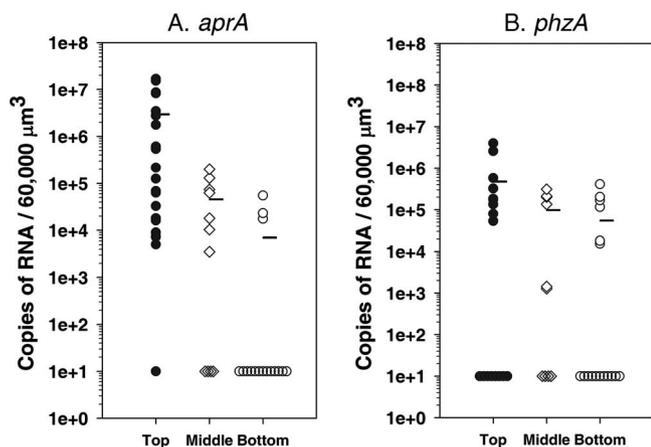
FIG. 4. Expression of QS-regulated genes *aprA* and *phzA1* in *P. aeruginosa* PAO1 biofilms that were cultivated in drip-flow reactors. (A) *aprA* mRNA obtained from the top (*n* = 20), middle (*n* = 11), and bottom (*n* = 17) of biofilms. The mRNA levels in some samples were below the level of detection (top, 1 sample; middle, 4 samples; bottom, 13 samples). (B) *phzA1* mRNA from the same samples. The mRNA levels in some samples were below the level of detection (top, 8 samples; middle, 4 samples; bottom, 11 samples). Two-tailed Mann-Whitney test results demonstrated significant differences in *aprA* expression (*P* < 0.01 for values for the top layer compared to those for the middle or bottom layer). Two-tailed Mann-Whitney test results demonstrated no significant differences in *phzA* expression (*P* > 0.1 for values for the top layer compared to those for the middle or bottom layer).

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 that ranged from 1×10^3 to 1×10^5 μm^3 ($r^2 = 0.94$ for *acpP*; $r^2 = 0.90$ for *gfp*) (Fig. 5A and B). Therefore, this tech-

nique 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 also were determined for exponentially growing planktonic cells, allowing for the comparison of the mRNA transcript counts to the viable cell counts. RNA was extracted from serially diluted planktonic *P. aeruginosa*(pAB1) cultures containing from 1 to 10^9 cells (determined as the number of CFU) (Fig. 5). Linear responses were observed for cell numbers and mRNA transcripts ($r^2 = 0.97$ for *gfp*; $r^2 = 0.99$ for *acpP*). A linear response also was 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 the 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 an equivalent volume of the biofilm. RNA amounts were approximately 40-, 250-, and 33-fold greater in planktonic cells for *gfp*, *acpP*, and 16S rRNA, respectively, than those from the top biofilm zone. Therefore, although mRNA amounts are highest at the top zone of these biofilms, the amounts in biofilms are less than those for exponentially growing planktonic cultures.

DISCUSSION

The goals of this research were (i) to develop methodology to quantify bacterial gene expression from small groups of
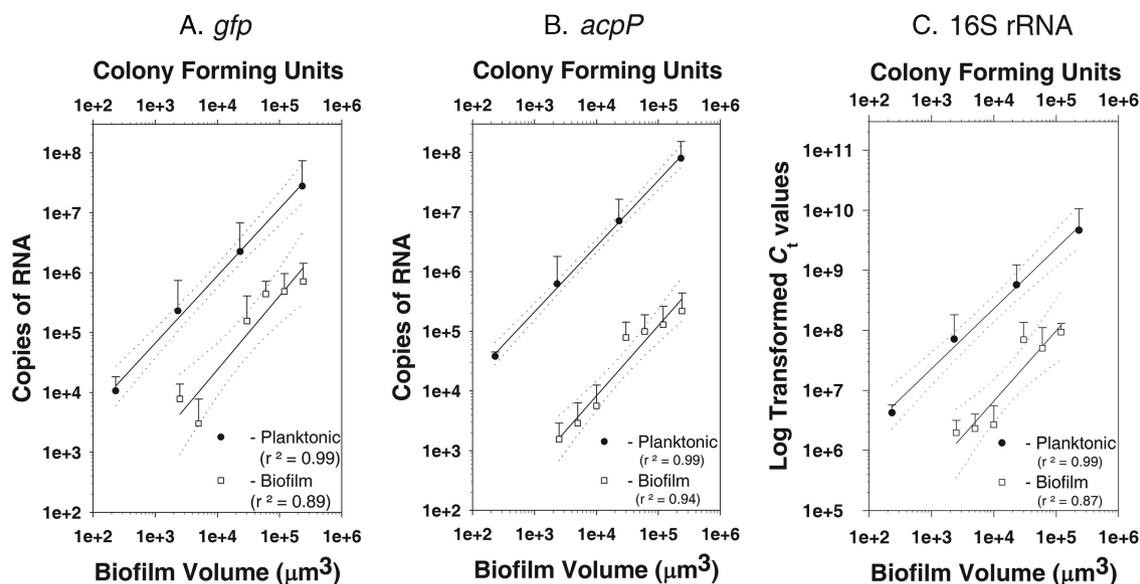

FIG. 5. Limits of detection for microdissected biofilm samples and for planktonic cultures for *gfp* (A), *acpP* (B), and 16S rRNA (C). Detection limits were based on biofilm volume using 5-μm-thick sections of the top zones of the biofilms. Detection limits for planktonic cells were based on the numbers of CFU of serially diluted cultures. Dual-labeled probes were used in multiplex reactions for *gfp* ($r^2 = 0.89$ for biofilm cells and $r^2 = 0.99$ for planktonic cells) and for *acpP* ($r^2 = 0.94$ for biofilm cells and $r^2 = 0.99$ for planktonic cells). The 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).

bacterial cells isolated from biofilms without the prior genetic manipulation of the cells, and (ii) to use the method to determine if local conditions found within biofilms result in the spatial variation of bacterial gene expression. By using the cryosectioning of biofilms and LCMM, we were able to isolate and extract RNA from small groups of bacterial cells that were isolated from different regions within biofilms. Multiplex qRT-PCR allowed the 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 QS. 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, for which 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 the 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 for IPTG-induced biofilm cultures than for noninduced samples, and they were greater for the GFP fluorescent zone than the nonfluorescent deeper areas of the biofilms. Variation in the amount *gfp* mRNA at different regions of the biofilms was expected, given the localized nature of the fluorescent zone. Interestingly, an active zone of *acpP* mRNA of similar size also was observed (Fig. 2). Housekeeping genes, such as *acpP*, often serve as internal controls in qRT-PCR experiments to normalize the 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 various metabolic activities 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 likely are the most active in membrane synthesis. The cells in the deeper portions of the biofilm likely are in the slow-growth state. These results are consistent with those of another study that indicates 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 primarily due to their slow growth rate. The results here, showing the localized expression of *acpP*, further support 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 the activity of genes that are 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 the vertical layers of the biofilm (Fig. 3). These results were similar in all conditions tested, including colony biofilms, drip-flow-cultivated biofilms, and biofilms supplemented with IPTG. The expression of ribosomal RNAs is highly regulated in bacteria (35). The level of 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 than for the exponentially growing planktonic cells. The 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 portion of the biofilms, may explain the relative uniformity in rRNA abundance throughout the biofilm layers observed here and in a previous study (63).

The results indicate that rRNA can 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 that of any of the mRNA species tested) is a drawback to using it as an internal control for quantifying the 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 QS are responsive to small diffusible molecules, such as acyl homoserine lactones, the concentrations of which increase with increasing cell density (20, 21, 36, 37). Since cells in biofilms are at high density, QS is one factor that plays a role during biofilm development (12, 47). Here, we assayed the expression of two genes regulated by QS and found differences in their expression patterns. The amounts of *aprA* mRNA showed stratification and were 100 to 1,000-fold greater at the top of the drip-flow biofilms thab those of the middle or bottom layer (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 those of *aprA*. The results demonstrate that genes that are regulated by high cell density show the stratification and heterogeneity of expression in response to growth in biofilm microenvironments. The results for *aprA* are in contrast to those of a previous study that used reporter genes to characterize the local expression of two other QS-regulated gene, *lasI* and *rhlI* (13). The prior studies demonstrated that most of the cells expressing *lasI* and *rhlI* were near the biofilm substratum. The differences between the QS-regulated genes shown here and those in the prior study likely are due to the differences in the biofilm cultivation systems. Whereas the *lasI*/*rhlI* study used flowthrough 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 the physiological differences between *P. aeruginosa* growing in biofilms and cells growing in planktonic cultures (44, 50, 58, 59, 62). Studies also have 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 assay only genes that are actively expressed at the time of sampling. The results here suggest that the transcription of individual genes varies 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.

ACKNOWLEDGMENTS

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