# Comparative transcriptome analyses of *Pseudomonas aeruginosa*

**Deepak Balasubramanian**[1] and **Kalai Mathee**[2],*

[1]Department of Biological Sciences, College of Arts and Science, Florida International University, Miami, FL 33199, USA

[2]Department of Molecular Microbiology and Infectious Diseases, College of Medicine, Florida International University, Miami, FL 33199, USA

## Abstract

One of the hallmarks of bacterial survival is their ability to adapt rapidly to changing environmental conditions. Niche adaptation is a response to the signals received that are relayed, often to regulators that modulate gene expression. In the post-genomic era, DNA microarrays are used to study the dynamics of gene expression on a global scale. Numerous studies have used Pseudomonas *aeruginosa*—a Gram-negative environmental and opportunistic human pathogenic bacterium—as the model organism in whole-genome transcriptome analysis. This paper reviews the transcriptome studies that have led to immense advances in our understanding of the biology of this intractable human pathogen. Comparative analysis of 23 *P. aeruginosa* transcriptome studies has led to the identification of a unique set of genes that are signal specific and a core set that is differentially regulated. The 303 genes in the core set are involved in bacterial homeostasis, making them attractive therapeutic targets.

## Keywords

*alginate*; *bacteria*; *biofilm*; *comparative transcriptome*; *copper*; *iron limitation*; *microarray*; *osmotic stress*; *oxidative stress*; *pathogenesis*; *PCP*; *Pseudomonas aeruginosa*; *sulphate*; *quorum sensing*

## Introduction

Bacteria rapidly adapt to changing conditions by altering gene expression. The process of bacterial adaptation has fascinated researchers, as it provides important insights into their physiology. This information is especially useful for human pathogens because it can be applied to combating infections more effectively. This premise has led to studies that examine gene expression on a global scale. An entire set of RNA transcripts produced by a population of cells at any given time during gene expression is referred to as a transcriptome.[1] Variations in the external environment or genome perturbations, such as mutations, can alter the transcriptome of a cell. Transcriptome studies thus provide us with a snapshot of the organism's response, based on the genes that are expressed. Although there is not always a linear relationship between the expressed genes and the proteins synthesised, such studies give us a reasonable insight into the state of the cell.

In the pre-genomic era, transcriptome analysis relied on techniques such as differential display and RNA arbitrary primed polymerase chain reaction (PCR),[2–4] cDNA-amplified restriction

*Correspondence to:* Tel: +1 305 348 0628; Fax: +1 305 348 2913; Kalai.Mathee@fiu.edu.

fragment length polymorphism,[5–7] random expressed sequence tag sequencing,[8] serial analysis of gene expression (SAGE),[9] LongSAGE,[10] SuperSAGE[11,12] and massively parallel signature sequencing.[13] Prior sequence information is not required for these methods but they are both labour- and time-intensive. Bacterial studies entered the genomic era with the sequencing of the *Haemophilus influenzae* genome in 1995.[14] Since then, over 770 bacteria have been fully sequenced. Large-scale sequencing has ushered in the development of high-throughput genome-wide expression techniques such as DNA microarrays. This powerful technology has been harnessed in numerous studies, under diverse conditions, to elucidate whole-genome expression in both prokaryotes and eukaryotes. One such prokaryote is *Pseudomonas aeruginosa*, for which DNA microarrays have been of immense help in understanding its complex biology.

## P. aeruginosa

*P. aeruginosa* is a Gram-negative human pathogen of the family *Pseudomonadaceae* and can switch from being an environmental isolate, found in the soil and water, to being a human pathogen.[15] It is typical of opportunistic human pathogens, being implicated in a wide range of infections of the pulmonary tract,[16,17] urinary tract,[18–21] burns[22–24] and wounds,[25,26] among others. It causes persistent nosocomial infections[27,28] and is also a major pathogen in cystic fibrosis (CF) patients. One of the major weapons in its arsenal is its resistance to antibiotics. It is inherently resistant but can also acquire resistance genes from other bacteria, making it a notorious pathogen. Understandably, numerous studies over the years have focused on elucidating the mechanisms of pathogenesis and antibiotic resistance of *P. aeruginosa*. Since the sequencing of the genome,[29] the emphasis of these studies has shifted from conventional pathogenesis research, where the focus was on individual virulence determinants, to a much larger scale, involving whole-genome analyses.

The prototypic reference strain of *P. aeruginosa* is PAO1, isolated from a burn wound, which was the first *P. aeruginosa* strain to be sequenced.[29] The most prominent feature of the PAO1 genome is its size. It has one of the largest bacterial genomes, with 6.26 million base pairs, comprising almost 90 per cent protein-coding genes. The genome complexity of *P. aeruginosa* explains its ability to adapt and thrive in a wide range of environments. The genome has 5,570 open reading frames (ORFs), a number that is close to those of simple eukaryotes such as *Saccharomyces cerevisiae*. Other features of the PAO1 genome include the presence of four ribosomal RNA loci (*rrn*), which are the regions of the longest repeats, and a high GC ratio, at 66.6 per cent.[29] Following the sequencing of the genome, high-density DNA microarrays were developed for *P. aeruginosa*[30] that offered us a chance to take snapshots of the dynamic responses of this versatile genome to various stimuli. An important study in this context analysed the genomes of both environmental and clinical isolates of *P. aeruginosa*,[31] and suggested the presence of a core genome in different strains and expression of different sets of genes to facilitate growth in particular environments. This calls for a robust control over transcription mediated by transcriptional regulators. Analysis revealed that about 10 per cent of the PAO1 genome comprises transcriptional regulators.[29] This suggests the existence of a tight control over the way that genes are expressed in *P. aeruginosa*.

Subsequent to PAO1, other *P. aeruginosa* strains, such as PA14,[32] PA2192 (a CF isolate),[33] C3719 (the Manchester epidemic strain)[34] and PACS2,[35] have been sequenced. Comparative genome analysis of these five sequenced *P. aeruginosa* strains has led to the identification of 5,021 genes that are conserved between the strains (core gene set), the majority of which are housekeeping genes.[35] In addition, there are at least 1,800 ORFs belonging to the accessory genome that comprises mostly hypothetical ORFs or those related to transposons and bacteriophages, and genes involved in niche adaptation specific to each of the five strains.[35] The accessory set also contains transcriptional regulators that may also control expression of

ORFs in the core genome. The currently available *P. aeruginosa* GeneChip (Affymetrix) is based on the PAO1 genome, which has 5,570 ORFs. In addition, this GeneChip contains probe sets for 117 ORFs from non-PAO1 strains, a gross under-representation of the accessory genome. Thus, performing microarray studies with non-PAO1 strains would seriously underestimate the number of genes in a transcriptional profile and would undoubtedly affect data interpretation. Thus the next major advance in *P. aeruginosa* microarrays would be the development of a pangenome array, comprising probe sets for the core and accessory genomes from all sequenced strains. This will greatly advance our understanding of the system by allowing researchers to study non-PAO1 strains and helping them better to appreciate the biology of this versatile bacterium.

## P. aeruginosa transcriptome studies

### P. aeruginosa and iron limitation

Iron is an important cofactor, which plays a critical role in many cellular processes. Low iron availability in host tissues is a major impediment that bacteria have to overcome in establishing an infection. Bacteria have evolved an elaborate system of acquiring iron from their surroundings. *P. aeruginosa* secretes siderophores (pyochelin and pyoverdine), which chelate iron and bring it back into the cell via specific cell-surface receptors.[36] Fur is a major repressor that tightly regulates this uptake process by controlling the expression of the regulators, PvdS and PchR.[37]

In one of the initial transcriptome studies of the response of *P. aeruginosa* to iron,[38] stationary phase *P. aeruginosa* cells were grown in the presence and absence of iron at 32°C, followed by gene expression analysis. The results indicated that 137 genes were upregulated on iron depletion.[38] A more systematic study was performed by Palma *et al.*, who addressed how the transcriptome changes when iron-starved *P. aeruginosa* cells are fed with iron.[39] Differential regulation was seen in the mRNA levels of 177 genes. Most of the genes were downregulated, especially genes involved in transport of small molecules, transcriptional regulators and hypothetical proteins. It was interesting to note that genes involved in virulence were upregulated, some of which probably help the cells to overcome the host innate immune system. [39] An important finding was that 38 genes, including those coding for siderophore receptors, iron transport, regulators, sensors and sigma factors, were identified whose transcription decreased on iron treatment. These 38 genes were proposed to be part of a regulon involved in the iron response in *P. aeruginosa*. In another study, Zheng and coworkers identified a hypothetical protein, PA2384, which regulates over 70 genes involved in the iron uptake process in a Fur-independent manner.[40] Moreover, deleting PA2384 also led to differential expression of many genes involved in cellular physiology, such as translation, metabolism and electron transfer, indicating a broader role for this protein.

### P. aeruginosa and oxidative stress

For bacteria successfully to infect a human host, they should be able to overcome many barriers. One primary defence mechanism is the production of reactive oxygen species (ROS) by the host cells that leads to oxidative stress in bacteria. Palma *et al.* studied the response of *P. aeruginosa* to a natural oxidant (hydrogen peroxide) in human systems, as an indication of the global transcriptional status of the cell's early response.[41] The study confirmed that some of the genes that were earlier found to be important in a cell's response to oxidants were upregulated, in addition to identifying 520 genes with a significant (more than fivefold) change in mRNA levels.[41] Of these, 216 genes were upregulated, seven of which (primarily hypothetical proteins of unknown function) were upregulated more than 50-fold. Other upregulated genes included cytotoxin genes such as exoenzymes S and T and PvdS, which control PrpL proteinase and exotoxin A expression and could potentially inhibit the cells of

the innate immune system. Most of the genes that were downregulated are involved in metabolic pathways and ribosomal synthesis, which is logical, as under oxidative stress the cell would need first to deal with the stress, before actively metabolising or growing.[41]

In another study looking at the response to hydrogen peroxide, stationary phase cells of three strains belonging to two clonal types were tested.[42] Two of the strains, TB and 892, were CF isolates and their response was compared with that of PAO1, which belongs to a different clonal type. The expression profiles of the CF isolates were more similar to each other than to PAO1, sharing more than 80 per cent of the differentially expressed genes with each other. This study highlighted the importance of clone-specific transcriptome profiles, and variations seen in both intraclonal (between the two CF isolates) and inter-clonal (between CF isolates and PAO1) strains.

Genome-wide transcriptomes of the three strains, *P. aeruginosa* PAO1 and the two CF isolates TB and 892, were also analysed for their response to superoxide-generating paraquat.[43] Fewer than 30 genes were differentially expressed in each of the three strains, of which only six were upregulated in all three. Upregulated genes belonged to the following functional categories: oxidative stress response, transport, and acetyl coenzyme A and acetoin metabolism. Four genes of a single operon coding for the superoxide dismutase SodB, catalase KatA and alkyl hydroperoxide reductase AhpC were upregulated in three strains, highlighting their importance in the *P. aeruginosa* superoxide stress response. Energy metabolism genes were downregulated to counter the superoxide stress.[43]

Two recent papers by Small et al.[44,45] looked at the oxidative stress response of *P. aeruginosa*. Both of these papers focused on the effect of oxidising agents routinely used in hospitals for surface sterilisation, and their effect on *P. aeruginosa*. The first was a transcriptome analysis of sodium hypochlorite (NaOCl)-induced oxidative stress.[44] In this study, Luria-Bertani (LB) broth-grown mid-log phase *P. aeruginosa* PAO1 cells were treated for 20 minutes with sub-lethal concentrations of bleach. RNA extraction and microarray analysis followed. The results were compared with similarly grown untreated cells. About 20 per cent of the 5,570 genes of *P. aeruginosa* were differentially expressed in response to the treatment, approximately 10 per cent each being up- and downregulated. Responses similar to that seen with hydrogen peroxide were generated, with upregulation of catalase, alkyl hydroperoxide reductase and glutathione peroxide/reductase genes indicating that the mode of action of bleach in inducing the oxidant defence system genes is similar to that of hydrogen peroxide. Genes involved in the transport of sulphur compounds were highly upregulated. A possible explanation for this observation is that the free electrons in the sulphydryl groups in the biological molecules react with the hypochlorous acid formed by exposure to NaOCl, a known strong oxidiser, countering its effect. This would increase the chances of cell survival. [44] Genes that are repressed are primarily those involved in oxidative phosphorylation and electron transport, indicating that the cells under NaOCl-induced stress would have lowered energy owing to impaired metabolic processes.

Another study compared and analysed the transcription patterns of three different oxidative damage-inducing agents, NaOCl, peracetic acid and hydrogen peroxide, on *P. aeruginosa*.[45] Over 550, 300 and 200 genes were differentially regulated as a result of NaOCl, peracetic acid and hydrogen peroxide, respectively. Of these, 63 genes were common to all three agents, 40 upregulated and 23 downregulated. About 25 per cent of the common upregulated genes were 'related to phage, transposon or plasmid' and eight per cent to membrane protein. The primary downregulated genes were those involved in the transport of small molecules. Other prominent gene categories included those encoding membrane proteins and putative enzymes. It is surprising to note that membrane protein genes were both up- and downregulated. NaOCl resulted in the greatest downregulation of genes involved in energy metabolism, suggesting

that NaOCl may be more effective than the other two agents in limiting energy production. Peracetic acid exposure resulted in the stimulation of the expression of genes involved in adaptation and protection, and the downregulation of transcriptional regulators. Several protective mechanisms were triggered after exposure of cells to hydrogen peroxide. A significant finding of this study was the positive expression of the pyocin genes of the F-, R- and S-types after hydrogen peroxide treatment but downregulation with the other two treatments.[45] This has been linked to DNA damage and RecA activation, which has been known to induce pyocins. RecA has been demonstrated to cleave single-stranded DNA with the help of hydroxyl radicals that are formed by the exposure of cells to hydrogen peroxide. The finding that pyocin genes are induced in response to hydrogen peroxide treatment, published separately,[46] demonstrates that NaOCl exposure leads to significant differences in the transcription pattern of *P. aeruginosa* and is more lethal to the cell than either hydrogen peroxide or peracetic acid.[45]

Nde *et al.* tested the response of *P. aeruginosa* to ortho-phenylphenol (OPP),[47] a widely used disinfectant and antimicrobial agent whose mode of action in bacteria has not yet been elucidated. Using two different exposure times (15 and 60 minutes), they identified genes coding for virulence, membrane and ribosomal proteins, upregulated at both time points. Forty-five genes involved in translation, post-translation modification and degradation were upregulated, primarily initiation, elongation and release factors.[47] Genes encoding components of the SecY system involved in the export of various proteins, toxins and enzymes showed enhanced expression, as did some membrane-associated proteins involved in small molecule transport, possibly indicating that the OPP-treated bacteria were transporting proteins across the cell membrane as part of an adaptive process. Also upregulated were genes governing type IV pili assembly proteins, fatty acid biosynthesis and energy metabolism, all of which indicate adaptation.[47]

## P. aeruginosa and pentachlorophenol stress

The response of *P. aeruginosa* to pentachlorophenol (PCP) was investigated recently.[48] PCP is thought to embed in the membrane, where it acts as a proton shuttle uncoupling oxidative phosphorylation.[49] In addition, since RpoS is the stationary phase sigma factor thought to be important in the cell's stress response, *rpoS* mutants were also examined under the same conditions. Sixty genes showed a twofold or greater increase in transcription. RpoS did not play a protective role in the cell's response to PCP-induced stress. The highlight of this study was the induction of multiple operons involved in multi-drug efflux, the first anthropogenic induction of the MexAB–OprM system with chemicals. Notably, two operons of the resistance–nodulation–cell division (RND) pumps were upregulated, in addition to one operon that positively regulates MexAB–OprM. These are involved in antibiotic and solvent efflux. Putative major facilitator superfamily (MFS) proteins were also upregulated in response to PCP-induced stress. Selective downregulation of genes involved in central metabolism, fatty acid metabolism, energy metabolism and adaptation/protection was observed.[48]

## P. aeruginosa response to copper

Copper is an essential micronutrient, and plays an important role in cellular respiration as part of the electron transport chain and many biological reactions.[50] Bacteria have to control cellular levels of copper tightly, to prevent toxicity or starvation. Two studies looked at how *P. aeruginosa* responds to varying levels of copper.

Tietzel *et al.* identified 41 genes that form a core response of *P. aeruginosa* to copper, comparing transcriptomes of cells exposed to high levels of copper for a short time (copper-shocked, 405 genes) with those grown in copper (copper-adapted, 331 genes).[51] Genes

involved in the active efflux of copper included those encoding transporters, copper oxidase, periplasmic thiol-disulphide interchange proteins, lyases and transcriptional regulators.

Copper starvation of *P. aeruginosa* under aerobic conditions[52] led to downregulation of 132 genes, while only ten genes were upregulated. The most strongly downregulated genes were the iron-acquisition genes, mostly those repressed by Fur, the iron-uptake regulator. One of the downregulated transcriptional regulators, PA3410, encodes a probable extracellular sigma factor.[51] The upregulated genes included mostly hypothetical genes and a bacterioferritin involved in iron storage. In fact, copper starvation and iron depletion[38,39] led to the expression of similar sets of genes but have opposite effects on them.

## P. aeruginosa and sulphate starvation

The CF lung mucin is highly sulphated, probably as a defence mechanism to prevent degradation by bacterial enzymes.[53,54] The metabolically versatile *P. aeruginosa* can utilise a variety of organic and inorganic compounds for growth. Under limiting conditions of inorganic sulphate, the cells can take up organosulphur compounds for growth without affecting the growth rate.[55] The global response to sulphate starvation in a non-mucoid CF isolate of *P. aeruginosa*, E601, and PAO1 was addressed.[56] The study was aimed at determining which genes are regulated by sulphur supply and whether this list was altered during CF infection, a condition simulated by exposure to mucin. Three groups of responses were observed: response to sulphur starvation, to mucin as a sulphur source and to mucin exposure. Functional analysis of differentially expressed genes revealed that both strains showed similar responses in the different functional classes, with maximal differential regulation of genes encoding to hypothetical proteins, transport of small molecules and putative enzymes. Unlike PAO1, the CF isolate E601 was shown to utilise mucin as a sulphur source. Moreover, several genes involved in the type III secretion system and exoenzyme S were induced, suggesting that cells recognise mucin not only as a sulphur source, but also as a host component: a dual response.

## P. aeruginosa and osmotic stress

*P. aeruginosa*, being a versatile pathogen, can survive in a wide range of environments, some of which can be osmotically stressful. Transcriptional profiling of osmotically stressed *P. aeruginosa* was studied.[57] *P. aeruginosa* PA14 cells, grown in glucose minimal media, were exposed to osmotic stress, either under steady-state conditions (0.3 M NaCl and 0.7 M sucrose) or osmotically up-shocked (0.3 M NaCl for a short time). A major difference in the expression pattern between these two conditions is the number of genes that are differentially regulated. While 431 genes were differentially regulated on osmotic up-shocking, only about 100 genes were more than threefold regulated under either condition of steady-state osmotic shock. These studies led to the identification of a steady-state osmotic shock regulon comprising 66 genes. Forty of these are virulence associated, primarily genes of the type III secretion system, two are type III exotoxins and two are chaperones. By contrast, the osmotically up-shocked cells showed repression of the type III secretion system genes that the authors hypothesise to be an energy-saving step. In addition, the sigma factor AlgT/U, previously known to be induced in various stress conditions, and genes known to be involved in osmoprotection, including hydrophilins, whose exact role in osmoprotection has not yet been conclusively proven in bacteria,[58] were induced in all the conditions of osmotic shocking.[57]

## P. aeruginosa and quorum sensing

*P. aeruginosa* senses the cell density for coordinated expression of virulence factors using a global regulatory system called quorum sensing (QS). Sensing is achieved using small diffusible molecules that in *P. aeruginosa* and other Gram-negative bacteria are the N-acylated homoserine lactones.[59] *P. aeruginosa* has two N-acylated homoserine lactone systems, *las* and *rhl*.[60] Transcriptome analysis of *lasI rhlI* mutants were performed by three groups.[61−63] Two

of these groups[61,62] identified about 300 genes that were induced by QS, while the other group found only 163 differentially expressed genes as a result of use of more stringent criteria. The three studies had approximately 100 common genes and the encoded products belonged to the functional class of secreted factors. This group included toxins and extracellular enzymes. In addition, above-average expression changes were seen in the genes involved in metabolism, biosynthesis of cofactors and fatty acid metabolism, implying that QS signals mediated by N-acylated homoserine lactones were involved in the regulation of many genes other than those involved in virulence. The third group, Hentzer *et al.*, targeted the QS system in an attempt to mitigate *P. aeruginosa* virulence[63] using a furanone isolated from the Australian seaweed *Delisea pulchra*. The authors elegantly showed, using transcriptome analyses, that about 80 per cent of the genes that were downregulated by the furanone were also repressed in an N-acylated homoserine lactones mutant. The furanone compound also affected biofilm formation and the persistence of infection in a mouse lung model. Together, the three studies led to the identification of a core set of 77 genes of the QS regulon that are positively regulated.[63] Schuster and Greenberg reviewed many other studies of regulators that further elucidate the complex QS system and the intricate interconnections.[64] They propose a complex network that involves 13 different regulators that interact with LasI and RhlI and form the QS regulon.

## P. aeruginosa biofilms

Quorum sensing has been implicated as an important step in the formation of ordered, antibiotic-resistant bacterial communities called biofilms.[65,66] Planktonic cells, in response to external stimuli, form biofilms in varied habitats, such as in the CF lung, on surgical implants and in various environmental situations, exhibiting drastically altered physiology; this led to the hypothesis that the gene expression between these two states of cells are very different. One of the earliest studies that employed microarrays to understand differences in gene expression patterns between planktonic and biofilm cells, in addition to antibiotic resistance mechanisms in biofilms, was by Whiteley *et al.*[67] Using chemostats, it was found that a surprisingly small number (1 per cent) of genes were differentially regulated between planktonic and biofilm cells. The most highly upregulated genes were those of the Pf1-like prophage, with nine of the 11 genes on the chromosome induced over 100–1,000 fold in the biofilm cells. This could imply higher rates of gene transfer in biofilms via transduction. Genes involved in bacterial motility were repressed, as was *rpoS*, the stationary phase sigma factor. Strains harbouring a mutant *rpoS* were shown to form larger, more drug-resistant biofilms. Exposure of the biofilm to the antibiotic tobramycin, followed by transcriptional analysis, showed that *tolA* was upregulated, which can serve as a barrier to aminoglycoside uptake. Cytochrome C oxidase genes were repressed, probably slowing growth and negating the antibiotic effect. In addition, antibiotic exposure also induced two probable efflux systems and many hypothetical genes.

The effect of the β-lactam imipenem on gene expression in planktonic and biofilm cells of *P. aeruginosa* was examined by Bagge *et al.*[68] Microarray analysis of imipenem-exposed planktonic and biofilm cells revealed that the chromosomal β-lactamase *ampC* was overexpressed in these cells under these conditions. Thirty-four genes were more than fivefold differentially regulated and included genes involved in alginate production, flagella biosynthesis, cell division, and peptidoglycan-related and type 4 pili biogenesis. In addition, biofilm cells also showed enhanced production of alginate, unlike in the Whiteley study with tobramycin. Bagge *et al.* could not ascribe higher antibiotic resistance of biofilm cells to a specific set of genes owing to similar expression patterns in planktonic and biofilm cells.[68]

A more recent study made a comprehensive analysis of transcriptomes of planktonic cultures (in log and stationary phase) and biofilms at eight, 14, 24 and 48 hours (microcolony and mature biofilms) using a static biofilm model.[67] This study showed a similar gene expression pattern

between developing biofilms and log phase planktonic cultures, while mature biofilms had expression patterns similar to stationary phase cells. The most highly upregulated genes in biofilm cells were those of a Pf1-related temperate bacteriophage, suggesting an important role for phage-mediated gene transfer in biofilm cells. Downregulated genes include those coding for pili, which suggests that, although pili are required for the establishment of biofilms, they are not essential in mature biofilms. Also, expression is relatively stable in developed biofilms, with only 1 per cent of the genes differentially expressed. This was the first study to look at global gene expression for two planktonic and two biofilm states of the cells, and the authors have generated a comprehensive dataset for reference that compares transcriptomes of planktonic, developing and mature biofilms.

## Transcriptomics of alginate production in P. aeruginosa

Alginate gene upregulation in *P. aeruginosa* is one of the primary modes of survival in the CF lung and converts the non-mucoid cells to a mucoid pheno-type[69] as a result of over-production of the extracellular polysaccharide alginate. Production of alginate is part of a stress response in *P. aeruginosa* and is mediated by the alternate sigma factor AlgT/U, a sigma E homologue. AlgT/U is held sequestered by the anti-sigma factor MucA. Release of AlgT/U leads to alginate production and is part of a much larger response to help the cells adapt.[70] Transcriptome analysis of *mucA*, *algT/U-mucA* mutants and the wild-type PAO1 showed that alginate was upregulated in the *mucA* mutant when there was no control on AlgT/U production. This control was lost in the double mutant, as expected.[71] Interestingly, many genes responsible for toxin production, enzymes and transport were also upregulated, indicating that AlgT/U was part of a regulon that coordinated the expression of many genes, ultimately enhancing *P. aeruginosa* survival in the harsh CF lung environment.

*P. aeruginosa*, like other bacteria, produces flagella only when needed, for example in biofilm formation, and has been implicated in establishing pulmonary infections.[72] AlgT/U was shown negatively to regulate flagella production in *P. aeruginosa* by downregulating an essential flagella regulator gene, *FleQ*.[73] There are many regulators in flagella biosynthesis, and a systematic study of several regulator mutants was performed by Dasgupta *et al.*[74] Using transcriptome analysis, they found that flagella are assembled in a four-step process that is controlled by the sequential expression of different regulators.

## Lipopolysaccharide as a virulence determinant

Lipopolysaccharides play a major role in *P. aeruginosa* virulence.[75] The bacteria are known to modify their lipopolysaccharide layer in response to the milieu. Lipid A, a major constituent of the bacterial liposaccharide, is hepta- or hexa-acetylated in strains causing acute infections, while it is penta-acetylated in chronic infections such as CF.[76] Lipopolysaccharide changes with alginate production,[77] and production of both of these important virulence factors are influenced by oxygen concentration.[78–80] Although we are not aware of any microarray studies that compare lipopolysaccharide mutants, Firoved *et al.* compared the expression patterns of a mucoid (PAO578II) and the isogenic non-mucoid parent (PAO6865), and demonstrated that lipoprotein genes are most highly upregulated when *P. aeruginosa* switches over to the mucoid phenotype.[81] Eight of the nine genes that were upregulated over 30-fold encoded lipoproteins, which can have toxic effects in environments such as the CF lung by disrupting host Toll-like receptor 2 signalling and are also antigenic, activating host defence mechanisms. Thus, some of these lipoproteins have been termed lipotoxins.[81] Elucidating mechanisms of co-regulation of lipopolysaccharide production/modification and conversion of the strains to mucoidy would help us better to understand the adaptation/pathogenesis of this versatile bacterium in chronic infections such as CF.

## Transcriptome of a post-transcriptional regulator

Burrowes *et al.* studied the role of a small post-transcriptional regulatory protein, RsmA.[82] RsmB, a small untranslated regulatory RNA, binds to RsmA, limiting its activity.[83] RsmA controls the expression of many virulence genes and QS signalling molecule synthesis,[83,84] and thus plays an important role in *P. aeruginosa* pathogenesis. Although some of the targets of RsmA were known, this study focused on determining the direct and indirect regulatory control exerted by RsmA in *P. aeruginosa*.[82] Transcriptional analysis of PAO1 and the *rsmA* mutant revealed that, of the 506 genes that had significantly altered expression, genes involved in iron acquisition and antibiotic resistance, especially those involved in the MexEF–OprN pump, were upregulated and genes involved in *Pseudomonas* quinolone signal biosynthesis and motility were downregulated. The data obtained suggest that RsmA is a global regulator.[82]

## P. aeruginosa interactions with human cells

When a bacterium comes into contact with a host cell for the first time, there is a drastic change in the lifestyle of the bacteria which aids in colonisation. The host, on the other hand, recognises this threat and mounts an immune response. As one might predict, this race between the pathogen and host to outdo each other will undoubtedly be accompanied by changes in the gene expression profile in both organisms. Numerous studies have looked at the response of the human immune system to *P. aeruginosa* exposure, and we have a fairly good idea of the immune signals and molecules involved. One of the first studies to employ transcriptomics to investigate host responses to initial contact with *P. aeruginosa* was carried out by Ichikawa *et al.*[85] Using a time course microarray experiment with lung epithelial cells exposed to *P. aeruginosa* and custom-made cDNA microarrays containing about 1,500 human clones with both characterised and uncharacterised genes, they determined that about 25 per cent of the human genes represented on the array were differentially regulated. Of interest was a lipopolysaccharide-independent twofold activation of the interferon regulatory factor-1 gene, with no activation of other downstream genes that are normally activated by interferon. This indicated that *P. aeruginosa* activation of interferon regulatory factor-1 is different from the normal mechanism of interferon regulatory factor-1 activation mediated by cognate receptor binding.[85] Using a type IV pili mutant, these authors demonstrated that PilA is the ligand responsible for lipopolysaccharide-independent activation of interferon regulatory factor-1. This study demonstrated the power of microarrays in understanding host–pathogen interactions by elucidating potentially new signalling pathways and characterising the roles of different virulence factors involved in pathogen recognition and mounting an immune response.

Two other studies have employed DNA microar-rays to study the interactions of *P. aeruginosa* to human epithelial cells, but the results are contradictory with respect to the regulation of iron-responsive genes. In the first study, by Frisk *et al.*,[86] *P. aeruginosa* was exposed to primary human airway epithelial cells for four and 12 hours *in vitro* and transcription was analysed post-exposure. Two major groups of genes were differentially regulated: phosphate uptake genes were upregulated, whereas iron-acquisition genes were downregulated. This was contrary to the notion that iron is an important limiting factor in the human host; one would have expected upregulation of the iron-acquisition genes. The authors concluded that *P. aeruginosa* was able to acquire enough iron for infection that led to deregulation of those genes. The reverse was true for phosphate.

In an attempt better to understand the initial adaptation processes of *P. aeruginosa*, especially on interaction with human epithelia, a study by Chugani and Greenberg[87] compared transcriptomes of *P. aeruginosa* PAO1 wild-type with a double mutant that was deficient in the type III secretion system and rhamnolipid biosynthesis. These two bacterial systems are key factors contributing to human airway epithelial cell damage. Strain-specific responses were

seen. Whereas virulence genes such as PA-1 galactophilic lectin were induced in the wild-type strain, alginate regulatory genes were induced in the mutant, along with *gacA*,[87] which is known to be upregulated in chronic *P. aeruginosa* infections.[30] Also, Vfr—a known upregulated regulator in acute infections—was downregulated, indicating the early stages of a chronic infection.

In contrast to the results obtained by Frisk *et al.*,[86] the Chugani and Greenberg study showed that iron-starvation genes were positively regulated, indicating that the infecting cells had little iron to utilise. This is in concordance with the belief that infecting bacterial cells have little access to iron in the human body. One possible explanation for these discordant results, as noted by the authors, is that the epithelial cell integrity in the previous study could have been compromised, leading to leakage of intracellular iron and resulting in iron-replete conditions. This would explain the increased expression of iron-dependent genes over time seen in the Frisk study. Epithelial cell integrity was monitored in the Chugani and Greenberg study by measuring the transepithelial electrical resistance (Rt). An interesting finding by Chugani *et al.* is that the general pattern of gene expression is similar to that seen in CF isolates from chronic human infections.

Many bacteria use type III secretion systems to inject proteins directly into host cells. An interesting whole-genome array study by Wolfgang *et al.*[31] looked at the conditions and regulatory mechanisms that control the expression of the type III secretion systems. They utilised the observation that type III secretion systems are induced by either growth in low-calcium-containing medium or by cell contact, and, by growing cells in low calcium, found 11 calcium-dependent regulators that were involved in the process. Analysis of these proteins led to the discovery that a membrane-associated adenylate cyclase initiates a signalling pathway that controls the expression of type III secretion system genes.[31]

The transcriptome studies of *P. aeruginosa* with human cells indicate the presence of as yet unidentified signals, from both the host and the bacteria, during interactions. Unravelling these signals could provide us with valuable information effectively to counter infections by *P. aeruginosa*: microarrays will undoubtedly prove vital in this endeavour.

## Comparative analysis of all transcriptomes

In order better to understand the response of *P. aeruginosa* to the different stress conditions, a comparison was carried out of significantly differentially regulated genes from the studies within each stress condition. This analysis was performed only for conditions for which there had been multiple studies and publicly available gene lists. This comparative analysis led to the identification of common sets of genes that are regulated in response to each particular stress condition across the different studies. Additionally, unique genes that are specifically regulated only in individual studies were also identified. These data are summarised in Table 1.

Analysis of significantly differentially regulated genes from all of the above studies led to the identification of 303 genes that form a core set and are differentially expressed in all the different conditions (Table S1). This suggests that this set of core genes is important for maintaining homeostasis in *P. aeruginosa*. Functional categorisation of the core set genes (Figure S1) reveals that, apart from the hypothetical genes (27 per cent), the most highly differentially regulated genes encode membrane proteins (15 per cent), followed by genes involved in the transport of small molecules (11 per cent), adaptation and protection (7 per cent) and transcriptional regulators (6 per cent), clearly indicating an adaptive response of *P. aeruginosa* to the changing environment. This microarray core set of 303 genes was then compared with the core genome of *P. aeruginosa*, which contains genes found in all *P. aeruginosa* sequenced strains.[35] Most of these genes (90 per cent) belong to the *P.*

*aeruginosa* core genome, but 29 genes belonging to the PAO1 accessory genome were also identified (Table S1). This set of 29 genes contains two type IV pili-related genes, genes involved in adaptation and protection (membrane proteins, transport of small molecules and metabolism) and hypothetical genes, among others. A caveat to this analysis is that, unlike PAO1, the other sequenced *P. aeruginosa* genomes do not have 100 per cent coverage.

After subtracting the core set, sets of unique genes that are specifically differentially regulated in each of the conditions examined were compiled. Functional categorisation of all the genes in the unique gene sets (Table S2) revealed that, overall, as expected, hypothetical genes were the most differentially regulated. In addition, genes involved in adaptation (membrane proteins, transport of small molecules, putative enzymes, transcriptional regulators and energy metabolism) showed the highest percentage of differential regulation.

Table S3 lists all the unique genes identified under the various conditions used in these studies. In the iron studies, for example, genes of the secreted factors group (encoding toxins, enzymes, alginate) constitute 7 per cent of the differentially regulated genes. This supports the view that *P. aeruginosa* upregulates virulence genes in response to iron limitation, to help survival in the host system.[39] Analysis of the data from response of *P. aeruginosa* to copper revealed that 33 per cent of the differentially regulated genes encode membrane proteins, a much higher percentage than for small molecule transport genes (19 per cent) or hypothetical genes (14 per cent), suggesting that copper stress primarily affects the membrane. By far the most interesting observation was from the osmotic shock study. In stark contrast to the other studies, in this study there was only a small percentage of hypothetical genes (3 per cent) that were differentially regulated.[57] Transcriptional regulators (37 per cent), protein secretion/export apparatus (33 per cent) and two-component regulatory systems (10 per cent) form over 70 per cent of the genes differentially regulated under osmotic stress. The osmotic study was performed using *P. aeruginosa* PA14, which is known to be more virulent than *P. aeruginosa* PAO1 in a wide range of hosts.[88–90] Since the gene chips used in the Aspedon *et al*'s osmotic study were those designed for PAO1 genome, which has 5,570 ORFs, compared with 5,905 in PA14—there could be an under-representation of the genes (potentially 335 genes that are present in PA14 and not in PA01) that are differentially regulated.

## Conclusions

The transcriptome analysis studies that have been reviewed here provide evidence of the immense power of the DNA microarray. Microarray profiling generates an enormous amount of data that add to our knowledge database. Genes that are differentially regulated by transcription factors, by the host environment, by changing physiological conditions (including the response to external stimuli, which could range from nutrients to other factors affecting growth) and by interactions with host cells have all been studied by microarrays. A previous review by Goodman and Lory[30] involving nine *P. aeruginosa* microarray studies identified about a quarter of the genome to be differentially regulated in response to various stimuli. The present authors' comparative analysis of the significantly differentially regulated genes from all of the various studies showed that about 35 per cent of the *P. aeruginosa* genome (1,932 genes) is regulated overall. To arrive at a more comprehensive understanding of how bacteria react to the ever-changing environment, physiological experiments need to be performed, with appropriate analysis of data, protocol standardisation and appropriate controls put in place.

The next major advances in DNA microarrays would be to develop technologies to analyse the changing transcriptome of the host–pathogen interaction process *in vivo*, and pangenome arrays that would give us insights into the workings of this versatile bacterium. Together, these will help us to devise ways better to counter *P. aeruginosa* infections.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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**Table 1**

Comparative analysis of significantly differentially regulated genes from various studies within each condition

| Stress condition | Number of studies | Differentially regulated genes | |
|---|---|---|---|
| | | Common | Unique |
| Iron | 3 | 69 | 220 |
| Quorum sensing | 3 | 121 | 862 |
| Copper | 2 | 26 | 209 |
| Oxidative stress | 7 | 83 | 386 |
| Biofilms | 3 | 55 | 650 |