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Dual-seq transcriptomics reveals the battle for iron during *Pseudomonas aeruginosa* acute murine pneumonia

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Determining bacterial gene expression during infection is fundamental to understand pathogenesis. In this study, we used dual RNA-seq to simultaneously measure *P. aeruginosa* and the murine host's gene expression and response to respiratory infection. Bacterial genes encoding products involved in metabolism and virulence were differentially expressed during infection and the type III and VI secretion systems were highly expressed *in vivo*. Strikingly, heme acquisition, ferric-enterobactin transport, and pyoverdine biosynthesis genes were found to be significantly up-regulated during infection. In the mouse, we profiled the acute immune response to *P. aeruginosa* and identified the pro-inflammatory cytokines involved in acute response to the bacterium in the lung. Additionally, we also identified numerous host iron sequestration systems upregulated during infection. Overall, this work sheds light on how *P. aeruginosa* triggers a pro-inflammatory response and competes for iron with the host during infection, as iron is one of the central elements for which both pathogen and host fight during acute pneumonia.

Pseudomonas aeruginosa is an opportunistic Gram-negative respiratory pathogen of major clinical significance causing acute and chronic respiratory infections as well as bloodstream, urinary tract, burn wound, and surgical site infections¹. According to the Center for Disease Control (CDC), 51,000 healthcare-associated *P. aeruginosa* infections occur in the United States each year². This bacterium is highly versatile, able to survive in harsh environments, and forms robust biofilms that are difficult to eradicate on medical devices. *P. aeruginosa* is inherently highly resistant to antibiotics, and effective antimicrobial treatment of infections caused by this bacterium is often challenging. Multidrug-resistant *Pseudomonas* cause 13% of all *P. aeruginosa* infections and were given a threat level of "serious" in the CDC Antibiotic Resistance Threat report in 2013². Overall, *P. aeruginosa* carries one of the highest case fatality rates of all Gram-negative infections³. The success of this microorganism is associated with its highly adaptive genome, ability to survive and thrive in diverse environments and the expression of a wide variety of virulence factors⁴⁻⁶. It is therefore important to develop new therapeutics for the treatment of *P. aeruginosa* infections.

To accomplish this objective, it is crucial to better understand the simultaneous pathogen and host transcriptional responses during infection. Several studies have attempted to define the interaction dynamics between the pathogen and the host by studying gene and protein expression of either one or the other⁷⁻¹¹. These studies have identified *P. aeruginosa* genes and virulence factors involved in bacterial attachment during colonization, nutrient acquisition, biofilm formation, or resistance to the immune system to facilitate persistence. Numerous studies have also identified how the host responds to infection¹²⁻¹⁴ and shown that pathogen-host interaction drives the selection and co-evolution of the two organisms¹⁵⁻¹⁷. More recently, Westermann *et al.* performed the first dual-RNA sequencing during tissue culture infection, demonstrating that dual-RNA sequencing is possible and a great tool to study pathogen-host interactions¹⁸.

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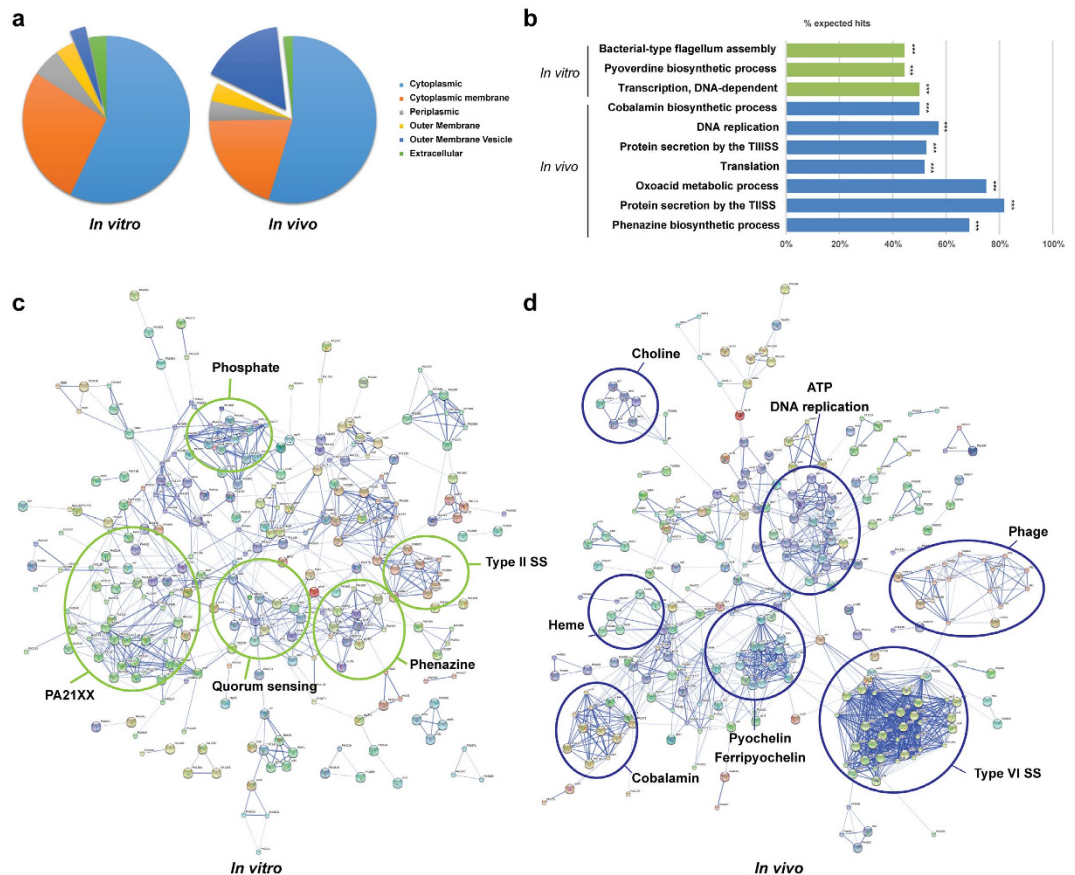


Figure 1. Characterization of *P. aeruginosa* gene expression *in vitro* and *in vivo*. (a) Primary cellular localization of the products of the genes of *P. aeruginosa* PAO1 differentially regulated *in vivo* and *in vitro*. The percentage of the total number of genes whose expression was significantly up-regulated *in vivo* compared to *in vitro* are represented on the left; the percentage of genes whose expression was significantly up-regulated *in vitro* compared to *in vivo* are represented on the right. Genes were classified according to their known and predicted cellular localization. (b) Hypergeometric test enrichment analysis on annotation of *P. aeruginosa* PAO1 genes differentially regulated *in vitro* and *in vivo*. Data are represented as the percentage of genes (hits) in each class which expression was significantly up-regulated *in vitro* (green) or *in vivo* (blue). The subsets in which the number of hits was significant are denoted with asterisks (** $p < 0.001$). STRING analysis representing the 300 most highly upregulated *P. aeruginosa* PAO1 genes expressed *in vitro* (c) or *in vivo* (d). Gene products are represented with circles and known associations between each gene or gene products are represented with a connecting line. Nodes of genes participating in similar functions are circled in green (expressed *in vitro*) or in blue (expressed *in vivo*).

However, none of these studies have characterized simultaneously the bacterial and host molecular factors involved in acute lung infection. It has been hypothesized that sequencing the RNA of both the host and the pathogen during *in vivo* infection would be feasible^{19–21} and was recently accomplished during *Toxoplasma gondii* infections²². Advances in DNA and protein sequencing during the past decade have now made dual analysis of RNA and protein expression possible, even in complex tissues. To characterize the armamentarium used in the battle between *P. aeruginosa* and the host during acute lung infection, we developed a methodology using dual RNA-seq to identify the transcriptional response of the pathogen and the host during infection.

Results

Dual genome-wide gene expression analysis during infection. In this study, we hypothesized that *in vivo* conditions influence gene expression in *P. aeruginosa* during infection and that the bacterium elicits transcriptional changes in the host. To test this hypothesis, we used a murine model of acute pneumonia to characterize the differences in *P. aeruginosa* gene expression during respiratory infection compared to *in vitro* growth. Using dual RNA-seq, we also gained insights into the effects of the bacterium on the host transcriptome. *P. aeruginosa* PAO1 was grown on *Pseudomonas* isolation agar (PIA) at 37 °C and 2×10^8 colony forming units (CFU) were used to infect outbred CD-1 mice by intranasal administration. The *P. aeruginosa* PAO1 grown on PIA was used to purify RNA for *in vitro* growth control. Mice were infected with PAO1, or PBS as control. After 16 h, mice were euthanized, lungs were removed and total RNA was purified. rRNA was depleted, and three libraries were

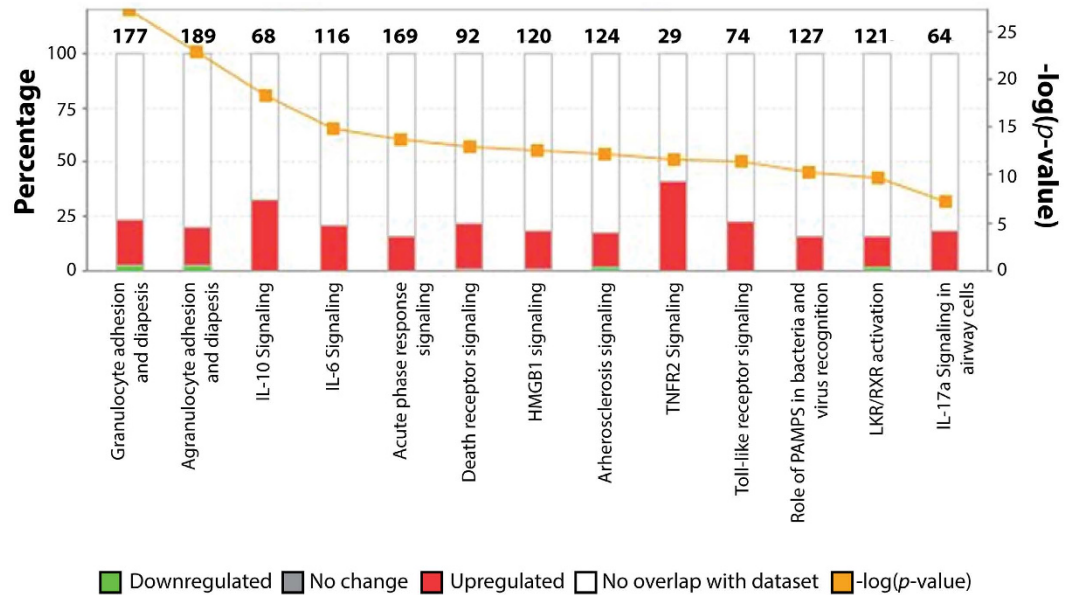


Figure 3. Comparison between host proteomes and transcriptomes in response to *P. aeruginosa* infection. Ingenuity Pathway Analysis (IPA) of canonical pathways significantly up-regulated during *P. aeruginosa* infection detected by transcriptome analysis. Murine genes significantly up- or down-regulated during infection were analyzed using IPA and classified according to their known and predicted function.

concentrations in PIA³². Overall these data suggest that *P. aeruginosa* adapts to changes in nutrient availability during infection, primarily as a result of its wide array of metabolic genes.

Non-coding RNA expression during infection. *P. aeruginosa* encodes within its genome numerous non-coding RNAs (ncRNA) involved in gene translation such as ribosomal RNAs and transfer RNAs. Other ncRNAs are involved in post-transcriptional regulation such as *crcZ*, *nalA*, *phrS*, *prfF1*, *prfF2*, *rsmY*, and *rsmZ*^{33–35}. The expression of *crcZ*, *prfF1*, *rsmY* and *rsmZ* was detected both *in vitro* and during infection (RPKM values between 9 and 97,710) (Table S1). The largest number of transcripts detected for a ncRNA in both datasets belonged to *crcZ*. This ncRNA is involved in carbon catabolite repression^{33,36} and was significantly down-regulated during infection (15 fold). The expression of the gene encoding for the RNA chaperone Hfq (−1.4 fold) was also found to be significantly down-regulated during infection (Table S1). Altogether, these data suggest that decreased expression of *hfq* and *crcZ* might play a role in carbon utilization changes observed during infection. *rsmZ* was the smallest ncRNA detected (116 bp), suggesting that the method used in this study for RNA extraction and sequencing allows for detection of small ncRNA expression during infection. However, since the RNA extraction method used in this study is optimal for fragments over the size of 200 bp, specialized purification procedures designed for RNA fragments of smaller sizes would likely enhance the characterization of small ncRNAs in this type of samples.

Expression of virulence factors during infection. *P. aeruginosa* encodes a wide variety of virulence factors for host colonization and to establish infections. As expected, we observed changes in *P. aeruginosa* virulence gene expression during acute murine pneumonia. The expression of genes encoding *P. aeruginosa* type III (T3SS) and type VI (T6SS) secretion systems which translocate effectors across the bacterial cell envelope and the eukaryotic cell membrane was significantly increased *in vivo* (Fig. 2, Table S1). The expression of genes encoding the T3SS secreted effectors ExoS, ExoT, and ExoY was also up-regulated during infection. These two systems are involved in eukaryotic cell targeting and *P. aeruginosa* virulence *in vivo*^{37,38}. It is important to note that T3SS and T6SS are usually known to be inversely controlled. Genes encoding the type I and type II secretion systems were more predominantly expressed *in vitro* (Fig. 2). Other genes encoding virulence factors such as the potent exotoxin A (*toxA*, 5.3 fold) or flagellar biosynthesis (1.62 to 3.55 fold) were up-regulated during infection. Unlike what was observed in burn wound infections¹¹, the expression of the majority of the genes involved in LPS biosynthesis (PA3146-PA3159; 1.55 to 3.51 fold) were also up-regulated during infection. However, multiple genes associated with virulence, which would have been predicted to be expressed during infection, were found to be down-regulated *in vivo*. For instance, the genes encoding the LasR and RhlR systems involved in quorum sensing (QS) and regulation of numerous virulence factors were significantly down-regulated *in vivo* compared to *in vitro* conditions. Not surprisingly, 82 QS-regulated genes identified by Wagner *et al.*³⁹ were also down-regulated, representing 46% of the QS regulon. This set includes genes involved in the biosynthesis of virulence factors such as alkaline protease (−1.67 to −4.70 fold), elastase (*lasB*, −154. fold) and protease IV (*piv*, −2.04 fold) (Table S1). This result might be due to differences in cell density between *in vitro* plate and *in vivo* growth conditions. Other non-QS regulated virulence factors were also down-regulated such as phospholipase C (*plcB*, −14.98 fold), alginate biosynthesis enzymes (−2.47 to −7.8 fold), and phenazines (−7.81 to −107.97 fold)

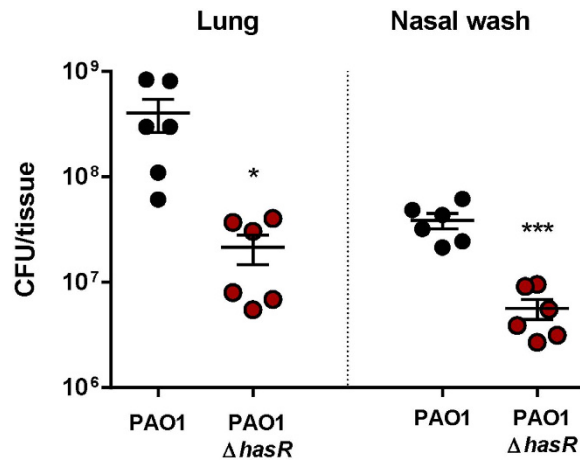


Figure 4. Role of HasR on bacterial burden in mice during acute infection. Mice were infected intranasally with 10^8 CFU of PAO1 Δ hasR and the parental PAO1 strain. Bacterial burden were determined in the nares and lung 16 hours post infection. Lung were resuspended in 1 ml PBS and nasal washes were performed with 1 ml of PBS. Data are represented as number of CFU detected in the total volume of sample. Experiments were performed with 6 mice per group and four technical replicates. Data were analyzed using a two-tailed student *t*-test and the software package Graphpad Prism. Statistically significant differences are denoted with asterisks (* $p < 0.05$; *** $p < 0.0001$).

(Table S1). These genes have been shown to be important for *P. aeruginosa* virulence in various models including keratitis⁴⁰, burn wound infections⁴¹, or chronic respiratory infections^{42,43}. Decreased expression of these virulence factors, together with the changes in T3SS and T6SS expression, could be associated with the down regulation during infection of the two-component system GacA/GacS (−2.0 and −1.5 fold respectively) which controls both acute and chronic virulence determinants such as biofilm formation, T6SS, or exopolysaccharide production^{44–46}. The gene encoding RetS, which controls the planktonic lifestyle and coordinates the production of toxins and the T3SS through the small RNAs *rsmZ* and *rsmY*⁴⁷, was significantly upregulated *in vivo*. Interestingly, the expression of the gene encoding for the lost adherence sensor LadS was not significantly changed during infection. Additionally, increases in expression of both T3SS and T6SS during infection could also be associated with population heterogeneity and changes in expression of other regulatory element, such as the up-regulation of the contact-dependent PpkA (3.13 fold) regulation of T6SS⁴⁴. The results obtained here suggest that some virulence factors might not be required for acute pulmonary infections, but might be expressed in later stages, for instance, in association with biofilms in chronic lung infections^{44,48}.

***M. musculus* host response during infection.** During infection, various host factors come into play to control and eliminate bacterial pathogens. To better understand the expression of these factors, EDGE statistical analysis was used to measure differences in gene expression in the lung of non-infected mice and mice infected with *P. aeruginosa*. The expression of 551 *M. musculus* genes increased and the expression of 151 decreased during infection compared to non-infected animals with a *p* value < 0.001 . Meta-analysis of the data using hyper-geometric tests indicated that 80% of the genes with increased expression during infection were associated with pro-inflammatory response, chemotaxis, response to antigens, stress response, and regulation of cell proliferation. Granulocyte colony-stimulating factor (G-CSF or CSF3) was the most up-regulated eukaryotic gene during infection with a fold change of 706.23 ($p = 8.93 \times 10^{-104}$). The expression of genes encoding other cytokines were also significantly increased during infection, including the pro-inflammatory cytokines IL1, IL8, tumor necrosis factor alfa (TNF- α), interferon gamma (IFN- γ), and CSF2. CXC-motif chemokines (CXCL1, 2, 5, 9, 10 and 16), CC-motif chemokines (CCL2, 3, 4, 5, 7, 9, 11, 17 and 20), and the chemokine receptors IL1R2, IL4R, IL17R, and IL18RAP. The genes encoding FAS and TNFR2 were also highly expressed during infection, together with genes whose products are involved in TNF and NF κ B signaling (Fig. 3, Table S3). These changes in chemokine expression were likely due to LPS and flagellin recognition by the Toll-like receptors (TLR) 2 and 4^{49–53}. The genes encoding CD14 (42.55 fold) and MyD88 (5.54 fold), two proteins involved in signaling through TLR4, had increased expression levels during infection (Fig. 3, Table S3). The expression of the gene encoding TLR2, associated with susceptibility to *P. aeruginosa* infections⁵⁴, was also found to be up-regulated during infection (10.85 fold). These data are in agreement with findings from numerous studies highlighting the importance of Toll-like receptors (TLR) in the recognition of *P. aeruginosa* during infection^{55–59}. Interestingly, while T3SS effectors, such as ExoS have been shown to inhibit the expression of IL-1 β ⁶⁰, the expression of this chemokine was up-regulated *in vivo* (13.69 fold). Altogether, chemokine expression is responsible for leukocyte differentiation, activation and recruitment, the production and release of pro-inflammatory cytokines, and play a major role in host defense against *P. aeruginosa*⁹. These chemokines can also be sensed by *P. aeruginosa* proteins such as OprF and affect gene expression in response to proinflammatory signals⁶¹.

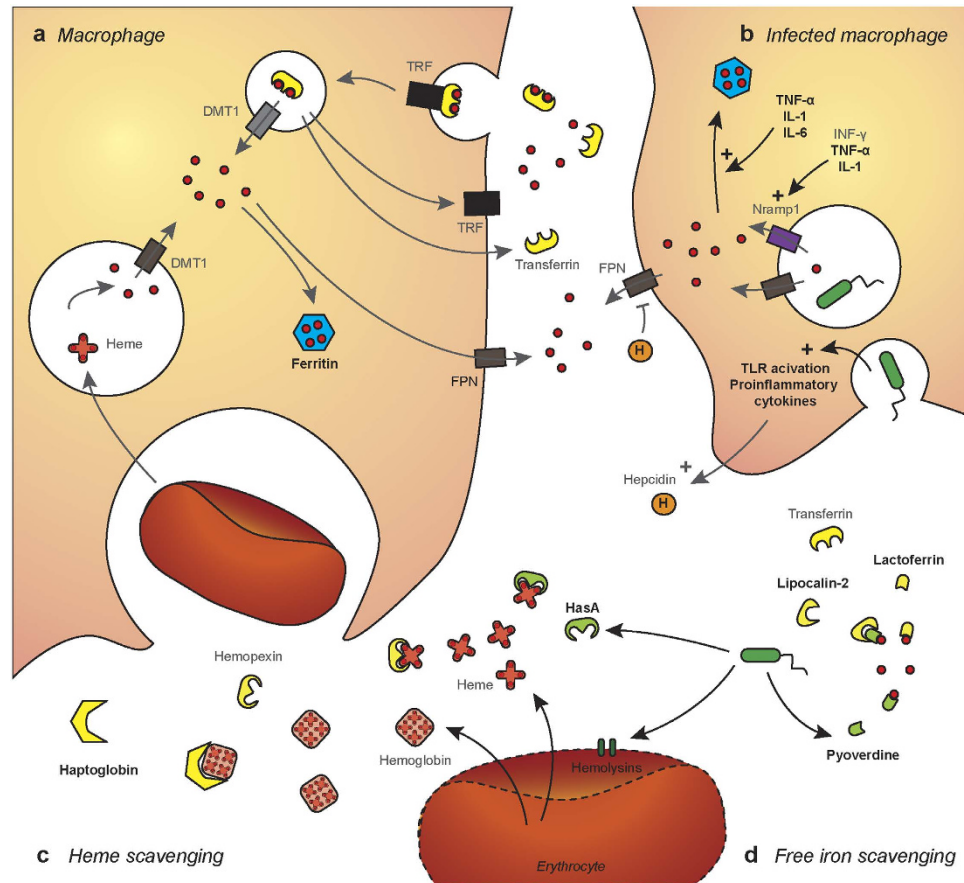


Figure 5. Iron scavenging and regulation *in vivo*. (a) Macrophages acquire iron via transferrin receptor (TRF)-mediated endocytosis of transferrin or via recycling of senescent erythrocytes. Macrophages degrade heme using heme oxygenase to generate iron, biliverdin and carbon monoxide. Iron is then transferred to the cytoplasm by DMT1 and either stored using ferritin or released using ferroportin (FPN). (b) During infection, bacterial recognition by pattern recognition receptors triggers activation of Toll-like receptors (TLR) and secretion of pro-inflammatory cytokines. As a result, cell release hepcidin to control iron release by FPN. Proinflammatory cytokines are also involved in the regulation of Nramp1, which together with FPN deprives internalized bacteria of iron. TNF- α , IL-1 and IL-6 also stimulate the storage of iron using ferritin. (c) During infection, bacteria can lyse erythrocytes using various hemolysins and toxins to release heme and hemoglobin. The host re-captures these iron-rich proteins using haptoglobin and hemopexin, while *P. aeruginosa* secretes hemophores such as HasA for heme scavenging. (d) The host uses lactoferrin and transferrin to limit iron availability during infection. *P. aeruginosa* secretes siderophores with a high iron affinity such as pyoverdine to circumvent iron sequestration. In response, the host releases siderocalin/lipocalin-2 to neutralize bacterial siderophores. Elements that were found to be up-regulated during *P. aeruginosa* infection in the lung are indicated in bold black letters. Elements that were unchanged are shown in grey.

Battle for iron during infection. *P. aeruginosa* iron acquisition during infection. Iron is a vital nutrient involved in a wide range of biological processes and enzymatic functions. In low iron conditions, *P. aeruginosa* uses siderophores such as pyoverdine and pyochelin to chelate free extracellular Fe³⁺^{62–64}. Pyoverdine binds Fe³⁺ to form ferripyoverdine. The *fpvA* gene encodes the ferripyoverdine TonB-dependent receptor (TBDR) FpvA. This receptor is bound to the anti- σ factor FpvR that sequesters the σ factors PvdS and FpvI to the bacterial membrane. Binding of ferripyoverdine to FpvA triggers the proteolysis of FpvR, releasing PvdS and FpvI. These σ factors then associate with the RNA polymerase and drive the expression of *fpvA* and the genes responsible for pyoverdine biosynthesis^{65,66}. In our RNA-seq analysis, the expression of *fpvA* was increased *in vivo* (7.41 fold), while the expression levels of *fpvI*, *fpvR* and *pvdS* were unchanged (Table S1). This result suggests that *in vivo*, ferripyoverdine binding to FpvA triggers the release of PvdS and FpvI. As a result, pyoverdine expression levels are significantly up-regulated during infection (*pvdADEFHJLNOPQRT*), supporting established *in vitro* models^{65,66}. The gene expression of the alternative ferripyoverdine receptor, encoding FpvB, was also up-regulated *in vivo* (4.86 fold) (Table S1). Previous studies have shown the importance of the expression of this system *in vivo*; pyoverdine and FpvA mutants show decreased virulence compared to the parental strain in establishing murine pulmonary infections^{67,68}. The expression of genes used to produce pyochelin, which has a lower affinity for iron than pyoverdine⁶³, were also up-regulated *in vivo*. The *pchABCD* operon was expressed 40 to 50 fold more *in vivo*

than *in vitro* and the genes *pchREFG* were expressed over 20 fold more *in vivo*. *pchA* was found amongst the genes most highly expressed *in vivo* (Table S1).

P. aeruginosa is also able to obtain heme from hemoproteins using the Has and Phu systems^{69,70}. In the Has system, the heme is extracted by the hemophore HasAp, which delivers the iron through the TBDR HasR. In the Phu system, the TBDR PhuR directly extracts the heme. In both pathways, heme is then transported to the cytoplasm by an ABC transporter, bound to a heme chaperone, PhuS, before being delivered to the heme oxygenase HemO⁷¹. In this RNA-seq study, *hasAp* was the most highly expressed gene *in vivo*, with differences in expression of 317.43 fold compared to *in vitro* settings. The genes *hasD* (228.00 fold), *hasE* (177.53 fold), *hasR* (72.16 fold), *phuR* (27.42 fold), *phuS* (15.00 fold), and *hemO* (6.39 fold) were also highly expressed *in vivo* (Table S1). HemO expression was shown to be necessary to drive the metabolic flow of heme into the cell, with PhuS participating in heme sensing and acting as a control valve for heme intake and degradation⁷². Overall, the Has heme acquisition system was the most highly expressed set of genes *in vivo*, suggesting the importance of iron acquisition from heme during infection. To validate these findings and determine the contribution of the Has system to *P. aeruginosa* pathogenesis, 6 weeks old female CD1 mice were infected intranasally with 10⁸ CFU of PAO1Δ*hasR* or the parental strain PAO1. Sixteen hours post infection, mice were euthanized by pentobarbital injection and the bacterial load in the respiratory tract was determined. In this model of acute respiratory infection, the bacterial load of PAO1Δ*hasR* was 18.89 fold lower in the lung, and 6.8 fold lower in the nasal washes than the parental strain (Fig. 4). These data indicate that a mutant lacking the heme receptor HasR was strongly attenuated during infection. Overall, both RNA-seq and animal infection data suggests, as other have hypothesized in the past⁷³, that pyoverdine and the Has/Phu systems are involved in acute respiratory infections and play a major role in *P. aeruginosa* pathogenesis (Fig. 5).

Iron sequestration by the host during infection. One of the first lines of defense against bacterial infection is the sequestration of nutrients such as iron to inhibit bacterial growth. During acute infection, the host decreases iron release into the circulation via hepcidin, increases intracellular iron storage using ferritin, decreases extracellular non-heme iron levels via ceruloplasmin and lactoferrin, and decreases extracellular-heme iron levels using hemopexin and haptoglobin. Vertebrates also produce lipocalin-2, or neutrophil gelatinase-associated lipocalin (NGAL), which is secreted by neutrophils in response to infection, and captures bacterial siderophores. In this study, we observed that RNA levels of genes encoding for the expression of ferritin, ceruloplasmin, lactoferrin and haptoglobin are increased 4 to 20 fold during *P. aeruginosa* acute pneumonia (Table 1). However, no changes were observed in ferroportin, hepcidin, hemopexin, transferrin, or transferrin receptor expression. Additionally, the expression of norepinephrine, which increases iron availability, was decreased. These results seem to indicate that during *P. aeruginosa* infection, the host attempts to sequester iron from the pathogen to limit growth and dissemination (Fig. 5).

Discussion

In this study, we determined the changes in *P. aeruginosa* and host gene expression in the context of an acute murine pneumonia. As expected, we observed that *P. aeruginosa* expressed numerous virulence factors during infection, including T3SS and T6SS. *P. aeruginosa* was found to up-regulate outer membrane vesicle formation and modify its metabolism in response to infection. In the past, we observed that arginine metabolism responded to changes of temperature corresponding to the transition from the environment to the host⁷. In this dataset, we were able to confirm that arginine metabolism gene expression is also increased during infection, suggesting that *P. aeruginosa* adapts its metabolism in response to nutrient availability such as arginine. Overall, there is an important overlap between the data obtained here and gene expression changes observed in acute and chronic burn wounds infections¹¹ (T3SS, T6SS, iron acquisition, amino-acid metabolism). This overlap suggests, as others have hypothesized, that while a core set of virulence genes are required to cause infection, *P. aeruginosa* adaptation to the lung requires the coordinated expression of genes involved in both virulence and metabolism^{11,74}.

Infection with *P. aeruginosa* triggered a strong innate immune response and a cytokine storm mediated by TLR and MyD88 signaling. These data are in agreement with various studies showing that *P. aeruginosa* and outer-membrane vesicles are strong agonists of TLR pathogen-associated molecular pattern receptors^{75–77}. Additionally, we observed that the expression of numerous host proteins involved nutritional immunity⁷⁸ were increased during infection. In particular, proteins participating in iron sequestration were up-regulated during infection, including ferritin, ceruloplasmin, lactoferrin, and haptoglobin. Iron is an essential nutrient required by both mammalian hosts and pathogens, and the race for iron is undoubtedly crucial during infection. In the mammalian host, iron is not freely available and is bound to heme molecules found in hemoproteins or chelated by extracellular proteins such as lactoferrin and transferrin⁷⁹. During infection, *P. aeruginosa* sequesters free iron using siderophores and scavenges iron from heme using the Has and Phu heme acquisition systems. One of the most striking observations in this study was the expression levels of the *P. aeruginosa* Has heme acquisition system during infection (over 300 fold up-regulated compared to *in vitro* conditions for *hasAp*). Pyoverdine and pyochelin siderophores, together with the Phu heme acquisition system were also up-regulated, suggesting that bacteria are starved for iron during infection. Up-regulation of both heme and siderophores systems *in vivo* is likely controlled by the master iron regulator Fur, as no significant changes in expression of the ncRNA *prfF1,2* and *prfH* were observed during infection⁸⁰. Additionally, both HasAp and PhuS could also be involved in heme sensing and might be up-regulated during infection in response to the presence of heme in the lung⁷². Numerous studies have demonstrated the importance of siderophores in bacterial virulence and colonization⁸¹, and authors have shown that pyoverdine production can be detected in the sputum of CF patients⁸². It has also been shown that *P. aeruginosa* interaction with epithelial cells *in vitro* in presence of iron is sufficient to up-regulate pyoverdine acquisition genes⁸³. Siderophores have a high affinity for iron, can displace iron from host NGAL and

Gene name	Description	Fold up-regulated during infection	p-value
Lcn2	Lipocalin 2	30.85	8.34E-04
Ltf	Lactotransferrin	14.34	2.16E-05
Fth1	Ferritin heavy chain 1	6.27	2.33E-03
Cp	Ceruloplasmin	4.82	2.83E-05
Hba-a1	Hemoglobin alpha, adult chain 1	2.11	3.12E-03
Hba-a2	Hemoglobin alpha, adult chain 2	2.96	3.03E-04
Hbb-bs	Hemoglobin beta adult s chain	2.07	1.98E-03
Hbb-bt	Hemoglobin beta adult t chain	2.54	1.89E-04
Steap4	Metalloreductase able to reduce Fe ³⁺ to Fe ²⁺ using NAD ⁺ as receptor	20.64	4.59E-05
Slc39a14	Soluble metal carrier protein	17.85	2.69E-05
Fbx15	F-box and leucin-rich repeat protein 5, iron sensor	5.72	1.65E-05
Picalm	Phosphatidylinositol binding clathri assembly protein, transferrin receptor internalization	3.74	3.45E-05
Scd2	Stearoyl-coenzyme A desaturase 2, iron binding and oxydoreductase activity	-1.9	5.54E-05
Mmp8	Metallopeptidase 8	34.28	2.57E-05
Mmp9	Metallopeptidase 9	13.82	2.28E-05

Table 1. Host iron-associated genes differentially regulated during *P. aeruginosa* infection. Fold changes were calculated by comparing infected to non-infected mice. Fold changes greater than 1 indicate that the gene was up-regulated during infection compared to *in vitro* growth conditions. Fold changes inferior to -1 indicate that the gene was down-regulated during infection compared to *in vitro* growth conditions.

transferrin for iron piracy, and have been shown to also act as toxins⁸⁴. While others have suggested that other iron acquisition systems such as Has and Phu play a role in *P. aeruginosa* pathogenesis⁷³, their use as an alternative iron acquisition system during infection has been underappreciated. The data presented here strongly suggests that heme acquisition by the Has/Phu systems play a crucial role for iron acquisition *in vivo*. Others have shown that iron and heme utilization by *P. aeruginosa* depends on disease progression, and suggested that heme acquisition is characteristic of chronic *P. aeruginosa* infections^{85,86}. Isolates from chronic CF respiratory infections were shown to become more efficient in heme utilization by up-regulating PrrF and HemO expression⁸⁶. In our dataset, heme acquisition also seems to play a major role in acute infection. For this reason, it would be interesting to further define the timing and synergy of heme and iron acquisition systems in *P. aeruginosa* during infection. While siderophores have been used as a therapeutic target^{87,88}, it is crucial to determine the therapeutic potential of heme acquisition inhibitors in *P. aeruginosa*. Seminal studies have paved the way to understand the role of gene expression in *P. aeruginosa* pathogenesis^{11,41,74}. Current advances in technology now allow us to study bacterial pathogenesis from a more systematic approach¹⁹. We anticipate that the methodology described in this study will further our understanding of pathogen-host interactions and be applicable in a wide range of systems to facilitate the development of novel therapeutics.

Experimental procedures. *Bacterial strains and growth media.* *P. aeruginosa* type strain PAO1 was originally obtained from Dr. Michael L. Vasil (University of Colorado). PAO1Δ*hasR* was obtained from Dr. Angela Wilks⁷⁰ (University of Maryland). *P. aeruginosa* was grown in Pseudomonas isolation agar (Becton Dickinson, Franklin Lakes, NJ) for 16h at 37 °C before infection.

Infection model. *P. aeruginosa* PAO1 was grown as described above. Bacteria were swabbed off the plate and resuspended in phosphate buffered saline (PBS) at a concentration of 10¹⁰ colony forming units (CFU) per ml. A fraction of that suspension was centrifuged and resuspended in RNA protect to measure bacterial gene expression *in vitro* before infection. Twenty outbred six weeks old female CD1 mice (Charles River, Frederick, MD) were anesthetized by injection of ketamine and xylazine. Ten microliters of the bacterial suspension were administered to each nostril (10⁸ CFU/mouse). Ten mice were infected with PAO1, and 10 mice were administered PBS vehicle control. Sixteen hours post-infection, mice were euthanized by exposure to carbon dioxide. Lungs were extracted from 4 mice per group and 3 immediately placed in bacterial RNA protect (Qiagen, Germantown, MD) to prevent RNA degradation. The remaining lung was homogenized in PBS and serial dilutions were plated to determine the load in the lung. Sixteen hours post infection, approximately 10⁹ CFU were detected in the lung of each mouse (data not shown). To determine the role of *hasR* on *P. aeruginosa* pathogenesis, mice were infected as described above with 10⁸ CFU of PAO1Δ*hasR* or the parental strain PAO1. After 16 hours of infection, mice were euthanized as described above, and nasal washes and lungs were obtained. Lungs were placed into 1 ml of PBS and homogenized. Nasal washes and lung homogenates were then diluted in PBS, and plated on Lysogeny agar (LA) to determine the number of viable bacteria. Data were analyzed using a two-tailed student *t-test* and the software package Prism 7 (GraphPad, La Jolla, CA). These experiments were performed in accordance with the National Institutes of Health guide for the care and use of laboratory animals. The protocols used were approved by the University of Virginia and West Virginia University Institutional Animal Care and Use Committees.

RNA purification and RNA-seq Illumina library preparation and sequencing. RNA was isolated from bacteria grown *in vitro* on PIA plates using RNeasy Mini Kit (Qiagen) as specified by the instructions of the manufacturer.

Briefly, cells were pelleted by centrifugation at 8,000 g and RNA protect was pipetted off. Cells were lysed using 200 μ l of TE lysozyme for 5 min at room temperature. Cells were then mixed with 700 μ l of RLT and 500 μ l of 70% ethanol. RNA was then applied to RNeasy purification columns and cleaned with RW1 and RPE buffers. RNA was eluted, treated with RNase-free DNase (Qiagen), and cleaned up on another RNeasy Mini column. RNA was isolated from the lung using the same kit with the following modifications: after removing the RNA protect, each lung was homogenized mechanically for 1 min in 667 μ l of TE with 1 mg/ml of lysozyme. A total of 2.33 ml of RLT buffer was then added and tissue was homogenized again for 1 min. Samples were centrifuged at 12,000 g for 10 min and supernatant was transferred to a fresh tube. Five ml of 70% ethanol were added and RNA was purified using 12 RNeasy columns (2 columns per sample). Columns were cleaned with RW1 and RPE buffers, and RNA was eluted. The RNA extracted from the two columns per sample was pooled, treated with RNase-free DNase (Qiagen), and cleaned up using an RNeasy Mini column.

The resulting RNA was quantified on a Nanodrop ND-1000 (Nanodrop, Wilmington, DE). Next, the RNA integrity was assessed using Agilent BioAnalyzer RNA Pico chip (Agilent, Santa Clara, CA). All samples were then submitted to two rounds of eukaryotic and prokaryotic Ribo-zero rRNA depletion (Illumina, San Diego, CA) and reassessed for RNA integrity. rRNA depleted mRNA samples were then fragmented and prepared into libraries using Illumina TruSeq RNA library prep kit v2. Libraries were checked for quality control with KAPA qPCR QC assay (KAPA Biosystems, Wilmington, MA). The libraries were then sequenced on an Illumina MiSeq by the University of Virginia Department of Biology Genomics Core using Illumina MiSeq v3 2 \times 75 bp reads. Three MiSeq lanes were used and each lane contained one biological sample of infected *P. aeruginosa* infected lung, one non-infected lung, and one *P. aeruginosa in vitro* sample. A total of three lanes were used to obtain for 81 million 2 \times 75 bp reads for the three biological replicates of each group. Sequencing data were deposited to the Sequence Read Archive (SRA) and are available under the reference number SRP090213, BioProject number PRJNA343201.

RNA-seq bioinformatics analyses. RNA-seq reads were analyzed using the software CLC Genomics workbench 7.5.1 (Qiagen). The *P. aeruginosa* PAO1 genome and annotations were downloaded from the *Pseudomonas* Genome Database and *Mus musculus* genome was downloaded from NCBI. Reads were mapped against the gene regions of the two genomes using the following settings for mapping: mismatch cost = 2, insertion cost = 3, deletion cost = 3, length fraction = 0.8, similarity fraction = 0.8. RPKM values were generated using default parameters for CLC Genomics. Fold changes in gene expression and statistical analyses were performed using an Extraction of Differential Gene Expression (EDGE) test with the Bonferroni correction⁸⁹.

Metabolic and gene set enrichment analyses. Genes with differences in gene expression with a *p* value < 0.001 were mapped on the Kyoto Encyclopedia of Genes and Genomes using KEGG Mapper v.2.5²⁴. Gene set enrichment analysis (GSEA) tests were performed using CLC Genomic workbench 7.5.1²³. Analyses were performed using gene ontology (GO) terms for biological processes²⁵, with a total of 10,000 permutations. Primary cellular localization of the gene products was obtained from the *P. aeruginosa* PAO1 genome annotation downloaded from the *Pseudomonas* Genome Database. The 300 genes most highly up and down regulated in the *P. aeruginosa* PAO1 gene expression dataset were analyzed and grouped using the STRING 9.1 database of known and predicted interactions²⁶ (string-db.org).

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Author Contributions

M.B. and F.H.D. were equally involved in experimental design, mouse infection and cell harvesting, RNA purification, preparation and next generation sequencing transcriptome analysis. Both authors also participated evenly in the preparation of this manuscript. A.O.-S. and A.W. participated in experimental design and the preparation of this manuscript.

Additional Information

Supplementary information accompanies this paper at <http://www.nature.com/srep>

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