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Heterogeneous *rpoS* and *rhlR* mRNA Levels and 16S rRNA/rDNA (rRNA Gene) Ratios within *Pseudomonas aeruginosa* Biofilms, Sampled by Laser Capture Microdissection[▽]

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The local environmental conditions in biofilms are dependent on the impinging aqueous solution, chemical diffusion, and the metabolic activities of cells within the biofilms. Chemical gradients established in biofilms lead to physiological heterogeneities in bacterial gene expression. Previously, we used laser capture microdissection (LCM) and quantitative reverse transcription (RT)-PCR to target defined biofilm subpopulations for gene expression studies. Here, we combined this approach with quantitative PCR of bacterial DNA to normalize the amount of gene expression per cell. By comparing the ratio of 16S rRNA to 16S rDNA (rRNA gene), we demonstrated that cells at the top of thick *Pseudomonas aeruginosa* biofilms have 16S rRNA/genome ratios similar to those of cells in a transition from the exponential phase to the stationary phase. Cells in the middle and bottom layers of these biofilms have ratios that are not significantly different from those of stationary-phase planktonic cultures. Since much of each biofilm appeared to be in a stationary-phase-like state, we analyzed the local amounts of the stationary-phase sigma factor *rpoS* gene and the quorum-sensing regulator *rhlR* gene per cell. Surprisingly, the amount of *rpoS* mRNA was largest at the top of the biofilms at the air-biofilm interface. Less than one *rpoS* mRNA transcript per cell was observed in the middle or base of the biofilms. The *rhlR* mRNA content was also greatest at the top of the biofilms, and there was little detectable *rhlR* expression at the middle or bottom of the biofilms. While the cell density was slightly greater at the bottom of the biofilms, expression of the quorum-sensing regulator occurred primarily at the top of the biofilms, where the cell metabolic activity was greatest, as indicated by local expression of the housekeeping gene *acpP* and by expression from a constitutive P_{trc} promoter. The results indicate that in thick *P. aeruginosa* biofilms, cells in the 30 μm adjacent to the air-biofilm interface actively express genes associated with stationary phase, while cells in the interior portions do not express these genes and therefore are in a late-stationary-phase-like state and may be dormant.

Bacterial biofilms consist of populations of cells that respond differently to the heterogeneous conditions that occur in the biofilm interstitial solution (5, 17, 49). Chemical gradients form throughout these biofilms due to nutrient consumption, bacterial waste product production, and generation of secondary metabolites and signaling compounds (28, 41, 49). Since chemical gradients may intersect or overlap, unique environmental conditions are established at localized sites within biofilms. Bacteria within the biofilms adapt to these local environmental conditions. The physiological responses of biofilm-associated bacteria depend not only on the impinging solution and the nature of the substratum but also on the location of the cells within the biofilm (11, 12, 18, 25, 27, 40, 48, 52, 64, 65). Biofilms are therefore composed of bacteria in many different physiological states, and even cells close to each other have unique physiological activities (49).

Quantitative real-time reverse transcription-PCR (qRT-PCR) is a sensitive approach for quantifying RNA transcripts from very low numbers of cells, possibly even at the single-cell level (19, 33). We recently combined qRT-PCR with laser capture microdissection (LCM) (3, 15, 47) to isolate subsets of bacterial cells from defined locations of *P. aeruginosa* biofilms and to study the levels of expression of individual genes in the subpopulations (27). This approach has the advantage that gene expression in cells may be analyzed without prior genetic manipulation of the cells. However, the LCM–qRT-PCR approach is less amenable to online monitoring of gene expression than microscopic analysis of fluorescent reporter gene expression. Since genetic manipulation of cells is not required for LCM–qRT-PCR, this approach may be used for gene expression studies of wild-type organisms or of natural multispecies biofilm assemblages. In addition, LCM–qRT-PCR provides a quantitative, rather than qualitative, assessment of the RNA transcripts in a sample. Our previous study demonstrated that the levels of mRNA for individual genes may vary by several orders of magnitude over relatively short distances within biofilms (27).

In typical qRT-PCR experiments internal controls are used to normalize the expression values for the genes being studied (53, 55, 56). Housekeeping genes are generally used as normalizing factors since their expression is uniform under a variety of environmental conditions. However, it was apparent from our initial LCM–qRT-PCR studies that it would be difficult to identify an mRNA transcript that could be used as an internal control. Even for a commonly used housekeeping gene, *acpP*, the levels of expression in different regions of the biofilms were vastly different (27). Since AcpP is involved in

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lipid metabolism and membrane biosynthesis, the variable expression of this protein likely reflected differences in the growth rates of bacteria in different regions of the biofilms. The results suggested that housekeeping genes may not be adequate internal controls for biofilm qRT-PCR studies. In contrast, the levels of 16S rRNA were relatively uniform throughout *P. aeruginosa* biofilms (27, 64). The uniformity of the rRNA levels was due to the nonoptimal growth rate of the most active cells at the biofilm periphery (60) and to the stability of the rRNA within ribosomes of cells in deeper regions of the biofilms (14).

Studies of rRNA, including 16S rRNA, have been particularly useful in environmental microbiology (1, 13, 29, 30, 61). 16S rRNA is used as a taxonomic indicator to examine species distributions in populations and as a target for fluorescent *in situ* hybridization (FISH). The relative levels and expression of rRNA in cells have also been used as indicators of bacterial growth rates in biofilms (4, 9, 23, 52).

Since regulation of rRNA synthesis has been shown to be dependent on the growth status of cells during planktonic growth (8, 34, 45), we hypothesized that the amount of rRNA per cell could be used as an indicator of the growth status of small groups of cells at discrete locations in biofilms. To test this hypothesis, we used quantitative PCR (qPCR) and qRT-PCR techniques with both 16S rDNA (16S rRNA gene) and 16S rRNA from laser-microdissected biofilm samples. The copy number of DNA molecules could then be used as an internal control for determination of the number of cells per sample. The rRNA content could be determined on a per cell basis, which allowed predictive assessment of the growth state of bacteria isolated from different locations within the biofilms. In addition, since many of the cells in the biofilms studied here appeared to be in the stationary growth phase, we quantified the mRNA of two genes typically associated with stationary-phase growth and quorum sensing, *rpoS* and *rhlR*. The results described here provide information concerning the growth status of cells at discrete locations within biofilms and evidence indicating that *rpoS* and *rhlR* are expressed primarily at the top periphery of *P. aeruginosa* biofilms rather than in the deeper biofilm regions, where the cell density is high and growth is slow.

MATERIALS AND METHODS

Strains and conditions. *P. aeruginosa* PAO1 cultures were grown to exponential or stationary phase at 37°C in Luria-Bertani broth (LB) (2) in an orbital shaker incubator at 220 rpm. For analysis of planktonic cultures, 18-h cultures were grown from frozen stocks of *P. aeruginosa* PAO1 at 37°C in LB in an orbital shaker incubator at 220 rpm for 10 to 12 h. Cultures were diluted 1:50 in LB and incubated for an additional 2 h. These cultures were then diluted to obtain an optical density at 600 nm (OD_{600}) of 0.3 and used to inoculate (1:100) logarithmic-phase (4 h) or stationary-phase (10 h) planktonic cultures. *P. aeruginosa*(pMF230) was used for analysis of local gene expression from a constitutive promoter. Plasmid pMF230 contains the green fluorescent protein (*gfp*) gene behind the strong P_{trc} promoter (32). Since neither *P. aeruginosa* nor pMF230 contains the *lacI* repressor, *gfp* is constitutively expressed from this promoter.

Biofilms were cultivated on stainless steel coupons placed in a drip flow reactors (64) which allowed continuous flow of biofilm minimal medium (BMM) (44). Prior to inoculation into the system, cells were incubated in BMM for 18 h at 37°C and then transferred to fresh BMM and incubated for 4 h to obtain an OD_{600} of 0.2. The inoculum was diluted 20-fold in BMM, and 5 ml was used to inoculate steel coupons that were kept under static conditions for 25 min. The flow of BMM was initiated, and the medium was pumped through the reactors at a rate of 1.2 ml/min. The temperature of the reactors was maintained at 37°C. In separate experiments, colony biofilms were cultivated on LB agar (60). *P. aeruginosa* cells from planktonic cultures grown overnight at 37°C in LB were used for inoculation. The OD_{600} of the inocula was adjusted to 0.4 (path length, 1 cm) by dilution in LB, and 25 μl was used to inoculate presterilized (by UV treatment) black polycarbonate membrane filters (diameter, 13 mm; pore size, 0.2 μm; GE Water & Process Technologies) placed on LB agar plates (Difco Laboratories). The plates were incubated at 37°C for 72 h, and the membranes were transferred to fresh LB agar every 12 h. The final transfer was 4 h prior to cryoembedding.

Cryogenic processing and LCM. Biofilms were cryoembedded on dry ice using Tissue-Tek O.C.T. compound (Sakura Finetechnical Co.). The embedded biofilms were thin sectioned (5 μm) using a cryomicrotome at −20°C. Thin sections were placed onto membrane-coated microscope slides (P.A.L.M. Microlaser Technologies) and kept on dry ice until observation and laser microdissection. Laser capture microdissection (LCM) was performed using the Zeiss/P.A.L.M. Laser-MicroBeam system and a ×20 objective lens (27). Laser dissection and laser catapult were used to dissect and capture 24,000-μm^2 areas from the top, middle, and bottom sections of the biofilms.

Nucleic acid extraction. For planktonic culture analysis, exponential- and stationary-phase cultures were serially diluted over 4 orders of magnitude (1:10 through 1:10,000), and 10 μl of each diluted culture was used for nucleic acid extraction. Cultures were extracted in 1 ml of TriReagent (Molecular Research Center, Inc.). For biofilm cultures, microdissected areas of the biofilms were captured using laser catapult in 30 μl of TriReagent in the caps of microcentrifuge tubes. Samples obtained by LCM were added to 470 μl of TriReagent. A 3-μl aliquot of polyacryl carrier (Molecular Research Center, Inc.) diluted 1:10 was added to each sample. *lucI* RNA (1.7×10^4 copies; Promega, Corp.) was added to the TriReagent just prior to extraction as an internal control to account for RNA loss and degradation. Each extraction mixture was heated at 65°C for 5 min with occasional vortexing. Samples were cooled before bromochloropropane was added. Isolation of RNA and DNA was performed as described by the manufacturer of TriReagent and by Pérez-Osorio and Franklin (37). Purified RNA was treated with DNase to remove contaminating genomic DNA, using a Turbo DNA-free kit (Ambion, Inc.). The organic layer was kept on ice until DNA extraction could be resumed. Polyacryl carrier (3 μl of a 1:10 dilution) was added to the organic layer to facilitate DNA precipitation. RNA and DNA were stored at −80°C until qRT-PCR experiments were performed.

***In vitro* transcription standards and PCR standards.** To generate standard curves for qRT-PCR and qPCR, target RNA and DNA were generated by *in vitro* transcription (IVT) using a MEGAscript T7 kit (Ambion, Inc.) (16, 27) and by PCR. Primers were designed using Primer3 (42) and were purchased from Integrated DNA Technologies. For IVT, forward primer 5′-TAATACGACTCACTATAGGGGGTGGTTCAGCAAGTTGGAT-3′ and reverse primer 5′-TAAGGTTCTTCGCGTTGCTT-3′ were used to amplify an internal fragment of the *P. aeruginosa* 16S rRNA gene. RNA standards for *lucI* and *acpP* were generated by IVT using primers described previously (27). To make RNA standards to quantify *rpoS*, forward primer 5′-TAATACGACTCACTATAGGGTCGACCTGGATCTGACGAA-3′ and reverse primer 5′-TGACCACGATGATGAAGTGC-3′ were used; to quantify *rhlR*, forward primer 5′-TAATACGACTCACTATAGGGCTGGATGTTCTTGTGGTGGA-3′ and reverse primer 5′-CTGGGCTTCGATTACTACGC-3′ were used. Following IVT, products were analyzed by electrophoresis using a Bioanalyzer 2100 and an RNA Nano chip (Agilent Technologies). RNA standards were stored as aliquots at −80°C and were quantified using UV absorption at 260 nm prior to each assay. DNA standards were prepared by PCR amplification of *P. aeruginosa* PAO1 genomic DNA using the same primers that were used for generating the RNA IVT standards. DNA standards were purified using phenol-chloroform and were analyzed by gel electrophoresis. The DNA standards were stored as aliquots at −80°C. DNA standards were quantified using a Rotor-Gene 6000 instrument (Corbett Research) and a PicoGreen nucleic acid quantification kit (Molecular Probes) prior to each assay. Standard curves were generated by performing qRT-PCR or qPCR with RNA or DNA serially diluted in 8 μl/ml of polyacryl carrier (Molecular Research Center, Inc.) to obtain 10^2 to 10^8 nucleic acid molecules per reaction mixture.

qRT-PCR. A one-step QuantiTect probe RT-PCR kit (Qiagen) was used to measure 16S rRNA and *lucI* mRNA in duplex 25-μl reaction mixtures, using the dual-label probe approach (38). The primers and probes used for 16S RNA were forward primer 5′-CAAAACTACTGAGCTAGAGTACG-3′, reverse primer 5′-GCCACTGGTGTTCCTTCCTA-3′ (29) and probe 5′-JOE (6-carboxy-4′,5′-dichloro-2′,7′-dimethoxyfluorescein)-TCTACGCATTTCACCGCTACACAGGA-BHQ1 (Black Hole Quencher 1)-3′. The primers and probes used for *lucI* and *acpP* have been described previously (27). A QuantiTect SYBR green RT-PCR kit was used for SYBR green labeling analysis of *rpoS* and *rhlR* in 25-μl reaction

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mixtures (38). The primers used for *rpoS* were forward primer 5′-CGATCATC CGCTTCCGACCAG-3′ and reverse primer 5′-CTCCCCGGGCAACTCCAA AAG-3′. The primers used for *rhlR* were forward primer 5′-CTCAGGATGAT GGCGATTTC-3′ and reverse primer 5′-AATTTGCTCAGCGTGCTTTC-3′. Reaction mixtures were assembled using a CAS1200 liquid-handling system (Corbett Research).

The final primer and probe concentrations were optimized for each reaction. For the 16S rRNA and *lucI* duplex reactions the concentrations were 100 nM 16S rRNA forward primer and probe, 300 nM 16S rRNA reverse primer, 400 nM *lucI* forward and reverse primers, and 200 nM *lucI* probe. The concentrations of the *acpP* primers and probe were adjusted to obtain a final concentration of 400 nM for each primer and a final concentration of 200 nM for the probe. All *rpoS* and *rhlR* reactions were performed with primers at a final concentration of 300 nM. The Rotor-Gene 6000 instrument (Corbett Research) was used for all assays.

The following parameters were used for the reactions with dual-label probes: one cycle of 50°C for 30 min, followed by denaturation at 95°C for 15 min and then 45 cycles of 94°C for 15 s and 59°C for 60 s. Data were acquired during the 59°C annealing step. The temperature used for the combined annealing and acquisition step for *acpP* was 60°C. For SYBR green reactions the parameters used were one cycle of 50°C for 30 min, followed by denaturation at 95°C for 15 min, 45 cycles of 94°C for 15 s, 60°C for 30 s, and 72°C for 30 s, and then an additional step for data acquisition of 84°C for 15 s, which allowed measurements to be taken at a temperature where nonspecific amplification products did not contribute to the fluorescence signal. Three technical replicates were performed for each standard, and two technical replicates were performed for each experimental sample. Primer specificity was confirmed by electrophoresis using a Bioanalyzer 2100 DNA 500 chip (Agilent Technologies). Reverse transcriptase negative controls, no-template controls, and a mixture of standards (positive controls) were included in each assay.

Transcript copy numbers were calculated for each standard using the following formula: number of molecules/μl = (g/μl RNA/molecular weight) × Avogadro's constant. Using the Rotor Gene software (version 1.7, build 75; Corbett Research), the log-transformed concentration of each standard was plotted against the cycle number at which the fluorescence exceeded the background value (threshold cycle [C_T]). The slope of the calibration curve was used to determine the reaction efficiency (E) of the C_T with the equation $E = 10^{-1/\text{slope}} - 1$. An E of 1 indicates that there was exponential amplification of the product. The slope of the calibration curve was also used to calculate the number of transcript copies per reaction mixture. The levels of *lucI* recovered after RNA extraction were used to control for RNA loss.

qPCR. The one-step QuantiTect probe PCR kit (Qiagen) was used to measure 16S rDNA levels (38). The primer concentrations for 16S rDNA assays were 100 nM for the forward primer, 300 nM for the reverse primer, and 100 nM for the dual-label probe. The reactions conditions were one cycle of 95°C for 15 min, followed by 45 cycles of 94°C for 15 s and 59°C for 60 s. Data were acquired during the 59°C annealing step. The DNA copy number was determined using the calibration curve generated by performing qPCR with known amounts of 16S rDNA. No-template and positive standards were included in every assay as negative and positive controls, respectively.

Statistical analysis. To determine if the duplex reactions could be used quantitatively, we evaluated several aspects of their performance. The correlation coefficient for the linear range of standards was excellent (0.999) for values between 1×10^1 and 1×10^8 copies of 16S rRNA and between 1×10^2 and 7×10^8 copies of 16S rDNA. The *lucI* assay was also linear for values between 1×10^2 and 1×10^7 copies ($r^2 = 0.999$). Similar linear results were obtained for duplex reactions when the 16S rRNA assay was used with *lucI*. The efficiencies for these assays were 0.94 for the 16S rRNA qRT-PCR and qPCR and 0.99 for both the 16S rRNA and *lucI* qRT-PCR assays with the duplex reactions. The *acpP* qRT-PCR assay was linear for values between 5×10^1 and 5×10^5 copies ($r^2 = 0.999$), and the efficiency was 0.94. The evaluation of the characteristics of the SYBR green assays indicated that the correlation coefficient was >0.995 for values between 2×10^2 and 2×10^6 copies of *rpoS* and *rhlR*, and the efficiency was 0.91 for each of these assays. The average variability within each assay (technical replicates in an assay) and between replicate assays (technical replicates from separate assays) was 5%.

The significance of differences between the means for the 16S rRNA-to-16S rDNA ratios for the top, middle, and bottom samples from biofilms and from planktonic cells harvested during the stationary and exponential growth phases was assessed using the two-tailed Mann-Whitney test. This test allowed us to determine the appropriate ratio and number of 16S rDNA copies per cell for planktonic cultures to use for estimation of the number of 16S rDNA copies per cell for biofilm samples. When the average level of expression of mRNA transcripts was reported, the geometric mean of all measurements was used. The significance of differences between means was calculated using the two-tailed Mann-Whitney test.

RESULTS

Genome copy number for *P. aeruginosa* during planktonic growth. Bacteria growing in biofilms are often organized into dense communities of cells that are surrounded by extracellular matrix material. Determination of the number of cells in biofilms is generally performed computationally using scanning confocal laser microscopy (SCLM) images of fluorescently stained cells (20). Numbers of cells may also be determined by microscopic direct counting (DC) or by counting the CFU of bacteria dispersed from the biofilms. One goal of this study was to determine the number of cells obtained from biofilm subsections using laser capture microdissection (LCM) so that gene expression could be normalized on a per cell basis. Determination of the numbers of CFU using these samples was not possible since the biofilms were frozen and cryoembedded. Microscopic DC is difficult due to the limits of detection of DC and because dispersion of cells is required. Therefore, we used qPCR as a sensitive approach to determine the number of genome copies in order to estimate the size of the cell population in each LCM sample. Since the TriReagent nucleic acid extraction method allows isolation of both RNA and DNA from individual samples, we evaluated the effectiveness of TriReagent for extracting nucleic acids from samples containing very low numbers of cells, such as samples obtained by LCM. This approach was first tested with serially diluted planktonic cultures containing from 2×10^3 to 1×10^8 cells. Following nucleic acid extraction, 16S rRNA and 16S rDNA were quantified using qRT-PCR and qPCR. Multiplex reactions were carried out with dual-label probes for 16S rRNA and the spike-in control RNA (*lucI*) to examine RNA loss and degradation during the nucleic acid extraction steps. Figure 1A shows that there was a linear relationship between the number of cells determined by microscopic DC and the number of genome copies determined by qPCR ($r^2 > 0.96$) for cells incubated planktonically until the exponential (4 h) and stationary (10 h) phases. The linear range was more than 5 orders of magnitude, and the method was sensitive for as few as 2,000 cells.

P. aeruginosa PAO1 has four copies of the 16S rDNA gene per genome (50). Therefore, if each cell contained one copy of the genome, we predicted that there were four 16S rDNA genes per cell. However, the genome copy number may vary depending on the growth rate of the bacteria; cells that are growing faster have multiple initiation events, and therefore there are multiple genomes per cell. Figure 1B shows that there was more DNA per cell in exponential-phase cultures than in stationary-phase cultures. Planktonic cells growing in exponential phase had an average of 11 copies of 16S rDNA per cell, demonstrating that rapidly growing *P. aeruginosa* cells contain between two and three copies of the genome per cell. The stationary-phase cells had an average of seven copies of 16S rDNA per cell, indicating that these cells contained between one and two copies of the genome per cell. While certain bacteria exhibit polyploidy (54), these values for *P. aeruginosa* are in good agreement with the genome content reported for *Escherichia coli* (31).

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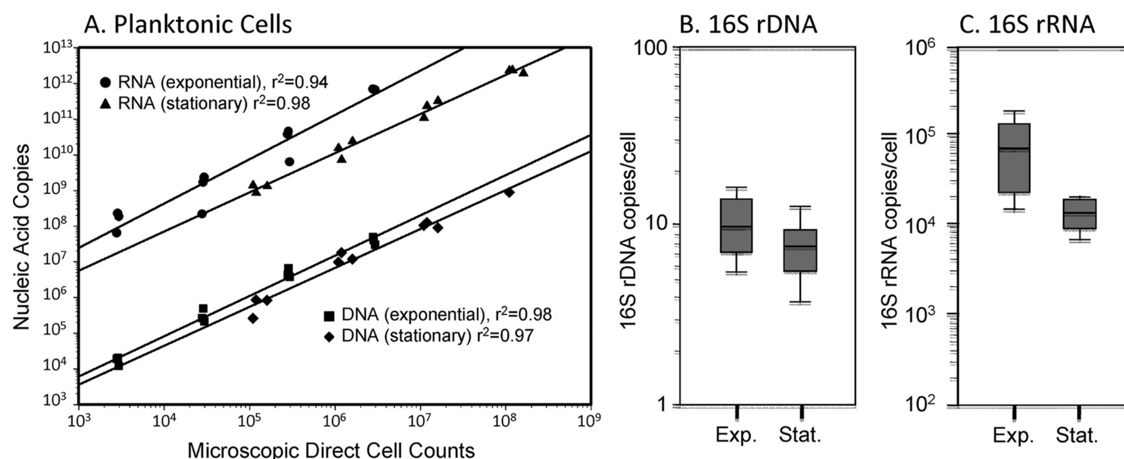

FIG. 1. Dynamic range and sensitivity of qPCR and qRT-PCR for *P. aeruginosa* planktonic cells. (A) RNA and DNA were obtained simultaneously from 2×10^3 to 2×10^8 *P. aeruginosa* cells. Concentrations of cells were determined by SYBR green staining and microscopic direct counting. The linear ranges were 5×10^3 to 5×10^8 copies of 16S rRNA ($r^2 > 0.94$) and 1×10^2 to 1×10^7 copies of 16S rDNA ($r^2 > 0.97$). The results of qRT-PCR and qPCR showed that the efficiency ($E > 0.97$) and linearity ($r^2 > 0.99$) were high for all assays. ●, 16S rRNA isolated from logarithmic-phase cells; ▲, 16S rRNA isolated from stationary-phase cells; ■, 16S rDNA isolated from logarithmic-phase cells; ◆, 16S rDNA isolated from stationary-phase cells. (B) Box plot showing the number of 16S rDNA copies per cell in the logarithmic (Exp.) and stationary (Stat.) phases, as determined by qPCR and microscopic direct counting. The vertical lines indicate the ranges for samples, and the filled boxes indicate the ranges for 75% of the samples. (C) Box plot showing the number of 16S rRNA copies per cell in the logarithmic and stationary phases, as determined by qRT-PCR and microscopic direct counting. The vertical lines indicate the ranges for all samples, and the filled boxes indicate the ranges for 75% of the samples.

Ribosome content of *P. aeruginosa* planktonic cultures. The 16S rRNA in planktonic cells growing in exponential and stationary phases was also measured to determine the ribosome copy number for these cells. As observed for the genome copy number, linear relationships were observed for microscopic DC and the number of rRNA copies ($r^2 > 0.93$) for both exponential- and stationary-phase cultures (Fig. 1A). As predicted, the cells in the exponential-phase cultures contained more rRNA per cell than the cells in the stationary-phase cultures. On average, exponential-phase cells had 7.3×10^4 copies of 16S rRNA per cell, as measured by qRT-PCR of the 16S rRNA (Fig. 1C). On average, stationary-phase cells had 7-fold less 16S rRNA (1.2×10^4 copies per cell). These values are in good agreement with previously published values for *E. coli* and demonstrate that the ribosome copy number was greater for rapidly growing bacteria than for cells in stationary phase (34).

rRNA/rDNA ratio used to estimate the growth status of cells in different regions of biofilms. The values for rRNA and rDNA from planktonic cells allowed us to estimate the ribosome copy number on a per cell basis for bacteria from defined locations in biofilms. For these experiments, colony biofilms were incubated for 72 h, and the medium was changed every 8 h. Colony biofilms receive nutrients from the bottom of the biofilm associated with the agar or membrane surface. The colony biofilms used in this study were up to approximately 400 μm thick. In addition, biofilms were cultivated in drip flow reactors for 4 days. Drip flow biofilms receive nutrients from the top layer at the air interface due to a continuous flow of minimal medium. Drip flow biofilms generally have more channels and mushroom-like shapes than colony biofilms, and the thickness can range from 200 to 500 μm (27).

The biofilms were cryoembedded and thin sectioned to obtain vertical transects. Areas of 2.4×10^4 μm² were obtained from the top (air-biofilm interface), bottom (adjacent to the substratum), and middle of the biofilms. Using the TriReagent extraction method, both the 16S rDNA content and the16S rRNA content were determined for individual laser-dissected samples. For the drip flow biofilms, the amounts of 16S rDNA per sample were similar for the top, middle, and bottom of the biofilms; the average amount was between 1×10^5 and 3×10^5 copies of 16S rDNA per LCM section (Fig. 2A). Similar amounts of 16S rDNA were also obtained from colony biofilms (Fig. 2B). The average amounts of 16S rRNA were determined by qRT-PCR; the results showed that the amounts were slightly larger at the top of both the drip flow and colony biofilms, but the values were not statistically significantly dif-

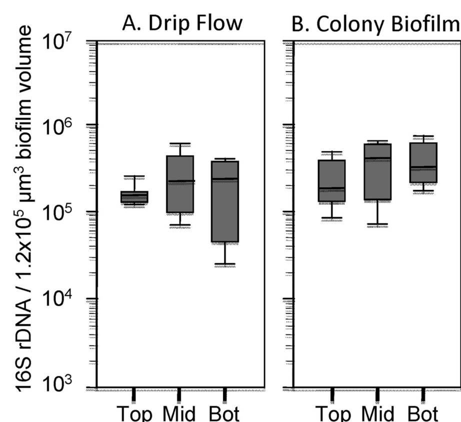

FIG. 2. 16S rDNA from biofilms. LCM was used to obtain 120,000-μm³ biofilm portions from the top, middle (Mid), and bottom (Bot) of the biofilms. The 16S rDNA in each sample was measured by using qPCR. (A) Drip flow biofilms. (B) Colony biofilms. The vertical lines indicate the ranges for samples, and the filled boxes indicate the ranges for 75% of the samples.

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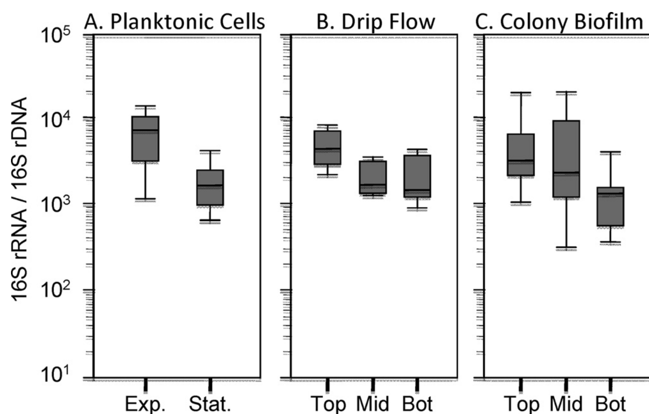

FIG. 3. Use of the 16S rRNA-to-rDNA ratio to estimate the growth states of cells in different regions of biofilms. (A) 16S rRNA/rDNA ratios for planktonic cells grown to the exponential (Exp.) and stationary (Stat.) phases. The ratios were significantly different ($P < 0.05$). (B) 16S rRNA/rDNA ratios for cells isolated from the top, middle, and bottom of drip flow biofilms. (C) 16S rRNA/rDNA ratios for cells isolated from the top, middle, and bottom of colony biofilms. Cells in the top of drip flow biofilms have a growth rate that is greater than the growth rates of cells in the middle and bottom layers, as well as the growth rate of planktonic stationary cells. The vertical lines indicate the ranges for samples, and the filled boxes indicate the ranges for 75% of the samples.

ferent from the values for the middle or bottom portions of the biofilms. Therefore, the ribosomal content in the biofilm layers is relatively uniform, in agreement with the findings of previous studies (27, 64).

In planktonic cultures the ribosomal content of exponentially growing cells is greater than the ribosomal content of stationary-phase cultures. The difference was quantified by using qPCR and qRT-PCR to obtain a ratio of 16S rRNA to 16S rDNA for cells growing in the exponential (4 h) or stationary phase (10 h) (Fig. 3A). Similar experiments were performed with the different biofilm layers to obtain the relative growth status of the bacteria in each of the different biofilm strata (Fig. 3B and C). Cells growing in the top layer of the drip flow biofilms had a 16S rRNA-to-16S rDNA ratio that was between the ratios for the exponential- and stationary-phase planktonic cells. This observation suggests that cells at the top of biofilms do not replicate as quickly as cells in exponential-phase planktonic cultures. In contrast, cells located in the bottom layer of the biofilms had a lower 16S rRNA/16S rDNA ratio that was not significantly different from the ratio for planktonic cells in stationary phase. The rRNA/rDNA ratio for cells at the bottom of the biofilms was significantly different from the ratio for planktonic cells in exponential phase ($P < 0.05$). Therefore, the cells at the top of the biofilms were likely in a transition state between exponential-phase growth and stationary-phase growth, while cells in the deeper portions of colony biofilms were in a stationary-phase-like growth state. Similar experiments were used to estimate the growth phase of cells isolated from colony biofilms. As observed with the drip flow biofilms, the rRNA/rDNA ratios for these cells indicate that the cells in the top layers were between the exponential growth phase and the stationary growth phase, while the cells in the deeper portions of the biofilms were not significantly different from cells in planktonic stationary-phase cultures (Fig. 3C).

The association between the 16S rRNA/16S rDNA ratios for planktonic and biofilm cells allowed us to determine the most appropriate estimator of the 16S rDNA content of each type of cells from the biofilms. The difference between the rRNA/rDNA ratio for planktonic cells growing exponentially and the rRNA/rDNA ratio for cells collected from the tops of the drip flow and colony biofilms was modest and not significant. Therefore, we estimated that these cells each had 11 copies of 16S rDNA, like exponentially growing cells. The rRNA/rDNA ratio for the middle layer of colony biofilms was not significantly different from the rRNA/rDNA ratio for either exponential or stationary planktonic cells; therefore, we estimated that the cells in this layer had approximately 9 copies of 16S rDNA per cell, as this number is between the measured values for exponential- and stationary-phase planktonic cells. The rRNA/rDNA ratios for cells harvested from the middle and bottom of drip flow biofilms and from the bottom of colony biofilms were significantly different from the rRNA/rDNA ratios for exponentially growing cells ($P < 0.01$) but not significantly different from the rRNA/rDNA ratios for cells in the stationary phase of planktonic growth. Therefore, for normalization, we estimated that these cells had seven 16S rDNA copies per cell, like stationary-phase planktonic cells.

To estimate the accuracy of our calculations, we calculated the biofilm volume occupied per cell based on qPCR results. The results were then compared to previously published transmission electron microscope (TEM) images of *P. aeruginosa* biofilms cultured in similar colony biofilms (60). Using the qPCR data, the estimated volume occupied by each cell at the top of colony biofilms was 4 μm^3. Cells in the deeper layers were more densely packed, and each cell occupied approximately 2 μm^3. These values are in general agreement with the TEM analyses that showed that cells each occupied approximately 1 to 2 μm^3 and that there was denser packing of cells in the deeper regions of the biofilms (60). The values calculated here that were slightly higher than the values based on the TEM analyses were likely due to the lack of 100% efficiency for either the laser capture of cells or the DNA extraction procedure.

Abundance of mRNA associated with stationary-phase growth and quorum sensing in biofilm strata. Cells isolated from subsections of biofilms using the LCM technique have been shown to contain various concentrations of mRNA depending on the location from which the sample was obtained (27). Since there are no known appropriate housekeeping genes that can be used as internal normalization factors for different biofilm strata, we normalized mRNA data by estimating the numbers of cells using the number of genome copies. By using this normalization technique with biofilm samples, we were able to determine levels of expression on a per cell basis. Since the cells in all regions of the biofilms had characteristics of stationary-phase growth or a transition to stationary phase, we analyzed expression of *rpoS* and *rhlR* as indicators of the stationary phase and quorum sensing. The sigma factor RpoS has been shown to regulate genes that respond to environmental challenges and to play a role in biofilm development (51, 58, 59, 63). In colony biofilms, expression of *rpoS* was localized in the top layer of the biofilms (Fig. 4A). The average number of *rpoS* transcripts per cell for the top of colony biofilms was

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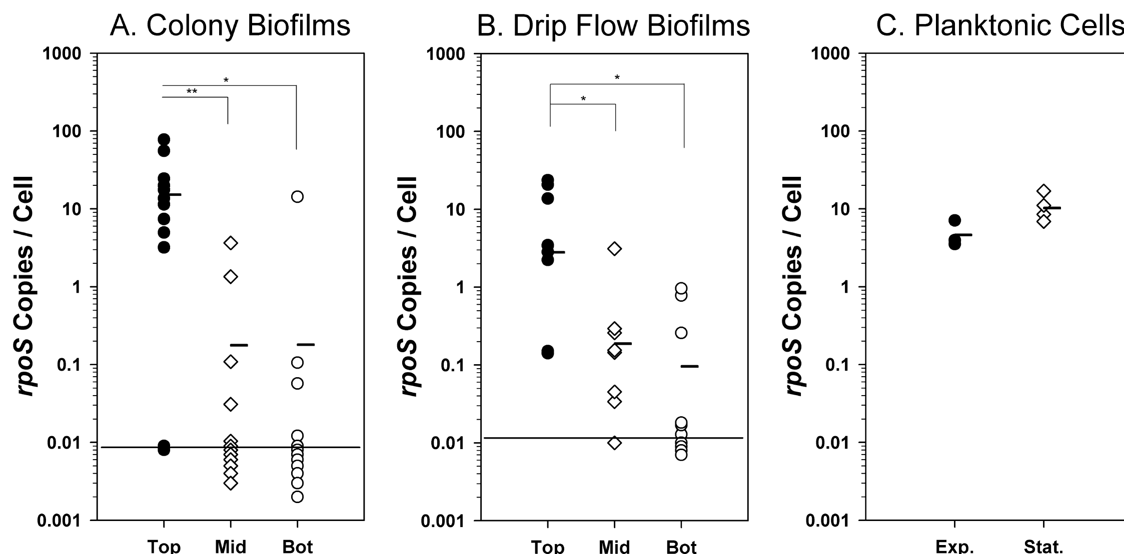

FIG. 4. Numbers of copies of the *rpoS* mRNA transcript per cell in biofilms. (A) LCM samples were obtained from the top, middle, and bottom of colony biofilms, and the number of copies of the *rpoS* transcript was compared to the number of cells, as determined by qPCR of 16S rDNA. The average numbers of *rpoS* copies per cell are indicated by bars. The horizontal lines indicate the limit of detection of the technique. *, $P < 0.05$; **, $P < 0.01$. The number of mRNA copies per cell was calculated as described in the text. (B) Analysis of *rpoS* for drip flow biofilms. (C) Amounts of *rpoS* per cell in exponential- and stationary-phase planktonic cultures. Mid, middle; Bot, bottom; Exp., exponential phase; Stat., stationary phase.

approximately 15 (range in this region of the biofilms, 3 to 90 *rpoS* mRNA transcripts per cell). This average number was approximately 2 orders of magnitude greater than that for the middle or bottom of the biofilms, in which the average was less than one *rpoS* transcript per cell. In most samples from the middle and bottom of the biofilms, the *rpoS* abundance was below the limit of detection of the technique. In drip flow biofilms, the *rpoS* abundance was generally lower than that in colony biofilms. However, as observed with colony biofilms, the *rpoS* mRNA abundance was greater in the top of the biofilms than in the middle or bottom of the biofilms (Fig. 4B). Less than one *rpoS* transcript per 10 cells was observed in the middle and bottom of the drip flow biofilms. The average level of *rpoS* transcripts in the top of the biofilms was similar to the levels found in planktonic cells, in which on average there were 5 *rpoS* transcripts per cell in exponential-phase cultures and 11 transcripts per cell in stationary-phase cultures (Fig. 4C).

The transcriptional regulator RhlR coordinates the expression of many pathogenic determinants in *P. aeruginosa* and is activated in a density-dependent manner by the autoinducer *N*-butyryl-homoserine lactone, a product RhlI (6, 36, 39). Since their expression is density dependent, RhlR and RhlI have been implicated in biofilm developmental processes (10, 46). Expression of *rhlR* and *rhlI* is maximal during the transition from logarithmic phase to stationary phase in planktonic cultures (7). For the colony biofilms, expression of *rhlR* was greatest in the top layer of the biofilms. On average, in the top layer of colony biofilms there were 25 *rhlR* transcripts per cell (Fig. 5A). The averages for the middle and bottom layers of these biofilms were 1 and 0.5 transcripts per cell, respectively, and many of the samples contained no detectable *rhlR* mRNA. The *rhlR* mRNA levels were greater in the top of these biofilms than in planktonic cells in the exponential phase and stationary phases, in which the average levels were 9 and 4 *rhlR* mRNA copies per cell, respectively (Fig. 5C). While drip flow biofilms had lower levels of *rhlR* mRNA than colony biofilms, they also had the highest levels of *rhlR* in the top of the biofilms (approximately 8 transcripts per cell, compared to less than 1 *rhlR* mRNA per cell in the middle or bottom layers of the biofilms) (Fig. 5B).

We previously reported that the highest level of mRNA of the housekeeping gene *acpP* was found at the top of drip flow and colony biofilms (27). Here we further characterized the amounts of *acpP* on a per cell basis by normalization using the genomic DNA content (Fig. 6). In drip flow biofilms, the concentration of *acpP* mRNA in cells collected from the top layer was approximately 10 *acpP* mRNA transcripts per cell, while the other layers did not contain more than 1 copy of *acpP* mRNA per cell, indicating that cells in the deeper regions of the biofilms were in a low metabolic state. To further characterize the metabolic status of cells throughout the biofilms, we analyzed the abundance of *gfp* mRNA when it was expressed from the constitutive P_{trc} promoter. The mean level of *gfp* mRNA ($n = 24$) was 33-fold greater at the top of the biofilm than at the base of the biofilm, and it was significantly different from the values for both the middle ($P = 0.002$) and bottom ($P = 0.009$) of the biofilms. The *gfp* mRNA levels for the middle and bottom of the biofilms were low and not significantly different from each other ($P = 0.65$). The small amounts of *acpP* mRNA and the *gfp* expression from a constitutive promoter in the middle and base of the thick *P. aeruginosa* biofilms indicate that the cells had very little transcriptional activity and may have been in a late-stationary-phase-like state.

DISCUSSION

Chemical gradients in biofilms result in discrete localized microenvironments (49). Bacteria respond to the local environmental conditions with diverse transcriptional and enzy-

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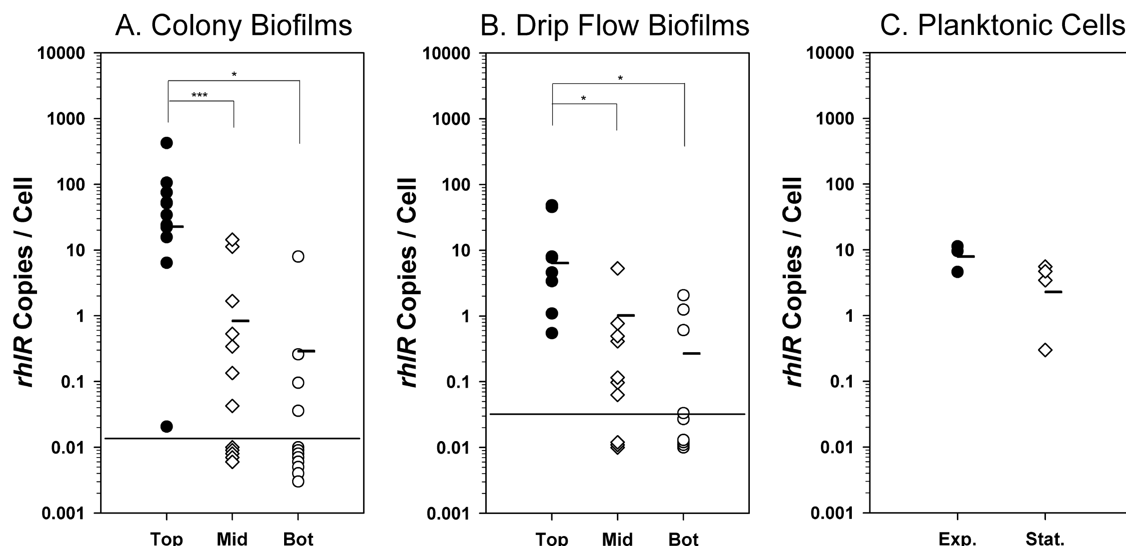

FIG. 5. Numbers of copies of the *rhlR* mRNA transcript per cell in biofilms. (A) LCM samples were obtained from the top, middle, and bottom of colony biofilms, and the number of copies of the *rhlR* transcripts per cell was determined based on qPCR of 16S rDNA, as described in the text. The bars indicate the average numbers of *rhlR* copies per cell, and the horizontal lines indicate the limit of detection of the technique. *, $P < 0.05$; ***, $P < 0.005$. The approximate numbers of copies per cell were calculated as described in the text. (B) Analysis of *rhlR* for drip flow biofilms. (C) Amounts of *rhlR* per cell in exponential- and stationary-phase planktonic cultures. Mid, middle; Bot, bottom; Exp., exponential phase; Stat., stationary phase.

matic activities. Techniques such as fluorescent *in situ* hybridization (FISH) combined with microautoradiography (MAR) have been developed to localize the bacteria and to characterize their enzymatic activities *in situ* (21, 24, 26, 35, 57). In addition, fluorescent reporter genes combined with SCLM have been used to characterize localized transcriptional activities of the biofilm bacteria (25, 48, 52, 62). With improvements in the sensitivity of techniques such as qRT-PCR for gene expression studies, it is now possible to analyze mRNA abundance for very small numbers of bacterial cells. Since this method does not require prior genetic manipulation to insert reporter genes, it is applicable to any microbial cell or biofilm, including cells or biofilms from natural multispecies communities. In a previous study, we combined qRT-PCR with laser capture microdissection microscopy (LCM) to study gene expression (based on mRNA abundance) for cells obtained from local sites in biofilms (27). The results of that study indicated that the mRNA abundance values for individual genes, including housekeeping genes, vary by orders of magnitude over relatively small distances within biofilms. The sharp differences in housekeeping transcript levels in biofilm strata resulted in a need to develop appropriate internal controls for normalization of gene data in qRT-PCR studies. Therefore, one goal of the present study was to determine if 16S rDNA and/or 16S rRNA could be used to normalize expression of genes responsive to the local environmental conditions. A second goal was to determine if qPCR could be used to estimate numbers of cells in biofilm samples by determining the genome copy number. By generating data for genome copy number and amounts of 16S rRNA, it was also possible to use the ratio of rRNA to rDNA to estimate the growth status of cells in different regions in biofilms, which has been done previously for planktonic cells (43).

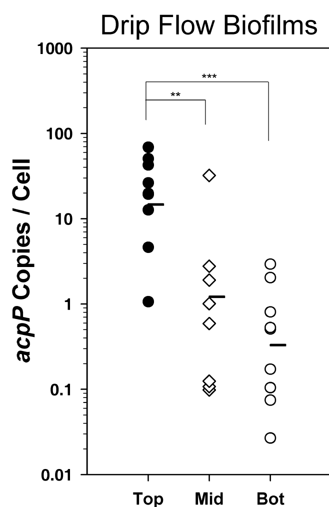

FIG. 6. Numbers of copies of the *acpP* mRNA transcript per cell in drip flow biofilms. LCM samples were obtained from the top, middle, and bottom of drip flow biofilms, and the number of copies of the *acpP* transcript per cell was determined as described for *rpoS* and *rhlR* in the legends to Fig. 4 and 5. The bars indicate the average numbers of *acpP* copies per cell. **, $P < 0.01$; ***, $P < 0.005$. Mid, middle; Bot, bottom.

We first established that both RNA and DNA could be efficiently extracted from a sample containing as few as 2,000 *P. aeruginosa* cells. We then evaluated the design of the quantitative assay for detection and quantification of 16S rDNA, 16S rRNA, and mRNA transcripts of *rpoS* and *rhlR*. The assays were validated and found to have excellent efficiency and sensitivity, as well as a broad dynamic range (>4 orders of magnitude). The uniform distribution of 16S rDNA in different regions of biofilms suggests that DNA may provide the most useful internal control for transcriptional studies of cells isolated from a biofilm by LCM. This possibility assumes that the

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growth rate of the cells in the biofilms can be determined, since there may be multiple genome copies per cell (31, 54). Given that qPCR, DC, and counting CFU yielded similar results for planktonic cultures, we concluded that normalization using DNA is a reliable option for transcriptional studies of samples with limited quantities of cells. The qPCR method has the advantage that cell cultivation is not required and that the lower limit of detection of qPCR is greater than that of direct microscopic counting. One disadvantage of this approach is that if cell material or DNA is lost during laser catapult or DNA extraction, the number of cells may be underestimated. A second disadvantage is that this approach cannot be used to distinguish viable cells from dead cells or cells that may have lysed and released extracellular DNA. However, even with these disadvantages, the qPCR approach appears to be accurate for estimating numbers of cells. The numbers of cells per biofilm volume described here were similar to direct counts obtained from previously published TEM images of similar biofilms (60). Therefore, DNA is a preferred internal control for these studies compared to less appropriate alternatives, such as housekeeping gene mRNAs, whose levels vary drastically throughout the biofilm depth (27).

Expression of rRNA can be used to estimate the growth status of cells. Exponentially growing cells have greater expression and larger amounts of 16S rRNA per cell than stationary-phase cultures, which was confirmed here. Cells in exponentially growing *P. aeruginosa* cultures had approximately 7×10^5 rRNA copies per cell, while cells in stationary-phase cultures contained approximately 2×10^5 rRNA copies per cell. The assays used here quantified the abundance of rRNA, which included both RNA expression and stability. The cells in the middle and bottom portions of both drip flow and colony biofilms contained approximately 2×10^5 rRNA copies per cell. This suggests that cells in the middle and bottom layers of these biofilms were likely in a stationary-phase-like growth state. It also suggests that even as these regions of a biofilm age, cells still contain a certain number of ribosomes. Since rRNA not associated with ribosomes is rapidly degraded (14), cells in the deeper portions of the biofilms contain approximately 20,000 ribosomes per cell. In *E. coli*, ribosomes are kept in an inactive state by ribosome hibernation factors (67). The relatively high levels of rRNA in the middle and bottom portions of the biofilms were likely due to the stability of rRNA in hibernating ribosomes of the late-stationary-phase cells instead of active expression of rRNA. While the top portions of the biofilms had higher levels of rRNA (approximately 4×10^5 rRNA copies per cell) than the middle and bottom portions of the biofilms, the ribosomal content was not as high as that in exponentially growing planktonic cultures. Therefore, cells at the air-biofilm interface were not in the exponential phase but were likely in transition from the exponential phase to the stationary phase.

Using estimates of cell numbers and the growth status for biofilm subsections, we determined localized levels of mRNA for genes previously shown to be important in biofilm development. The levels of mRNA obtained for *P. aeruginosa* planktonic cells and for the top portions of the biofilms are in agreement with other results indicating that each bacterial cell contains a few copies of individual mRNAs (55). The results for localized levels of *rpoS* and *rhlR* were not intuitive, since the levels of both of these genes were highest at the top of the biofilms. *rpoS* is expressed in both the exponential and stationary growth phases, but the expression in stationary phase is greater. The results described here provide further evidence that cells at the top of a biofilm may be in transition between these phases. Cells at the top of the biofilms at the air-biofilm interface contained between a few copies and several copies of *rpoS* mRNA per cell. The cells in the middle and bottom portions of the biofilms contained little or no *rpoS* mRNA. Therefore, while cells in the deeper regions of a biofilm still contained many ribosomes, they had transitioned beyond the stationary phase and no longer contained mRNA for the stationary-phase sigma factor.

The results for *rhlR* mRNA levels in different regions of a biofilm seem to contradict the results of a previous study in which reporter gene analysis of *rhlI* expression in biofilms was used (11). The previous study indicated that greater numbers of cells express *rhlI* in the deeper regions of a biofilm. The differences in the results described here and the results reported previously are likely due to the different biofilm cultivation systems. De Kievit et al. (11) used a flowthrough system that resulted in biofilms that were approximately 30 to 50 μm thick. In the present study, biofilms were up to 10-fold thicker when both the drip flow and colony biofilm formats were used. Expression of the quorum-sensing regulator *rhlR* was greatest at the top of these biofilms. While the density of cells in the deeper regions of these biofilms was high where one would expect the greatest quorum-sensing activity, the cells in the deeper regions were probably less active metabolically due to oxygen limitation or lack of another essential nutrient (22, 41, 66). The slow metabolism of cells in the bottom of the biofilms likely inhibited the transcription of genes, even those involved in stationary-phase metabolism and quorum sensing. This possibility was substantiated by the lower ratio of rRNA to rDNA in the deeper portions of the biofilms, as well as by the low levels of expression of the housekeeping *acpP* gene and the normally strong constitutive P_{trc} promoter. *acpP* is considered a housekeeping gene that is involved in the synthesis of membrane lipids. Since *acpP* was not expressed in the deep portions of the biofilms, cells in this region were probably not actively dividing. Therefore, even the cells in the deep portions of the biofilm were densely packed, which may have induced quorum sensing, and they were not in a metabolic state conducive to expression of genes involved in quorum sensing. These results are in agreement with our previous finding that the abundance of mRNA of *aprA*, a gene regulated by quorum sensing, was greatest at the top of thick biofilms (27).

Transcriptional profiling studies provide valuable insights into gene expression in biofilms. Biofilms have multiple phenotypic traits even within individual biofilm communities. While global transcriptional profiling provides average gene expression values for the entire community, LCM in combination with qRT-PCR provides a tool for characterizing local gene expression patterns. Analysis of rRNAs allows this approach to be used for studies of natural biofilm communities, including investigations of genetic processes in mixed-species biofilms on a per cell basis.

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