signalling. Separation behaviour induced by Fz7 in FGF-stimulated animal cap cells was inhibited by pertussis toxin, and this block was released by coexpression of PKCα. This indicates that, as in the lower lip, the PKC branch of the Fz signalling cascade is involved, and that the animal cap system is a valid model for endogenous involuted mesoderm. Induction of separation behaviour is specific to *Xenopus* Fz7, as similar ectopic expression of Fz3 or Fz8 did not promote it.

The response to Fz7 expression is ligand-dependent. A Fz7 deletion construct lacking the extracellular amino terminus (ΔNFz7) induced a low level of separation behaviour, which could not be further increased by FGF. Coexpression of Fz7 with a secreted, putative ligand-binding domain (NXfz7-fun) or a construct lacking the intracellular carboxy-terminus (ΔCFz7) prevented the separation response in FGF-stimulated animal cap cells, demonstrating that these constructs could act as dominant-negative proteins.

Together, our results with dorsal lips and animal caps show that the PCP and the Fz/PKC branches are clearly separable pathways. In particular, the Fz/PKC pathway does not involve Dsh function (Fig. 4c). During gastrulation this pathway controls tissue separation in the anterior mesoderm through the *Xenopus* Fz7 receptor, which is critical for normal development. This type of boundary formation is an important process in embryogenesis and its regulation is of general significance. Further experiments will clarify whether Fz/PKC signalling is also involved in other tissue separation events in *Xenopus* as well as in other vertebrate and invertebrate species. □

Methods

DNA constructs

Two hundred and one base pairs of the 5′ UTR and the complete coding sequence of *Xenopus* Fz7 were amplified by polymerase chain reaction (PCR) and cloned into *Bam*HI/*Cla*I sites of the expression plasmid pCS2+–MT to generate Myc-tagged UTR Fz7. For the N-terminal deletion of Fz7 (ΔNFz7), a fragment from the coding region (residues 215–549) containing the seven transmembrane domains and the carboxyl tail was amplified by PCR and cloned into the *Eco*RI/*Xba*I sites of the pCS2+ vector. A fragment coding for the signal peptide (residues 1–52) was joined with this clone (residues 215–549) at the *Bam*HI/*Eco*RI sites. Sense RNA for ΔNFz7 was transcribed from template that was linearized with Asp 718, using the SP6 promoter.

Sequence of Fz7Mo and transcription/translation of Fz7

Fz7Mo oligonucleotide (5′-CCAACAAGTGATCTCTGGACAG CAG-3′) and unspecific control morpholino oligonucleotide (Gene-Tools). The target sequence for Fz7Mo is not present in the 5′ UTR and the coding region of the Fz8 complementary DNA. For *in vitro* transcription/translation of Fz7 we used the TNT Coupled Reticulocyte Lysate System (Promega) according to the manufacturer's instructions.

Whole-mount analysis and immunofluorescence

Expression of *brachyury* and *chordin* was analysed in embryos at the gastrula stage as described[7]. Immunostaining and colorimetric detection using horseradish peroxidase has been described[17]. Protein extracts were separated on SDS–polyacrylamide gel electrophoresis (PAGE) gels, and EGFP-Myc and Fz7-Myc protein was detected with the 9E10 antibody as described[17].

Embryos were fixed in Dent's solution (methanol/DMSO; 4:1). Cryosections (10 μm) and immunodetection of the phosphorylated form of PKCα (anti-phospho-PKCα Ser 657; Upstate Biotechnology) and Myc-epitope (9E10) was performed as described[18].

Separation behaviour and dorsal lip elongation

In vitro separation assay was performed as described[8]. Convergent extension in dorsal lips was assayed as described[2].

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Correspondence and requests for materials should be addressed to H.S. (e-mail: herbert.steinbeisser@tuebingen.mpg.de).

Gene expression in *Pseudomonas aeruginosa* biofilms

Marvin Whiteley*, M. Gita Bangera†, Roger E. Bumgarner‡, Matthew R. Parsek§, Gail M. Teitzel§, Stephen Lory† & E. P. Greenberg*

** Department of Microbiology, University of Iowa College of Medicine, Iowa City, Iowa 52242, USA*
† Department of Microbiology and Molecular Genetics, Harvard University, Boston, Massachusetts 02115, USA
‡ Department of Microbiology, University of Washington, Seattle, Washington 98195, USA
§ Department of Civil Engineering, Northwestern University, Chicago, Illinois 60208, USA

Bacteria often adopt a sessile biofilm lifestyle that is resistant to antimicrobial treatment[1–5]. Opportunistic pathogenic bacteria like *Pseudomonas aeruginosa* can develop persistent infections[1–3]. To gain insights into the differences between free-living *P. aeruginosa* cells and those in biofilms, and into the mechanisms underlying the resistance of biofilms to antibiotics, we used DNA microarrays. Here we show that, despite the striking differences in lifestyles, only about 1% of genes showed differential

expression in the two growth modes; about 0.5% of genes were activated and about 0.5% were repressed in biofilms. Some of the regulated genes are known to affect antibiotic sensitivity of free-living *P. aeruginosa*. Exposure of biofilms to high levels of the antibiotic tobramycin caused differential expression of 20 genes. We propose that this response is critical for the development of biofilm resistance to tobramycin. Our results show that gene expression in biofilm cells is similar to that in free-living cells but there are a small number of significant differences. Our identification of biofilm-regulated genes points to mechanisms of biofilm resistance to antibiotics.

Many persistent infections are caused by biofilms[2]. One particularly devastating example is the lung infections caused by *Pseudomonas aeruginosa* biofilms in people with the genetic disease cystic fibrosis. Once *P. aeruginosa* colonizes the cystic fibrosis lung it cannot be eradicated by even the most aggressive antibiotic therapy[2,3,6,7]. Bacteria growing in biofilms possess characteristics distinct from their free-floating or swimming (planktonic) counterparts. Biofilm bacteria are resistant to antimicrobial treatments, and to the immune defences of hosts[1,2]. Biofilm bacteria form structured communities of cells embedded in an extracellular polymeric (EPS) matrix[1,4,5]. Biofilm formation by *P. aeruginosa* occurs in discrete steps: surface attachment and multiplication; microcolony formation; and differentiation into mature, structured, antibiotic-resistant communities[2,8]. The differences in populations of biofilms and planktonic bacteria have led to the hypothesis that there are major differences in gene expression between the two growth modes[1]. We used DNA microarray technology to test this hypothesis with *P. aeruginosa* as a model, and to gain insights into possible mechanisms of the ability of biofilm bacteria to withstand exposure to antibiotics.

Gene expression may vary during development, but the first question is how gene expression differs in a mature biofilm and planktonic cells. This is particularly relevant because of the resistance of mature biofilms to antimicrobial treatment. We grew *P. aeruginosa* using continuous-culture techniques with an effort to minimize differences in the conditions to which the bacteria were exposed. Planktonic bacteria were grown in a chemostat near the maximum growth rate for *P. aeruginosa*. Biofilms were also grown in chemostat vessels; however, they were attached to granite pebbles (Fig. 1). The growth medium and dilution rate for the biofilm were the same as for the chemostat culture of planktonic bacteria.

We used a *P. aeruginosa* microarray to compare gene expression in planktonic and biofilm cells[9]. The array contained 5,500 of the predicted 5,570 *P. aeruginosa* genes. Most genes in *P. aeruginosa* strain PAO1 were expressed at levels that allowed an analysis with reasonably small statistical variation (74% of the 5,500 genes arrayed). Few of these genes showed differential expression in biofilms compared with planktonic cultures (Fig. 2). This result argues strongly against the proposal that existence in a biofilm results in dramatic differences in the overall make-up of bacterial cells[1]. However, our analysis averages gene expression in biofilms, which are heterogeneous groups of cells exhibiting different activities[1,10]. It may be that certain subpopulations in the biofilm had substantially different patterns of gene expression than did the homogeneous planktonic population or most of the metabolically active cells in the biofilm. This could be true for planktonic cultures too.

More interesting than the genes that did not show biofilm-specific gene regulation is the small number of genes (73) that did show differential expression (at least a twofold difference; Table 1). In all, 34 were activated and 39 were repressed in biofilm populations. We validated the array data by analysing expression of several genes by northern blotting and ribonuclease protection assays (see Methods). About 34% of the 73 biofilm-regulated genes code for hypothetical proteins of unknown function. This is slightly lower then the overall percentage of such genes (44%) derived from the genome-sequencing project of *P. aeruginosa*[11].

To show that the microarray results applied to biofilms and planktonic cells grown under different conditions, we used ribonuclease protection assays to compare the expression of three genes in a once-flow-through biofilm system[7] with the expression in a batch culture of planktonic *P. aeruginosa*. The genes were PA2128, gene for probable fimbrial protein; PA1080, gene for flagellar hook protein; and PA0971, *tolA*. We also tested expression of one gene (PA3622, *rpoS*) by using a *lacZ* reporter. Gene expression patterns appeared similar to those obtained in our microarray analysis of *P. aeruginosa* grown in chemostat vessels. For PA2128, we observed 7-fold biofilm repression in tube biofilms versus 16-fold repression in the microarray experiment; for PA1080, 2.5-fold repression versus 2-fold repression; for PA0971, 3-fold induction versus 4-fold induction; and for PA3622, 2-fold repression versus 2.3-fold repression. We do not predict that all of the genes identified in the microarray analysis will show biofilm regulation under all experimental conditions, but these four genes show similar patterns of regulation in at least two different experimental systems.

The most highly activated genes in *P. aeruginosa* biofilms were those of a temperate bacteriophage that is closely related to the filamentous bacteriophage Pf1 (ref. 12). The genome of *P. aeruginosa* PAO1 contains 11 out of 14 Pf1 genes[11]. We assessed the abundance of Pf1-like phage in the fluid over the biofilms and in the planktonic chemostat culture fluid by using a plaque-formation assay on a Pf1-sensitive strain of *P. aeruginosa*. The induced levels of

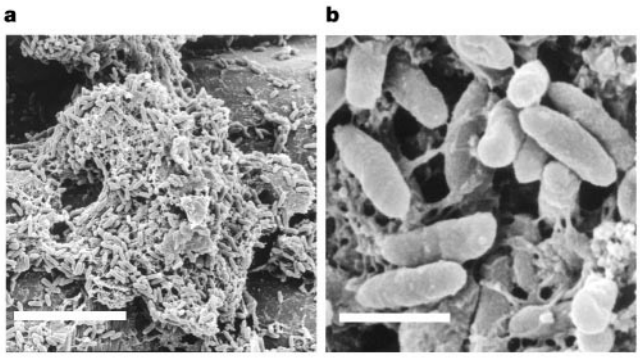

Figure 1 Scanning electron micrographs of a *P. aeruginosa* biofilm on the surface of a granite pebble. **a**, A biofilm on the surface of a pebble. Scale bar, 10 μm. **b**, High magnification of a biofilm showing rod-shaped *P. aeruginosa* and strings of dehydrated EPS connecting bacterial cells. Scale bar, 1 μm.

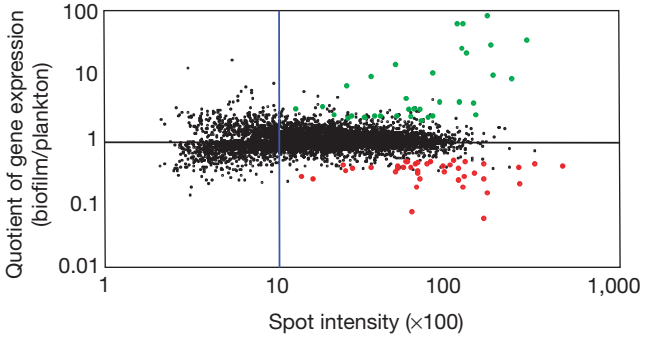

Figure 2 Gene expression and spot intensities of the 5,500 *P. aeruginosa* genes analysed with the microarray. Biofilm-activated genes are in green and biofilm-repressed genes are in red. Black dots represent genes that showed less than twofold regulation, or genes for which the data were not statistically different. Spot intensity refers to average total spot fluorescence (mean of eight independent spots). Spot intensities below 1,000 are shown but were not included in subsequent analyses because of statistical variability[30].

Pf1 transcripts in biofilm cells were reflected by a 100–1,000-fold greater abundance of Pf1 in the biofilm reactor than in the plankton reactor. Phage induction might be important for gene transfer within biofilms, it could function in exclusion of other strains of *P. aeruginosa* from a biofilm, or perhaps, as is true for certain temperate bacteriophage in other bacteria, the phage genome contains a gene specifying a toxin[13,14]. Many phage gene remnants and transposon-related sequences seem to be induced in *Escherichia coli* biofilms (A. Hay, personal communication).

Table 1 Genes differentially expressed in *P. aeruginosa* biofilms

P. aeruginosa ORF	Number	Fold activation (mean ± s.e.m.)
Bacteriophage genes		
Coat protein B of bacteriophage Pf1	PA0723	83.5 ± 10.3
Hypothetical protein of bacteriophage Pf1	PA0722	64.2 ± 5.6
Helix-destabilizing protein of bacteriophage Pf1	PA0720	35.2 ± 2.7
Hypothetical protein of bacteriophage Pf1	PA0721	26.6 ± 4.1
Protein of bacteriophage Pf1	PA0718	22.6 ± 2.9
Hypothetical protein from bacteriophage Pf1	PA0727	14.6 ± 2.4
Probable coat protein A of bacteriophage Pf1	PA0724	10.1 ± 0.6
Hypothetical protein of bacteriophage Pf1	PA0725	9.9 ± 1.1
Hypothetical protein of bacteriophage Pf1	PA0726	8.9 ± 0.5
Motility and attachment		
Probable fimbrial protein	PA2128	–16.5 ± 1.5
Pilin protein PilA	PA4525	–6.6 ± 0.8
Flagellar basal-body rod modification protein FlgD	PA1079	–2.7 ± 0.3
Probable pili assembly chaperone	PA2129	–2.4 ± 0.2
Flagellin type B	PA1092	–2.3 ± 0.3
Flagellar capping protein FliD	PA1094	–2.1 ± 0.3
Flagellar hook protein FlgE	PA1080	–2.0 ± 0.1
Translation		
50S ribosomal protein L28	PA5316	4.4 ± 0.5
50S ribosomal protein L19	PA3742	2.7 ± 0.1
50S ribosomal protein L4	PA4262	2.4 ± 0.2
50S ribosomal protein L18	PA4247	2.3 ± 0.3
50S ribosomal protein L23	PA4261	2.3 ± 0.2
30S ribosomal protein S7	PA4267	2.2 ± 0.3
Translation initiation factor IF-2	PA4744	2.1 ± 0.1
Ribosome modulation factor	PA3049	–5.3 ± 0.7
ATP-binding protease component ClpA	PA2620	–2.1 ± 0.1
Metabolism		
Urease β subunit	PA4867	63.1 ± 8.1
Ferredoxin (4Fe-4S)	PA0362	2.9 ± 0.3
Lipoate-protein ligase B	PA3997	2.8 ± 0.4
Glycerol-3-phosphate dehydrogenase	PA3584	–4.1 ± 0.3
Cytochrome *c* oxidase, subunit III	PA0108	–2.9 ± 0.3
Cytochrome *c* oxidase, subunit II	PA0105	–2.9 ± 0.2
Cytochrome *c* oxidase, subunit I	PA0106	–2.7 ± 0.2
Leucine dehydrogenase	PA3418	–2.5 ± 0.2
Membrane proteins or secretion		
Translocation protein TatB	PA5069	6.9 ± 1.4
TolA protein	PA0971	3.9 ± 0.4
Translocation protein TatA	PA5068	2.4 ± 0.2
Outer membrane lipoprotein OmlA	PA4765	2.4 ± 0.7
Probable porin	PA3038	–3.5 ± 0.5
Type III secretion central regulator	PA1710	–2.5 ± 0.3
Probable sodium:solute symporter	PA3234	–2.3 ± 0.1
Regulation		
Probable transcriptional regulator	PA2547	3.1 ± 0.1
σ-factor RpoH	PA0376	2.3 ± 0.3
σ-factor RpoS	PA3622	–2.3 ± 0.3
Probable two-component response regulator	PA4296	–2.2 ± 0.2
Other		
Rod shape-determining protein MreC	PA4480	3.1 ± 0.5
Probable DNA-binding protein	PA5348	–4.6 ± 0.4
Probable glycosyl hydrolase	PA2160	–2.3 ± 0.2
Methylated-DNA-protein-cysteine methyltransferase	PA0995	–2.1 ± 0.2
Conserved hypothetical		
PA0990, 4.0 ± 0.4; PA0579, 3.3 ± 0.7; PA2971, 2.4 ± 0.2 PA3785, –3.9 ± 0.5; PA3235, –3.2 ± 0.2; PA4738, –3.1 ± 0.2; PA2621, –3.0 ± 0.3; PA0107, –2.7 ± 0.2; PA0588, 2.6 ± 0.3; PA1533, –2.6 ± 0.3; PA0587, –2.3 ± 0.2		
Hypothetical		
PA1870, 29.7 ± 1.2; PA3884, 11.1 ± 1.6; PA3231, 3.8 ± 0.4; PA0714, 2.5 ± 0.3; PA1372, 2.5 ± 0.3 PA3411, –12.8 ± 1.7; PA1676, –5.2 ± 0.6; PA1830, –3.9 ± 0.9; PA4638, –3.7 ± 0.8; PA1244, –3.1 ± 0.4; PA1855, –2.9 ± 0.2; PA4607, –2.6 ± 0.3; PA4661, –2.4 ± 0.2; PA3922, –2.1 ± 0.2		

Positive values represent activation in biofilms. Negative values represent repression.

Genes for synthesis of pili and flagella are repressed in biofilms (Table 1). Pili and flagella are reported to be involved in the initial steps (attachment and microcolony formation) of development of *P. aeruginosa* biofilms[8]. Our results suggest that these appendages may not be required for maintenance of a mature biofilm, but that they are involved in committed steps in biofilm development. Once development has proceeded through these steps, pili and flagella are no longer required.

We asked whether the array analysis provides insights into antibiotic resistance in *P. aeruginosa* biofilms. An analysis of the *P. aeruginosa* genomic sequence revealed more than ten gene clusters seeming to encode efflux pumps of the resistance nodulation division (RND) type [11]. Activation of some or all of these gene clusters are suggested to be involved in biofilm resistance to antibiotics[15]. Our data show that none were induced in the biofilm. However, some of the genes that are activated or repressed in biofilms are known to affect antibiotic sensitivity in *P. aeruginosa*. Cationic antibiotics such as tobramycin and gentimicin bind to the negatively charged lipopolysaccharide (LPS) of the outer membrane[16,17], and subsequent transport into *P. aeruginosa* correlates with the level of the transmembrane electrical potential[18–20].

The major aminoglycoside-resistance mechanism of *P. aeruginosa* is impermeability of the bacteria to antibiotic entrance[21,22]. This impermeability involves several factors, including the *tolA* gene product[23] and terminal electron transport proteins[18,19]. The *tolA* gene product affects LPS structure, resulting in decreased aminoglycoside affinity for the outer membrane. Evidence indicates that *tolA* is an essential *P. aeruginosa* gene, but mutants that underproduce *tolA* are hypersensitive to aminoglycoside antibiotics[23]. The

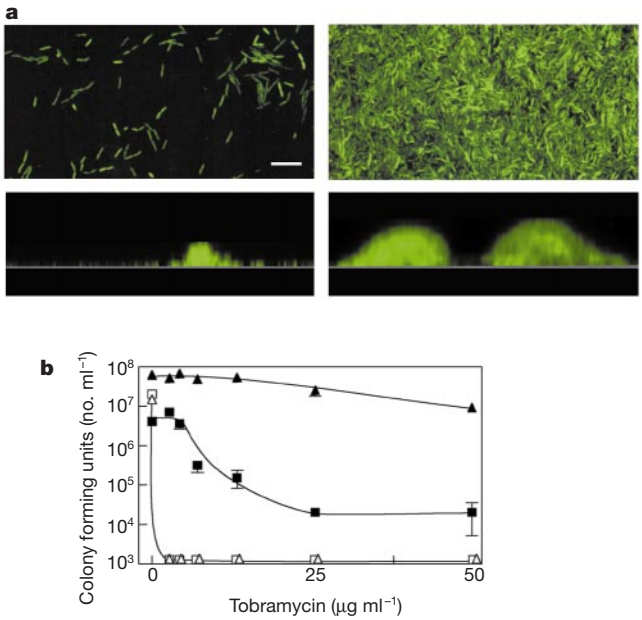

Figure 3 Comparison of wild-type and *rpoS*-mutant *P. aeruginosa* biofilms. **a**, Epifluorescent photomicrographs (top) and scanning confocal saggital reconstructions (bottom) of wild-type (left) and *rpoS*-mutant (right) *P. aeruginosa* containing a GFP-expression plasmid. Scale bar, 5 μm. **b**, Survival of cells in wild-type and *rpoS*-mutant biofilms after treatment with the antibiotic tobramycin. Open symbols, planktonic cultures; filled symbols, biofilms; squares, parent strain PAO1; triangles, *rpoS* mutant. Error bars show s.e.m. and are too small to be seen in most cases. At tobramycin concentrations above 5 μg ml^{-1} there were <2 viable cells ml^{-1} in planktonic cultures. Complementation of the *rpoS* mutant with the plasmid pMW105 containing a functional *rpoS* gene[26] restored biofilm formation rates and tobramycin survival to levels similar to wild-type levels (data not shown).

tolA gene was activated in *P. aeruginosa* biofilms (Table 1). This clearly could contribute to the resistance of the biofilms to aminoglycosides. The cytochrome *c* oxidase genes were repressed. Cytochrome *c* oxidase is the terminal electron acceptor during aerobic growth, and repression of cytochrome *c* oxidase should decrease sensitivity of *P. aeruginosa* to aminoglycoside antibiotics[18,19]. These are just two examples of genes that may be involved in biofilm resistance to one class of antibiotics. Many other genes in Table 1 might be involved as well (for example, the porin genes). The genes coding for proteins of unknown function are particularly interesting candidates as antibiotic-resistance factors. If such a gene is involved in antibiotic resistance, functional studies may reveal previously unknown biofilm resistance mechanisms.

If the microarray experiment (Table 1) has identified genes important in biofilm development or antibiotic resistance, then mutants defective in some of these genes should show aberrant biofilm structure and antibiotic sensitivity. We studied a mutant defective in one of the biofilm-regulated genes, *rpoS*. The *rpoS* gene codes for an RNA polymerase σ subunit and it influences transcription of other *P. aeruginosa* genes[15]. Previous studies indicated that an *rpoS*-deletion mutant of *P. aeruginosa* was hypervirulent in a mouse model[24], and that *rpoS* may serve some role in biofilm development[25]. The *rpoS* gene was repressed in our array experiment (Table 1) and in the once-flow-through tube biofilm reactor system.

To examine the involvement of *rpoS* in biofilm development, we grew the wild-type strain and an isogenic *rpoS* mutant[26], both tagged with a *gfp* plasmid (which expresses green fluorescent protein (GFP)), in flow-cell reactors. The *P. aeruginosa* biofilms that developed in the flow cells were examined by scanning confocal laser microscopy (Fig. 3a). Within 4 h, differences in the *rpoS* mutant and wild-type biofilms were evident. The mutant had attached to and covered much more of the glass surface. In a quantitative microtitre dish assay[8], biofilm formation of the parent strain was 38% of the *rpoS* mutant. After 24 h, the mutant biofilm had matured and large structured groups of bacteria were evident. The wild-type biofilm showed smaller structures (Fig. 3a), and after a further incubation the wild-type biofilm remained thinner than the mutant biofilm. This is consistent with a previous examination of our *rpoS* mutant: a 6-day-old *rpoS* mutant biofilm showed a mean thickness of 17 μm, and the thickness of the parent biofilm was 6 μm (ref. 25).

To assess whether *rpoS* influenced the susceptibility of *P. aeruginosa* biofilms to antibiotics, we treated biofilms of the *P. aeruginosa* wild type and *rpoS* mutant with increasing amounts of the aminoglycoside antibiotic tobramycin. Tobramycin is a front-line antibiotic used in the treatment of *P. aeruginosa* infections[27]. Biofilms of the *rpoS* mutant were much more resistant to killing by tobramycin than were wild-type *P. aeruginosa* biofilms (Fig. 3b). These results indicate that the *rpoS* gene is important for biofilm formation and in susceptibility to the antibiotic tobramycin. These studies also indicate that our array analysis identified a gene important for biofilm development and antibiotic sensitivity. It seems likely that other genes identified in the microarray analysis will be important in biofilm development and antibiotic susceptibility.

We hypothesize that existence in a biofilm induces moderate levels of resistance to all antimicrobial treatments. This could afford cells in a biofilm the opportunity to respond to an antibiotic by inducing genes more specific to that antibiotic. We compared biofilms exposed to tobramycin with untreated biofilms. Consistent with our hypothesis, 20 genes were differentially expressed in tobramycin-treated biofilms (Table 2): 14 were activated and 6 were repressed by tobramycin (seven times the minimum inhibitory concentration for planktonic cells). Of these 20 genes, 12 were classified as genes coding for hypothetical proteins of unknown function. As expected, treatment with tobramycin, which causes errors in protein synthesis, seemed to induce a stress response, with activation of *dnaK* and *groES* genes, for example. Tobramycin strongly induced several genes coding for hypothetical proteins. It also induced two probable efflux systems (a probable non-RND drug efflux system and a P-type ATPase). These are candidate tobramycin-resistance loci. Four genes that were expressed at a higher level in biofilms than in plankton were repressed by tobramycin treatment of biofilms, and two that were repressed in biofilms were activated in tobramycin-treated biofilms.

This initial microarray analysis of *P. aeruginosa* biofilms shows that, on average, gene expression in biofilm cells is remarkably similar to gene expression in planktonic cells maintained under similar environmental conditions. We identified only 73 genes that were differentially expressed in biofilms. Our studies of one biofilm-regulated gene, *rpoS*, showed it to be involved in the morphology and antibiotic sensitivity of biofilms. Further studies will reveal which of the biofilm-induced genes are activated under different environmental conditions, and which are important in biofilm biology. Such a subset of genes will be of great use in the development of rapid screens for agents that block biofilm maintenance. □

Table 2 Genes regulated by tobramycin in *P. aeruginosa* biofilms

P. aeruginosa ORF	Number	Fold activation (mean ± s.e.m.)
Ribosome modulation factor	PA3049	24.7 ± 11.4
Hypothetical protein	PA4326	23.2 ± 7.5
Hypothetical protein	PA2703	19.3 ± 3.7
Probable DNA-binding protein	PA5348	18.1 ± 4.9
Hypothetical protein	PA1110	8.7 ± 1.7
Conserved hypothetical protein	PA3463	8.3 ± 3.2
GroES	PA4386	5.8 ± 1.1
Conserved hypothetical protein	PA3785	5.0 ± 1.3
Probable drug-efflux protein	PA1541	4.7 ± 1.4
Probable metal-transporting P-type ATPase	PA3920	3.6 ± 1.1
Probable transcriptional regulator	PA3574	3.3 ± 0.6
Conserved hypothetical protein	PA2498	2.9 ± 0.6
DnaK	PA4761	2.7 ± 0.4
Conserved hypothetical protein	PA3819	2.1 ± 0.3
Hypothetical protein	PA1893	–2.4 ± 0.3
Hypothetical protein of bacteriophage Pf1	PA0725	–3.1 ± 0.5
Urease β subunit	PA4867	–3.7 ± 1.1
Hypothetical protein of bacteriophage Pf1	PA0721	–3.9 ± 1.0
Hypothetical protein	PA1870	–6.4 ± 1.5
Hypothetical protein	PA3884	–14.7 ± 5.7

Positive values represent activation by tobramycin. Negative values represent repression.

Methods

Planktonic and biofilm culture conditions

For array experiments, *P. aeruginosa* strain PAO1 was grown at 37 °C with aeration in chemostat vessels (100 ml of medium, dilution rate 0.2 h^{-1}). The growth medium consisted of 0.5% NH_4Cl, 0.25% NaCl, 0.025% Mg SO_4. $7H_2O$, 0.015% KH_2PO_4, 1.5% MOPS buffer at pH 7.0, non-chelated trace elements and 0.015% casamino acids. For biofilms, the chemostat vessel contained 100 g of sterilized granite pebbles (~70 pebbles). Vessels were inoculated with 10^6 *P. aeruginosa* cells. For biofilm cultures, bacteria were allowed to attach to the pebbles for 24 h before the flow of medium was initiated, which was at a high rate (100 ml h^{-1}). After 4 h at the high flow rate, >99% of the unattached bacteria were washed away. The flow was then decreased to a dilution rate identical to that in the planktonic chemostat. After 5 d, cell numbers in the biofilm and planktonic chemostats were similar (10^{10} cells) as determined by standard plate-counting techniques. To remove biofilm bacteria for plate counting, pebbles were vortexed for 2 min in 5 ml phosphate-buffered saline. A small planktonic population was present in the biofilm reactors throughout the experiment, but never represented more than 0.1% of the total chemostat population.

To validate the microarray experiments in one way, biofilms were grown in a once-flow-through system in silicone tubing as previously described[7], except that 20% Luria Bertani (LB) broth was used as the growth medium. For studies of biofilm development, *P. aeruginosa* containing the *gfp* plasmid pMRP9-1 were grown in flow-cell reactors and examined by scanning confocal microscopy[28]. For tobramycin-susceptibility studies, we grew biofilms using a spinning disk reactor and treated them with tobramycin as outlined elsewhere[29].

To assess the influence of tobramycin on biofilm gene expression, we added the antibiotic (5 μg ml^{-1}) to the influent medium after 4 d of biofilm growth. After 24 h in the presence of tobramycin, we removed biofilms and isolated the RNA. A concentration of 5 μg ml^{-1} of tobramycin is about seven times the minimum inhibitory concentration for planktonic *P. aeruginosa* strain PAO1. Standard plate counting indicated that 5 μg ml^{-1}

tobramycin did not measurably affect biofilm cell numbers. However, this level of tobramycin effectively killed planktonically cultured *P. aeruginosa* (Fig. 3b).

Scanning electron microscopy

Biofilm samples were prepared for scanning electron microscopy by fixation with 2.5% glutaraldehyde and staining with 1% osmium tetroxide. The samples were dehydrated in ethanol and hexamethyldisilizane, air dried, mounted on aluminium stubs, and sputter coated with gold and palladium (60 : 40). Imaging was conducted with a Hitachi S-4000 scanning electron microscope. Scanning confocal microscopy was performed as previously described[28].

Microarray analysis

We isolated RNA from planktonic and biofilm bacteria using the Trizol reagent (Life Technologies). Planktonic cells were formed into pellets by centrifugation (7,000 *g* for 10 min) at 4 °C. For isolation of biofilm RNA, rocks containing biofilm bacteria were rinsed with sterile medium and suspended in Trizol reagent. After vortexing for 1 min, the rocks were removed and the remaining cell material was sonicated for 10 s. Insoluble material was removed by centrifugation and RNA was isolated as described above. Contaminating DNA was eliminated by DNase treatment, and RNA was isolated by phenol-chloroform extraction and precipitation with LiCl. Complementary DNA probes were produced from RNA with random primers (NSNSNSNSNS) and Cy5-dCTP or Cy3-dCTP (Amersham) according to previously described procedures[30]. To avoid complications associated with Cy5-dCTP and Cy3-dCTP incorporation rates into resulting cDNA, each RNA comparison was performed with both dye combinations on separate microarrays. The microarrays were glass microscope slides containing representative gene-specific DNA fragments from 5,500 of the estimated 5,570 open reading frames (ORFs) of *P. aeruginosa*[9]. The microarrays were printed with a Generation II Array printer (Molecular Dynamics), and the hybridized microarrays were imaged with a Generation II scanning confocal fluorescent microscope (Molecular Dynamics). A full description of the microarray will be published later. Statistical analysis of microarray data was performed with previously described computer software[30].

The data in Tables 1 and 2 represent results of two independent experiments (means of eight individual comparisons). *Pseudomonas aeruginosa* ORF numbers and homologies were obtained from the *Pseudomonas* Genome Project (http://www.pseudomonas.com). Classifications are based on those described by Stover *et al.*[11]. Northern blot analysis and ribonuclease protection assays were performed as outlined by the manufacturer (Ambion) to verify microarray data for four activated and repressed genes. These techniques revealed fold regulations of 75 ± 10, 3.6 ± 0.7, -25 ± 5, and -3.0 ± 0.7 for PA4867, PA0971, PA2128, and PA1080, respectively.

Gene expression in once-flow-through tube biofilms (5 d old) was compared with gene expression in mid-logarithmic-phase planktonic cultures grown in LB broth. Analysis was either by quantitative ribonuclease protection assays or by monitoring β-galactosidase activity in a strain with a *lacZ* reporter, as indicated.

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Correspondence and requests for materials should be addressed to E.P.G. (e-mail: everett-greenberg@uiowa.edu).