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Toxicogenomic response of Pseudomonas aeruginosa to ortho-phenylphenol

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Abstract

Background: Pseudomonas aeruginosa (P. aeruginosa) is the most common opportunistic pathogen implicated in nosocomial infections and in chronic lung infections in cystic fibrosis patients. Ortho-phenylphenol (OPP) is an antimicrobial agent used as an active ingredient in several EPA registered disinfectants. Despite its widespread use, there is a paucity of information on its target molecular pathways and the cellular responses that it elucidates in bacteria in general and in P. aeruginosa in particular. An understanding of the OPP-driven gene regulation and cellular response it elicits will facilitate more effective utilization of this antimicrobial and possibly lead to the development of more effective disinfectant treatments.

Results: Herein, we performed a genome-wide transcriptome analysis of the cellular responses of P. aeruginosa exposed to 0.82 mM OPP for 20 and 60 minutes. Our data indicated that OPP upregulated the transcription of genes encoding ribosomal, virulence and membrane transport proteins after both treatment times. After 20 minutes of exposure to 0.82 mM OPP, genes involved in the exhibition of swarming motility and anaerobic respiration were upregulated. After 60 minutes of OPP treatment, the transcription of genes involved in amino acid and lipopolysaccharide biosynthesis were upregulated. Further, the transcription of the ribosome modulation factor (rmf) and an alternative sigma factor (rpoS) of RNA polymerase were downregulated after both treatment times.

Conclusion: Results from this study indicate that after 20 minutes of exposure to OPP, genes that have been linked to the exhibition of anaerobic respiration and swarming motility were upregulated. This study also suggests that the downregulation of the rmf and rpoS genes may be indicative of the mechanism by which OPP causes decreases in cell viability in P. aeruginosa. Consequently, a protective response involving the upregulation of translation leading to the increased synthesis of membrane related proteins and virulence proteins is possibly induced after both treatment times. In addition, cell wall modification may occur due to the increased synthesis of lipopolysaccharide after 60 minutes exposure to OPP. This gene expression profile can now be utilized for a better understanding of the target cellular pathways of OPP in P. aeruginosa and how this organism develops resistance to OPP.

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**Background**

Hospital-acquired infections caused by opportunistic pathogens present a serious threat to public health. Nosocomial infections are estimated to occur in 5% of all acute-care hospitalizations and in more than 2 million cases each year [1]. *P. aeruginosa* is the most common opportunistic pathogen responsible for hospital acquired burn wound infections, urinary tract infections and ventilator-associated pneumonia [2-5]. In cystic fibrosis patients, *P. aeruginosa* is implicated in chronic lung infections, leading to high rates of illness and death [6]. In the increasing AIDS population, 50% of deaths have been linked *P. aeruginosa* bacteremia [7]. The increasing prevalence of nosocomial infections has been associated to the growing problem of antimicrobial and detergent-resistant pathogens [8,9]. As such, proper use of effective disinfecting strategies in hospitals is necessary to abate this growing problem [10].

Ortho-phenylenophenol is used as a fungicide and as an antibacterial agent in a wide variety of settings. OPP is used as a hospital disinfectant, and as a fungicide and disinfectant for wood preservation, the treatment of vegetables and citrus fruits and textile production [11,12]. Results from toxicological studies indicate that OPP administered in diet, leads to the formation of tumors in the urinary bladder of rats [13]. OPP has also been reported to cause sister-chromatid exchanges and chromosomal aberrations in Chinese hamster ovary cells (CHO-K1 cells) [14].

Despite the aforementioned detrimental effects of OPP and its many uses to combat microbial contamination, to our knowledge, the mechanism of action of OPP on bacterial pathogens has not been elucidated. Moreover, the use of OPP as a hospital disinfectant necessitates an understanding of the cellular functions that it affects in different pathogenic bacteria. This will facilitate the determination of its mode of action such that it can be more effectively utilized. Further, such information will expedite the development of efficient antimicrobials which target specific pathogenic bacteria and exert nominal effects on other species. In previous studies, whole genome microarrays have been successfully used to analyze the global transcriptomic response of *P. aeruginosa* to different antimicrobials. From these studies, specific cellular functions affected by the application of these antimicrobials were elucidated through the identification of signature genes that were up or downregulated [15-19].

To our knowledge, for the first time, we investigated the genome-wide changes in *P. aeruginosa* gene transcription upon exposure to 0.82 mM OPP for 20 and 60 minutes using Affymetrix *P. aeruginosa* GeneChip arrays. Our findings show that: (i) the transcription of genes encoding ribosomal, virulence and membrane proteins (including membrane transport systems) were upregulated after 20 and 60 minutes (ii) the transcription of genes that may allow transient switches to anaerobic respiration and swarming motility as stress responses were upregulated after 20 minutes (iii) after 60 minutes, amino acid anabolism and lipopolysaccharide synthesis were upregulated. (iv) after both 20 and 60 minutes of OPP treatment, the transcription of the genes encoding the ribosome modulation factor and the alternative sigma factor, RpoS were significantly downregulated.

**Results and discussion**

**Growth inhibition of *P. aeruginosa* by OPP**

In order to determine a suitable sublethal concentration of OPP that will produce strong growth inhibition, *P. aeruginosa* was exposed to six concentrations of OPP dissolved in DMSO (0.58, 0.82, 0.94, 0.99, 1.05 and 1.18 mM), and growth inhibition was monitored at intervals of 10 minutes for 60 minutes. Note that the concentration of OPP that inhibits 90% of *P. aeruginosa* isolates (MIC<sub>90</sub>) has been reported to be 2000 mg/L (1.18 mM) [20]. In figure 1, the highest concentration of OPP used (1.18 mM) produced marked growth inhibition. Therefore, a lower sublethal concentration of 0.82 mM was selected as the test concentration since this concentration caused a non-drastic sublethal growth inhibition as seen in figure 1.

**Changes in the transcriptional profiles of *P. aeruginosa* in response to OPP**

Four separate microarray experiments were performed in the absence (control) and in the presence (experimental) of 0.82 mM OPP. In order to investigate early and late changes in transcription in response to OPP, RNA was isolated after 20 and 60 minutes exposure to 0.82 mM OPP. To determine which genes showed significant changes in transcript level in response to OPP, the following criteria were applied: (i) the <i>p</i>-value for a Mann-Whitney test should be less than 0.05, (ii) an absolute fold change in transcript level should be equal to or greater than 2 (iii) a gene should have a present or marginal call (Affymetrix, Inc.) from 50% or more replicates on both experimental and control replicate sets. After a one-way ANOVA was performed, 1012 out of the 5900 genes that make up the *P. aeruginosa* genome were found to be statistically significant. Further analysis revealed that a total of 509 genes showed statistically marked upregulation (≥2-fold) or downregulation (≤2-fold) after 20 minutes and after 60 minutes exposure to OPP. The expression levels of the 5900 genes in the *P. aeruginosa* genome obtained from control experiments and after treatment with OPP (20 and 60 minutes) have been deposited in NCBI’s gene Expression Omnibus [21] and can be accessed through the GEO series accession number: GSE10604 [22] (additional file 1).
Functional classification of upregulated and downregulated genes

In order to relate the up and downregulated genes to their functions, the 509 statistically significant genes were classified into different functional classes. Functional classes were obtained from the \textit{P. aeruginosa} Community Annotation Project \cite{23,24}. Figure 2 illustrates the grouping of up and downregulated genes at 20 and 60 minutes into different functional classes and the total number of genes in each class for the two treatment times. Note that a total of 137 genes were classified as "hypothetical, unclassified, unknown".

Figure 2 illustrates that in general at 60 minutes, there were more upregulated and downregulated genes in the functional classes, when compared to 20 minutes. In particular, genes belonging to the functional classes of "adaptation and protection", amino acid biosynthesis and metabolism", "biosynthesis of cofactors, prosthetic groups and carriers", "carbon compound catabolism", "cell division", "cell wall/LPS/capsule", "central intermediary metabolism", "chaperones and heat shock proteins", "DNA replication, recombination and repair", "energy metabolism", "fatty acid and phospholipid metabolism", "membrane proteins", "nucleotide biosynthesis and metabolism", "putative enzymes", "transcription", "transcriptional regulators", "translation, post-translational modification, degradation", and "transport of small molecules contained significantly more upregulated genes at 60 minutes.

Among the downregulated genes, the functional classes of: "adaptation and protection", "amino acid biosynthesis and metabolism", "carbon compound catabolism" and "energy metabolism" contained significantly more downregulated genes at 60 minutes compared to 20 minutes. The marked differences between the numbers of upregulated and downregulated genes after 20 minutes exposure compared to 60 minutes of treatment may be related to the growth inhibition observed following exposure to OPP.

Grouping of functionally classified up and down regulated genes

To further analyze the 509 upregulated and downregulated genes, we removed the 137 genes belonging to the class designated as "hypothetical, unclassified, unknown". The remaining 372 genes were placed in six groups based on their transcription directions. Figure 3 illustrates the six different groups and the total number of genes in each group. Group I contains genes that were upregulated after 20 and 60 minutes. Group II is made up of genes that were upregulated after 20 minutes only. Group III contains genes that were downregulated upon 20 minutes exposure to OPP. Group IV contains genes that were upregulated after 60 minutes only. Group V is made up of genes that were downregulated only after 60 minutes exposure to OPP. Group VI contains genes that were downregulated after both 20 and 60 minutes exposure to OPP. All of the genes discussed in this report can be found in additional file 2. However, for clarity and to facilitate the reading of this report, the genes discussed below in the six groups are indicated in table 1.

\textbf{Group I: genes upregulated at 20 and 60 minutes exposure}

Group 1 consisted of genes that were induced both at 20 and 60 minutes exposure to OPP (additional file 2). The most distinctive functional class in this group was "translation, post-translational modification and degradation" which contained 45 genes (additional file 2). This functional class contained several 30 and 50S ribosomal proteins. The two most upregulated 30 and 50S ribosomal proteins are indicated on table 1. A complete list of ribosomal proteins in this group can be found in additional file 2. Group 1 also contained genes coding for translation initiation factors: PA2619, PA4744 and PA2743 (\textit{infA}, \textit{infB} and \textit{infC}), elongation factors G and Ts: PA4266, PA3655
(fusA1, tsf) and peptide chain release factor: (prfA) PA4665 (table 1). The upregulation of these genes after both 20 and 60 minutes suggests that protein synthesis is affected in *P. aeruginosa* upon exposure to OPP and even after prolonged exposure (60 minutes). This may reflect a cellular protective response, whereby proteins involved in stress response are synthesized. The upregulation of the cold shock protein: PA4743 (rbfA) and the heat shock protein: PA4263 (suhB) which are involved in stress response supports this hypothesis. The observed upregulation of translation may also indicate an increase in the synthesis of virulence factors, which can be produced in response to environmental stress. Previous studies have suggested that the pathogenesis of *Staphylococcus aureus* is stimulated as a protective response against antimicrobial treatments [25,26]. In line with this hypothesis was the upregulation of PA5117 (typA), which has been suggested to be relevant for pathogenesis in *Escherichia coli* when it is tyrosine phosphorylated [27].

Among the genes in the functional class of "membrane proteins" in this group, we observed three proteins of the SecY system: PA4243 (secY), PA4276 (secE) and PA4747 (secG) were upregulated. In gram negative bacteria, the Sec system is utilized for the secretion of degradative enzymes, virulence factors, toxins and proteins across the cytoplasmic membrane and for the insertion of proteins into the cytoplasmic membrane [28], allowing for growth and survival. The concomitant upregulation of genes involved in the Sec system and genes involved in translation is possibly indicative of the transport of synthesized proteins across the cell membrane.

Concurrent with the induction of genes of the Sec system was the upregulation of genes involved in membrane associated transport of small molecules notably, PA4687: ferric iron-binding periplasmic protein (hitA), PA1964: probable ATP-binding component of ABC transporter, PA2760: probable outer membrane protein precursor and PA4688: Iron III-transport system permease (hitB). These results are in agreement with those of a recent study that demonstrated that the hitA and hitB genes were 2- to 8-fold upregulated in *P. aeruginosa* in response to a two hour exposure to 10 mM hydrogen peroxide [17]. HitA, HitB
and HitC (a nucleotide binding protein) encoded by the hitABC operon facilitate iron acquisition from the periplasm [29]. In contrast, a previous study indicated that the hitAB genes were downregulated approximately 7- and 6-fold in *P. aeruginosa* treated with sodium hypochlorite for 20 minutes [18]. Therefore the upregulation of hitA (ferric iron-binding periplasmic protein) and hitB (Iron III-transport system permease) in this study is suggestive of active iron uptake, which is essential for bacterial growth and an important determinant of bacterial virulence [29].

It is worth noting that eight type IV pilus assembly proteins belonged to this group including pil C, D, G, I, M, N, O and P. Type IV pilus have been implicated in the pathogenicity of gram negative bacteria, and mediate cellular functions such as twitching motility, host-cell adhesion and cell signaling [30]. The expression of type IV pilus is necessary for colonization and maturation of *P. aeruginosa* biofilms on a variety of surfaces [31]. Considering that the cellular functions noted above generally mediate virulence and cell survival, it is therefore possible that the upregulation of these genes may be associated with protection and concomitant survival of *P. aeruginosa* when treated with OPP.

Five genes involved in fatty acid biosynthesis (*fab* A, B, D, G and Z) were also categorized under group I. A previous study that investigated the proteomic response of *P. putida* to phenol-induced stress noted that several enzymes involved in fatty acid biosynthesis, including FabB and FabH2 were upregulated approximately 2 fold and 4 fold respectively when treated with phenol [32]. Although FabA was not mapped in the aforementioned study, it was suggested that under phenol stress, the expression level of FabA correlated with the upregulation of FabB. Our study corroborates this theory, as both *fabA* and *fabB* were upregulated approximately 3 and 4 fold respectively following 60 minutes exposure to OPP (t). In contrast, *fabH2* was downregulated in *P. aeruginosa* after 20 minutes of treatment with peracetic acid [16].

With respect to energy metabolism, the genes: PA5561 (*atp1*), PA2354, (probable cytochrome) and PA5556 (ATP synthase alpha chain), which are involved in ATP synthesis associated with oxidative phosphorylation and the electron transport chain were upregulated at both 20 and 60 minutes. This result suggests that oxidative phosphorylation is possibly a major route for energy production in *P. aeruginosa* treated with OPP.

**Group II: genes upregulated upon 20 minutes exposure**

Group II contained 13 genes, the least number of genes among the six groups (additional file 2). The most upregulated gene in this group was norB (nitric oxide reductase subunit B), with an approximately four fold increase in transcription (table 1). The nitric oxide reductase enzyme is a membrane bound cytochrome bc complex which has been reported to be expressed under anaerobic conditions in *P. stutzeri* [33-35]. NorB is the catalytic component of the NorBC complex and harbors low and high spin and low spin ferric heme proteins [36]. Recent experimental evidence suggests that coupled with electron transfer, proton uptake by NorB occurs from the periplasmic side of the bacterial cell membrane [33]. As such, nitrate can be used as the terminal electron acceptor instead of oxygen under anaerobic conditions, with nitric oxide being one of the intermediates in the reduction of nitrate to dinitrogen in the denitrification process [33]. Nitric oxide produced during denitrification is highly toxic to the cell and relies on the scavenging activity of nitric oxide reductase for cell survival [34]. A previous study [37] demonstrated that several genes involved in anaerobic respiration were upregulated in *S. aureus* after 20 minutes of exposure to peracetic acid, suggesting the possibility of a shift to anaerobic respiration in response to oxidative stress.

Another gene of interest in this group was PA3479: rhamnosyl transferase chain A (*rhlA*). The *rhlAB* operon catalyzed the first glycosyl transfer reaction required for the synthesis of rhamnolipids [38,39]. Rhamnolipids have been found in high levels in the sputum of cystic fibrosis patients and are classified as virulence factors [40]. Rhamnolipids have been postulated to act as biosurfactants that facilitate surface colonization [41]. The multicellular nature of both biofilms and cells undergoing swarming motility indicate that both phenomena are related [42,43]. Rhamnolipid transferase chain A has been found to be critical for the exhibition of swarming motility by *P. aeruginosa*, which is important for environmental adaptation [44,45]. It has also been demonstrated that *P. aeruginosa* mutants lacking type IV pili and flagella are unable to swarm [45]. The upregulation of type IV pili assembly genes at 20 and 60 minutes (group I) supports the possibility that *P. aeruginosa* treated with OPP may exhibit swarming motility as a stress response. The finding that the *rhlA* gene exhibited no change in its expression level at 60 minutes also supports this hypothesis. Moreover, the probable purine binding chemotaxis protein, PA0177, which belongs to the group of flagellar assembly proteins, was also upregulated in this group.

Previous studies have revealed that low iron levels significantly stimulated swarming motility, thereby preventing biofilm formation [44,46]. From this observation, it was hypothesized that in an unfavorable nutritional environment, biosurfactant production and surface motility are over expressed in order to prevent *P. aeruginosa* from settling into a biofilm [44]. Our results are in line with this hypothesis, considering that the ferric iron-binding periplasmic protein and the iron III-transport system permease (*hitA* and *hitB*) were upregulated after both 20 and 60 minutes (group I) of exposure to OPP, suggesting active iron uptake.
Table 1: List of significantly up or downregulated \emph{P. aeruginosa} genes that are discussed in this report

| Affymetrix ORF # | Probe ID   | \(<20\text{ minutes}\) | \(<60\text{ minutes}\) | Description                                                                 | Symbol | Functional class             |
|------------------|------------|-------------------------|-------------------------|----------------------------------------------------------------------------|--------|-----------------------------|
| **Group I: Upregulation (20 min) – Upregulation (60 min)** |            |                         |                         | probable ATP-binding component of ABC transporter                           |        | Transport of small molecules |
| PA1964_at        | PA1964     | 2.029                   | 2.381                   | P value 0.0112                                                             |        |                             |
| PA2760_at        | PA2760     | 2.152                   | 2.94                    | P value 0.0416                                                             |        |                             |
| PA4687_hitA_at   | PA4687     | 2.219                   | 2.069                   | P value 0.00808                                                             |        |                             |
| PA2743_infC_at   | PA2743     | 2.044                   | 2.634                   | P value 0.00327                                                             |        |                             |
| PA2619_infA_at   | PA2619     | 2.14                    | 3.644                   | P value 0.000526                                                            |        |                             |
| PA4266_fusA1_at  | PA4266     | 2.18                    | 3.88                    | P value 0.012                                                             |        |                             |
| PA3655_tsf_at    | PA3655     | 2.197                   | 5.495                   | P value 0.017                                                             |        |                             |
| PA4665_prfA_at   | PA4665     | 2.426                   | 2.774                   | P value 0.00398                                                             |        |                             |
| PA4273_rplA_at   | PA4273     | 2.725                   | 6.018                   | P value 0.0349                                                             |        |                             |
| PA3656_rpsB_at   | PA3656     | 2.543                   | 7.267                   | P value 0.00953                                                             |        |                             |
| PA4744_infB_at   | PA4744     | 2.783                   | 3.826                   | P value 0.0134                                                             |        |                             |
| PA4934_rpsR_at   | PA4934     | 2.894                   | 6.619                   | P value 0.000276                                                            |        |                             |
| PA4255_rpmC_at   | PA4255     | 2.927                   | 6.655                   | P value 0.00331                                                             |        |                             |
| PA4528_pilD_at   | PA4528     | 2.144                   | 2.65                    | P value 0.014                                                             |        | Motility & Attachment       |
| PA4008_pilG_at   | PA4008     | 2.294                   | 4.026                   | P value 0.0144                                                             |        | Chemotaxis                  |
| PA5041_pilP_at   | PA5041     | 2.169                   | 2.232                   | P value 0.00817                                                            |        | Motility & Attachment       |
| PA0410_pill_at   | PA0024     | 2.188                   | 2.267                   | P value 0.0484                                                             |        |                             |
Table 1: List of significantly up or downregulated *P. aeruginosa* genes that are discussed in this report (Continued)

| Gene Symbol | Description | Group | Fold Change | p-value | Gene Symbol | Description | Group | Fold Change | p-value |
|-------------|-------------|-------|-------------|---------|-------------|-------------|-------|-------------|---------|
| PA5042_pilO_at | PA5042 | 2.26 | 0.00345 | type 4 fimbrial biogenesis protein PilO | pilO | Motility & Attachment |
| PA4527_pilC_at | PA4527 | 2.27 | 0.00205 | still frameshift type 4 fimbrial biogenesis protein PilC | pilC | Motility & Attachment |
| PA5043_pilN_at | PA5043 | 2.34 | 0.000628 | type 4 fimbrial biogenesis protein PilN | pilN | Motility & Attachment |
| PA5044_pilM_at | PA5044 | 2.893 | 0.00702 | type 4 fimbrial biogenesis protein PilM | pilM | Motility & Attachment |
| PA4688_hitB_at | PA4688 | 2.282 | 0.0261 | iron (III)-transport system permease HitB | hitB | Membrane proteins |
| PA4747_secG_at | PA4747 | 2.294 | 0.00628 | secretion protein SecG | secG | Membrane proteins |
| PA4243_secY_at | PA4243 | 2.914 | 9.26E-05 | secretion protein SecY | secY | Membrane proteins |
| PA4276_secE_at | PA4276 | 2.274 | 2.07E-05 | secretion protein SecE | secE | Protein secretion/export apparatus |
| PA2968_fabD_at | PA2968 | 2.137 | 0.0131 | malonyl-CoA-[acyl-carrier-protein] transacylase FabD | fabD | Fatty acid and phospholipid metabolism |
| PA1609_fabB_at | PA1609 | 2.373 | 0.0147 | beta-ketoacyl-ACP synthase FabB | fabB | Fatty acid and phospholipid metabolism |
| PA2967_fabG_at | PA2967 | 2.864 | 0.00295 | beta-hydroxydecanoyl-ACP dehydrase FabA | fabA | Fatty acid and phospholipid metabolism |
| PA3645_fabZ_at | PA3645 | 2.854 | 2.25E-05 | (3R)-hydroxymyristoyl-[acyl carrier protein] dehydratase FabZ | fabZ | Cell wall/LPS/capsule |
| PA5556_atpA_at | PA5556 | 2.202 | 0.00136 | ATP synthase alpha chain AtpA | atpA | Energy metabolism |
| PA5491_at | PA5491 | 2.354 | 0.0049 | probable cytochrome | | Energy metabolism |
| PA5351_atpl_at | PA5351 | 2.558 | 0.0015 | ATP synthase protein I AtpI | | Energy metabolism |
| PA3818_at | PA3818 | 2.746 | 0.00337 | extragenic suppressor protein SuhB | SuhB | Adaptation, protection |
| PA4743_rhbA_at | PA4743 | 2.824 | 0.0177 | ribosome-binding factor A RhbA | RhbA | Adaptation, protection |
| PA5117_typA_at | PA5117 | 3.136 | 0.000343 | regulatory protein TypA TypA | TypA | Adaptation, protection |

**Group II: Upregulation (20 min) – No change (60 min)**

| Gene Symbol | Description | Group | Fold Change | p-value |
|-------------|-------------|-------|-------------|---------|
| PA0524_norB_at | PA0524 | 3.869 | 0.0456 | norB | Energy metabolism |
| PA3479_rhaA_at | PA3479 | 2.376 | 0.0364 | rhamnosyltransferase chain A RhaA | RhaA | Secreted Factors (toxins, enzymes, alginate) |
| PA0177_at | PA0177 | 2.683 | 0.00294 | probable purine-binding chemotaxis protein | | Adaptation, protection |

**Group III: Downregulation (20 min) – No change (60 min)**

| Gene Symbol | Description | Group | Fold Change | p-value |
|-------------|-------------|-------|-------------|---------|
| PA2193_hcnA_at | PA2193 | -2.268 | 0.0324 | hydrogen cyanide synthase HcnA | HcnA | Central intermediary metabolism |
| PA2194_hcnB_at | PA2194 | -2.762 | 0.018 | hydrogen cyanide synthase HcnB | HcnB | Central intermediary metabolism |
| PA2195_hcnC_at | PA2195 | -2.183 | 0.0437 | hydrogen cyanide synthase HcnC | HcnC | Central intermediary metabolism |
| PA4385_groEL_at | PA4385 | -2.16 | 0.00513 | GroEL protein GroEL | | Chaperones & heat shock proteins |
Table 1: List of significantly up or downregulated *P. aeruginosa* genes that are discussed in this report (Continued)

| Gene ID       | Description                   | Log2 Fold Change | P-value | ClpB protein         | clpB Modification                                      |
|---------------|--------------------------------|-----------------|---------|----------------------|--------------------------------------------------------|
| PA4542_clpB_at| PA4542 ClpB protein            | -2.77           | 0.00253 | ClpB protein          | Translation, post-translational modification, degradation |
| PA4272_rplJ_at| PA4272 50S ribosomal protein L10 | 5.918           | 0.00886 | rplJ                 | Translation, post-translational modification, degradation |
| PA4271_rplL_at| PA4271 50S ribosomal protein L17/ L12 | 5.898           | 0.00319 | rplL                 | Translation, post-translational modification, degradation |
| PA2740_pheS_at| PA2740 phenylalanyl-tRNA synthetase, alpha-subunit | 3.323           | 0.00548 | pheS                 | Translation, post-translational modification, degradation |
| PA4240_rpsK_at| PA4240 30S ribosomal protein S11 | 3.283           | 0.0292  | rpsK                 | Translation, post-translational modification, degradation |
| PA4267_rpsG_at| PA4267 30S ribosomal protein S7 | 3.104           | 0.0138  | rpsG                 | Translation, post-translational modification, degradation |
| PA2739_pheT_at| PA2739 phenylalanyl-tRNA synthetase, beta subunit | 2.142           | 0.0375  | pheT                 | Translation, post-translational modification, degradation |
| PA3987_leuS_at| PA3987 leucyl-tRNA synthetase   | 2.632           | 0.0216  | leuS                 | Amino acid biosynthesis and metabolism                 |
| PA0009_glyQ_at| PA0009 glycyl-tRNA synthetase alpha chain | 2.568           | 0.00361 | glyQ                 | Amino acid biosynthesis and metabolism                 |
| PA3525_argG_at| PA3525 argininosuccinate synthase | 2.45            | 0.0411  | argG                 | Amino acid biosynthesis and metabolism                 |
| PA3167_serC_at| PA3167 3-phosphoserine aminotransferase | 2.345           | 0.0396  | serC                 | Amino acid biosynthesis and metabolism                 |
| PA0904_lysC_at| PA0904 aspartate kinase alpha and beta chain | 2.337           | 0.00528 | lysC                 | Amino acid biosynthesis and metabolism                 |
| PA5263_argH_at| PA5263 argininosuccinate lyase | 2.29            | 0.00853 | argH                 | Amino acid biosynthesis and metabolism                 |
| PA4007_proA_at| PA4007 gamma-glutamyl phosphate reductase | 2.282           | 0.0231  | proA                 | Amino acid biosynthesis and metabolism                 |
| PA5277_lysA_at| PA5277 diaminopimelate decarboxylase | 2.247           | 0.0334  | lysA                 | Amino acid biosynthesis and metabolism                 |
| PA5143_hisB_at| PA5143 imidazolglycerol-phosphate dehydratase | 2.246           | 0.00461 | hisB                 | Amino acid biosynthesis and metabolism                 |
| PA5039_aroK_at| PA5039 shikimate kinase         | 2.234           | 0.013   | aroK                 | Amino acid biosynthesis and metabolism                 |
| PA0018_fmt_at | PA0018 methionyl-tRNA formyltransferase | 2.222           | 0.0137  | fmt                  | Amino acid biosynthesis and metabolism                 |
| PA5067_hisE_at| PA5067 phosphoribosyl-ATP pyrophosphohydrolase | 2.212           | 0.0214  | hisE                 | Amino acid biosynthesis and metabolism                 |
| PA3482_metG_at| PA3482 methionyl-tRNA synthetase | 2.043           | 0.0312  | metG                 | Amino acid biosynthesis and metabolism                 |
| PA5119_glnA_at| PA5119 glutamine synthetase      | 2.038           | 0.0442  | glnA                 | Amino acid biosynthesis and metabolism                 |
| Gene ID       | Description               | log2 Fold Change | p-value | Category                      |
|--------------|---------------------------|-----------------|---------|-------------------------------|
| PA4439_trpS_at | tryptophanyl-tRNA synthetase | 2.002           | 0.0155  | Amino acid biosynthesis and metabolism |
| PA0903_alaS_at | serine hydroxymethyltransferase | 2.099           | 0.0102  | Aminocyclitols               |
| PA4602_glyA3_at | FadA protein             | 3.347           | 2.83E-05 | Proteins                     |
| PA0971_tolA_at | TolA protein              | 2.249           | 0.000381 | Transport of small molecules |
| PA5479_gltP_at | proton-glutamate symporter | 2.413           | 0.00978 | Membrane proteins            |
| PA3821_secD_at | secretion protein SecD   | 3.516           | 0.00324 | Membrane proteins            |
| PA3820_secF_at | secretion protein SecF   | 2.206           | 0.0411  | Membrane proteins            |
| PA5070_tatC_at | TatC protein              | 2.312           | 0.0209  | transport protein TatC        |
| PA0973_oprL_at | Peptidoglycan precursor   | 2.281           | 0.00672 | Membrane proteins            |
| PA0280_cysA_at | probable binding protein | 2.141           | 0.00709 | Transport of small molecules |
| PA3644_lpxA_at | UDP-N-acetylglucosamine 1-carboxyvinyltransferase | 2.855 | 0.00044 | Transport of small molecules |
| PA5276_lppL_i_at | Lipopeptide LppL precursor | 2.786           | 0.0149  | Cell wall/LPS/capsule        |
| PA3643_lpxB_at | lipid A-disaccharide synthase | 2.561           | 0.0247  | Cell wall/LPS/capsule        |
| PA5012_waaF_at | heptosyltransferase II    | 2.092           | 0.00978 | Cell wall/LPS/capsule        |
| PA3337_rfsD_at | ADP+L-glycero-D-mannohexose 6-epimerase | 3.397 | 0.0498 | Cell wall/LPS/capsule        |
| PA5129_gtxA_at | glutaredoxin              | 3.855           | 0.0334  | Energy metabolism            |
| PA5554_atpG_at | ATP synthase gamma chain  | 3.527           | 0.0287  | Energy metabolism            |
| PA3621_fdxA_at | ferredoxin I              | 3.011           | 0.00384 | Energy metabolism            |
| PA5559_atpE_at | ATP synthase C chain      | 2.734           | 0.0205  | Energy metabolism            |
| PA2995_nqrE_at | NADH:quinone oxidoreductase subunit Nqr5 | 2.216 | 0.00617 | Energy metabolism            |
| PA5553_atpC_at | ATP synthase epsilon chain | 2.175           | 0.00502 | Energy metabolism            |
| PA0362_fdxI_at | ferredoxin [4Fe-4S]       | 2.125           | 0.000346| Energy metabolism            |
Table 1: List of significantly up or downregulated *P. aeruginosa* genes that are discussed in this report (Continued)

| Gene ID       | Description                                      | Fold Change | P-value   | Function                          | Group                  |
|---------------|---------------------------------------------------|-------------|-----------|-----------------------------------|------------------------|
| PA3527_pyrC_at| dihydroorotase                                    | 2.218       | 0.0211    | pyrC Nucleotide biosynthesis and metabolism | Group V: No change(20 min) – Downregulation(60 min) |
| PA3531_pyrE_at| orotate phosphoribosyltransferase                 | 2.156       | 0.0166    | pyrE Nucleotide biosynthesis and metabolism |                        |
| PA3654_pyrH_at| uridylate kinase                                  | 2.434       | 0.0212    | pyrH Nucleotide biosynthesis and metabolism |                        |
| PA3763_pyrL_at| phosphoribosylformylglycinamidine synthase        | 2.279       | 0.00792   | purL Nucleotide biosynthesis and metabolism |                        |
| PA1174_napA_at| periplasmic nitrate reductase protein NapA        | -3.425      | 0.0251    | napA Energy metabolism            |                        |
| PA1175_napD_at| NapD protein of periplasmic nitrate reductase     | -2.949      | 0.0134    | napD Energy metabolism            |                        |
| PA1176_napF_at| ferredoxin protein NapF                           | -2.949      | 0.024     | napF Energy metabolism            |                        |
| PA1173_napB_at| cytochrome c-type protein NapB precursor          | -3.077      | 0.00344   | napB Energy metabolism            |                        |
| PA2248_bkdA2_at| 2-oxoisovalerate dehydrogenase (beta subunit)    | -5.076      | 0.0072    | bkdA2 Amino acid biosynthesis and metabolism |                        |
| PA2247_bkdA1_at| 2-oxoisovalerate dehydrogenase (alpha subunit)   | -9.434      | 0.00376   | bkdA1 Amino acid biosynthesis and metabolism |                        |
| PA2250_lpdV_at| lipoamide dehydrogenase-Val                      | -3.497      | 0.00576   | lpdV Amino acid biosynthesis and metabolism |                        |
| PA3049_rmf_at | ribosome modulation factor                        | -6.25       | 0.000723  | mlf Translation, post-translational modification, degradation | Group VI: Downregulation (20 min) – Downregulation (60 min) |
| PA3622_rpoS_at| sigma factor RpoS                                 | -2.653      | 0.00961   | rpoS Transcriptional regulators |                        |
| PA4762_grpE_at| heat shock protein GrpE                          | -2.915      | 0.00501   | grpE DNA replication, recombination, modification and repair |                        |
| PA5054_hslU_at| heat shock protein HslU                          | -3.226      | 0.000316  | hslU Chaperones & heat shock proteins |                        |
| PA5053_hslV_at| heat shock protein HslV                          | -2.597      | 0.00113   | hslV Chaperones & heat shock proteins |                        |
| PA1596_htpG_at| heat shock protein HtpG                          | -2.695      | 0.00706   | htpG Chaperones & heat shock proteins |                        |

Genes are categorized by their transcription patterns and related functions. The microarray results are the mean of four replicates of each gene.

*The microarray results are the mean of four replicates of each gene.*

*bThe fold change is a positive number when the expression level in the experiment increased compared to the control and is a negative number when the expression level in the experiment decreased.*
Group III: genes downregulated upon 20 minutes exposure

One of the characteristics of this group was the downregulation of genes involved in hydrogen cyanide production: PA2193 (hcnA), PA2194 (hcnB), PA2195 (hcnC). The hcnABC genes encode a cyanide synthase, which forms hydrogen cyanide from glycine [47]. These results are similar to those of a previous study where the hcnA and B genes were downregulated in *P. aeruginosa* treated with peracetic acid for 20 minutes [16]. Hydrogen cyanide is considered an extracellular virulence factor of *P. aeruginosa* and its production is transcriptionally regulated by the anaerobic regulator ANR and the quorum-sensing regulators LasR and PhlR [48]. It has been established that hydrogen cyanide is optimally produced when cell densities are high during the transition from exponential to stationary phase [49]. *P. aeruginosa* does not produce cyanide when it is grown under anaerobic conditions, with nitrate being used as the terminal electron acceptor [50]. The down regulation of genes responsible for cyanide production, therefore, supports the possibility that *P. aeruginosa* experiences an oxygen limiting state characterized by a transient switch to anaerobic metabolism. The upregulation of the nitric oxide reductase enzyme (*norB*) in group II supports this theory. Further, the hcnABC genes did not exhibit a change in expression levels at 60 minutes, indicating resumption of aerobic metabolism.

Also in this group was the groEL gene which encodes a heat shock protein. In a previous study, the expression of groEL was unchanged in *P. putida* exposed to phenol [32]. The cplB gene which encodes an ATP dependent protease that functions as part of a chaperone network necessary for the recovery of stress induced protein aggregates was downregulated 2.7 fold. In contrast, the cplB gene has been shown to be upregulated 2.4 fold in *P. putida* treated with phenol [32].

Group IV: genes upregulated upon 60 minutes exposure

Group IV consists of genes whose expression levels increased only in response to 60 minutes of exposure to OPP (additional file 2). This group contained the highest
number of genes (227 genes) among the six groups generated based on gene transcription direction. The significantly higher number of genes in this group compared with the number of genes in group II (genes upregulated upon 20 minutes exposure), suggests that *P. aeruginosa* significantly adjusts its transcriptional profile after 60 minutes of treatment with OPP.

The most dominant classes in this group were "amino acid biosynthesis and metabolism", "membrane proteins", "nucleotide biosynthesis and metabolism", "translation, post-translational modification and degradation" and "transport of small molecules".

Compared to the other functional classes in this group, a higher number of genes were involved in amino acid biosynthesis and in translation, post-translational modification and degradation. Active protein synthesis was reflected in the upregulation of several genes coding for 30S and 50S ribosomal proteins, aminoacyl-tRNA synthetases associated with alanine (alaS), formylmethionine (fmet), glycine (glyQ), leucine (leuS), methionine and selenomethionine (merG), phenylalanine (phenS and pheT) and treptophan (trpS). In addition, genes involved in the biosynthesis of several amino acids: arginine and proline (argG, argH), glutamine (glnA), lysine (lysA, lysC), glutamate (proA, gltP), histidine (hisB, hisE), phenylalanine (aroK), serine (serC, glyA3), were also upregulated. These results are similar to those of a previous study [32] which indicated that the following enzymes were induced under phenol stress in *P. putida*: ArgD, ProA, GhtD (involved in the glutamate biosynthetic pathway), TrpB and TrpS (belonging to aromatic amino acid biosynthetic pathways), and CysK and Gly-A2 (involved in the serine biosynthetic pathway). Based on these results, the authors suggested that phenol-stressed cells may experience amino acid deficits, and hence a shortage of proteins required for growth and survival [32]. Interestingly it has been shown that *E. coli* can adjust its rate of tryptophan biosynthesis following a shift to stressful nutritional conditions [51]. In contrast, a similar study in our laboratory investigating the effect of OPP on *S. aureus* [84] revealed that several genes involved in amino acid anabolism and specifically lysine and diaminopimelic acid (DAP) biosynthesis were markedly downregulated. This suggests that the effect of 0.82 mM OPP on amino acid metabolism in *P. aeruginosa* and *S. aureus* differ.

Among the genes coding for proteins involved in the transport of small molecules was the tolA gene, which codes for the TolA protein. The TolA protein is an inner membrane protein belonging to the TolQRAB protein complex [52] and is necessary for the uptake of the group A colicins and Tol-dependent phage [53,54]. Tol proteins are also required to maintain the integrity of the bacterial cell envelope structure [52,55]. Mutations in TolA have been shown to cause increased sensitivity to detergents and certain antimicrobials and the leakage of periplasmic proteins [56]. The upregulation of *tolA* after 60 minutes of exposure to OPP suggests a protective role for TolA, possibly related to the maintenance of the cell membrane structure.

In line with the upregulation of genes in the class of "transport of small molecules" after 60 minutes, was the induction of several genes belonging to the classes of "membrane proteins" and "protein secretion/export apparatus". The upregulation of the glycerol-3-phosphate transporter gene (gltP) and the proton glutamate symporter (gltP) is indicative of active transport across the cell membrane. Further evidence of translocation was seen in the upregulation of the components of translocation pathways such as the Sec dependent pathway (secD, secF) which is driven by ATP hydrolysis and the twin-arginine translocation (Tat) pathway (tatC) which uses energy derived from the proton motive force to translocate proteins across the cytoplasmic membrane [57,58]. Interestingly, *E. coli* with mutations in *tatC*, which is critical for the functioning of the Tat system, show pleitropic defects in the cell envelope, leading to hypersensitivity to some detergents and drugs [59,60]. The peptidoglycan associated lipoprotein precursor (oprL), which has been reported to play a protective role against hydrogen peroxide treatment in biofilms of *P. aeruginosa* [61] was also upregulated. Further, several genes encoding proteins in the ABC transport system, including the sulfate transport protein (cysA), probable protein binding component of iron ABC transporter (PA5217), probable periplasmic polyanime binding protein (PA0295) and probable ATP binding component of ABC transporters (PA4461, PA5503) were also upregulated in this group. These findings possibly imply that membrane components of *P. aeruginosa* were altered and that activated and or facilitated transport of ions, sugars, amino acids and other solutes necessary for cell survival was boosted after 60 minutes of exposure to OPP. It therefore appears that both the maintenance of active transport across and the integrity of the cell membrane are necessary for cell survival after 60 minutes of OPP treatment.

This group also contained six genes: rfaD, murA, lpxA, lppL, lpxB and waaF that are involved in the lipopolysaccharide (LPS) biosynthetic pathway. LPS is the main component of the outer cell wall and upregulation of its synthesis suggests that adaptation to OPP treatment in *P. aeruginosa* may involve cell wall modification. Similar results were obtained in *P. putida*, where the LpxC, MurA and the Vac1 (a putative lipoprotein) proteins were upregulated after exposure to phenol [32].
Another predominant functional group in this class contained genes involved in energy metabolism. Genes encoding several components of the F$_{1}$-ATP synthase (atpB, atpC, atpD, atpG) involved ATP generation by oxidative phosphorylation and elements mediating electron transfer (glutaredoxin (PA5129), ferredoxin I (PA3621), ferredoxin (PA0362) and the Na$^+$-translocating NADH:quinone oxidoreductase subunit Nqr5 (PA2995) were upregulated. Other components involved in the oxidative phosphorylation pathway: (PA5561 (atp1) and PA5556 (ATP synthase alpha chain) were upregulated after both 20 and 60 minutes (PA5561 (atp1) and PA5556 (ATP synthase alpha chain). This corroborates the theory that energy production through this route is essential for OPP-treated cells.

An interesting observation was the upregulation of several genes involved in the biosynthesis of purines and pyrimidines after 60 minutes of treatment with OPP. The genes: pyrC, pyrE, pyrH, belonging to the pyrimidine biosynthetic operon that has been described in Bacillus subtilis [62] and the purI. gene involved in purine biosynthesis [63] were upregulated, suggesting that an increase in nucleotide biosynthesis may contribute to the adaptive response of upregulated, suggesting that an increase in nucleotide bio-

Group V: genes downregulated upon 60 minutes exposure

This group contained a total of 70 genes (additional file 2) that were downregulated after 60 minutes of treatment with OPP. Genes belonging to the functional classes of "amino acid biosynthesis and metabolism", "energy metabolism" and "putative enzymes" contained a relatively higher number of genes. It was interesting to find the napA, B, D and F genes among the genes in the class of energy metabolism. These genes are some of the components of the nap operon that has been identified in many gram negative bacteria [64]. The E. coli nap operon (napFDAGHBC) encodes a periplasmic nitrate reductase [65]. The respiratory periplasmic nitrate reductase in denitrifying P. sp. Strain G-179 has been reported to support anaerobic growth in the presence of nitrate [66]. Most Nap enzymes consist of a large catalytic sub subunit (NapA) and a small diheme cytochrome c (NapB). NapC is a membrane bound tetraheme cytochrome c that transfers electrons from the quinol pool in the cytoplasmic membrane to NapAB. NapD is found in the cytoplasm and plays a role in the maturation of the enzyme prior to export [67] and NapF is a non-heme iron-sulfur protein [68]. The downregulation of the nap A, B, D and F genes in this study suggests that after 60 minutes of OPP treatment, P. aeruginosa probably maintains aerobic growth. This is in contrast to after 20 minutes of treatment when the nitric oxide reductase gene was upregulated, suggest-

ing a possible transient switch to anaerobic respiration (table 1).

This group also contained several genes involved in valine, leucine and isoleucine degradation. In particular, PA2247 (bkdaA1), PA2248 (bkdaA2), and 2250 (lpdV) which are involved in the conversion of valine, leucine and isoleucine to alkyl-CoA derivatives that feed into the TCA cycle, pyrimidine metabolism and propanoate metabolism were downregulated. Similarly, the bkdaA1, bkdaA2 and lpdV genes were downregulated in P. aeruginosa after exposure to peracetic acid for 20 minutes [16]. The results of the present study suggest that the synthesis of these amino acids was being inhibited after 60 minutes of OPP treatment with concomitant inhibition of energy production through the TCA cycle. Further, several genes involved in the synthesis of acetylCoA were present in this group. In particular, PA2013 and PA0745 (probable enolylCoA hydratases) involved in the synthesis of acetyl-CoA from Lysine and butanoate respectively and PA3417 (probable pyruvate dehydrogenase E1 component) which catalyzes the transformation of pyruvate to acetylCoA were downregulated. These results support the fact that energy production through the TCA cycle was being inhibited after 60 minutes of exposure to OPP.

Group VI: genes downregulated at 20 and 60 minutes exposure

The most downregulated gene in this class was the ribosome modulation factor gene (rmf), which exhibited a fold change of -6.25 after 20 minutes of OPP treatment and -25.9 after 60 minutes. The ribosome modulation factor (RMF) is a ribosome associated protein that is produced by E. coli during slow growth at exponential phase [69] and upon entry into stationary phase [70]. RMF is considered a protective factor of ribosomes against stresses such as heat shock, acidic/basic pH and high osmolarity during stationary phase [71]. Mutant stationary phase E. coli strains without functional RMF are more susceptible to osmotic stress [72] and heat stress [73]. The production of RMF by stationary phase cells has been linked to the detection of the dimerized form of 70S ribosomes: 100S ribosomes with no translational activity [71,74,75].

In exponentially growing E. coli cells not treated with any chemicals and in those treated with acidifying agents, it was found that rmf expression was growth rate dependent and there was an inverse relationship between rmf expression and growth rate [69,76]. It has been postulated that the function of RMF in slow growing exponential phase cells is to promote more efficient protein synthesis through the inactivation of excess ribosomes, thereby reducing competition for protein synthesis factors [76]. Our results contrast those of previous studies which have reported that rmf is expressed in slow growing cells during exponential phase [69,76]. It was surprising that rmf
expression was downregulated upon both treatment times, but more significantly after 60 minutes, given the fact that OPP-treated *P. aeruginosa* cells in this study were also slow growing during exponential phase. However, more investigation is required to determine the mechanism by which OPP treatment influences transcriptional control, leading to downregulation of the expression of the *rmf* gene in *P. aeruginosa*.

The *rpoS* gene which encodes RpoS, an alternative sigma factor of RNA polymerase was also downregulated after both exposure times. RpoS is known to participate in the stress response of both *E. coli* and *P. aeruginosa* [77,78]. Although RpoS is more widely considered a global regulator in a complex regulatory network that controls the expression of several stationary-phase inducible genes, it has been demonstrated that RpoS also acts as a master regulator of gene expression in exponentially growing *E. coli* cells exposed to osmotic stress [79]. Further, a previous study revealed that cell viability was slightly decreased in *E. coli* cells containing mutations in *rmf* and *rpoS* [80]. The downregulation of the *rmf* and *rpoS* genes may therefore be indicative of the mechanism of action by which OPP causes a growth inhibition in *P. aeruginosa*.

This group also contained four genes that are involved in the *P. aeruginosa* heat shock response: *grpE*, *hslU*, *hslV* and *htpG*. In contrast to these results, the HtpG and GrpE proteins have been reported to be upregulated in *P. putida* exposed to phenol and to hydrochloric acid [32,81]. The *hslUV* operon encodes two heat shock proteins, HslV and HslU that function together as an ATP-dependent protease [82]. The *hslUV* operon was upregulated in *E. coli* after exposure to acid stress for 10 minutes [81]. This result suggests that transcriptional regulation of the expression of these heat shock proteins during stress may vary depending on the nature of the environmental stress.

**Validation of microarray data using real-time PCR**
In order to validate the relative transcript levels obtained by the microarray analysis, we employed quantitative real-time PCR on six genes. These genes were selected because they displayed a wide range of mRNA level changes (-25-to 6-fold). Table 2 indicates that our microarray results were in agreement with quantitative real-time PCR analyses of the selected genes.

**Conclusion**
The present study represents the first genome-wide response of *P. aeruginosa* exposed to 0.82 mM OPP. The results from this study indicate that after 20 minutes of OPP exposure, genes involved in anaerobic metabolism and swarming motility were upregulated. We suggest that *P. aeruginosa* undergoes a switch to denitrification as indicated by downregulation of cyanide production which is indicative of anaerobic respiration. OPP treatment also caused the downregulation of the genes encoding the ribosome modulation factor (*rmf*) and an alternative sigma factor (*rpoS*) of RNA polymerase which have been linked to decreases in cell viability when mutated. The repression of these genes may be contributory to the growth inhibition observed after *P. aeruginosa* was exposed to OPP and may reflect the mechanism of action by which OPP reduces the viability of *P. aeruginosa* cells, leading to the observed growth inhibition. We suspect that the continuous marked upregulation of translation after both 20 and 60 minutes and of amino acid biosynthesis following 60 minutes exposure to OPP are consequential responses to combat this growth repression. Our results suggest that these responses may involve the upregulation of genes involved in the synthesis of membrane transport and virulence proteins and also proteins involved in the maintenance of the integrity of the cell membrane. In addition, after 60 minutes of OPP treatment, the adaptive response to OPP treatment may involve cell wall modification evidenced by the upregulation of lipopolysaccharide biosynthesis genes. It is worth noting that in contrast to the results of this study, we have observed that in *S. aureus*, OPP treatment led to downregulation of amino acid anabolism in general and specifically lysine and diaminopimelic acid (DAP) biosynthesis genes (unpublished data). It is therefore apparent that OPP exerts differential effects on amino acid metabolism in *P. aeruginosa* and *S. aureus*.

This gene expression profile can now be employed to more profoundly understand the mechanisms by which OPP exerts a killing effect on *P. aeruginosa* and how this organism develops resistance to phenolic disinfectants in general and to OPP in particular. The information from this study provides useful information that will benefit further research on the toxicogenomic impact of phenolic biocides on *P. aeruginosa*. Further, considering that multicellular behavior in bacteria such as swarming motility is an adaptation to environmental stress, it will be interesting to investigate the comparative response of sessile cells versus cells exhibiting swarming motility to OPP treatment.

**Methods**

**Bacterial growth and treatment with OPP**
*Pseudomonas aeruginosa* PA01 was grown at 37°C for 17 hours on Luria-Bertani (LB) agar. An isolated colony was inoculated into 100 ml of sterilized LB broth (10 g of tryptone, 5 g of yeast extract and 10 g of sodium chloride per liter) and incubated overnight for 17 hours at 37°C with shaking at 250 rpm. A 1:100 dilution of the culture was performed using pre-warmed LB broth. The diluted culture was incubated at 37°C with shaking at 250 rpm until a final optical density (OD600) of 0.8 (early logarithmic
phase) was attained. A further 1:10 dilution was performed using LB broth and incubated at 37 °C with shaking at 250 rpm. When the OD600 of the 1:100 dilution reached 0.8, the culture was incubated at 37 °C with shaking at 250 rpm with various concentrations of OPP (Sigma-Aldrich, Inc., St Louis, MO) and the OD600 of the growth culture was determined at intervals of ten minutes for a total time of 60 minutes. A concentration of 0.82 mM OPP, with treatment times of 20 and 60 minutes were targeted for this study.

**RNA isolation**

Total RNA was extracted after 20 and 60 minutes incubation with 0.82 mM OPP and without OPP (control). The RNA extraction procedure was carried out using the RNeasy mini kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions. Briefly, 1 ml of bacterial culture was added to 2 ml of RNP reagent (Qiagen, Inc., Valencia, CA). Centrifugation (5000 g for 10 minutes) of the mixture was performed to precipitate the cells. The harvested cells were incubated in TE buffer with 1 mg/ml lysozyme (Fisher Scientific, Pittsburgh, PA). Total RNA was eluted in 50 ml of RNase free water (Ambion Inc., Austin Texas) using kit supplied columns containing silica gel membranes. The quantity of eluted RNA was determined using the NanoDrop spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE). RNA quality was examined using the RNA 6000 Nano Labchip with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA).

**cDNA synthesis and labeling**

cDNA was synthesized from 12 µg of total RNA using random primers (Invitrogen, Carlsbad, CA) and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) following the Affymetrix *P. aeruginosa* GeneChip arrays protocol (Affymetrix, Inc., Santa Clara, CA). Spike controls containing RNA transcripts from several *Bacillus subtilis* genes were included in the RNA mixtures as internal controls to monitor the efficiency of labeling, hybridization and staining. The reaction mixture was incubated at 25 °C for 10 minutes, 37 °C for 60 minutes and 42 °C for 60 minutes followed by inactivation of the enzyme at 70 °C for 10 minutes. Purification of cDNA was carried out using the Quick PCR purification kit (Qiagen, Inc., Valencia, CA). The cDNA was fragmented at 37 °C for 10 minutes in a reaction mixture consisting of purified cDNA and DNase I (Roche Applied Science, Indianapolis) in One-Phor-All buffer (Invitrogen, Carlsbad, CA) in the order of 0.06 U DNase/µl of cDNA. The quality of fragmented cDNA was examined using the RNA 6000 Nano Labchip with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Labeling of the 3' termini of fragmented cDNA was performed using the Enzo BioArray terminal labeling kit with Biotin-ddUTP (Enzo Life Sciences, Inc., Farmingdale, NY).

**Hybridization, processing and scanning**

Array hybridization and processing were carried out according to instructions provided in the affymetrix expression analysis technical manual: chapters 5 and 6

| Gene | 3′mRNA level change with microarray | 5′mRNA level change with real-time PCR | Forward primer sequence (5′-3′) | Reverse primer sequence (5′-3′) |
|------|-----------------------------------|--------------------------------------|---------------------------------|---------------------------------|
|      | 20 min  | 60 min | 20 min  | 60 min |                                     |                                   |
| PA4243 | 2.914   | 6.799   | 3.605 (± 1.55) | 7.378 (± 0.7) |
| PA2193 | -2.268  | 1.967 (± 1.06) | -3.03 (± 5.64) |
| PA1173 | -6.25   | -25.907 | -10.196 (± 0.14) | -48.503 (± 0.35) |
| PA3049 | -2.762  | -10.256 | -5.856 (± 0.21) | -14.928 (± 1.27) |
| PA3622 | -2.653  | -2.967  | -1.954 (± 1.95) | -5.924 (± 1.60) |
| 3′PA3001 | 1.00   | 1.00    | 1.00     | 1.00   |

The real time PCR results are the mean of three biological replicates with three technical replicates for each gene. The microarray results are the mean of four replicates of each gene.

The microarray results are the mean of four replicates of each gene.

The real time PCR results are the mean of three biological replicates with three technical replicates for each gene.

Internal control: glyceraldehyde-3-phosphate dehydrogenase
[83]. The hybridization solution consisted of the fragmented/labeled cDNA, B2 control oligonucleotide, MES hybridization buffer, bovine serum albumin, and dimethyl sulfoxide (DMSO) in a final volume of 200 μl. The mixture was hybridized onto \textit{P. aeruginosa} GeneChip arrays (Affymetrix, Inc., Santa Clara, CA) at 50°C for 16 hours with tumbling. The arrays were washed and stained using the Affymetrix GeneChip hybridization, wash and stain kit containing the stain cocktails 1 and 2 and the array holding buffer. The array staining and washing process was performed using the GeneChip Fluidics station 450 (Affymetrix). Processed arrays were scanned with the Affymetrix GeneChip Scanner 3000.

**Data analysis**

Data analysis was carried out using the Affymetrix GeneChip Operating Software (GCOS), version 1.0 and GeneSpring Version 7.3 (Agilent Technologies). The following parameters were employed for expression analysis using GCOS: $\alpha_1 = 0.04$, $\alpha_2 = 0.06$, $\tau = 0.015$ and target signal was scaled to 150. Genes that were assigned "absent calls" from 50% or more of the replicates in GeneSpring were not included in the analysis. Gene expression changes with statistical significance were identified by 1-way ANOVA ($p$ cutoff value = 0.05). Fold changes were calculated as the ratios between the signal averages of four untreated (control) and four OPP-treated (experimental) cultures. Genes with a two-fold or more induction or repression were used in this analysis.

**Real-Time PCR analysis**

Transcript level changes obtained from microarray analysis (six genes) were evaluated using quantitative real-time PCR. The genes and primer sequences employed for the real-time PCR analysis are listed in table 2. The housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (PA3001) was used as an endogenous control. Real-time PCR was performed using the iCycler IQ Real-Time PCR Detection System with iScript cDNA Synthesis Kit and IQ SYBR Green Supermix (BioRad Laboratories, Inc., Hercules, CA). For each gene, three biological replicates and three technical replicates were employed. Reaction mixtures were incubated for 3 minutes at 95.0°C, followed by 40 cycles of 10 seconds at 95.0°C, 30 seconds at 55.0°C, and 20 seconds at 72.0°C. PCR efficiencies were also derived from standard curve slopes in the iCycler software v. 3.1 (BioRad Laboratories, Inc., Hercules, CA). To evaluate PCR specificity, melt curve analysis was performed and this resulted in single primer-specific melting temperatures. In this report, relative quantification based on the relative expression of a target gene versus the glyceraldehyde-3-phosphate dehydrogenase gene was utilized to determine the transcript level changes.

**Authors’ contributions**

CWN performed microarray experiments, and data analysis, and drafted the manuscript. HJ performed microarray experiments, and data analysis, and reviewed the manuscript. FT initiated and supervised the study and reviewed the manuscript. WEB reviewed the manuscript.

**Additional material**

**Additional File 1**

The raw data representing the entire genome (5900 genes) under control conditions (0 minutes) and experimental (after 20 and 60 minutes exposure to 0.82 mM OPP). This data has also been deposited in NCBI’s Gene Expression Omnibus [http://www.ncbi.nlm.nih.gov/geo and can be accessed through the GEO series accession number: GSE10604 http://www.ncbi.nlm.nih.gov/projects/geo/query/acc.cgi?token=md8ay&acc=GSE10604&form=html&lang=ja&view=quick]

**Additional File 2**

List of 509 functionally classified \textit{P. aeruginosa} genes that were significantly up and downregulated after 20 and 60 minutes of OPP exposure. The genes were categorized into six groups based on their transcription patterns.

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**References**

1. Wenzel RP, Edmond MP: The impact of hospital-acquired bloodstream infections. Emerg Infect Dis 2001, 7: [http://www.cdc.gov/ncidod/dvbid/volf002/wenzel.html].
2. Bielecki G, Glick A, Kawecki M, Martins dos Santos VAP: Towards understanding \textit{Pseudomonas aeruginosa} burn wound infections by profiling gene expressions. Biotechnol Lett 2008, 30:777-790.
3. Brewer SC, Wunderink RG, Jones CB, Leeper KV Jr: Ventilator associated pneumonia due to \textit{Pseudomonas aeruginosa}. Chest 1996, 109:1019-1029.
4. Saltzstein D, Wachs B, Perroncel R, Benson A, Herrington J, Haverstock D, Pertel P: Complicated urinary tract infections treated with extended-release ciprofloxacin with emphasis on \textit{Pseudomonas aeruginosa}. J Chemother 2007, 19:694-702.
5. Van Delden C, Igleswki Bt: Cell-to-cell signaling in \textit{Pseudomonas aeruginosa} infections. Emerg Infect Dis 1998, 4: [http://www.cdc.gov/ncidod/dvbid/volf004/vandelden.htm].
6. Govan JR, Deretic V: Microbial pathogenesis in cystic fibrosis: mucoid \textit{Pseudomonas aeruginosa} and Burkholderia cepacia. Microbial Rev 1996, 60:539-574.
7. Mendelson MH, Gurtman A, Szabo S, Neibert E, Meyers BR, Polcar M, Cheung TW, Lillienfeld D, Reddy S, et al.: \textit{Pseudomonas aerugi-
nose bacteremia in patients with AIDS. Clin Infect Dis 1994, 18:886-895.
8. Darzins A, VC. Nickerson KW, Hamlett NV, O’Hara C: Prevalence of extreme detergent resistance among the Enterobacteria.
Can J Microbiol 1984, 30:711-713.

9. Lautenbach E, Polk RE. Resistant gram-negative bacilli: a neglected health-care crisis? Am J Health Syst Pharm 2007, 64(Suppl 14):S3-52.
10. Dettenkofer M, Spencer RC: Importance of environmental decontamination-a critical review. J Hosp Infect 2007, 65(Suppl 2):55-57.

11. Bomhard EM, Brendler-Schwaab SY, Freyberger A, Herbold BA, Lelic KH, Richter M: O-pheynylphenol and its sodium and potassium salts: a toxicological assessment. Crit Rev Toxicol 2002, 32:551-625.
12. Stouten H: Toxicological profile of o-phenylphenol and its sodium salt. J Appl Toxicol 1998, 18:261-270.
13. Chang W, Small DA, Toghril F, Bentley WE: Microarray analysis of Pseudomonas aeruginosa reveals induction of pyocin genes in response to hydrogen peroxide. BMC Genomics 2005, 6:115.

14. Chang W, Small DA, Toghril F, Bentley WE: Microarray analysis of toxicogenic effects of peracetic acid on Pseudomonas aeruginosa. Environ Sci Technol 2005, 39:5893-5899.
15. Salunkhe P, Topfer T, Buer J, Tumbiler B: Genome-wide transcriptional profiling of the steady-state response of Pseudomonas aeruginosa to hydrogen peroxide. J Bacteriol 2003, 185:2565-2572.

16. Chang W, Small DA, Toghril F, Bentley WE: Toxicogenic analysis of sodium hypochlorite antimicrobial mechanisms in Pseudomonas aeruginosa. Appl Microbiol Biotechnol 2007, 74:176-185.
17. Chang W, Small DA, Toghril F, Bentley WE: Comparative global transcriptome analysis of sodium hypochlorite, peracetic acid, and hydrogen peroxide on Pseudomonas aeruginosa. Appl Microbiol Biotechnol 2006, 73:105.

18. Lambert RJW: Comparative analysis of antibiotic and antimicrobial biocide susceptibility data in clinical isolates of methicillin-sensitive Staphylococcus aureus, methicillin-resistant Staphylococcus aureus and Pseudomonas aeruginosa between 1999 and 2003. Can J Microbiol 2007, 53:69-711.

19. NCBi’s Gene Expression Omnibus [http://www.ncbi.nlm.nih.gov/geo]
20. NCBi’s Gene Expression Omnibus series accession number: GSE10604 [http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE10604&form=/detail&term=ms=1]
21. Pseudomonas aeruginosa Community Annotation Project [http://v2.pseudomonas.com/]
22. Winsor GL, Sui J, Jung KS, Huang S, Cheng D, Ching WK, Hancock RE, Brinkman FS: Pseudomonas aeruginosa Genome Database and PseudoCAP: facilitating community-based, continually updated, genome annotation. Nucleic Acids Res 2005, 33:D338-D343.
23. Chang W, Small DA, Toghril F, Bentley WE: Global transcriptome analysis of Staphylococcus aureus response to hydrogen peroxide. J Bacteriol 2006, 188:1484-1489.

24. Jang HJ, Chang MW, Toghril F, Bentley WE: Microarray analysis of toxicogenic effects of triclosan on Staphylococcus aureus. Appl Microbiol Biotechnol 2008, 78:695-707.
25. Hueck CJ: Type III secretion systems in bacterial pathogens of animals and plants. Microb Mol Biol Rev 1998, 62:379-433.

26. Adhikari P, Kirby SD, Nowalk AJ, Veraldi KL, Schyryers AB, Mietzner TA: Biochemical Characterization of a Haemophilus influenzae periplasmic iron transport operon. J Biol Chem 1995, 270:25142-25149.
27. Darzins A: Characterization of a Pseudomonas aeruginosa gene cluster involved in pilus biosynthesis and twitching motility: sequence similarity to the chemotaxis proteins of enterics and the gliding bacterium Myxococcus Xanthus. Mol Microbiol 1994, 11:137-153.

28. O’Toole GA, Colter RW: Flagellar and twitching motility are necessary for Pseudomonas aeruginosa biofilm development. Mol Biol 1998, 30:293-304.
29. Santos P, Bennendorf D, Sa-Correia I: Insights into Pseudomonas putida KT2440 response to phenol-induced stress by quantitative proteomics. Proteomics 2004, 4:2624-2625.
30. Flock U, Thorndycroft FH, Matorin AD, Richardson DJ, Watmough NJ: Defining the proton entry point in the bacterial respiratory nitric-oxide reductase. J Biol Chem 2007, 283:3839-3845.
31. Kato-Itoh DHW, Heiss TJ, Kromeck MH, Zumft WG: Nitric oxide from Pseudomonas stutzeri, a novel cytochrome bc complex. Phospholipid requirement, electron paramagnetic resonance and redox properties. Eur J of Biochem 1994, 222:293-303.
32. Kormer H: Expression of nitric oxide reductase from denitrifying Pseudomonas stutzeri. Adv Biochem Eng Biotechnol 1997, 53:89-118.
33. Kowalszuki R, Tumbiler M, Doring G: Rhamnolipid of Pseudomonas aeruginosa in sputum of cystic fibrosis patients. Lancet 1987, 1:1026-1027.
34. Beal R, Betts WB: Role of rhamnolipid biosurfactants in the uptake and mineralization of hexadecane in Pseudomonas aeruginosa. J Appl Microbiol 2000, 89:158-166.
35. Eberl L, Soren M, Giskovik M: Surface Motility of Sorretia liquefaciens MG1. J Bacteriol 1999, 181:1703-1712.
36. Harshay RM: Bees aren’t the only ones: swarming in gram-negative bacteria. Mol Microbiol 1994, 13:389-394.
37. Beal R, Betts WB: Role of rhamnolipid biosurfactants in the uptake and mineralization of hexadecane in Pseudomonas aeruginosa. J Appl Microbiol 2000, 89:158-166.
38. Eberl L, Soren M, Giskovik M: Surface Motility of Sorretia liquefaciens MG1. J Bacteriol 1999, 181:1703-1712.
39. Pessi G, Haas D: Transcriptional control of the hydrogen cyanide biosynthetic genes hcnABC by the anaerobic regulator ANR and the quorum-sensing regulator LasR and RhlR. J Bacteriol 2002, 182:6940-6949.
40. Castric PA, Ebert RF, Castric KF: The relationship between growth phase and cyanogenesis in Pseudomonas aeruginosa. Can J Microbiol 1997, 2:287-292.
41. Castric PA: Hydrogen cyanide, a secondary metabolite of Pseudomonas aeruginosa. Can J Microbiol 1975, 21:613-618.
42. Yanofsky C, Horn V: Role of regulatory features of the trp operon of Escherichia coli in mediating a response to a nutritional shift. J Bacteriol 1991, 173:624-629.
43. Vianney A, Lezou TM, Beyer WF, Jr, Lazzaroni JC, Portailier R, Webster RE: Membrane topology and mutational analysis of the ToIQ protein of Escherichia coli required for the uptake of macromolecules and cell envelope integrity. J Bacteriol 1994, 176(9):822-829.
44. LeenGood SK, Beyer WF, Webster RE: ToIQ: A membrane protein involved in colicin uptake contains an extended helical region. Proc Natl Acad Sci USA 1991, 88:5939-5943.
45. Journet L, Bouvet E, Rigel A, Llobes R, Lazdunski C, Benedetti H: Import of collcins across the outer membrane of Escherichia coli.
coli involves multiple protein interactions in the periplasm. Mol Microbiol 2001, 42:331-344.
55. Dunnick JI, Lafortunato ER, Sokolol PA: Identification and characterization of the tolQRA genes of Pseudomonas aeruginosa. J Bacteriol 2001, 173:7059-7068.
56. Schendel SL, Click EM, Webster RE, Cramer WA: The TolA protein interacts with colicin E1 differently than with other group A colicins. J Bacteriol 1997, 179:3638-3640.
57. Manting EH, Driessen AJM: Escherichia coli translocase: the unraveling of a molecular machine. Mol Biol 2000, 37:226-238.
58. Yahr TL, Wickner WT: Functional reconstitution of bacterial Tat translocation in vitro. EMBO J 2001, 20:2472-2479.
59. Ize B, Stanley NR, Buchanan G, Palmer T: Role of the Escherichia coli Tat pathway in outer membrane integrity. Mol Microbiol 2003, 48:1183-1193.
60. Stanley NR, Findlay K, Berks BC, Palmer T: Escherichia coli strains blocked in Tat-dependent protein export exhibit pleiotropic defects in growth and survival during planktonic and biofilm culture. J Bacteriol 2008 in press.
61. Turner RJ, Lu Y, Switzer RL: Regulation of the Bacillus subtilis pyrimidine biosynthetic (ppy) gene cluster by an autogenous transcriptional attenuation mechanism. J Bacteriol 1994, 176:3708-3722.
62. Alacantra RB, Read RDA, Valderas MW, Brown TD, Roop RM II: The peptidoglycan-associated lipoprotein, OprL, helps protect Pseudomonas aeruginosa mutant devoid of the transactivator, OxyR, from hydrogen peroxide-mediated killing during planktonic and biofilm culture. J Bacteriol 2008 in press.
63. Potter LC, Cole JA: Roles of NapF, NapG and NapH, subunits of the Escherichia coli periplasmic nitrate reductase in ubiquinol oxidation. Mol Microbiol 2002, 44:245-255.
64. Bedzyk L, Wong T, Ye RW: The periplasmic nitrate reductase of Pseudomonas putida G-179 catalyzes the first step of denitrification. J Bacteriol 1999, 181:2802-2806.
65. Potter LC, Cole JA: Essential roles for the products of the napABCD genes, but not napFGH, in periplasmic nitrate reduction by Escherichia coli K-12. Biochem J 1999, 346:57-66.
66. Mukherjee A, Brondijk THC, Overton TW, Richardson DJ, Leach ER, Cole JA: The NapF protein of Escherichia coli periplasmic nitrate reductase system: demonstration of a cytoplasmic location and interaction with the catalytic subunit, NapA. Microbiol 2006, 152:3227-3237.
67. Yamagishi M, Matsushita H, Wada A, Sakagami M, Fujita N, Ishihama A: Regulation of the Escherichia coli rmf gene encoding the ribosome modulation factor: growth phase- and growth rate-dependent control. EMBO J 1993, 12:625-630.
68. Wada A, Yamazaki Y, Fujita N, Ishihama A: Structure and probable genetic location of a ribosome modulation factor associated with 100S ribosomes in stationary phase Escherichia coli cells. Proc Natl Acad Sci USA 1990, 87:2657-2661.
69. Izutsu K, Wada A, Wada C: Expression of ribosome modulation factor (RMF) in Escherichia coli requires pGpP. Genes Cells 2001, 6:655-667.
70. Niven GW: Ribosome modulation factor protects Escherichia coli during heat stress but this may not be dependent on ribosome dimerization. Arch Microbiol 2004, 182:60-66.
71. El-Sharoud WM, Niven GW: The influence of ribosome modulation factor on the survival of stationary-phase Escherichia coli during acid stress. Microbiol 2007, 153:247-253.
72. Yoshida H, Maki Y, Kato H, Fujisawa H, Izutsu K, Wada C, Wada A: The ribosome modulation factor (RMF) binding site on the 100S ribosomes of Escherichia coli. J Biochem 2002, 132:983-989.
73. Yahr TL, Wickner WT: Functional reconstitution of bacterial Tat translocation in vitro. EMBO J 2001, 20:2472-2479.
74. El-Sharoud WM, Niven GW: The activity of ribosome modulation factor during growth of Escherichia coli under acidic conditions. Arch Microbiol 2005, 184:18-24.
75. Yoshida H, Maki Y, Kato H, Fujisawa H, Izutsu K, Wada C, Wada A: The ribosome modulation factor (RMF) binding site on the 100S ribosomes of Escherichia coli. J Biochem 2002, 132:983-989.
76. Sir Paul Nurse, Cancer Research UK

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