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## The Influence of Human Respiratory Epithelia on *Pseudomonas aeruginosa* Gene Expression

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### Abstract

The opportunistic pathogen *Pseudomonas aeruginosa* can cause acute or chronic infections in humans. Little is known about the initial adaptation of *P. aeruginosa* to host tissues and the factors that determine whether a *P. aeruginosa*-epithelial cell interaction will manifest as an acute or a chronic infection. To gain insights into the initial phases of *P. aeruginosa* infections and to identify *P. aeruginosa* genes regulated in response to respiratory epithelia we exposed *P. aeruginosa* to cultured primary differentiated human airway epithelia. We used a *P. aeruginosa* strain that causes acute damage to the epithelia and a mutant with defects in Type III secretion and in rhamnolipid synthesis. The mutant did not cause rapid damage to epithelia as did the wildtype. We compared the transcriptomes of the *P. aeruginosa* wildtype and the mutant to each other and to *P. aeruginosa* grown under other conditions, and we discovered overlapping sets of differentially expressed genes in the wildtype and mutant exposed to epithelia. A recent study reported that exposure of *P. aeruginosa* to epithelia is characterized by a repression of the bacterial iron-responsive genes. These findings were suggestive of ample iron availability during infection. In contrast, we found that *P. aeruginosa* shows an iron-starvation response upon exposure to epithelial cells. This observation highlights the importance of the iron starvation response in both acute and chronic infections and suggests opportunities for therapy.

### Keywords

Acute infection; chronic infection; transcriptome; regulatory network

## 1. Introduction

*Pseudomonas aeruginosa* is an opportunistic pathogen that can cause clinically diverse diseases in humans with impaired immune defenses. For example, *P. aeruginosa* can cause acute infections such as pneumonia or bacteremia in patients with severe burns, and it can cause chronic infections in the lungs of patients with the genetic disease cystic fibrosis (CF) [1].

The adaptation of *P. aeruginosa* to the human host involves among other things, the expression of numerous virulence determinants. Furthermore, it appears that *P. aeruginosa* expresses distinct sets of virulence factors in acute and chronic infections. For example, experiments with diverse animal models implicate the Type III secretion system (TTSS) as a key virulence determinant in acute infections [2–4]. In contrast, *P. aeruginosa* strains isolated from chronic

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infections are often variants that express decreased levels of the TTSS or lack a functional TTSS [5]. Chronic *P. aeruginosa* infections are often biofilm infections, which are recalcitrant and rarely resolved [6]. The cues that determine bacterial commitment toward an acute or a chronic infection are not known.

Information about the factors involved in the initial adaptation of *P. aeruginosa* to host tissues is scarce, and currently available animal infection models do not permit a comparison of bacterial gene expression patterns in the initial phases of acute and chronic *P. aeruginosa* infections. Previously, Frisk *et al* examined the transcriptional profile of *P. aeruginosa* after exposure to primary normal human airway epithelial cells and found that iron-regulated genes were repressed and that the number of repressed iron-regulated genes increased over the time of exposure [7]. These findings contradict the long-held view that in the host, pathogens exist in an environment where there is intense limitation for iron [8,9]. To help resolve the conflicting reports and to identify genes regulated in *P. aeruginosa* upon exposure to cultured primary respiratory epithelia, we performed a transcriptome analysis using experimental conditions that closely controlled for epithelial damage. We found that the expression profiles in strains initiating chronic vs acute infections are overlapping but distinct from one another. Furthermore, the set of overlapping genes includes virtually all of the known and putative *P. aeruginosa* iron-responsive genes and the expression pattern of these genes is indicative of a strong iron-starvation response.

## 2. Results

### 2.1. A TTSS-rhamnolipid mutant shows attenuated epithelial cytotoxicity

Previous studies indicate the TTSS and rhamnolipids are key factors in causing damage to polarized epithelia following infection [10,11]. We first examined the ability of the wildtype and a TTSS, rhamnolipid synthesis double mutant to damage epithelial integrity. To assess epithelial integrity we measured trans-epithelial resistance ( $R_t$ ). With wildtype *P. aeruginosa* the  $R_t$  begins to decrease within about two h of co-incubation. In comparison, epithelia infected with the mutant remain undamaged for at least 18 h (Fig. 1). Epithelia retain their integrity and there is not detectable leakage of medium from the basolateral to the apical surface until the resistance drops well below 50% of the starting resistance [33]. By using standard plate count procedures we determined that both wildtype and mutant bacteria undergo about one doubling during the initial 5 h of incubation with epithelia (data not shown). Thus exposure of epithelia to the wildtype resulted in a more acute injury than exposure to the mutant, which did not injure the epithelium rapidly. In this way the infection of epithelia with the mutant *P. aeruginosa* resembled a chronic infection.

### 2.2. A transcriptome analysis of the *P. aeruginosa* response to epithelia

To identify genes regulated in the initial phase of an acute *P. aeruginosa* infection, we performed transcriptome analyses of wildtype PAO1 co-incubated with respiratory epithelia. For our initial comparison we used wildtype *P. aeruginosa* PAO1 grown in Trypticase Soy Broth (TSB) (Fig. 2). We appreciate that with these experiments the use of other media or growth conditions would alter the data, and the results would be different, but this analysis provides a basis on which to build further analyses (described below). There is not a perfect control for the experiments performed on epithelia. The bacteria on the epithelia are exposed to a thin layer of epithelium-produced fluid at an interface with the ambient atmosphere. They are not exposed to the medium for epithelial growth (Dulbecco's Modified Eagle Medium). As described in Section 2.3 we ultimately compared our transcriptome results to many other transcriptome analyses performed under a variety of growth conditions in a variety of different media. But for the initial comparison of *P. aeruginosa* grown on epithelia to *P. aeruginosa* grown in the TSB medium used to grow bacteria used to inoculate epithelia we identified 1025

genes and 31 intergenic regions that were differentially expressed (Supplementary Table 1). Of the 1025 genes, 70% (721) were induced and 30% (304) repressed by exposure to epithelia. To identify genes regulated in the initial phase of a chronic *P. aeruginosa* infection (an infection that does not result in acute injury to the epithelium), transcriptome analyses were done with the TTSS, rhamnolipid synthesis mutant co-incubated with respiratory epithelia in our in vitro infection model. We identified 1570 genes and 44 intergenic regions as differentially regulated in bacteria exposed to epithelia vs bacteria grown in TSB (Supplementary Table 2). Of the 1570 genes, 58% (906) were induced and 42% (664) were repressed when co-incubated with epithelia. There were 722 genes and 23 intergenic regions common to our acute and chronic in vitro infection models (wildtype vs TTSS, rhamnolipid synthesis mutant) (Supplementary Table 3 and Fig. 3). In addition to the subset of overlapping genes, a unique set of 303 genes and 8 intergenic regions was regulated in the acute infection (Supplementary Table 4) and a unique set of 848 genes and 21 intergenic regions was regulated in the *P. aeruginosa* mutant (Supplementary Table 5).

When we used the wildtype exposed to epithelia and the mutant exposed to epithelia for comparison we found 515 genes and 8 intergenic regions that were differentially regulated (Supplementary Table 6 and Fig. 3). Of these 40% (210) were induced and 60% (305) were repressed in the mutant. When grown in TSB we found only 48 genes that were differentially expressed in mutant vs wildtype. Of these, 28 were either known or putative members of the TTSS. Thus, with the exception of genes we expected to be influenced by the mutations only a small number were affected when cells were grown in TSB rather than exposed to epithelia. Taken together, it is apparent from the transcript profiles that compared to the parent, the mutant strain which is unable to initiate an acute infection shows significant differences in transcriptional regulation upon exposure to epithelia but not during growth in broth culture (Fig. 2).

One would expect that all genes in an operon be regulated in a similar manner. In fact, genes in known operons appear to be coregulated. For example, all genes in the *hcnABC* operon were repressed by epithelia in the mutant. Another observation that validates our analyses is that *exoS*, a TTSS gene whose expression is induced upon contact with eukaryotic host cells, was induced in the acute infection (wildtype infection) and not in the chronic infection (mutant infection).

### 2.3. Relationship between epithelia-regulated *P. aeruginosa* genes and regulons defined in previous transcriptome analyses

To gain an insight into the physiology of *P. aeruginosa* growing on epithelial cells we compared the sets of genes identified in our acute and chronic infection models with sets of genes identified as differentially regulated in previous microarray analyses of various *P. aeruginosa* strains grown under a variety of conditions. We obtained from published articles lists of quorum-regulated genes [12], genes belonging to the RpoS regulon [13], RetS-regulated genes [14], iron-responsive genes [15], SadARS-regulated genes [16], and the transcript profile of *P. aeruginosa* grown within the rat peritoneum in a dialysis membrane [17]. Additionally, we analyzed experiments available through the GeNet database (<http://cfgenomics.unc.edu>), and chose for comparison, lists of genes that met our filtering criteria as defined in the *Materials and methods*. Thus, we were able to compare our lists of differentially expressed genes with regulons defined using *P. aeruginosa* strains that were mutants in different genes; these include regulators of alginate biosynthesis (*algU* and *mucA*) (GeNet, Deretic\_May03) [18], regulators of flagellar motility (*fleQ*, *fleR*, and *fliA*) (GeNet, Ramphal\_Jun02) [19], several components of the TTSS (*exsA*, *vfr*, and *cyaAB*) (GeNet, Lory\_Nov02) [20], and mutants in *mvfR* (GeNet, Rahme\_Mar03) [21], and *algR* (GeNet, Schurr\_May02) [22]. We also compared our lists with previously reported transcript profiles of *P. aeruginosa* grown under experimental conditions

designed to identify genes regulated in response to iron availability (GeNet, Vasil\_Jul02) [23], and genes regulated under nitrosative stress conditions (GeNet, Deretic\_May03) [24].

In our comparisons with other analyses, we found overlaps of varying extents with genes identified in our experiments. Because our experimental protocol exposed the bacterial cells to an environment that mimics the apical surface of a lung we believe that the overlaps with responses to known stimuli are a reflection of how *P. aeruginosa* perceives the epithelium. The most overlap was seen with a comprehensive list of iron-responsive genes generated using a set of experiments from the GeNet database (Vasil\_Jul02). Among the list of 375 genes identified as iron-responsive, 26% (98) were induced and 74% (277) were repressed in iron-depleted conditions. The subset of 98 induced genes encodes most known or putative iron acquisition and receptor systems. A comparison revealed that of this set of 98 induced genes, 82% were induced in the wildtype and 78% were induced in the mutant after 5 hours on epithelia. The iron-responsive genes induced in both the parent and mutant include genes required for synthesis of the iron binding siderophores pyoverdine and pyochelin, membrane receptors, and transcriptional regulators. Among the 277 genes repressed in iron-depleted conditions were several genes involved in energy metabolism and iron storage. Of the 277 repressed genes, 18% were shared with genes repressed by epithelia in the wildtype and 30% were shared with genes repressed in the mutant.

### 3. Discussion

In this analysis we employed microarrays to examine *P. aeruginosa* transcript levels on a genome-wide basis in a strain incapable of initiating rapid epithelial destruction and its parent, which does cause rapid injury to the epithelium (Fig. 1). We have used primary differentiated human epithelial cultures. It is clear that the interaction between *P. aeruginosa* and the host is complex, involves cells other than epithelial cells, and evolves over time. The system we employed can be viewed as a model for the initial response of *P. aeruginosa* to the epithelial surface of the respiratory tract. We have not attempted to simulate aspects of a chronic infection that require more time to develop (for example, the organization of bacterial cells into structured biofilms). Cystic fibrosis lung infections are chronic but again, our model only reflects what might happen early in the colonization of any given region of a CF lung.

Our experiments show that there is a common response to epithelia regardless of whether we examine the strain causing acute injury or the more benign strain. There is also transcript regulation unique to one strain or the other. It is reasonable to assume some of the responses unique to one strain or the other are important in the process of bifurcation towards acute vs chronic infection. In this regard it is of interest that extracellular virulence factors like the PA-1 galactophilic lectin were induced in the wildtype strain, whereas alginate regulatory genes and *gacA* were induced in the mutant strain. The PA-1 galactophilic lectin is involved in acute *P. aeruginosa* infections and it has been reported to be induced by host factors, which are released in response to injury [25]. Alginate synthesis is a hallmark of chronic *P. aeruginosa* lung infections in CF [26,27]. Additionally, several gene clusters associated with motility were repressed by epithelia in the mutant *P. aeruginosa*. These findings correlate with the nonpiliated and nonflagellated phenotypes typical of isolates from chronic CF infections [28].

A recent study described a multicomponent signaling network that functions to regulate contrasting sets of virulence factors during acute and chronic infections [14]. This study indicates that the transcriptional regulators RetS and Vfr activate factors that promote an acute infection whereas the alternate activation of the GacS-GacA pathway promotes persistence. It is interesting to note that *vfr* was repressed and *gacA* was induced in the mutant *P.*

*aeruginosa*, our chronic in vitro infection model. Several genes controlled by Vfr and GacA in vitro showed a response when exposed to the epithelia.

A significant finding was that in either the wildtype or the mutant *P. aeruginosa* a battery of genes previously shown to be activated by iron starvation were activated upon exposure to epithelia. This indicates that *P. aeruginosa* on epithelia rapidly sense an iron limitation, and it is of some importance for several reasons: As described in the *Introduction*, although it has long been known that supplies of iron are limiting in the host and that the success of pathogens hinges on their ability to effectively employ iron acquisition mechanisms, a recent analysis of *P. aeruginosa* on epithelia has brought this into question [7]. One explanation, suggested by the authors for their findings, was that iron is made available during interaction with primary normal human airway epithelial cells. We believe that a more likely explanation, and one that the authors invoke to explain the increasing number of repressed iron-responsive genes over time, is that the response of the bacteria was measured in experimental conditions where the epithelium had been breached and iron was available from the culture medium or from lysed epithelial cells. We believe that the contrasting findings between our study and that of Frisk *et al* might have to do with our attempt to closely control epithelial damage. We believe our experiments reflect a situation where there is minimal damage to the epithelium whereas this was likely not the case in the previous study. Either situation can occur during *P. aeruginosa* infections, but the initial interaction with many strains of *P. aeruginosa* likely does not involve significant epithelial damage. There is considerable evidence in the literature highlighting the importance of iron scavenging mechanisms of pathogens to overcome the low iron availability in the host [9]. There is also accumulating evidence that iron acquisition and utilization might be a target for therapeutic intervention. The envisioned therapies, which involve inert metals like zinc or gallium, and iron chelators, show maximal effects under iron limiting conditions.

A sorting of genes by comparing the transcriptomes of the wildtype PAO1 and the mutant strain PAO-SC11 growing on epithelia yielded a relatively small list of 515 differentially regulated genes, about 10% of the genome. This eliminated genes arising in comparisons of growth of either strain on epithelia to growth in laboratory media. Thus we saw few genes involved in metabolism or in starvation (including iron starvation) among the 515 genes differentially expressed in the mutant and wildtype on epithelia.

By comparing our data to the wealth of *P. aeruginosa* transcriptome data available from other studies some interesting patterns emerge that suggest the function of complex regulatory networks involving multiple inputs. For example, the *hcnABC* operon has been shown to be quorum-sensing induced [29] and repressed in iron depleted laboratory media (GeNet, Vasil\_Jul02). What about *hcnABC* transcription on epithelia where many quorum-sensing genes are activated and where iron appears to be limiting? One would predict that *hcnABC* would be induced by exposure to epithelia but it was repressed in both the wildtype and mutant strain. This indicates *hcnABC* regulation is more complicated than previously believed. Likewise, the *phz* operon is induced by quorum sensing and one might anticipate that it would be induced with other quorum-sensing activated genes by epithelia. This is the case with the wildtype *P. aeruginosa* but not with the mutant. All of this points to the notion that there are epithelial cell modulators of gene expression in *P. aeruginosa*.

Overall, our findings suggest that *P. aeruginosa* has evolved a network to control the expression of alternative sets of coregulated genes in acute and chronic infections. A particularly interesting finding is that the pattern of changes of several sets of genes in our chronic infection model is reminiscent of phenotypic or genotypic changes associated with CF isolates. The trend for these changes may be set early in the chronic infection process. One might imagine that different host signals are emitted depending on the degree of injury caused by the infecting strain. Our work and the work of others [25] strongly suggest the existence of signals produced

by the host that lead to the development of acute vs chronic *P. aeruginosa* infections. These hypothetical signals remain to be identified. We hope to develop reporters based on some of the genes we have discovered to be differentially regulated in the mutant vs the wildtype *P. aeruginosa* upon exposure to epithelia, and to use these reporters in efforts to identify important host-generated signals.

## 4. Materials and methods

### 4.1. Bacterial strains and growth conditions

A *rhlA* mutant was obtained from the *P. aeruginosa* Transposon Mutant Library at the University of Washington Genome Center (strain ID 54908) [30]. The mutation was moved into *P. aeruginosa* PAO1 to generate the strain PAO-SC10. To construct a PAO1 *rhlA*, *exsA* mutant, PCR-amplified DNA fragments flanking *exsA* were cloned into pEX18Tc [31] resulting in a deletion of codons 8 to 245 in *exsA*. This plasmid was digested with *Bam*HI and ligated with a gentamicin-resistant ( $Gm^R$ )-GFP FRT cassette from pPS858 [31] to generate pSAC201. We mobilized pSAC201 from *E. coli* SM10 [32] into PAO-SC10 and isolated  $Gm^R$  colonies. A tetracycline-sensitive ( $Tc^S$ ) recombinant was identified and the  $Gm^R$ -GFP marker was deleted using pFLP2 [31]. Loss of pFLP2 following excision was confirmed by the carbenicillin-sensitive and sucrose-resistant phenotype of the mutant, PAO-SC11. The *rhlA* mutation was confirmed by PCR analysis and the *exsA* mutation was confirmed by Southern analysis. PAO-SC11 had no discernible growth defect compared with wildtype.

### 4.2. Culturing primary respiratory epithelia and infecting the epithelia with *P. aeruginosa*

Human lung epithelial cells were obtained from the Iowa Donor Network and prepared as described previously [33]. All cell layers were incubated for at least 15 days before use to allow development of morphological and functional properties of airway epithelia. Four days prior to infection with bacteria, the epithelia were transferred to antibiotic-free medium and washed daily with Dulbecco's Modified Eagle Medium to remove antibiotics. To mimic the milieu of the apical surface of respiratory epithelia, a 24-h period was allowed between the final wash and infection. For infections, *P. aeruginosa* strains were grown in TSB to an optical density ( $OD_{600}$ ) of 1.0, washed once, and suspended in Dulbecco's phosphate-buffered saline (D-PBS) (Invitrogen, cat. no. 14287). Ten  $\mu$ l of bacterial cell suspension ( $2 \times 10^9$  CFU  $ml^{-1}$ ) was applied to the apical surface of epithelium at a multiplicity of infection of approximately 25. The fluid in this small application volume is absorbed within a short time leaving the bacteria in contact with the airway cells or the normal airway surface fluid. Unless otherwise specified, the bacteria and epithelia were co-incubated for 5 h. Epithelial integrity was assessed by measuring the transepithelial electrical resistance ( $R_t$ ) using an Ohm meter and 250  $\mu$ l D-PBS. Only bacteria washed from epithelia that had  $R_t$  measurements  $\geq 50\%$  of the starting values were used for transcription profiling experiments. By using standard plate counting techniques we found that over 90% of the total bacteria on an epithelium were recovered in the wash with the remaining bacteria exhibiting a tighter binding to the epithelium.

### 4.3. Transcript profiling

As a baseline comparison, duplicate bacterial cultures of the wildtype *P. aeruginosa* PAO1 or the isogenic mutant PAO-SC11 were grown in TSB from an  $OD_{600}$  of 0.10 to an  $OD_{600}$  of 0.85 and  $2 \times 10^9$  cells were mixed with RNA Protect Bacteria reagent (Qiagen). For infection experiments, bacteria were washed from the epithelia in 250  $\mu$ l of D-PBS and the suspension was mixed with RNA Protect Bacteria reagent. RNA was isolated using RNeasy mini columns (Qiagen) according to the manufacturer's recommendations. Bacteria pooled after washing from 9 epithelial layers yielded approximately 3.5  $\mu$ g RNA. The purified RNA was treated with DNase I to ensure removal of genomic DNA followed by purification using RNeasy mini columns. Chromosomal DNA contamination was monitored by PCR as described previously

[12]. To enable transcriptome analysis using low amounts of RNA, we used a modification of existing protocols for cDNA synthesis (Affymetrix protocol and a protocol suggested by M. Wolfgang, Harvard University) as described below. For the primer annealing step of the cDNA synthesis reactions we used 1.2  $\mu\text{g}$  of purified RNA at a final concentration of 0.04  $\mu\text{g}/\mu\text{l}$  and semirandom hexamer primers with an average G+C content of 75% at a final concentration of 25  $\text{ng}/\mu\text{l}$ . We could synthesize about 2.5  $\mu\text{g}$  cDNA from 1.2  $\mu\text{g}$  RNA using our modified protocol. This allowed us to adhere to the Affymetrix recommendations for the subsequent procedure, which requires about 3  $\mu\text{g}$  cDNA for GeneChip analysis. Samples were hybridized to GeneChip *P. aeruginosa* Microarrays (Affymetrix) at the University of Iowa Bioinformatics Core Facility. To validate our modified protocol, we performed a microarray experiment to compare expression profiles of *P. aeruginosa* grown in broth culture under conditions inducing or non-inducing for the TTSS. Analysis using cDNA synthesized from 12  $\mu\text{g}$  RNA with the recommended protocol was compared to analysis done using cDNA synthesized from 1.2  $\mu\text{g}$  of the same RNA with our modified protocol. A 98% correlation coefficient between the two analyses established the validity of our modified protocol. Replicates were biological replicates (the entire biological experiment was repeated). The microarray data have been deposited at <http://www.ncbi.nlm.nih.gov/geo> under the accession number GSE4614.

#### 4.4. Analysis of microarray data

Initial data analysis was done by using the Affymetrix Microarray Software Suite (MAS) version 5.0. All genes showing expression levels above the lower detection level of the chips (present calls) in MAS 5.0 in at least one of the two conditions (baseline vs experiment) were checked for differential expression using Cyber-T (<http://www.visitor.ics.uci.edu/genex/cybert/>). The Bayesian prior estimate was 10, the sliding window size was 100, and the  $\beta$ -fit iteration value was 2. We defined the cutoff value for differential gene expression as transcripts that showed a  $>2.5$ -fold change and a t-test  $P$  value of  $<0.001$ . The posterior probability of differential expression (PPDE) ( $< p$ ) value for the genes identified was  $>0.97$ .

For experiments available through the Cystic Fibrosis Foundation Therapeutics, Inc.-GeNet shared workspace we obtained the original raw data files from the Signet server (<http://cfgenomics.unc.edu>). Analysis was as described above except for the following modifications: For experiments with three replicates, only those genes with present calls in at least 2 out of 3 replicates in either condition (experiment vs baseline) were analyzed further using Cyber-T. The  $\beta$ -fit iteration value for Cyber-T analysis was 1. The cutoff value for differential gene expression was defined as transcripts that showed a  $>2.5$ -fold change and a t-test  $P$  value of  $<0.05$ . A comparison of gene expression patterns between different experiments was done by using GENESPRING (<http://www.silicongenetics.com>).

## Supplementary Materials

Refer to Web version on PubMed Central for supplementary material.

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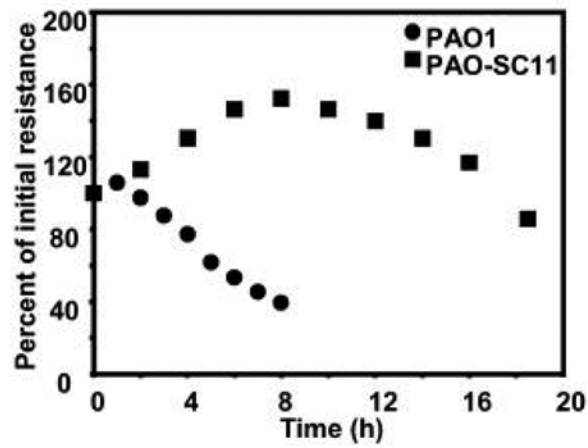
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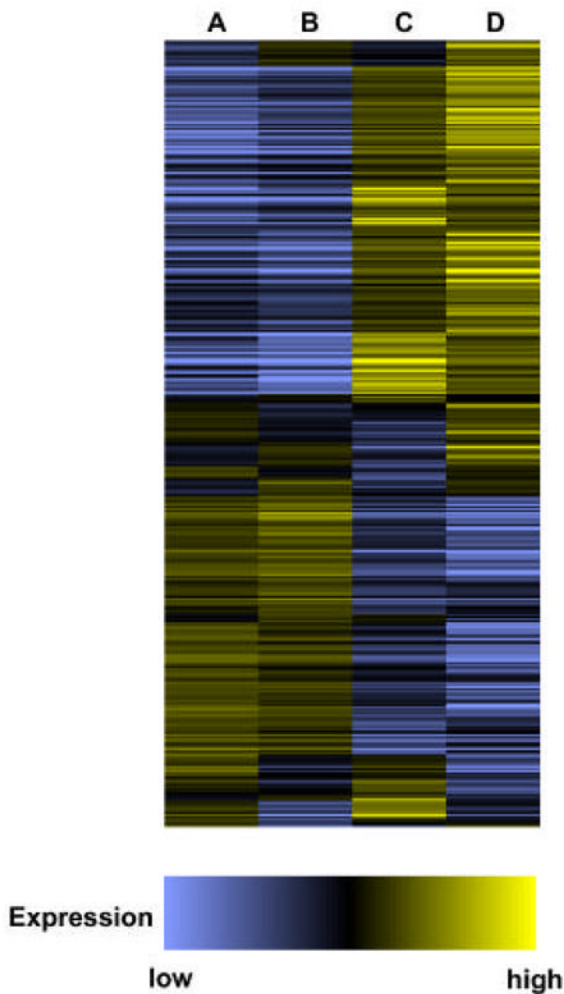
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**Figure 1. Measurements of transepithelial electrical resistance ( $R_t$ ) of airway epithelia grown in millicells and infected with the parent PAO1 or the mutant PAO-SC11**  
The inoculum size is  $\sim 2 \times 10^7$  CFU/millicell.  $R_t$  values are reported as averages of 5 millicells at each time point expressed as a percentage of the starting values. The range was  $\pm 15\%$ .



**Figure 2. Absolute expression profiles of genes during growth of *P. aeruginosa* in TSB and on epithelia**

Wildtype *P. aeruginosa* PAO1 in TSB (A), the mutant PAO-SC11 in TSB (B), wildtype *P. aeruginosa* PAO1 on epithelia (C) and, the mutant PAO-SC11 on epithelia (D). The profile was generated by combining lists of genes assigned a present call in at least one of the two replicates for each growth condition. Depicted are the averaged expression values normalized to the median. Genes are displayed in the order of the hierarchical clustering of their expression profiles as determined using the Spearman correlation in GENESPRING.



**Figure 3. Venn diagram showing overlaps between genes regulated in the parent PAO1 and the mutant PAO-SC11 upon exposure to epithelia**

Blue, genes regulated in wildtype PAO1 exposed to epithelia versus grown in TSB. Yellow, genes regulated in the mutant PAO-SC11 exposed to epithelia versus grown in TSB. Red, genes regulated in a comparison of wildtype PAO1 exposed to epithelia and the mutant PAO-SC11 exposed to epithelia.