Transcriptome Analysis of *Pseudomonas aeruginosa* Growth: Comparison of Gene Expression in Planktonic Cultures and Developing and Mature Biofilms†

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*Pseudomonas aeruginosa* is a versatile organism that can survive in soil, marshes, and marine habitats, on plant and animal tissue, and on nonliving surfaces (24). At 6.3 million base pairs (5,570 open reading frames [ORFs]), the genome of *P. aeruginosa* is one of the largest bacterial genomes that have been sequenced (24), and its size and complexity are thought to enable survival in diverse environments. Central to this survival is the ability to adopt and switch between free-living (planktonic) and biofilm (surface-attached) lifestyles. Biofilms are populations of microorganisms adhered to a surface or interface, and *P. aeruginosa* biofilms arise in a variety of clinical settings, including the cystic fibrosis lung, urinary catheters, and contact lenses (22, 23, 29). Biofilm infections are notoriously difficult to eradicate, even after prolonged antimicrobial therapy, and it is well documented that biofilms are less susceptible to antimicrobial agents than free-living (planktonic) bacteria and provide protection from the host immune response (5, 9, 11).

Control of gene expression in *P. aeruginosa* is the key determinant of its flexibility, and a variety of highly integrated regulatory mechanisms have been described previously. These include the LasR-LasI and RhlR-RhlI cell density-dependent quorum-sensing (QS) systems and a large number of two-component regulatory systems (6, 13, 16, 24, 27). Knowledge of the specific genes required by *P. aeruginosa* for survival under different environmental conditions will improve our understanding of the biology of this organism and form the basis for the rational design of novel therapeutic approaches. The recent development of an Affymetrix GeneChip microarray representing 5,549 ORFs on the *P. aeruginosa* chromosome now enables the analysis of global gene expression of this organism under defined growth conditions and the comparison of different growth states. Here we provide the first study in which the transcriptional profiles of two bacterial planktonic phases (logarithmic phase [LP] and stationary phase [SP]) and multiple biofilm time points (8, 14, 24, and 48 h) are compared.

**Biofilm characterization.** Previous studies using continuous culture biofilm systems (10, 17) have shown that biofilms form in a sequential process: (i) attachment, (ii) microcolony formation, and (iii) biofilm maturation. Using a Zeiss LSM510 confocal laser scanning microscope (40× magnification), we characterized biofilm development of wild-type *P. aeruginosa* strain PAO1 (7) tagged with green fluorescent protein (pUCP18 containing gfpmut3* constitutively expressed from a *lacZ* promoter) by using a static model in which biofilms are grown on nitrocellulose filters placed on agar. LB broth (peptone, 10 g/liter; yeast extract, 5 g/liter; NaCl, 5 g/liter) was grown on nitrocellulose filters placed on agar. LB broth (peptone, 10 g/liter; yeast extract, 5 g/liter; NaCl, 5 g/liter) was purchased from Invitrogen (Paisley, United Kingdom), and reduced strength LB broth was used in order to reflect a low-nutrient environment which would be encountered in vivo. Nitrocellulose filters (diameter, 25 mm; Millipore) placed on 20 ml LB (20%) agar were inoculated with 105 CFU of an overnight culture and were incubated at 37°C. From single cells (Fig. 1A), microcolonies began to develop after 2 h (Fig. 1B) and increased in size with time (4, 6, and 8 h [Fig. 1C, D, and E, respectively]) and eventually formed a confluent biofilm after 14 h (approximately 20 μm deep [Fig. 1F]) which did not change in architecture when examined after 24 and 48 h. Hence, in this static biofilm model, *P. aeruginosa* biofilms develop in a similar sequential process to that in continuous culture biofilm systems. The two advantages of this static model are (i) biofilm cells can be removed from filters and lysed immediately without any previous processing and (ii) on harvesting of each biofilm, there is no possibility of simultaneously collecting planktonic cells that could be present if a flowthrough model was used.

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†Supplemental material for this article may be found at http://jb.asm.org/.
Comparison of biofilm and planktonic culture gene expression. *P. aeruginosa* wild-type strain PAO1 (7) was used for biofilm and planktonic culture expression analysis. Overnight cultures were grown in LB broth (37°C, 200 rpm). Three separate RNA samples were extracted under each condition. Therefore, three biological replicates were used, and data from triplicate arrays were obtained for each condition. For biofilm samples, five filters were removed for each RNA extraction after 8, 14, 24, and 48 h. Immediately after removal from the agar surface, filters were placed directly into TRIzol reagent (Invitrogen) and cells were removed from each filter by scraping with a sterile loop. For planktonic RNA samples, 100 ml LB (20%) broth in 250-ml Erlenmeyer flasks was inoculated with 1 ml PAO1 overnight LB culture (diluted 10-fold), and the resulting cultures were grown at 37°C with agitation (200 rpm). After 4-h (optical density at 600 nm [OD<sub>600</sub>, 0.1]) (LP) and 24-h growth (OD<sub>600</sub>, 0.3) (SP), 25-ml aliquots were pelleted by centrifugation (5 min, 8,000 rpm, 4°C). Cells were then resuspended in TRIZol reagent (Invitrogen). All samples (planktonic and biofilm) were subjected to vortexing and sonication before chloroform extraction and isopropanol precipitations were carried out. RNA pellets were then washed with 70% ethanol and resuspended in water. Residual DNA was then removed by use of a DNA-free kit (Ambion) and RNA of <200 nucleotides removed by use of an RNeasy (QIAGEN) RNA extraction kit. The quantity and purity of RNA were calculated through OD<sub>260/280</sub> spectrometry and agarose (1%) gel electrophoresis. cDNA synthesis (from 11 μg RNA), hybridization, and scanning were performed according to the manufacturer’s instructions for the *P. aeruginosa* GeneChip array (Affymetrix).

All data were globally scaled to set the average signal intensity of each array to a target signal of 100. Data analysis and normalizations were performed by using GeneSpring software (version 5.1; Silicon Genetics). For comparisons between two different conditions, genes that displayed an expression value of under 50 (considered background) for all replicates were removed. By use of the expression data from all six conditions (four biofilm time points and two planktonic phases), an experiment tree was drawn by using GeneSpring software (version 5.1; Silicon Genetics) (Fig. 2). This shows the relationships between the expression levels of the six different conditions and clusters together conditions with similar expression profiles. This procedure clustered the LP planktonic culture with
the 8-h-time point developing biofilm, whereas the SP planktonic culture clustered with the confluent biofilms formed at the 14-, 24-, and 48-h time points. Quantitative reverse transcriptase PCR of four genes ($fliE$, PA0020, PA2184, and PA5555) was used to provide independent verification of the microarray results (data not shown).

In order for a gene to be considered to be differentially expressed, two criteria had to be fulfilled: the average change in expression ($n$-fold) must be $\geq 2.5$ and $P$ values must be $< 0.05$ (one-way analysis of variance). Table 1 illustrates that 19.4% of the PAO1 genome is differentially expressed (10.5% genes up-regulated and 8.9% genes down-regulated) when gene expression in LP planktonic culture is compared with that in SP planktonic culture. However, when gene expression in LP planktonic culture is compared with gene expression in 8-h developing biofilms, only 3.1% of the genome is differentially expressed (0.8% genes up-regulated, 2.3% genes down-regulated), and when SP planktonic culture gene expression is compared with gene expression of 14-, 24-, and 48-h confluent biofilms, $\leq 14.3\%$ of the genome is differentially expressed. However, gene expression among the confluent biofilm time points (14, 24, and 48 h) was found to be very conserved ($< 1\%$ of genes are differentially expressed) (Table 1), whereas gene expression in developing biofilms (8 h) differs considerably from the confluent biofilms (14, 24, and 48 h), as at least 15.5% of genes are differentially expressed.

Recent advances in genomics and proteomics have accelerated our understanding of the physiology of biofilms, but two recent studies have yielded conflicting data (17, 28). A microarray study in which gene expression in strain PAO1 biofilms grown on stones in a chemostat was compared with that of a chemostat planktonic population unexpectedly showed that only 1% of the genome was differentially expressed (28). Conversely, in a recent PAO1 proteomic study, the difference in proteomes was $> 50\%$ when a mature 6-day biofilm grown inside silicone tubing was compared to a planktonic culture (17). Protein patterns from different stages of biofilm development were also found to be profoundly different (17). Recently, a gene expression study using *Escherichia coli* Affymetrix DNA arrays compared a single biofilm stage with LP and SP planktonic cultures (18). When biofilm gene expression was compared with that obtained in LP planktonic culture, 4.8% of genes were up-regulated ($\geq 2.5$-fold change) and 0.63% were down-regulated, whereas a comparison with SP planktonic culture found 9.7% of genes were up-regulated and 4.48% were down-regulated (18). Another *E. coli* biofilm microarray study using a different strain compared a mature biofilm and LP planktonic culture and found that 1.9% of the genome was up- or down-regulated by a factor of 2 or more (2). Considering these *P. aeruginosa* and *E. coli* studies, together with the results from our study, this emphasizes the importance of comparing transcriptomic profiles of more than one planktonic state of

| % of genes (of indicated culture) up-regulated or down-regulated | 8 | 14 | 24 | 48 |
|---|---|---|---|---|
| LP | 10.5 | 0.8 | 12.4 | 12.1 | 11.3 |
| SP | 8.9 | 9.8 | 7.1 | 6.1 | 5.5 |
| 8 | 2.3 | 8.1 | 8.8 | 8.5 | 8.3 |
| 14 | 11.1 | 10.9 | 10.9 | 10.9 | 10.9 |
| 24 | 8.7 | 4.5 | 7.0 | 7.0 | 7.0 |
| 48 | 9.0 | 5.1 | 8.5 | 8.5 | 8.5 |

*LP, LP planktonic culture; SP, SP planktonic culture. Numbers (8, 14, 24, and 48) refer to biofilm time points. To obtain the percentage of genes significantly up-regulated between two culture conditions, the first condition is chosen from those listed in the first column and the second condition is chosen from those listed as a row (average change in expression [$n$-fold] $\geq 2.5$). To obtain the percentage of genes significantly down-regulated, this process should be reversed (average change in expression [$n$-fold] $\leq 2.5$). ($P$ values of $< 0.05$.)
growth with more than one biofilm structure. These studies also highlight the fact that differential gene expression between biofilm and planktonic culture will also differ among strains, culture conditions, and technologies used (glass slide array, GeneChip array, and proteomics) and that more of these studies are needed in order for us to fully understand the genetics of bacterial growth.

**Genes up-regulated in 8-h developing biofilms.** Filtering by use of gene lists generated for Table 1 enabled us to generate a list of 20 genes (Table 2) that are up-regulated by ≥2.5-fold in the 8-h developing biofilms compared to the five other conditions. Eight of these genes have no putative function previously assigned to them (www.pseudomonas.com), although additional functional information was obtained from various resources (us.expasy.org and www.tigr.org) for seven of these genes (PA0446, PA2080, PA4697, and PA5139) (see Table S1 in the supplemental material).

The list of genes up-regulated in 8-h developing biofilms contains three genes from different chromosomal regions involved in type IV pilus production (pilB, pilY2, and pilP). This was not surprising, given that type IV pilus have previously been shown to play a role in microcolony formation (12). Nearly half of the genes in this list have a putative role in transport; 94 genes that are ≥2.5-fold up-regulated in all three confluent biofilms (at the 14-, 24-, and 48-h time points) compared to the three other conditions. Sixty-eight of these genes have previously been found to be QS (20, 26) and/or RpoS (19) up-regulated in SP planktonic culture by using P. aeruginosa GeneChips. Of the 26 remaining genes (Table 3), only one gene in this list has previously been characterized: algD, which encodes an enzyme (GDP-mannose-6-dehydrogenase) involved in biosynthesis of alginate (4, 25). Seventeen have no putative function previously assigned to them (www.pseudomonas.com), although further functional information was obtained (us.expasy.org and www.tigr.org) for seven of these genes (PA0290, PA0557, PA2168, PA2179, PA2181, PA3461, and PA4870) (see Table S2 in the supplemental material).

Three of the genes in this list (PA0290, PA2718, and PA4870) may have a regulatory role in mature biofilms. PA2718 encodes a probable transcriptional regulator of the MerR family. Many of the regulators in this family respond to environmental stimuli often associated with biofilms: oxidative stress, heavy metals, and antibiotics (3). PA0290 encodes a protein with a GGDEF domain and is 54% similar to the C terminus of PleD, a two-component system response regulator of Caulobacter crescentus. This organism can switch between a sessile-adhesive (stalked) and motile-planktonic (swarmer) cell phenotype in its life cycle (14), and PleD has been shown to be required during the swarmer-to-stalked cell transition for flagellar ejection and efficient stalk biogenesis (1). It is possible that PA0290 plays a similar role in the transition from microcolony to mature P. aeruginosa biofilm phenotype, in which it might be involved in processes such as pilus retraction and cell differentiation. A previous study has found that when a Salmonella enterica serovar Typhimurium protein (AdrA) containing a GGDEF domain was expressed in P. aeruginosa, motility was repressed and biofilm formation enhanced (21).

PA4870 encodes a putative prokaryotic DksA/TraR C4-type zinc finger protein. DksA has been shown to repress ribosomal

### Table 2. Genes that are up-regulated by ≥2.5-fold in developing biofilms (8 h) compared to the five other conditions

| ORF     | Product name                  | Gene name | Functional class                   |
|---------|-------------------------------|-----------|------------------------------------|
| PA0049  | Hypothetical protein          |           | HUU                                |
| PA0446  | Conserved hypothetical protein|           | HUU                                |
| PA0447  | Glutaryl-CoA dehydrogenase    | gcdH      | aa biosynthesis and metabolism     |
| PA0952  | Hypothetical protein          |           | HUU                                |
| PA2080  | Hypothetical protein          |           | HUU                                |
| PA2114  | Probable MFS transporter      |           | Transport of small molecules       |
| PA2782  | Hypothetical protein          | csA        | Chaperones and HS proteins         |
| PA3221  | CsaA protein                  |           | Transport of small molecules       |
| PA4501  | Probable porin                |           | Transport of small molecules       |
| PA4502  | Probable binding protein       | pilB      | Motility and attachment            |
| PA4503  | Probable permease of ABC transporter | pilY2 | Motility and attachment           |
| PA4504  | Probable permease of ABC transporter |        | HUU                                |
| PA4505  | Probable ATP-binding component of ABC transporter | pilP | Motility and attachment           |
| PA4506  | Probable ATP-binding component of ABC transporter |        | HUU                                |
| PA4526  | Type 4 fimbrial biogenesis protein |        |                                      |
| PA4555  | Type 4 fimbrial biogenesis protein |        |                                      |
| PA4697  | Hypothetical protein          |           | HUU                                |
| PA5033  | Hypothetical protein          |           | HUU                                |
| PA5041  | Type 4 fimbrial biogenesis protein |        |                                      |
| PA5139  | Hypothetical protein          |           | HUU                                |

*Genes are identified by ORF designation, gene name, and product name (www.pseudomonas.com). HUU, hypothetical, unclassified, and unknown; aa, amino acid; HS, heat shock. See Table S1 in the supplemental material.*
gene transcription in *P. aeruginosa* by interacting with RNA polymerase on ribosomal promoters (15) and is also a novel regulator of virulence factor production (8). Likewise, PA4870 could play a similar regulatory role in the nutrient limiting conditions of mature biofilms. The roles of the other genes in this list in mature biofilms are unclear, although the functional classes of many of these genes (central intermediary metabolism, putative enzymes, carbon compound catabolism, and peptidase activity) indicate a possible metabolic role (Table 3; see Table S2 in the supplemental material).

Therefore, we have identified 26 genes that are up-regulated in mature biofilms and have not previously been found to be induced by cell density and stationary-phase conditions in planktonic cultures. However, it is possible that these genes are (i) QS or RpoS regulated but not detected in the previous planktonic studies (e.g., PA2155 is in an operon, the majority of genes of which are QS regulated), (ii) up-regulated by RpoS, QS, or both but only under confluent biofilm conditions, or (iii) expressed uniquely in confluent biofilms and their regulation is independent of the RpoS and QS systems.

This study is the first to examine global gene expression profiles for two planktonic phases (LP and SP) and two biofilm structures (microcolony and confluent biofilms) of *P. aeruginosa*. These data have shown that gene expression in developing biofilms (8 h) is more closely related to LP planktonic culture than to biofilms at the other time points, while gene expression in confluent biofilms (14, 24, and 48 h) is more closely related to SP planktonic culture than to developing biofilms (8 h). It is also clear that once a uniform biofilm is formed after 14 h, gene expression remains relatively stable for a further 34 h (<1% genes are differentially expressed [Table 1]). This is a reflection of the observation that there were no changes in biofilm architecture between the three different confluent time points (14, 24, and 48 h). These results not only provide further evidence to the participation of type IV pili and QS (data not shown) in biofilm formation but also provide clues to other cellular processes and regulatory mechanisms involved in biofilm development. The role of these novel genes, which are up-regulated in microcolonies and confluent biofilms, in the formation of these structures is the subject of ongoing studies within our laboratory.

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### REFERENCES

1. Aldridge, P., and U. Jenal. 1999. Cell cycle-dependent degradation of a flagellar motor component requires a novel-type response regulator. Mol. Microbiol. 32:379–391.

2. Beloin, C., J. V. Stoyanov, S. P. Kidd, and J. L. Hobman. 2003. The MerR family of transcriptional regulators. FEMS Microbiol. Rev. 27:145–163.

3. Balestrino, J. A. Haagensen, S. Molin, G. Prensier, B. Arbeille, and J. M. Ghigo. 2004. Global impact of mature biofilm lifestyle on *Escherichia coli* K-12 gene expression. Mol. Microbiol. 55:659–674.

4. Derelic, V., J. F. Gill, and A. M. Chakrabarty. 1987. *Pseudomonas aeruginosa* infection in cystic fibrosis: nucleotide sequence and transcriptional regulation of the algD gene. Nucleic Acids Res. 15:4567–4581.

5. Drenkard, E. 2003. Antimicrobial resistance of *Pseudomonas aeruginosa* biofilms. Microbes Infect. 5:1213–1219.

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**TABLE 3. Genes up-regulated in confluent biofilms (14, 24, and 48 h) and not previously found to be QS or RpoS up-regulated**

| ORF    | Product name | Gene name | Functional class |
|--------|--------------|-----------|------------------|
| PA0290 | Hypothetical protein | HUU | Central intermediary metabolism |
| PA0557 | Hypothetical protein | HUU | Central intermediary metabolism |
| PA1471 | Hypothetical protein | HUU | Central intermediary metabolism |
| PA1931 | Probable ferredoxin | HUU | Central intermediary metabolism |
| PA1932 | Probable hydroxylase molybdopterin-containing subunit | HUU | Central intermediary metabolism |
| PA1933 | Probable hydroxylase large subunit | HUU | Central intermediary metabolism |
| PA2021 | Hypothetical protein | HUU | Central intermediary metabolism |
| PA2107 | Hypothetical protein | HUU | Central intermediary metabolism |
| PA2136 | Hypothetical protein | HUU | Central intermediary metabolism |
| PA2140 | Probable metallothionein | HUU | Central intermediary metabolism |
| PA2149 | Hypothetical protein | HUU | Central intermediary metabolism |
| PA2155 | Probable phospholipase | HUU | Central intermediary metabolism |
| PA2168 | Hypothetical protein | HUU | Central intermediary metabolism |
| PA2179 | Hypothetical protein | HUU | Central intermediary metabolism |
| PA2181 | Hypothetical protein | HUU | Central intermediary metabolism |
| PA2184 | Conserved hypothetical protein | HUU | Central intermediary metabolism |
| PA2186 | Probable alcohol dehydrogenase (Zn-dependent) | HUU | Central intermediary metabolism |
| PA2187 | Probable transcriptional regulator | HUU | Central intermediary metabolism |
| PA2188 | Probable acetyltransferase | HUU | Central intermediary metabolism |
| PA2189 | Conserved hypothetical protein | HUU | Central intermediary metabolism |
| PA2191 | GDP-mannose 6-dehydrogenase AlgD | HUU | Central intermediary metabolism |
| PA2197 | Hypothetical protein | HUU | Central intermediary metabolism |
| PA3460 | Probable metallothionein | HUU | Secreted factors |
| PA3461 | Conserved hypothetical protein | HUU | Secreted factors |
| PA3540 | GDP-mannose 6-dehydrogenase AlgD | HUU | Secreted factors |
| PA4570 | Hypothetical protein | HUU | Secreted factors |
| PA4870 | Conserved hypothetical protein | HUU | Secreted factors |
| PA4877 | Hypothetical protein | HUU | Secreted factors |
| PA4860 | Hypothetical protein | HUU | Secreted factors |

* Genes are identified by ORF designation, gene name, and product name (www.pseudomonas.com). HUU, hypothetical, unclassified, and unknown. See Table S2 in the supplemental material.

