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Contribution of Stress Responses to Antibiotic Tolerance in *Pseudomonas aeruginosa* Biofilms

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Enhanced tolerance of biofilm-associated bacteria to antibiotic treatments is likely due to a combination of factors, including changes in cell physiology as bacteria adapt to biofilm growth and the inherent physiological heterogeneity of biofilm bacteria. In this study, a transcriptomics approach was used to identify genes differentially expressed during biofilm growth of *Pseudomonas aeruginosa*. These genes were tested for statistically significant overlap, with independently compiled gene lists corresponding to stress responses and other putative antibiotic-protective mechanisms. Among the gene groups tested were those associated with biofilm response to tobramycin or ciprofloxacin, drug efflux pumps, acyl homoserine lactone quorum sensing, osmotic shock, heat shock, hypoxia stress, and stationary-phase growth. Regulons associated with Anr-mediated hypoxia stress, RpoS-regulated stationary-phase growth, and osmotic stress were significantly enriched in the set of genes induced in the biofilm. Mutant strains deficient in *rpoS*, *relA* and *spoT*, or *anr* were cultured in biofilms and challenged with ciprofloxacin and tobramycin. When challenged with ciprofloxacin, the mutant strain biofilms had 2.4- to 2.9-log reductions in viable cells compared to a 0.9-log reduction of the wild-type strain. Interestingly, none of the mutants exhibited a statistically significant alteration in tobramycin susceptibility compared to that with the wild-type biofilm. These results are consistent with a model in which multiple genes controlled by overlapping starvation or stress responses contribute to the protection of a *P. aeruginosa* biofilm from ciprofloxacin. A distinct and as yet undiscovered mechanism protects the biofilm bacteria from tobramycin.

The enhanced antibiotic tolerance of microorganisms growing in biofilms has been recognized for decades (1) yet remains an unsolved problem. The problem is unsolved both at the level of a fundamental understanding of the tolerance mechanisms and at the development of improved therapies for biofilm infections. These two aspects are interrelated. Insight into the mechanisms of biofilm antibiotic tolerance might lead to the identification of new targets for curing persistent biofilm infections. Therapies that work *in vivo* would provide clues about the mechanisms of biofilm antibiotic tolerance. Several studies have reported on the increased tolerance of *Pseudomonas aeruginosa* growing in biofilms compared to that of the same strain growing planktonically (2–4). Published data on biofilm and planktonic susceptibility of *P. aeruginosa* to ciprofloxacin and tobramycin, which is also the model system examined in this work, are summarized in Table 1. These comparisons consistently demonstrate enhanced tolerance of biofilm cells to these two drugs. We note that the results of Folsom et al. (2), who used an experimental system identical to that reported in this article, establish the suitability of the drip-flow biofilm model as a basis for investigating biofilm antibiotic tolerance. When biofilms grown in this model were mechanically dispersed into a suspension and then treated with antibiotic, susceptibility was fully restored (2). This result confirmed that the reduced susceptibility of these biofilm cells was not due to the accumulation of antibiotic-resistant mutants in the biofilm.

Most antibiotics penetrate biofilms, suggesting that diffusion of the antibiotics into the biofilm is not the major source of biofilm tolerance (5, 6). Direct measurement of the delivery of fluoroquinolone antibiotics, such as ciprofloxacin, has consistently demonstrated the ready access of these agents to the biofilm bacteria (7–11). In comparison, aminoglycoside antibiotics have slower biofilm penetration (10, 11). The retarded delivery of these drugs is hypothesized to derive from binding of the cationic aminoglycoside to negatively charged polymers in the biofilm matrix. Sorption, however, is not a mechanism of exclusion. Over longer exposure times comparable to those realized *in vivo*, even aminoglycosides are expected to penetrate biofilm cells (5, 10).

Biofilm tolerance is not primarily an issue of resistance mechanisms, such as the acquisition of a mutation or mobile genetic element that confers heritable protection (12). A common feature of biofilm tolerance is that antibiotic susceptibility can be restored by physically dispersing microbial cells from the biofilm and growing them in planktonic mode. This suggests that biofilm tolerance to antibiotics is associated with a reversible phenotypically derived state. Heritable resistance mechanisms may arise in biofilms over the long term (13, 14), just as they do in planktonic cultures.

A number of gene products with enzymatic or structural properties, including PA0084 (*tssC1*) (15), PA1163 (*ndvB*) (16), PA1875 to PA1877 (17), and PA3063 (*pelB*) (18), have been reported to modulate *P. aeruginosa* biofilm susceptibility to antibiotics. However, if multiple functions are required for biofilm

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TABLE 1 Antibiotic tolerance of biofilms of *P. aeruginosa* strain PAO1

Model system	Agent[a]	Log reduction in: Biofilm cells	Log reduction in: Planktonic cells	Tolerance factor[b]	Reference
Drip-flow	TOB	0.72	3.18	4.4	2
Drip-flow	CIP	1.37	4.84	3.5	2
Colony biofilm	TOB	0.52	5.67	10.9	3
Colony biofilm	CIP	1.13	5.05	4.5	3
Silicone tube	TOB	0.35	3.20	9.1	4

[a] TOB, tobramycin; CIP, ciprofloxacin.
[b] The tolerance factor is the log reduction in viable counts for planktonic cells divided by the log reduction in viable counts for biofilm cells.

TABLE 2 Bacterial strains

Strain	Relevant characteristics	Source
PAO1	Wild type	MJF archive
PW3784	*anr*-E05::IS*lacZ/hah*	39
PW7151	*rpoS*-B03::IS*lacZ/hah* Δ*relA* Δ*spoT*	39 19
PW2812	*mvfR*-G11::IS*lacZ/hah*	39
PW9205	*brlR*-C05::IS*lacZ/hah*	39
PAO216	Δ*lasI* Δ*rhlI*	40

antibiotic tolerance or if there is redundancy in protective gene functions, a single mutation may show little detectable phenotypic difference in biofilm antibiotic tolerance. It is likely that the genetic basis of biofilm protection from antibiotics derives from multiple overlapping responses involving dozens of different genes. Several regulators of multiple genes have been shown to affect *P. aeruginosa* antibiotic tolerance, including PA0934 and PA5338 (*relA* and *spoT*) (19), PA4878 (*brlR*) (4, 20), and PA5332 (*crc*) (21).

The focus of antibiotic tolerance thus shifts to an understanding of the physiology of the cells within a biofilm, which are inherently heterogeneous. Individual biofilms may contain subpopulations of actively growing cells, nongrowing cells (10, 22–27), bacteria undergoing stress responses (19, 28–35), and cells in a persister state (36, 37). Since differential gene expression precedes entry of the cells into each of these physiological states, we predict that as biofilm cells enter protected phenotypic states, a subset of the differentially expressed genes will contribute to antibiotic tolerance. Therefore, the goal of this research was to identify classes of genes that are differentially expressed in *P. aeruginosa* biofilms during the biofilm antibiotic-tolerant state and to determine if induction of those groups of genes plays a role in the protection of *P. aeruginosa* from antibiotic treatments.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and antibiotics. *P. aeruginosa* strain PAO1 and its mutant derivatives (Table 2) were cultured in *Pseudomonas* basal mineral medium (PBM) (38) containing 0.2 g liter^{-1} glucose for experiments measuring growth or antibiotic susceptibility. Inocula were grown in the same medium containing 1 g liter^{-1} glucose. Mutant strains with transposon insertions in *anr*, *rpoS*, *mvfR*, and *brlR* were obtained from the University of Washington *Pseudomonas aeruginosa* two-allele library (39). The *P. aeruginosa* Δ*relA* Δ*spoT* mutant was graciously provided by Pradeep Singh (19), and the *P. aeruginosa* Δ*lasI* Δ*rhlI* mutant was graciously provided by Herb Schweizer (40). Cultures were prepared in shake flasks at 37°C with agitation at 200 rpm. Tobramycin sulfate and ciprofloxacin hydrochloride were obtained from Sigma-Aldrich. Viable cell numbers were determined as CFU on tryptic soy agar (TSA) (Becton Dickinson).

Drip-flow biofilm growth. Biofilms were formed in the continuous flow drip-flow biofilm reactor (41) using PBM supplemented with 0.2 g liter^{-1} glucose. This reactor contained four parallel chambers that were covered with polycarbonate lids containing septa for the introduction of medium using 22-gauge needles and a filtered air vent. Medium was pumped into the chambers at a flow rate of 60 ml h^{-1}, dripping onto the stainless steel slides (8.5 cm by 1.3 cm) placed in the chambers. The reactors were placed on a stand inclined at 10° from horizontal, and PBM flowed along the length of the coupon and drained from the reactor. The reactors were inoculated by adding 1 ml of an overnight culture to 15 ml of fresh PBM to cover the slides (inoculum optical density at 600 nm [OD_{600}], ≈0.3) in PBM (1 g liter^{-1} glucose). The reactor was sealed by clamping the effluent tubes, and the inoculum was allowed to incubate in the reactor for 18 to 24 h on a level surface. After the inoculation period, the reactor was inclined, and the medium flow was initiated. The entire drip-flow reactor system was maintained at 37°C. The biofilms were cultured in the drip-flow reactors for 72 h after the static inoculation phase.

Antibiotic susceptibility of biofilms. After 72 h of growth in the absence of antibiotic, antibiotics were added to the growth medium, and the flow with the medium containing antibiotic was continued for an additional 12 h. Tobramycin was applied at 10 μg ml^{-1} and ciprofloxacin at 1.0 μg ml^{-1}. After treatment, the stainless steel coupons were removed from the reactor, and the number of viable cells was determined by scraping the biofilms into 10 ml of phosphate-buffered saline and then vortexing, sonicating, and vortexing again to disperse cell aggregates. The resulting cell suspensions were serially diluted and plated on TSA. Killing was reported in log reductions. The log reduction was calculated relative to the cell count at the onset of treatment. The experiments were performed at least in triplicate.

Preparation of biofilms and planktonic cultures for microarrays. Biofilms were grown in the drip-flow reactor for 72 h and then treated with either tobramycin ($n = 3$) or ciprofloxacin ($n = 3$) for 12 h, as detailed above. Immediately after treatment, biofilms were scraped directly into 1 ml of RNA*later* (Ambion). Clumps were dispersed by repeated pipetting with a micropipette, and the recovered biofilms were stored at 4°C for 1 day prior to removal of the RNA*later* by centrifugation (15 min, 4°C, and 14,000 × *g*) and freezing of the biofilm cells at −70°C.

The antibiotic-treated biofilm cells were thawed on ice, resuspended in 300 μl of 1 mg lysozyme ml^{-1} and Tris-EDTA (TE) buffer (10 mM Tris, 1 mM EDTA [pH 8.0]), and divided into three aliquots. Each aliquot was sonicated for 15 s, incubated at room temperature for 15 min, and then combined for application onto one extraction column. RNA was extracted with an RNeasy minikit (Qiagen Sciences) with on-column DNA digestion. RNA concentrations and purity were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). RNA quality was evaluated using the RNA 6000 NanoChip assay on a 2100 Bioanalyzer (Agilent Technologies).

Four *P. aeruginosa* planktonic cultures were grown from frozen stocks in *Pseudomonas* basal medium containing 1.0 g liter^{-1} glucose overnight. Thirty microliters of each of these cultures was used to inoculate 30 ml of PBM (1 g liter^{-1} glucose). The cultures were allowed to incubate at 37°C with orbital shaking at 200 rpm for 5 h. Exponential-phase cells (OD_{600}, ~0.26) were collected by centrifugation at 11,000 × *g* and 4°C for 3 min. The supernatant was carefully removed, and 1 ml of RNA*later* (Ambion) was added to the cell pellet and mixed thoroughly by a pipette. RNA extraction was then performed as described for the biofilm samples, except that samples were not divided or sonicated. Four planktonic biological replicates were processed independently.

Microarray hybridization. Isolated total RNA (10 μg for antibiotic-treated biofilms and 8 μg for planktonic cultures) was reverse transcribed, fragmented using DNase I, and biotin end labeled according to the Affymetrix prokaryotic target labeling protocol (GeneChip expression anal-

ysis technical manual, November 2004). For each *Pseudomonas* genome array (catalog no. 900339; Affymetrix), labeled fragmented cDNA (4.5 μg from antibiotic-treated biofilms or 3 μg from planktonic cultures) was hybridized to the arrays at 50°C for 16 h with constant rotational mixing at 60 rpm. Washing and staining of the arrays were performed using the Affymetrix GeneChip fluidics station 450. The arrays were scanned using an Affymetrix GeneChip scanner 7G and GCOS software version 1.4.

Analysis of biofilm and planktonic microarray data. The Affymetrix .CEL files were imported into FlexArray version 1.6.1 for quality control, normalization, and data analysis (M. Blazejczyk, M. Miron, R. Nadon, Génome Québec, Montreal, Canada) Previously published data from untreated biofilms grown in the drip-flow reactor for either 72 h ($n = 3$) or 84 h ($n = 3$) were imported into FlexArray and pooled. Microarray data were background corrected and normalized using the GeneChip robust multiarray averages (GC-RMA) algorithm. Gene expression in untreated biofilms and planktonic cells was compared with an analysis of variance (ANOVA) and false-discovery rate (FDR) correction using the Benjamini Hochberg algorithm to generate a list of genes with expression changes of >4-fold at an adjusted *P* value of ≤0.01 (see Table S1 in the supplemental material). A similar analysis was performed to identify changes in gene expression between untreated biofilms and antibiotic-treated biofilms of >4-fold at a *P* value of ≤0.01 (see Table S2 in the supplemental material).

To identify the physiological activities represented by the gene lists, the lists were compared to lists of genes associated with particular responses or activities, such as iron limitation, quorum sensing, or nitrosative stress. These activity gene lists were compiled from the literature, as documented in Table S3 in the supplemental material. *P* values for assessing the statistical significance of gene set enrichment were calculated using a negative binomial distribution. The gene lists were also uploaded into the Database for Annotation, Visualization and Integrated Discovery (DAVID) for the identification of enriched biological themes with the functional annotation clustering tool (43, 44).

Microarray data accession no. The data discussed in this publication have been deposited in the NCBI Gene Expression Omnibus (42) and are accessible through GEO series accession no. GSE65882.

RESULTS AND DISCUSSION

Anabolic and catabolic pathways are downexpressed in *P. aeruginosa* biofilms. Microarray-based transcriptional profiling was used to develop insight into the physiological activities of biofilm bacteria. Based on a 4-fold difference in transcript abundance and an adjusted *P* value of ≤0.01, *P. aeruginosa* had 340 genes with increased expression in 3-day drip-flow biofilms, compared to exponential-phase planktonic cells, and 683 genes with reduced expression in the biofilms (see Table S1 in the supplemental material).

We used DAVID analysis (43, 44) to identify the metabolic pathways associated with these differentially expressed genes (Table 3). Clusters of genes that were significantly enriched in the list of genes with increased expression in biofilm samples included genes for nitrogenous compound oxidoreductase activity, heme or heme-copper binding, and organic acid catabolic processes (Table 3). The first two of these groups are likely associated with anaerobic and microaerophilic respiration, respectively. Numerous pathways were significantly reduced in expression in the biofilm samples. Catabolic pathways, including genes for the tricarboxylic acid (TCA) cycle, NADH dehydrogenase activity, and cellular respiration, were downexpressed in biofilms. Transcription was also reduced for many anabolic pathways, including amino acid and nucleotide biosynthesis, translation, lipid biosynthesis, and DNA metabolism. The downexpressed pathways were not surprising based on our previous studies of heterogeneity in biofilms (26, 45). In those studies, we demonstrated that in thick *P. aeruginosa* biofilms, the lower portion of the biofilm had transitioned into an oxygen-starved low metabolic state, while the upper portion of the biofilm was in a transition state to stationary-phase growth. This would result in reduced overall anabolic and catabolic activities for the entire biofilm. Since the analysis here was performed on whole biofilms, the average expression of the catabolic and anabolic pathways showed reduced expression compared to that of exponential-phase planktonic cells.

TABLE 3 Gene enrichment analysis of *P. aeruginosa* biofilms compared to planktonic cells based on Gene Ontology groups (43, 44)

Gene Ontology term	Gene count	*P* value
Upexpressed in biofilms		
Nitrogenous compound/oxidoreductase activity	8	2.3×10^{-10}
Heme binding	16	1.2×10^{-8}
Heme-copper terminal oxidase activity	5	1.8×10^{-3}
Organic acid catabolic processes	6	3.2×10^{-3}
Downexpressed in biofilms		
Nitrogen compound biosynthetic processes	107	9.0×10^{-29}
Translation	71	1.3×10^{-42}
tRNA aminoacylation	20	3.0×10^{-12}
Nucleotide biosynthesis	39	7.7×10^{-16}
Tricarboxylic acid cycle	12	5.8×10^{-6}
Histidine biosynthesis	11	1.2×10^{-7}
NADH dehydrogenase activity	12	3.4×10^{-8}
GTP binding	15	3.6×10^{-6}
RNA processing	24	3.5×10^{-4}
Lipid biosynthetic processes	37	1.6×10^{-9}
Aspartate family amino acid biosynthesis	14	4.8×10^{-6}
Glutamine family amino acid biosynthesis	18	2.2×10^{-4}
Nucleotide kinase activity	5	2.0×10^{-4}
DNA topoisomerase activity	5	7.1×10^{-5}
Glycolysis	10	5.0×10^{-5}
Coenzyme/folic acid metabolic processes	33	2.8×10^{-7}
Metal ion binding	99	7.6×10^{-4}
Alcohol biosynthetic processes	7	1.2×10^{-4}
Purine biosynthetic processes	23	2.1×10^{-9}
Protein folding	11	3.3×10^{-3}
Aromatic amino acid biosynthesis	16	9.9×10^{-4}
Fatty acid biosynthesis	11	3.3×10^{-3}
Cellular respiration	20	7.2×10^{-5}
Pyrimidine biosynthetic processes	9	4.4×10^{-5}
Nucleoside metabolic processes	14	5.4×10^{-4}
DNA replication	1	5.7×10^{-3}
Protein transmembrane transport	5	2.3×10^{-3}

Gene sets associated with drug efflux, oxidative stress, SOS response, and heat shock do not overlap genes upexpressed in *P. aeruginosa* biofilms. We hypothesized that some of the differentially expressed genes identified above contribute to the protection of biofilm from antibiotics. We also expected that many of those genes do not make a difference for the antibiotic tolerance of the biofilm. The challenge was to find an unbiased approach to analyze the data without focusing on a few known genes and neglecting poorly characterized genes. The Gene Ontology results in Table 3 focus primarily on catabolic or biosynthetic pathways. Since the exposure of bacteria to antibiotics likely induces stress responses in the bacteria, other specialized pathways, such as those involved in stress response or antibiotic resistance, may be induced. These pathways may not cluster with the pathways represented in the DAVID analysis. Therefore, to identify particular

TABLE 4 Gene set enrichment analysis of hypothesized protective mechanisms and other stress responses in drip-flow reactor *P. aeruginosa* biofilms

Mechanism[a]	*P* value for overlap with genes		Reference(s)
	Up in biofilm	Down in biofilm	
Efflux pumps	0.916	0.994	53
Peroxide stress	0.071	1.000	48–51
SOS response	1.000	0.861	52
Heat shock and chaperones	0.852	0.012	53
Fe limitation	0.896	0.997	55, 55
Cyclic-di-GMP	0.910	0.957	56
Toxin-antitoxin modules	1.000	1.000	57
Planktonic sensitivity to CIP	0.364	0.256	58
Planktonic tolerance to CIP	0.965	0.020	59
Planktonic sensitivity to TOB	1.000	0.017	59
Response to TOB in DFB, up	0.993	1.63×10^{-8}	Table S2
Response to TOB in DFB, down	$<1 \times 10^{-14}$	0.994	Table S2
Response to CIP in DFB, up	0.993	0.051	Table S2
Response to CIP in DFB, down	1.50×10^{-9}	0.861	Table S2
Oxygen limitation	$<1 \times 10^{-14}$	1.000	62
Oxygen downshift	$<1 \times 10^{-14}$	1.000	63
Stationary phase	$<1 \times 10^{-14}$	1.000	62, 96–98
HSL quorum sensing	0.004	1.000	69–71
Osmotic stress	1.11×10^{-4}	1.000	72

[a] CIP, ciprofloxacin; TOB, tobramycin; DFB, drip-flow biofilm; HSL, homoserine lactone.

subsets of genes that might be important for *P. aeruginosa* biofilm antibiotic tolerance, we compiled from the literature lists of genes associated with particular hypothesized antibiotic-protective mechanisms (see Table S3 in the supplemental material). We then compared those lists of genes to differentially expressed genes in the biofilm cells to determine if groups of genes associated with a particular stress response or environmental condition were statistically significantly enriched in the biofilm transcriptomes.

For example, one explanation for biofilm antibiotic tolerance is that drug efflux pumps are expressed at elevated levels in the biofilm, even prior to antibiotic treatment. We examined the overlap between a list of 39 genes associated with drug efflux in *P. aeruginosa* and the 340 genes expressed at higher levels in biofilm (Table 4). Only a single gene was common to both lists (PA4206, *mexH*), and this degree of overlap between the efflux pumps and genes upexpressed during biofilm growth was not statistically significant. This suggests that increased expression of multiple drug efflux pumps is likely not responsible for the antibiotic tolerances of these biofilm bacteria.

Oxidative stress has been shown to play a role in antibiotic sensitivity both in planktonic (32, 33) and biofilm cells (19), although the generality of this relationship has recently been questioned (46, 47). To test for an oxidative stress response in the biofilm, we compared the genes reported to be induced by hydrogen peroxide exposure in *P. aeruginosa* (48–51) with the genes upexpressed in the biofilm. The overlap was borderline significant ($P = 0.07$; Table 4).

We tested for the involvement of other putative protective mechanisms in drip-flow biofilms. In the absence of antibiotics, there was no evidence of expression of the classes of genes associated with the SOS response (52), heat shock (53), iron limitation (54, 55), or cyclic-di-GMP metabolism (56) in these biofilms (Table 4).

Toxin-antitoxin modules have been associated with the formation of antibiotic-tolerant persister cells in biofilms (35, 37). In the *P. aeruginosa* PAO1 genome, several putative toxin-antitoxin modules have been identified by bioinformatic analysis (57). The predicted toxin-antitoxin genes were not among those differentially expressed in the drip-flow reactor biofilms (Table 4). This is not surprising, since our analyses were performed on whole biofilms, whereas the putative persister cells would only make up a small portion of the population. Therefore, the enhanced expression of these genes is not likely to be identified by this global analysis of whole biofilms.

Global screens of the transposon mutant libraries for *P. aeruginosa* mutants altered in their susceptibility to tobramycin or ciprofloxacin have been reported (58, 59). Those studies were done exclusively with planktonic bacteria. Nevertheless, it is plausible that a gene that modulates the antibiotic sensitivity of a planktonic cell might also modulate antibiotic susceptibility in a biofilm. In particular, a gene that when mutated renders a cell more sensitive to an antibiotic would be a candidate for a protective gene if it were constitutively upexpressed in the biofilm. No evidence for this general mechanism was found (Table 4). Another version of this hypothesis posits downregulation in the biofilm of a gene whose inactivation renders a cell more tolerant to the drug. A borderline-positive result (two genes, $P = 0.02$) was found for ciprofloxacin (Table 4).

A number of gene products (with corresponding gene in parentheses) have been reported to modulate *P. aeruginosa* biofilm susceptibility to antibiotics: PA0084 (*tssC1*) (15), PA0934 and PA5338 (*relA spoT*) (19), PA1163 (*ndvB*) (16), PA1875 to PA1877 (17), PA3063 (*pelB*) (18), PA4878 (*brlR*) (4, 20), and PA5332 (*crc*) (21). None of these genes appear on our list of 340 upexpressed genes, and just one (*spoT*) appears on our list of 680 downexpressed drip-flow biofilm genes.

Genes induced by treatment with tobramycin or ciprofloxacin do not overlap genes upexpressed in *P. aeruginosa* biofilms. Biofilm bacteria may evade killing by antibiotics by deploying adaptive responses. For example, *P. aeruginosa* cells in the outer region of a biofilm have been shown to actively respond to treatment with colistin by expressing specific genes that protect these cells from the antimicrobial (34). Therefore, we also characterized the transcriptomic response of 3-day-old drip-flow reactor biofilms of *P. aeruginosa* treated with either tobramycin or ciprofloxacin. The microarray results of the biofilm cells that survived treatments with antibiotics indicated that 78 genes had increased expression, and 15 genes had reduced expression (4-fold increase, adjusted $P < 0.01$) following 12 h of treatment with ciprofloxacin (see Table S2 in the supplemental material). Treatment with tobramycin resulted in the increased expression of 111 genes and decreased expression of 70 genes (4-fold increase, $P \leq 0.01$) (see Table S2).

A DAVID analysis identified the pathways induced in biofilm cells by antibiotic exposure (Table 5). Ciprofloxacin induced genes for the SOS response, non-membrane-bound organelles, including *dnaT*, *rimM*, and *recN*, and genes for cytolysis, including genes for bacteriocin biosynthesis. Also induced by ciprofloxacin was the large prophage gene product cluster from PA0612 to PA0648. The gene pathways upexpressed by tobramycin included those for genes for ribosome biosynthesis and RNA metabolism, while the downexpressed genes included those for energy production, such as those for glycolysis, TCA cycle, and oxidative phosphorylation (Table 5).

TABLE 5 Gene enrichment analysis of *P. aeruginosa* biofilms treated with antibiotics compared to untreated biofilms based on Gene Ontology groups

Gene Ontology term	Gene count	*P* value
Upexpressed in biofilms by ciprofloxacin		
Non-membrane-bound organelle	5	8.3×10^{-5}
Cytolysis	3	1.9×10^{-3}
SOS response	3	8.0×10^{-4}
Downexpressed in biofilms by ciprofloxacin		
Arginine metabolic process	3	3.1×10^{-5}
Heme binding	4	1.2×10^{-3}
Upexpressed in biofilms by tobramycin		
Ribosome	12	3.2×10^{-13}
RNA binding	11	2.8×10^{-7}
RNA processing	9	7.1×10^{-5}
SOS response	3	4.2×10^{-3}
Downexpressed in biofilms by tobramycin		
Generation of precursor metabolites for energy	12	5.8×10^{-10}
TCA cycle	5	7.8×10^{-5}
Glycolysis/gluconeogenesis	5	1.7×10^{-4}
Cellular respiration	5	8.9×10^{-4}
Arginine metabolic process	3	8.0×10^{-3}

TABLE 6 Gene set enrichment analysis of regulatory gene activity in the 340 genes upexpressed in drip-flow reactor *P. aeruginosa* biofilms

Gene set[a]	Total no.	Overlap	*P*
$MvfR^+$	115	34	10^{-15}
$MvfR^-$	18	3	0.094
$RpoS^+$	423	88	10^{-15}
$RpoS^-$	176	14	0.189
Anr^+	191	40	10^{-12}
Crc^+	19	0	1.00
Crc^-	47	5	0.158

[a] +, genes positively regulated by the indicated protein; −, genes negatively regulated by the indicated protein.

One possible mechanism of biofilm protection from an antibiotic is that the genes that do provide adaptive protection are constitutively expressed in the biofilm state, even prior to antibiotic exposure. We tested for this possibility by comparing the genes induced in biofilms by tobramycin or ciprofloxacin with those upexpressed prior to antibiotic exposure and found no support for this hypothesis (Table 4). To the contrary, we found a statistically significant overlap between the genes expressed at higher levels in biofilm and the genes repressed in response to treatment with either antibiotic (Table 4).

Another interesting conjecture is that biofilms, in response to antibiotic treatment, express a set of genes that is distinct from the genes that are induced in antibiotic-exposed planktonic cells. Twenty-one genes were induced in drip-flow biofilms by both ciprofloxacin and tobramycin. Of these 21 genes, 16 have been reported to be induced by either ciprofloxacin or tobramycin (60, 61). This suggests that the transcriptional response of *P. aeruginosa* to ciprofloxacin and tobramycin is similar for cells growing planktonically and for biofilm cells.

Genes associated with the responses to hypoxia, osmotic stress, growth arrest, and quorum sensing overlap genes upexpressed in *P. aeruginosa* biofilms. Alvarez-Ortega and Harwood (62) characterized the transcriptional response of planktonic *P. aeruginosa* to oxygen limitation. A list compiled from their work of 159 genes expressed at elevated levels under some low-oxygen condition (in comparison to an air-saturated culture) overlaps extensively with the 340 genes expressed in our drip-flow biofilms (Table 4). These two lists have 58 genes in common, and the enrichment is highly statistically significant ($P < 10^{-14}$). Similarly, from a list of 117 genes induced by a shift from aerobic to anaerobic conditions (63), 51 genes overlap this biofilm list ($P < 10^{-14}$). These two comparisons show that bacteria in a biofilm experience hypoxia. These results on whole biofilms confirm our prior studies on localized gene expression in biofilms, which showed that cells at the top of colony biofilms were likely in a transition phase to hypoxia-induced stress, whereas cells at the bottom of biofilms had likely transitioned to anaerobic conditions (26). Microelectrode profiling of the oxygen concentrations of similar biofilms also showed that the concentration of oxygen rapidly decreased within about 50 μm from the top of the biofilms (10, 23, 64).

We also found a statistically significant enrichment of genes associated with the stationary phase (see Table S3 in the supplemental material, $P < 10^{-14}$), which again was similar to the top of the biofilms from our previous study. Acyl homoserine lactone quorum sensing has been shown to be important for *P. aeruginosa* biofilm formation (65–67) and to contribute to biofilm tolerance to tobramycin (68). We compiled a consensus list of quorum-sensing-activated genes from three independent studies (69–71; see also Table S3) and compared this list to genes expressed in *P. aeruginosa* drip-flow reactor biofilms. The enrichment of quorum-sensing-regulated genes was statistically significant in these biofilms ($P = 4 \times 10^{-3}$) (Table 4).

Genes associated with osmotic stress (72) ($P = 1 \times 10^{-4}$) were also significantly enriched in expression from the 340 genes upexpressed in biofilm growth (Table 4).

In summary, global gene expression patterns provide evidence of the following physiological activities or conditions in 3-day-old drip-flow biofilms: oxygen limitation, stationary-phase growth state, osmotic stress, and quorum sensing.

Global gene expression patterns are consistent with activation of the RpoS, MvfR, and Anr regulons but not the Crc regulon. To gain insight into the role of specific regulatory mechanisms in determining the gene expression in these biofilms, we compared lists of genes up- or downregulated by selected regulatory genes to those upexpressed in the biofilm transcriptome (Table 6). Both the hypoxia-responsive Anr regulon (63) and genes positively regulated by the stationary-phase sigma factor RpoS (73) were significantly enriched in the 340 genes upexpressed in biofilms (Table 6). Given the stationary-phase character of the biofilm, the stringent response is another likely regulatory system active in the biofilm. However, the complete stringent response regulon has not yet been mapped in *P. aeruginosa*, so this comparison could not be made.

Because the catabolite repression regulator (Crc) has been reported to modulate biofilm tolerance to ciprofloxacin (21), we tested for enrichment of Crc-regulated genes (74) in the biofilm transcriptome. No statistically significant overlap was found with the 340 genes upexpressed in biofilms (Table 6). Curiously, both

TABLE 7 Antibiotic susceptibility of selected *P. aeruginosa* mutants in biofilms

Strain/mutant	Locus	LR with CIP[a]	*P*[b]	LR with TOB[a]	*P*[b]	X_o[a,c] (log_{10} CFU cm^{-2})
MPAO1	WT[d]	0.9 ± 0.3		0.6 ± 0.2		9.8 ± 0.2
anr mutant	PA1544	2.6 ± 0.3	0.001	0.3 ± 0.4	0.52	8.8 ± 0.2
rpoS mutant	PA3622	2.9 ± 0.9	0.027	0.6 ± 0.3	0.96	10.1 ± 0.3
relA spoT mutant	PA0934, PA5338	2.4 ± 0.4	0.003	0.9 ± 0.4	0.30	9.9 ± 0.2
mvfR mutant	PA1003	1.2 ± 0.5	0.46	ND[e]		9.5 ± 0.4
brlR mutant	PA4878	ND		0.4 ± 0.4	0.54	9.8 ± 0.1
lasI rhlI mutant	PA1432, PA3476	1.7 ± 0.4	0.07	0.5 ± 0.4	0.95	8.9 ± 0.2

[a] Uncertainty estimates are the standard deviations. LR, log reduction.
[b] *P* values are for the comparison to the log reduction measured for the wild type.
[c] X_o, areal cell density of untreated biofilms.
[d] WT, wild type.
[e] ND, not determined.

genes that were negatively and positively regulated by Crc were enriched in the 683 genes downexpressed in biofilms (data not shown). Finally, we noticed a significant enrichment of genes positively regulated by MvfR (75) among the genes upexpressed in the biofilm (Table 6). A connection between MvfR and the downregulation of translational capacity, postulated to be involved in persister cell formation, was recently reported (76).

None of these regulatory genes themselves (*anr*, *rpoS*, *crc*, *relA*, *spoT*, or *mvfR*) were among the 340 upexpressed genes in the biofilm. In a similar *P. aeruginosa* biofilm experimental system, it was reported that the *rpoS* gene and RpoS protein were expressed in biofilm at levels comparable to those in stationary-phase planktonic cells (77).

In summary, in this biofilm system, global gene expression patterns are consistent with activation of the RpoS, MvfR, and Anr regulons but not the Crc regulon.

Ciprofloxacin susceptibility of mutant strains grown as biofilms confirms a role for hypoxia and growth arrest stress responses in biofilm tolerance. Based on the transcriptomics analysis, regulatory genes associated with oxygen limitation and growth arrest were hypothesized to contribute to biofilm antibiotic tolerance. We therefore tested the antibiotic susceptibility of strains with mutations that inactivated the global regulator involved in the hypoxia response, *anr*, and the stationary-phase sigma factor *rpoS*. The *anr*-E05::IS*lacZ*/*hah* and *rpoS*-B03::IS*lacZ*/*hah* mutant strains were obtained from the *P. aeruginosa* two-allele transposon mutant library (39) and were grown as biofilms (Table 7). Since both *anr* and *rpoS* are likely monocistronic, the transposon insertions are unlikely to be polar on downstream genes and affect only the expression of genes under their regulatory control. We also tested a stringent response-deficient double mutant, Δ*relA* Δ*spoT*, which was previously shown to be impaired in its ability to produce the stringent response signaling compound ppGpp (19). The *rpoS* and *relA spoT* mutants formed biofilms with cell densities similar to those of the wild type. The areal cell density of the *anr* mutant was an order of magnitude less than that of the wild-type strain. All three of these mutant strains were statistically significantly more susceptible to ciprofloxacin in the biofilm state. Whereas wild-type biofilms exhibited a 0.9-log reduction with ciprofloxacin challenge, the *anr*, *rpoS*, and *relA spoT* mutants yielded 2.4- to 2.9-log reductions. A quorum-sensing mutant (*lasI rhlI*) deficient in the production of both acyl homoserine lactone synthases was borderline more susceptible to ciprofloxacin (Table 7).

Even though MvfR positively regulates 10% of the genes upexpressed in biofilms, an *mvfR* mutant biofilm did not show increased susceptibility to ciprofloxacin (Table 7).

These results show that genes regulated by oxygen limitation, specifically via Anr, and by growth arrest, via both RpoS and the stringent response, contribute to biofilm tolerance to ciprofloxacin. This analysis does not identify the specific functional genes that participate in this effect, but it seems likely that multiple gene products are involved.

Tobramycin susceptibility of mutant strains grown as biofilms failed to confirm any genetic basis for biofilm tolerance to tobramycin. In contrast to the results with ciprofloxacin, the *anr*, *rpoS*, and *relA spoT* mutants yielded no differential susceptibility phenotype when grown as biofilms and challenged with tobramycin (Table 7), and neither did the double mutant deficient in acyl homoserine lactone quorum sensing.

The transcriptional regulator BrlR has been reported to govern antimicrobial susceptibility in *P. aeruginosa* biofilms (4, 20). Neither the *brlR* gene (PA4878) itself nor the drug efflux pump genes regulated by *brlR* were among the top 340 genes expressed more highly in drip-flow biofilms than in planktonic cells. Despite the lack of transcriptomic support for this mechanism, we tested the susceptibility of a *brlR* mutant grown as a biofilm to tobramycin (the antibiotic for which a biofilm phenotype was most extensively investigated in prior work). As with all other mutants examined, no reduced tobramycin tolerance was seen (Table 7). We note that our result does not contradict the findings of Liao and Sauer (4); at the identical dose concentration and duration, those researchers also reported an insignificant log reduction difference between the wild type and mutant. It was only at much higher tobramycin concentrations that these investigators discerned a phenotype.

In this work, we were not able to reproduce previously reported tobramycin biofilm susceptibility phenotypes for mutants affected in quorum sensing, stringent response, or the *brlR* gene. No mutant that we tested had a different susceptibility from that of the wild type when grown as a biofilm. One conclusion that could be made is that the particular set of genes that modulate antibiotic susceptibility in a biofilm may depend on parameters such as the strain, growth medium, biofilm age, and antibiotic dosing parameters. Another conclusion we can draw is that some other protective mechanism must be at work in a 3-day-old drip-flow biofilm challenged with tobramycin. Here, we propose an alternative explanation that has not yet been tested experimentally.

We hypothesize that many of the bacteria in 3-day-old drip-flow reactor biofilms have diminished membrane potential and that these cells are protected from tobramycin independent of

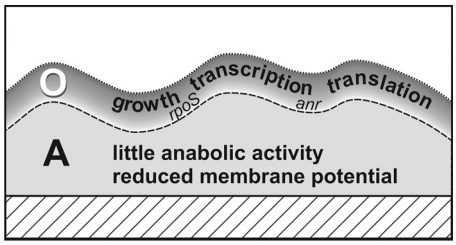

FIG 1 Hypothesized physiological activities within mature *P. aeruginosa* drip-flow reactor biofilms. An oxic zone (O) approximately 50 μm thick overlies an anoxic zone (A) approximately 100 μm thick. The shading denotes the oxygen concentration gradient, with darker shading indicating higher oxygen concentration and lighter shading indicating anaerobic conditions.

their genetic makeup or gene expression pattern. Tobramycin, like other aminoglycosides, is actively transported into the cytoplasm. This uptake is dependent on the cellular membrane potential (78). Bacterial cells in which the membrane potential is reduced will not take up tobramycin, and the drug will therefore not reach its intracellular target (78). This hypothesis is concordant with the evidence of oxygen starvation and arrested growth, both of which are plausible physiological preludes to diminished membrane potential. This hypothesis also predicts that genotype will be immaterial, as the pattern of gene expression in a cell is of no significance if the antibiotic never enters the cell in the first place. If reduced membrane potential is responsible for tobramycin tolerance, it could be possible to enhance antibiotic efficacy by adding a substrate that restores the proton motive force and membrane potential (79, 80).

In this work, we tested only two antibiotics, and so we caution against extrapolating the results to other agents, particularly antibiotic classes other than fluoroquinolones or aminoglycosides.

Role of physiological heterogeneity in biofilm antibiotic tolerance. Most mature biofilms harbor considerable microscale physiological heterogeneity (81). We have described elsewhere the characterization of the significant chemical and biological heterogeneity in *P. aeruginosa* drip-flow biofilms (2, 82). These analyses included oxygen concentration gradients measured with microelectrodes (2), stratified protein synthetic activity revealed using an inducible green fluorescent protein (GFP) strain (2), and stratified mRNA content measured by laser microdissection, followed by either quantitative reverse transcription-PCR (44, 82) or microarray analysis (26). The hypothesized physiological activities and their spatial localization are diagrammed in Fig. 1. Transcription and translation are active in the oxic zone, which constitutes the top one-third of the biofilm. Because of the steep oxygen concentration gradient in this region, bacteria in the oxic zone express genetic responses to hypoxia and growth arrest, including those

TABLE 8 Genes and proteins reported to be more highly expressed in *P. aeruginosa* biofilms than in planktonic cells in three or more experimental investigations

Locus ID[a]	Gene	T[b]	P[c]	Note[d]	Annotation
PA0139	*ahpC*	0	3	H_2O_2	Alkyl hydroperoxide reductase subunit C
PA0263	*hcpC*	1	2		Secreted protein Hcp
PA0515		3	0	O_2	Probable transcriptional regulator
PA0588		2	2	O_2, SP	Conserved hypothetical protein
PA0713		3	0	O_2, SP	Hypothetical protein
PA1123		3	0	O_2, SP	Hypothetical protein
PA1546	*hemN*	3	0	O_2, SP	Oxygen-independent coproporphyrinogen III oxidase
PA1555	*ccoP2*	3	0	O_2, SP	Cytochrome *c* oxidase, cbb_3 type, CcoP subunit
PA1556	*ccoO2*	3	0	O_2	Cytochrome *c* oxidase, cbb_3 type, CcoO subunit
PA1673		3	0	O_2	Hypothetical protein
PA1746		3	0	O_2, SP	Hypothetical protein
PA1904	*phzF2*	3	0	PHZ, SP	Probable phenazine biosynthesis protein
PA1905	*phzG2*	2	1	PHZ, SP	Probable pyridoxamine 5′-phosphate oxidase
PA2274		3	0	O_2	Hypothetical protein
PA2386	*pvdA*	0	3		L-Ornithine N^5-oxygenase
PA2782		3	0		Hypothetical protein
PA3126	*ibpA*	3	1	O_2	Heat shock protein IbpA
PA3309	*uspK*	2	2	O_2	Usp-type stress protein essential for survival during pyruvate fermentation
PA3572		3	0	O_2, SP	Hypothetical protein
PA4067	*oprG*	2	1	O_2	Outer membrane protein OprG precursor
PA4211	*phzB1*	3	1	PHZ, SP	Probable phenazine biosynthesis protein
PA4217	*phzS*	2	1	PHZ	Flavin-containing monooxygenase
PA4236	*katA*	1	2	O_2, H_2O_2, SP	Catalase
PA4352		3	2	O_2, SP	Conserved hypothetical protein
PA4610		3	0	O_2	Hypothetical protein
PA4765	*omlA*	2	1		Outer membrane lipoprotein OmlA precursor
PA5427	*adhA*	3	1	O_2, SP	Alcohol dehydrogenase
PA5460		3	0		Hypothetical protein
PA5475		3	0	O_2, SP	Hypothetical protein

[a] ID, identification.
[b] T, number of studies with transcriptomic identification.
[c] P, number of studies with proteomic identification.
[d] H_2O_2, peroxide stress; SP, stationary phase; O_2, oxygen limitation; PHZ, phenazine biosynthesis.

mediated by *anr*, *rpoS*, and the stringent response. In a prior work, we showed strong expression of the ribosome hibernation factors *rmf* and PA4463 in this region (26). In the anoxic zone, which occupies the bottom two-thirds of the biofilm, anabolic activity, including transcription, is drastically reduced, and the cellular mRNA content is much lower than that in the oxic zone. Because they have such diminished amounts of mRNA, cells from the anoxic zone contribute little to the whole-biofilm transcriptome. Cells in the anoxic zone were at one time exposed to oxygen before being buried in the biofilm interior by continued cell growth. These cells therefore passed through the state of gene expression captured by the activity of cells in the oxic zone. The inactive cells in the anoxic zone may lose membrane potential over time and thereby evade killing by aminoglycosides. These cells may also be partially protected from killing by ciprofloxacin through the stress response genes that were expressed at an earlier time as the cells responded to oxygen limitation and a reduced growth rate.

What this model suggests is that the whole-biofilm transcriptome provides a snapshot of the gene expression occurring in the oxic zone of the biofilm. This snapshot is useful in that it suggests the direction in which these cells are headed, i.e., a dormant state for which they prepare by expressing overlapping stress responses, such as *anr*, *rpoS*, and the stringent response.

Meta-analysis of genes and proteins expressed more highly in biofilm than in planktonic cells. We compiled lists from six transcriptomic (2, 83–87) and five proteomic (88–92) investigations of differential expression between biofilm and planktonic cells. This expands on previous analyses (93–95). Across the 11 reports, 807 unique genes or proteins were reported to be more highly expressed in biofilms than in planktonic cells. There were 110 genes or proteins that were identified in two or more studies as being more highly expressed in biofilm and 29 genes or proteins identified in three or more of these studies (summarized in Table 8). When tested for overlap with the same set of gene lists named in Table 4, statistically significant enrichment was found for oxygen limitation ($P = 10^{-13}$), oxygen downshift ($P = 10^{-15}$), stationary phase ($P = 10^{-10}$), and phenazine biosynthesis ($P = 10^{-6}$). In contrast to our results with the drip-flow biofilms, no statistically significant evidence for homoserine lactone quorum sensing ($P = 0.32$) or osmotic stress response ($P = 1.00$) was detected. Six of the 11 papers reported genes expressed at lower levels in biofilm than in planktonic cells, with a total of 104 unique genes or proteins. No genes or proteins were identified that were more highly expressed in planktonic cells than in biofilms in three or more reports. Only three genes, PA1092 (*fliC*), PA4661 (*pagL*), and PA5348 (a probable DNA-binding protein), appeared on two lists. This meta-analysis supports the possibility that oxygen limitation and growth arrest are common physiological responses in *P. aeruginosa* biofilms and so may contribute to antibiotic tolerance, as described above.

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