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In Vivo Evidence of *Pseudomonas aeruginosa* Nutrient Acquisition and Pathogenesis in the Lungs of Cystic Fibrosis Patients[▽]†

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One of the hallmarks of *Pseudomonas aeruginosa* infection in cystic fibrosis (CF) patients is very-high-cell-density (HCD) replication in the lung, allowing this bacterium to induce virulence controlled by the quorum-sensing systems. However, the nutrient sources sustaining HCD replication in this chronic infection are largely unknown. Here, we performed microarray studies of *P. aeruginosa* directly isolated from the lungs of CF patients to demonstrate its metabolic capability and virulence in vivo. In vivo microarray data, confirmed by real-time reverse transcription-PCR, indicated that the *P. aeruginosa* population expressed several genes for virulence, drug resistance, and utilization of multiple nutrient sources (lung surfactant lipids and amino acids) contributing to HCD replication. The most abundant lung surfactant lipid molecule, phosphatidylcholine (PC), induces key genes of *P. aeruginosa* pertinent to PC degradation in vitro as well as in vivo within the lungs of CF patients. The results support recent research indicating that *P. aeruginosa* exists in the lungs of CF patients as a diverse population with full virulence potential. The data also indicate that there is deregulation of several pathways, suggesting that there is in vivo evolution by deregulation of a large portion of the transcriptome during chronic infection in CF patients. To our knowledge, this is the first in vivo transcriptome analysis of *P. aeruginosa* in a natural infection in CF patients, and the results indicate several important aspects of *P. aeruginosa* pathogenesis, drug resistance, nutrient utilization, and general metabolism within the lungs of CF patients.

Pseudomonas aeruginosa is the major cause of morbidity and mortality in lung diseases, including cystic fibrosis (CF) (6, 11, 32) and nosocomial pneumonia (3, 40). Over 93% of CF patients between 18 and 24 years old have been reported to have *P. aeruginosa* infections (11). In addition, nosocomial pneumonia is the second most common of all nosocomial infections, and *P. aeruginosa* was the most frequently isolated microbe involved from 1992 to 1997 (37). The pathogenesis of this organism has been intensively studied with respect to virulence and virulence expression (5, 26, 31, 36, 39), biofilm production (10, 43), and quorum sensing (15, 27, 28, 30). Several virulence factors that *P. aeruginosa* expresses (e.g., exotoxin A, exoenzyme S, cytotoxin, proteases, lipases, phospholipases, alginate, and hydrogen cyanide) all contribute to severe lung damage. High-cell-density (HCD) replication is necessary for many of these events to occur, and $>10^9$ bacteria/ml of sputum have been found in the lungs of CF patients (43, 46). The ability of *P. aeruginosa* to obtain nutrients in the lung for HCD replication and maintenance is the quintessential factor leading to quorum-sensing-induced virulence expression, which is a hallmark of chronic lung infections in CF patients. However, the nutrient requirements of *P. aeruginosa* in vivo are unknown. A recent in vitro study by Palmer et al. (25), in which *P. aeruginosa* was grown on minimal medium supplemented with lyophilized sputum, suggested that one of the nutrient sources in the lung is amino acids. Therefore, because the enigmatic nutrient source(s) in vivo has long been ignored and until now has been presumptive, an area of research that should be explored is the metabolic capability of this organism with respect to nutrient acquisition in the lungs of CF patients. Understanding the nutrient requirements that contribute to HCD in the lungs of CF patients may yield new insights into the pathogenesis and potential treatment of infections in CF patients caused by *P. aeruginosa*.

Recovery of an HCD *P. aeruginosa* population from the lungs of CF patients presents a unique opportunity to analyze the transcriptome of this organism in vivo. The primary focus of our approach was to reveal bacterial in vivo gene expression prior to collection of expectorate from a patient. Specifically, we examined bacteria immediately after they were collected from fresh sputum samples in order to decipher their metabolic activities in vivo. This was achieved by isolating bacterial mRNA immediately after a single expectoration from a 42-year-old CF patient ($>10^9$ bacteria/ml of sputum). The sample was used for microarray and real-time reverse transcription (RT)-PCR, and the results of gene expression profiling experiments were compared to the results for a clinical isolate pool from the same patient grown in vitro in 1× M9 medium containing citrate. This comparison, performed using Affymetrix *P. aeruginosa* GeneChips, revealed hundreds of genes that were induced ≥2-fold ($P \leq 0.05$) in vivo compared to in vitro growth. Constitutively expressed genes were also observed by comparing the expression profile of the clinical isolate pool to that of PAO1, both grown in 1× M9 medium containing citrate. In this report, our data provide evidence showing that *P.*

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aeruginosa as a population exhibited a specific repertoire of highly expressed genes related to virulence, drug resistance, and nutrient utilization in vivo.

MATERIALS AND METHODS

Media and bacterial culturing conditions. All in vitro studies were carried out using *P. aeruginosa* laboratory strain PAO1 and the clinical isolate pools. In this study, we utilized minimal medium with a defined carbon source (e.g., citrate) for comparisons to the lung environment rather than a rich medium, because the nutrient source metabolized by *P. aeruginosa* in the lung may also be present in rich medium (e.g., amino acid in LB medium) and therefore may abolish differential expression of genes essential for nutrient utilization in vivo. This medium was amenable to the identification of *P. aeruginosa* pathways induced in the lung that are not necessarily "on" when a single defined carbon source is metabolized. To obtain cultures for RNA isolation, starter cultures of PAO1 or the clinical isolate pools were grown in *Pseudomonas* isolation broth (Difco) overnight and inoculated (1:200 dilution) into 200 ml of 1× M9 minimal medium (38) with 0.4% (wt/vol) phosphatidylcholine (PC), 0.4% (wt/vol) $C_{16:0}$, or 20 mM citrate and grown at 37°C with shaking at 225 rpm; in addition, all media contained 0.2% Brij 58 (Sigma, St. Louis, MO). When cells reached mid-log phase (optical density at 540 nm [OD_{540}], ~0.6), cells were harvested for RNA isolation as described below.

Bacterial RNA isolation from sputum and bacterial culture. Harvested cell cultures described above and 30 ml of a fresh sputum sample (single expectoration with >10^9 CFU/ml sputum) collected from a 42-year-old CF patient were kept on ice and processed immediately as follows. The approach and method used for sputum collection were approved by the Institutional Review Board Committee on Human Studies at the University of Hawaii at Manoa, and full informed consent was obtained from the patient. The cell pellets from cultures and the fresh sputum sample were treated on ice for 20 min with an equal volume of Sputolysin reagent (Calbiochem, La Jolla, CA) plus Sigma DNase I (10 U/ml of cell suspension) and proteinase K (600 μg/ml of cell suspension) with intermittent mixing to remove extracellular chromosomal DNA and proteins. Samples were then centrifuged at 8,000 × *g*, and the cell pellets were washed twice with ice-cold sterile double-distilled water (DDW) to lyse eukaryotic cells and remove soluble cell debris in the sputum. For the sputum sample, an aliquot of the washed cells was serially diluted and plated on no-salt Luria-Bertani medium (Teknova, Hollister, CA) and *Pseudomonas* isolation agar (Difco) to obtain a clinical isolate pool and for determining bacterial counts. The use of a clinical isolate pool, as opposed to a single clinical isolate, eliminated the possibility of any bias towards a particular isolate, and the pool was more representative of the *P. aeruginosa* population in vivo. Total RNA was isolated from *P. aeruginosa* using the established protocol of Stephen Lory (http://cfgenomics.unc.edu/protocols_rna_prep.htm), with a few minor modifications. Briefly, cells were harvested at 4°C and then resuspended and sonicated in Trizol reagent (Invitrogen, Carlsbad, CA) and treated with chloroform. An additional phenol-chloroform extraction was performed, and total nucleic acids were precipitated with ethanol. DNA was digested with DNase I (Promega, Madison, WI), and the RNA was phenol-chloroform extracted, ethanol precipitated, and resuspended in diethyl pyrocarbonate (DEPC)-treated DDW. tRNA was removed using QIAgen RNeasy purification columns as recommended by the manufacturer (QIAGEN, Valencia, CA), with an additional on-column DNase I digestion. The final RNA concentrations and purities were determined with a Beckman DU7500 spectrophotometer (A_{260}/A_{280}, between 1.8 and 2.0). The RNA yield for the sputum sample was ~55 μg and was sufficient for performing up to three GeneChip analyses and several real-time PCR runs. In our experience, the volume of sputum was not as critical as the *P. aeruginosa* cell counts in the sputum for successful microarray and real-time RT-PCR experiments. In particular, we ensured that processed sputum had an HCD (≥10^9 CFU/ml of sputum).

cDNA synthesis, labeling, and hybridization and microarray data analyses. cDNA was synthesized, fragmented, labeled, and processed as recommended by Affymetrix (Affymetrix, Santa Clara, CA). Hybridization to *P. aeruginosa* GeneChips was performed at the Greenwood Biotechnology Facility, University of Hawaii. Raw data were obtained using the Affymetrix GeneChip Operating System 1.4 software (Affymetrix, Santa Clara, CA). The tab-delimited files were then imported into the GeneSpring 7.0 software (Agilent Technologies, Redwood City, CA) for further analysis. Analysis was done by conducting pairwise comparisons between duplicate or triplicate GeneChips for two conditions at a time (i.e., PAO1 grown in PC versus PAO1 grown in citrate, PAO1 grown in $C_{16:0}$ versus PAO1 grown in citrate, in vivo sputum versus in vitro-grown clinical pool, in vitro-grown clinical pool versus PAO1 grown in citrate). The list of genes was subjected to statistical analysis using analysis of variance (ANOVA) and including the two-tailed Student *t* test, and only significant expression data ($P \leq 0.05$) were kept. The gene list was further analyzed based on fold changes, where only genes showing a change of twofold or greater were kept, and all hypothetical genes were removed from the final list. Fold change values were averaged for nine independent pairwise comparisons (three GeneChips per in vitro condition) with *P* values of ≤0.05. However, for in vivo conditions, fold change values were averaged for six independent pairwise comparisons (two GeneChips for in vivo conditions and three GeneChips for in vitro conditions) with *P* values of ≤0.05. Average expression levels of a few selected genes (*glp* genes, *mexY*, and *plcH*) that did not pass the statistical stringency test ($P \leq 0.05$) were analyzed individually by determining the relative expression level for each pairwise comparison, and the average expression levels were calculated (see Fig. 2).

Gene description and pathway designation. Gene descriptions and pathway designations were assigned according to the Kyoto Encyclopedia of Genes and Genomes (http://www.genome.ad.jp/kegg/) (24).

cDNA synthesis for real-time RT-PCR. Three micrograms of the same purified mRNA for each condition used for microarrays was used for cDNA synthesis. An iScript cDNA synthesis kit was used as recommended by the manufacturer (Bio-Rad, Hercules, CA). A "no reverse-transcriptase" control was included for each sample during cDNA synthesis to ensure that there was no DNA carryover contamination from the RNA isolation. Final volumes were brought to 1,400 μl with DDW (with no DEPC), and 10 μl was used for each real-time PCR as described below.

Bacterial mRNA processing of a sample from a second CF patient for real-time RT-PCR. A sputum sample was obtained from a young adult (an 18-year-old patient). The total volume of sputum was approximately 2 ml, and the *P. aeruginosa* plate count was 2.3×10^8 CFU/ml. Total RNA was initially isolated as described above, but the subsequent cDNA synthesis procedure was slightly modified as follows. The total RNA on a QIAGEN RNeasy column was eluted in 30 μl of DEPC-treated DDW and protected with 40 U of rRNasin (Promega, Madison, WI). A 2.75-μg aliquot of RNA was treated with 2 U of RQ1 RNase-free DNase I (Promega) for a second time in 2 μl of iScript cDNA synthesis buffer (Bio-Rad) for 10 min at 37°C. The DNase I was inactivated at 70°C for 10 min, and the sample was chilled on ice. Then 14 μl of iScript cDNA synthesis buffer, 4 μl of iScript reverse transcriptase (Bio-Rad), and 23 μl of DEPC-treated DDW were added to the same tube. cDNA was synthesized according to the recommendations of Bio-Rad. The volume of the final product was adjusted to 1,200 μl with DDW (with no DEPC) rather than 1,400 μl because there was less input RNA, and 10 μl was used as a template for each quantitative real-time TaqMan PCR as described below.

Primers and TaqMan probe design. Primers and probes (see Table S3 in the supplemental material) for each gene in the real-time PCRs were designed using Integrated DNA Technologies Primer Quest software (http://www.idtdna.com). Briefly, the amplicon sizes ranged from 67 to 86 bp and the primer melting temperatures were designed for 62°C, with a melting temperature difference of less than 4°C for each primer pair. The probe melting temperatures were designed to be 5 to 10°C higher than the melting temperatures for the corresponding primer pairs. Primer and probe sequences were also subjected to BLAST analysis against the *P. aeruginosa* PAO1 genome to eliminate the possibility of nonspecific binding. It was particularly ensured that the primers had no significant complementarity at the 3′ end and the probe had no significant complementarity at the 5′ end to other nonspecific locations on the PAO1 genome.

Real-time PCR. Supermixtures for all reactions were made and aliquoted into subsupermixtures for each gene assayed. Essentially, each real-time PCR mixture (final volume, 25 μl) contained 10 μl of cDNA, 12.5 μl of iQ Supermix (Bio-Rad), 120 nM of each forward and reverse primer, and 12 nM probe. Real-time PCR was performed with an iCycler iQ (Bio-Rad) using the following protocol: denaturation at 95°C for 10 min and then 55 cycles of amplification and quantification at 95°C for 20 s and at 65°C for 45 s. To control for variations between runs, all three housekeeping genes and the various target genes for each individual condition were amplified at the same time on a 96-well plate.

Real-time PCR data analyses. For analysis we used the method of Peirson et al. (29) as previously reported, which gives more accurate quantitative real-time PCR data. Because normalization by geometric averaging using multiple housekeeping genes has been shown to yield more accurate fold changes than normalization using a single housekeeping gene (47), we opted to use three housekeeping genes for our normalization analyses. Real-time PCR was conducted for each of the 11 target genes and the three housekeeping genes in eight replicates. Real-time PCR fold change values were averaged for eight replicates for each gene and were determined by comparison with the geometric mean of three housekeeping genes. A list of housekeeping genes was determined based on in vivo and in vitro microarray data from over 40 GeneChips for 16 different growth

conditions in various growth phases, from mid-log phase to stationary phase (data not shown). These housekeeping genes were analyzed using GeneSpring 7.0 and were found to be expressed consistently across all conditions (fold change, <2; $P \leq 0.05$). Three housekeeping genes (PA1769, PA1795, and PA1805) which showed the least amount of variability and were expressed consistently across all conditions (as determined using Spearman's correlation coefficient calculations) were selected. Real-time PCR fold changes were calculated using the amplification plot method and the available macro for data analyses of real-time PCR (DART-PCR) (29). Our requirement for real-time PCR is that the efficiencies of each gene (including housekeeping genes) are within 5% from one condition to the next, but similar efficiencies are not necessarily required for the different genes. Accordingly, the average efficiencies of each gene in this study were very similar for the conditions compared (<4.8% efficiency differences), allowing accurate analysis. We recommend not using the standard curve method for clinical samples, since it requires at least three serial dilution points in order to generate a reasonable standard curve. Due to the initial limited amount of input RNA isolated directly from sputum and the possibility of rare transcripts and low transcript abundance within bacterial cells, we were unable to use the dilution-standard curve method to obtain consistent standard curves and efficiencies (<5% difference) for different conditions for many genes. Using the amplification plot analysis method solves this problem, and this method has been demonstrated (29) to yield very good results comparable to those of standard curve analysis and the absolute quantification method, and it is much more accurate than the $\Delta\Delta C_T$ and the efficiency correction method.

Assay for constitutive alginate production. Starter cultures of PAO1 and the clinical isolate pool from the 42-year-old CF patient were grown overnight in *Pseudomonas* isolation broth and then subcultured (1:200 dilution) into no-salt Luria-Bertani medium (Teknova) and incubated at 250 rpm and 37°C. Cell density (OD_{540}) was measured at regular intervals to monitor cell growth and the growth phases, and aliquots were taken for a simultaneous alginate assay. The amount of alginate produced for each strain was normalized to the corresponding OD_{540}. Cells were harvested at early, mid-, and late-log and early stationary phases, and the alginate assays with carbazole reagent were performed as previously described (13, 16). Extracellular alginate production was quantified in triplicate (± standard error of the mean) at various growth phases. The amount of alginate produced by the clinical isolate pool at early stationary phase was defined as 100%, and all other alginate quantities were normalized to this value.

Microarray data accession number. Our microarray data have been deposited in the NCBI Gene Expression Omnibus (GEO) repository under accession number GSE7704.

TABLE 1. Summary of genes induced in vivo[a]

Pathway induced	No. of genes
Fatty acid degradation	11
Fatty acid and lipid metabolism	8
Choline metabolism	4
Amino acid degradation	26
Amino acid transport systems	10
Amino acid biosynthesis	24
Nucleotide and nucleic acid metabolism	19
Virulence factors	
Biofilm	3
Iron metabolism	5
Flagella	8
Other virulence factors	7
Drug resistance/RND and efflux transporters	8
General metabolism	
Tricarboxylic acid cycle	7
Starch and sucrose metabolism	2
Glycolysis/gluconeogenesis	3
Nitrogen metabolism	15
Pentose phosphate pathway	6
Glyoxylate and dicarboxylate metabolism	4
Pyruvate metabolism	3
Propanoate metabolism	2
Glutathione metabolism	2
Vitamin and cofactor metabolism	11
Oxidative phosphorylation	20
ATP synthesis	7
Sensory and two-component system	19
Protein secretion, chaperones, heat shock proteins, and secreted factors	19
ABC transporters	14
Ribosome	38
Cell wall metabolism and cell division	9
Transcriptional regulators and sigma factors	40
DNA/RNA modification and processing	20
Protein translation	8
Transporters, porins, and outer membrane proteins	18
tRNA processing	8
Others	29
Total	437

[a] Data summarized from Table S1 in the supplemental material, based on a microarray analysis indicating the total number of genes in different pathways induced twofold or more ($P \leq 0.05$) in vivo compared to the results for the same *P. aeruginosa* sputum isolate pool grown in 1× M9 medium containing 20 mM citrate.

RESULTS AND DISCUSSION

Virulence expression and deregulation of *P. aeruginosa* genes in vivo. Affymetrix *P. aeruginosa* GeneChips were used for monitoring the profile of gene expression in *P. aeruginosa*, based on hybridization with mRNA directly isolated from the sputum of a chronically infected patient. In vivo induced genes were determined by comparing the gene expression of bacteria obtained directly from the sputum sample to that of the isolated bacterial pool from the same sputum sample grown in vitro. The large data set collected in the whole-genome expression profile experiments for *P. aeruginosa* from a 42-year-old CF patient with chronic lung infection is summarized in Table 1 (see Table S1 in the supplemental material). There was a limited set of induced genes that were involved in virulence; this set included three genes for alginate synthesis, five genes for iron acquisition, eight genes for flagellar assembly and function, and four genes for neutralizing oxidative stress (see Table S1 in the supplemental material).

The lack of induction of more virulence genes in this initial comparison, especially biofilm synthesis genes (e.g., *alg* and *muc* genes) that create an obvious mucoid phenotype, was quite puzzling (Table 1). Mucoid (biofilm) *P. aeruginosa* is by far the predominant organism in the lungs of most CF patients and is the cause of high morbidity and mortality associated with CF (17). Interestingly, several genes expected to be upregulated (e.g., more *alg* genes for alginate biosynthesis) were not present in the initial list (Table 1; see Table S1 in the supplemental material), although we clearly isolated organisms with the mucoidy phenotype among the clinical isolates. Perhaps, in our attempt to detect in vivo induced genes by comparing microarray data for in vivo organisms to data for the in vitro-grown isolate pool (Table 1; see Table S1 in the supplemental material), we may have missed many constitutively expressed genes that have lost regulatory mechanisms (e.g., *alg* genes). Therefore, we hypothesized that many genes were constitutively expressed or deregulated, and there were no differences in the expression of many virulence genes between the in vivo condition and the isolated bacterial pool grown in vitro. The loss of these regulatory mechanisms likely represents the

TABLE 2. Summary of constitutively expressed genes[a]

Pathway induced	No. of genes
Fatty acid degradation	7
Fatty acid biosynthesis	6
Choline metabolism	3
Glycerol metabolism	4
Amino acid degradation	12
Amino acid transport systems	14
Amino acid biosynthesis	5
Nucleotide and nucleic acid metabolism	4
Virulence factors	
Phospholipase/lipase	3
Biofilm	19
Iron metabolism	7
Proteases	7
Other virulence factors	6
Drug resistance/RND and efflux transporters	9
General metabolism	
Tricarboxylic acid cycle	9
Starch and sucrose metabolism	7
Glycolysis/gluconeogenesis	4
Porphyrin and chlorophyll metabolism	3
Nitrogen metabolism	5
Glycolate metabolism	3
Vitamin and cofactor metabolism	2
Oxidative phosphorylation	12
Quinone biosynthesis	4
Sensory and two-component system and chemotaxis	22
Protein secretion, chaperones, heat shock proteins, and secreted factors	9
ABC transporters	13
Cell wall metabolism and cell division	3
Transcriptional regulators and sigma factors	45
DNA/RNA modification and processing	11
Protein translation	1
Transporters, porins, and outer membrane proteins	20
Osmotically inducible proteins	2
Others	42
Total	323

[a] Data summarized from Table S2 in the supplemental material, based on a microarray analysis indicating the total number of genes in different pathways constitutively expressed twofold or more ($P \leq 0.05$) in the *P. aeruginosa* sputum isolate pool compared to the results for PAO1 when both cultures were grown in 1× M9 medium containing 20 mM citrate.

many years of evolution of *P. aeruginosa* during the chronic infection within the host.

This observation prompted our efforts to analyze the GeneChip expression profiles of the in vitro-grown clinical pool to those of prototype strain PAO1, both grown in 1× M9 medium containing citrate. Our results revealed hundreds of genes constitutively expressed compared to the expression in PAO1, where the expression of these genes in PAO1 is more controlled (Table 2; see Table S2 in the supplemental material). As suspected and summarized in Table 2 (see Table S2 in the supplemental material), the clinical isolate pool obtained from the 42-year-old patient had higher constitutive expression levels of several classical virulence factors (*muc* and *alg* genes for biofilm synthesis, lipase, phospholipase, and several different proteases, *rhl* for rhamnolipid hemolysin, *hcn* for hydrogen cyanide production, and *pch* for pyochelin). The significance of biofilms in the lungs of CF patients cannot be emphasized enough, since the levels of expression of many *alg* genes were several-hundred-fold; the results for one of these genes (*algK*) were confirmed by real-time RT-PCR (Table 3). This constitutive expression of biofilm synthesis genes in the CF patient isolate pool relative to the basal biofilm production in PAO1 translated into greater alginate production at all growth phases (Fig. 1). The lack of virulence gene (*plcH*, encoding phospholipase C) expression, despite the presence of *plcR* expression in vivo, was due to larger variations in the microarray data ($P >$ 0.05) for this gene, since PlcR is the accessory protein that is required for PlcH secretion (see Table S2 in the supplemental material) (9). Although not detected by microarrays at high stringency ($P \leq 0.05$), *plcH* was expressed in vivo, and this was confirmed by real-time RT-PCR (Table 3 and Fig. 2F). There were nonidentical fold changes in some genes that may be organized in an operon (e.g., *bet* genes and PA5372 to PA5375; and *alg* genes and PA3540 to PA3551) (see Tables S1 and S2 in the supplemental material), which are normal and typical of many microarray data sets described previously (41, 48, 50). However, the general trends for genes expressed in the same direction (positive increase) were observed for genes arranged in operons. In a few cases (e.g., *mexX*, *arcC*, and *arcD*) (see

TABLE 3. Verification of microarray data by real-time RT-PCR for genes required for in vivo PC degradation, drug resistance (*mexY*), and biofilm biosynthesis (*algK*) by *P. aeruginosa*

PA no.	Gene	Description	Fold change		In vivo expression[b]
			Microarray[a]	Real-time PCR	
PA3014	*fadB5*	Fatty acid oxidation complex α-subunit	7.1	6.9	Induced
PA3299	*fadD1*	LCFA coenzyme A ligase	2.5	2.8	Induced
PA3300	*fadD2*	LCFA coenzyme A ligase	2.3	4.7	Constitutive
PA3924	*fadD4*	Medium-chain fatty acid coenzyme A ligase	3.6	5.2	Constitutive
PA5372	*betA*	Choline dehydrogenase	65.2	9.9	Induced
PA3582	*glpK*	Glycerol kinase	4.4	7.5	Constitutive
PA3584	*glpD*	Glycerol-3-phosphate dehydrogenase	3.7	7.5	Constitutive
PA2862	*lipA*	Lactonizing lipase precursor	6.8	2.1	Constitutive
PA0844	*plcH*[c]	Hemolytic phospholipase C precursor	2.8	15.4	Induced
PA2018	*mexY*[c]	RND multidrug efflux transporter	2.2	4.5	Induced
PA3543	*algK*	Alginate biosynthetic protein AlgK	408.3	271	Constitutive

[a] Microarray fold change values obtained from Tables S1 and S2 in the supplemental material ($P \leq 0.05$).
[b] Induced expression was based on a comparison of an in vivo sample to an in vitro-grown sputum isolate pool in 1× M9 medium containing 20 mM citrate (see Table S1 in the supplemental material); constitutive expression was based on a comparison of an in vitro-grown sputum isolate pool to PAO1, both grown in 1× M9 medium containing 20 mM citrate (see Table S2 in the supplemental material).
[c] Genes expressed on average >2-fold ($P > 0.05$) in Fig. 2, which was confirmed by real-time RT-PCR.

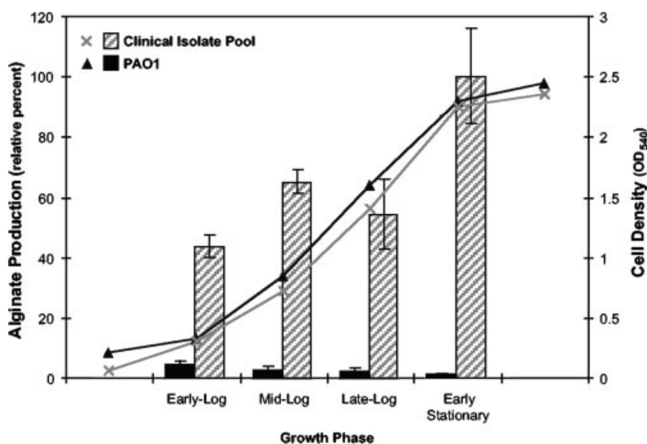

FIG. 1. Constitutive expression of *P. aeruginosa* biofilm/alginate biosynthetic genes results in high production of alginate at all growth phases by the clinical isolate pool from the CF patient relative to the production by strain PAO1. Although the growth of the pooled isolates from the CF patient and the growth of PAO1 are comparable, the pooled isolates from the CF patient showed excessive alginate production at all growth phases.

below), gene expression was observed both in vitro and in vivo (see Tables S1 and S2 in the supplemental material). We reasoned that these genes were expressed in a gradient fashion, with the expression highest in vivo, lower in the in vitro clinical isolate pool, and lowest or repressed in PAO1. Therefore, Tables 1 and 2 represent a summary of in vivo expressed genes for in vivo survival and virulence; some genes are regulated (Table 1; see Table S1 in the supplemental material), while others have evolved to be constitutive (Table 2; see Table S2 in the supplemental material).

The expression of many genes would have been overlooked if we had not considered the potential constitutive expression and evolution of the organism, by deregulation of the transcriptome, within the lungs of CF patients with chronic infections. A recent study by Smith et al. (45), who compared the genomes of two isolates in the same CF patient 7.5 years apart, identified several differences between the two isolates, which suggested that there was in vivo evolution of *P. aeruginosa* during chronic lung infection in a CF patient. Further detection of the same mutations in several other isolates of the population in one CF patient over time indicated that there was significant evolution and population diversity of *P. aeruginosa* within the lungs of this CF patient (45). Our data complement and support this important genomic finding (45) with

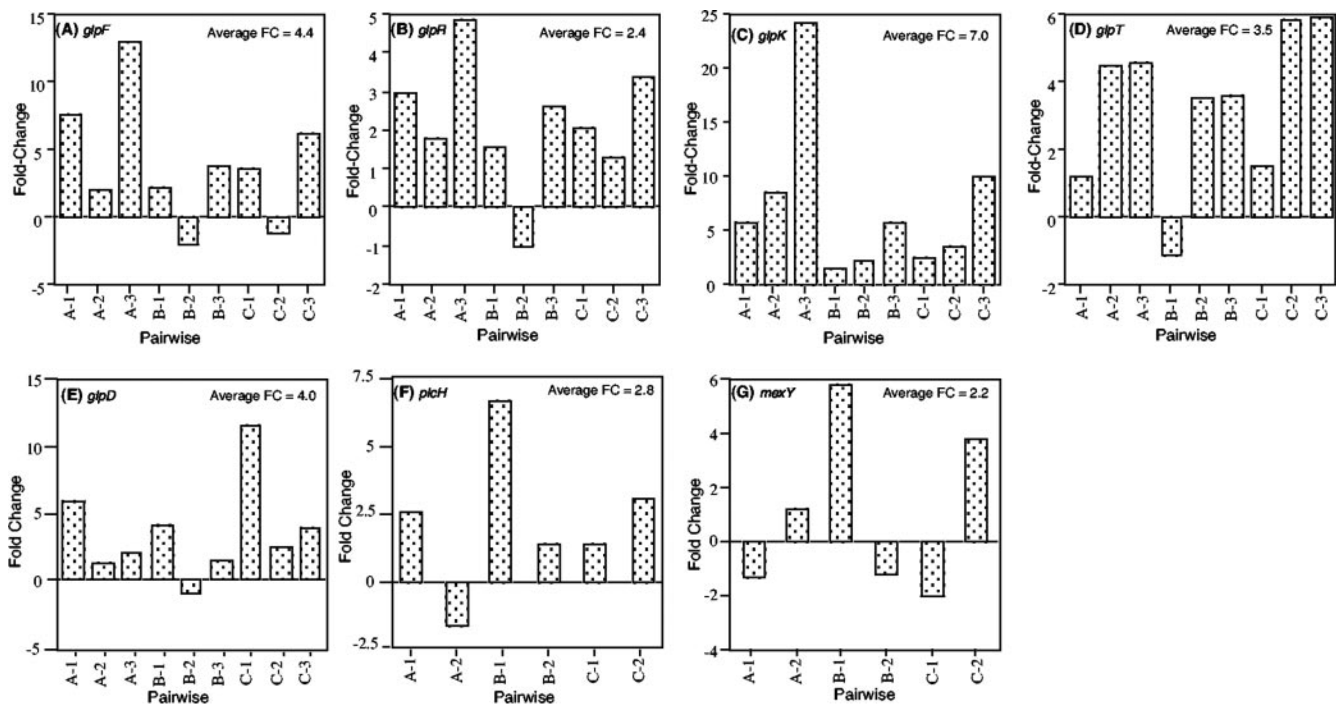

FIG. 2. Average fold changes for pairwise comparisons of *glp* genes, *plcH*, and *mexY*, which were not detected due to the statistical stringency ($P \leq 0.05$) imposed on the GeneChip data for *glp* genes (Table 4) and *plcH* and *mexY* (see Table S1 in the supplemental material). (A to E) Pairwise comparisons of three data sets for PAO1 grown in 1× M9 medium containing 0.4% PC (indicated by the numbers 1 to 3 on the *x* axis) to three data sets for PAO1 grown in 1× M9 medium containing 20 mM citrate (indicated by the letters A to C on the *x* axis). (F and G) Pairwise comparisons of two data sets for clinical in vivo samples (indicated by the numbers 1 and 2 on the *x* axis) to three data sets for the clinical isolate pool grown in 1× M9 medium containing 20 mM citrate (indicated by the letters A to C on the *x* axis). The glycerol uptake facilitator gene *glpF* (A), the glycerol metabolism regulator gene *glpR* (B), the glycerol kinase gene *glpK* (C), the glycerol-3-phosphate transporter gene *glpT* (D), and the glycerol-3-phosphate dehydrogenase gene *glpD* (E) all demonstrated induction when PAO1 was grown on PC compared to when PAO1 was grown on citrate, yielding average changes of 4.4-, 2.4-, 7.0-, 3.5-, and 4.0-fold, respectively. The hemolytic phospholipase C precursor gene *plcH* (F) and the RND multidrug efflux transporter gene *mexY* (G) were induced more on average in vivo than in the clinical isolate pool grown in 1× M9 containing 20 mM citrate, with average changes of 2.8- and 2.2-fold, respectively. Real-time RT-PCR confirmation data for *glpK* and *glpD* are shown in Table 4, and confirmation data for *plcH* and *mexY* are shown in Table 3.

evidence for deregulation of the transcriptome in the bacterial population. Smith et al. (45) also showed that isolates from CF patients have mutations in genes important for O-antigen biosynthesis, type III secretion, twitching motility, exotoxin A regulation, multidrug efflux, osmotic balance, phenazine biosynthesis, quorum sensing, and iron acquisition. Most importantly, they found that in many, but not all, of the 29 CF patients examined, *P. aeruginosa* isolates showed mutations in *lasR* (one of the major virulence and biofilm activators at HCD) to abolish virulence in order to evade immune detection, which was paradoxical in view of the current belief that extracellular virulence factors and biofilm synthesis genes are important during chronic infection in patients with CF. However, immune evasion seems paradoxical, considering the chronic inflammation in the lungs of CF patients during chronic infection (32). Our transcriptome data indicated that many virulence and biofilm synthesis genes, controlled by LasR, were expressed in the bacterial population (see Tables S1 and S2 in the supplemental material), which may also seem paradoxical in view of the previous study (45). However, this finding does not contradict the findings of Smith et al. (45), and it agrees with current dogma concerning infection in CF patients, considering population diversity and the diverse microenvironment of each individual bacterium in the population. Therefore, looking at gene expression of individual isolates from CF patients, although important, may not reveal the complete picture of the whole cooperative population. In support of the concept of a cooperative *P. aeruginosa* population during infection of CF patients, it has also been shown that different *P. aeruginosa* isolates in the same CF patient could produce completely different extracellular protein profiles, whereas the cellular protein profiles are quite conserved (51). This difference in the extracellular protein profiles of different isolates in the same CF patient suggests that there is cooperation between members of the bacterial population, where each member may have specific roles that serve the whole population. For example, secreted virulence elastases (LasA and LasB) may benefit both producers and nonproducers. In addition, the presence of a heterogeneous environment in the lungs of CF patients through space and time has recently been suggested by Nguyen and Singh (21), which may contribute to population expression diversity. As complex as the population diversity is, we agree that the diverse microenvironments in the lungs of CF patients, within which distinct isolates occur and adapt, signal individual bacteria to behave distinctly and may even select for certain mutations. For example, for osmotic regulation, twitching, or type III secretion, bacteria found on the surface of the biofilm and in contact with epithelial cells may express the genes encoding these properties or even deregulate these genes differently than bacteria growing and surviving inside the biofilm.

In vivo expression of drug resistance genes. In terms of mechanisms of resistance to antibacterial agents, *P. aeruginosa* is second to none, having several such mechanisms in its huge arsenal, including biofilm production, target modification and overexpression, drug modification, and more than 12 multidrug efflux pumps (1, 18, 22, 33). All possible mechanisms of drug resistance were observed in our data (see Tables S1 and S2 in the supplemental material), with the exception of target modification since our approach cannot detect such changes. At the time of sputum collection, the patient was being treated with tobramycin (an aminoglycoside) by inhalation, tobramycin and cefepime (a fourth-generation cephalosporin) intravenously, and Bactrim (trimethoprim-sulfamethoxazole) orally. Certainly, the highly expressed biofilm biosynthesis genes could present biofilm as the first barrier for drug resistance. The target of tobramycin is at the level of the ribosome to inhibit protein translation, and we observed significant overexpression of 38 different ribosomal proteins (target overexpression) (see Table S1 in the supplemental material). In addition to the induction of two aminoglycoside drug modification proteins, PA1409 (*aphA*) and PA4119 (*aph*), we also observed induction of genes for the aminoglycoside efflux pump, MexXY-OprM (19) (Table 3 and Fig. 2G; see Table S1 in the supplemental material). Mutations in *mexZ*, a negative regulator of *mexX* and *mexY*, have been found in the majority of isolates from several CF patients (45). It has been previously demonstrated that MexXY-OprM also effluxes trimethoprim (7). Therefore, expression of this pump may also provide resistance against administered trimethoprim. Increased expression of five folate biosynthetic genes was also observed (cofactor [see Table S1 in the supplemental material]), likely due to the inhibition of folate synthesis by the trimethoprim-sulfamethoxazole, and one of the proteins overexpressed was dihydropteroate synthase (FolP, PA4750), which is the target of sulfamethoxazole (target overexpression). For potential resistance to the β-lactam cefepime, overexpression of β-lactamases was observed in vivo (*ampC*, 87.9-fold [see Table S1 in the supplemental material]; PA5514, 13.9-fold [see Table S2 in the supplemental material]). A previous study showed that antibiotic resistance mechanisms arise from hypermutable *mutS* variants (44), and isolates from CF patients do harbor *mutS* mutations (45). Some of the antibiotic resistance mechanisms discussed here in the population may have arisen from such *mutS* variants. Regardless, our important findings suggest that *P. aeruginosa* utilizes several mechanisms in vivo to deal with antibacterial agents in a natural infection, by inducing many genes to counteract the negative effects of the administered drugs (see Tables S1 and S2 in the supplemental material).

General metabolism. Various central pathways expressed by *P. aeruginosa* (Tables 1 and 2) indicate active metabolism in vivo. Gene expression in vivo for central pathways, including those of saccharide metabolism, the Krebs and glyoxylate cycles, the pentose phosphate pathway, and oxidative phosphorylation, along with vitamin/cofactor biosyntheses and nutrient transport, indicates that *P. aeruginosa* is very metabolically active in vivo. The carbon sources utilized by *P. aeruginosa* seem to be both lipids of lung surfactant and amino acids, as discussed below. All these activities may contribute to replication and division in vivo, as evident from the expression of genes for cell wall metabolism and division. The data also indicate that *P. aeruginosa* actively senses and interacts with the in vivo environment by expressing many genes involved in two-component systems, secretion, and transport. In addition, various processes are controlled by induction of a large number of transcriptional regulators and sigma factors. Finally, in an animal model, the expression of the *napEFDABC* and *arcDABC* gene clusters has been monitored to indicate anaerobic respiration by reflecting an increase in nitrate and arginine turnover (4). Our data demonstrate that there is significant expression of one of these two gene clusters, *arcDABC*

(13.1-, 28.0-, 8.8-, and 4.2-fold changes, respectively) (see Table S1 in the supplemental material). Together with expression of genes involved in oxidative respiration, this suggests that anaerobic respiration also occurs in the population, which may result from the diverse microenvironment within the lung or biofilm (21). As alluded to by Nguyen and Singh (21), we believe that in a sputum biofilm in a CF patient there may be subpopulations of *P. aeruginosa* undergoing oxidative respiration on the biofilm surface, while other subpopulations are anaerobically respiring within the biofilm or oxygen-deficient areas, as indicated by the expression of both aerobic and anaerobic respiration genes in the population.

Active amino acid degradation contributing to HCD replication in vivo. Our data indicate that *P. aeruginosa* metabolizes amino acids as a nutrient source in vivo. Previous studies utilizing sterilized mucopurulent respiratory liquid or sputum from CF patients to grow *P. aeruginosa* in vitro have discovered several sputum-induced genes (25, 53). One of these in vitro studies (25), in which *P. aeruginosa* was grown on a pool of several lyophilized sputum samples, showed that there was induction of a subset of 20 genes for amino acid degradation, suggesting that *P. aeruginosa* metabolizes amino acids as nutrient sources in vivo. However, this hypothesis requires in vivo confirmation. Our in vivo data (see Tables S1 and S2 in the supplemental material), obtained by isolating mRNA directly from a single expectoration source of sputum, indicate that there is induction of a much larger set of genes for amino acid transport and degradation (36 genes induced and 26 genes constitutively expressed). This set represents a total of 60 different genes (since *arcC* and *arcD* are present in both Table S1 and Table S2 in the supplemental material) that are involved in amino acid degradation, which suggests that amino acid degradation in vivo could be much more important than previously thought (25). Since proteases were expressed in vivo (LasA, LasB, and others) (see Table S2 in the supplemental material) to cleave proteins into peptides and amino acids, it was significant to find that many amino acid and peptide transporters were also expressed in our data. However, these data indicate that *P. aeruginosa* may utilize more than amino acids and that lung surfactant lipids may also serve as a great nutrient source in vivo.

Degradation of lung surfactant lipids by *P. aeruginosa*. Lung surfactant components, especially lipids, are essential for proper lung functions, and the lack of these components can lead to respiratory distress syndromes in adults and prematurely born infants. Pulmonary surfactant consists of ~10% surfactant proteins (SP-A, SP-B, SP-C, and SP-D) and ~90% lipids (PC, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, and small amounts of other lipids and free fatty acids), and PC accounts for ~80% of the lipids (2, 14). Thus, the most plentiful sources of potential bacterial nutrients in lung surfactant are lipids, especially PC.

We initially performed GeneChip studies to determine which genes were induced by *P. aeruginosa* strain PAO1 during growth on PC in vitro as the sole carbon source (Table 4). By identifying the genes involved in PC degradation in vitro, we could relate and search for the expression of the same set of genes in vivo to determine if *P. aeruginosa* metabolizes PC in vivo. Through the action of lipases (Fig. 3), the richest nutrient obtained from the lung surfactant PC molecule in vivo is fatty acids, mostly palmitic acid ($C_{16:0}$) (50 to 60%) but also approximately 10 to 20% each $C_{14:0}$, $C_{16:1}$, $C_{18:1}$, and $C_{18:2}$ (34). Accordingly, the PC utilized in our experiments (Sigma, St. Louis, MO) contained mostly long-chain fatty acids (LCFA) ($C_{16:0}$, 33%; $C_{18:1}$, 30%; $C_{18:2}$, 14%; $C_{18:0}$, 14%). These LCFA can be metabolized via β-oxidation in the fatty acid degradation (Fad) pathway (Fig. 3C). As observed for the relevant genes in Table 4, several key genes signal the degradation of PC and LCFA in vitro. Specifically, lipases (LipA and LipC) and phospholipases (PlcH and the accessory protein PlcR) were expressed to break down PC into smaller constituents: fatty acid, glycerol, and phosphorylcholine. These constituents could be further metabolized in vitro, via the expression of Bet enzymes (choline head group metabolism) (49), Glp enzymes (glycerol metabolism) (42), and Fad enzymes (fatty acid degradation) (8). Fold changes for the glycerol degradation genes in Table 4 were determined with less stringency, because of the larger variations ($P > 0.05$) between the pairwise comparisons in our microarray data for these genes, and all showed positive induction (Fig. 2). Expression of the *fadBA5* operon was observed only with $C_{16:0}$ and not with PC relative to citrate (Table 4), although these genes were involved in PC degradation in vitro (Fig. 4) and were expressed in vivo (Table 3). FadB5 has 73% homology to the well-characterized *Escherichia coli* FadB protein (35). To demonstrate that FabBA5 is involved in PC degradation, we created a Δ*fadBA5* deletion mutant. This mutant showed a decrease in the ability to grow on LCFA and PC (Fig. 4). The Δ*fadBA5* mutant could still grow on LCFA, because there are at least two other *fadBA* operons in *P. aeruginosa* (unpublished data). Of course, there was less of a defect for this mutant to grow on PC than for it to grow on palmitate, since the PC molecule still contains two other nutrient sources (i.e., glycerol and choline) (Fig. 3).

In vivo (see Tables S1 and S2 in the supplemental material), we observed that the majority of the same genes were induced for PC degradation. Our microarray data were confirmed, both in vitro and in vivo, by real-time RT-PCR for several of these genes (Tables 3 and 4). The *fadD2*, *betA*, *glpK*, *glpD*, *lipA*, and *plcH* genes for PC degradation were also expressed in vitro when *P. aeruginosa* was grown on PC as the sole carbon source (Table 4). FadD1 and FadD2 have very high homology (72%) to *E. coli* FadD and are also involved in β-oxidation (unpublished data), and all three FadDs (FadD1, FadD2, and FadD4) have a very conserved ATP/AMP binding signature motif compared to the *E. coli* signature motif (52). The in vivo gene expression data (Table 3; see Tables S1 and S2 in the supplemental material) strongly suggest that PC is metabolized in vivo. *P. aeruginosa* can metabolize PC and all three components of PC (fatty acid, glycerol, and choline) (Fig. 3), at least in vitro (data not shown). The individual constituents are further imported and degraded, via high levels of expression of many *fad*, *glp*, and *bet* genes (Fig. 3). With the exception of one lipase gene, previous in vitro growth of *P. aeruginosa* in pooled and lyophilized sputum did not reveal any other genes induced for PC degradation (25). We reasoned that the previous study was performed under in vitro conditions and could have missed less soluble nutrients (such as PC and other lipids) in the lung surfactant that coats the surface of the lung and are continuously being replenished due to its essentiality in vivo.

TABLE 4. In vitro microarray analysis of relevant genes expressed twofold or more in *P. aeruginosa* strain PAO1grown on 1× M9 medium containing 0.4% PC or 0.4% palmitate ($C_{16:0}$) compared to the expression with 20 mM citrate

PA no.	Gene	Description	Fold change	Carbon source
Phospholipases and lipases				
PA0843	*plcR*	Phospholipase accessory protein PlcR	14.1	PC
PA0844	*plcH*[a]	Hemolytic phospholipase C precursor	37.2 (6.6)	PC
PA2862	*lipA*[a]	Lactonizing lipase precursor	37.4 (11.2)	PC
PA2863	*lipH*	Lipase modulator protein	7.9	PC
PA2949		Probable lipase	3	PC
PA4813	*lipC*	Lipase LipC	3.9	PC
Fatty acid degradation				
PA0507	*fadE*	Probable acyl-coenzyme A dehydrogenase	7.2	PC
PA1284	*fadE*	Probable acyl-coenzyme A dehydrogenase	26	PC
PA1628	*fadB*	Probable 3-hydroxyacyl-coenzyme A dehydrogenase	5.9	PC
PA1629	*fadB*	Probable enoyl-coenzyme A hydratase/isomerase	4.2	PC
PA2889	*fadE*	Probable acyl-coenzyme A dehydrogenase	2	PC
PA2893	*fadD3*	Probable very-long-chain acyl-coenzyme A synthetase	10.7	PC
PA3013	*fadA5*	Fatty acid oxidation complex beta-subunit	3.2	$C_{16:0}$
PA3014	*fadB5*[a]	Fatty acid oxidation complex alpha-subunit	6.2 (14.3)	$C_{16:0}$
PA3300	*fadD2*[a]	LCFA coenzyme A ligase	3.9 (4.9)	PC
PA3454	*fadA*	Probable acyl-coenzyme A thiolase	6.1	PC
PA4199	*fadE*	Probable acyl-coenzyme A dehydrogenase	2.1	PC
PA4785	*fadA4*	Probable acyl-coenzyme A thiolase	4.6	PC
PA4814	*fadH2*	2,4-Dienoyl-coenzyme A reductase FadH2	3.4	PC
PA4994	*fadE*	Probable acyl-coenzyme A dehydrogenase	4.7	PC
PA4995	*fadE*	Probable acyl-coenzyme A dehydrogenase	8.1	PC
Choline degradation				
PA3933		Probable choline transporter	25	PC
PA5372	*betA*[a]	Choline dehydrogenase	11.9 (6.3)	PC
PA5373	*betB*	Betaine aldehyde dehydrogenase	17.1	PC
PA5374	*betI*	Transcriptional regulator BetI	18.8	PC
PA5375	*betT1*	Choline transporter BetT	25.6	PC
Glycerol degradation[b]				
PA3581	*glpF*	Glycerol uptake facilitator protein	4.4	PC
PA3582	*glpK*[a]	Glycerol kinase	7 (22.1)	PC
PA3583	*glpR*	Glycerol-3-phosphate regulon repressor	2.4	PC
PA3584	*glpD*[a]	Glycerol-3-phosphate dehydrogenase	4 (23.5)	PC
PA5235	*glpT*	Glycerol-3-phosphate transporter	3.5	PC

[a] Genes confirmed by real-time RT-PCR; fold changes are indicated in parentheses.
[b] Fold changes for the glycerol degradation genes were determined with less stringency because of the larger variations ($P > 0.05$) in the pairwise comparisons, but all genes showed positive induction (Fig. 2).

Expression of PC degradation genes in a second CF patient as determined by real-time RT-PCR. To further demonstrate the importance of potential PC degradation in vivo through gene expression, we analyzed the expression of *lipA*, *plcH*, *fadB5*, *fadD1*, *fadD2*, *fadD4*, *betA*, *glpD*, and *glpK* from *P. aeruginosa* isolated directly from an 18-year-old CF patient by real-time RT-PCR. However, not armed with microarray data for this patient, we wanted to perform more thorough and careful analyses to clearly show gene expression as it relates to PC degradation. Table 5 indicates that genes required for PC degradation in vitro were also expressed in vivo, compared to both the same clinical isolate pool or PAO1 grown in vitro in 1× M9 medium with 20 mM citrate. As Table 5 shows, using gene expression in PAO1 as a reference is essential to get a baseline for quantifying the relative expression of many genes. In this second CF patient, as in the first patient described above, *P. aeruginosa* clearly expressed genes essential for the degradation of PC. It is interesting that *glp* genes, *fadD2*, and *fadD4* in the first patient were deregulated (Table 3). Likewise, these same genes were also constitutively expressed in the second patient (Table 5). These gene expression data further support our overall hypothesis that *P. aeruginosa* may utilize PC as one of the major nutrient sources in vivo.

Future analyses of the available microarray data. The microarray data have been deposited in the NCBI Gene Expression Omnibus repository for future analyses. Low signals in microarray data have previously been observed for some genes, for example, the exotoxin A-encoding gene (23). Due to the inherent variability in microarray data that could lead to missing some expressed genes shown in this study (Fig. 2), we suspect that much more could be gleaned from our microarray data by investigators interested in certain aspects of *P. aeruginosa* beyond those that are discussed here. For example, we did not observe expression of the virulence factor PA1249 for alkaline protease (*aprA*), but the accessory proteins encoded in the same operon (PA1248 [*aprF*] and PA1250 [*aprI*]) were constitutively expressed (see Table S2 in the supplemental

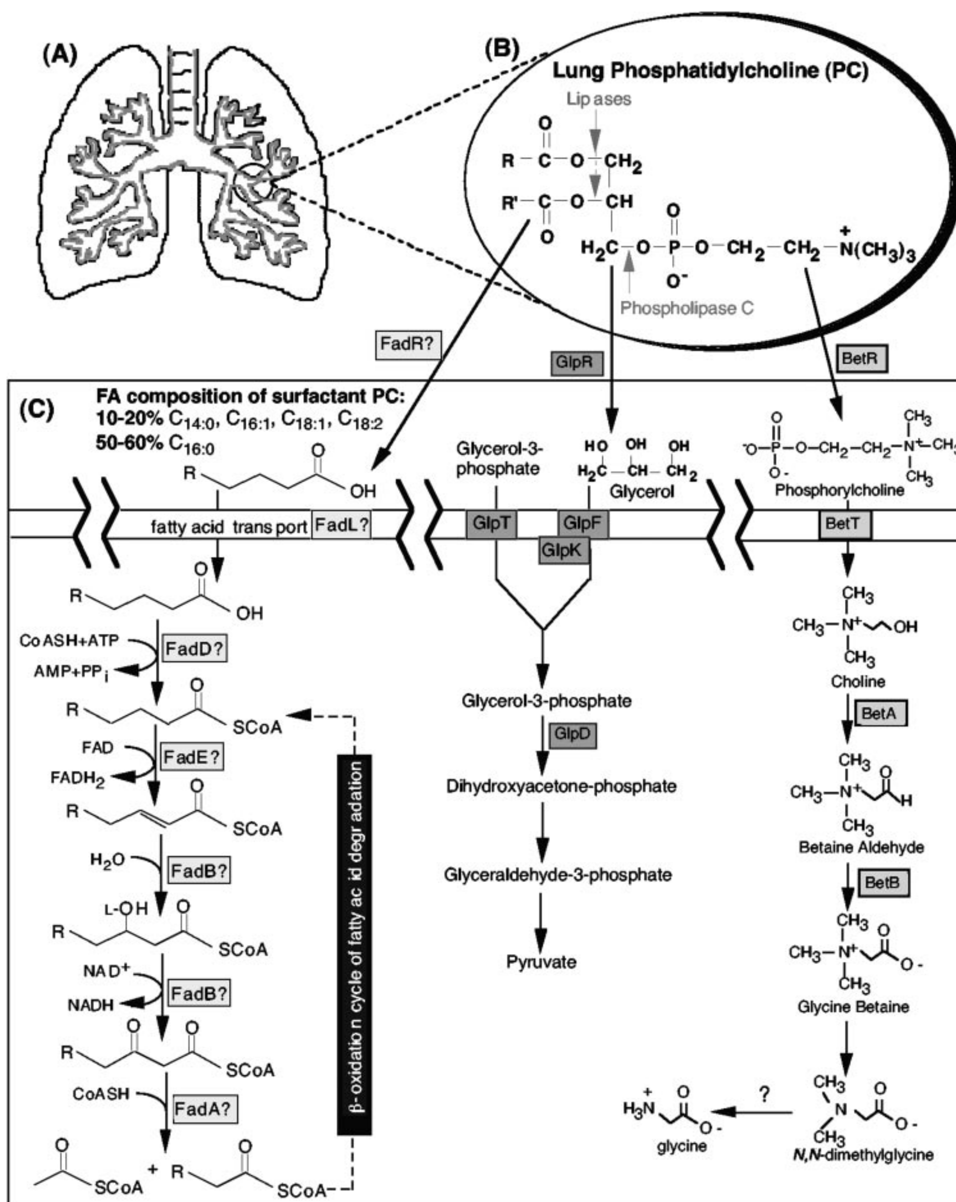

FIG. 3. (A) Lung surfactant, made of 90% lipids and 10% proteins, coats the trachea, bronchioles, and alveoli of the lung. (B) Of the 90% lipids, 80% consists of PC, which can be cleaved by *P. aeruginosa* lipases and phospholipase C into three constituents, including fatty acids (FA), glycerol, and phosphorylcholine. (C) Glycerol and phosphorylcholine constituents are further metabolized by known Glp and Bet enzymes of *P. aeruginosa* (42, 49). The regulators GlpR and BetR control the expression of these enzymes. GlpT and GlpF of the cytoplasmic membrane facilitate glycerol-3-phosphate and glycerol transport, respectively. Based on the *E. coli* model (8), predicted steps in the Fad pathway of *P. aeruginosa*, which has not been characterized, are shown. In addition, the regulation of the *fad* genes in *P. aeruginosa* is an enigma. FadL, an outer membrane protein, is involved in fatty acid transport. FAD, flavin adenine dinucleotide; $FADH_2$, reduced flavin adenine dinucleotide; CoA, coenzyme A.

material). Perhaps *aprA* is expressed, and this may be a situation similar to the situation for *plcH* and *plcR* discussed above. Since performing pairwise comparisons for all genes in the *P. aeruginosa* genome is an enormous undertaking, we selected a few genes to examine in this study (Fig. 2). On average, these genes were induced in pairwise comparisons (Fig. 2). Indeed, these genes were induced when we performed real-time RT-PCR (e.g., *glp* genes in Table 4 and *mexY* and *plcH* in Table 3). Therefore, we hope that the deposited microarray data may be of some use in future analyses performed by other investigators who may use lower stringency (higher *P* values) or pairwise comparisons and further testing.

In summary, our study revealed several important aspects of *P. aeruginosa* pathogenesis, drug resistance, and metabolism during infection in CF patients in relation to population diversity. *P. aeruginosa* has a huge arsenal of metabolic capabilities, which allows it to exploit many possible nutrients in the lung environment. This is the first study that defines the metabolic capability of *P. aeruginosa* in vivo and the host pulmonary nutrient factors that may contribute to bacterial replication.

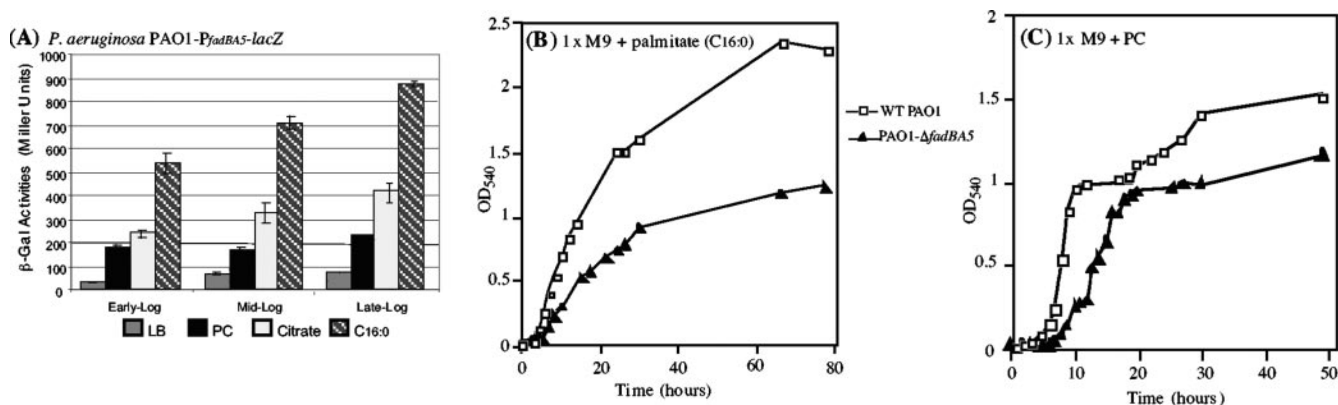

FIG. 4. *fadBA5* operon is involved in PC degradation. (A) *P. aeruginosa* fusion strain PAO1-P*fadBA5-lacZ* was grown in LB, PC, citrate, and palmitate ($C_{16:0}$), and the β-galactosidase (β-Gal) assay was performed (20). The results indicate the reason for no apparent difference in the fold change in the *fadBA5* operon expression shown in Table 4 for growth on PC relative to growth on citrate. The lack of *fadBA5* induction when *P. aeruginosa* strain PAO1 was grown on 1× M9 medium plus PC compared to growth on citrate seems to be due to the high expression of this operon when bacteria were grown in 1× M9 medium plus citrate compared to the expression during growth on PC. (B and C) Δ*fadBA5* mutant showed a defect in growth on palmitate compared to wild-type strain PAO1 (WT) (B), and this mutant had a defect in PC degradation compared to wild-type strain PAO1 (C), although the defect was not as dramatic as the defect with palmitate, probably due to growth on other carbon sources (glycerol and phosphorylcholine of PC). Both panel B and panel C show that the Δ*fadBA5* mutant had a lower growth rate and overall lower final cell density, which were due to a partial defect in the ability to degrade fatty acid as one of the components of PC as a nutrient. Data for construction of the mutant and fusion are not shown.

Our objective in this study was to identify the expression of metabolic pathways which may contribute to nutrient acquisition, leading to replication and maintenance of *P. aeruginosa* in the lung at an HCD. Our data suggested that *P. aeruginosa* degrades amino acids as a nutrient source in the lung. In addition, *P. aeruginosa* also induces genes in vivo to potentially metabolize lung surfactant lipids, adapting to the many possible nutrient sources.

TABLE 5. Expression of genes involved in PC degradation in a second CF patient by real-time RT-PCR

Gene analyzed	Fold change[a]		
	In vivo vs in vitro[b]	In vitro vs PAO1[c]	In vivo vs PAO1[d]
lipA	2.7	2.5	**8.8**
plcH	**6.2**	0.3	2.5
fadB5	2.8	3.1	**11.4**
fadD1	1.8	3.3	**7.1**
fadD2	0.5	11.3	**7.0**
fadD4	0.5	9.3	**5.9**
betA	17.3	5.4	**105.2**
glpD	1.0	10.5	**12.1**
glpK	0.8	6.4	**6.5**

[a] Bold type indicates that genes required for PC degradation in vitro were also expressed in vivo.

[b] The values for in vivo versus in vitro (in vivo induced) represent the expression levels of in vivo mRNA isolated directly from CF sputum relative to those of the same clinical isolate pool grown in 1× M9 medium containing 20 mM citrate. For *plcH*, an expression value of 6.2 is more indicative of in vivo expression and induction.

[c] The values for in vitro versus PAO1 (in vivo constitutive) represent the expression levels of mRNA isolated from the clinical isolate pool relative to those of mRNA isolated from PAO1, where the two cultures were grown identically in 1× M9 medium containing 20 mM citrate. There was constitutive expression of *glpD* and *glpK*, whereas there was no in vivo induction (1.0- and 0.8-fold changes), but the expression of these genes was evident (10.5- and 6.4-fold changes) when we compared the strain from the CF patient to PAO1, in which these genes were more repressed.

[d] The values for in vivo versus PAO1 represent the expression levels of in vivo mRNA directly isolated from sputum from the CF patient relative to those of mRNA of PAO1 grown in 1× M9 medium containing 20 mM citrate. This was a direct comparison to PAO1, where we predicted that some of the genes were more regulated. The direct comparison values (in vivo versus PAO1) are similar and exhibited the same trends as the theoretical values (when values from the in vivo induced column were multiplied by values from the in vivo constitutive column).

The gene expression of *P. aeruginosa* in vivo may be influenced by years of evolution during chronic lung infection and by the microenvironment of each bacterium, which leads to a diverse infectious population. Our study revealed a different level of evolution (i.e., deregulation) for several genes in the transcriptome rather than the deletion or acquisition of genes previously demonstrated in the genome (12, 54). We suggest that the population diversity and microenvironment might influence an individual bacterium's behavior, perhaps contributing to a cooperative and diverse infectious population. The shift in thinking of the bacteria in a chronic infection in a CF patient as a diverse infectious population and not as individual isolates may yield novel insight into future treatment. For example, the existence of hypermutable *mutS* variants coupled to the bacterial population diversity found in the lungs of CF patients with chronic lung infections as opposed to acute infections has important ramifications for future testing for effective antibiotic combinations and concentrations. Thus, future tests of antibiotics on planktonic cells or biofilm would be performed more effectively with a clinical population or a pool of isolates than with a single isolate, in order to obtain the true resistance potential of the infectious *P. aeruginosa* population. Therefore, both the diversity of the population and the microenvironment may contribute to the full potential of *P. aeruginosa* within the lungs of CF patients, such that each member of the population plays a distinct role in order to contribute to the pathogenesis of the infectious population. However, this view awaits novel technology to dissect the metabolism and virulence of individual bacteria in the population relative to the microenvironment, which should answer some questions concerning the extent of bacterial cooperation.

The results of this study should serve as an initial model for

understanding (i) how *P. aeruginosa* reacts to antibacterial treatment in vivo, (ii) nutrient acquisition for extracellular pathogens of the lung, and (iii) the detrimental effects of nutrient metabolism in the lung. Finally, this study opens opportunities for further and more thorough analyses of these important pathogenesis aspects with clinical samples from other CF patients in order to determine the transcriptome conservation and heterogeneity of *P. aeruginosa* populations in vivo.

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