

Omics Analyses of Sulfur Starvation Response in *Pseudomonas aeruginosa*: Linking Oxidative Stress to Iron Homeostasis

by

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Abstract

Inorganic sulfur, primarily in the form of sulfate, is the preferred sulfur source utilized by bacteria. However, sulfate availability in terrestrial environments is severely limited, comprising less than 1–5% of the total available sulfur, with the majority existing in organic (organosulfur) forms. Consequently, soil bacteria have evolved strategies to utilize a diverse range of organosulfur compounds. Previous nutritional studies have established that sulfate limitation triggers extensive metabolic reprogramming characterized predominantly by the upregulation of sulfur-scavenging genes. Particularly, sulfur deprivation also consistently induces the expression of antioxidant genes, yet the underlying mechanisms remain poorly understood.

To address this knowledge gap, we employed an integrative omics approach combining RNA sequencing (RNA-seq), label-free proteomics, fluorometric assays, and computational network inference analyses, using the opportunistic pathogen *Pseudomonas aeruginosa* PAO1 cultured in sulfur-free media (SFM) as our experimental model. As expected, RNA-seq and proteomics datasets revealed pronounced overexpression of genes and proteins associated with scavenging and assimilation of organosulfur compounds (*ssu*, *msu*, and *sfn* operons), alongside antioxidant genes encoding organic hydroperoxide resistance protein (Ohr), 1-Cys peroxiredoxin (LsfA), and iron-dependent superoxide dismutase (SodB).

Surprisingly, we uncovered a previously unreported response: sulfur starvation significantly downregulated numerous iron-acquisition pathways, including siderophore biosynthesis (pyoverdine, pyochelin operons), heme uptake clusters (*has*, *phu*), and genes related to xenosiderophore transport. Concomitantly, sulfur-deprived cells displayed significant

upregulation of iron-storage proteins (BfrB, Dps, PA4880), indicative of an Fe-replete cellular state. Quantitative Fe measurements using inductively coupled plasma optical emission spectroscopy (ICP-OES) demonstrated a significantly diminished iron uptake capacity in sulfur-starved cells, aligning with our omics findings. Complementary quantitative HPLC analyses further corroborated these results by revealing decreased extracellular concentrations of pyoverdine siderophore.

To understand the oxidative stress dynamics under sulfur deprivation, we employed fluorometric reactive oxygen species (ROS) assays using 2',7'-dichlorofluorescein diacetate (DCFH-DA), revealing a sustained, time-dependent increase in ROS levels in sulfur-starved cells. Additionally, the assessment of intracellular labile Fe using Calcein-AM fluorescence demonstrated a biphasic response, with an initial rise in intracellular Fe at early starvation (2.5 h), followed by a notable decline below control levels at later time points (5–7.5 h). While initially perplexing, this biphasic iron response is consistent with the physiological adaptations observed, namely downregulated iron uptake and increased iron sequestration, which collectively diminish intracellular unincorporated Fe levels over time. Time-course proteomics provided additional insights, demonstrating a progressive, sustained elevation of antioxidant proteins (Ohr, LsfA, SodB), and Fe-storage proteins (BfrB, PA4880, PA0962), underscoring their crucial roles in mitigating oxidative damage through prolonged sulfur limitation.

To complement our experimental findings, we conducted a computational analysis to elucidate the regulatory circuitry underpinning the sulfur starvation response. This approach was necessitated by the observed differential expression of key regulatory elements, including siderophore biosynthesis regulators (*pvdS*), and the iron-responsive small RNAs (PrrF). Leveraging our transcriptomic dataset, we employed GENIE3, a machine-learning-based network

inference tool, to construct gene regulatory networks aimed at identifying key transcription factors and regulatory hubs involved in mediating the sulfur deprivation response. Our computational analyses identified MexL as a putative regulatory hub, potentially orchestrating gene expression changes associated with sulfur limitation. Although the predictive accuracy of our inferred network was constrained by the limited sample size and suboptimal precision-recall metrics, MexL emerged as a biologically relevant candidate due to its well-established regulatory roles. Specifically, MexL is known to influence phenazine biosynthesis pathways and the expression of the MexJK-OprM efflux pump system, both significantly altered under sulfur-starved conditions in our omics analyses. Therefore, despite methodological limitations, the identification of MexL as a central regulator is both plausible and biologically compelling. Nonetheless, these computational predictions should be interpreted cautiously and require rigorous experimental validation.

Overall, our integrative analyses support a model wherein sulfur deprivation triggers Fe dyshomeostasis in *Pseudomonas aeruginosa*, initially elevating intracellular Fe pools and subsequently activating Fe-storage mechanisms to mitigate Fe-induced oxidative stress. This adaptive response offers a mechanistic explanation for the induction of antioxidant genes consistently observed during sulfate limitation, shedding light on a longstanding puzzle regarding oxidative stress responses under sulfur scarcity. Additionally, our study also reveals that sulfur starvation significantly suppresses multiple virulence determinants, including quorum-sensing regulators, RND-type efflux pumps, phenazine biosynthetic enzymes, and hydrolytic proteases, many of which are established targets of the ferric uptake regulator (Fur) and iron-responsive PrrF small RNAs.

In conclusion, our findings position sulfur availability as a central modulator of Fe metabolism, oxidative stress responses, and virulence gene expression in *P. aeruginosa*. These

mechanistic insights significantly advance our understanding of bacterial adaptation to sulfur-limiting environments and highlight sulfur metabolism as a promising therapeutic target. Exploiting bacterial sulfur dependency could represent an innovative nutritional immunity strategy, potentially addressing limitations associated with traditional Fe-targeted interventions that inadvertently risk enhancing bacterial virulence.

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List of Abbreviations

ABC – ATP-Binding Cassette

ANOVA – Analysis of Variance

APS – Adenosine 5'-Phosphosulfate

BfrB – Bacterioferritin B

CF – Cystic Fibrosis

CFTR – Cystic Fibrosis Transmembrane Conductance Regulator

CoA – Coenzyme A

CPM – Counts Per Million

Cys – Cysteine

DAVID – Database for Annotation, Visualization and Integrated Discovery

DCF – 2',7'-Dichlorofluorescein

DCFH-DA – 2',7'-Dichlorodihydrofluorescein Diacetate

DEG – Differentially Expressed Genes

DFO – Deferoxamine

DMS – Dimethyl Sulfide

DMSO – Dimethyl Sulfoxide

DMSO₂ – Dimethyl Sulfone

DNA – Deoxyribonucleic Acid

DTPA – Diethylenetriaminepentaacetic Acid

DTT – Dithiothreitol

ECF – Extracytoplasmic Function Sigma Factors

EN – Entner–Doudoroff Pathway

ESKAPE – *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter species*

Fe – Iron

Fe-S – Iron–Sulfur cluster
FMNH₂ – Reduced Flavin Mononucleotide
FpvA – Ferripyoverdine Receptor A
FpvI – Ferripyoverdine Sigma Factor I
FN – False Negatives
FP – False Positives
Fur – Ferric Uptake Regulator
GENIE3 – GENE Network Inference with Ensemble of Trees
GO – Gene Ontology
GRN – Gene Regulatory Network
GSH – Reduced Glutathione
H₂O₂ – Hydrogen Peroxide
HPLC – High-Performance Liquid Chromatography
ICP-OES – Inductively Coupled Plasma–Optical Emission Spectroscopy
IM – Inner Membrane
KEGG – Kyoto Encyclopedia of Genes and Genomes
LFQ – Label-Free Quantification
MBR – Match Between Runs
MS/MS – Tandem Mass Spectrometry
NADPH – Nicotinamide Adenine Dinucleotide Phosphate (Reduced)
ncRNA – Non-Coding RNA
NGS – Next-Generation Sequencing
OM – Outer Membrane
P2TF – Predicted Prokaryotic Transcription Factor Database
PBS – Phosphate-Buffered Saline
PCH – Pyochelin
PCN – Phenazine-1-Carboxamide
PCA – Phenazine-1-Carboxylic Acid
PPI – Protein–Protein Interaction
PP – Pentose Phosphate

PVD – Pyoverdine
PYO – Pyocyanin
pvdI – Pyoverdine Biosynthesis Gene
qPCR – Quantitative Polymerase Chain Reaction
RCF – Relative Centrifugal Force
RNA – Ribonucleic Acid
RNA-seq – RNA Sequencing
RNS – Reactive Nitrogen Species
ROS – Reactive Oxygen Species
SC – Sulfur-Containing
SF – Sulfur-Free
SFM – Sulfur-Free Media
sRNAs – Small Regulatory RNAs
SOD – Superoxide Dismutase
TCA – Tricarboxylic Acid
TF – Transcription Factor
TFA – Trifluoroacetic Acid
TMM – Trimmed Mean of M-values
UHPLC – Ultra-High-Performance Liquid Chromatography

INTRODUCTION

***Pseudomonas aeruginosa*: a versatile and clinically significant pathogen**

Pseudomonas aeruginosa is a highly adaptable Gram-negative bacterium found in a broad range of environments, including soil, water, and both human and plant hosts.^{1,2} Clinically, it is recognized as an opportunistic pathogen responsible for severe infections in immunocompromised individuals, such as patients with cystic fibrosis (CF), burn wounds, cancers, and those undergoing invasive medical procedures.^{3,4} In CF lungs, *P. aeruginosa* establishes chronic infections that contribute substantially to patient morbidity and mortality.⁵ The bacterium's innate resistance to multiple antibiotics and ability to thrive under diverse conditions underscore its importance as a public health concern.^{6,7} Due to its resistance to conventional antibiotics, *P. aeruginosa* is classified among the ESKAPE pathogens, which are a group of bacteria that are predominant causes of nosocomial infections worldwide and exhibit significant antimicrobial resistance.⁸ *P. aeruginosa*'s pathogenicity arises from a suite of virulence factors, including adhesive structures (pili, fimbriae), exotoxins, degradative enzymes, and the capacity to form biofilms, all of which confer protection against host immune responses and antibiotic treatments.^{9,10}

The biological importance and biogeochemistry of sulfur

Sulfur is an essential macronutrient integral to a wide range of cellular processes in all living organisms. It is a key component of sulfur-containing amino acids (cysteine and methionine), vitamins (thiamine and biotin), coenzymes (coenzyme A and lipoic acid), and iron-sulfur (Fe-S) clusters critical for electron transport and enzymatic reactions.^{11,12} Sulfur's unique ability to cycle between oxidation states from -2 to +6 underpins its vital role in redox reactions,

thereby contributing to cellular redox balance and metabolic versatility.^{13,14} This redox versatility is reflected in molecules such as glutathione, a cysteine-containing tripeptide that scavenges reactive oxygen species (ROS) and helps maintain redox homeostasis.¹⁵

In natural environments, sulfur undergoes a dynamic biogeochemical cycle encompassing mineralization, oxidation, and reduction processes mediated by microorganisms.¹⁶ Microbial assimilation of inorganic sulfate and its subsequent reduction to sulfide, followed by incorporation into organic molecules, is a central component of this cycle.¹² When inorganic sulfate becomes limited, many terrestrial bacteria can switch to alternative sulfur sources, including sulfonates or sulfate esters, by inducing specialized transporters and enzymes^{17,18} involved in the desulfurization of these compounds. This metabolic flexibility is finely tuned by regulatory proteins sensing intracellular sulfur levels,¹⁹ enabling bacteria to adapt to limiting environmental sulfate supplies.

***Pseudomonas aeruginosa* as a model for sulfur metabolism studies**

Several features make *P. aeruginosa* an excellent organism for exploring sulfur metabolism: its clinical relevance and remarkable metabolic flexibility owing to its comparatively large genome (~6.3 Mb).²⁰ *P. aeruginosa* encodes numerous transporters, regulatory proteins, and enzymes that confer remarkable metabolic versatility, enabling it to utilize an extensive array of carbon substrates and nitrogen sources.^{21–23} Studies have shown that *P. aeruginosa* can utilize organosulfur substrates, including taurine, thiocyanates, cyclamates, and alkanesulfonates, when inorganic sulfate, the preferred sulfur source, is unavailable.^{24–26} However, these studies essentially focused on the effect of sulfate limitation, or the organism's capacity to utilize organosulfur compounds, rather than the effects of complete sulfur starvation. Moreover, the regulatory pathways governing sulfate limitation and organosulfur utilization in *P. aeruginosa* are poorly understood. Most available insights come from studies in *Escherichia coli*, which identified

transcription factors like CysB and Cbl as key regulators of sulfate starvation-induced (SSI) gene expression.^{18,27,28} Consequently, understanding how the SSI response is orchestrated in *P. aeruginosa* remains an important research endeavor.

In addition, sulfur metabolism potentially intersects with virulence in *P. aeruginosa*: sulfur-containing molecules, including S-adenosyl methionine, are crucial for synthesizing quorum-sensing autoinducers like homoserine lactones, which govern biofilm formation and virulence gene expression.²⁹ Interference with sulfur assimilation can, therefore, potentially compromise bacterial virulence, pointing to sulfur metabolism as a potential target for novel antimicrobial strategies.

Harnessing the power of high-throughput omics to understand bacterial sulfur metabolism

Omics technologies, encompassing genomics, transcriptomics, proteomics, and metabolomics, have significantly enhanced our ability to explore complex biological systems.³⁰ Such high-throughput methods enable comprehensive mapping of gene expression, protein abundance, and metabolite changes under specific physiological or environmental conditions.³¹

Transcriptomics using RNA sequencing (RNA-seq) identifies differentially expressed genes, proteomics provides insight into protein-level changes and post-translational modifications, and metabolomics reveals shifts in metabolic fluxes.^{32–34} By combining these datasets, researchers can build regulatory networks that pinpoint key transcription factors (TFs) orchestrating gene expression responses.³⁵ Moreover, integrative omics approaches can offer a powerful tool to examine how bacteria, including *P. aeruginosa*, detect and respond to changes in sulfur availability by capturing global changes in gene expression, protein levels, and metabolic fluxes. Some recent multi-omics studies on *P. aeruginosa* have focused on respiratory infection models, the sensitivity

of multidrug-resistant (MDR) strains to polymyxin–rifampicin, or the metabolic advantages conferred by plasmids through horizontal gene transfer.^{36–38} However, to the best of our knowledge, no investigation has examined the impact of sulfur starvation using a comprehensive multi-omics framework. Hence, sulfur metabolism in *P. aeruginosa* remains understudied at the systems level. A few decades-old study in the area relied on relatively low-resolution methods such as protein gel electrophoresis²⁴ and microarray-based transcriptome profiling²⁶ to explore aspects of sulfur metabolism under sulfate-limited conditions in *P. aeruginosa*. Additionally, some of these investigations focused only on a few genes or pathways connected to sulfur metabolism,^{39,40} without capturing the broader regulatory interactions that might influence the organism’s adaptive capacity. Using modern, high-resolution multi-omics strategies, insights can be gained on how *P. aeruginosa* coordinates global gene expression to cope with sulfur scarcity.

Research question: Mechanisms underlying sulfur starvation-induced oxidative stress

Sulfur-starved bacteria, including *P. aeruginosa*, upregulate SSI genes involved in sulfur acquisition.¹² Intriguingly, sulfate limitation also prompts the expression of antioxidant genes, including *ahpC*, *katB*, and *lsfA*—implying a potential connection between sulfur metabolism and cellular redox equilibrium.^{24,25,41} One proposed mechanism involves the accumulation of reduced flavin mononucleotide (FMNH₂) in the absence of suitable substrates for monooxygenases, leading to ROS generation by autoxidation.^{26,41} However, a recent structural biology investigation of *P. aeruginosa* alkanesulfonate monooxygenase revealed that the monooxygenase prevents FMNH₂ autoxidation through conformational shielding, suggesting that additional factors underlie the observed oxidative stress during sulfur starvation.⁴²

Additionally, previous studies in *E. coli* indicate a close interplay between disrupted Fe-S cluster assembly and elevated reactive oxygen species (ROS) levels, ultimately leading to an

accumulation of free Fe. This surplus iron can further exacerbate oxidative stress through Fenton-like reactions.^{43,44} Therefore, understanding the consequences of sulfur deficiency on these processes can elucidate the link between bacterial sulfur stress physiology and antioxidant response.

Objectives and hypothesis

Central hypothesis

This research tested the hypothesis that the ROS response observed during sulfur starvation in *P. aeruginosa* is, at least partly, the result of disrupted iron (Fe) metabolism. Since sulfur is critical for Fe-S cluster assembly,^{11,45} its deficiency will potentially lead to Fe-S cluster disruption, excessive free Fe, and enhanced ROS production.^{46,47}

Objective 1: Cataloging sulfate starvation-induced (SSI) genes

By applying transcriptomic (RNA-seq) and proteomic approaches, we profiled the global changes in gene and protein expression in *P. aeruginosa* during sulfur starvation. We confirmed the upregulation of SSI genes while identifying previously uncharacterized repression of Fe uptake pathways (including siderophores, heme-based transport, and Feo systems) and concurrent induction of Fe storage genes. Fe-dependent virulence pathways, such as PQS and phenazine biosynthesis, were similarly downregulated, suggesting a link between sulfur starvation and reduced pathogenicity.

Objective 2: Elucidating the connection between Fe metabolism and the SSI oxidative stress response

To determine how sulfur depletion influences Fe homeostasis and ROS generation, we measured intracellular and extracellular Fe concentrations using inductively coupled plasma optical emission spectroscopy (ICP-OES), monitored the labile Fe pool and ROS generation via fluorescence-based assays, and tracked key Fe-associated metabolites (siderophores and phenazines) using high-performance liquid chromatography (HPLC). Time-course proteomic data focusing on antioxidant enzymes (e.g., catalases, peroxidases) and Fe-storage proteins allowed us to correlate sulfur availability, Fe dysregulation, and the activation of ROS defenses. The results established a link: sulfur starvation triggered a transient Fe overload in the cells, which potentially acts as an initial trigger for ROS generation.

Objective 3: Identifying Transcription Factors Governing the SSI ROS Response

We integrated multi-omics datasets using computational gene regulatory network analysis, leveraging various bioinformatics tools and databases, including GENIE3, STRINGDB, and Cytoscape. While Fur (ferric uptake regulator) and OxyR (oxidative stress regulator) are well-known transcription factors involved in Fe and oxidative stress responses, we identified (albeit with low confidence) additional transcription factors that may play essential roles in modulating sulfur and iron homeostasis under sulfur limiting conditions. These implicated regulators offer potential targets for further investigation into how *P. aeruginosa* orchestrates complex stress responses and virulence under sulfur limitation.

Significance of the study

This study combines high-throughput omics methodologies to provide a comprehensive view of how *P. aeruginosa* adapts to sulfur starvation. It reveals a mechanistic link between sulfur availability, iron homeostasis, and oxidative stress. This integrative approach enriches fundamental insights into bacterial physiology and highlights metabolic vulnerabilities that could be therapeutically exploited. Moreover, identifying the metabolic interplay between sulfur and iron offers a strategic angle for attenuating *P. aeruginosa* virulence, particularly where multidrug resistance complicates clinical management.^{48,49}

These findings illuminate how *P. aeruginosa* balances nutrient acquisition and redox homeostasis during sulfur limitation and underscore the broader relevance of sulfur-iron interactions in bacterial pathogenesis. Further exploration of these pathways could pave the way for innovative therapeutic approaches that disrupt nutrient sensing and metabolic regulation in persistent, drug-resistant bacteria species.

CHAPTER ONE: Literature Review

1.1 Introduction to Sulfur Metabolism in Bacteria

1.1.1 An overview of sulfur Biochemistry

Sulfur is a macronutrient that constitutes up to 1% of the dry mass of bacterial cells, underscoring its importance for growth and survival.⁵⁰ It is integral to various biochemical processes, primarily through its incorporation into sulfur-containing amino acids, vitamins, and cofactors. Cysteine and methionine, the two sulfur-containing amino acids, are fundamental building blocks for proteins, playing critical roles in maintaining protein structure and function.¹² Beyond their structural roles, these amino acids are involved in enzymatic catalysis, redox reactions, and signal transduction pathways.⁵¹

Sulfur's versatility is further demonstrated by its presence in various cofactors. For instance, thiamine (vitamin B₁) and biotin (vitamin B₇) contain sulfur within their molecular structures. In thiamine, sulfur is part of the thiazole ring, stabilizing the structure required for its role in decarboxylation reactions mediated by thiamine pyrophosphate. In biotin, sulfur is a component of the tetrahydrothiophene ring, which maintains the cofactor's conformation for carboxylation reactions in fatty acid synthesis.^{52,53} More directly, coenzyme A (CoA) and lipoic acid rely on sulfur for their biochemical functions. Coenzyme A contains a thiol (-SH) group, which is crucial for its role in acyl-group transfer reactions.⁵³ Lipoic acid, another sulfur-containing cofactor, functions in oxidative decarboxylation reactions as part of the pyruvate dehydrogenase complex and similar enzyme systems, where its disulfide bonds participate in electron transfer and redox balance.⁵⁴

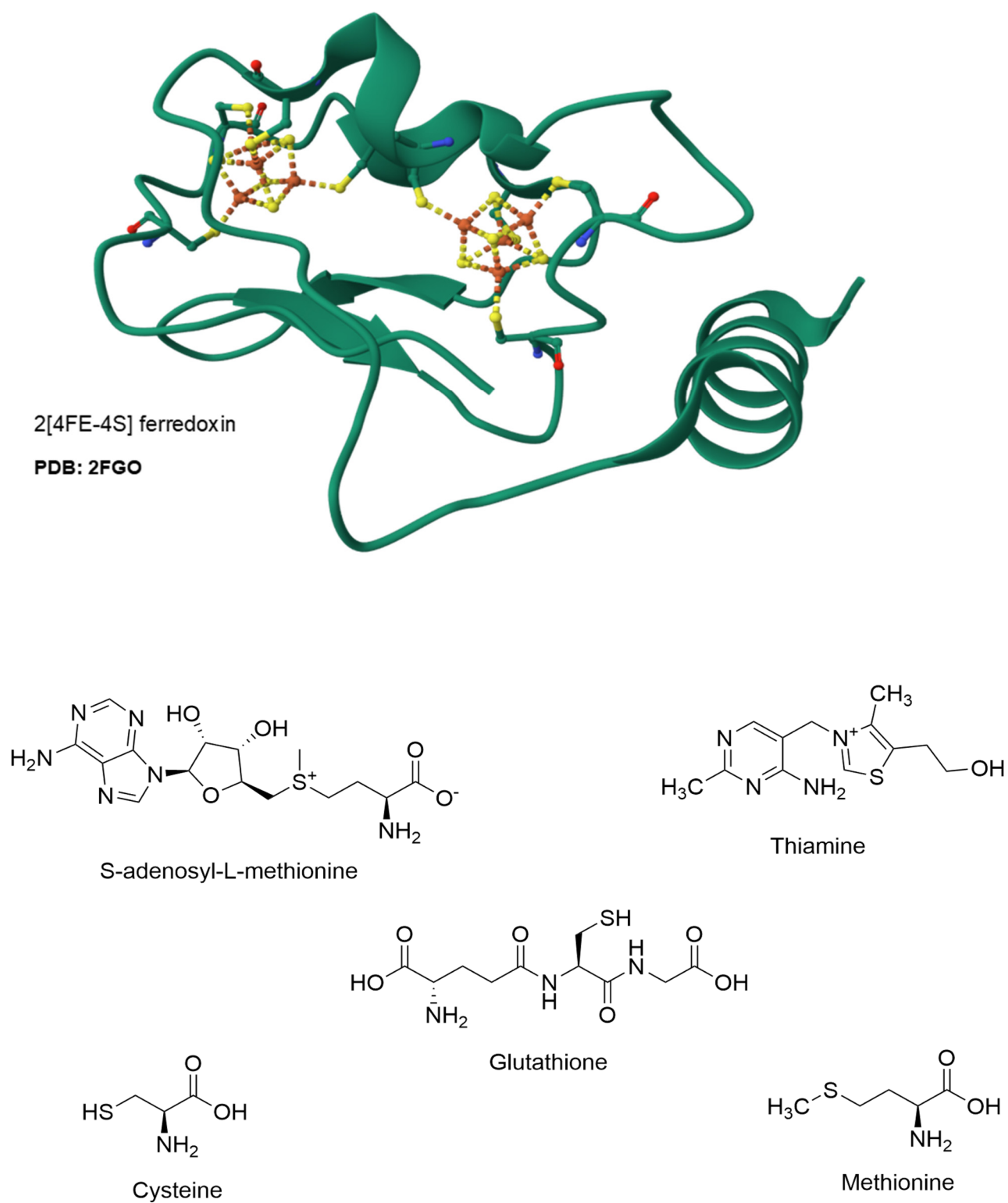


Figure 1.1. Structures of common biological molecules containing sulfur

1.1.2 Sulfur-containing compounds in Bacteria

In bacteria, sulfur is incorporated into various compounds that are essential for cellular function. Sulfur-containing amino acids, cysteine and methionine, serve as precursors for other critical sulfur-containing molecules. For instance, cysteine is a precursor for glutathione, a major antioxidant that protects cells from oxidative damage by neutralizing reactive oxygen species (ROS).¹⁵ Additionally, cysteine is required in the formation of disulfide bonds, which are important for stabilizing protein tertiary structures.

Moreover, sulfur is a key component of iron-sulfur (Fe-S) clusters, which are essential cofactors in enzymes involved in electron transport, DNA repair, and metabolic regulation.^{11,45} These Fe-S clusters facilitate electron transfer in processes such as respiration and photosynthesis, highlighting sulfur's role in energy metabolism and redox balance.⁴⁷

1.1.3 Bacteria Acquisition and Assimilation of Sulfur

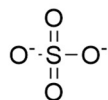
Bacteria have evolved mechanisms to acquire and assimilate sulfur from their environment, ensuring an adequate supply of the nutrient even under limiting conditions. The primary source of sulfur for most terrestrial bacteria is inorganic sulfate (SO_4^{2-}), which is actively transported into the cell through high-affinity sulfate transport systems.^{17,24}

In terrestrial environments, however, the most abundant forms of sulfur available (> 95%) are as substituents or functional groups in organic compounds. These include sulfur-containing amino acids, dimethylsulfide (DMS), and its oxidation products—dimethyl sulfone (DMSO_2) and methanesulfonate (MS), along with other anthropogenic organosulfur compounds (Figure 1.2).^{12,39} In contrast, inorganic sulfate is typically limited. When inorganic sulfate is not present in sufficient quantities to support demand, most bacteria can utilize alternative sulfur sources, such as

organosulfonates and sulfate esters, by inducing specific assimilation and desulfurization pathways.^{17,18}

These pathways involve specialized transporters and enzymes that desulfurize organosulfur compounds into usable inorganic forms. For example, the *ssu* (sulfonate-sulfur utilization) operon encodes proteins responsible for the uptake and desulfonation of alkanesulfonates, providing sulfur for assimilation.⁵⁵ Key enzymes in these pathways include monooxygenases and dioxygenases that catalyze the oxidative cleavage of organosulfur compounds, releasing sulfite or sulfide for incorporation into an organic backbone.²⁴ These enzymes are often regulated by sulfate availability, with transcription factors such as CysB, Cbl, and SfnR modulating the expression of sulfur assimilation and scavenging genes in response to sulfur levels.^{27,40}

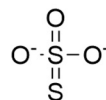
Inorganic sulfur sources



sulfate

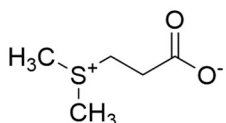


sulfite

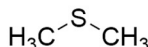


thiosulfate

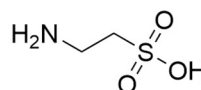
A select group of naturally occurring organosulfur compounds



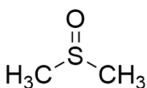
DMSP: dimethylsulfoniopropionate



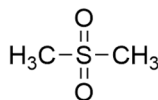
DMS: dimethyl sulfide



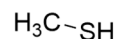
Taurine



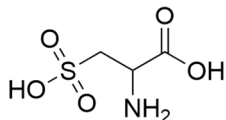
DMSO: dimethyl sulfoxide



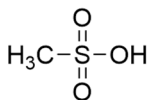
DMSO₂:dimethyl sulfone



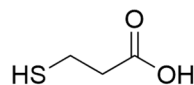
MeSH: methanethiol



cysteate

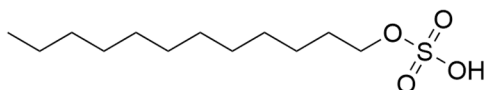


MSA: methanesulfonic acid

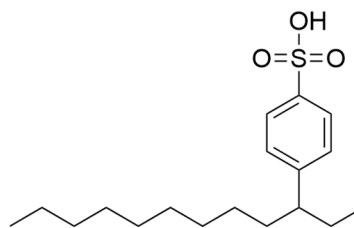


MPA: 3-Mercaptopropionic acid

Xenobiotic organosulfur compounds



Dodecyl sulfate



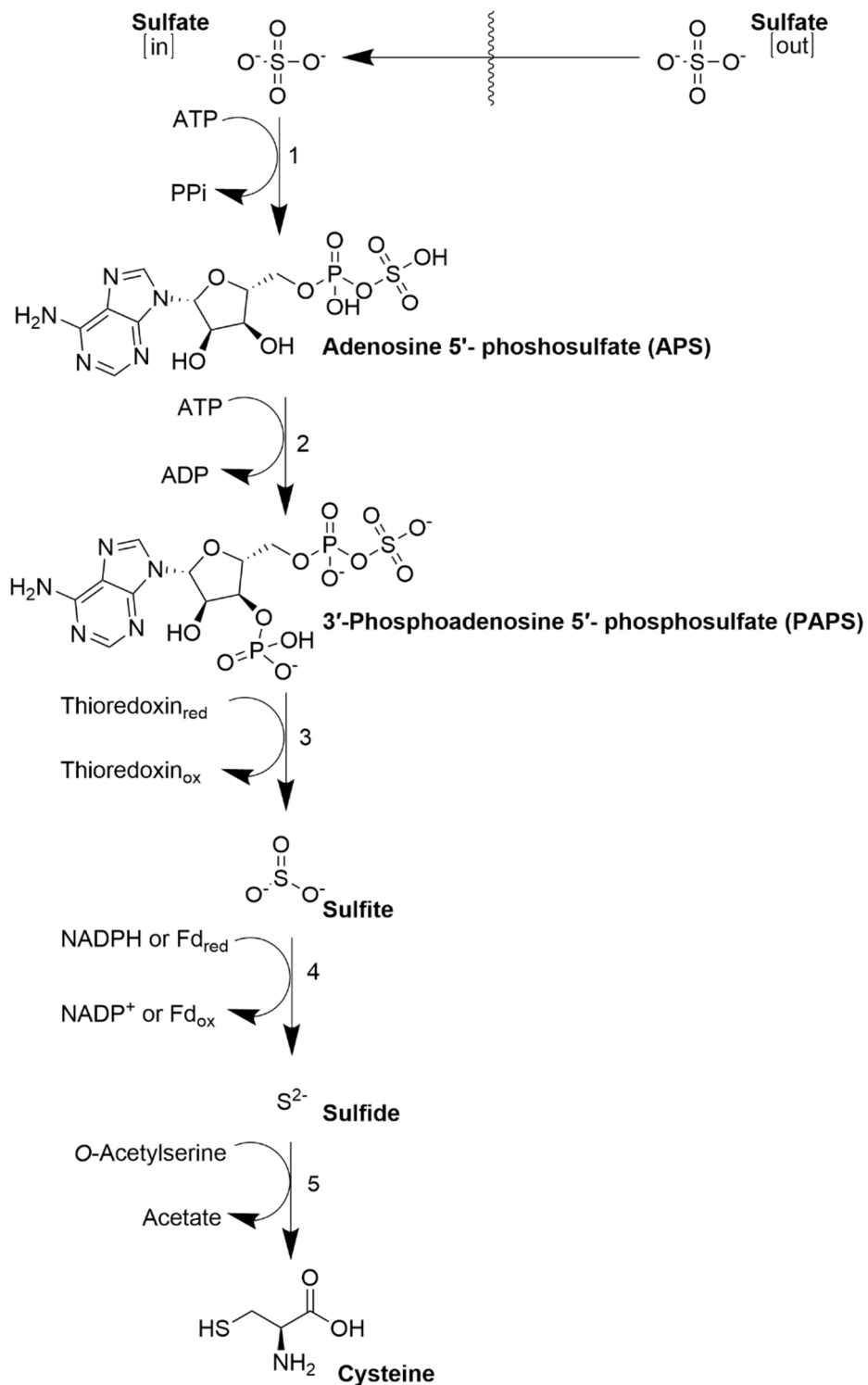
LAS: p-(3-dodecyl)benzenesulphonic acid

Figure 1.2: Natural and anthropogenic sources of entry for sulfur into bacteria metabolism

1.1.4 Sulfur Acquisition in *Pseudomonas aeruginosa*

P. aeruginosa stands out among bacteria for its remarkable metabolic flexibility, contributing to its adaptability and pathogenicity. This metabolic plasticity is primarily attributed to its relatively large genome (~6 Mbp). By comparison, the genome for a common strain of *E. coli* (e.g., K-12) is 4.6 Mbp. This larger genetic capacity encodes an extensive array of transporters, regulatory proteins, and metabolic enzymes.²⁰ This genetic repertoire allows *P. aeruginosa* to utilize a wide variety of carbon, nitrogen, and sulfur sources, enabling it to thrive in diverse ecological niches and host tissues.^{21,23,56}

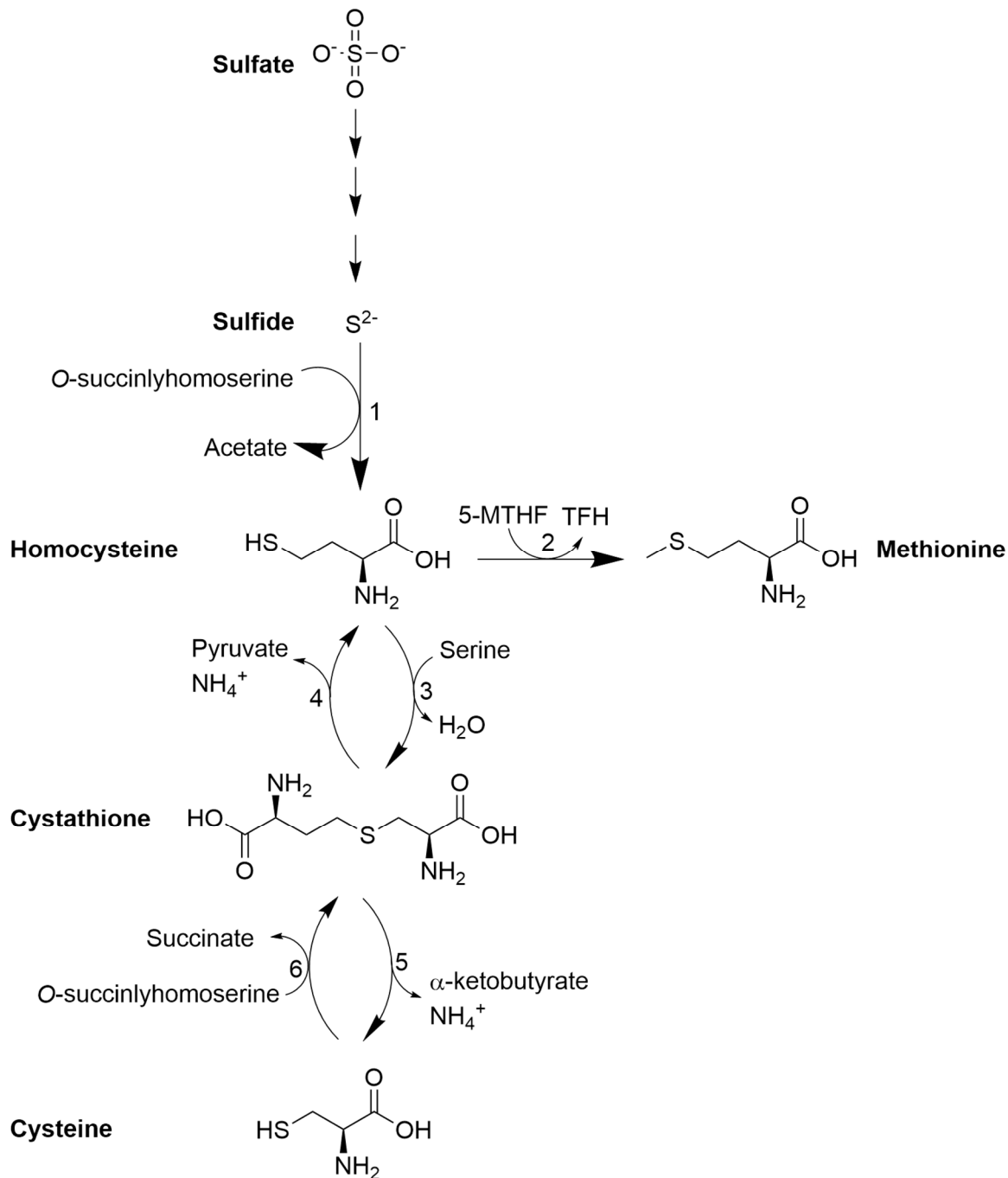
When sulfate, the organism's preferred sulfur source, is available, the assimilation process begins with the sulfate activation by ATP sulfurylase (Figure 1.3), which converts sulfate into adenosine 5'-phosphosulfate (APS). This intermediate is then sequentially reduced to sulfite and subsequently to sulfide, following a pathway analogous to that in other enteric bacteria, including *E. coli*. In *E. coli*, sulfide is typically incorporated into *O*-acetylserine, derived from serine, by the action of *O*-acetylserine sulfurylase, producing cysteine.¹²



1. ATP sulfurylase 2. APS kinase 3. PAPS reductase 4. Sulfite reductase 5. O-acetylserine sulfhydrylase

Figure 1.3: Overview of sulfur assimilatory pathway in bacteria. Adapted from Grein et al.⁵⁷

In contrast, the pathway in *P. aeruginosa* diverges at the point of sulfide assimilation. Rather than transferring sulfide to *O*-acetylserine, *P. aeruginosa* predominantly utilizes *O*-succinylhomoserine (generated from homoserine) as the acceptor for sulfide (Figure 1.4). The reaction yields homocysteine, which is then metabolized via one of two routes: a one-step methylation reaction that produces methionine or a two-step transsulfuration pathway involving cystathionine- β -synthase and cystathionine- γ -lyase, converting homocysteine to cysteine.^{58,59} This divergence in sulfur assimilation illustrates the organism's metabolic diversity and marks a significant evolutionary departure from the canonical pathways observed in enteric bacteria.



1. O-succinylhomoserine sulfhydrylase 2. methionine synthase 3. cystathionine β -synthase 4. cystathionine β -lyase 5. cystathionine γ -lyase 6. cystathionine γ -synthase.
 5-MTHF: 5-Methyltetrahydrofolate ; MTHF: Tetrahydrofolate

Figure 1.4: *P. aeruginosa* sulfate assimilation pathway

1.2 Sulfur Starvation Response in Bacteria

During sulfur limitation, bacteria activate coordinated regulatory and metabolic responses to scavenge sulfur, including utilizing organosulfur sources,¹² these genes, as alluded to above, are loosely called sulfate starvation-induced (SSI) genes.

In *E. coli*, in which sulfur metabolism has been more extensively studied, sulfur starvation induces the *cysteine regulon* governed by the LysR-type regulator CysB,²⁷ which works in concert with the small molecule N-acetyl-L-serine (NAS).⁶⁰ When sulfur (e.g. sulfate or cysteine) is scarce, NAS accumulates from the cysteine biosynthetic pathway and serves as an inducer for CysB, leading to robust transcriptional activation of genes for sulfate uptake, reduction, and cysteine synthesis.⁶¹ This allows *E. coli* to increase the import of sulfate (via ABC transporters like CysTWA and the periplasmic binding protein Sbp) and to increase the enzymatic reduction of sulfate to sulfide and incorporation into cysteine.⁶² In addition to CysB, *E. coli* employs a second regulator, Cbl (a CysB paralog), to expand its sulfur-scavenging capacity.⁶³ The CysB/Cbl regulon includes not only the conventional sulfate assimilation genes but also pathways for utilizing alternative sulfur sources, including, for instance, taurine degradation genes (*tauABCD*)²⁷ and the alkanesulfonate utilization operon (*ssuEADCB*), induced to scavenge organosulfur compounds.⁶⁴ These regulatory mechanisms collectively enable *E. coli* to scavenge sulfur from terrestrial environments that are often deficient in inorganic sulfur forms. Notably, the activation of the CysB/Cbl regulon in *E. coli* induces a coordinated, global response in which most genes involved in sulfur assimilation are upregulated during sulfur limitation.⁴¹

1.2.1 Sulfur Starvation Response in *Pseudomonads*

Pseudomonas aeruginosa exhibits a complex metabolic response to sulfate limitation compared to *E. coli*, though fewer studies have specifically investigated sulfur limitation in this organism. Therefore, this discussion broadly covers findings from the genus *Pseudomonas*, which includes *P. aeruginosa* but also covers data from other pseudomonads.

Under sulfur deprivation, both *E. coli* and *pseudomonads* express high-affinity sulfate transport systems (e.g., *cysPTWA*) and enzymes such as sulfate adenylyltransferase (*cysND*) and sulfite reductase (*cysIJ*). These enzymes facilitate sulfate reduction to sulfide, which is subsequently incorporated into amino acids like cysteine.¹² However, transcriptomic analyses evaluating *P. aeruginosa* show that the organism only weakly expresses the genes of the *cys* operon when grown in cyclamate, an organosulfur compound. This demonstrates that the organism's sulfate starvation response is more nuanced than observed in *E. coli* and may potentially involve a more diverse gene set.

Drilling down to organosulfur compound utilization, both *E. coli* and *pseudomonads* possess pathways to metabolize alkanesulfonates via the *ssu* operon. However, the *pseudomonad* *ssu* operon (*ssuEADCBF*) enables a broader substrate specificity and is efficient at cleavage of C–S bonds of complex organosulfur compounds, including aromatic sulfonates, releasing sulfite.^{65,66} Conversely, *E. coli*'s *ssu* operon (*ssuEADCB*) is comparatively limited to metabolizing simpler alkanesulfonates.^{64,67}

Both *E. coli* and *Pseudomonas* species harbor the *tau* operon for taurine metabolism. The key enzyme, TauD, an α -ketoglutarate-dependent dioxygenase, cleaves taurine, releasing sulfite.^{12,27} Despite this similarity, *pseudomonads* generally exhibit broader substrate utilization

capabilities beyond taurine, reflecting their enhanced metabolic flexibility. For instance, certain *Pseudomonas* species uniquely contain the *msu* operon (*msuEDC*), which is dedicated to methanesulfonate (MS) metabolism. Within this operon, MsuDC, flavin-dependent monooxygenases, catalyze first the oxidation of methanesulfinate to methanesulfonate and subsequently to sulfite, while MsuE provides the reduced FMN required for this reaction.³⁹ Furthermore, *pseudomonads* possess the *sfnG* gene encoding dimethyl sulfone monooxygenase, which facilitates the conversion of dimethyl sulfone (DMSO₂) to methanesulfonate. This pathway effectively links sulfur metabolism with the environmental cycling of dimethyl sulfide.⁴⁰

As addressed above, SSI response is mainly coordinated by CysB and analogous CysB-like regulators in *E. coli*. However, additional regulators, including transcription factors such as SfnR proteins, play a role in the *pseudomonad* SSI response. Specifically, the transcriptional activator SfnR2 induces the expression of the *msu* and *sfn* operons in *pseudomonads* under sulfur-limiting conditions, increasing the range of organosulfur compounds that can be utilized during sulfur nutrient stress.⁴⁰

1.2.2 Sulfur Starvation Induced Antioxidant Genes

It has been well documented that SSI response in bacteria includes the expression of antioxidant genes involved in scavenging reactive oxygen species (ROS).^{24–26} In *E. coli*, sulfur stress triggers overexpression of the *ahpCF* operon, encoding alkyl hydroperoxide reductase.^{25,41} This enzyme, composed of AhpC (peroxiredoxin) and AhpF (NADH-flavin reductase), plays a crucial role in removing endogenously generated hydrogen peroxide (H₂O₂). AhpC is noted to be the primary scavenger of H₂O₂ in *E. coli*.^{68,69}

In *P. aeruginosa*, a couple of antioxidant genes have been reported to be expressed as part of SSI response, including *ahpC*, *katB*, *ohr*, and *lsfA*.^{26,70} Again, it appears that *P. aeruginosa* expresses a much more robust repertoire of antioxidant genes than *E. coli*, further highlighting its metabolic complexity. To more fully illustrate their importance in the organism's antioxidant defense armament, the roles of some of these genes in the organism are briefly discussed here.

The *katB* gene encodes catalase B, one of the catalase enzymes in *P. aeruginosa*. Catalases decompose hydrogen peroxide into water and oxygen. *P. aeruginosa* has multiple catalases; KatB is known to be induced under stress conditions and is regulated by OxyR in response to oxidative signals.⁷¹ *katB* expression has been shown to increase when the organism is provided with cyclamate as the sole sulfur source,²⁶ indicating an adaptive increase in the bacterium's capacity to eliminate H₂O₂. The upregulation of *katB* (but not the housekeeping catalase, *katA*) suggests that *P. aeruginosa* specifically bolsters inducible peroxide defenses when sulfate is limited, which would mitigate potential oxidative damage resulting from any imbalance in cellular redox chemistry.

The *ohr* gene encodes an organic hydroperoxide resistance protein (Ohr). Ohr is a hydroperoxide reductase specialized for detoxifying organic peroxides (such as fatty acid hydroperoxides) rather than hydrogen peroxide.⁷² It protects cell membranes and other cellular components from the accumulation of lipid peroxidation products. *E. coli* lacks an *ohr* homolog, relying instead on AhpCF and other peroxidases for organic peroxide defense. The presence of Ohr in *P. aeruginosa* provides an extra line of defense specifically against organic hydroperoxides, complementing the H₂O₂-focused defenses governed by OxyR.

LsfA (Low-Sulfur Factor A) is a thiol-specific antioxidant protein induced under sulfur limitation. Initially characterized in *P. putida*, *lsfA* encodes a 212-amino-acid protein of the peroxiredoxin family.⁶⁵ It is a 1-Cys peroxiredoxin, and it uses a single cysteine residue to reduce peroxides. Accordingly, it is a low-sulfur-cost enzyme for antioxidant defense. Sulfur starvation strongly induces LsfA production in *pseudomonads* and is potentially part of a strategy to replace or supplement high-sulfur enzymes with low-sulfur alternatives. The upregulation of *lsfA* during sulfate starvation^{24,26} underscores the link between sulfur metabolism and oxidative stress: LsfA can potentially help eliminate peroxides when cysteine and glutathione (typically performing thiol-based detoxification) are in short supply.

1.2.3 Role of OxyR in Coordinating ROS Response

OxyR serves as the master regulator for antioxidant gene expression in many bacteria, including *E. coli* and *P. aeruginosa*.^{73–75} As intracellular H₂O₂ concentration rises, OxyR's sensor cysteines become oxidized, activating OxyR as a transcription factor.^{76,77} Activated OxyR swiftly induces the expression of its regulon. In *E. coli*, this regulon includes *ahpCF* and *katG* and genes for glutaredoxins, thioredoxins, and the alternative Fe-S assembly machinery *suf*.⁷⁵ Notably, OxyR activation under oxidative stress turns on the *suf* operon to replace the compromised Isc pathway for Fe-S cluster biogenesis – a crucial adaptation when sulfur and iron metabolism intersect during stress.⁷⁸ In *P. aeruginosa*, OxyR similarly upregulates a broad set of defense genes; studies have identified OxyR-binding sites upstream of *katB*, *ahpB*, and *ahpC* promoters, implying a direct involvement in regulating these genes.⁷¹ Without functional OxyR, *P. aeruginosa* cannot induce these antioxidant enzymes and suffers severe oxidative damage even under normal conditions.^{71,79}

Activating OxyR and allied regulators (like OhrR for *ohr*) is potentially a pivotal part of the sulfur starvation *stimulon*. The cell effectively senses the redox imbalance caused by sulfur limitation and mounts an oxidative stress response similar to that triggered by external peroxides. By boosting peroxide scavengers (AhpC, catalases, and peroxiredoxins like LsfA), bacteria, including *P. aeruginosa*, can potentially preserve cellular components during sulfur stress.

1.2.4 Potential Link Between Sulfur Starvation and Oxidative Stress

Sulfur is indispensable for maintaining redox balance through the synthesis of antioxidant molecules like glutathione and thioredoxins.^{15,80} During sulfur limitation, the synthesis of these antioxidants can be compromised, potentially rendering cells more susceptible to ROS-induced damage.

Additionally, sulfur starvation can disrupt the formation and maintenance of Fe-S clusters in proteins, which are essential for electron transport and other critical enzyme-catalyzed transformations.^{11,45} The impairment of Fe-S cluster assembly can increase the cellular levels of unincorporated, and therefore, unregulated Fe. Such pools of low-molecular weight iron are able to catalyze the Fenton reaction, generating highly reactive hydroxyl radicals from hydrogen peroxide. This increase in ROS exacerbates oxidative stress, creating a feedback loop that further impairs cellular functions.⁴⁷

Although abounding evidence links sulfur starvation to activating oxidative stress response pathways in bacteria, the mechanistic details of how sulfur limitation induces oxidative stress remain to be fully understood. The traditional view is that ROS accumulation during sulfur starvation is primarily due to the adventitious autooxidation of reduced flavin cofactors in reductases and oxidases when their cognate substrate is deficient, as is the case with the SSI

response.^{26,41,81} However, a recent study examining the structure of SsuD, a flavin-dependent monooxygenase, reveals that conformational changes can prevent FMNH₂ autoxidation even in the absence of cognate organosulfur substrates.⁴² Hence, it is plausible that while autoxidation triggers the ROS response, other mechanisms, including impaired Fe homeostasis, contribute to the observed ROS response during sulfate limitation.

1.3 Iron Homeostasis and Oxidative Stress in Bacteria

1.3.1 Iron Metabolism and Homeostasis

Iron is an essential micronutrient for living organisms, including bacteria. Its unique redox properties are critical in cellular metabolism, DNA synthesis, electron transport, and energy production.⁸² Despite its importance, iron is not freely available in many environments: under aerobic conditions, Fe typically exists as insoluble ferric oxides, which limits its bioavailability. Moreover, while iron is necessary for bacterial survival, high intracellular concentrations can drive Fenton chemistry and produce toxic hydroxyl radicals that damage proteins, nucleic acids, and lipids.^{83,84} Therefore, bacteria must balance efficient iron acquisition and strict regulation to prevent oxidative stress.

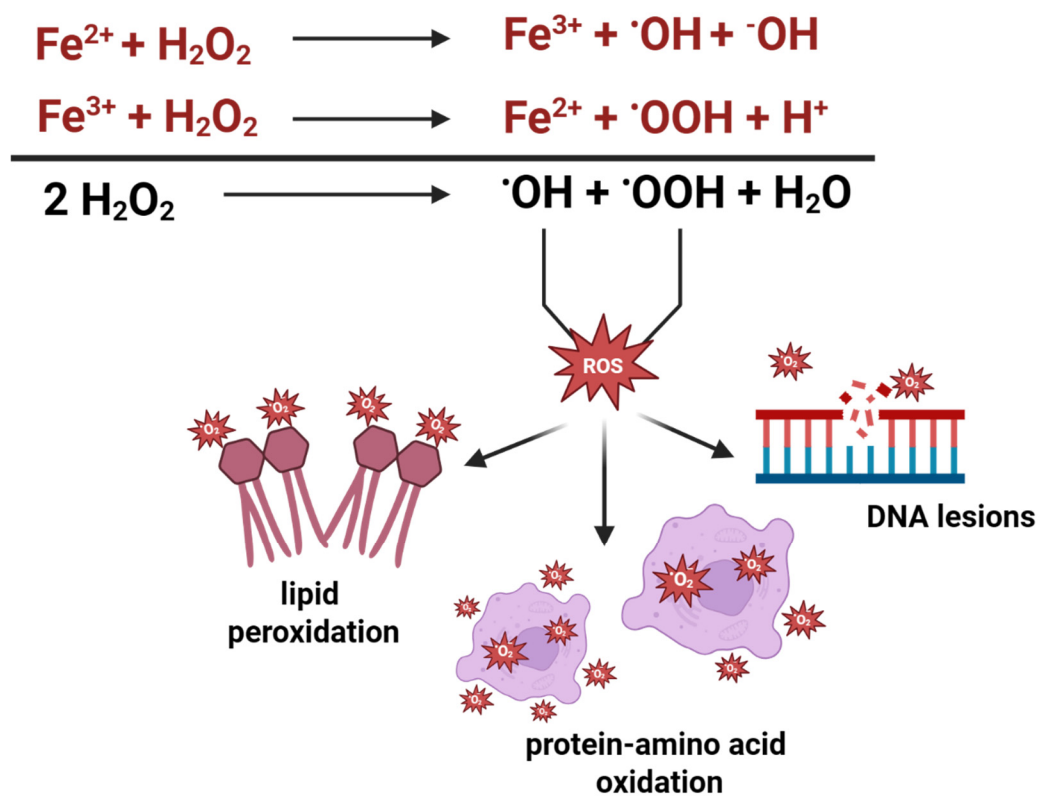


Figure 1.5: Free iron can cause cellular damage through the Fenton reaction process

P. aeruginosa has garnered significant attention in clinical settings due to its remarkable adaptability, multidrug resistance, and propensity to cause chronic infections in immunocompromised individuals. Two of the most common conditions that draw *P. aeruginosa* infection are cystic fibrosis and severe burn wounds.⁸⁵ Iron availability strongly influences bacterial fitness and virulence, particularly in host tissues where proteins like transferrin, lactoferrin, and ferritin sequester iron.^{86,87} Consequently, *P. aeruginosa* employs various mechanisms to acquire, store, and regulate iron. The section below will attempt to give an overview of the diverse iron uptake strategies used by *P. aeruginosa* and the regulatory networks that maintain iron homeostasis.

1.3.2 Iron Uptake and Storage Strategies in *Pseudomonas aeruginosa*

Siderophore-Mediated Uptake

One of the most extensively studied aspects of iron metabolism in *P. aeruginosa* is its ability to synthesize and utilize siderophores, which are small, high-affinity iron-chelating molecules. These siderophores are critical in scavenging ferric iron (Fe^{3+}) from the environment, particularly under iron-limited conditions. Once secreted, siderophores tightly bind extracellular Fe^{3+} , forming complexes that are recognized and actively transported back into the bacterial cell via specific receptor-mediated uptake systems, thus facilitating iron acquisition essential for growth, virulence, and survival. *P. aeruginosa* synthesizes two major siderophores, pyoverdine and pyochelin. Interestingly, the organism can also utilize siderophores produced by other microorganisms.⁸⁸

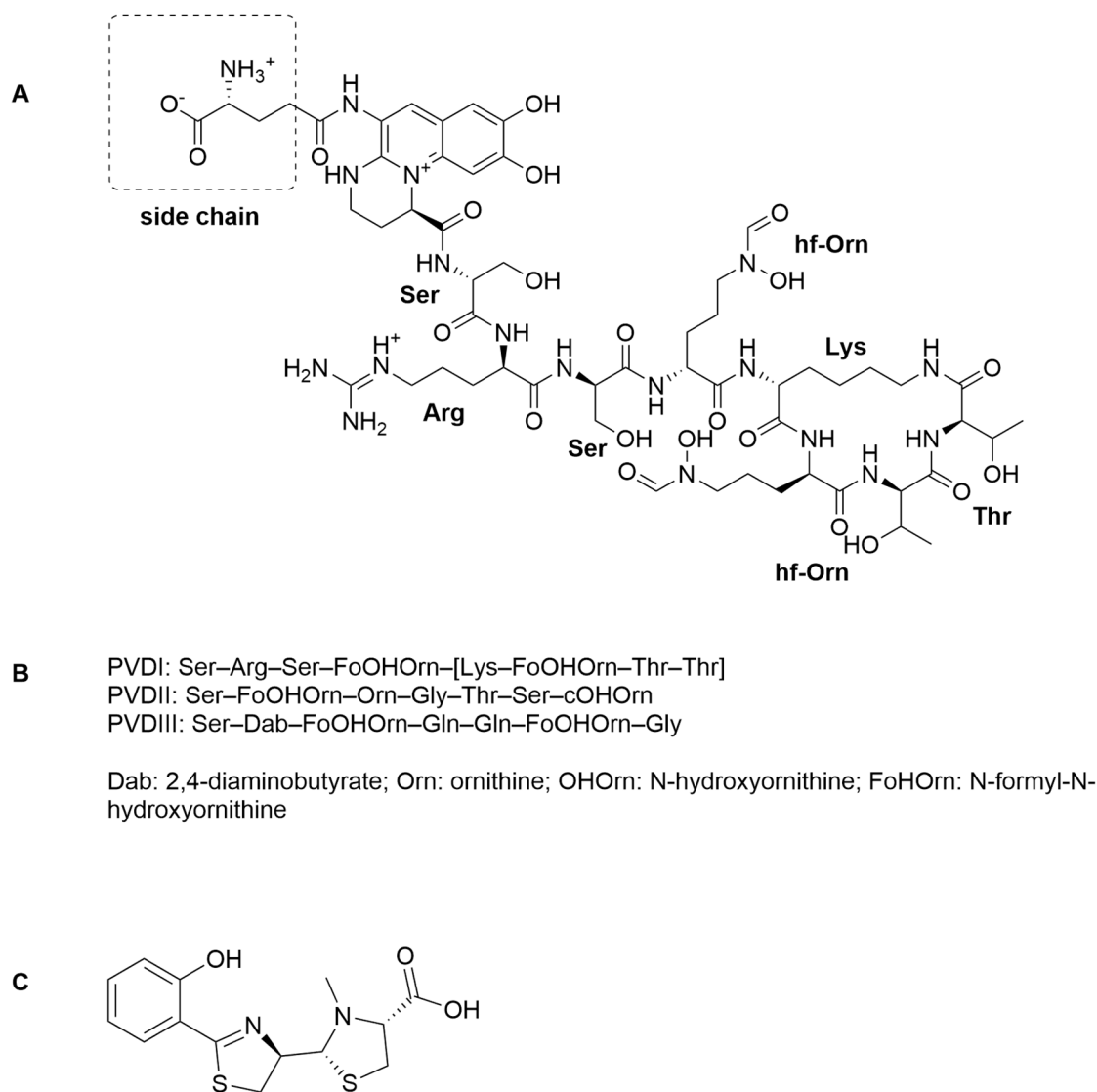


Figure 1.6: (A) Structure of *P. aeruginosa* type I pyoverdine (B) Sequence of the peptide chains from *P. aeruginosa* type I, II, and III pyoverdines (C) Structure of the second *P. aeruginosa* siderophore pyochelin. Adapted from Cornelis & Dingemans.⁸⁸

Pyoverdine Metabolism in Pseudomonas aeruginosa

Pyoverdine (PVD) is a high-affinity siderophore produced by *P. aeruginosa*, which is critical for survival in iron-limiting conditions found in various environments, including host tissues. Pyoverdine biosynthesis involves a complex enzymatic pathway primarily governed by nonribosomal peptide synthetases (NRPSs). The core biosynthetic machinery encoded by the *pvd* operon consists of several NRPS enzymes, including PvdL, PvdI, PvdD, and PvdJ, that sequentially assemble the peptide backbone and incorporate a dihydroxyquinoline chromophore derived from 2,3-dihydroxybenzoate (DHB)(Figure 1.7). Tailoring enzymes then introduce hydroxylations and cyclizations, which are essential for pyoverdine's iron-binding affinity and fluorescence properties.^{89–93}

Mature pyoverdine is secreted through the ATP-binding cassette (ABC) exporter transporter PvdE into the periplasm, where additional oxidations by PvdN, PvdO, PvdP, and PvdQ finalize chromophore maturation to yield PVDI.^{93,94} The ATP-dependent efflux pump PvdRT–OpmQ facilitates the export of PVDI to the extracellular environment.⁹⁵ Once in the extracellular space, pyoverdine chelates ferric iron (Fe^{3+}) with exceptional affinity ($K_d \approx 10^{32}$ M), allowing for scavenging of Fe from host proteins such as transferrin and lactoferrin.^{96,97}

Upon extracellular Fe chelation, the Fe^{3+} –PVDI complex binds specifically to the outer membrane TonB-dependent receptor FpvA. Transport across the outer membrane involves energy coupling provided by the TonB–ExbBD complex, driven by proton motive force that ferries the Fe^{3+} –PVDI complex into the periplasm.^{98,99} Within the periplasm, the ferric complex is processed by periplasmic proteins (FpvC, FpvG), facilitating reduction and iron release. Released ferrous

iron (Fe^{2+}) is subsequently transported into the cytoplasm through ABC transporters (FpvDE) (Figure 1.7) to replenish the intracellular iron pool.^{86,100}

Regulation of pyoverdine synthesis is primarily governed by the Ferric uptake regulator (Fur) and the alternative sigma factor PvdS. Under iron-replete conditions, Fur represses pvdS transcription, preventing unnecessary synthesis. Conversely, iron limitation relieves repression, activating siderophore production.^{97,101} Additionally, environmental factors such as quorum sensing and oxidative stress modulate pyoverdine production, linking iron acquisition closely to virulence expression and biofilm formation.¹⁰²

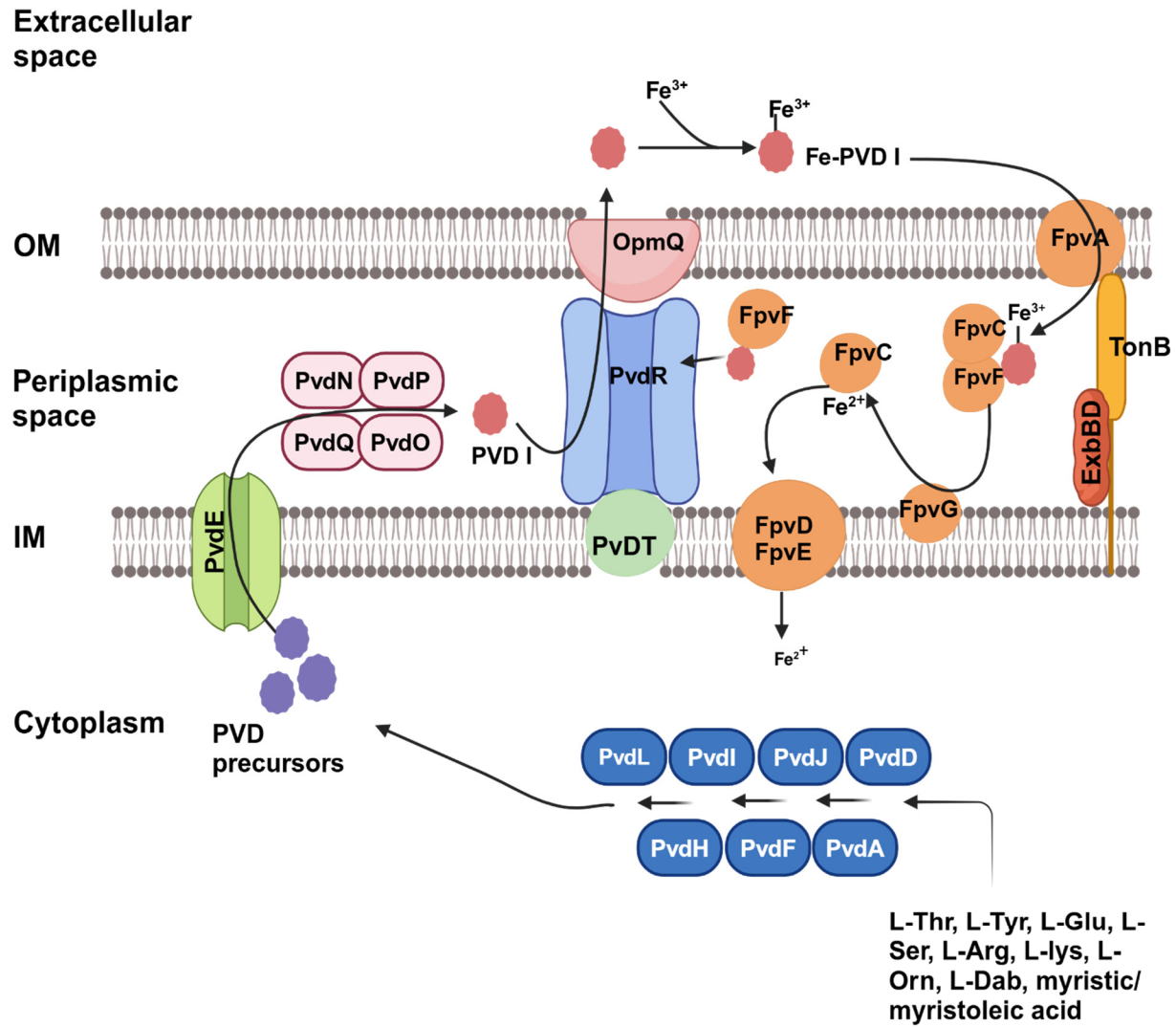


Figure 1.7: *P. aeruginosa* pyoverdine-mediated Fe uptake pathway, adapted from Schalk et al.¹⁰³

Pyochelin Metabolism in Pseudomonas aeruginosa

Pyochelin (PCH) is a secondary siderophore produced by *P. aeruginosa*. Despite its lower affinity for ferric iron (Fe^{3+}) compared to pyoverdine ($K_d \approx 10^{-5}$ vs $K_d \approx 10^{-32}$ M), PCH plays a crucial role in environments with moderate iron limitation.^{104–106} Its significance becomes particularly evident when pyoverdine production is energetically costly or restricted, allowing the bacterium to conserve metabolic resources.⁸⁸

Pyochelin biosynthesis is encoded by genes organized within two divergently transcribed operons, *pchDCBA* and *pchEFGHI*, located on the *P. aeruginosa* chromosome (Figure 1.8).^{107–109} The biosynthetic pathway starts with chorismate, which is converted to salicylate by the salicylate synthase PchA. Subsequently, PchD activates salicylate by adenylation, followed by enzymatic coupling of salicylate with two molecules of cysteine, catalyzed sequentially by nonribosomal peptide synthetases (NRPSs) PchE and PchF.¹¹⁰ This assembly line involves sequential adenylation, thiolation, condensation, and cyclization steps that ultimately yield pyochelin.¹¹¹

Once synthesized in the cytoplasm, pyochelin is exported to the extracellular environment through specific membrane-associated efflux pumps that have yet to be fully characterized. Studies have identified the membrane proteins PchHI as part of a putative ATP-binding cassette (ABC) transport complex responsible for pyochelin secretion.^{112,113} Once in the extracellular environment, pyochelin binds ferric iron, forming a ferric-pyochelin complex (Fe^{3+} -PCH). The specific outer membrane receptor FptA, a TonB-dependent β -barrel transporter, recognizes this complex, facilitating transport into the bacterial periplasm.^{107,114} Similar to pyoverdine-mediated iron uptake, Fe^{3+} -pyochelin translocation through FptA relies on energy coupling provided by the TonB-ExbB-ExbD complex, which harnesses the proton motive force from the inner membrane to facilitate siderophore uptake.^{88,108}

Upon reaching the periplasm, iron is released from the pyochelin complex via reduction of Fe^{3+} to Fe^{2+} , mediated by periplasmic reductases such as FptX, or indirectly through cellular reductive activities.^{104,108} The released ferrous iron is then transported across the inner membrane into the cytoplasm by dedicated transporters such as FeoB or ABC-type permeases, thereby contributing to the labile iron pool and subsequently incorporated into metabolic processes and enzyme cofactors.⁸⁸

Pyochelin synthesis and uptake genes are tightly regulated by iron availability through the ferric uptake regulator (Fur)(Figure 1.8). Under iron-rich conditions, Fur binds Fe^{2+} and directly represses transcription of the pyochelin biosynthetic machinery (*pch* and *fpt* operons), whereas low-iron conditions alleviate this repression, allowing active siderophore synthesis.^{112,115}

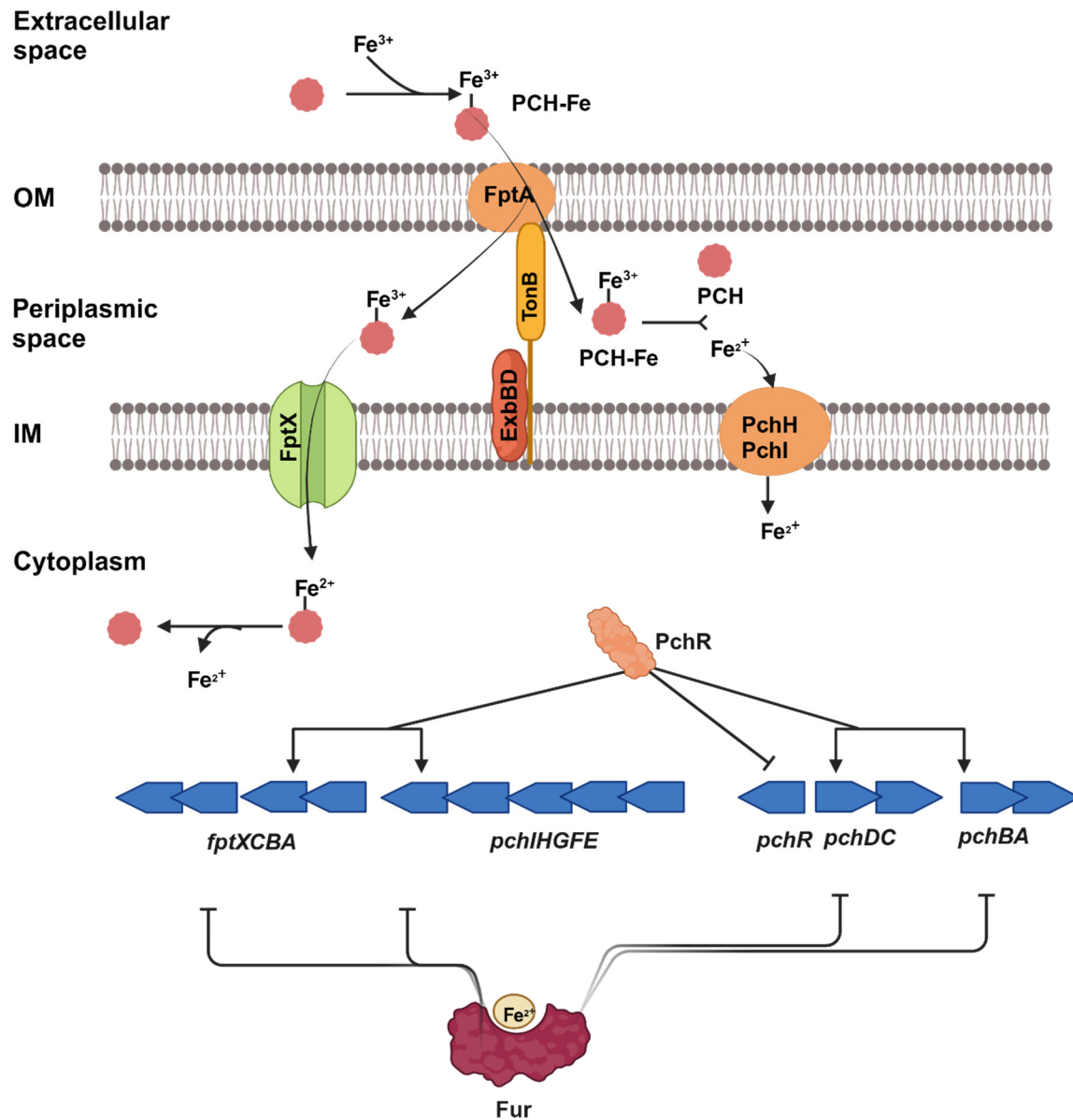


Figure 1.8: Pyochelin-mediated Fe uptake and the roles of Fur and PchR in regulating pyochelin metabolism. Adapted from Ghssein & Ezzeddine.⁸⁹

Ferrichrome and Xenosiderophores

In addition to its endogenously synthesized siderophores, *P. aeruginosa* can exploit ferrichrome (produced by fungi) and other xenosiderophores, once again highlighting its metabolic versatility.¹¹⁶ This strategy provides *P. aeruginosa* with a competitive advantage in polymicrobial communities, allowing it to scavenge iron complexed by siderophores that it does not synthesize.¹¹⁷ The transportation of ferrichrome-Fe³⁺ complexes similarly requires TonB-dependent outer membrane receptors, which ensure the bacterium can access iron from multiple exogenous sources.¹¹⁸ This ability necessitates the synthesis of specific TonB-dependent transporters (TBDTs) capable of identifying and transporting the xenosiderophore. Notably, *P. aeruginosa* generates 34 distinct TBDTs, with many likely playing a role in iron acquisition.^{115,119} Clearly, *P. aeruginosa* has adapted versatile mechanisms to draw iron from various sources.

Heme Acquisition Systems

Much of the iron available in host tissues is complexed in heme and utilized by proteins such as hemoglobin and myoglobin. *P. aeruginosa* has evolved dedicated heme uptake pathways, including the has (heme assimilation system) and phu (Pseudomonas heme uptake) system, to acquire iron from these heme-containing molecules.^{120,121} Following binding by specific receptors (HasR, PhuR), the heme is transported across the outer membrane through TonB-dependent translocation. Once internalized, intracellular heme oxygenases break down the porphyrin ring to release Fe²⁺, which is used for metabolic processes and incorporated into cellular components, including Fe-S and bacterioferritin complexes.¹²² This heme uptake is particularly advantageous

in the competitive environment of host tissues, where heme is abundant, but free iron is tightly regulated.

1.3.3 Regulation of Iron Homeostasis

Fur (Ferric Uptake Regulator) and Other Transcriptional Controls

Central to iron homeostasis in *P. aeruginosa* is the ferric uptake regulator (Fur). Under iron-sufficient conditions, Fur-Fe²⁺ complexes bind to “Fur boxes” in the promoters of iron-responsive genes, repressing transcription of siderophore biosynthetic genes and outer membrane receptor genes.¹²³ When intracellular iron becomes scarce, Fur dissociates from DNA, allowing the upregulation of genes necessary for iron acquisition.¹¹⁷ Notably, it has recently been demonstrated that purified Fur from *P. aeruginosa* binds to DNA sequences other than the well-characterized Fur box, demonstrating a plasticity that potentially enables it to bind to diverse sets of substrates, even those lacking the canonical Fur box sequence.¹²⁴

Additional regulatory layers include small non-coding RNAs (sRNAs) such as the PrrFs (PrrF1 and PrrF2), which negatively modulate mRNA stability and translation of over 50 iron-responsive genes.^{125,126} Through this, the PrrFs conserve iron during scarcity (iron-sparing response). This mechanism is conserved across diverse bacteria, emphasizing its importance in iron homeostasis. Conversely, Fur positively regulates the PrrFs repressed genes indirectly, relieving them of inhibition by the sRNAs during Fe-replete conditions.¹²⁵

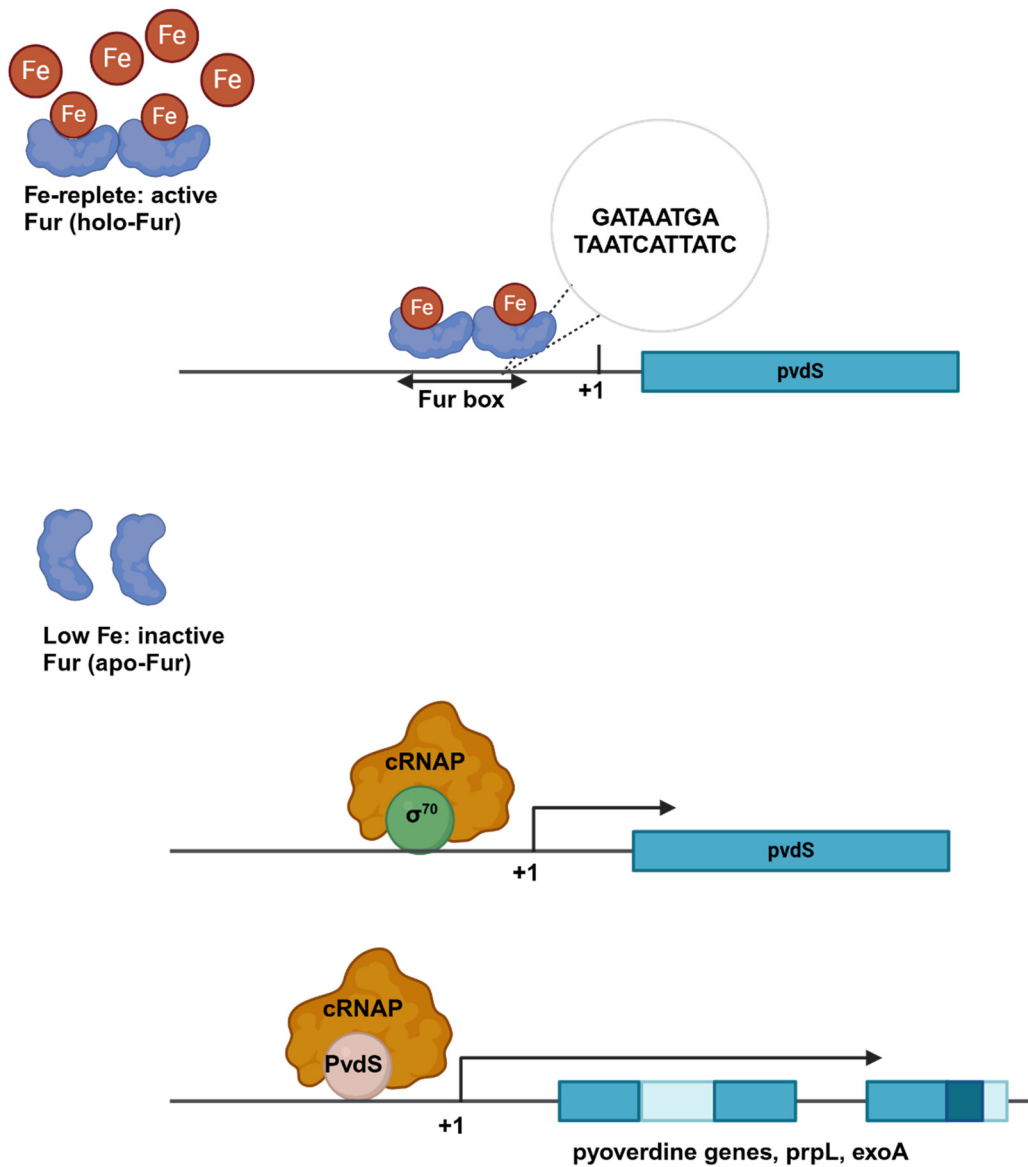


Figure 1.9: Schematic illustrating how Fur functions as an Fe sensor by repressing genes related to Fe metabolism. This example shows the regulation of PvdS and the genes under its regulatory control by Fur. Adapted from Leoni et al.¹⁰¹

Fe storage through ferritins

Due to the toxicity of iron, even in minute amounts, its levels must be stringently and meticulously regulated. Ferritins, iron storage proteins, are a vital factor in managing intracellular iron levels in bacteria.¹²⁷ The ferritin family includes ferritins (Ftn), bacterioferritins (Bfr), and DNA-binding proteins from starved cells (Dps).¹²⁸ Ftn is present in animals, plants, fungi, archaea, and bacteria, while Bfr is exclusive to bacteria and archaea.¹²⁹ Both Ftn and Bfr are made up of 24 subunits arranged in a spherical shell, containing pores and channels that facilitate the movement of iron, chelators, oxidants, and reductants, with a central cavity able to store approximately 3000 Fe^{3+} ions.¹³⁰ Dps proteins consist of 12 identical subunits that also create a spherical structure enclosing a cavity that can hold about 500 iron ions.¹³¹ *P. aeruginosa* possesses genes for ferritin (*ftnA*) and bacterioferritin (*bfrB*).¹³² The mobilization of stored Fe^{3+} ions from these molecules necessitates interaction with the Bfd ferredoxin, which transfers electrons to reduce Fe^{3+} , facilitating the release of Fe^{2+} ions.^{129,132} Bfd attaches to BfrB at the interface between two BfrB subunits above a heme molecule but does not associate with FtnA. Direct interaction between FtnA and Bfd hasn't been experimentally confirmed in *P. aeruginosa*; the Bfd–BfrB axis provides a model that FtnA may mirror, possibly using a different electron donor.¹³⁰ *P. aeruginosa* also contains a gene for a Dps protein (PA0962). It has recently been structurally characterized as a 12-mer with 12 ferroxidase centers that can bind divalent cations, along with an additional ability to oxidize ferrous Fe^{2+} to Fe^{3+} *in vitro* using H_2O_2 instead of O_2 . Consequently, it is proposed to engage in mitigating oxidative stress induced by H_2O_2 , while also potentially acting as an Fe storage protein during stress.¹³³

1.4 The Link Between Iron Metabolism and Virulence in *Pseudomonas aeruginosa*

1.4.1 *P. aeruginosa*, an Important Human Pathogen

P. aeruginosa is a Gram-negative bacterium recognized as a significant human pathogen and a primary public health concern.^{8,134} *P. aeruginosa* can cause severe infections, particularly in immunocompromised individuals. Cystic fibrosis and severe burn patients are two of the most prominent examples.^{135,136} Additionally, the bacterium is the leading cause of bacteremia in cancer patients¹³⁷ and a significant contributor to wound ulceration in diabetic patients.¹³⁸ Clearly, this organism is a clinically important pathogen.

P. aeruginosa is notorious for multidrug resistance, leading the World Health Organization to classify it as a high-priority pathogen alongside other ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, ***Pseudomonas aeruginosa***, *Enterobacter* species) pathogens resistant to first-line antibiotics.^{8,134} Indeed, *P. aeruginosa* is a leading cause of death attributed to antimicrobial resistance.¹³⁴ A key factor contributing to *P. aeruginosa*'s antibiotic resistance is its remarkable metabolic flexibility and adaptability owing to its large genome and multiple, sometimes redundant, metabolic pathways. These pathways enable the bacterium to circumvent or overcome the mechanisms of action employed by antibiotics.^{20,139} Additionally, *P. aeruginosa* possesses diverse pathways for acquiring essential nutrients within the host environment, where nutrient availability can be limited, thereby enhancing its overall fitness during infection.²¹

1.4.2 The Critical Nature of Iron Homeostasis

Iron is a crucial micronutrient for bacterial survival and virulence, playing a key role in various cellular metabolic processes such as respiration, electron transport, energy metabolism, and DNA replication and repair.¹⁴⁰ The ability of iron to cycle between its ferrous (Fe^{2+}) and ferric (Fe^{3+}) states is fundamental to the formation of mononuclear iron centers, as well as the biosynthesis of heme and iron–sulfur clusters—key cofactors for a wide range of metalloenzymes essential to core cellular functions.^{140,141}

Nevertheless, Fe's redox cycling ability also poses a challenge in cells, as Fe can induce toxicity through the production of reactive oxygen species (ROS) and nitrogen species (RNS), byproducts of basal cellular metabolism. The reaction of these species with Fe typically generates more deleterious ROS/RNS, which are harmful and can damage biomolecules and induce cell death through the well-studied Fenton chemistry.^{45,141,142} For these reasons, cells and bacteria must maintain relatively low (10^{-5} - 10^{-8} M) Fe levels to avoid Fe toxicity.^{143,144}

1.4.3 Iron Acquisition Strategies in *P. aeruginosa* and their Connection to Virulence

The host immune system utilizes a strategy called *nutritional immunity*, which involves sequestering essential nutrients such as iron from invading pathogens like *P. aeruginosa* to restrict their growth and survival.¹⁴⁵ Despite this host defense mechanism, *P. aeruginosa* has developed multiple iron-acquisition systems to navigate iron limitation within the host.¹⁴⁶ Interestingly, there is evidence that iron depletion or low intracellular iron levels in mammalian host tissues can paradoxically signal the organism to overexpress virulence genes.¹⁴⁷

Heme Acquisition: Heme, the most abundant form of iron storage in humans, can be scavenged by *P. aeruginosa* using outer membrane receptors such as HasR and PhuR. Once

internalized, heme is degraded by cytoplasmic heme oxygenase HemO to release iron, which is utilized for diverse cellular functions or stored in ferritins.¹¹⁵

Siderophores: As discussed earlier, *P. aeruginosa* produces two primary siderophores, pyoverdine and pyochelin, both of which are small molecules with a high affinity for ferric iron (Fe^{3+}). These siderophores are secreted into the extracellular environment, where they effectively chelate iron from host iron-binding proteins such as lactoferrin and transferrin.^{87,96} Generally, pyoverdine shows a higher affinity for iron compared to pyochelin and is typically secreted under conditions of severe iron scarcity, playing a crucial role in iron acquisition during acute infections.¹⁴⁸ In addition to its role in iron uptake, pyoverdine functions as a signaling molecule that triggers the expression of other virulence determinants.^{88,96,149} Under iron-limiting conditions, Fe^{3+} -PVD binds to FpvA at the outer membrane, initiating a signal transduction cascade that leads to the proteolytic degradation of the anti-sigma factor FpvR. This degradation event liberates FpvI and another ECF sigma factor, PvdS, both of which are normally sequestered by FpvR. Once released, FpvI associates with RNA polymerase to drive the transcription of *fpvA* and possibly other genes involved in ferripyoverdine uptake, effectively establishing a positive feedback loop that enhances iron acquisition^{150,151} (Figure 1.10). In parallel, PvdS activates genes involved in pyoverdine biosynthesis as well as virulence factors such as *toxA* (exotoxin A) and *prpL* (protease IV). This coordinated regulatory response ensures that the bacterium not only enhances its ability to acquire iron but also triggers virulence programs upon the successful detection and internalization of iron-bound siderophores. The FpvI–FpvR–FpvA axis thus acts as a dedicated module that connects environmental iron sensing to the transcriptional regulation of iron uptake systems, creating a tightly controlled network vital for iron homeostasis and pathogenicity.^{92,100,149}

While its role in acute infections may be less prominent than that of pyoverdine, pyochelin is believed to aid in iron acquisition during chronic infections and might help modulate the host's inflammatory response alongside pyocyanin, another redox-active compound secreted by the bacteria.^{5,152}

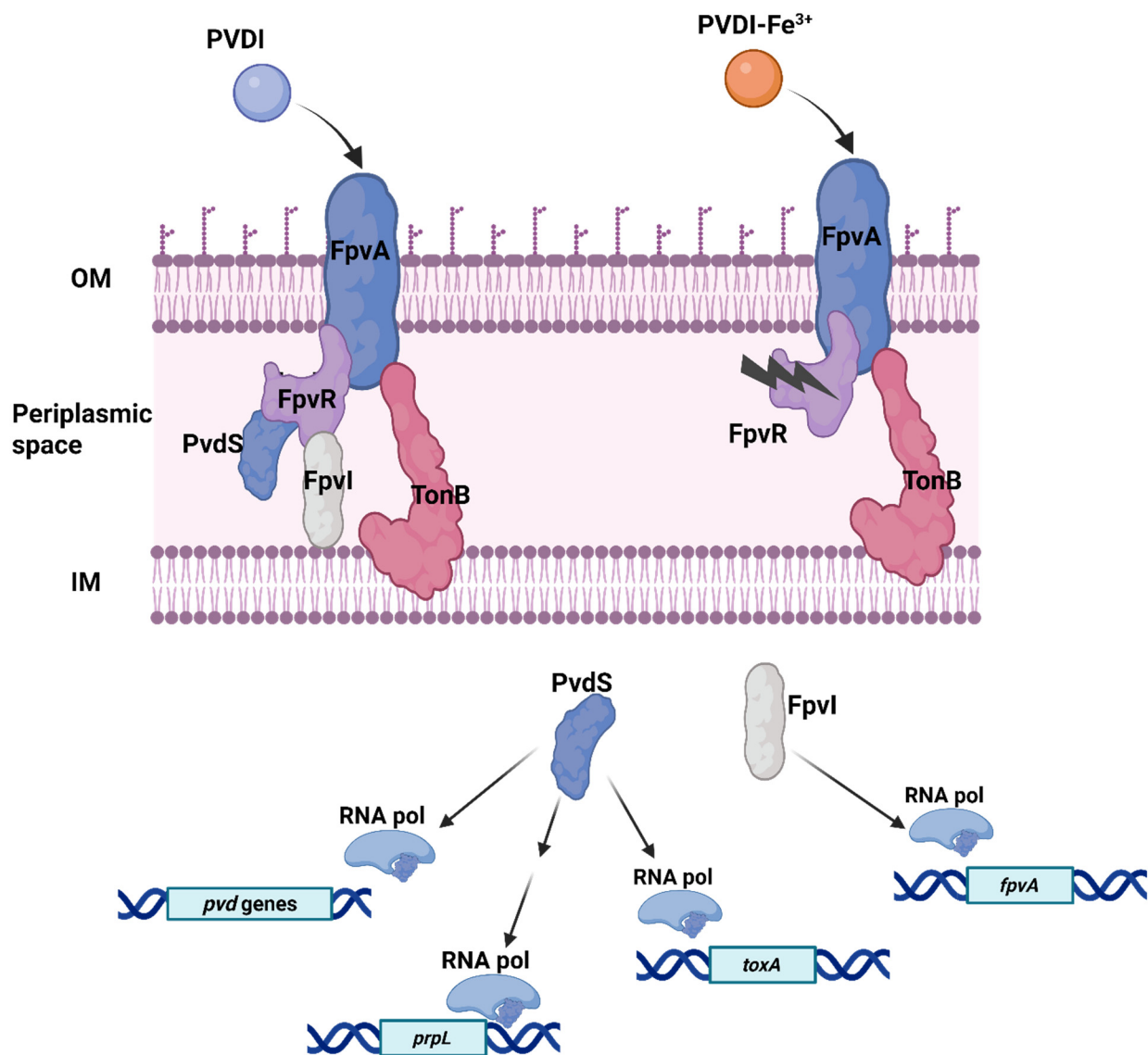


Figure 1.10: Schematic illustrating how ferripyoverdine functions as a signaling molecule that induces the expression of pyoverdine biosynthesis and virulence proteases genes. Adapted from Cornelis & Dingemans.⁸⁸

Ferrous Iron Uptake: In *P. aeruginosa*, ferrous iron (Fe^{2+}) is acquired through the Feo system, a transport mechanism comprising the membrane permease FeoB and its accessory protein FeoA.¹⁵³ This system is crucial under microaerobic or anaerobic conditions, such as those found in the viscous mucus of CF lungs, where ferric iron is poorly soluble and oxygen-dependent iron acquisition systems are less effective.¹⁵⁴ In this environment, *P. aeruginosa* not only adapts to low oxygen but also modifies its metabolic strategies to ensure sustained iron availability. A key adaptation involves the biosynthesis and use of phenazines, redox-active secondary metabolites that facilitate iron reduction and uptake.

The biosynthesis of phenazines in *P. aeruginosa* begins with *chorismic acid*, a product of the shikimate pathway, and proceeds through the action of two homologous seven-gene operons: *phz1* and *phz2*. These operons encode a set of conserved enzymes that convert chorismate into phenazine-1-carboxylic acid (PCA) (Figure 1. 11), the central phenazine scaffold.^{155,156} PCA functions both as a final product and as a precursor for several phenazine derivatives, depending on the activity of downstream tailoring enzymes. Among these derivatives, pyocyanin, a blue-green pigment and virulence factor, is the most well-characterized. The biosynthetic conversion of PCA to pyocyanin involves two key enzymes: PhzM, a methyltransferase, and PhzS, a flavin-dependent monooxygenase. First, PhzM catalyzes the methylation of PCA to form 5-methylphenazine-1-carboxylic acid betaine (5-Me-PCA). PhzS then hydroxylates this intermediate at the 1-position to generate pyocyanin.¹⁵⁷ Other enzymes, including PhzH and PhzS, act on PCA to produce the alternative phenazine derivatives phenazine-1-carboxamide (PCN) and 1-hydroxyphenazine (1-HP), expanding the phenazine chemical repertoire.

These phenazine derivatives serve not only as pigments but also as redox mediators. PCA and pyocyanin can accept electrons and reduce extracellular Fe^{3+} (bound to host proteins, such as

transferrin or lactoferrin), resulting in the formation of soluble Fe^{2+} . This reduced iron can then be transported into the cell via the FeoB-mediated system, supporting bacterial growth in iron-restricted environments such as the CF lung.^{158–160} The capacity of phenazines to sustain redox cycling also promotes metabolic activity and biofilm development under oxygen-limited conditions. Indeed, the accumulation of both Fe^{2+} and phenazines has been observed in biofilm-dominated regions of the CF lung, suggesting a synergistic interaction between redox-active metabolites and iron acquisition pathways that supports persistent infection.¹⁶¹

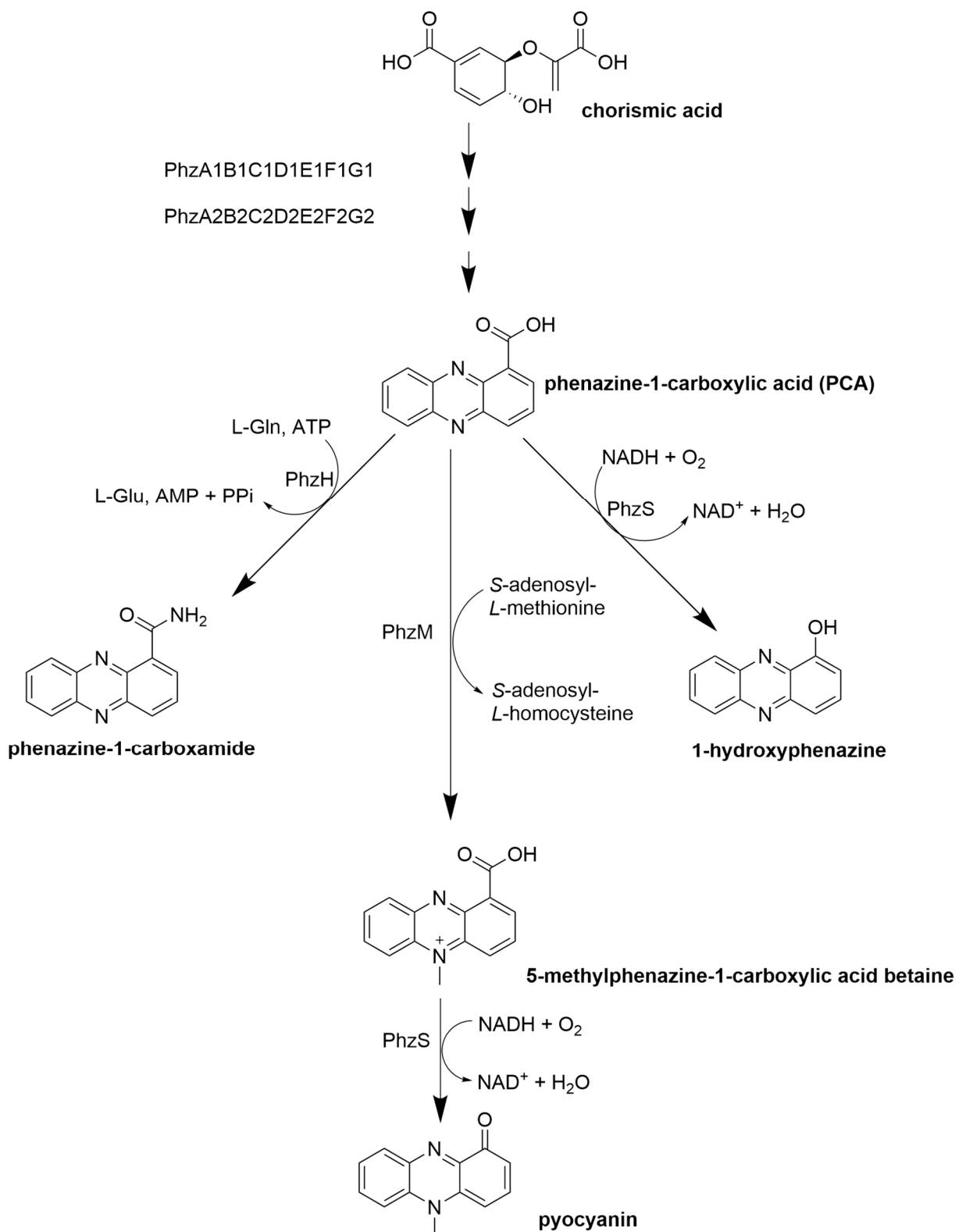


Figure 1.11: A proposed pathway for the synthesis of phenazines in *P. aeruginosa*. Adapted from Movrodi et al.¹⁵⁵

1.4.4 The Role of Iron-Responsive Regulators in Virulence

The intricate link between iron availability and virulence in *P. aeruginosa* is mediated mainly by key regulatory proteins and small non-coding RNAs (sRNAs).

Fur : The Ferric uptake regulator (Fur) is a central iron sensor in *P. aeruginosa*, playing a crucial role in regulating not only iron homeostasis but also the organism's virulence response ¹⁶². Under iron-replete conditions, Fur, in complex with iron, acts as a transcriptional repressor, binding to specific DNA sequences (Fur boxes) in the promoter regions of target genes, inhibiting their expression. Conversely, under iron-limiting conditions, Fur dissociates from these promoter regions, leading to the expression of genes involved in iron acquisition and virulence. For instance, Fur indirectly regulates the expression of exotoxin A (toxA), a major virulence factor involved in tissue invasion by facilitating entry into host cells.^{163,164} This regulation is achieved through the extracytoplasmic function (ECF) sigma factor PvdS and the ferripyoverdine receptor FpvR, which are both controlled by Fur.

PrrF/PrrH sRNAs: The *prfF* gene, which encodes small regulatory RNAs (PrrF1 and PrrF2), is also regulated by Fur and is implicated in the iron-sparing response, as noted before. During iron scarcity, PrrF sRNAs inhibit the expression of non-essential iron-utilizing genes, thereby prioritizing iron for critical cellular processes.^{125,126} Notably, PrrF sRNAs have also been linked to the production of Pseudomonas quinolone signal (PQS), a quorum-sensing molecule that regulates the expression of numerous virulence factors.¹⁶⁵ This effect is achieved through the repression of the *antABC* mRNA, which encodes enzymes involved in the metabolism of anthranilate, the precursor for PQS synthesis.¹⁶⁶ Both Fur and PrrF/PrrH sRNAs play a critical role in supporting the progression of *P. aeruginosa* infections by carefully managing intracellular iron

levels and ensuring sufficient iron for metabolic needs while preventing iron toxicity. Notably, PrrF1 and PrrF2 sRNAs are essential for *P. aeruginosa* infection in a murine model of acute lung infection¹²⁵ and are regularly expressed in the lungs of cystic fibrosis (CF) patients, indicating their significance during *P. aeruginosa* infections in CF airways.

1.4.5 Iron and Biofilm Formation

The formation of biofilms, surface-associated communities of bacteria encased in a self-produced matrix, is a critical aspect of *P. aeruginosa* infections, contributing significantly to antimicrobial resistance and evasion of host immune defenses.^{167,168} Several studies have demonstrated that iron starvation can disrupt biofilm formation in *P. aeruginosa*.^{102,102,128,169} For example, one study demonstrated that iron sequestration by the host's iron transporter, lactoferrin, enhances *P. aeruginosa* twitching motility while simultaneously suppressing biofilm formation.¹⁶⁹ Additionally, a study by Glick and coworkers¹⁷⁰ suggests that iron limitation promotes the synthesis and secretion of rhamnolipids early in the biofilm formation process, leading to the development of an unstructured rather than a mature biofilm, and also increases bacterial surface motility on abiotic surfaces. Also, Iron has been shown to promote the formation of the exopolysaccharide Psl,¹⁷¹ a key component of the biofilm matrix. Furthermore, the connection between Fe metabolism and biofilm formation is reinforced by Fe deprivation studies, in which synthetic Fe chelators or the Fe³⁺ mimic gallium (Ga³⁺) have been shown to disrupt biofilm formation and maturation.^{172–174}

In the CF lung environment, characterized by mutations in the CFTR gene (notably $\Delta F508$ -CFTR), there is an aberrant increase in iron secretion into the airway surface liquid. This elevated iron availability fosters the formation of robust biofilms by *P. aeruginosa*, contributing to chronic

infections and heightened antibiotic resistance.¹⁷⁵ Studies have demonstrated that airway epithelial cells expressing $\Delta F508$ -CFTR release more iron compared to those with wild-type CFTR, and this excess iron enhances biofilm development.¹⁷⁶ Also, *P. aeruginosa* employs multiple iron acquisition systems to thrive in the iron-rich yet hostile environment of the CF lung. Among these, the FeoB transporter facilitates the uptake of ferrous iron (Fe^{2+}), a form prevalent under the anaerobic conditions often found in thick CF mucus.^{154,177} Interestingly, the FeoB protein has been shown to enhance biofilm formation in the presence of phenazine, likely by enabling the oxidation of ferrous iron to ferric iron.^{160,178}

1.4.6 Therapeutic Implications

Given the critical role of iron in both the metabolism and virulence of *P. aeruginosa*, targeting iron acquisition and homeostasis has emerged as a potential strategy for developing novel antimicrobial therapies. For example, studies have suggested that depriving cells of iron can reduce virulence, and the FDA-approved iron chelator deferoxamine (DFO) has been shown to enhance the bactericidal activity of tobramycin against *P. aeruginosa* in an *in vitro* model of cystic fibrosis.¹⁷⁴ However, it has been suggested that depriving the organism of iron might inadvertently worsen infection by triggering the upregulation of virulence factors and iron scavenging pathways, potentially leading to increased tissue damage. Therefore, a more nuanced approach may be required. One proposed strategy is to specifically target the uptake of ferrous iron, which is more abundant in the lung tissue of CF patients.⁹⁷ Another promising approach involves targeting regulatory sRNAs using antisense RNA or by knocking down the expression of specific proteins involved in iron metabolism and virulence, as has been successfully applied in other pathogenic bacteria.¹⁷⁹ The energy-dependent uptake of iron through siderophores, mediated by TonB-

dependent receptors and the proton motive force,¹⁸⁰ also presents a potential target for therapeutic intervention.

In summary, iron homeostasis is intricately linked to the virulence of *P. aeruginosa*. The bacterium has evolved sophisticated mechanisms to acquire iron from the host environment, and iron-responsive regulators like Fur and PrrF sRNAs tightly regulate these mechanisms. These regulators also control the expression of key virulence factors and influence biofilm formation. Understanding the complex interplay between iron metabolism and virulence provides valuable insights for developing new strategies to combat this pathogen.

1.5 Omics Approaches in Studying Bacterial Stress Responses

Omics technologies have revolutionized the study of bacterial physiology by enabling comprehensive, high-throughput analyses of genes, transcripts, proteins, and metabolites. These approaches provide a holistic view of cellular processes, allowing researchers to decipher complex regulatory networks and metabolic pathways involved in stress responses.³⁰

Transcriptomics focuses on the global analysis of gene expression by measuring RNA transcripts. Techniques like RNA sequencing (RNA-seq) enable the identification of differentially expressed genes under various conditions, revealing how bacteria adjust their gene expression in response to environmental stresses.¹⁸¹ **Proteomics** involves the large-scale study of proteins, including their expression levels, modifications, and interactions. Mass spectrometry-based proteomics allows for the quantification of protein abundance and the identification of post-translational modifications that regulate protein activity.¹⁸²

By integrating transcriptomic and proteomic data, comprehensive insights can be obtained into bacterial stress responses, revealing transcriptional shifts and their corresponding protein-level effects. This combined approach clarifies complex regulatory networks and the molecular mechanisms of adaptation. For example, a transcript may be significantly upregulated, yet the corresponding protein could be rapidly degraded or require post-translational modifications to become fully functional. Therefore, multi-omics approaches, utilizing diverse omics techniques, have increasingly become indispensable for gaining a holistic view of bacterial stress response.

Recent advancements in next-generation sequencing (NGS) and mass spectrometry have improved the capacity for conducting highly sensitive transcriptomic and proteomic analyses using high-throughput and precise instrumentation. Moreover, integrative omics approaches, combining transcriptomics, proteomics, and metabolomics, offer a more comprehensive understanding of the interconnected metabolic networks and regulatory mechanisms involved in sulfur starvation responses. Finally, the rapid growth in the computational infrastructure of the last few decades makes it more feasible to analyze the expansive datasets that are often generated by omics technologies.¹⁸³

1.5.1 Omics Studies on Sulfur Metabolism and Starvation

Despite sulfur's critical role in bacterial physiology, omics studies targeting sulfur metabolism and starvation responses in *P. aeruginosa* are relatively limited. Early studies utilizing low-resolution techniques like two-dimensional gel electrophoresis and microarrays identified a subset of SSI genes involved in organosulfur compound uptake and metabolism^{24,26}. However, these approaches lacked the sensitivity and comprehensiveness needed to capture the full extent of the cellular response to sulfur starvation.

Addressing these gaps requires the adoption of integrative omics approaches that combine high-resolution transcriptomic, proteomic, and metabolomic data with advanced computational tools for network analysis and regulatory modeling. Such comprehensive studies can provide deeper insights into the global cellular responses to sulfur starvation, uncovering novel genes, regulatory elements, and metabolic pathways involved in bacterial adaptation and stress management.

1.6 Summary of Current Knowledge

The existing literature provides a foundational understanding of sulfur metabolism and its importance in bacterial physiology. Sulfur is essential for the synthesis of key biomolecules, including amino acids, vitamins, and cofactors, and plays a critical role in maintaining redox balance through its involvement in antioxidant systems and Fe-S cluster formation.^{12,15} *P. aeruginosa*, with its extensive genetic repertoire, demonstrates remarkable metabolic flexibility, enabling it to utilize diverse sulfur sources and adapt to sulfur-limited environments.^{20,24,26}

Sulfur starvation triggers the expression of SSI genes involved in organosulfur compound uptake and metabolism, as well as antioxidant genes that mitigate ROS-induced damage.^{25,26,41} However, the mechanistic link between sulfur limitation and oxidative stress remains elusive. While it is widely accepted that ROS accumulation is due to the autoxidation of reduced flavin cofactors, recent evidence challenges this hypothesis, indicating that there are potentially other sources of ROS generation under sulfur starvation.⁴²

Additionally, the relationship between sulfur metabolism and iron homeostasis during sulfur starvation has not been investigated previously. Our experimental data indicate that sulfur

starvation disrupts iron metabolism, resulting in iron dysregulation that could potentially trigger the observed ROS response associated with sulfur starvation.

1.7 Conclusion

This literature review highlights the essential role of sulfur in bacterial metabolism, particularly in *P. aeruginosa*, and underscores the known connections between sulfur metabolism and ROS response. Furthermore, the existing understanding of Fe metabolism and its potential link to oxidative stress and virulence in *P. aeruginosa* has also been provided. While significant progress has been made in understanding sulfur metabolism and its regulation, the causal mechanisms linking sulfur starvation to oxidative stress and iron dysregulation remain poorly understood. This body of work leveraged a comprehensive omics approach to address these knowledge gaps, providing a holistic view of the cellular responses to sulfur starvation and uncovering the connection between sulfur starvation, Fe homeostasis, and virulence in *P. aeruginosa*.

CHAPTER TWO: Materials and Methods

2.1 Cultivation of *Pseudomonas aeruginosa*

2.1.1 Preparation of Sulfur-free Media

Sulfur-free media (SFM) was prepared as previously described¹⁶⁶, using double-deionized water (18.2 MW/cm resistivity) (Barnstead™ GenPure™, ThermoScientific, USA), with glassware rinsed using 3 M HCl followed by distilled water to prevent contamination. The final prepared media contained Tris-HCl (50 mM), sodium succinate (25 mM), NH₄Cl (20 mM), KH₂P0₄ (0.5 mM), MgCl₂ (0.5 mM), FeCl₃ (2 μM), MnCl₂ (1 μM), CaCl₂ (1 μM), ZnCl₂ (1 μM), and 50 μg/mL of each proteinogenic amino acid except for cysteine and methionine. Cells grown in this media supplemented with 500 μM sodium sulfate served as the control group. Our SFM and sulfate-supplemented control media were pH-adjusted (7.3) and filtered through 0.2 μm pore-size syringe strainers (VWR, USA).

2.1.2 Growth Conditions and Modelling

Colonies of *P. aeruginosa* PAO1 cells were used to inoculate 5 mL Luria-Bertani (LB) media, and the culture was allowed to grow overnight at 37 °C with constant agitation (220 rpm). Following this, 10 μL of bacterial suspension was used to inoculate a 48-well microtiter plate (Corning, Fisher Scientific, USA) containing either 990 μL of SFM (Sulfur-starved group - test) or SFM supplemented with 500 μM sulfate (Sulfur-containing group - control) to a final dilution of 1:100. The plate was incubated with continuous agitation (250 rpm) in a Cytation™ 3 multimode plate reader (BioTek Instruments, Winooski, VT, USA). Growth rates were monitored by optical density (OD₆₀₀) recorded every 10 min for 14h.

Gompertz and logistic growth models were used to accurately identify exponential growth characteristics for both test and control cells. Exponential growth parameters, including growth rate, lag time, and maximal optical density, were derived using R software (v 4.4.1). The identified exponential phases (~2-7 h for the test condition and ~4-8 h for the control condition) were used for subsequent downstream analyses.

2.2 Omics Analyses

2.2.1 Cell Culture

PA PAO1 stock culture stored at -80 °C was plated on LB-agar and incubated overnight (~ 16h) at 37 °C. To create biological replicates of *P. aeruginosa* cells, independent colonies from the LB-agar plate were used to inoculate tubes containing 4 mL of LB and grown overnight. The overnight cultures were then diluted 1:100 into either SFM (test group) or SFM supplemented with 500 µM sulfate (control group) and incubated at 37 °C in a shaking incubator until growth reached mid-exponential phase (~ 5 for test and 7h for control). Cultures were split equally for RNA-seq and proteomics, pelleted at $5,000 \times g$ for 5 min at 4 °C, flash-frozen on dry ice, and stored at -80 °C until analysis.

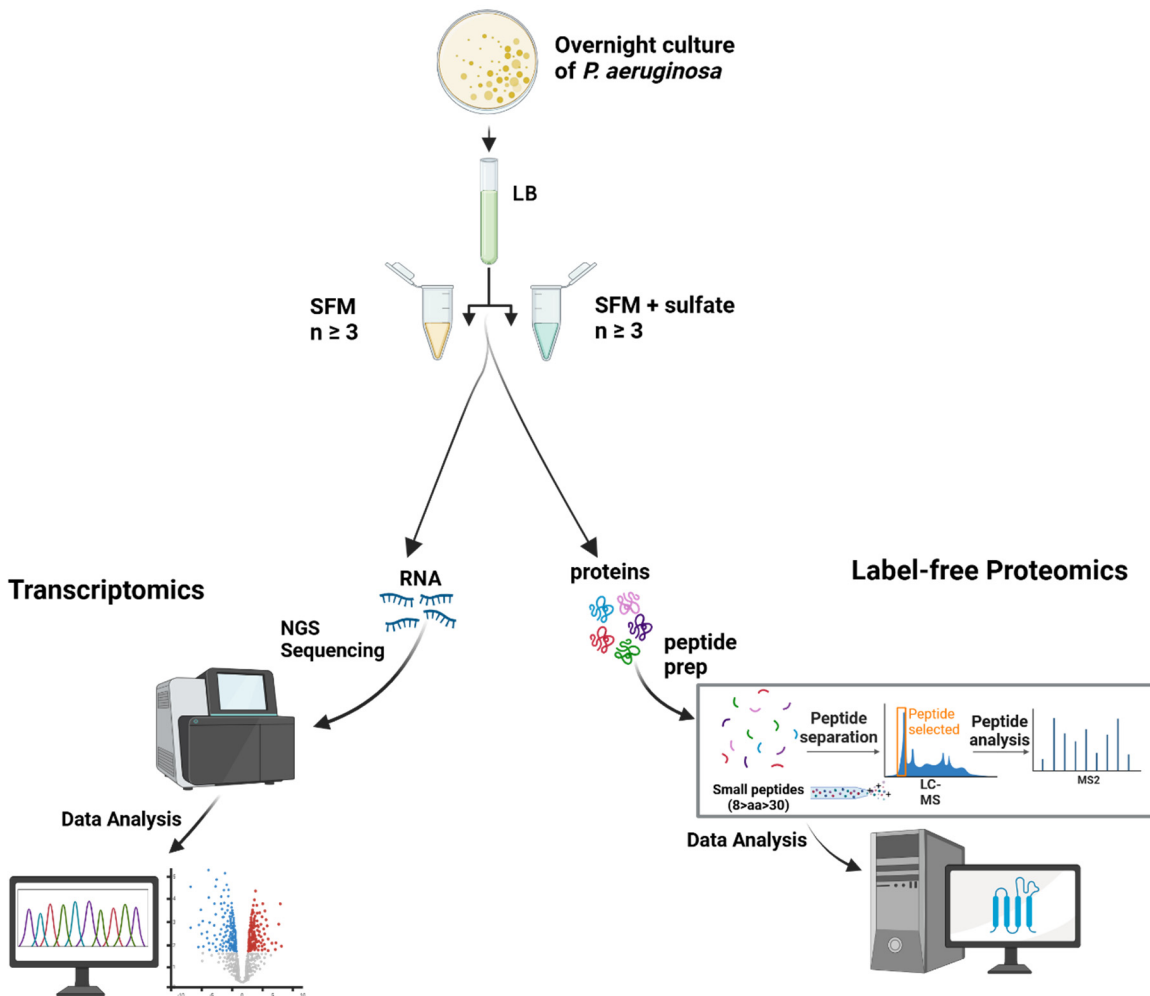


Figure 2.1: Overview of omics analyses—from cell culture through transcriptomics and proteomics

2.2.2 RNA Extraction

We employed RNA-seq to quantify transcriptional changes resulting from sulfur starvation in *P. aeruginosa* PAO1 cells. Three replicates of each test and control *P. aeruginosa* pellets were resuspended by vortexing in 100 μ L TE (10 mM Tris-HCl, 1 mM EDTA, pH 8) buffer containing 0.2 mg/mL lysozyme. This was followed by incubating the mixture at 37 °C for 10 min to ensure cell lysis. Total RNA (> 200 nt) was then purified from the mixture using the Illustra™ RNAspin Mini RNA isolation kit (GE Healthcare, UK), following the manufacturer's instructions.

The quality and concentration of extracted RNA were determined using agarose gel electrophoresis (1.25% agarose, 120V, 35 min) and a Nanodrop spectrophotometer (ThermoFisher Scientific, USA). The RNA samples were then shipped to a next-generation sequencing (NGS) facility (Admera Health, NJ, USA) for sequencing. The integrity of the samples was assessed using an Agilent Bioanalyzer, with all six samples (three biological replicates per group) meeting the minimum requirement of an RNA Integrity Number (RIN) cutoff of seven.

2.2.3 RNA-seq Library Preparation and Sequencing

To enrich messenger RNA (mRNA), ribosomal RNA was depleted from the samples using the QIAset FastSelect –5S/16S/23S Kits (Qiagen, Germantown, MA, USA). The depleted samples were then prepared for sequencing using the NEBNext® Ultra™ II RNA Library Prep kit (NEB, Ipswich, MA, USA). Library quality control was performed using Qubit® dsDNA HS (High Sensitivity) assay (ThermoFisher, USA), High Sensitivity DNA ScreenTape (Agilent, Santa Clara, CA, USA), and qPCR (KAPA SYBR® FAST qPCR Master Mix kit). Paired-end (PE) sequencing by synthesis was conducted on the Illumina NovaSeq S4 platform (Illumina, San Diego, USA) using a 2×150 bp, generating approximately 20 million reads per sample (10 million reads per end).

2.2.4 RNA-seq Data Analyses and Visualization

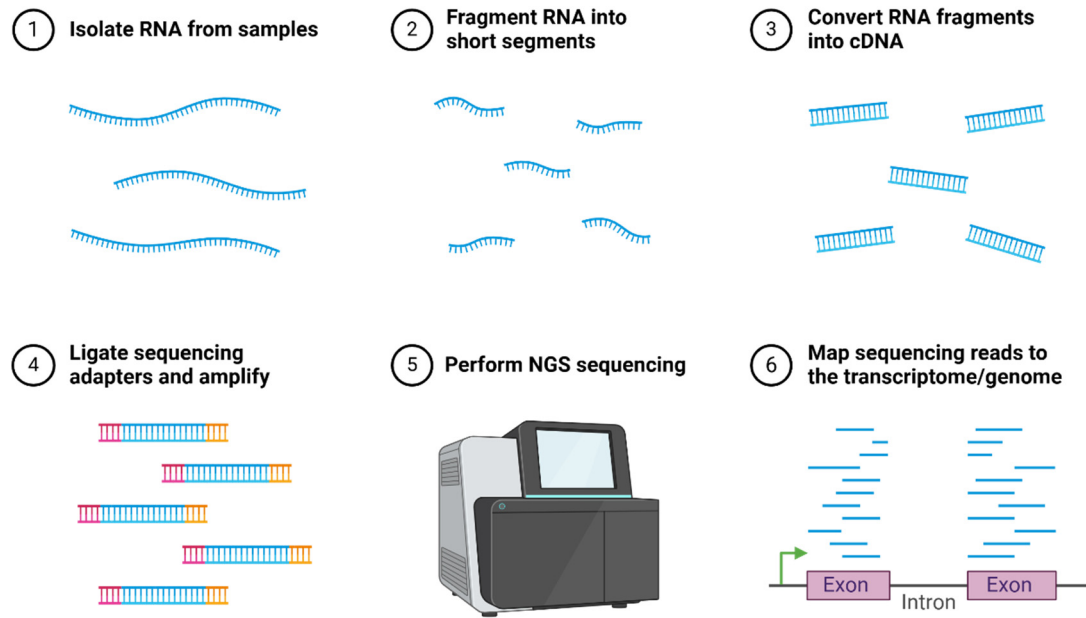
Downstream bioinformatics analyses were conducted on raw reads on the Galaxy platform¹⁸⁴ and Alabama HPC clusters. Therein, FastQC (v 0.11.9) was employed for quality assessment of raw reads, Trimmomatic¹⁸⁵ (v 0.38.0) was used in removing low-quality reads (Phred score < 30), HISAT2¹⁸⁶ (v 2.2.1) was utilized for read alignment and mapping to reference *P. aeruginosa* PAO1 genome (genome-build-accession: GCA_000006765.1), and featureCounts¹⁸⁷ (v 2.0.1) employed for counting aligned reads. Differential expression analysis of gene counts was performed using the R Bioconductor package *limma*¹⁸⁸ (v 3.48.0). Differential expression analysis was conducted using the limma-voom method. Genes with counts per million (CPM) less than 0.5 in at least two samples were considered insignificant and filtered out, resulting in the removal of 19 out of 5,677 genes (0.33%). To assess gene expression changes between sulfur-starved and control samples, raw library sizes were first normalized using the Trimmed Mean of M-values (TMM) method, which adjusts for differences in sequencing depth and RNA composition across samples. This normalization ensures that comparisons of gene expression are accurate and not biased by technical variation. Following normalization, the voom function was applied to transform the count data into log₂ counts per million (CPM), a common scale used in RNA-seq analysis. In addition to this transformation, voom estimates precision weights for each observation, enabling the analysis to account for the typical mean-variance relationship observed in RNA-seq data, where genes with low expression tend to exhibit higher variability.

Linear models were then fitted to the transformed data to test for differential expression between treatment groups. Specifically, the effect of sulfur starvation was compared relative to the control condition. To improve the reliability of the statistical estimates, empirical Bayes moderation of the standard errors was performed using the eBayes function with robust settings.

This approach stabilizes variance estimates, particularly for genes with lower counts, making the results more dependable. Genes were considered differentially expressed if they showed an absolute $\log_2$ fold change ($\log_2FC$) of at least one and a false discovery rate (FDR)-adjusted p -value of 0.05 or lower. Genes with positive $\log_2FC$ values were classified as upregulated under sulfur starvation, while those with negative $\log_2FC$ values were classified as downregulated relative to the control condition.

Enrichment analysis was performed using the DAVID database, which aggregates annotation and enrichment information from various databases, including the Gene Ontology (GO) database. Enrichment p -values were calculated using Fisher's Exact test. For our analyses, we corrected for multiple comparisons using the FDR method and a threshold of ≤ 0.05 . Count and DE tables were used in producing graphical outputs using GraphPad Prism and R-based packages (ggplot2 v 2.0.0).

RNA Sequencing



Bioinformatics workflow

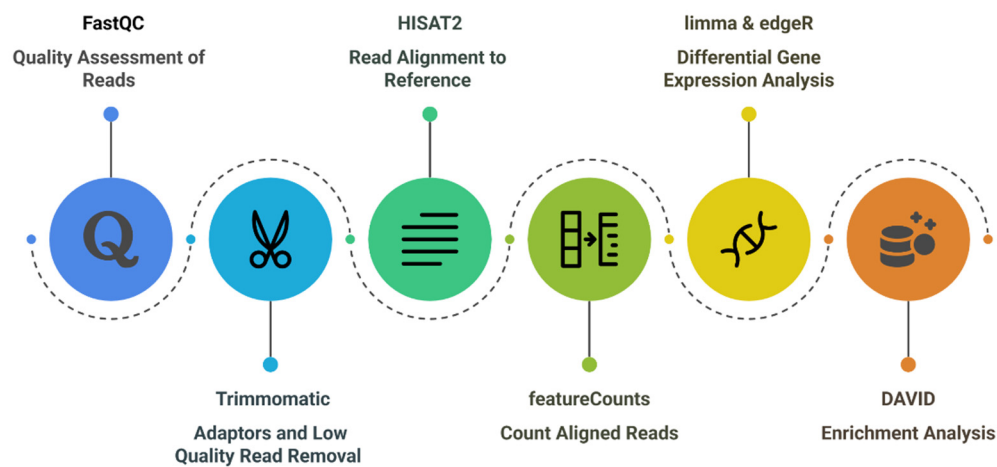


Figure 2.2: Summary of RNA-seq procedure and downstream bioinformatics analyses

2.2.5 Label-free Proteomics

Label-free quantification (LFQ) was utilized to understand the effect of sulfur starvation on the proteome of *P. aeruginosa* PAO1. Briefly, cell pellets obtained from cultures of *P. aeruginosa* PAO1 grown in SFM with or without sulfate supplementation were lysed using urea lysis buffer (50 mM Tris, pH 8.0, 8 M urea, 40 mM NaCl, 2 mM MgCl₂, and 1X HALT protease inhibitor) prior to sonication (4', 30% amplitude, 10 sec, 4 °C). Lysed cells were centrifuged at 10,000 ' g for 10 min to isolate proteins. The total protein in the resulting supernatant was determined using bicinchoninic acid (BCA) assay with PierceTM BCA protein assay kits (Thermo Scientific, Rockford, IL, USA), and 200 µg of each sample was diluted to a total volume of 200 µL using the lysis buffer without urea. Samples were reduced (5 mM DTT) at 32 °C for 30 min and then alkylated (15 mM iodoacetamide) in the dark for 30 min. Denatured, alkylated proteins were digested using trypsin (1: 100 trypsin:protein) at 37 °C overnight after which the samples were acidified with 0.1% FA (final concentration). The resulting peptides were desalted using solid-phase extraction (Sep-Pak® C18 Plus Short Cartridges, Waters) and eluted first with a solution of acidified (formic acid) ACN:H₂O (25:75) followed immediately by a second elution step with 50:50 H₂O:ACN + 0.1% FA. Samples were lyophilized to dryness overnight and then reconstituted in loading buffer (98:2 H₂O:ACN, 0.1% FA) to a final concentration of 0.25 µg/µL. Peptide concentrations were verified using a colorimetric peptide assay (Pierce Quantitative Peptide Assay).

Purified peptides were analyzed by nanoLC-MS/MS using an UltiMate 3000 RSLCnano system coupled to a Q Exactive Plus Hybrid Orbitrap mass Spectrometer (Thermo Fisher Scientific) via nanoelectrospray ionization. Peptides were separated using an effective linear gradient of 4-35% acetonitrile (0.1% formic acid) over 135 min. For data-dependent acquisition,

MS spectra were acquired in positive mode. MS1 scans were performed at a resolution of 70,000 with an AGC target of 2×10^5 ions and a maximum injection time of 100 ms. Data-dependent acquisition was used to collect MS2 spectra on the top 20 most abundant precursor ions with a charge >1 and an isolation window of 1.5 m/z, a fixed first mass of 140 m/z, and a normalized collision energy for MS2 scans of 30. MS2 spectra were acquired at 17,500 resolution with a maximum injection time of 60 ms, an AGC target of 1×10^5 and a dynamic exclusion of 30 sec.

2.2.6 Proteomics Data Analyses and Visualization

Fragpipe^{189,190} (v 20.0) was used for raw data analyses with default parameters for label-free quantification, match between runs (LFQ-MBR) workflows. Precursor ion m/z tolerance was ± 20 ppm with three missed cleavages for Trypsin. Variable modifications included oxidation (+15.5995 Da on Met), carbamylation (+42.025 Da on Lys), and fixed modification carbamidomethyl (+57.025 Da on Cys). MS data were searched against the PA PAO1 reference proteome (Uniprot Proteome ID: UP0000002438, accessed 7/2023) with modifications for common contaminants and decoy (50%) ions. Biological replicates for each experiment were identified in the search. The search results were filtered by a 1% false discovery rate (FDR) at the peptide level. Label-free quantification was carried out using IonQuant.¹⁹¹ Match between runs FDR rate at the ion level was set to 1% for the top 500 runs. High-confidence proteins (protein probability >0.95 , at least two peptides per protein) were filtered and median normalized.

Downstream statistical analysis, quality checks, and results visualization were conducted using a combination of R-based web tools (Fragpipe Analyst),¹⁹² R-statistical packages (tidyverse suite v 1.3.2), and GraphPad Prism on output files from Fragpipe (*combined_protein.tsv* and *sample annotation* files). Differential expression analysis was performed using a protein-wise linear model combined with empirical Bayes using *limma*¹⁸⁸. Perseus-type imputation¹⁹³ was used

to establish relative abundance values for low sampling proteins, and replacing them with random numbers drawn from a normal distribution of 1.8 sd downshift with a width of 0.3 for each sample. The significance threshold was set at the adjusted *p*-value (Benjamini-Hochberg method) and absolute log₂ fold change thresholds of $\leq .05$ and ≥ 1 , respectively. Result graphics were produced with ggplot2 and GraphPad Prism. Functional enrichment analysis was performed using Gene Ontology (GO)¹⁹⁴ and Kyoto Encyclopedia of Genes and Genomes (KEGG)¹⁹⁵ tool on STRINGApp¹⁹⁶ plugin of Cytoscape to identify enriched biological processes and pathways. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium¹⁹⁷ via the PRIDE partner¹⁹⁸ repository with the dataset identifier PXD062994.

2.3 Iron Inventory Investigations

2.3.1 Measurement of Iron Levels Using ICP-OES

Inductively coupled plasma optical emission spectroscopy (ICP-OES) was used to measure Fe levels in spent media (Figure 2.3). For these experiments, *P. aeruginosa* PAO1 cells were grown for 2.5, 5, and 7.5 h in SFM (test) or SFM supplemented with 500 μ M sulfate (control) at 37 °C with constant agitation (220 rpm). Samples were harvested by centrifugation (5000 RCF, 4 °C, 10 min), and the supernatant was collected into 15 mL Falcon tubes following filtration through a 0.22 μ m syringe filter (VWR, Radnor, PA, USA).

Cell pellets were processed further by washing them twice in phosphate-buffered saline containing 100 μ M DTPA followed by two additional washes with Milli-Q (i.e., 18.2 MW/cm resistivity) water before drying the cells to remove excess water. After this, the dried pellets underwent acid digestion for 30 minutes in 1 mL of concentrated trace-metal grade nitric acid

(EMD Millipore, Switzerland) at 90 °C, followed by the addition of 4 mL Milli-Q water in 15 mL Falcon tubes.

Quantitation of Fe in the samples was carried out using an Avio 220 Max ICP-OES spectrometer (PerkinElmer, Waltham, MA, USA), operated in argon mode. The instrument was calibrated before each analysis using a five-point (5-200 µg/L) Fe calibration curve prepared using 2% HNO₃ (v:v). The presence of Fe was monitored at an emission of 238.204 nm, with one ppm of manganese (TruQ Multi-Element Standard, PerkinElmer, USA) used as an internal standard for axial torch alignment with a viewing height observation set at 15 mm. Plasma, auxiliary, and nebulizer gas flows were adjusted to 8, 0.2, and 0.7 L/min, respectively. Plasma power was set to 1500 W. During analysis, the peristaltic pump ran at 1 mL/min while it operated at 2 mL/min during flushing. The read delay, flush, and wash times were set to 45, 90, and 120 seconds, respectively.

2.3.2 Data Analyses and Visualization

An average of three reads per replicate ($N \geq 3$) was obtained from the instrument and analyzed in GraphPad Prism (v 10.4.1) evaluating statistical differences using an unpaired t-test with a significance threshold set at $p \leq 0.05$. To account for differences in Fe abstraction from media due to cell density, Fe concentrations amongst groups were normalized using A_{600} . The abstraction of Fe from spent media was calculated with the formula given below, whereas for Fe in lysate, a straightforward normalization (dividing Fe concentration by mass of pellet) regime was utilized.

$$Relative [Fe]_{spent\ media} = ([Fe]_{media} - [Fe]_{spent\ media})/A_{600}$$

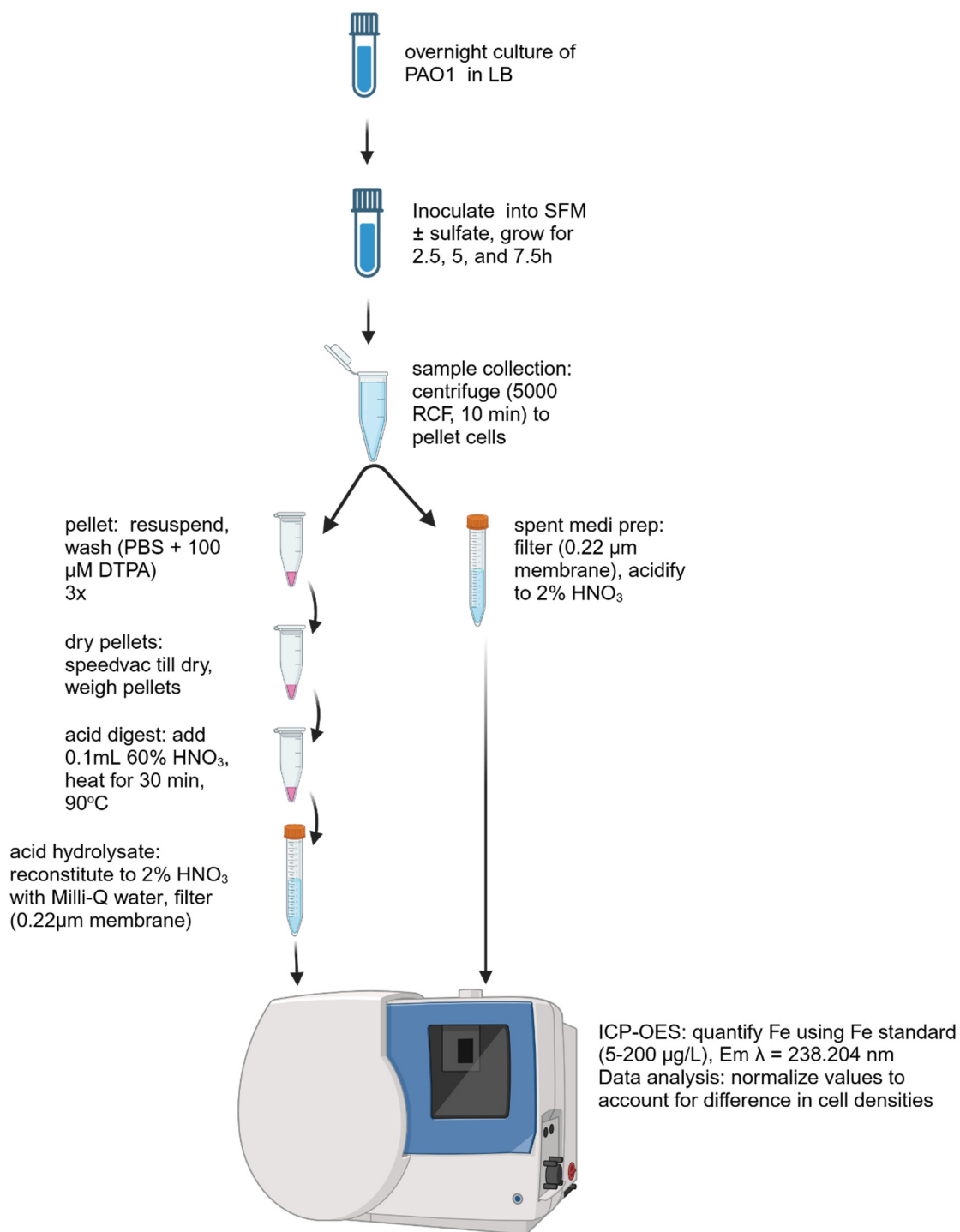


Figure 2.3: Schematic depiction of Fe assay in *P. aeruginosa* cells using ICP-OES

2.4 Monitoring Labile Iron and ROS Levels

2.4.1 Labile Fe assay employing Calcein-AM dye

2.4.1.1 Preparation of Calcein-AM stock solution

Calcein-AM (see Figure 2.4) (ENZO Life Sciences, Farmingdale, NY, USA) was utilized to assay labile iron (Fe^{2+}) levels in *P. aeruginosa* PAO1. Calcein-AM is a cell-permeable, non-fluorescent dye hydrolyzed by intracellular esterases to fluorescent calcein, which remains in the cytoplasm. Calcein fluorescence is quenched by Fe^{2+} binding, providing a measure of intracellular labile Fe^{2+} .

To prepare a one mM Calcein-AM stock solution, 1 mg of Calcein-AM was dissolved in 1 mL anhydrous DMSO. Prior to storage at -20°C , the stock solution was aliquoted into smaller volumes (i.e., 50 μL) to minimize reagent deterioration across multiple freeze-thaw cycles.

2.4.1.2 Experimental Procedure

Overnight cultures of *P. aeruginosa* were inoculated at a 1:1000 dilution into freshly prepared SFM (test) or SFM supplemented with 500 μM sulfate (control). Cultures were incubated aerobically at 37°C with constant agitation (220 rpm). Samples were collected at three time points (2.5, 5, and 7.5 h) post-inoculation to monitor temporal changes in intracellular labile iron levels. At each time point, 1 mL aliquots were withdrawn and centrifuged at 6700 RCF for 5 minutes using an Eppendorf MiniSpin® plus centrifuge (Eppendorf SE, Hamburg, Germany).

Thereafter, harvested cells were washed and resuspended in 1 mL phosphate-buffered saline (PBS, pH 7.4), and Calcein-AM was added to a final concentration of 5 μM . The samples were incubated in the dark at ambient temperature for 30 minutes to allow intracellular cleavage and fluorescence generation. After incubation, cells were pelleted by centrifugation (6700 RCF)

and washed twice with PBS (pH 7.4) to remove unincorporated Calcein-AM. Pellets were then resuspended in PBS.

Fluorescence measurements were conducted using a Varioskan Flash spectral scanning multimode reader (ThermoFisher Scientific, Waltham, MA, USA). For fluorescence detection, 150 μ L aliquots of each cell suspension were transferred into sterile, black, flat-bottom 96-well Fluorotrac plates (Greiner Bio-One, Frickenhausen, Germany). Each experimental condition was measured in quadruplicate to enable robust statistical evaluation. Untreated cells (washed with PBS without Calcein-AM) served as controls to establish baseline fluorescence.

The fluorescence was measured kinetically over a 10-minute period at one-minute intervals using excitation and emission wavelengths of 495 nm and 515 nm, respectively. The excitation bandwidth was set to 5 nm. The instrument was programmed to maintain constant agitation and an incubation temperature of 37°C throughout measurements to ensure consistent cell suspensions and physiological conditions.

A parallel experiment was set up to monitor absorbance (OD_{600}) to assess cell growth using clear-bottom 96-well plates (Greiner Bio-One, Frickenhausen, Germany).

2.4.1.3 Data Analyses

Fluorescence data were extracted and processed using a custom R script (accessible on the GitHub repo). Fluorescence intensity values obtained during the kinetic assays were summed over the 10-minute measurement period for each replicate, averaged, and the standard deviation was calculated. To control for variability in cell density between samples, fluorescence values were normalized to their corresponding A_{600} readings.

Background fluorescence obtained from blank wells (containing PBS without Calcein-AM) was subtracted from each experimental sample reading to obtain corrected fluorescence

values. Labile iron levels in *P. aeruginosa* under test and control conditions were visualized by plotting normalized fluorescence values against sampling time points (2.5, 5, and 7.5 h).

Statistical differences between test and control conditions were analyzed using the Welch two-sample t-test, which is suitable for groups with unequal variances and was performed with GraphPad Prism software (v 10.4.1). Statistical analyses were conducted to test differences in means between conditions at specified time points, with results presented in bar charts displaying the mean $\pm$ standard deviation of the measurements.

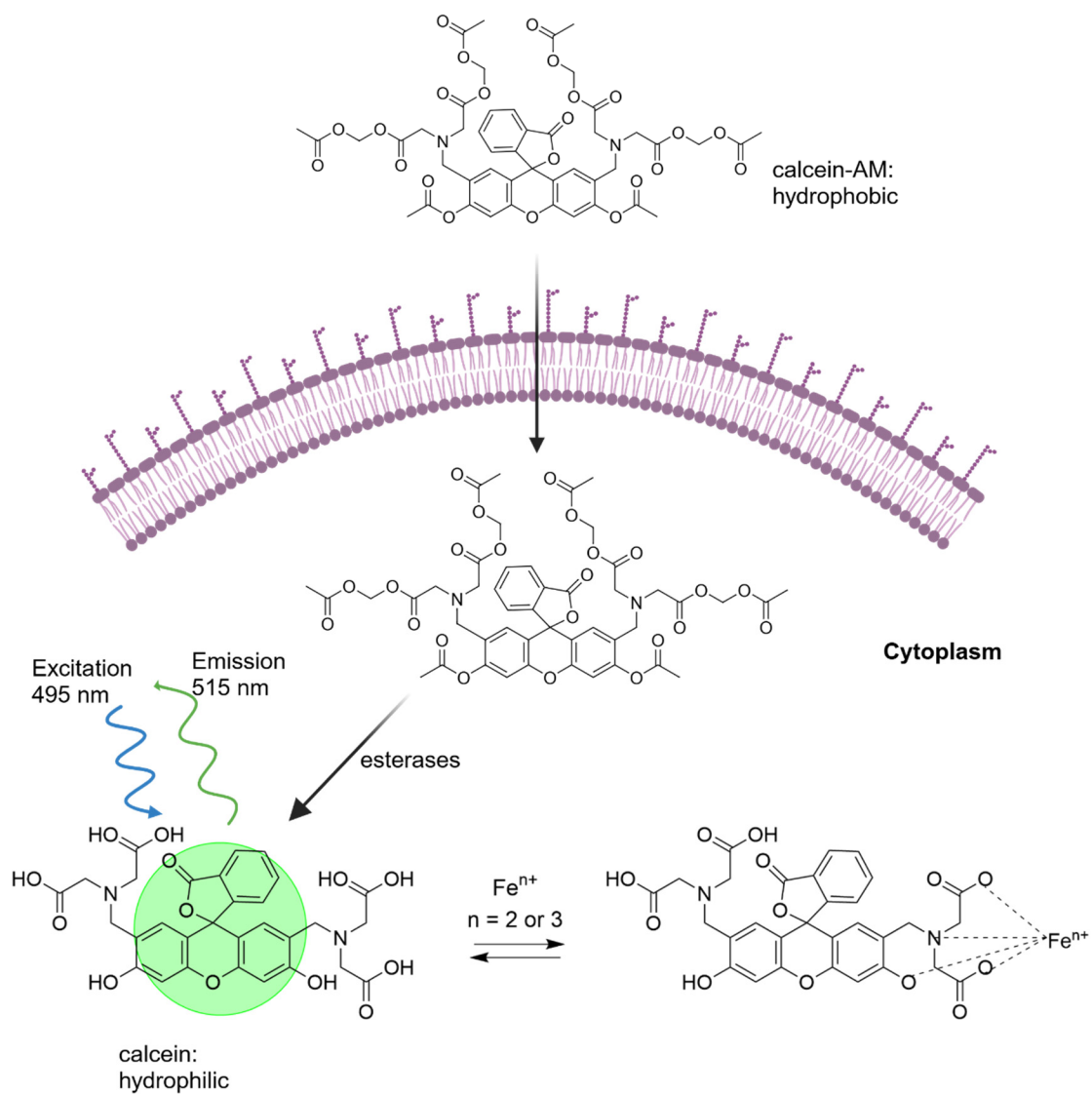


Figure 2.4: Calcein-AM, a membrane-permeable fluorescent dye, is used in labile Fe assays

2.4.2 ROS Assay using DCFH-DA Dye

Total intracellular reactive oxygen species (ROS) levels in *P. aeruginosa* PAO1 were also evaluated by a fluorescence-based method using the broad-spectrum ROS fluorophore 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) (AAT Bioquest, Pleasanton, CA, USA). DCFH-DA (25 mg) was dissolved in anhydrous DMSO to prepare a 51.3 mM stock solution. Prior to storage at -20°C , this solution was aliquoted into smaller volumes (50 μL each) to minimize reagent deterioration with repeated freeze-thaw cycles. With minor modifications, the same methodology and instrumentation were used to monitor labile Fe with Calcein-AM (see above). In particular, cells were incubated with DCFH-DA at a final concentration of 10 μM (instead of 5 μM for Calcein-AM). For the DCFH-DA fluorophore, the excitation wavelength was set to 505 nm and the emission wavelength to 526 nm.

All other experimental steps, including bacterial culture conditions, cell harvesting, incubation, washing, resuspension, OD normalization, and data analyses, were the same as those described previously for Calcein-AM.

2.5 Quantification of Key Fe-Uptake Metabolites Using HPLC

Quantitative analysis of Fe-uptake metabolites, specifically phenazines and pyoverdine, was performed using High-Performance Liquid Chromatography (HPLC). Filtered spent media samples from test and control cultures, prepared as described above, were utilized for these assays.

2.5.1 Phenazine Quantification (Pyocyanin; PYO)

Pyocyanin (PYO) levels were quantified using reversed-phase HPLC (Agilent 1100 Series, Agilent Technologies, USA). Briefly, samples (20 μ L injection volume) were separated on a reversed-phase Pinnacle II C18 column (Zorbax, Agilent, USA; 5 μ m particle size, 4.6 $\times$ 150 mm). Chromatographic separation was achieved with a gradient mobile phase composed of solvent A (0.1% trifluoroacetic acid [TFA] in water) and solvent B (0.1% TFA in acetonitrile) at a flow rate of 1 mL/min and a total run time of 25 min. The column temperature was maintained at 25°C under isothermal conditions. Absorption was monitored at 690 nm using a diode array detector (DAD). For accurate identification and quantification, a pyocyanin standard (10 μ M) was run alongside experimental samples, allowing for the identification and quantification of PYO in both test (i.e., SFM) and control (i.e., sulfate-supplemented SFM) samples by comparison of retention times and peak areas. This method was adapted and modified from Recinos et al.¹⁹⁹

2.5.2 Pyoverdine Quantification

Pyoverdine was quantified using an HPLC instrument (Agilent 1260 Infinity II, Agilent Technologies, USA) with variable wavelength (absorption) and fluorescence detectors. Chromatographic separation was performed on a C18 column (Pinnacle II, ThermoFisher Scientific, USA; 5 μ m particle size, 4.6 $\times$ 250 mm). The same gradient solvents (solvent A: 0.1% TFA in water, solvent B: 0.1% TFA in acetonitrile) used for phenazine separation were employed,

with a 1 mL/min flow rate. The injection volume for each sample was 20 μ L. Absorbance was monitored at 405 nm, and fluorescence detection utilized an excitation wavelength of 400 nm and emission at 450 nm.

Although no pyoverdine standard was used, validation of pyoverdine detection was conducted by analyzing spent media from *P. aeruginosa* PAO1 cultured in rich media (LB broth) both with and without the addition of the iron-chelating agent diethylenetriaminepentaacetic acid (DTPA). Cultures grown in LB media supplemented with DTPA (known to induce pyoverdine production) demonstrated increased fluorescence when normalized by optical density (A_{600}), thus confirming that the fluorescence observed corresponded to pyoverdine. This validation approach ensured reliable identification and relative quantification of pyoverdine, despite the absence of an external pyoverdine standard. This protocol was modified from Zygiel et al.²⁰⁰

For both metabolites (pyocyanin and pyoverdine), quantification was performed by normalizing obtained peak areas to the optical density (A_{600}) of bacterial cultures to account for differences in cell density between experimental conditions.

2.6 Time-course Label-free Quantitative Proteomics

A time-based proteomics experiment investigated temporal changes in antioxidant protein expression in *P. aeruginosa* during sulfur stress. The experimental design consisted of independent cultures harvested at three time points (2.5, 5, and 7.5 h) after inoculation. Each time point represented an independent experiment comparing cultures grown in SFM to control cultures grown in SFM supplemented with 500 μ M sulfate.

2.6.1 Sample preparation

At each sampling time (2.5, 5, and 7.5 h), 1 mL aliquots of each culture were harvested by centrifugation (5000 RCF, 10 min). The cell pellets were processed for proteomic analysis using the PreOmics iST kit (PreOmics GmbH, Germany), following the manufacturer's protocol with minor modifications.

Briefly, cell pellets were lysed using 50 μ L of the provided lysis buffer from the PreOmics iST kit and incubated at 95 °C in a heat block (VWR, USA) for 10 minutes. Protein concentrations were determined via bicinchoninic acid (BCA) assay with the Pierce™ BCA Protein Assay kit (Thermo Scientific, Rockford, IL, USA). Bovine serum albumin (BSA) was used as a protein standard. Specifically, 10 μ L aliquots of lysed samples were diluted tenfold in PBS, mixed with 100 μ L BCA reagent, and compared against a BSA calibration curve (2.0–0.25 mg/mL).

Based on BCA quantification, 50 μ g protein from each sample was trypsin-digested using 50 μ L PreOmics DIGEST buffer. Samples were digested at 37 °C for 3h. Following this, 100 μ L of STOP buffer was added to end digestion. Peptides were purified using the kit-provided spin-columns and eluted using ELUTION buffers. This was followed by resuspension in 50 μ L LC-LOAD buffer. Peptide concentration was estimated using a nanodrop spectrophotometer (Implen, Westlake, CA, USA). Moreover, A_{205} was measured to estimate the relative concentration of the peptide backbone in samples.

2.6.2 LC-MS/MS Analyses

Samples were analyzed on an Orbitrap Exploris 240 mass spectrometer (ThermoFisher Scientific) coupled to an UltiMate 3000 RSClnano system (Dionex, Germering, Germany), which included a binary pump, column compartment, and autosampler. Chromatographic separation was carried out on an EASY-Spray PepMap RSLC C18 column (2 μ m, 100 Å, 50 μ m $\times$ 15 cm) in

tandem with an Acclaim PepMap C18 trapping column (3 μm , 100 $\text{\AA}$). The mobile phases consisted of 0.1% (v/v) formic acid in water (Buffer A) and 0.1% (v/v) formic acid in 80% acetonitrile and 20% water (Buffer B). Peptides were eluted at a flow rate of 300 nL/min using the following gradient program: 3.5% Buffer B for 15 minutes, followed by a linear increase to 25% Buffer B at 100 minutes, then to 50% Buffer B at 120 minutes, and to 98% Buffer B at 128 minutes with a 2-minute hold. The solvent was then returned to 3.5% Buffer B over 2 minutes, followed by a 10-minute re-equilibration at 3.5% Buffer B.

Full MS scans were acquired over an m/z range of 375–1500 at a resolving power of 120,000, with advanced peak width determination and RunStartEasyIC in peptide application mode. The normalized automatic gain control (AGC) target was set to 300%, the radio frequency (RF) lens was adjusted to 70%, and one microscan was used. MS/MS acquisition was performed in top 10 data-dependent mode, restricting the charge state range to 2–5. Dynamic exclusion was enabled after one occurrence, with a 10-ppm tolerance and an exclusion duration of 45 seconds. An intensity filter with a minimum of 5000 was applied. Fragment ions were detected at a resolution of 30,000 using a normalized fixed HCD collision energy of 30%, and the isolation window was set to 1.6 m/z .

2.6.3 Data Analyses and Visualization

Raw data obtained from LC-MS/MS runs were processed as previously described for label-free proteomics, with modifications to the downstream statistical analyses for comparing means between groups. The processed count file from LFQ-Analyst was analyzed using an R-based custom script (Github Repo). Briefly, a 2×2 factorial design was employed to compare antioxidant protein expression under two growth conditions. Each condition was analyzed at three independent

sampling points (2.5, 5, and 7.5 h). A two-way analysis of variance (ANOVA) was performed separately for each protein to assess the main effects of treatment and time, as well as their interaction. Post-hoc pairwise comparisons were conducted using t-tests, with p -values adjusted for multiple testing using the Benjamini-Hochberg false discovery rate (FDR) method.²⁰¹ Comparisons with an adjusted p -value of 0.05 or lower were considered statistically significant.

Box plots were generated to display the $\log_2$ -transformed LFQ intensity values distribution for each gene across the four experimental groups. The plots were faceted by protein, with individual replicate data points overlaid as jittered dots to depict variability. Statistical annotations were added to the plots to indicate the significant pairwise comparison p -values ($p \leq 0.05$).

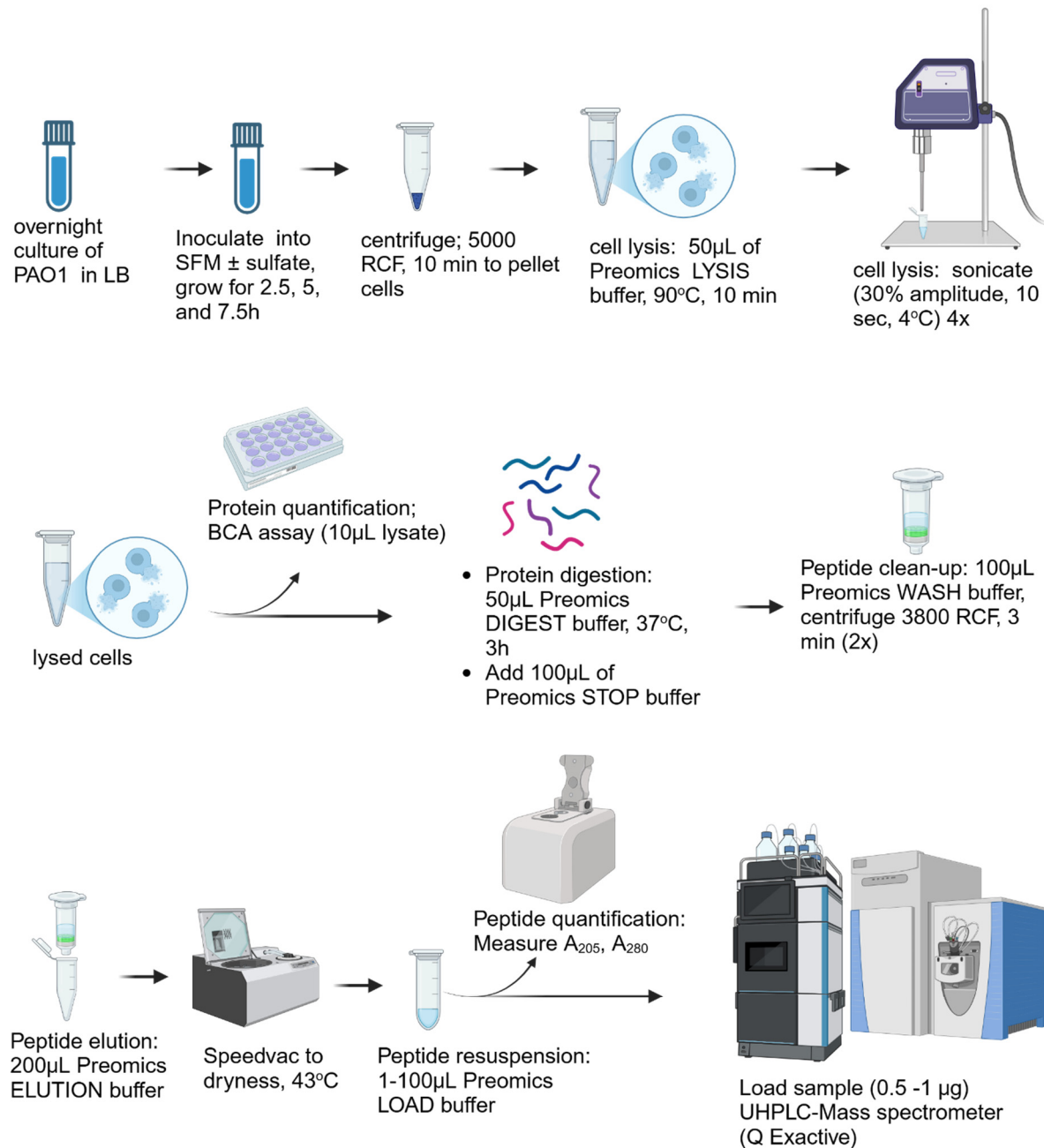


Figure 2.6: Schematic outlining sample preparation steps for time-course proteomics

2.7 Computational Gene Regulatory Network Analysis

To elucidate the key genes involved in regulating the sulfur starvation response in *P. aeruginosa*, RNA sequencing (RNA-seq) data were analyzed to construct a gene regulatory network (GRN). The count table was obtained from RNA-seq (refer to methodology section 2.3) and was employed for analysis. All analyses were performed using custom R and Python scripts (included in Github repository).

2.7.1 Identification of Potential Regulators

The normalized count data from RNA-seq analyses were used as gene *expression data* input for analyses. A list of probable and validated transcription factors was compiled to focus on potential regulatory genes within the differentially expressed genes (DEG) list from our RNA-seq result. Gene annotations were sourced from the Pseudomonas Genome Database²⁰² and the Predicted Prokaryotic Transcription Factors (P2TF) database.²⁰³ A search query was formulated using keywords associated with transcriptional regulation: '*transcriptional*,' '*sigma*,' and '*two-component*.' This query targeted genes annotated as transcription factors, sigma factors, and two-component response regulators. The search yielded 74 potential regulatory genes within the DEG subset.

2.7.2 Gene Regulatory Network Inference Using GENIE3

The gene expression data were formatted into a matrix suitable for input into the Gene Network Inference with Ensemble of Trees (GENIE3) algorithm (v1.24.0).³⁵ GENIE3 is a machine learning (ML) method that infers gene regulatory networks by solving regression problems for

each target gene, using expression levels of potential regulators as predictors. It employs the ensemble method, Random Forests, to model complex, nonlinear gene interactions.

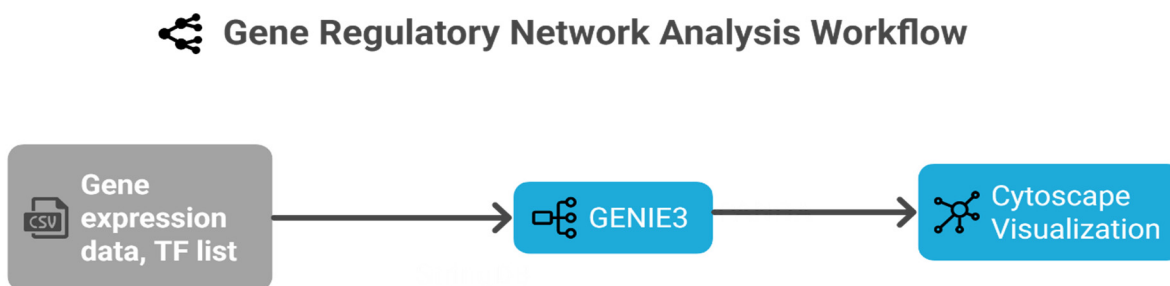


Figure 2.7: Flowchart of tools used for Gene Regulatory Network (GRN) analysis

The GENIE3 algorithm was executed with the following parameters: **Regulators:** Set to the list of 74 potential transcription factors identified from the DEGs. **Targets:** Included all 1,053 DEGs to ensure comprehensive network inference among genes responsive to sulfur starvation. **nTrees:** Set to 1,000, which indicates the number of trees in the ensemble for each regression task. The algorithm generated a weight matrix, where each entry represents the **importance** score of a regulatory link between a regulator and a target gene. Based on the expression data, these scores reflect the strength and likelihood of regulatory interactions.

2.7.3 Network Extraction and Formatting

From the weight matrix produced by GENIE3, the top regulatory interactions were extracted for network analysis. The *importance* scores were sorted in descending order, and the top 1,000 regulatory links were selected based on their scores. The list of regulatory links was formatted into an edge list with 'source' (regulator) and 'target' (target gene) columns to ensure

compatibility with Cytoscape software.²⁰⁴ Additionally, the weight of each interaction was included as an edge attribute to represent the strength of the predicted regulatory link.

2.7.4 Evaluating GRN Model Performance

To evaluate the predictive performance and biological accuracy of the final gene regulatory network (GRN), standard metrics, including precision and recall, were calculated. This assessment focused specifically on transcription factors (TFs) that have experimentally validated target genes. Initially, experimentally confirmed targets for a selected TF were compiled from established literature and curated regulatory databases, creating a reference dataset for performance evaluation.

Subsequently, predicted target genes for the TF were extracted from the GRN output, selecting top-ranked interactions based on prediction weights to define a set of high-confidence predicted interactions. Predicted targets were then compared against the experimentally validated targets, facilitating the identification of true positives (TP; predicted targets with experimental validation), false positives (FP; predicted targets lacking experimental evidence), and false negatives (FN; experimentally confirmed targets not captured by the GRN).

These comparisons enabled the calculation of *precision*, defined as $TP/(TP + FP)$, reflecting the accuracy of the predicted interactions, and *recall*, defined as $TP/(TP + FN)$, indicating the model's ability to recover experimentally validated interactions. Higher precision reflects fewer false-positive predictions, whereas higher recall indicates greater sensitivity in capturing known regulatory interactions. Both metrics were interpreted jointly to provide a balanced and integrated assessment of the GRN model's predictive reliability and biological relevance.

2.7.5 Visualization Using Cytoscape

Cytoscape (v3.10.1) was employed for network visualization and analysis.²⁰⁴ The network data were imported into Cytoscape, wherein the *Network Analyzer* tool was utilized to compute network statistics, including node degree, betweenness centrality, and clustering coefficients. Visual mapping was applied based on node degree metrics to identify key hub genes within the regulatory network. Nodes were visually mapped using the out-degree metric, representing the number of outgoing edges from a node (i.e., the number of targets regulated by a transcription factor). Nodes with higher out-degree values were assigned larger sizes and colored by their degree of differential expression.

2.8 Data and Code Access

Raw RNA sequencing reads (fastq), count data, and metadata files have been deposited at the National Center for Biotechnology (NCBI) under Bioproject ID [PRJNA1109844](#) with sequence read archive (SRA) accession numbers: [SRR28980933-SRR28980938](#) and will be publicly accessible upon manuscript publication. Proteomics files (.Raw files, fasta, and associated filtered protein files and metadata) have been deposited in the PRIDE database with an accession number (PXD062994). All codes used in data analyses, including result tables, are deposited in the project's [GitHub](#) repository and will be made publicly accessible once the core of this work is published.

CHAPTER THREE: Results

3.1 RNA-seq Analyses of *P. aeruginosa* PAO1 Under Sulfur Starvation

Our study aimed to investigate the expression of genes, particularly antioxidant genes, during sulfur starvation in bacteria, using *P. aeruginosa* PAO1 as a model organism. We utilized functional genomics tools to obtain a comprehensive overview of gene expression changes associated with sulfur starvation. To fully evaluate the correlation between sulfur starvation and activation of reactive oxygen species (ROS) response genes, we first compared the growth of *P. aeruginosa* PAO1 in minimal media lacking sulfur (test group) to the growth of PAO1 in media supplemented with 500 μ M sulfate (control) (Figure 3.1a). Following this, we modeled the growth of our samples using Gompertz and logistic growth curves, respectively (Figure 3.1b). This step was crucial for determining the exponential growth phase of our samples, during which metabolic activity is at its maximum. We then conducted next-gen RNA sequencing (RNA-seq) on cells collected at the exponential growth phase.

Results from RNA-seq showed extensive changes in gene expression patterns between sulfur-starvation and sulfate-supplemented conditions, with just over 1,000 genes differentially expressed (Figure 3.2b; [Supplementary Table S1](#)), using an FDR-adjusted p -value threshold of ≤ 0.05 and an absolute $\log_2$ fold change (LogFC) of ≥ 1 . Gene Ontology (GO) enrichment analysis of differentially expressed genes (DEGs) revealed that many of the genes with an increase in expression (fold change ≥ 10) were involved in sulfur and organosulfur compound uptake and metabolism, referred to as sulfate starvation-induced (SSI) genes (Figure 3.3, Figure 3.4; [Supplementary Table S2](#)). These include genes of the *cys*, *ssu*, and *msu* operons that encode ABC transporters, FMN-dependent reductases/monooxygenases, dioxygenases, and sulfatases.

Together, they form the sulfur starvation regulon required for scavenging and desulfurizing organosulfur compounds in diverse bacteria, including *P. aeruginosa* PAOI.^{24,27,39,67}

Over 500 genes showed decreased expression, with approximately half of these genes directly or indirectly involved in iron (Fe) metabolism based on their GO terms (Figure 3.4; [Supplementary](#) Table S2). A defined link between iron metabolism and sulfur starvation has not been previously reported.

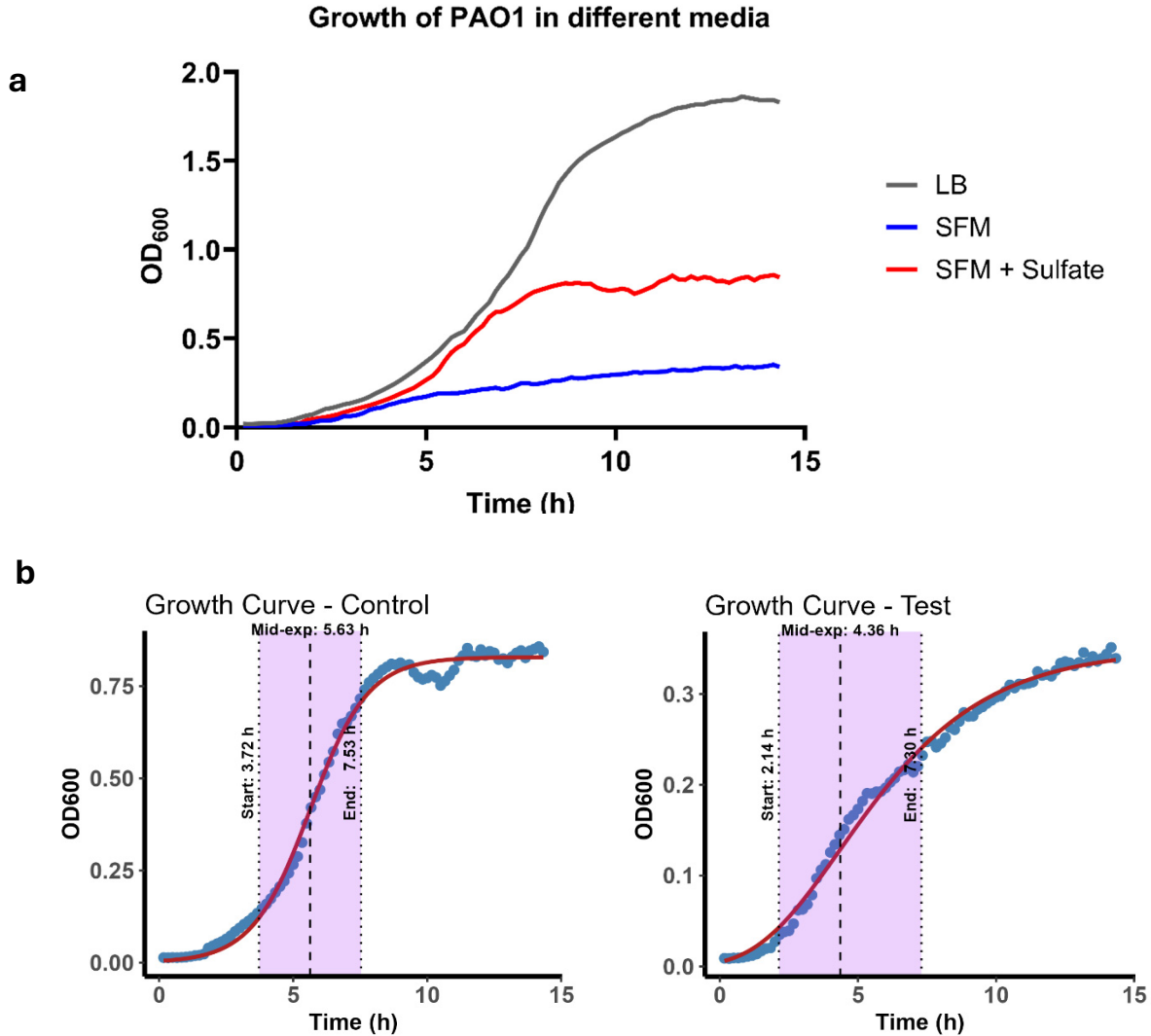


Figure 3.1: Sulfur starvation alters *P. aeruginosa* growth and defines the exponential phase **a)** Growth curves of *P. aeruginosa* PAO1 in sulfur-free minimal medium (SFM) or SFM supplemented with 500 μ M sulfate (SFM + sulfate) both show marked growth depression relative to rich LB medium, with the sulfur-deprived condition exhibiting the slowest increase in OD₆₀₀ **b)** To define the log-phase window, we fitted both Gompertz and logistic models to each dataset and selected the model minimizing the residual sum of squares (RSS). The logistic model best described the sulfate-supplemented (control) data, whereas the Gompertz model was optimal for sulfur-starved (test) cells. From these best-fit curves (red lines), the exponential phase (70% of maximal growth rate) spans **3.7–7.5 h** for controls and **2.1–7.3 h** for the test condition

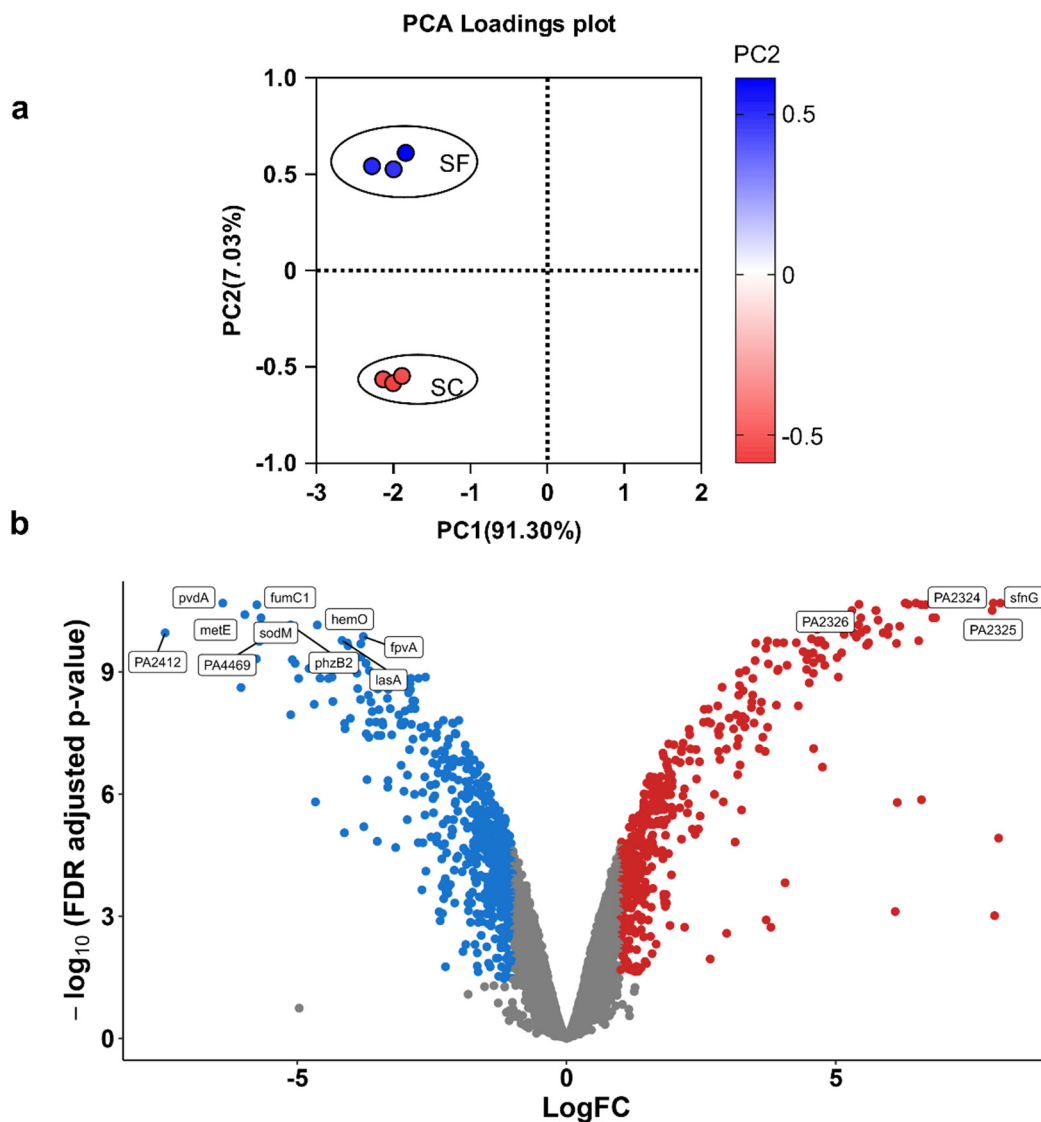


Figure 3.2: Transcriptome divergence induced by sulfur starvation **a)** Principal component analysis (PCA) was performed to assess sample-to-sample variance. The first two components together explain ~98.3% of the total variance (PC1 = 91.3%, PC2 = 7.03%). Biological triplicates cluster tightly, indicating high reproducibility, and PC2 separates the test (blue) from the control (red) samples. The absence of overlap between the test and control clusters highlights a strong treatment effect **(b)** The volcano plot displays the $\log_2$ fold change (LogFC) between PAO1 cells grown without sulfur (test) and with 500 μM sulfate (control) on the x-axis, and the negative $\log_{10}$ of the false discovery rate (FDR)-corrected p -value on the y-axis. This analysis revealed 468 upregulated genes (red), 585 downregulated genes (blue), and 4,605 unchanged genes (grey) based on an absolute $\text{LogFC} \geq 1$ and corrected $p\text{-value} \leq 0.05$. The top 14 differentially expressed genes, ranked by both p -value and LogFC, are highlighted in the plot, with *pvdA* and *sfnG* being among the most downregulated and upregulated candidates, respectively, in the test versus control group

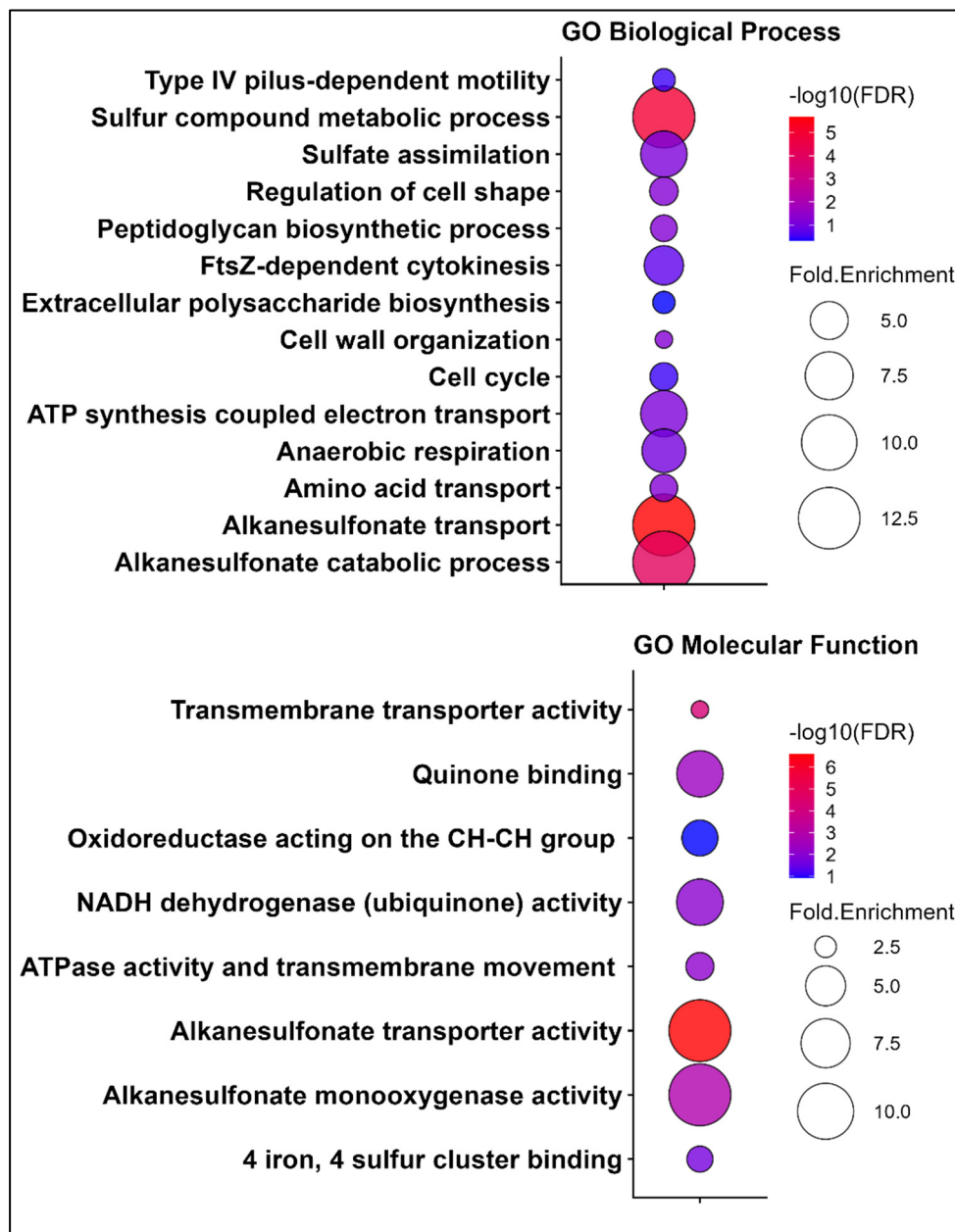


Figure 3.3: GO enrichment among genes upregulated by sulfur starvation. Bubble plots of the top Gene Ontology terms in Biological Process (upper) and Molecular Function (lower) categories enriched among genes induced under sulfur starvation conditions ($FDR \leq 0.05$). Each bubble's size reflects fold enrichment over background, and its color intensity corresponds to $-\log_{10}(\text{adjusted } P\text{-value})$. Sulfur-compound metabolic processes emerge as the most highly enriched biological pathway

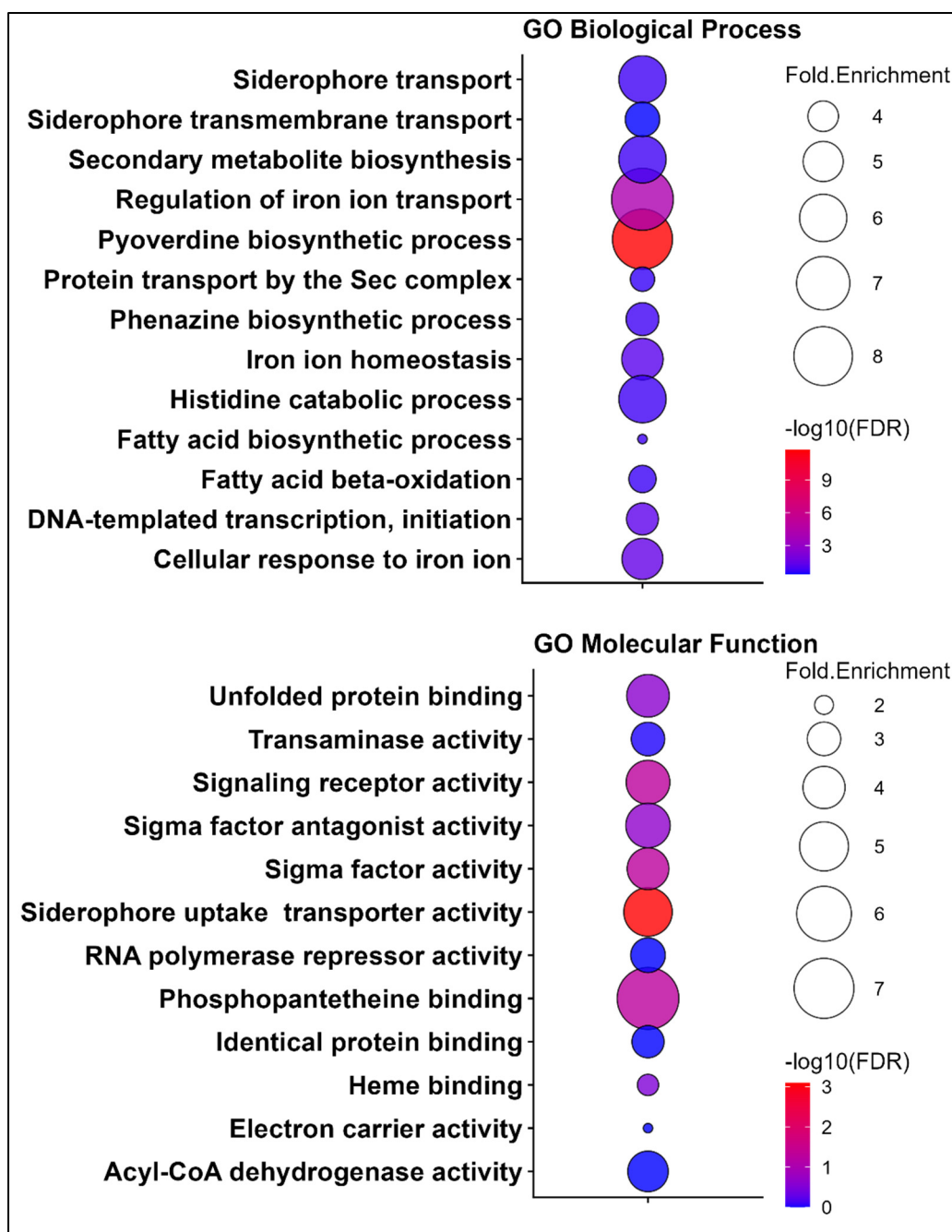


Figure 3.4: GO enrichment among genes downregulated during sulfur starvation Bubble plots of the top Gene Ontology terms in Biological Process (upper) and Molecular Function (lower) categories enriched among genes repressed under sulfur starvation conditions ($\text{FDR} \leq 0.05$). Bubble size indicates fold-enrichment relative to the genome background, and bubble color reflects $-\log_{10}(\text{adjusted } P\text{-value})$. Iron-related processes dominate the enriched terms among down-regulated genes

3.1.1 Impact of Sulfur Starvation Conditions on Sulfur Acquisition and Assimilation

Prior studies on sulfur metabolism in pseudomonads have established the expression of sulfur-scavenging genes when challenged with alternative sulfur sources. Major gene clusters involved in organosulfur metabolism include the *msu*, *cys*, *ssu* and *sfn* operons, each of which encodes monooxygenases, ABC transporters, and transcriptional regulatory genes. Notably, *sfnG*, which encodes an FMNH₂-dependent monooxygenase involved in utilizing alkanesulfonate, was among the most upregulated genes in response to sulfur starvation (~260-fold increase) (Figure 3.6a). Other upregulated genes involved in sulfur scavenging include *PA0183* (*atsA*), an arylsulfatase (> 95-fold increase); *msuD* and *msuE*, involved in methanesulfonate metabolism, both increased by over 50-fold during sulfur starvation (Figure 3.5a). Although CysB is a putative regulator for the *msu* and *sfn* operons, there was only a marginal increase in *cysB* expression (*PA1754*) (nearly twofold increase). Additional genes involved in cysteine metabolism and sulfur assimilation (*cysAWT*, *cysND*, *cysI*, *cysP*, and *cysH*) also regulated by CysB showed varying changes in expression levels with fold changes ranging from 2.5 to 25× (Figure 3.6a). Similar modest increases in expression levels have previously been observed for *P. aeruginosa* PAO1 in the absence of sulfate with alternative sulfur sources.²⁶

3.1.2 Oxidative Stress Response During Sulfur Starvation

Expression of genes involved in the oxidative stress response increases when alternative sources are used to provide sulfur.²⁶ A similar oxidative stress response was observed under sulfur starvation conditions as in the present work. In our hands, one of the most upregulated antioxidant genes was 1-Cys peroxiredoxin (*lsfA*, *PA3450*) (>45 fold increase) (Figure 3.5b). The *LsfA* protein detoxifies ROS generated by NADPH oxidase produced by macrophages. This detoxification leads

to increased virulence in *P. aeruginosa* strains.²⁰⁵ The *ohr* gene (*PA2850*) involved in detoxifying different fatty acid hydroperoxides and peroxyxynitrite, was strongly upregulated in our results (>40 fold increase).⁷² A previous transcriptomics study also noted upregulation of the *ohr* gene with alternative sulfur sources.²⁶

Surprisingly, our results showed downregulation of a few antioxidant genes, including the catalases (*kataA* and *kataB*), as well as the alkyl hydroperoxide reductases (*ahpC*, *ahpF*, *ahpB*). All of these showed modest decreases in expression of 2-3-fold (Supplementary Table S1). These antioxidant genes are regulated by OxyR, an H₂O₂ sensor in *P. aeruginosa* PAO1. Our RNA-seq data revealed that *oxyR* was unchanged (fold change = 1.5, FDR = 0.62). This may explain why the transcription of genes under its direct regulatory control remained unchanged or marginally downregulated. The increased expression of the *ohr* gene (Figure 3.5b), whose regulation is independent of OxyR, suggests an oxidative stress mechanism that is potentially independent of H₂O₂ as a stressor. At the time point used to evaluate differential expression, lipid hydroperoxides could be generated in the absence of a sulfur source, leading to an increase in *ohr* expression. The upregulation of antioxidant and sulfur-scavenging genes coincides with enhanced expression of iron storage genes, suggesting a coordinated response to mitigate oxidative stress and maintain iron homeostasis.

Other genes involved in the ROS response that were differentially expressed include genes expressing superoxide dismutase (*sod*) (Figure 3.5 b). In *P. aeruginosa* PAO1, the principal SODs are SodA (Mn-SOD) and SodB (Fe-SOD). The *sodB* gene showed >2-fold increase in expression while *sodA* showed a ~50-fold decrease in expression in response to sulfur-starved test compared to sulfate-supplemented control. As will be discussed later, expression of the *sodB* gene is

dependent on Fe-replete conditions and would show increased expression relative to *sodA* when iron is abundant. Additionally, genes involved in the pentose phosphate and Entner–Doudoroff pathways, including glucose-6-phosphate dehydrogenase (*zwf*) and phosphogluconate dehydratase (*edd*), were upregulated (Figure 3.5c). The role of these proteins in generating a redox buffer during oxidative stress through the generation of NADPH and glutathione in *Pseudomonas* species has been previously reported.^{206,207}

Overall, we observe a significant overexpression of antioxidant genes that have been typically known to be expressed during sulfur limitation or starvation in bacteria, with some exceptions—the slight depression of some catalases and alkyl hydroperoxide reductases.

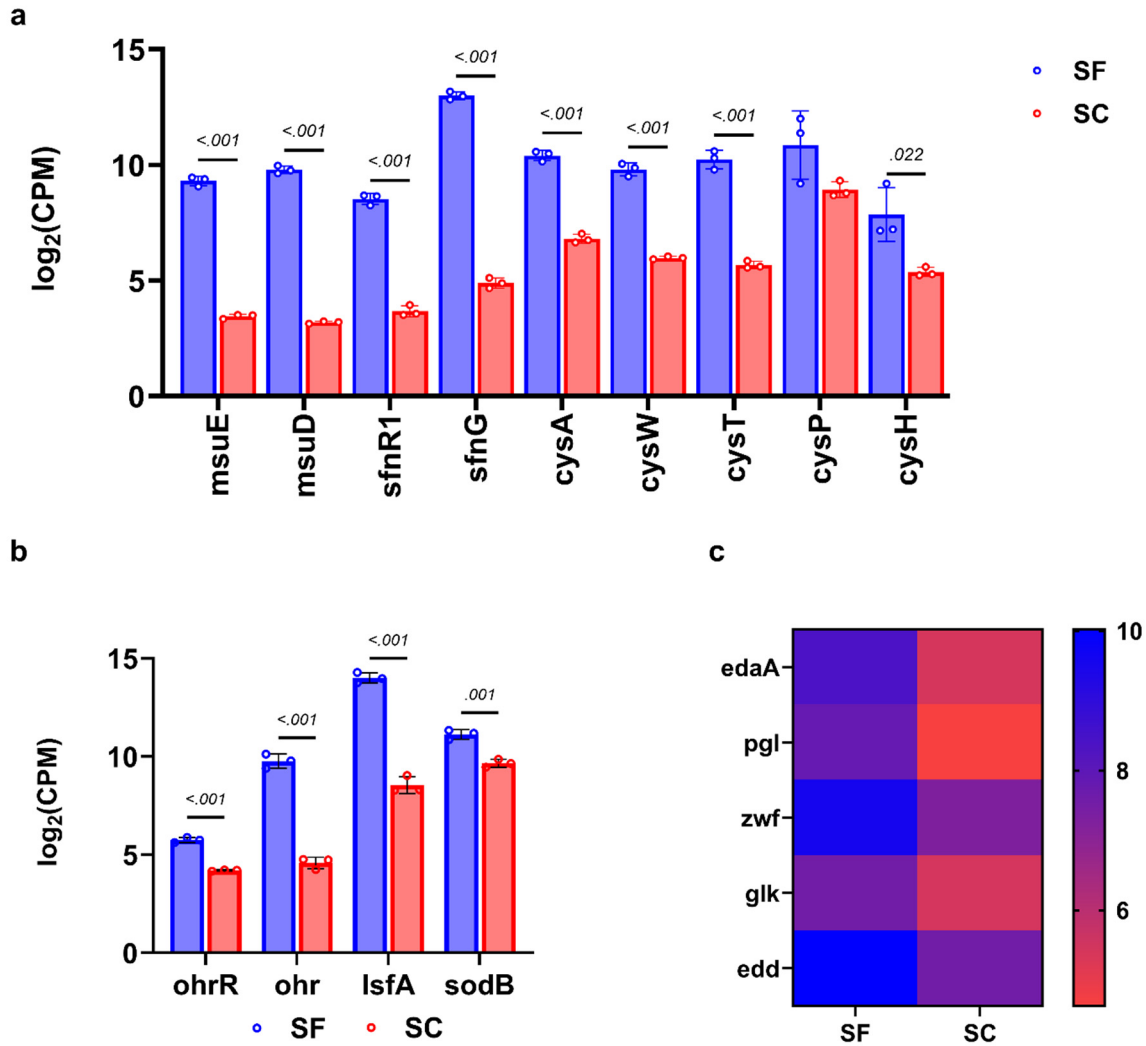


Figure 3.5: Co-overexpression of sulfur scavenging and antioxidant genes during sulfur starvation. a) Overexpression of genes from the *cys* and *msu* operons and transcription factors from the *sfn* family involved in regulating genes associated with organosulfur uptake across the test (SF) and control group (SC) **b)** Upregulated antioxidant genes, including *lsfA* (encoding a l-cys peroxiredoxin), and *ohr* (and its transcription regulator *ohrR*), both involved in metabolizing organic hydroperoxides **c)** Heatmap displaying upregulation of genes in the pentose phosphate (PP) and Entner-Doudoroff (ED) pathways. Gene expression levels were compared between SF and SC conditions using unpaired t-tests. Results are reported as mean $\pm$ SD. Multiple comparisons were corrected using FDR ($Q = 5\%$). Statistical significance was defined as $p \leq 0.05$

3.1.3 Iron Replete Conditions During Sulfur Starvation

Interestingly, we observed differential expression of genes involved in iron (Fe) metabolism during sulfur starvation. Two general features characterized this. First, there was a strong downregulation of genes involved in Fe uptake. Second, there was a pronounced upregulation of genes involved in Fe storage.

With respect to iron uptake by *P. aeruginosa* PAO1, iron is scavenged via the use of siderophores, siderophore piracy (utilization of xenosiderophores), utilization of host heme proteins (cytochromes and hemoglobin through Phu and Has uptake systems), and direct internalization of ferrous iron using redox-active phenazines and the Feo system.^{88,115} *P. aeruginosa* PAO1 produces two major classes of siderophores: pyoverdine (PVDI) and pyochelin (PCH). To utilize PVDI, the major pyoverdine-class siderophore produced in *P. aeruginosa* PAO1, FpvA (*PA2398*) is required. FpvA is a TonB-dependent outer membrane receptor involved in iron uptake, ferrying PVDI-Fe^{III} from the extracellular space into the periplasm.⁸⁶ In addition to FpvA, at least three other accessory proteins are involved in the uptake of PVDI, including FpvG (*PA2403*), FpvH (*PA2404*), and FpvC (*PA2407*), with the latter two acting as a reductase and an Fe²⁺ chelator, respectively, in a process that allows PVDI-Fe dissociation in the periplasm of *P. aeruginosa* PAO1.²⁰⁸ The genes encoding these receptor proteins, except *fpvA*, are part of a large operon (*PA2403–PA2410*) involved in ferripyoverdine uptake.²⁰⁸ Interestingly, we observed that the operon showed decreased expression, with changes ranging from 8- to 13-fold decreases. (Supplementary Table S1). Moreover, *fpvA* (*PA2398*), upstream of the operon, was also strongly downregulated. Separating the *fpvA* gene from the downstream *PA2403–PA2410* cluster is another operon encoding genes necessary for pyoverdine biosynthesis (*pvdG*, *pvdJ*, and *pvdL*). These were also strongly downregulated, with decreases from 10- to 30-fold observed (Figure 3.6a).

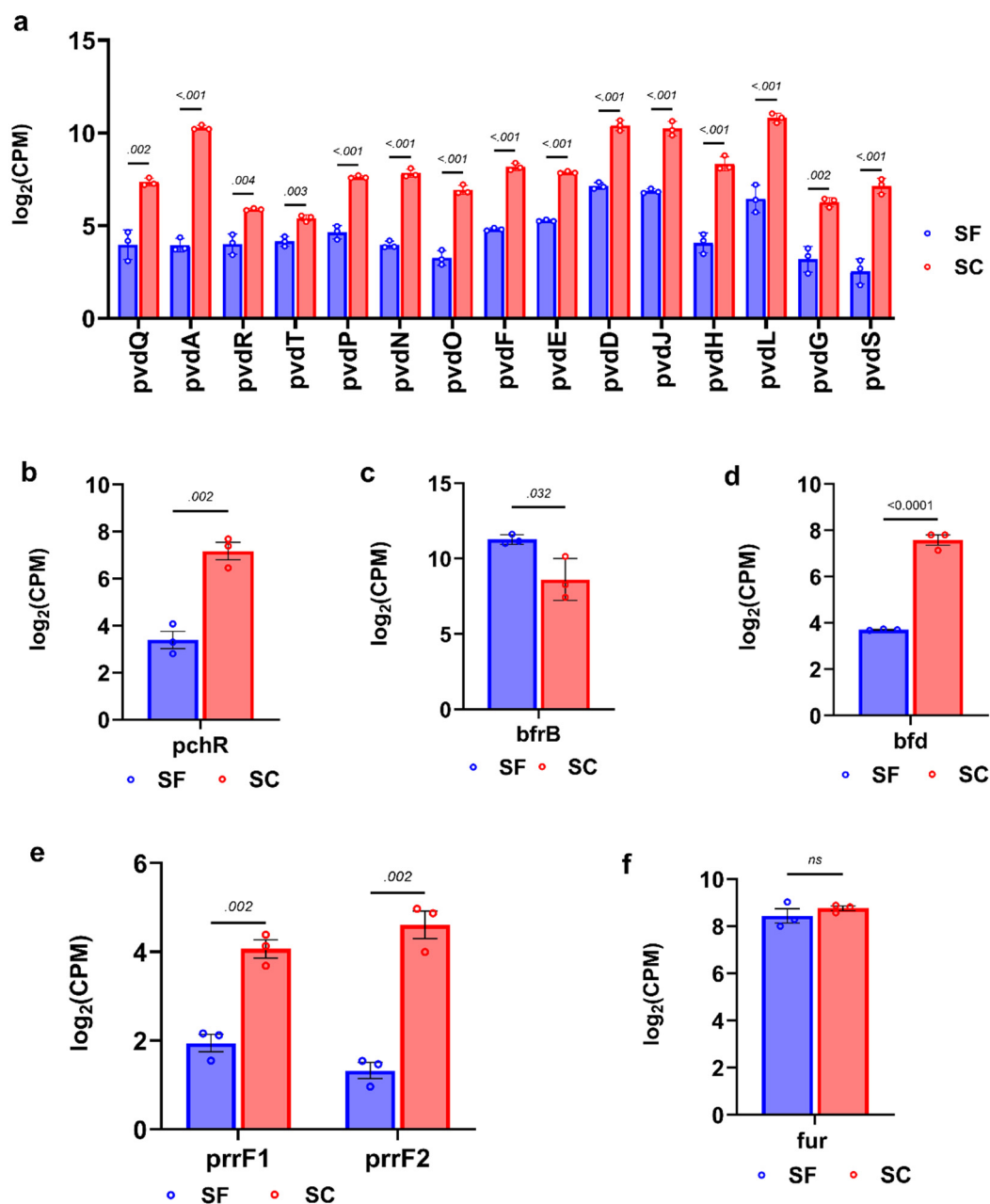


Figure 3.6: Sulfur starvation induces an Fe-replete response marked by repressed iron uptake and elevated storage **a)** Significant downregulation of *pvd* genes encoding pyoverdine biosynthetic machinery **b)** Reduced expression of *pchR*, a key regulator of pyochelin synthesis and uptake **c)** Strong induction of *bfrB*, encoding an iron storage protein (~7.8-fold increase) **d)** Repression of *bfd*, involved in mobilizing iron from BfrB. **e)** Decreased levels of small non-coding RNAs *prfF1* and *prfF2*, both involved in iron homeostasis. **f)** *fur* transcript levels remain unchanged between SF and SC groups, suggesting constitutive expression regulated post-transcriptionally by intracellular Fe. Gene expression was compared between SF and SC using unpaired t-tests. Results are reported as mean $\pm$ SD; multiple comparisons were FDR-corrected ($Q = 5\%$), with statistical significance defined as $p \leq 0.05$

Notably, the gene demonstrating the largest decrease in expression, *PA2412* (>150-fold decrease) (Supplementary Table S1), encodes an MbtH-like protein which is necessary for the production and secretion of pyoverdine.²⁰⁹ This further highlights the suppression of pyoverdine synthesis and accessory genes in response to sulfur starvation.

Like pyoverdines, the pyochelin (PCH) siderophore is secreted into the environment, where it binds ferric iron to form the PCH-Fe^{III} complex. The complex is internalized through the membrane transporters FptA and FptX.¹¹³ The genes for the biosynthesis and transport of pyochelin (*PA4218–PA4231*) are housed in two operons, which include the core biosynthetic clusters *pchDCBA* and *pchEFGHI* and their activator *pchR*, an AraC-type transcriptional regulator.^{106,210} In our results, *pchR* (*PA4227*) showed decreased expression (>10-fold decrease) (Figure 3.7b), as were many of the genes in the two adjacent operons (*PA4218–PA4231*) involved in pyochelin synthesis and transport. The Fe(III)-pyochelin outer membrane receptor, including *fptA* (*PA4221*), and the inner membrane permease *fptX* (*PA4218*), both showed a >2-fold decrease (Supplementary Table S1).

Other genes involved in Fe uptake that showed decreased expression in our RNA-seq data include those involved in xenosiderophore uptake, such as *femA*, *fiuA*, and *chtA* (fold changes ranging from 2- to 4-fold decrease) (Supplementary Table S1), and genes involved in ferrous Fe uptake, including *feoB* and *feoA* (7-fold and 17-fold decrease, respectively). Additionally, we observed decreased expression (>10-fold decrease) of the *PA3407* gene encoding the heme acquisition protein HasAp (Supplementary Table S1). Along with other proteins encoded by the *has* operon, HasAp is implicated in virulence by mediating the uptake of host heme to scavenge iron during Fe-limiting conditions.²¹¹

As noted earlier, we also observed the upregulation of genes involved in iron storage, reinforcing that sulfur limitation induces an Fe-replete response. The principal iron storage proteins in *P. aeruginosa* PAO1 are bacterioferritin BfrB (*PA3531*) and FtnA (*PA4235*). While the gene encoding BfrB showed increased expression (~ 7-fold increase) (Figure 3.6c), there was no significant change in *ftnA*. BfrB is the major iron-storage protein in *P. aeruginosa* PAO1 and associates with Bfd (*PA3530*; bacterioferritin-associated ferredoxin) to mobilize Fe³⁺ stored in BfrB when cytosolic iron levels drop below a certain threshold. This mobilization is facilitated by ferredoxin reductase (*PA3397*, FprA).²¹² FprA reduces Fe³⁺ to the soluble ferrous form for cellular release. It is particularly striking that the genes for Bfd and its accessory protein FprA were strongly downregulated in our results (~ 10-fold decrease) (Figure 3.6d). Decreased *bfd* and *fprA* expression would prevent premature iron mobilization in cells already overburdened with unincorporated Fe. Additionally, in the context of sulfur starvation, depressing the levels of Bfd, a 2Fe-2S ferredoxin, would help conserve sulfur for critical sulfur-containing proteins.

The ferric uptake regulator Fur is a key transcriptional repressor that controls iron metabolism in bacteria, regulating most iron metabolism-associated genes discussed earlier. Fur tightly regulates siderophore- and non-siderophore-mediated iron uptake to minimize toxicity induced by excess iron.²¹³ Under Fe-replete conditions, apo-Fur dimers form a ferrous iron complex (which acts as a co-repressor) and bind to a consensus target sequence, the Fur box, in the promoter regions of target iron uptake genes.¹⁶³ Although essential for iron homeostasis, the expression level of the *fur* transcript was unchanged during sulfur stress in our RNA-seq data (Figure 3.6f).

We observed that a few noncoding RNAs (ncRNAs) were also differentially expressed. Two of them, *prfF1* and *prfF2*, are involved in iron metabolism, inhibiting the expression of nonessential iron-containing proteins during iron-limiting conditions.^{126,214} Fur represses expression of PrrF sRNAs, acting as a positive regulator for genes that the PrrF sRNAs inhibit. We observed a decrease in expression of the *prfF* genes (> 4-fold decrease) (Figure 3.6e), suggesting Fur may be involved in orchestrating the response even though it was not differentially expressed. Superoxide dismutase B (*sodB*) and the bacterioferritin gene (*bfrB*), are also positively regulated through the action of the PrrF sRNAs. In our results, *sodB* and *bfrB* demonstrated increased expression that likely correlates with decreased expression of the *prfF* genes.

In summary, we observed an overall response that includes the overexpression of genes involved in iron storage, a response typically seen in Fe-replete conditions. Conversely, there was a strong downregulation of genes involved in the various pathways used by *P. aeruginosa* to scavenge iron, including those for siderophores, heme metabolism, and ferrous iron uptake.

3.2 Label-free Proteomics Result Supports RNA-Seq Data

To validate the findings from our RNA-seq experiments, we conducted label-free proteomics on *P. aeruginosa* PAO1 cultures grown in sulfur-free media with or without sulfate supplementation. Our proteomics data identified 729 differentially abundant proteins compared to the 1,053 differentially expressed transcripts in our RNA-seq data, ([Supplementary Table S3](#)), with the core enriched pathways being consistent between the two datasets (Figure 3.7 b-c; Figure 3.8; Figure 3.9).

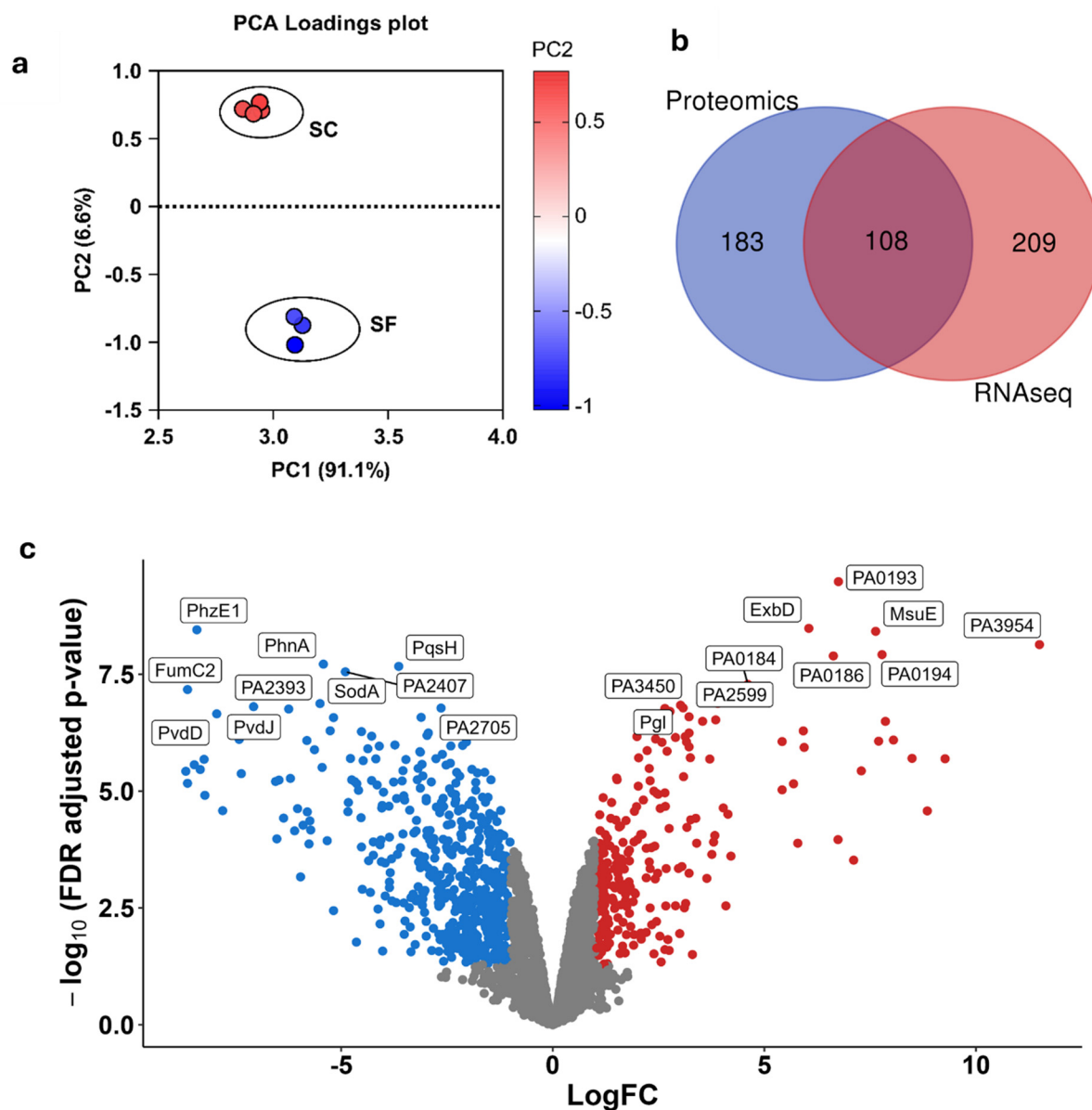


Figure 3.7: Label-free proteomics confirms transcriptomic changes under sulfur starvation **a)** PCA of proteomics data reveals strong separation between sulfur-free (SF, blue) and sulfur-containing (SC, red) samples along PC2 (6.6%), with PC1 accounting for 91.1% of total variance. Tight clustering of biological replicates indicates high reproducibility. **b)** Overlap of strongly differentially expressed features ($|\text{LogFC}| \geq 2$, $\text{FDR} \leq 0.001$) between RNA-seq and proteomics datasets **c)** Volcano plot showing differential protein abundance between SF and SC conditions. A threshold of $|\text{LogFC}| \geq 1$ and $\text{FDR} \leq 0.05$ identified 246 upregulated (red), 483 downregulated (blue), and 1,904 unchanged (grey) proteins

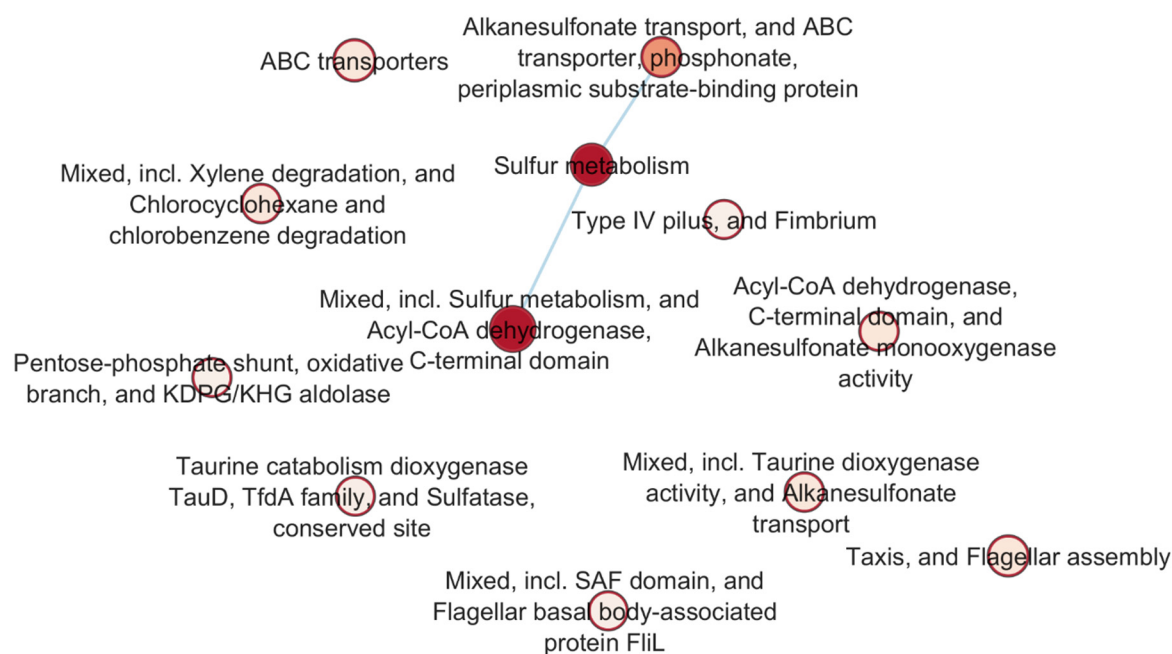


Figure 3.8: Functional enrichment of upregulated proteins during sulfur starvation Upregulated proteins were analyzed in Cytoscape using StringAPP and KEGG pathway enrichment, applying a redundancy cutoff of 0.5. EnrichmentMap was used to visualize significantly enriched terms (FDR $q < 0.05$). Each labeled node represents a pathway or biological process, with size proportional to the number of associated proteins and color indicating statistical significance (darker = lower q -value). Edges reflect shared proteins between terms. Consistent with our RNA-seq result, sulfur metabolism pathways emerged as the most significantly enriched

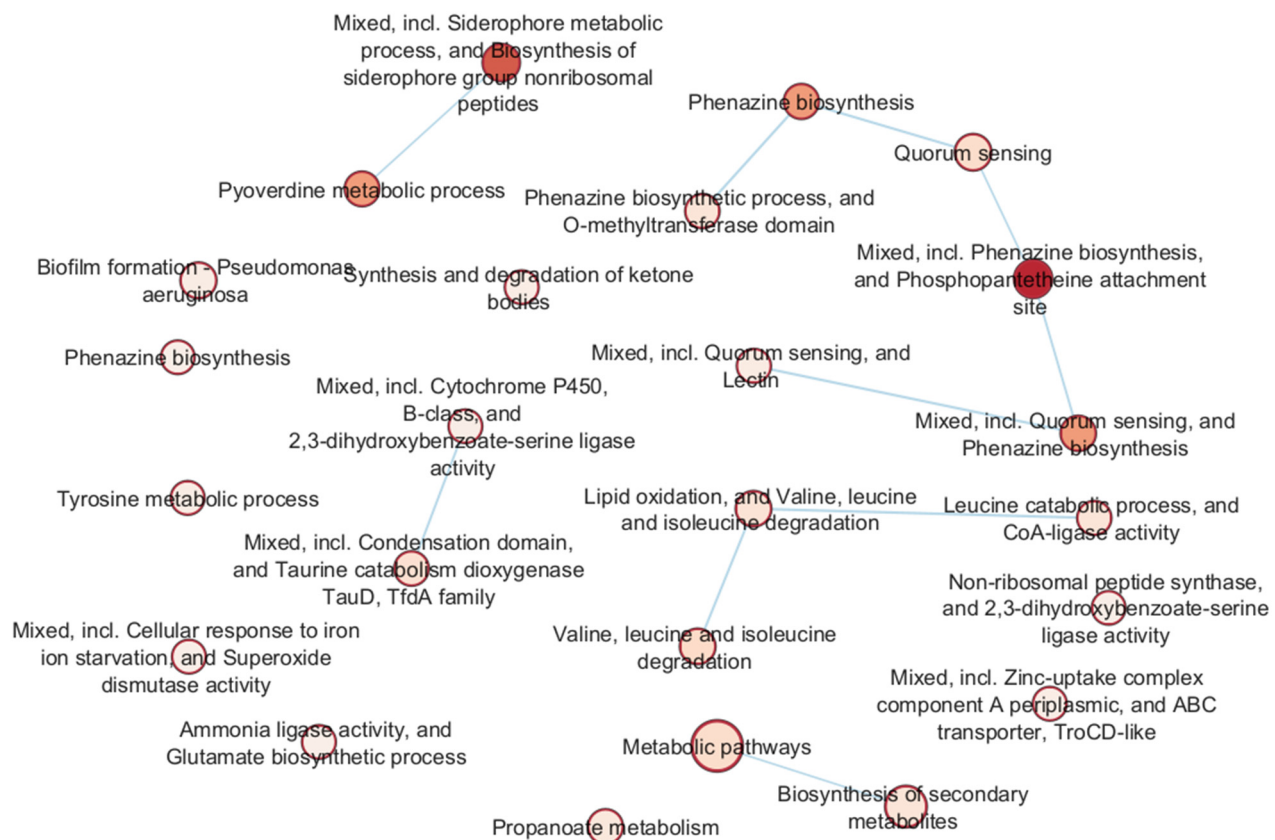


Figure 3.9: Functional enrichment of depressed proteins during sulfur starvation downregulated proteins were analyzed in Cytoscape using StringAPP and KEGG pathway enrichment, applying a redundancy cutoff of 0.5. EnrichmentMap was used to visualize significantly enriched terms (FDR $q < 0.05$). Each labeled node represents a pathway or biological process, with size proportional to the number of associated proteins and color indicating statistical significance (darker = lower q -value). Edges reflect shared proteins between terms. Siderophore, phenazine and quorum-sensing pathways were the most enriched

As with our RNA-seq data, we observed upregulation of proteins involved in the utilization of alternative sulfur compounds, with SfnG showing increased expression ($>2,800$ -fold change) in the sulfur-starved test versus control. Other sulfur-scavenging proteins that were overexpressed include those from the *msu* (SfnR1, PA2355, MsuDE) and *ssu* (SsuBDE) operons, with fold changes ranging from 2- to 470-fold (Supplementary Table S3). We also observed upregulation (2- to 6-fold change) of permease proteins involved in taurine metabolism (TauAC), as well as modest upregulation of proteins involved in cysteine metabolism, including CysW and the adjacent sulfate-binding protein (Sbp) (Supplementary Table S3).

We observed upregulation of the cognate proteins corresponding to the overexpressed antioxidant genes from our RNA-seq data. Some of the upregulated antioxidant proteins include Ohr ($\sim 2\times$) and LsfA ($8\times$) (Supplementary Table S3). However, SodB showed a marginal increase that was not statistically significant (fold change < 2 , $q > 0.05$). Conversely, we observed a strong downregulation of SodA (~ 45 -fold decrease), which may serve as a means of conserving manganese and ensuring that only iron-requiring isoforms of proteins are expressed during Fe-replete conditions. Consistent with our RNA-seq data, we also observed increased expression of proteins involved in the pentose phosphate and Entner-Doudoroff pathways, including phosphogluconate aldolase (Eda) and 6-phosphogluconolactonase (Pgl), with approximately 6-fold and 8.5-fold increases, respectively (Supplementary Table S3).

Notably, our proteomics data revealed the same pattern of downregulation of iron uptake proteins and pathways, as well as an increase in proteins involved in iron storage, consistent with our RNA-seq results. This reinforces the notion of an Fe-replete condition induced by sulfur starvation. Moreover, the downregulation of proteins involved in siderophore-mediated

(pyoverdine and pyochelin) uptake pathways was accompanied by a strong (3.5- to 50-fold) downregulation of proteins involved in heme metabolism (Figure 3.10a-c), including proteins of the Phu and Has systems involved in heme uptake and metabolism. Conversely, iron storage proteins, including the major *P. aeruginosa* PAO1 bacterioferritin BfrB (PA3531), were upregulated (four-fold increase) (Figure 3.12d). Furthermore, we observed overexpression of two proteins, PA0962 (Dps) and PA4880 (DpsL), which have been noted to serve as stores for ferric iron during iron-induced oxidative stress in *P. aeruginosa* PAO1.²¹⁵

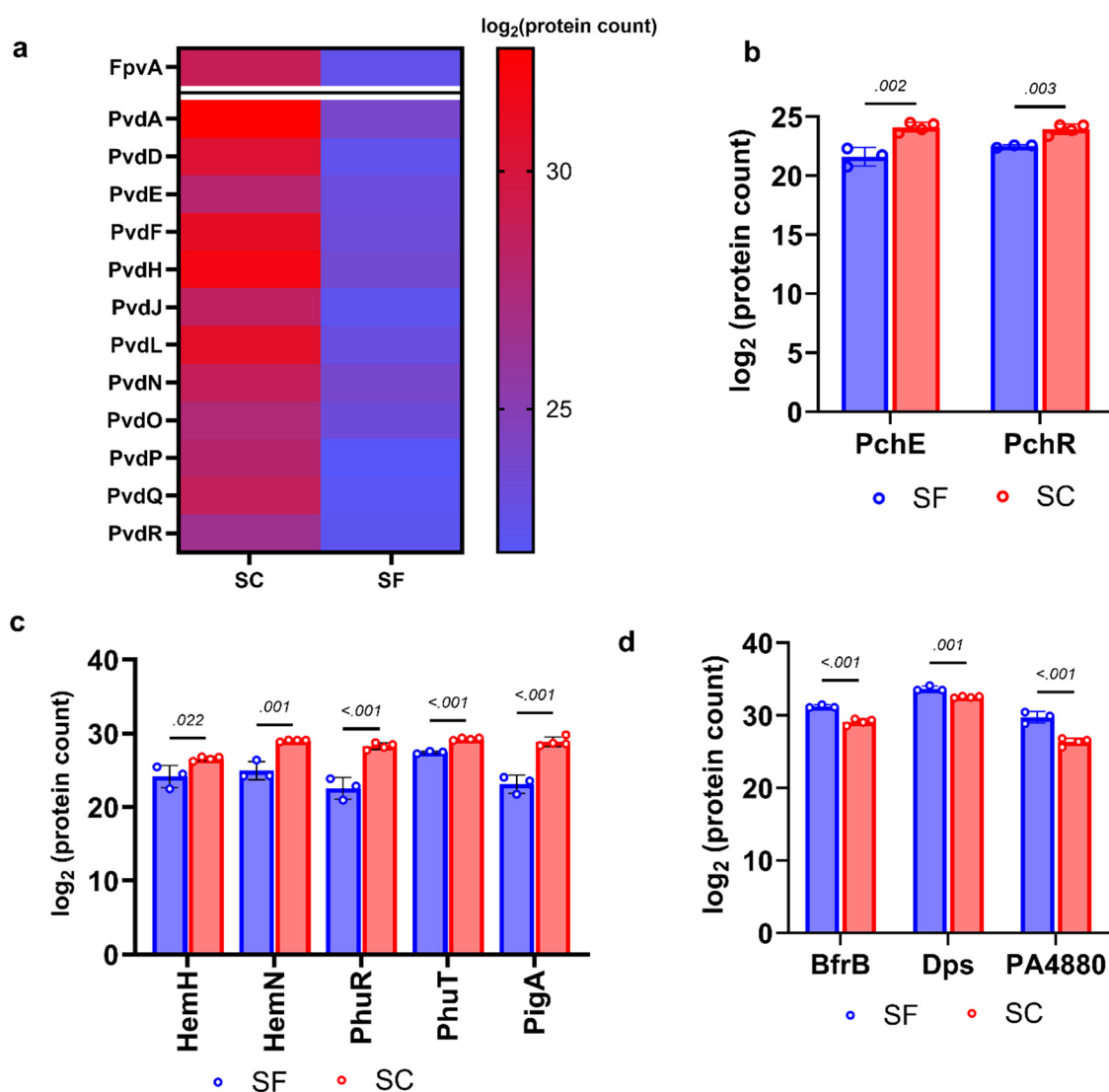


Figure 3.10: Proteomics data reinforce an Fe-replete response during sulfur starvation a) Heatmap showing downregulation of pyoverdine biosynthesis proteins under sulfur starvation (SF) conditions. **b)** Bar chart indicating reduced levels of PchE and PchR, involved in pyochelin metabolism. **c)** Downregulation of Phu and Has system proteins associated with heme uptake. **d)** Upregulation of iron storage proteins BfrB, Dps, and PA4480 under SF conditions. Protein expression (mean $\pm$ SD) was compared between SF (n = 3) and sulfur-containing (SC, n = 4) conditions using unpaired t-tests with FDR correction (Q = 5%). Variance was assessed by F-test, and Welch's correction was applied due to unequal sample sizes

3.3 Sulfur Starvation Induces Downregulation of Iron-Linked Virulence Genes

A key observation from our transcriptomic and proteomic profiling is the significant downregulation of gene products involved in virulence in *P. aeruginosa* PAO1, many of which are linked to iron metabolism.^{121,216–218} Several genes encoding transcription factors, transport proteins, sigma factors, and small RNAs associated with virulence were differentially expressed in our results.

3.3.1 Multidrug resistance (MDR) Efflux Pumps

Multidrug resistance (MDR) efflux pump transcripts, including *mexR* (PA0424), *mexA* (PA0425), *mexG* (PA4205), *mexH* (PA4206), *mexI* (PA4207), and *opmD* (PA4208), were downregulated with fold changes ranging from 2- to 13-fold (Figure 3.11a) in our transcriptomics data. The *mexGHI-opmD* operon encodes an efflux pump involved in resistance to multiple antibiotics and is induced by endogenously produced phenazines.²¹⁹ The downregulation of the *mexGHI-opmD* operon is likely linked to the observed decrease in phenazine biosynthesis genes. The MexAB-OprM efflux pump system, which has a broad substrate range, is regulated by the MexR repressor located upstream of the operon.^{220,221} In our results, while *mexA* was downregulated (fold change >2), *mexB* and *oprM* remained relatively unchanged. Interestingly, *mexR* was also downregulated (fold change >2) in our RNA-seq data (Figure 3.11a).

Our proteomic data showed a similar trend; however, only MexH, OpmD, and MexT were significantly downregulated, with corresponding fold changes of 14, 3, and 2.8, respectively (Supplementary Table S3). MexT, a LysR-type transcriptional regulator, acts as a transcriptional activator for another *P. aeruginosa* multidrug efflux pump system, *mexEF-OprN*, and also as a repressor for the outer membrane porin, OprD.²²² Moreover, it has been identified as a significant

contributor to type III secretion (T3SS) systems in *P. aeruginosa*, which is important for virulence.²²³

3.3.2 Phenazines and Pyocyanin

Phenazines are aromatic, redox-active secondary metabolites produced predominantly by *Pseudomonas* species^{155,224} and have been implicated in virulence, iron assimilation, and primary energy metabolism in *P. aeruginosa*.²²⁵ In *P. aeruginosa* PAO1, phenazines are initially synthesized as phenazine-1-carboxylic acid (PCA) via two independently acting, homologous operons: *phz1* (*phzA1B1C1D1E1F1G1*) and *phz2* (*phzA2B2C2D2E2F2G2*) that each encode seven enzymes. Three additional modifying enzymes—PhzH, PhzS, and PhzM—are required to convert the core PCA molecule into complex phenazine derivatives, including pyocyanin.^{155,224}

Our RNA-seq results showed decreased expression of four of the seven genes in both phenazine clusters, with fold changes ranging from 2- to 80-fold (Figure 3.11b), with more significant downregulation observed in the *phz2* cluster. Notably, the three remaining genes of the clusters (*phzD*, *phzE*, *phzF*) were absent from our results. This omission is potentially an artifact of our read-counting algorithm, which excludes multi-mapping reads to minimize potential over- or underestimation of paralogous genes. The high sequence identity of paralogous genes in both *phz* clusters (*phz1* and *phz2*) may lead to certain genes being entirely omitted. Additionally, the three genes encoding the tailoring enzymes required for the biosynthesis of PCA derivatives—*phzH*, *phzS*, and *phzM*—were downregulated with a fold change of at least two. Results from our proteomics experiments further validate this trend, showing strong downregulation of proteins encoded by genes from both clusters of the phenazine biosynthetic operons, with fold changes ranging from 8- to 340-fold (Figure 3.12a).

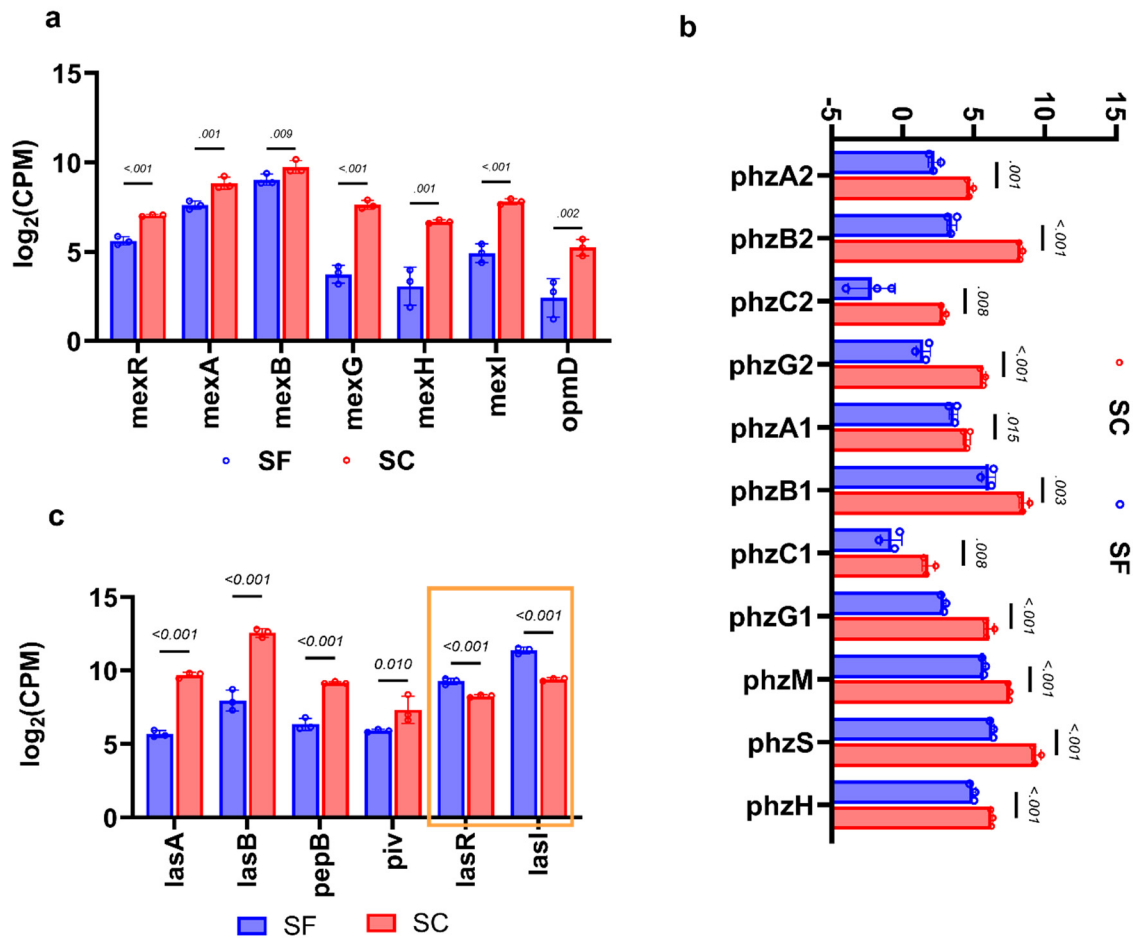


Figure 3.11: Depression of transcripts associated with virulence during sulfur stress **a)** Downregulation of genes of the *mex* operon involved in efflux pump biosynthesis in the sulfur starvation (SF) group **b)** significant downregulation of genes in both phenazine biosynthetic clusters in the sulfur starvation versus control **c)** Barchart showing decreased expression of genes encoding virulence-associated proteases, including *lasA*, *lasB*, *pepB*, and *prpL*. Orange triangle highlights upregulation of *lasR* and *lasI*, thought to control the expression of some of the aforementioned proteases. Where applicable, gene transcript levels were compared between SF and SC using unpaired t-tests. Results are reported as mean $\pm$ SD; multiple comparisons were FDR-corrected ($Q = 5\%$), with statistical significance defined as $p \leq 0.05$

3.3.3 Quorum Sensing and Regulation of Virulence Proteases

P. aeruginosa PAO1 utilizes a complex quorum sensing (QS) network to regulate the expression of numerous virulence factors, including secreted proteases, in response to environmental cues. The primary QS systems in *P. aeruginosa* PAO1 include the *Pseudomonas* quinolone signal (PQS), Las, and Rhl systems, each with distinct regulatory components and signaling molecules.²²⁶

The PQS system, specifically 2-heptyl-3-hydroxy-4-quinolone, plays a pivotal role in quorum sensing (QS) and controls the expression of virulence factors across various infection models.^{227,228} PQS synthesis originates from anthranilate via the biosynthetic gene cluster *pqsABCDE*, an operon activated by the LysR-type transcriptional regulator MvfR.¹⁶⁶ Additionally, the small non-coding RNA PhrS, in conjunction with the RNA chaperone protein Hfq, enhances *mvfR* transcription through a base-pairing mechanism that activates *mvfR* mRNA translation.²²⁹ In our RNA-seq results, genes within the *pqsABCDE* operon were downregulated at least two-fold under sulfur-starved conditions (Supplementary Table S1). While the expression of *mvfR* remained unchanged, both *phrS* and *hfq* exhibited a greater than two-fold decrease, suggesting a potential mechanism for the downregulation of the *pqs* operon. This indicates that multiple regulatory factors may influence the transcription of *pqs* genes. Notably, the sister non-coding RNAs PrrF1 and PrrF2 enhance PQS production by inhibiting the translation of *antR*, a transcriptional activator of the *antABC* genes involved in anthranilate catabolism.^{165,218} Interestingly, our data showed an over two-fold increase in *antR* expression, although the downstream *antABC* genes did not display significant changes. Our proteomic data also showed the downregulation of proteins associated with the *pqs* operon (PqsABCDEHL) and the adjacent *phn* operon, which is involved in anthranilate biosynthesis, a precursor of PQS (Figure 3.12b).

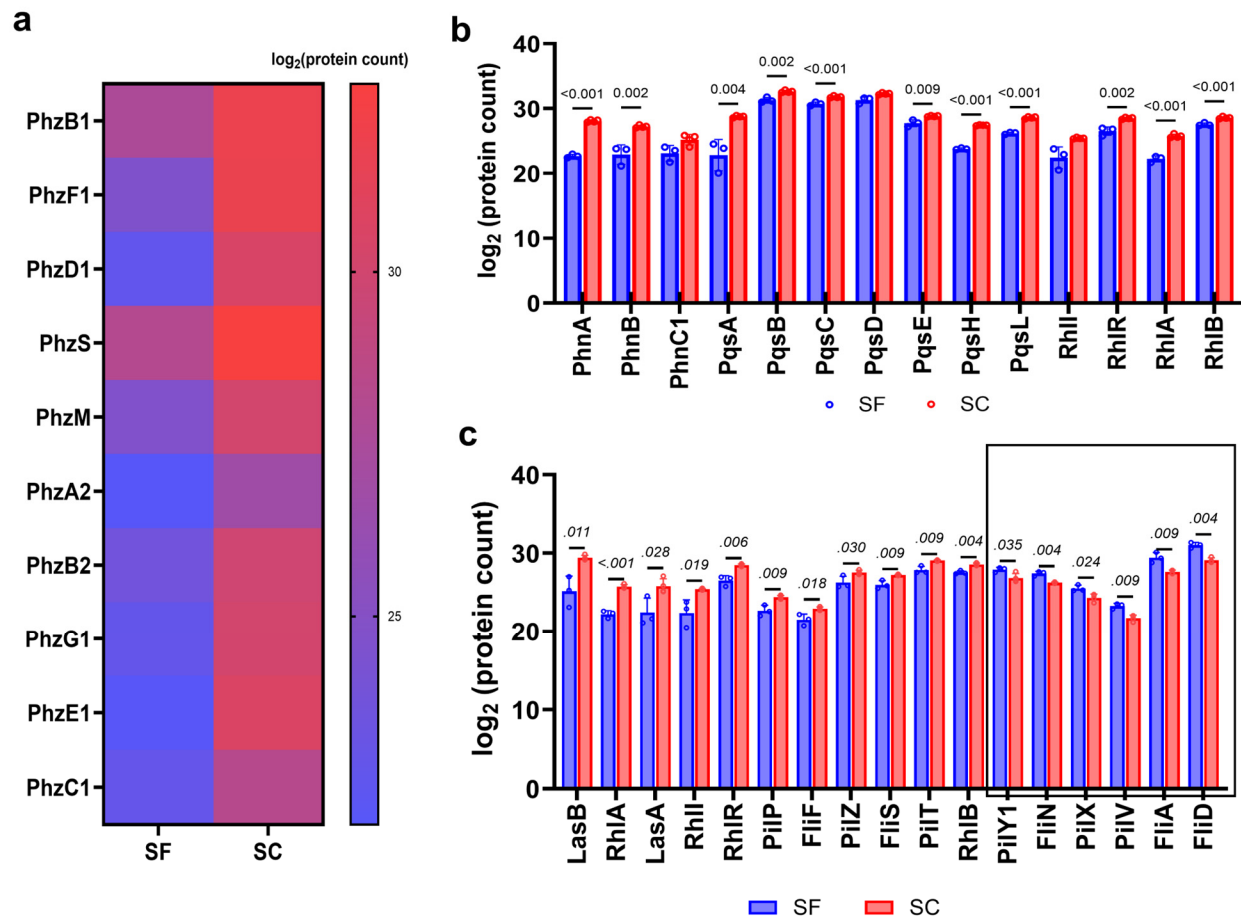


Figure 3.12: Depression of transcripts associated with virulence during sulfur stress a) Heatmap showing the depression of proteins from both phenazine clusters in the test compared to control b) Bar chart showing downregulation of proteins involved in quorum sensing, including those of the *phn*, *pqs*, and *rhl* operons c) Chart showing a mixed response in proteins involved in biofilm formation, including the Las, Rhl, Fli, and Pil proteins. Black box highlights the upregulated proteins in the test (SF). Significance was defined as $p \leq 0.05$. Welch's correction was applied to the proteomics t-test analysis due to unequal sample sizes. Results are reported as mean $\pm$ SD.

The Las QS system, consisting of LasR and LasI, is at the top of the QS hierarchy and controls the expression of various downstream genes, including those encoding the secreted proteases LasA and LasB.²³⁰ LasA exhibits both elastolytic and staphylytic activities, whereas LasB is primarily an elastase that can degrade host elastin, collagen, and immunoglobulins.^{231,232} These proteases play crucial roles in the pathogenicity of *P. aeruginosa* PAO1. Our RNA-seq data showed a greater than two-fold increase in *lasR* and *lasI* transcripts under sulfur-starved conditions. Intriguingly, this upregulation was accompanied by a strong downregulation (~17- to 30-fold) of virulence genes thought to be under their control, including *lasA* and *lasB* (Figure 3.11c). Similarly, proteomic data reflected a significant decrease in LasA and LasB proteins (Figure 3.12c), despite no significant changes in the levels of LasR and LasI. This suggests that LasA and LasB may be co-regulated through a complex interplay among QS systems, potentially involving post-transcriptional mechanisms or additional regulatory factors.

Other peptidases implicated in virulence in *P. aeruginosa* PAO1 include the lysyl endopeptidase PrpL (also called PIV), involved in degrading lactoferrin, and the aminopeptidase PepB (Figure 3.11c).²³³ The genes coding for these proteolytic enzymes (*prpL* and *pepB*) were strongly downregulated in our RNA-seq results, with fold changes as high as 30-fold. It is interesting that despite being located on different loci and operons in the *P. aeruginosa* PAO1 genome, these genes were both downregulated, implying that a common global regulator may be responsible for their modulation. A likely candidate transcription factor that may play this role is PvdS, an extracytoplasmic function (ECF) sigma factor that has been shown to directly control the transcription of *prpL*.²³⁴ We observed strong downregulation (>20-fold) of the *pvdS* gene, which may be responsible for the decreased expression of *prpL* and possibly other proteases. Moreover,

these proteases are also thought to be QS-regulated,⁹ and the depression of the QS system lends support to our observation.

In our proteomics data, we observed a strong downregulation of LasA and LasB proteins (Figure 3.12c), whereas PrpL levels remained unchanged and PepB was undetected. This discrepancy between transcriptomic and proteomic data for PrpL and PepB likely reflects methodological limits. Because only cell pellets were analyzed in the proteomics experiment, efficiently secreted proteins are underrepresented, reducing the reliability of transcriptome–proteome comparisons involving extracellular enzymes.

The Rhl QS system comprises the regulatory protein RhlR and the synthase RhlI, responsible for producing N-butanoyl-L-homoserine lactone (C4-HSL). The RhlAB proteins are involved in rhamnolipid production, which plays a crucial role in biofilm architecture and dispersion.^{235,236} Our RNA sequencing data revealed no significant changes in *rhlR* and *rhlI* expression levels under sulfur starvation. However, proteomic analyses indicated a significant downregulation (2- to 11-fold) of key Rhl system proteins, including RhlR, and RhlAB (Figure 3.12c), highlighting a notable difference between transcriptomic and proteomic responses.

These findings collectively underscore the comprehensive impact of sulfur starvation on QS-regulated virulence factors, including secreted proteases, at both the transcriptomic and proteomic levels. The downregulation of key QS systems and associated virulence factors suggests that sulfur limitation may attenuate the pathogenic potential of *P. aeruginosa* PAO1 by disrupting QS-mediated regulation. However, because QS activation is strongly dependent on cell density, we cannot rule out that the differences in *las*, *rhl*, and *pqs* expression reflect differences in growth yields between conditions rather than sulfur availability. Accordingly, the apparent sulfur-

dependent regulation of QS genes and their downstream targets should be interpreted in the context of these limitations and warrant further interrogation to draw a definite conclusion.

3.3.4 Biofilm Formation

The ability of *P. aeruginosa* PAO1 is a significant determinant of its persistence in CF patients. Studies have shown that iron starvation leads to increased twitching motility that prevents biofilm formation. This is whether iron starvation is induced through growth in Fe-limited media, chelating Fe from the media using lactoferrin, or via mutation of genes involved in the synthesis of pyoverdines or uptake of ferric siderophores, including *pvdS*, *pvdA*, and *fpvA*; all.^{102,169} In our RNA-seq result, we observed a mixed response in the expression of genes that have been implicated in biofilm formation. There was a 2-fold increase in the expression of five genes of the *psl* operon involved in cell-cell interaction and biofilm formation (*pslF*, *pslH*, *pslI*, *pslJ*, *pslK*). Some genes of the *pil* operon (*pilAYIY2*) were also upregulated; they encode Type IV pili and mediate the initial attachment, surface motility (twitching), and biofilm maturation.²³⁷ Other genes important in biofilm formation, including those of the *fli* (involved in flagellar biosynthesis and biofilm maturation)²³⁸ and *alg* (responsible for alginate synthesis, a critical component of biofilm matrix)²³⁹ operons were unchanged or undetected. Our proteomics data showed a similar trend; however, we observed strong downregulation of Rhl proteins and a mixed response in the expression of Fli and Pil proteins (Figure 3.12c).

3.4 Fe and Fe-Associated Metabolite Levels Corroborate an Fe-Replete Condition

Having observed dysregulation in iron metabolism, suggesting an Fe-replete condition during sulfur starvation, we further evaluated the total Fe levels in the spent media and lysate of the samples using inductively coupled plasma optical emission spectrometry (ICP-OES). We

hypothesized that an Fe-replete condition would result in reduced Fe uptake from our experimental sulfur-free media in the sulfur-starved test group compared to the control when normalized for cell density. Therefore, we anticipated higher Fe levels in the spent media from the test group compared to the control. Accordingly, we observed a significant difference in the levels of Fe in the spent media between the two groups, with the test group showing more Fe at the later growth time points (5 and 7.5h) compared to the control (Figure 3.13a). For the cell lysate, we anticipated a decreased total cellular Fe level in the test compared to the control. However, we observed an increased level of Fe in the lysate of the test groups, showing a contradictory response to our hypothesis. However, it is important to note that the observed increase was not statistically significant.

Additionally, we investigated the downregulation of siderophore production observed in our omics investigations by assaying the levels of pyoverdine in spent media using high-performance liquid chromatography (HPLC). Elution times and peak shapes for pyoverdine were consistent across all samples (Appendix: Figure A9-10). To confirm pyoverdine identity, an additional experiment was conducted using LB medium supplemented with the iron chelator DTPA, which induces pyoverdine production in *P. aeruginosa* PAO1. Overnight cultures grown in LB incubated with DTPA resulted in a threefold increase in the suspected pyoverdine peak (Appendix: Figure A11). Overall, there was a decrease in pyoverdine levels in the test compared to the control at later timepoints (5 and 7.5h) (Figure 3.13d).

Because we observed strong downregulation of phenazine gene products in our omics data, we conducted additional experiments to validate these findings. We measured pyocyanin levels, the most abundant form of phenazine, in the spent media of control and test groups and found that the levels were depressed in the sulfur-starved group compared to the control (Figure 3.13c). This aligns with what we observed with our RNA-seq and proteomics results.

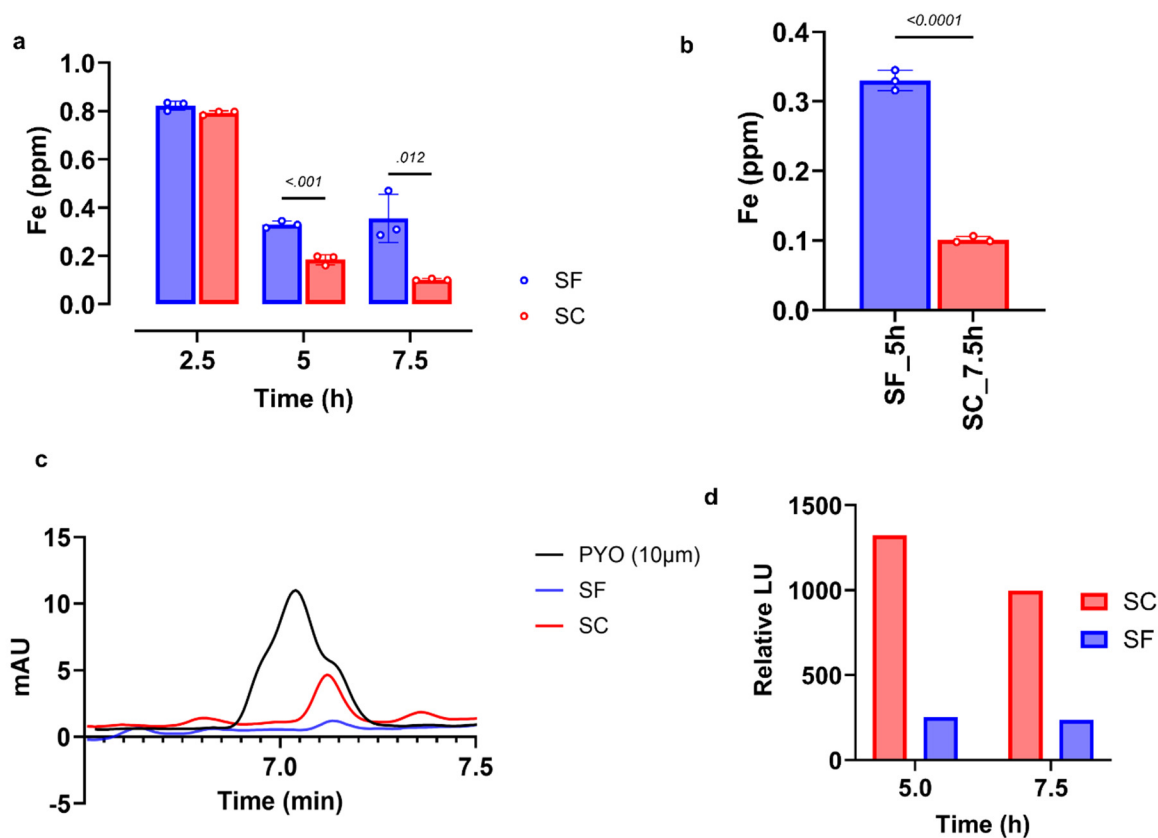


Figure 3.13: Levels of Fe and Fe-associated metabolites correlate with omics data **a)** Fe levels in spent media over time for sulfur starvation (SF) and control (SC) conditions. No significant difference was observed at the initial time point (2.5 h); however, at the later time points (5h and 7.5h), Fe levels remained higher in the SF group compared to the SC group, indicating reduced Fe uptake by the sulfur-starved cells. At time points 5 h and 7.5 h, Fe levels in the SF group remained constant, reinforcing the shutdown of Fe uptake pathways due to Fe stress **b)** A significant difference in Fe levels between the two groups at time points 5 and 7.5h, corresponding to the exponential phase when cells were collected for omics analyses for both groups **c)** HPLC traces display pyocyanin (PYO) levels in the SF and SC groups and a 10 µm PYO standard. Reduced PYO levels were observed in the sulfur-starved test compared to the sulfate-supplemented group. **d)** Observed decrease in peak areas of pyoverdine under SF versus SC conditions at different time points. The SC group exhibited significantly higher peak areas compared to the SF group when normalized by A_{600} , indicating greater pyoverdine production. Three independent assays were conducted and averaged. Unpaired t-test was employed to assess statistical significance using GraphPad Prism, with significance defined as $p \leq 0.05$, where a significance test was appropriate.

3.5 Fluorometric Assays Indicate Time-dependent Changes in Labile Fe and ROS Levels

Given the observed overexpression of antioxidant genes involved in ROS metabolism in our test group (a phenomenon already documented in sulfur limitation studies in bacteria), we conducted further investigations to gain mechanistic insight into the potential triggers of ROS gene overexpression and their connection to Fe metabolism.

First, we used the fluorogenic probe 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) to measure ROS levels in test and control cells at three different time points (2.5, 5, and 7.5 h). Once inside the cells, DCFH-DA is oxidized by reactive oxygen species (ROS) to produce the fluorescent compound 2',7'-dichlorofluorescein (DCF). The intensity of DCF fluorescence is directly proportional to the ROS levels in the cells. We observed that the test cells deprived of sulfur produced more ROS compared to the control when normalized for cell density (Figure 3.16a). However, at the earliest time point measured (2.5 h), no significant difference in ROS levels was found between the groups. A similar observation was made regarding Fe levels in our spent media, as the Fe levels at that time-point remained unchanged for both groups.

An additional assay was used to measure levels of labile Fe in the test and control groups using the turn-off dye Calcein-AM. Calcein is a green fluorescent dye whose fluorescence is quenched intracellularly by labile metals, including Fe. For our study, we monitored the green fluorescence quenching of the dye to assess levels of unincorporated Fe between the groups. Interestingly, we observed that at the earliest time point (2.5 h), the test group showed less fluorescence (implying potentially more Fe). However, this trend was reversed at the 5 and 7.5 h time points, where the test cells exhibited more fluorescence (presumably indicating less labile Fe) than the control (Figure 3.14c). This was unexpected; we attribute this response to the test cells initially having more unincorporated Fe due to a lack of sulfur, which triggers the Fe-replete

response involving the shutdown of Fe uptake pathways and, more crucially, the overexpression of genes involved in Fe storage, including the bacterioferritin genes that we noted earlier. In combination, the likely effect of this metabolic switch will be a reduction in the intracellular labile Fe pool over time in the test group to prevent the toxicity associated with unincorporated Fe through Fenton chemistry.

In summary, our data show a time-dependent increase in ROS levels in the test groups, which is indicative of enhanced oxidative stress due to sulfur deprivation. Conversely, the level of unincorporated Fe was initially higher in the test group but declined over time compared to the control. This biphasic response carries profound implications, suggesting that while Fe may initially trigger ROS generation during sulfur starvation, other mechanisms may also contribute to this process as time progresses.

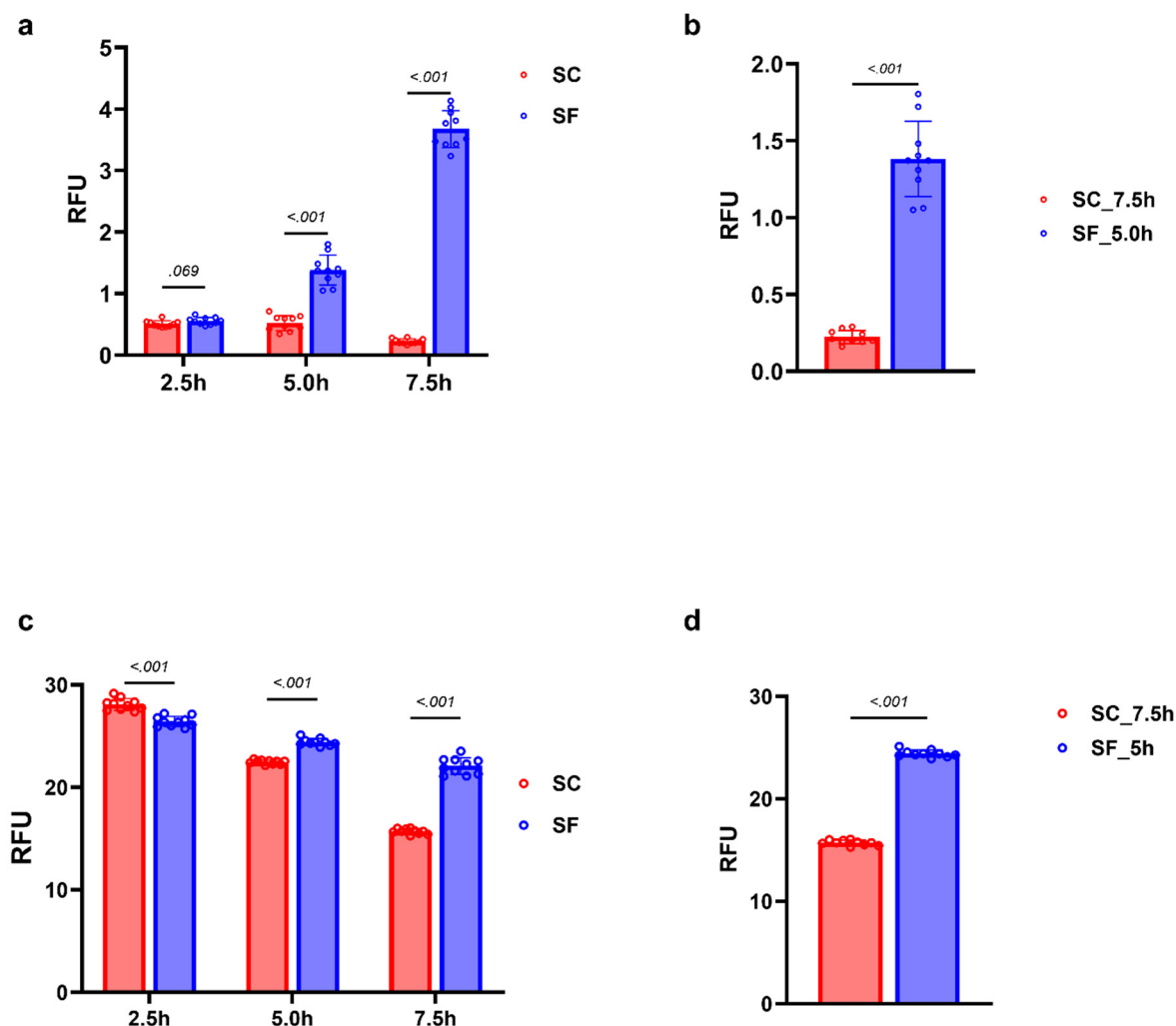


Figure 3.14: Time-dependent assay for ROS and labile-Fe using fluorescent dyes **a)** Intracellular ROS levels assayed over time for sulfur starvation (SF) and control (SC) conditions using DCFH-DA. No significant difference was observed at the initial time point (2.5 h); however, at the later time points (5h and 7.5h), ROS levels remained higher in the SF group compared to the SC group, indicating increased ROS production in the sulfur-starved cells. **b)** A significant difference in ROS levels between the two groups at time points 5 and 7.5h, corresponding to the exponential phase when cells were collected for omics analyses for both groups **c)** Labile-Fe levels were monitored in the test compared to the control group using calcein-AM. The test cells, deprived of sulfate, exhibited a significantly higher level of unincorporated Fe than the control group at 2.5h. However, at later time points (5 and 7.5 h), the control cells showed significantly more labile-Fe. **d)** Significant difference in labile-Fe levels at 5 and 7.5 h between the test and control was observed, corresponding to the exponential phase when cells were collected for omics analyses for both groups. An unpaired t-test with Welch correction was employed to assess statistical significance using GraphPad Prism, with significance defined as $p \leq 0.05$

3.6 Time-Course Proteomics Reveal Dynamic Antioxidant and Fe Storage Gene Expression

After identifying an overexpression of antioxidant genes in sulfur-deprived test cultures compared to sulfate-supplemented controls (Figure 3.5b), along with a marked, time-dependent increase in ROS production in the test relative to the control (Figure 3.14a), we hypothesized that ROS generation might drive the overexpression of antioxidant genes. If this hypothesis holds, we would expect a positive, time-dependent correlation between ROS levels and antioxidant gene expression, particularly in the test group. Conversely, we would not expect a time-dependent increase if the observed overexpression were a preemptive mechanism associated with sulfur starvation but not necessarily related to intracellular ROS levels.

3.6.1 Time-dependent Antioxidant Induction Aligns with Single-sampling Omics Findings

Our time-course proteomics investigation revealed differential expression of the antioxidant genes we had previously identified when analyzing the exponential growth phase data (5 versus 7.5 h for the test and control, respectively). *P. aeruginosa*, as noted previously, produces three catalases (KatA, KatB, and KatC). In our time-course experiment, we observed that the levels of katA mostly remained unchanged between the test and control groups, reaffirming its potential role as a housekeeping catalase ([Supplementary Table S5](#)). We observed a significant increase in the level of KatB, a peroxide-inducible catalase believed to play a central role in detoxifying hydrogen peroxide (H₂O₂) in *P. aeruginosa*²⁴⁰. KatB levels were differentially expressed across the test and control groups at 2.5, 5, and 7.5 h (Figure 3.15), with a general increase over time observed in both groups. This trend aligns with our ROS measurements, which showed a time-dependent increase in ROS generation in the sulfur-starved test (Figure 3.14a). The increase in KatB levels in the control, despite a relatively stable level of ROS generation at the equivalent time-points investigated, likely reflects a natural cellular response, wherein KatB enzymes

accumulate as cells transition from exponential growth to stationary phase under nutrient-limited conditions. This suggests that KatB expression may be part of a general oxidative stress response rather than being exclusive to sulfur limitation.

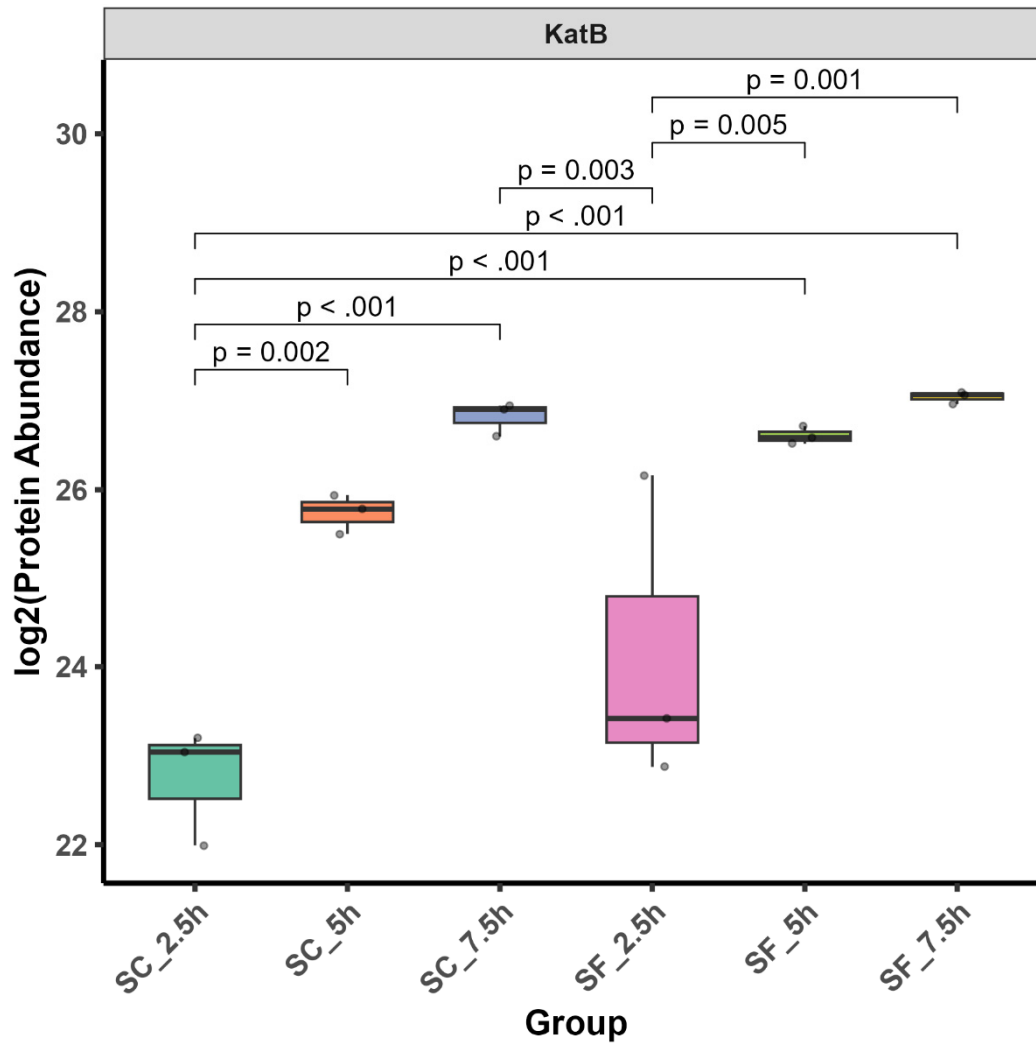


Figure 3.15: Boxplot showing the time-dependent increase in KatB levels. The levels of KatB exhibited a significant increase over time within and between the test (SF) and control (SC) groups. Notably, there was a marked increase in the level of KatB after the 2.5-h time point, with a less pronounced rise between the later time points (5 and 7.5 h) for both test and control conditions. Protein abundance levels were compared using a two-way analysis of variance (ANOVA) to assess the main effects of treatment and time. Multiple comparisons were adjusted using the FDR method, with statistical significance defined as $p \leq 0.05$

Furthermore, we observed a significant increase in the expression of organic hydroperoxide resistance (Ohr) protein between the test and control groups (Figure 3.16). Notably, the levels Ohr of the sulfur-starved test cells were significantly higher than those in the control at the later time points (5 and 7.5 h). The observed increase in the abundance of Ohr proteins correlates with our earlier findings from single sampling time proteomics and RNA-seq data, where Ohr was one of the most upregulated antioxidant features. On the other hand, OhrR, a transcriptional repressor that regulates the expression of Ohr, exhibited a relatively stable level both between and within the test and control groups (Figure 3.16).

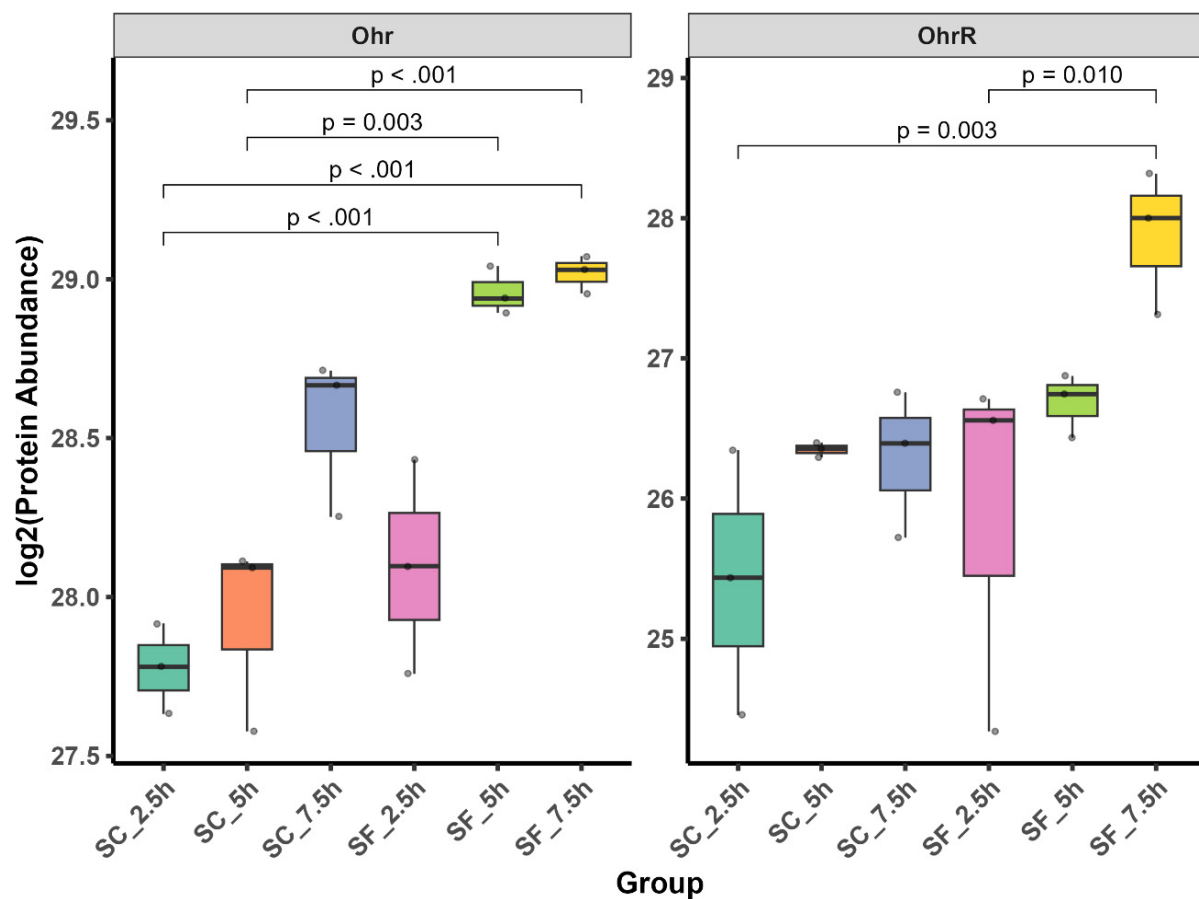


Figure 3.16: Boxplots showing the time-dependent levels of Ohr and OhrR. The levels of Ohr exhibited a pattern of increase over time within and between the test (SF) and control (SC) groups. There was a significant increase in the level of OhrR after the later time points (5 and 7.5 hours) between the test and control groups. OhrR, a repressor that modulates *ohr* expression, showed a relatively stable level across time and groups, except for a significant increase in the test group at the 7.5h versus 5h. For each protein, abundance levels were compared using a two-way analysis of variance (ANOVA) to assess the main effects of treatment and time. Multiple comparisons were adjusted using the FDR method, with statistical significance defined as $p \leq 0.05$

PA3450 (LsfA), a 1-Cys peroxiredoxin previously reported to be upregulated under sulfur-limiting conditions,^{24,26} exhibited strong differential expression between test and control groups, with significantly elevated levels in the test cells at later time points (5 and 7.5 h) (Figure 3.17). This pattern is consistent with our earlier single sampling time RNA-seq and proteomics analyses. The preferential expression of PA3450, which contains only a single cysteine residue, likely reflects a sulfur-sparing strategy by *P. aeruginosa*, allowing the bacterium to conserve cysteine for other essential cellular functions during sulfur starvation.

In addition, we observed a significant time-dependent increase in the Fe-containing superoxide dismutase, SodB, specifically in the test group. This supports SodB's proposed dual function as both an antioxidant enzyme and a mechanism for sequestering intracellular Fe under sulfur-limited conditions (Figure 3.17). In contrast, SodA, the Mn-dependent isoform, exhibited a general increase over time in both the test and control groups; however, the changes were not statistically significant (Supplementary Table S5). Although a modest reduction in SodA expression was observed in the test group relative to the control at corresponding time points, this difference was not statistically significant in the time-course data. Notably, this contrasts with our earlier single sampling time RNA-seq and proteomic datasets, which revealed a significant decrease (> 40-fold) in SodA expression under sulfur-limiting test conditions.

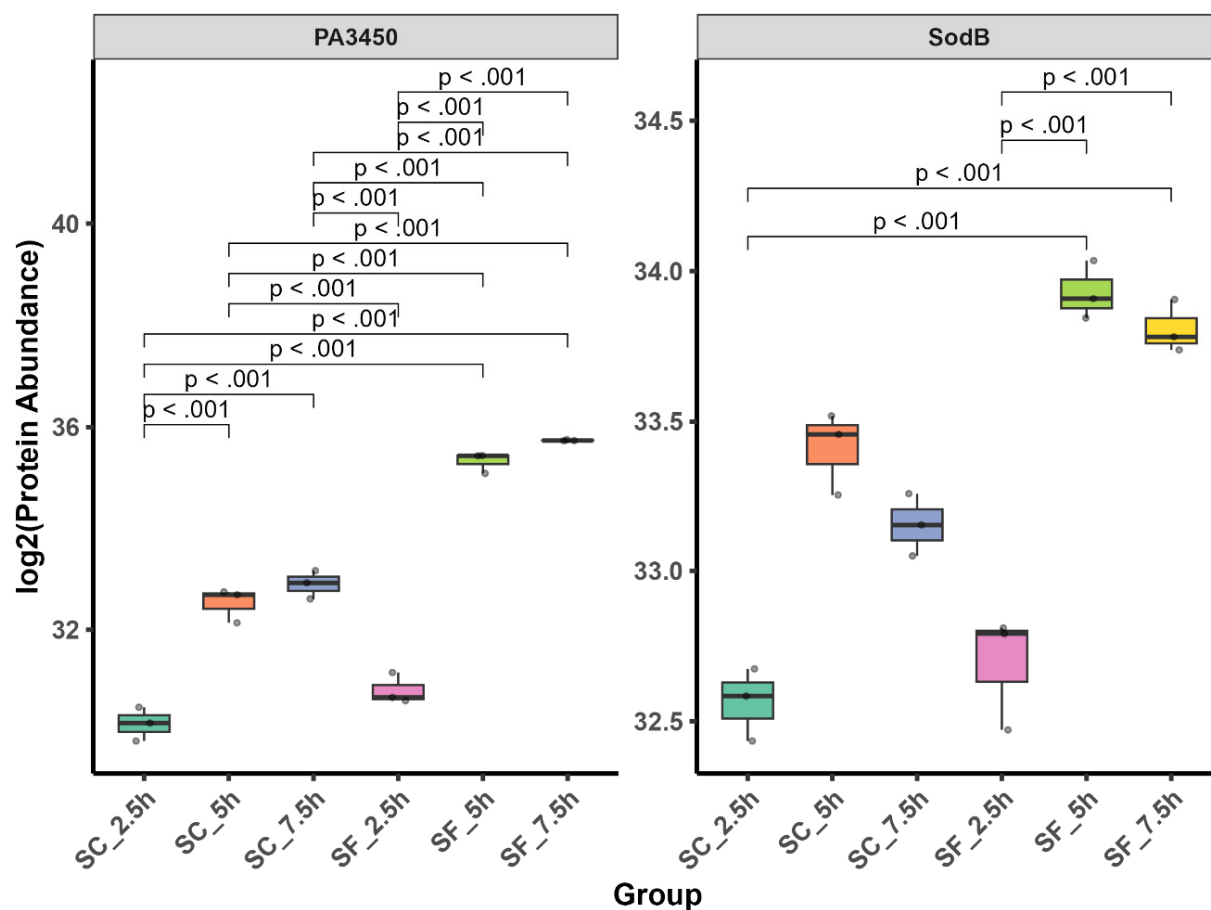


Figure 3.17: Boxplot showing the time-dependent protein levels of PA3450 (LsfA), and SodB in test and control groups. PA3450, a 1-Cys peroxiredoxin, exhibited a marked increase in the test (SF) group at the later time points (5 and 7.5h), consistent with its known upregulation under sulfur starvation conditions. SodB, the Fe-containing superoxide dismutase, also showed a significant time-dependent increase in the test group, supporting its dual role in oxidative stress defense and iron sequestration. For each protein, abundance levels were analyzed using two-way ANOVA to assess the main effects of treatment and time. Multiple comparisons were corrected using the FDR method, and statistical significance was defined as $p \leq 0.05$

Consistent with our earlier observations from single sampling datasets, we observed differential expression of key enzymes from the pentose phosphate pathway (PPP) and the Entner–Doudoroff (ED) pathway in response to sulfur starvation. These pathways are well-documented to be upregulated under oxidative stress conditions, primarily due to their role in generating NADPH, a critical reducing equivalent used in antioxidant defense.^{207,241} We observed significant, time-dependent increases in the abundance of Pgl (6-phosphogluconolactonase), Zwf (glucose-6-phosphate dehydrogenase), and EddA (2-keto-3-deoxy-6-phosphogluconate aldolase) in the test group relative to the control, especially at 5 and 7.5 h (Figure 3.18).

While the precise role of upregulating these enzymes during sulfur starvation remains to be elucidated, this response likely enhances NADPH production to support cellular antioxidant systems, including the biosynthesis of reduced glutathione (GSH) and thioredoxins. However, both GSH and thioredoxins are sulfur-containing molecules, which complicates their use as primary ROS-buffering systems under sulfur stress conditions. Interestingly, despite the deprivation of sulfur, we found that the levels of several enzymes involved in glutathione metabolism remained relatively stable between the test and control groups. This suggests a tightly regulated response to preserve reduced glutathione pools even under sulfur stress, possibly prioritizing its biosynthesis due to its essential role in maintaining redox balance.

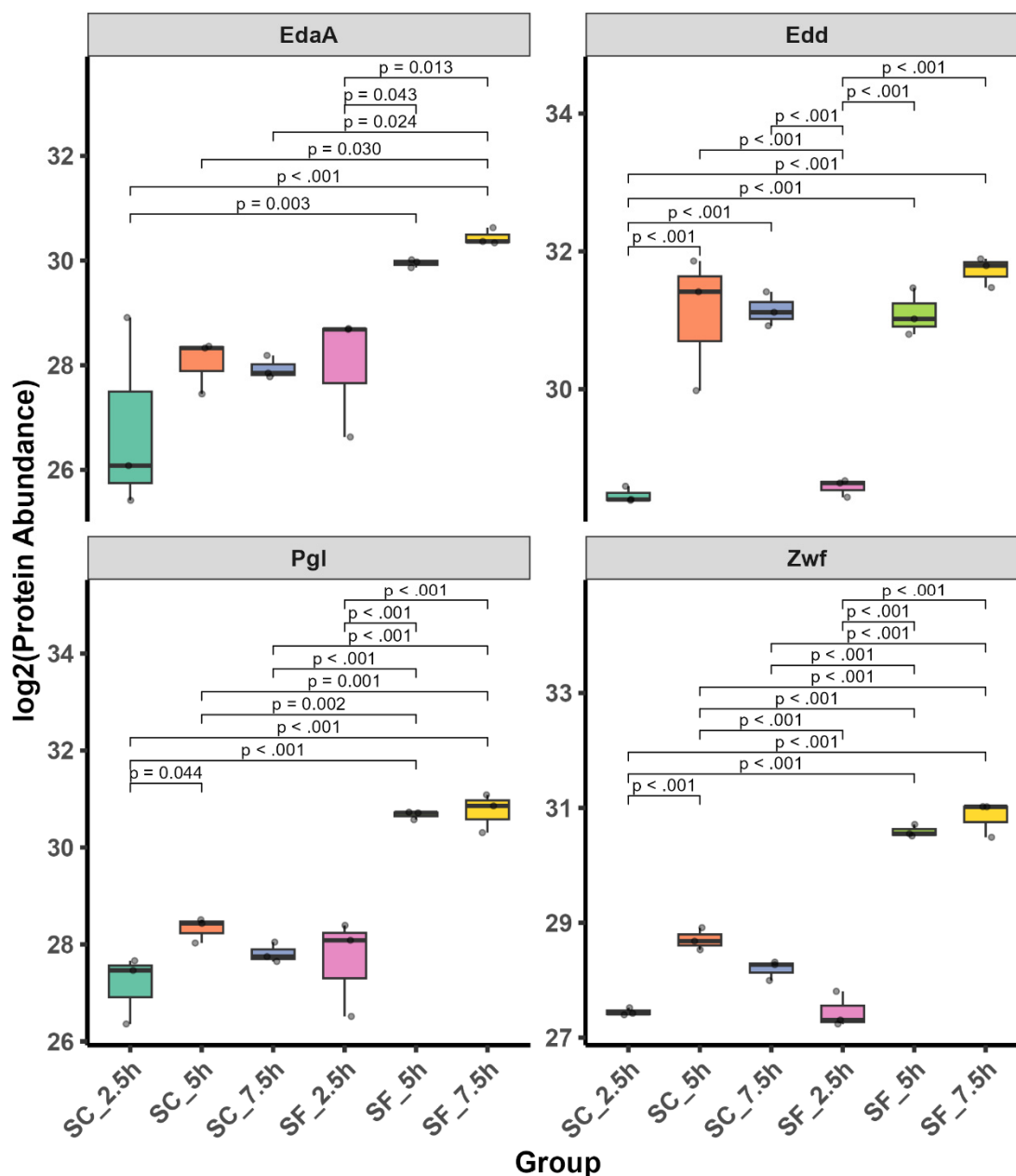


Figure 3.18: Boxplot showing the time-dependent protein levels of EdaA, Edd, Pgl, and Zwf in test and control groups. These enzymes (except for Edd), which belong to the pentose phosphate and Entner–Doudoroff pathways, exhibited significant increases in the test (SF) group at the 5 and 7.5h time points compared to the control (SC). Their upregulation is consistent with a cellular response to oxidative stress, likely aimed at boosting NADPH production to support antioxidant defenses during sulfur stress. For each protein, abundance levels were analyzed using two-way ANOVA to evaluate the main effects of treatment and time. Multiple comparisons were corrected using the FDR method, with statistical significance defined as $p \leq 0.05$.

3.6.2 Changes in Fe Storage Proteins Align with Single Sampling Omics Observations

We observed a clear time-dependent increase in the abundance of iron storage proteins, consistent with trends previously identified in our single sampling time analyses. Notably, the levels of BfrB, the primary bacterioferritin in *P. aeruginosa*, rose sharply in the test group at 5 and 7.5 h, indicating a shift toward an iron-replete state under sulfur-limited conditions (Figure 3.19). This pattern was mirrored by other iron-sequestering proteins, including PA4880, a putative bacterioferritin, and PA0962, annotated as a DNA-binding stress protein (Dps). Both proteins exhibited significant time-dependent increases in the test group, while their levels remained relatively stable in the control group (Figure 3.20). These findings suggest a coordinated cellular response to limit free intracellular iron, potentially as a protective mechanism against iron-driven oxidative stress during sulfur starvation.

In conclusion, the time-course data not only corroborate our single sampling time findings but also provide some insight into the dynamic nature of the oxidative stress response under sulfur starvation. The progressive upregulation of antioxidant genes over time closely mirrors the increase in ROS levels observed in the test group, highlighting a sustained oxidative burden. Additionally, time-dependent overexpression of iron storage proteins, especially in the sulfur-starved test cells, further supports the model of an Fe-replete state that necessitates intracellular iron sequestration as a protective mechanism. These findings highlight the interconnection between sulfur metabolism, oxidative stress, and iron homeostasis in *P. aeruginosa*.

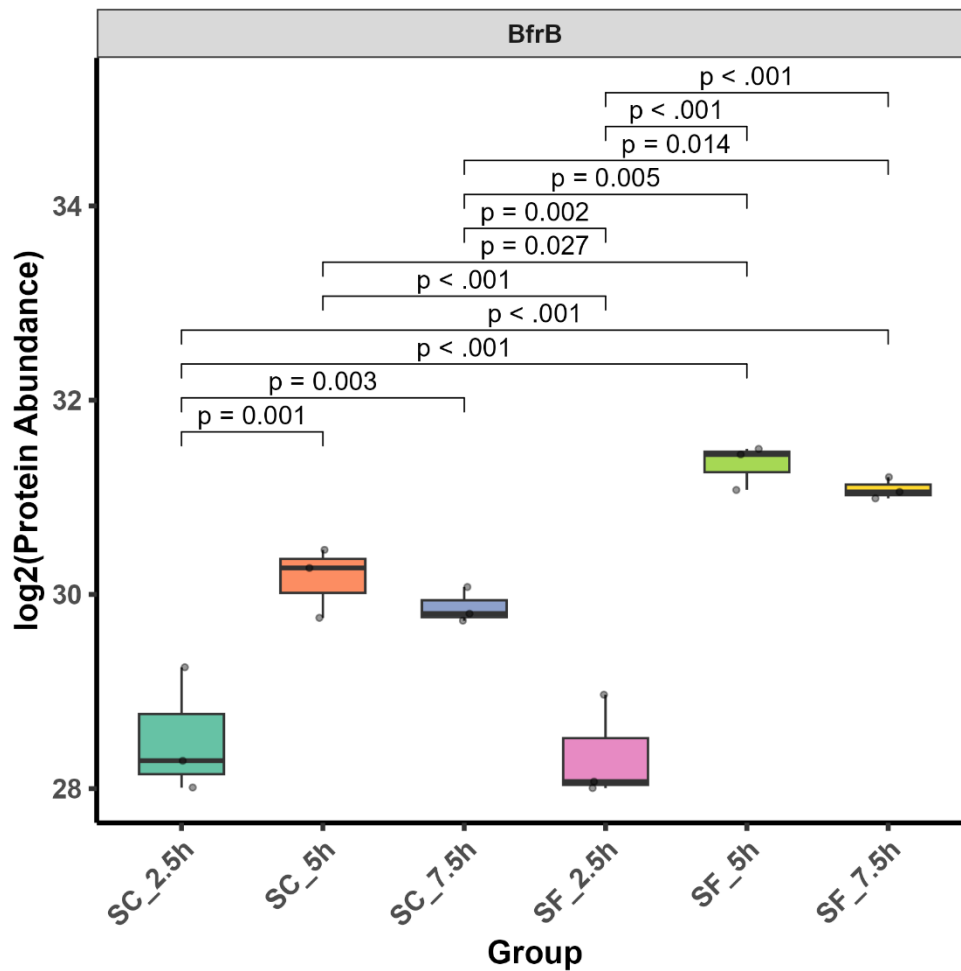


Figure 3.19: Boxplot depicting the time-dependent protein levels of BfrB in test and control groups BfrB, the primary bacterioferritin in *P. aeruginosa*, showed a significant increase in abundance in the test group at the 5 and 7.5h time points compared to the control, which aligns with an Fe-replete response triggered by sulfur limitation. The upregulation of BfrB likely indicates a protective mechanism that sequesters excess intracellular iron and reduces iron-catalyzed oxidative damage during sulfur stress. Protein abundance levels were analyzed using two-way ANOVA to evaluate the main effects of treatment and time. Multiple comparisons were adjusted using the FDR method, with statistical significance defined as $p \leq 0.05$

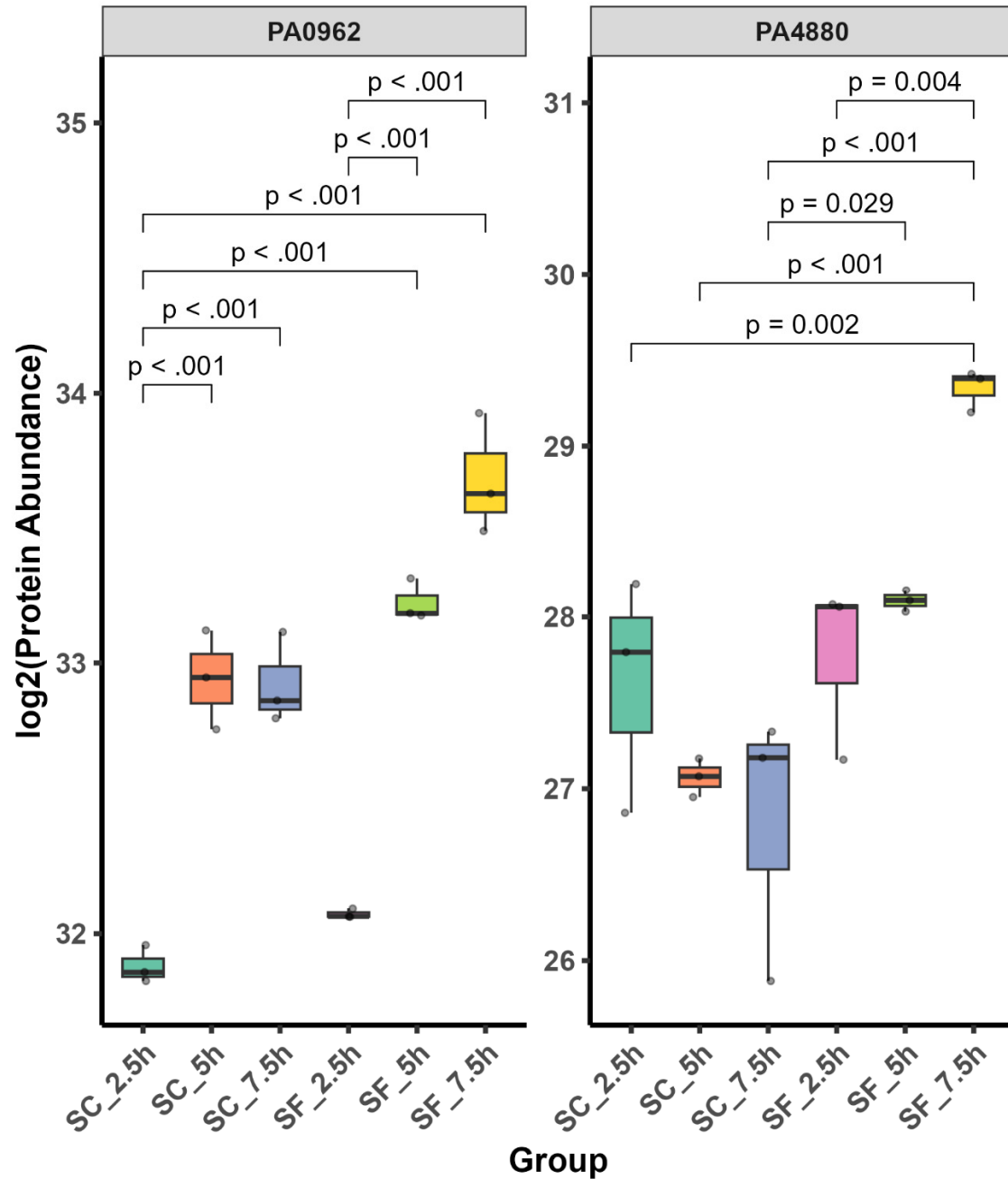


Figure 3.20: Boxplot showing the time-dependent protein levels of PA0962 and PA4880 in test and control groups. PA0962, annotated as a DNA-binding stress protein (Dps), and PA4880, a putative bacterioferritin, both demonstrated significant time-dependent increases in the test group at 5 and 7.5 h. The expression patterns of PA0962 mirror those of BfrB and indicate a coordinated iron storage response to sulfur limitation. This likely helps reduce intracellular free iron and mitigate oxidative stress. Protein abundance levels were analyzed using two-way ANOVA to evaluate the main effects of treatment and time. Multiple comparisons were corrected using the FDR method, with statistical significance defined as $p \leq 0.05$

3.7 Insights into the Gene Regulatory Network of SSI Response

The large genome of *P. aeruginosa* supports a highly complex regulatory network comprising numerous transcription factors, two-component response regulators, and extracytoplasmic sigma factors. Collectively, these elements enable the organism's metabolic flexibility. Indeed, *P. aeruginosa* is reported to have more than 400 transcription factors, 24 extracytoplasmic sigma factors, and various other regulatory elements, including non-coding small RNAs (ncRNAs).²⁴² Notably, many of these putative regulatory elements remain unverified experimentally, having been annotated primarily through computational predictions.²⁴³

Our RNA-seq and proteomic analyses underscore the intricate interplay of genes, regulatory factors, and pathways during sulfur starvation. This condition exerts a pronounced effect on Fe homeostasis, suggesting a key role for the ferric uptake regulator (Fur) and other regulators through functional association. Additionally, some virulence-related regulators were differentially expressed under sulfur-starvation conditions, including *pvds*, *mexT* and *mexR* (associated with multidrug efflux pumps), and *lasR*, *rhlR* (linked to quorum sensing).

Because many of these differentially expressed genes are annotated as putative and lack experimental validation, we conducted a gene regulatory network (GRN) analysis using the GENIE3 tool to gain insight into their potential role in modulating observed response. From our list of differentially expressed genes, we identified 74 regulatory elements ([Supplementary Table S6](#)) classified as transcription factors, two-component response regulators, or sigma factors in the P2TF database. Table 3.1 below presents a subset of these 74 transcription factors for illustrative purposes.

Table 3.1: Table showing a subset of the list of regulatory elements used for GRN analysis

The top differentially expressed transcriptional regulators and sigma factors under sulfur starvation, ranked by log fold-change (logFC). Adjusted p-values (adj.P.Val) were calculated using the Benjamini-Hochberg method.

GeneID	Name	logFC	adj.P.Val
PA2359	transcriptional regulator (PA2359)	6.668	2.25e-11
PA2312	transcriptional regulator (PA2312)	5.468	1.08e-10
PA2050	RNA polymerase sigma factor (PA2050)	5.109	3.40e-10
PA2354	transcriptional regulator (PA2354)	4.671	1.72e-10
PA3932	transcriptional regulator (PA3932)	4.394	3.17e-10
PA2299	transcriptional regulator (PA2299)	3.933	8.58e-10
PA2093	RNA polymerase sigma factor (PA2093)	3.28	2.89e-09
PA2334	transcriptional regulator (PA2334)	3.211	2.16e-09
PA0279	transcriptional regulator (PA0279)	2.336	7.31e-06
PA0535	transcriptional regulator (PA0535)	1.813	3.50e-04
PA2849	transcriptional regulator (ohrR)	1.625	3.86e-07
PA2332	transcriptional regulator (PA2332)	1.616	5.61e-06
PA0191	transcriptional regulator (PA0191)	1.609	9.02e-07
PA4843	two-component response regulator (PA4843)	1.58	6.13e-05
PA5116	transcriptional regulator (PA5116)	1.56	3.15e-03
PA3192	two-component response regulator GltR (gltR)	1.549	4.76e-06
PA2489	transcriptional regulator (PA2489)	1.544	2.01e-05
PA2917	transcriptional regulator (PA2917)	1.542	5.02e-07
PA2337	transcriptional regulator MtlR (mtlR)	1.467	3.09e-06
PA2270	transcriptional regulator (PA2270)	1.374	9.57e-06

Table 3.2: Top 10 predicted Regulatory elements identified by GENIE3

The table lists the top 10 regulatory elements predicted by GENIE3 based on frequency of appearance among the top-ranked regulatory edges. Each entry includes the gene ID, regulatory class (TR: transcriptional regulator, RR: response regulator, OCS: one-component system), predicted family type, and P2TF-based functional annotation.

GeneID	Frequency	Class	Type	P2TF Description
PA3678	41	TR	TetR	Transcription factor, TetR family contains 1 TetR_N
PA0942	39	TR	MarR	Transcription factor, MarR family contains 1 MarR
PA2220	36	OCS	LysR	Transcription factor, LysR family contains 1 HTH_1,1 LysR_substrate
PA4032	36	RR	OmpR	Response regulator, OmpR family contains 1 Response_reg,1 Trans_reg_C
PA2100	35	OCS	GntR	Transcription factor, GntR family contains 1 GntR,1 Aminotran_1_2
PA4135	34	TR	MarR	Transcription factor, MarR family contains 1 MarR
PA2588	33	TR	AraC	Transcription factor, AraC family contains 1 HTH_AraC
PA5431	33	OCS	GntR	Transcription factor, GntR family contains 1 GntR,1 Aminotran_1_2
PA3192	30	RR	OmpR	Response regulator, OmpR family contains 1 Response_reg,1 Trans_reg_C
PA2267	29	OCS	LysR	Transcription factor, LysR family contains 1 HTH_1,1 LysR_substrate

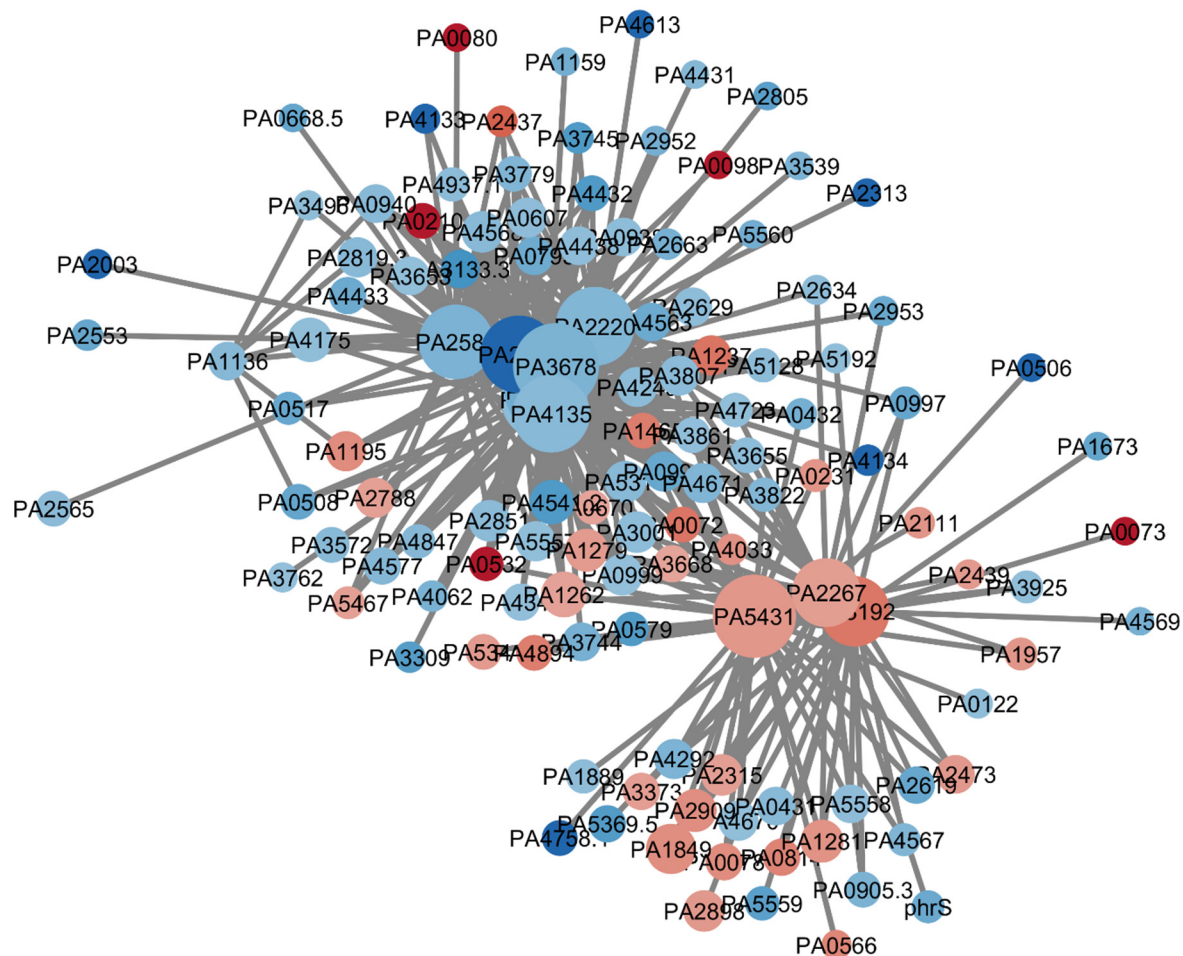


Figure 3.22: Subnetwork visualization of the top 10 predicted regulatory elements based on GENIE3 output, rendered in Cytoscape. The network includes only those regulators with the highest number of predicted targets and their respective downstream genes. Node color corresponds to the $\log_2$ fold-change in gene expression under sulfur starvation conditions: red indicates upregulated regulators, while blue indicates downregulated ones. Node size reflects the out-degree, representing the number of predicted targets regulated by each element, with larger nodes indicating more extensive regulatory influence. Edges represent predicted regulatory interactions, and network metrics including out-degree and betweenness centrality were computed using Cytoscape’s Network Analyzer. This focused subnetwork highlights key transcriptional hubs likely to play central roles in the gene regulatory response of *P. aeruginosa* under sulfur-limited conditions

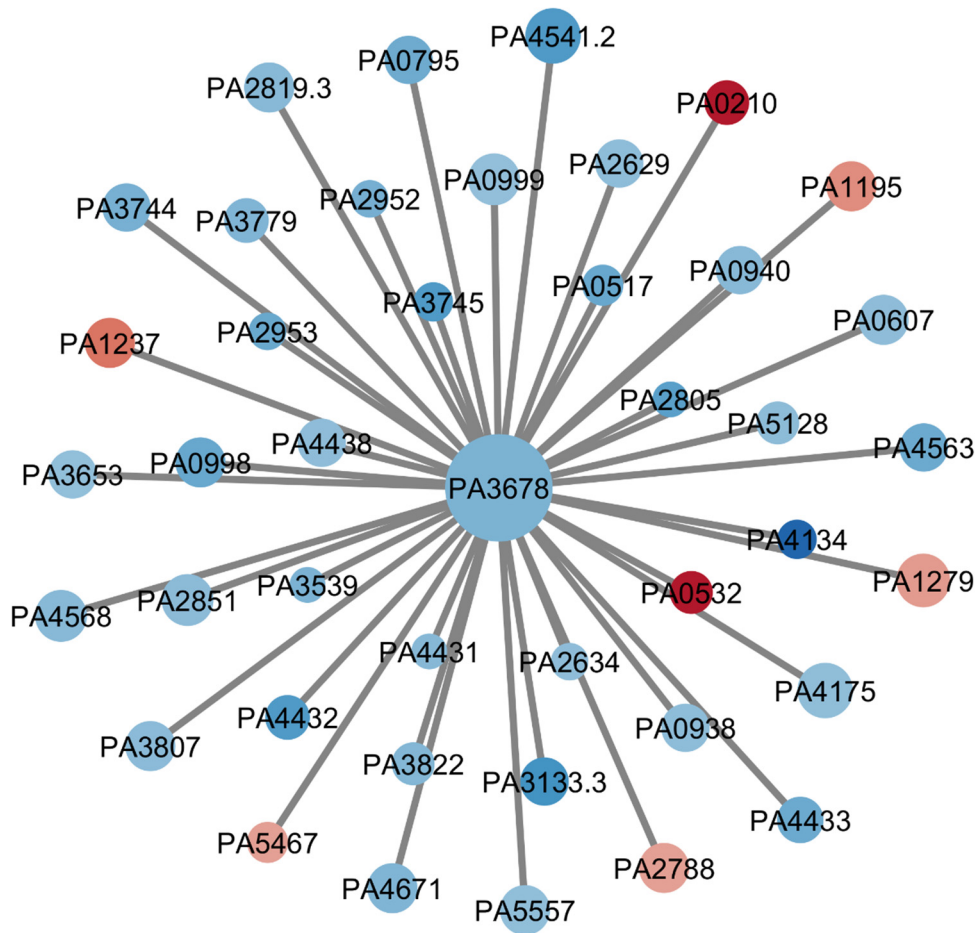


Figure 3.23: Subnetwork visualization of PA3678 (MexL), the top-ranked regulatory element predicted by GENIE3 based on interaction frequency. The network shows all predicted downstream genes regulated by PA3678, as visualized in Cytoscape. Node color reflects $\log_2$ fold-change in expression under sulfur starvation: red indicates upregulated genes, while blue indicates downregulated ones. PA3678 is positioned centrally, with edges representing directed regulatory predictions toward target genes. Node size is proportional to out-degree, emphasizing the extensive regulatory influence of PA3678 in this context. Network metrics were computed using Cytoscape’s Network Analyzer. This visualization highlights MexL as a potential transcriptional hub in the sulfur starvation response of *P. aeruginosa*

3.7.1 Model Evaluation of GENIE3-inferred Network

We evaluated the performance of GENIE3-inferred targets for PA3678 (MexL) using a gold standard composed of experimentally validated genes, including the *mexJK* operon and *phzA–G* clusters. None of the top 41 predicted targets overlapped with the gold standard, likely due to the small RNA-seq sample size ($n = 6$) and limited regulatory annotations for *P. aeruginosa* transcription factors. Despite the low precision and recall, the prominence of MexL in the network is biologically supported, as our transcriptomic and proteomic data revealed strong differential expression of *mex* efflux and phenazine biosynthetic genes, both of which are known to be regulated by MexL.

Table 3.3: Comparison of Gold Standard vs. GENIE3-Predicted Targets for PA3678

Table comparing known experimentally validated targets of PA3678 (MexL) with those predicted by GENIE3. The gold standard includes genes from the *mexJK* operon and phenazine biosynthesis clusters.

Gold Standard Targets	GENIE3-Predicted Targets
PA3676, PA3677, PA4210, PA4211	PA0210, PA0517, PA0532, PA0607
PA4212, PA4213, PA4214, PA4215	PA0795, PA0938, PA0940, PA0998
PA4216, PA1899, PA1900, PA1901	PA0999, PA1195, PA1237, PA1279
PA1902, PA1903, PA1904, PA1905	PA2629, PA2634, PA2788, PA2805
—	PA2819.3, PA2851, PA2952, PA2953
—	PA3133.3, PA3539, PA3653, PA3744
—	PA3745, PA3779, PA3807, PA3822
—	PA4134, PA4175, PA4431, PA4432
—	PA4433, PA4438, PA4541.2, PA4563
—	PA4568, PA4671, PA5128, PA5467
—	PA5557

CHAPTER FOUR: Discussion and Conclusion

4.1 Discussion

4.1.1 Sulfur Starvation Triggers an Iron-Replete Response

This work explores the regulatory and metabolic changes in *P. aeruginosa* PAO1 under sulfur starvation, with a focus on oxidative stress, iron homeostasis, and transcriptional regulation. By utilizing a combination of RNA-seq, label-free proteomics, fluorometric assays, and gene network inference, we present a comprehensive overview of how sulfur limitation affects cellular physiology and gene regulation in this opportunistic pathogen. Our findings reveal a complex interplay between sulfur metabolism, iron homeostasis, oxidative stress response, and virulence factor regulation. These data indicate that sulfur limitation induces an Fe-replete state, resulting in a significant decrease in iron uptake pathways and an increase in iron storage proteins. This response has profound implications for the bacterium's pathogenic potential and survival strategies.

Through single sampling time RNA-seq and proteomic analyses, we demonstrated a significant decrease in the abundance of genes involved in iron uptake, including siderophore biosynthesis (*pvd* and *pch* clusters), siderophore receptors (*fpvA* and *fptA*), and heme acquisition systems (*phu* and *has* operons). Conversely, genes encoding iron storage proteins, particularly bacterioferritin *bfrB*, were increased. Moreover, we observed a decrease in *bfd* (*PA3530*) and *fpr* (*PA3397*), which are ferritins involved in mobilizing stored iron from BfrB.²¹² The depression of these ferritins may serve to prevent the release of iron when the cell cannot safely utilize it, thus mitigating iron-induced toxicity. Additionally, decreasing the abundance of *bfd*, a 2Fe-2S ferredoxin, could help conserve sulfur for other critical processes. Overall, the pattern suggests

that sulfur starvation triggers an Fe-replete response, leading to reduced iron acquisition and increased intracellular iron sequestration. Iron is essential yet potentially toxic due to its ability to catalyze the formation of reactive oxygen species (ROS) through Fenton chemistry and other related redox processes.¹²⁷ Hence, the tight regulation of iron metabolism is crucial in preventing oxidative damage by ROS.

The Ferric Uptake Regulator (Fur) is key in controlling iron metabolism by repressing iron uptake genes under Fe-replete conditions.²⁴⁴ Although *fur* transcript levels remained unchanged, the widespread decrease in Fur-regulated genes implies enhanced Fur activity. Under sulfur starvation, increased intracellular free iron can activate Fur, leading to the repression of iron acquisition systems and the modulation of genes involved in oxidative stress and virulence. The absence of increased *fur* expression supports the idea that Fur operates as a housekeeping gene, rapidly responding to iron levels through post-translational mechanisms.¹⁴⁶

4.1.2 Canonical Antioxidant Gene Induction Accompanies Sulfur Starvation

A significant observation from our study is the increase in antioxidant genes during sulfur starvation, a phenomenon previously reported but lacking a strong mechanistic explanation. We observed increased abundances of several antioxidant genes, including 1-Cys peroxiredoxin LsfA and organic hydroperoxide resistance protein (Ohr), which are involved in detoxifying ROS.^{205,245} These increases are likely a direct response to the increased oxidative stress resulting from iron accumulation and impaired Fe-S cluster synthesis. Without sufficient sulfur, the cell cannot properly assemble Fe-S clusters, resulting in the accumulation of unincorporated Fe that can catalyze the production of ROS via Fenton-related chemistry. In addition, the formation of reactive oxygen species can lead to the oxidation of enzyme metallocenters, leading to a disruption in iron

homeostasis.²⁴⁶ The expression of antioxidant defenses mitigates this oxidative damage. Therefore, the two mechanisms work synergistically, leading to the expression of antioxidant proteins and an Fe-replete condition. Interestingly, we noted upregulation of SodB (Fe-SOD) and downregulation of SodA (Mn-SOD). A decrease in SodA aligns with an Fe-replete condition, where the cell favors iron-dependent antioxidant defenses. The downregulation of *sodA* may reflect a conservation strategy to prioritize iron-utilizing enzymes over manganese-dependent ones when unincorporated iron is abundant.^{247,248} These shifts in antioxidant enzyme expression further support the notion that sulfur limitation leads to iron-induced oxidative stress.

Additionally, sulfur starvation promoted the upregulation of enzymes from the pentose phosphate and Entner–Doudoroff pathways, particularly Pgl, Zwf, and EddA. These pathways are crucial for NADPH production, supporting glutathione and thioredoxin-based antioxidant systems. Interestingly, while glutathione biosynthesis involves sulfur, the levels of genes involved in its biosynthesis remained stable between groups, suggesting a prioritization of redox balance despite sulfur limitation.

Finally, our time-course proteomics data indicated significant temporal increases in KatB, PA3450 (LsfA), Ohr, and SodB under sulfur-starved conditions, which were much more pronounced compared to the control and showed substantial elevation at later time points (5 & 7.5h). SodB's upregulation in the test group further supports its potential role in antioxidant defense and Fe sequestration. Meanwhile, iron storage proteins, including BfrB, PA4880, and PA0962, showed increased abundance, consistent with a cellular strategy to mitigate oxidative stress through iron sequestration. In summary, we observed strong agreement in the expression patterns of antioxidant and iron-storage proteins between our time-course and single sampling proteomics

data, further reinforcing the observed overexpression of antioxidant genes accompanied by enhanced iron sequestration capabilities in the organism.

4.1.3 Iron May Initiate ROS Generation, but Other Mechanisms Sustain It

Initially, we hypothesized that the ROS burst is driven primarily by the accumulation of unincorporated Fe, due to the impaired assembly of Fe-S clusters under sulfur-limiting conditions. The unincorporated Fe catalyzes the generation of ROS through classical Fenton chemistry, a widely recognized source of oxidative stress in bacteria.²⁴⁹ Fluorometric analysis with Calcein-AM revealed a biphasic pattern of intracellular Fe dynamics, where initial measurements (2.5h) indicated elevated intracellular Fe levels in sulfur-starved cells compared to controls, consistent with Fe overload. However, at later time points (5 & 7.5h), Fe levels diminished below control levels. This pattern was anticipated due to the simultaneous shutdown of Fe-uptake systems and upregulation of Fe storage proteins, notably bacterioferritins, characteristic of an Fe-replete response.^{127,212}

Despite the observed drop in intracellular Fe levels at later time points, intracellular ROS levels continued to rise. This sustained ROS generation is particularly intriguing, suggesting the involvement of other oxidative mechanisms beyond simple Fe-mediated Fenton reactions. Several oxidases, including NADH oxidases and sulfite oxidases, have been implicated as significant contributors to ROS generation during nutrient and oxidative stress.^{81,249,250} Furthermore, the induction of monooxygenases under sulfur stress conditions, as previously reported, provides additional plausible sources of ROS generation.^{26,41,251}

Consequently, our findings suggest a scenario in which Fe initially serves as a trigger for ROS generation, prompting rapid cellular adaptive responses to mitigate Fe toxicity. However,

under prolonged sulfur limitation, the compromised synthesis of sulfur-containing antioxidant molecules such as glutathione and glutaredoxins eventually diminishes the bacterium's ability to effectively neutralize ROS. This underscores the broader complexity of oxidative stress management during sulfur starvation, highlighting multiple ROS-generating pathways that warrant further exploration through targeted biochemical and genetic studies.

4.1.4 Sulfur Starvation Attenuates *P. aeruginosa* Virulence

Our transcriptomic and proteomic analyses reveal a marked downregulation of virulence-associated genes during sulfur starvation, many of which are tightly connected to iron metabolism. Several multidrug resistance (MDR) efflux pump components, including the *mexGHI-opmD* operon and *mexR*, were significantly repressed at the transcript level. The repression of these genes has significant implications and broader effects on both antibiotic resistance and type III secretion pathways of the organism.^{221,223} In parallel, genes involved in phenazine biosynthesis, including those encoding core PCA-synthesizing enzymes (*phz* clusters) and tailoring enzymes (*phzH*, *phzS*, and *phzM*), were strongly repressed at both transcript and protein levels. Given phenazines' dual roles in redox metabolism and pathogenesis in *P. aeruginosa*,^{159,252} these findings suggest that sulfur limitation indirectly attenuates virulence by disrupting Fe-dependent secondary metabolism.

Furthermore, we observed significant depletion of quorum-sensing (QS) systems and associated virulence factors. The *P. aeruginosa* PAO1 quinolone signal (PQS) system genes (*pqsABCDE*) and regulatory RNAs (*phrS* and *hfq*) were decreased, potentially leading to decreased PQS production. We also observed that the Rhl QS system proteins, including RhlR, RhlI, and RhlAB, were depressed at the proteomic level. Intriguingly, our RNA-seq data showed increased levels of *lasR* and *lasI* (see Figure 3.13), central components of the Las QS system, in the sulfur-

starved cells. Despite this, their downstream target genes *lasA* and *lasB*, which encode key virulence proteases, were primarily depressed. This paradox suggests a complex regulatory interplay. One possible explanation is that sulfur starvation activates stress response pathways that override the QS-mediated activation of these virulence genes. Alternatively, post-transcriptional mechanisms or involvement of small RNAs might suppress *lasA* and *lasB* expression despite an increase in *lasR* and *lasI* transcripts.²⁵³ The repression of these virulence factors may be a strategic energy conservation measure under nutrient-limited conditions.

Biofilm formation is crucial for *P. aeruginosa* persistence, especially in specific disease states. Our data reveal a mixed response in the expression of biofilm-associated gene products. While some genes in the *psl* operon increased at the transcript and protein levels, the observed decrease in Rhl system components suggests potential impairment in biofilm maturation and maintenance. Rhamnolipids produced by the Rhl system are essential for biofilm architecture and dispersion.²³⁶ Reduced rhamnolipid production could lead to altered biofilm structures, affecting the bacterium's ability to withstand environmental stresses. These observations suggest that sulfur starvation impacts QS-regulated virulence factors and may influence biofilm formation, possibly through the modulation of QS systems and iron metabolism pathways. The integration of these regulatory networks underscores the complex response of *P. aeruginosa* PAO1 to environmental stressors and highlights potential targets for therapeutic intervention.

We note that the observed transcriptional and proteomic shifts in these virulence factors are hypothesis-generating rather than definitive with respect to phenotype. Direct phenotypic validation of antibiotic susceptibility, biofilm formation, and *in vivo* virulence was beyond the

scope of the present investigation and is required to confirm the consequences of sulfur restriction on virulence phenotypes.

4.1.5 Gene Regulatory Network Inference and Evaluation

To assess the reliability of our GENIE3-inferred gene regulatory network, we evaluated the predicted targets of PA3678 (MexL), a TetR-family transcriptional repressor, against a curated gold standard of experimentally validated target genes. Known MexL-regulated genes include the *mexJK* efflux operon and phenazine biosynthesis operons (*phzA1-G1* and *phzA2-G2*).²⁵⁴ However, among the top 41 predicted targets of MexL generated by GENIE3, none overlapped with the 16 known gold-standard targets, resulting in zero precision and recall at this threshold.

Several important limitations may explain this lack of overlap. First, the small sample size ($n = 6$ total; 3 test and 3 control) used for our RNA-seq dataset likely constrained the statistical power of the GENIE3 algorithm to infer high-confidence regulatory edges. Gene regulatory network inference tools, including GENIE3, typically perform better with larger sample sizes that capture sufficient expression variability across biological states.²⁵⁵ Second, unlike human or model organisms such as *E. coli* or *S. cerevisiae*, *P. aeruginosa* transcription factors and regulatory networks remain poorly annotated. Databases like TRANSFAC or JASPAR offer extensive regulatory annotations for eukaryotic systems but are limited in prokaryotic coverage, making it more difficult to validate predictions.^{256–258}

Despite the poor overlap with the gold standard at this stage, the identification of MexL as a top regulatory element in the network is biologically plausible. Both transcriptomic and proteomic data showed strong differential expression of genes encoding components of the *mex* efflux system and phenazine biosynthetic gene clusters under sulfur-limiting conditions—systems

known to be transcriptionally regulated by MexL.^{254,259} This functional coherence between experimental data and GENIE3 predictions suggests that while the exact gene targets may not have matched, the network inference captured biologically relevant regulators. Further refinement with expanded datasets, improved annotations, or integrative approaches may enhance the predictive power of GENIE3 for less-characterized bacterial systems.

4.2 Conclusion

Our study highlights the intricate connection between sulfur and iron metabolism in *P. aeruginosa* PAO1, emphasizing the critical role sulfur plays in maintaining cellular redox balance and iron homeostasis. Through our work, we show that sulfur starvation induces an Fe-replete response, characterized by the downregulation of iron uptake systems and the upregulation of iron storage proteins. This adaptation likely serves to prevent iron-induced oxidative stress due to the bacteria's impaired ability to metabolize Fe when sulfur is in short supply. Moreover, we provide a robust mechanistic model that explains the often-observed overexpression of antioxidant genes during sulfur limitation. Our findings suggest a scenario in which Fe initially serves as a trigger for ROS generation, prompting rapid cellular adaptive responses to mitigate Fe toxicity. However, under prolonged sulfur limitation, the compromised synthesis of sulfur-containing antioxidant molecules, such as glutathione and glutaredoxins, eventually diminishes the bacterium's ability to effectively neutralize ROS. Additionally, we demonstrate that disrupting sulfur metabolism could attenuate virulence and increase sensitivity to intervention with currently available antibiotics, offering multiple potential interventions against *P. aeruginosa* PAO1 infections. By elucidating the mechanistic links between sulfur limitation, iron homeostasis, and oxidative stress response, our work advances the understanding of bacterial adaptation to nutrient stress. It underscores the

importance of sulfur in bacterial physiology and pathogenicity, highlighting potential targets for the development of antimicrobial agents.

4.2.1 Future Directions

Beyond Iron: The Case for Sulfur in Nutritional Immunity

Our work has identified a critical connection between sulfur starvation, oxidative stress, and virulence gene expression in *P. aeruginosa*, suggesting potential new avenues for nutritional immunity strategies. Nutritional immunity traditionally targets Fe sequestration to limit pathogen growth, using Fe chelators such as deferoxamine and iron mimics like gallium.^{174,260,261} While effective, these approaches inadvertently trigger compensatory virulence responses in bacteria, representing significant therapeutic drawbacks.⁹⁷

The insights from our study position sulfur starvation as an alternative nutritional immunity strategy. Unlike iron-based methods, limiting sulfur availability could potentially disrupt critical bacterial survival mechanisms without triggering heightened virulence. Specifically, our data indicate that sulfur deprivation has a significant impact on quorum sensing and biofilm formation, which are crucial factors in *P. aeruginosa*'s resistance to frontline antibiotics. We observed a marked reduction in genes integral to initial biofilm formation and maturation processes, including components of the *psl* operon, quorum sensing regulators, and motility-associated genes such as *fli* and *pil*. These findings open compelling avenues for further exploration. For instance, future investigations could comprehensively assess the impact of sulfur limitation on biofilm dynamics—formation, maturation, and surface attachment—using techniques like confocal microscopy, crystal violet assays, flow cell systems, and transcriptomic or proteomic profiling.^{262,263} Prior

studies in bacterial models further support the potential efficacy of targeting nutrient pathways for biofilm disruption.^{84,264,265}

However, the implementation of sulfur limitation as a therapeutic strategy faces notable challenges. Bacteria, including *P. aeruginosa*, possess multiple, redundant pathways for sulfur acquisition, enabling the uptake and utilization of various sulfur-containing compounds beyond inorganic sulfate, such as methionine, cysteine, taurine, and even complex sulfur-rich carbohydrates like mucins.^{26,41} These extensive metabolic capabilities underline the complexity of effectively restricting sulfur availability. Nevertheless, targeting specific sulfur acquisition or utilization pathways through inhibitor-based approaches might prove beneficial.

Honing in on Potential Anti-Virulence Factors Linked to Sulfur Starvation

Interestingly, our observations indicate that classical antioxidant enzymes such as catalase (particularly the housekeeping enzyme KatA) play only minor roles under sulfur-limiting conditions. In contrast, our proteomic and transcriptomic analyses identified proteins like organic hydroperoxide resistance protein (Ohr) and the 1-Cys peroxiredoxin (LsfA) as central antioxidant defenses prominently induced during sulfur starvation. The sustained overexpression of these enzymes, particularly evident in our time-course proteomic experiments, suggests they might serve as attractive anti-virulence targets.

Future research could exploit these proteins by exploring their potential as therapeutic targets. Key questions remain: Do Ohr and LsfA have functional human orthologs, and if so, how divergent are their active sites? A study has already identified LsfA as being involved in virulence in *P. aeruginosa* by serving as the primary buffer against ROS generated by NADPH oxidase in an investigation utilizing mice.²⁰⁵ Detailed bioinformatics analyses followed by combinational

drug screenings and computational docking studies could assess the feasibility and specificity of targeting these enzymes. Such studies would clarify whether these bacterial antioxidant proteins represent viable candidates for novel antimicrobial or anti-virulence therapeutics without adverse off-target effects in human cells.

Computational Predictions Provide Insight but Require Experimental Validation

Our computational analyses provided intriguing insights into the regulatory networks governing sulfur starvation responses in *P. aeruginosa*, indicating MexL as a putative regulatory hub. However, these results must be interpreted with caution due to inherent limitations associated with computational inference approaches.

The network inference tool GENIE3, despite its established utility for regulatory network construction, yielded sub-optimal precision and recall values in our study. Efforts to improve predictive accuracy, including integration of protein-protein interaction (PPI) data from the STRING database and utilization of PANDA (Passing Attributes between Networks for Data Assimilation),²⁶⁶ unexpectedly resulted in diminished model performance. This decline highlights a critical limitation—the dependency of machine learning algorithms on both the quantity and quality of input data. Our analyses were significantly constrained by the relatively small RNA-seq sample size, underscoring the need for extensive datasets to generate robust and reliable network predictions.²⁵⁵

Nonetheless, the identification of MexL as a regulatory hub remains biologically plausible. MexL has known regulatory roles, either directly or indirectly, in modulating critical pathways such as phenazine biosynthesis and the MexJK-OprM efflux system, both implicated in bacterial

virulence and stress responses.^{254,259} Thus, despite computational limitations, MexL warrants further investigation as a potential central regulator mediating sulfur starvation response.

To conclusively validate these computational predictions, experimental methodologies with higher specificity and accuracy should be employed. Techniques such as electrophoretic mobility shift assays (EMSA) and chromatin immunoprecipitation sequencing (ChIP-seq) are well-established methods for experimentally validating regulatory interactions. EMSA allows for direct assessment of DNA-binding specificity and affinity, while ChIP-seq enables comprehensive mapping of transcription factor binding sites across the genome. These methods have successfully elucidated complex regulatory networks in *P. aeruginosa*, including studies of quorum-sensing regulators (LasR, RhlR) and iron-responsive regulator (Fur), providing high-confidence validation of computational predictions.^{267–270} Therefore, although computational approaches remain invaluable for hypothesis generation, our results reinforce the importance of complementary experimental validation. Future research should prioritize employing these rigorous experimental methods to definitively confirm regulatory interactions identified *in silico*.

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Appendix

RNA-seq Quality Assessment

Table A1. RNA concentration and purity metrics for test (S1–S4) and control (SS1–SS4) *P. aeruginosa* samples. Concentration is given in ng/μl, and purity is assessed using A260/A280 and A260/A230 absorbance ratios

Sample ID	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230
S1	159.60	ng/μl	3.99	1.84	2.16	2.30
S3	174.70	ng/μl	4.37	2.00	2.18	2.04
S4	180.10	ng/μl	4.50	2.06	2.19	1.89
SS1	575.30	ng/μl	14.39	6.75	2.13	2.20
SS3	578.70	ng/μl	14.47	6.71	2.16	2.21
SS4	624.60	ng/μl	15.61	7.21	2.26	2.19

Table A2. RNA quality control results from Admera Health for test (S1–S4) and control (SS1–SS4) *P. aeruginosa* samples. Reported values include RNA concentration, total RNA quantity, and RNA Integrity Number (RIN), confirming suitability for sequencing

Sample ID Table & QC Results

Sample #	Admera Health Sample ID	Customer Sample ID	Sample Type	Sample Volume (ul)	Admera Health Concentration (ng/ul)	Admera Health Total Quantity (ng)	RIN
1	22054R-01-01	S1	Total RNA	14	63.50	889	9.4
2	22054R-01-02	S3	Total RNA	14	65.30	914	9.3
3	22054R-01-03	S4	Total RNA	14	68.80	963	9.4
4	22054R-01-04	SS1	Total RNA	14	318.00	4452	7.7
5	22054R-01-05	SS3	Total RNA	14	299.00	4186	9.2
6	22054R-01-06	SS4	Total RNA	14	397.00	5558	8.4

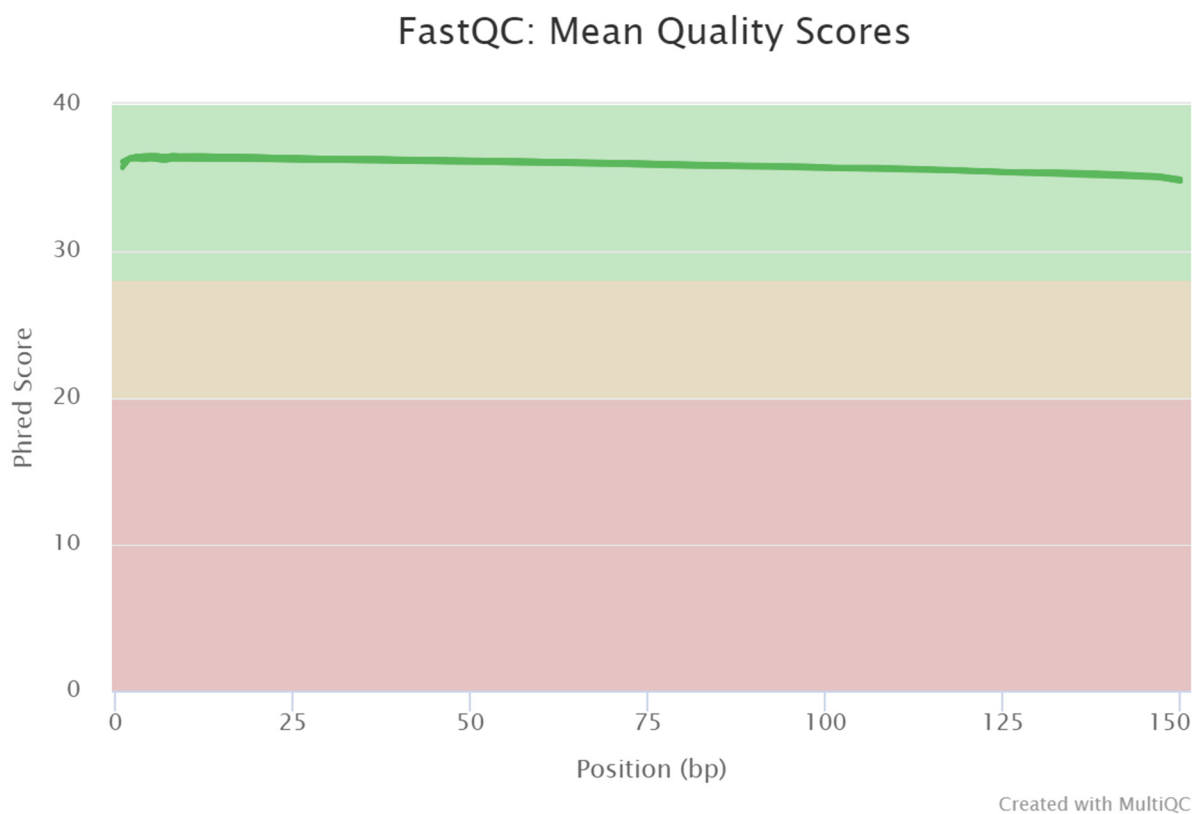
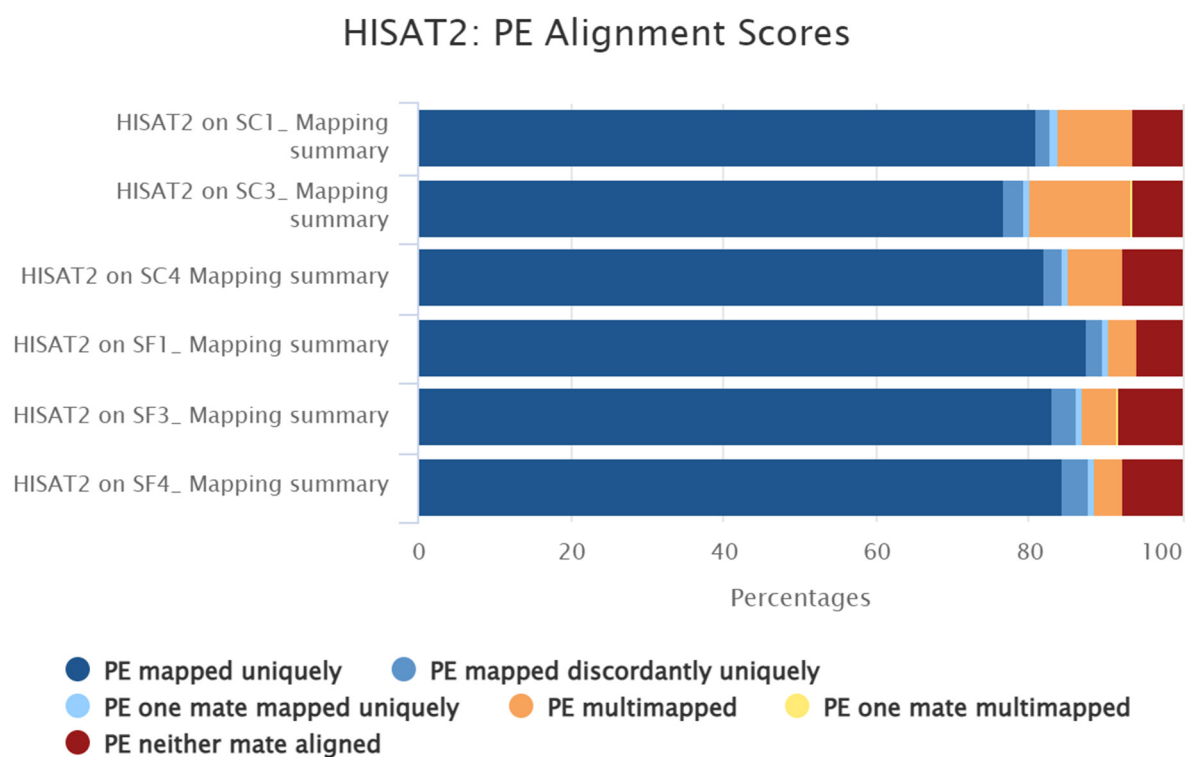


Figure A1. Per-base sequence quality scores from FastQC analysis of raw RNA-seq reads. High mean Phred scores (>35) across all positions indicate good base-calling accuracy suitable for downstream analysis



Created with MultiQC

Figure A2. HISAT2 alignment statistics for paired-end RNA-seq reads from sulfur-starved (SF) and control (SC) *P. aeruginosa* samples. Most reads mapped uniquely to the reference genome, indicating high alignment quality across all replicates

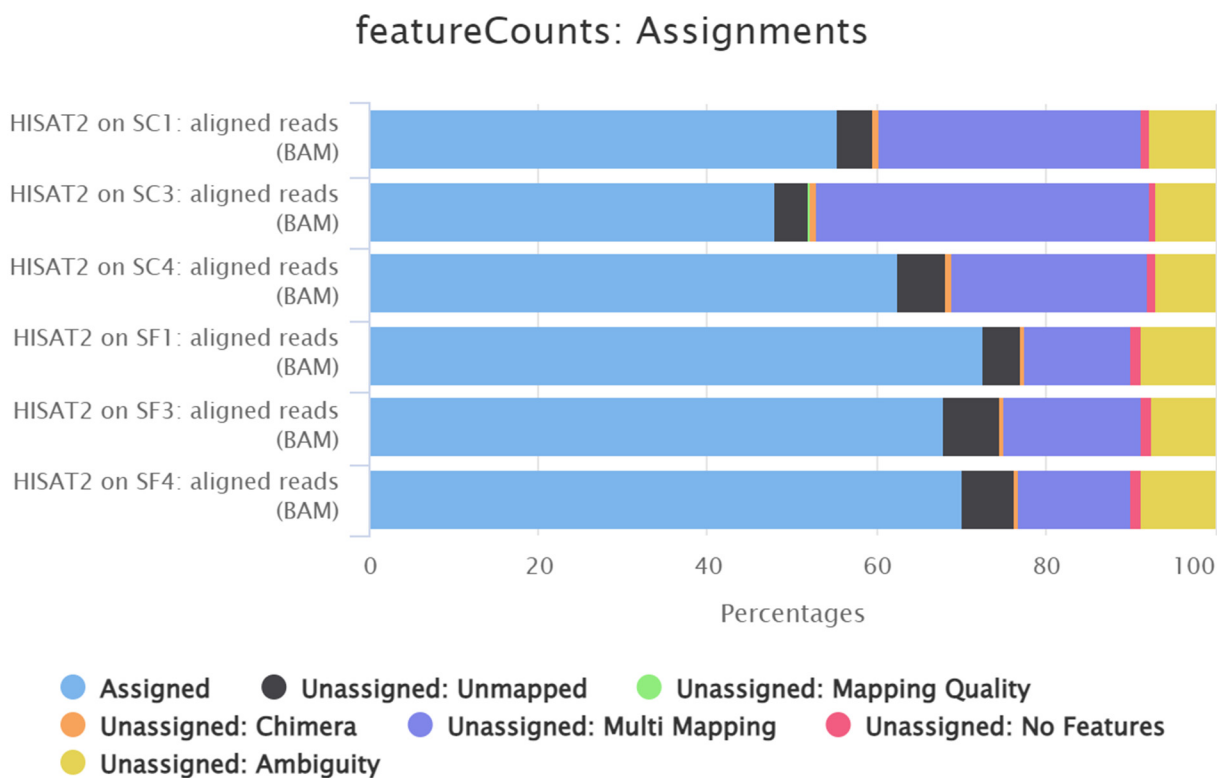


Figure A3. Summary of featureCounts assignments for aligned RNA-seq reads from control (SC) and sulfur-starved (SF) *P. aeruginosa* samples. Most reads were successfully assigned to genomic features, while unassigned reads were mainly due to ambiguity, multimapping, or lack of annotation

Proteomics Quality Assessment

Proteomics Experiment Summary

Protein quantified per sample (after pre-processing).

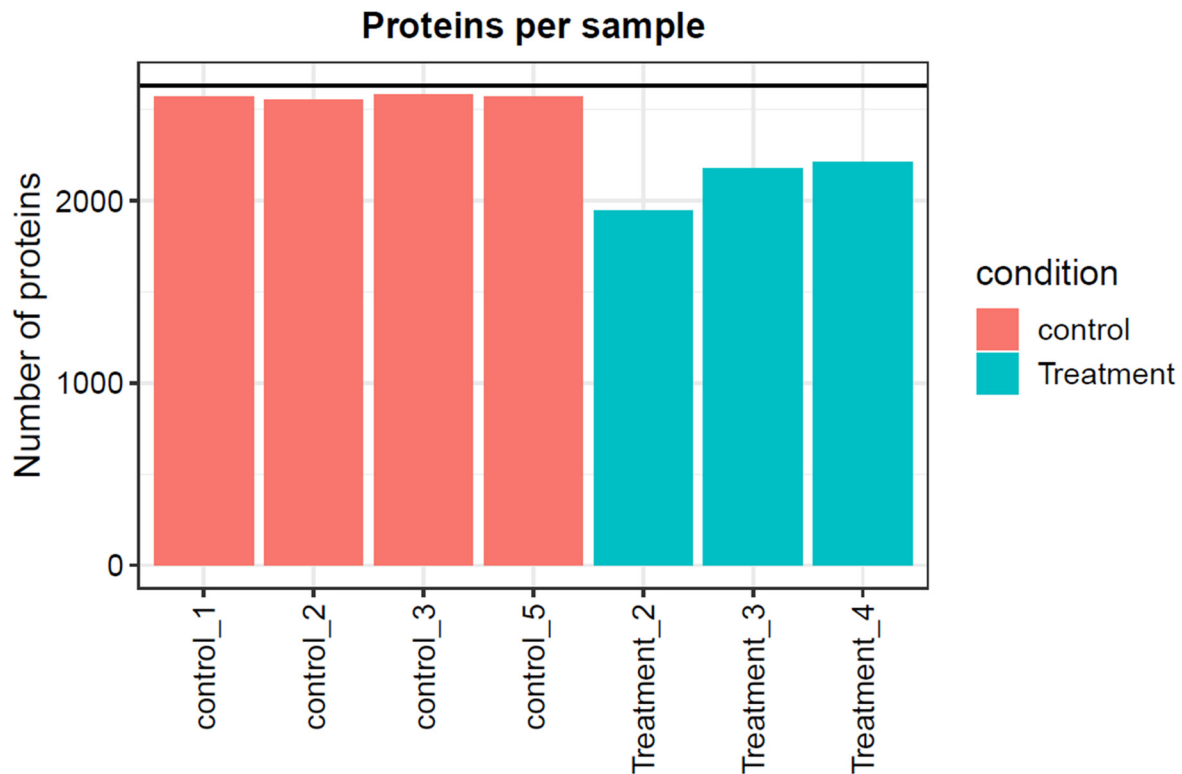


Figure A4. Bar chart showing the number of proteins quantified per sample after preprocessing. Control samples consistently yielded over 2,400 proteins, while treatment samples showed slightly lower counts, ranging from ~1,950 to ~2,200 proteins

Missing Value handling

Missing value heatmap

A heatmap for proteins with missing value in each dataset. Each row represent a protein with missing value in one or more replicate. Each replicate is clustered based on presence of missing values in the sample.

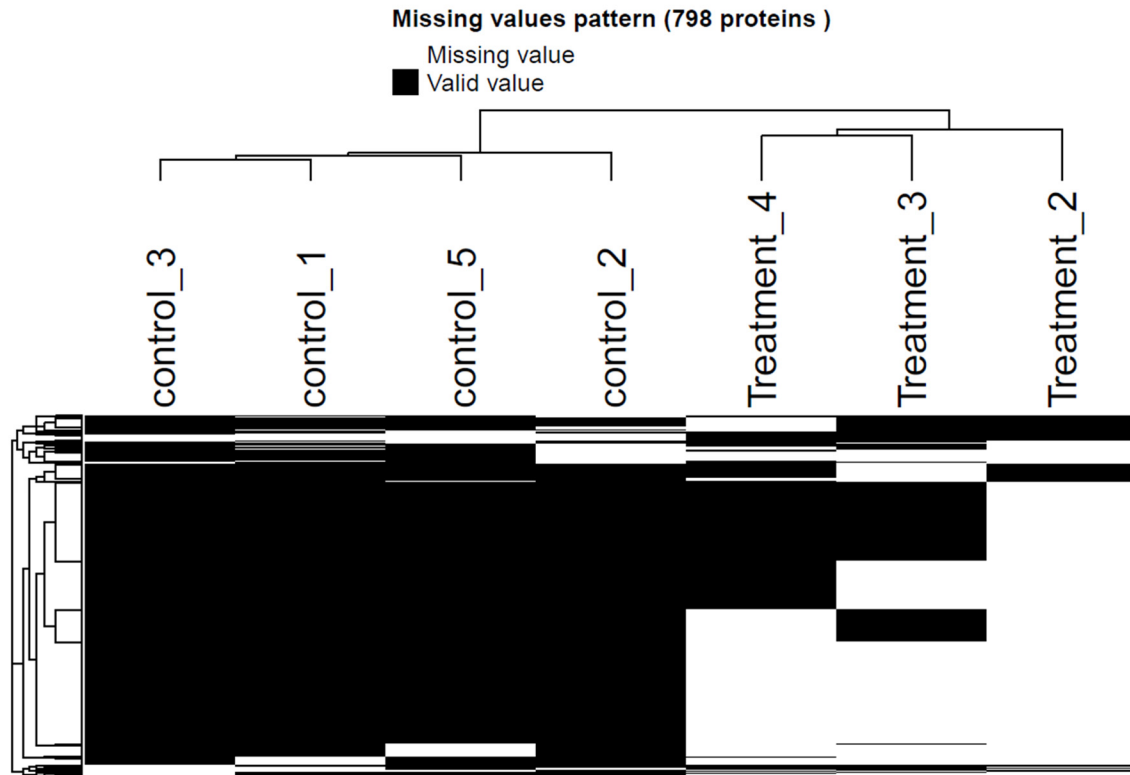


Figure A5. Heatmap displaying the distribution of missing values across samples. Each row represents a protein, with black indicating valid data and white denoting missing values. Clustering reveals that most missing values are *missing not at random* (MNAR), particularly enriched in treatment samples

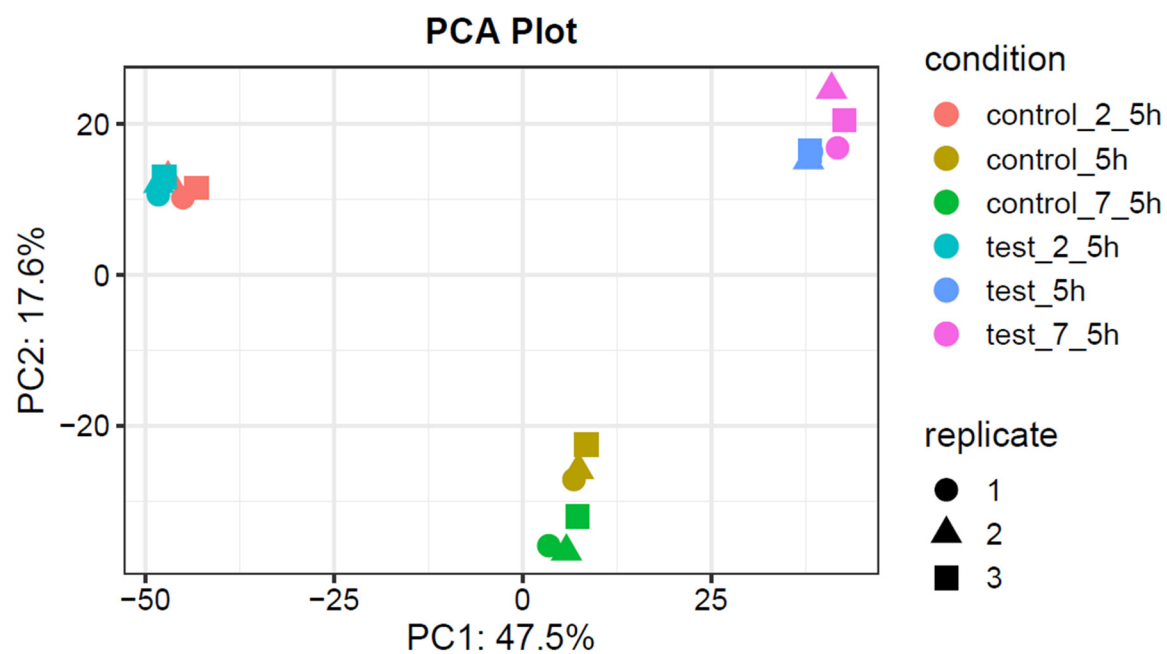


Figure A6. PCA plot from time-course proteomics showing clear separation of samples by condition and time point. PC1 (47.5%) and PC2 (17.6%) capture treatment- and time-dependent variance in protein expression

Proteomics Experiment Summary

Protein quantified per sample (after pre-processing).

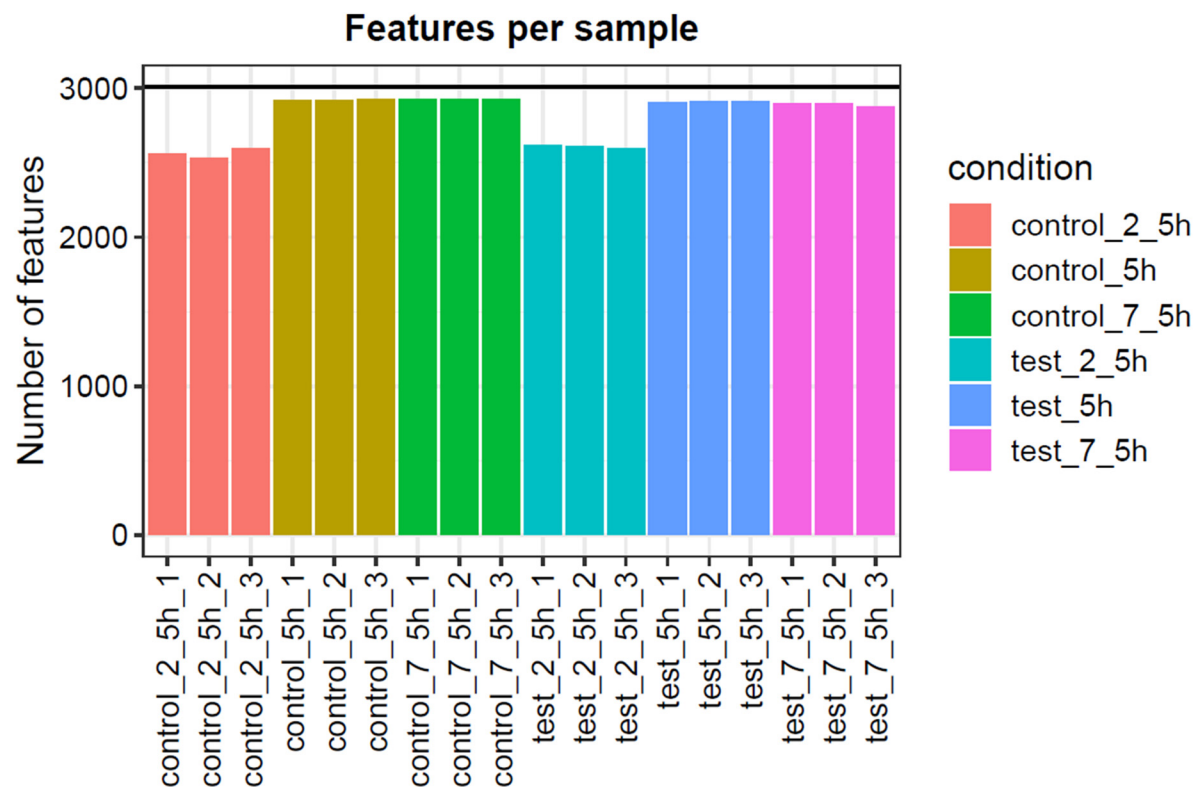


Figure A7. Barplot showing the number of quantified proteomic features per sample across time-course conditions. All samples show consistent feature counts following preprocessing, indicating uniform proteome coverage across conditions

Missing Value handling

Missing value heatmap

A heatmap for proteins with missing value in each dataset. Each row represent a protein with missing value in one or more replicate. Each replicate is clustered based on presence of missing values in the sample.

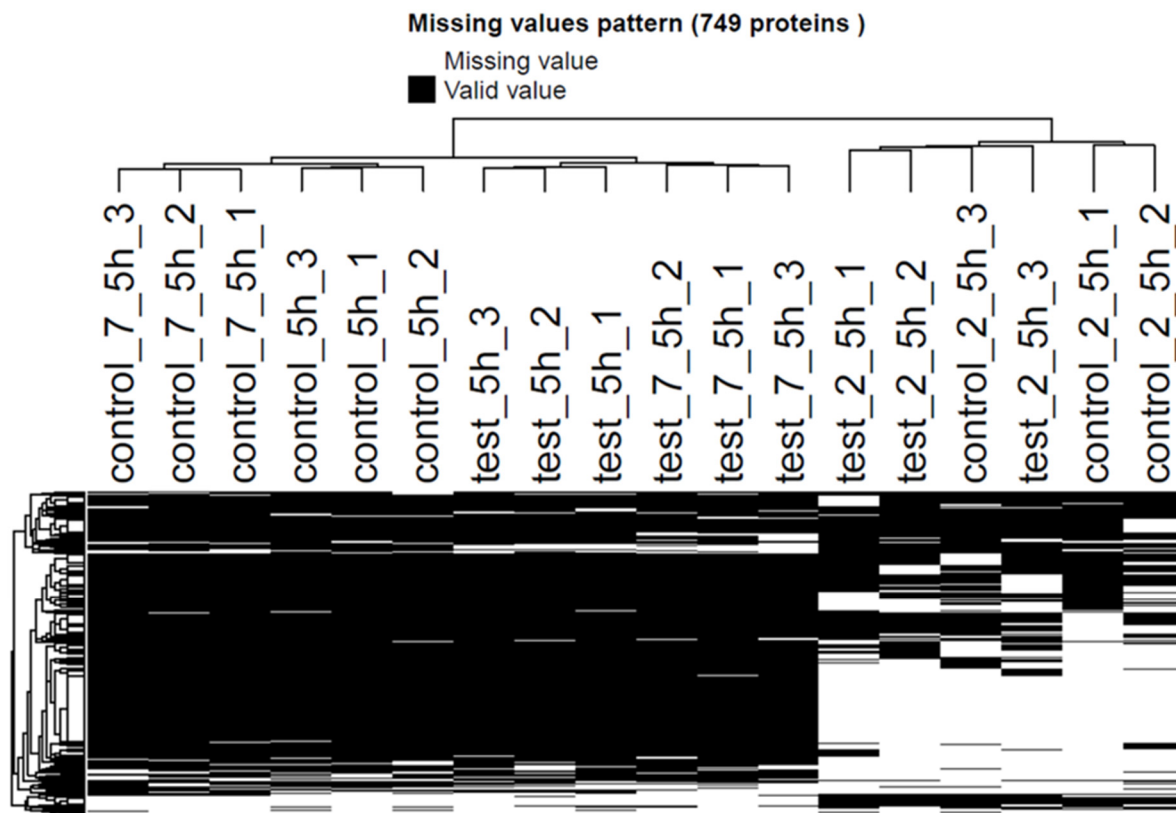


Figure A8. Heatmap showing the distribution of missing proteomic values across time-course samples. Most missing values occur non-randomly (MNAR), with clustering indicating systematic differences in missingness across conditions and replicates

HPLC Profiles for Pyoverdine Assay

Data file:	SC_control.dx	Project Name:	JA
Sequence Name:	chidozie_011025	Operator:	SYSTEM
Sample name:	SC_control	Injection date:	2025-01-10 17:51:25-06:00
Instrument:	NightHawk	Location:	1
Inj. volume:	20.000	Type:	Sample
Acq. method:	Chidozie_pyoverdine_4_24.amx	Sample amount:	0.00
Processing method:	*3D UV Quantitative_DefaultMethod.pmx		
Manually modified:	None		

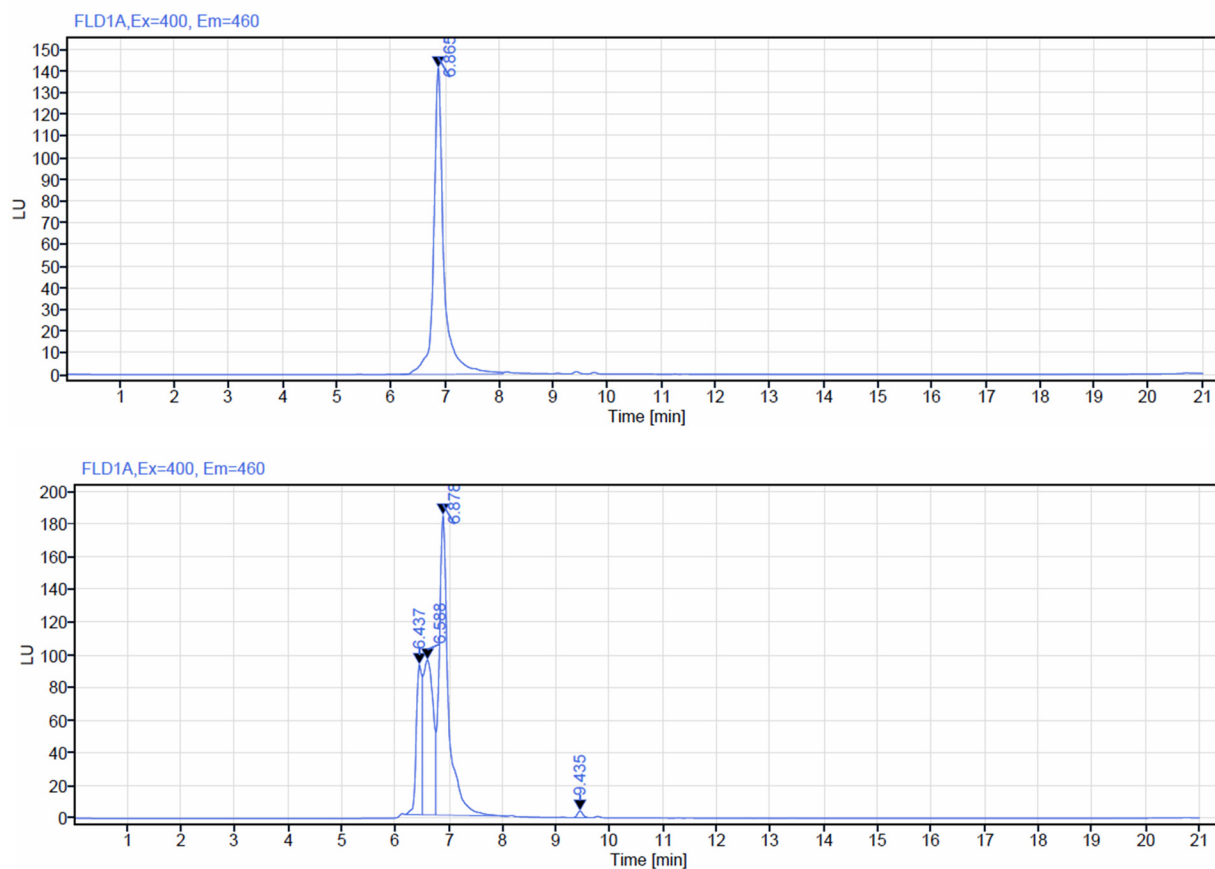


Figure A9. HPLC-FLD chromatograms for pyoverdine quantification in the control group. The upper panel shows the chromatogram at 5 h, while the lower panel shows the 7.5h timepoint. Fluorescence was monitored at Ex: 400 nm, Em: 460 nm

Data file:	SF_treatment.dx	Project Name:	JA
Sequence Name:	chidozie_011025	Operator:	SYSTEM
Sample name:	SF_treatment	Injection date:	2025-01-10 17:08:23-06:00
Instrument:	NightHawk	Location:	1
Inj. volume:	20.000	Type:	Sample
Acq. method:	Chidozie_pyoverdine_4_24.amx	Sample amount:	0.00
Processing method:	*3D UV Quantitative_DefaultMethod.pmx		
Manually modified:	None		

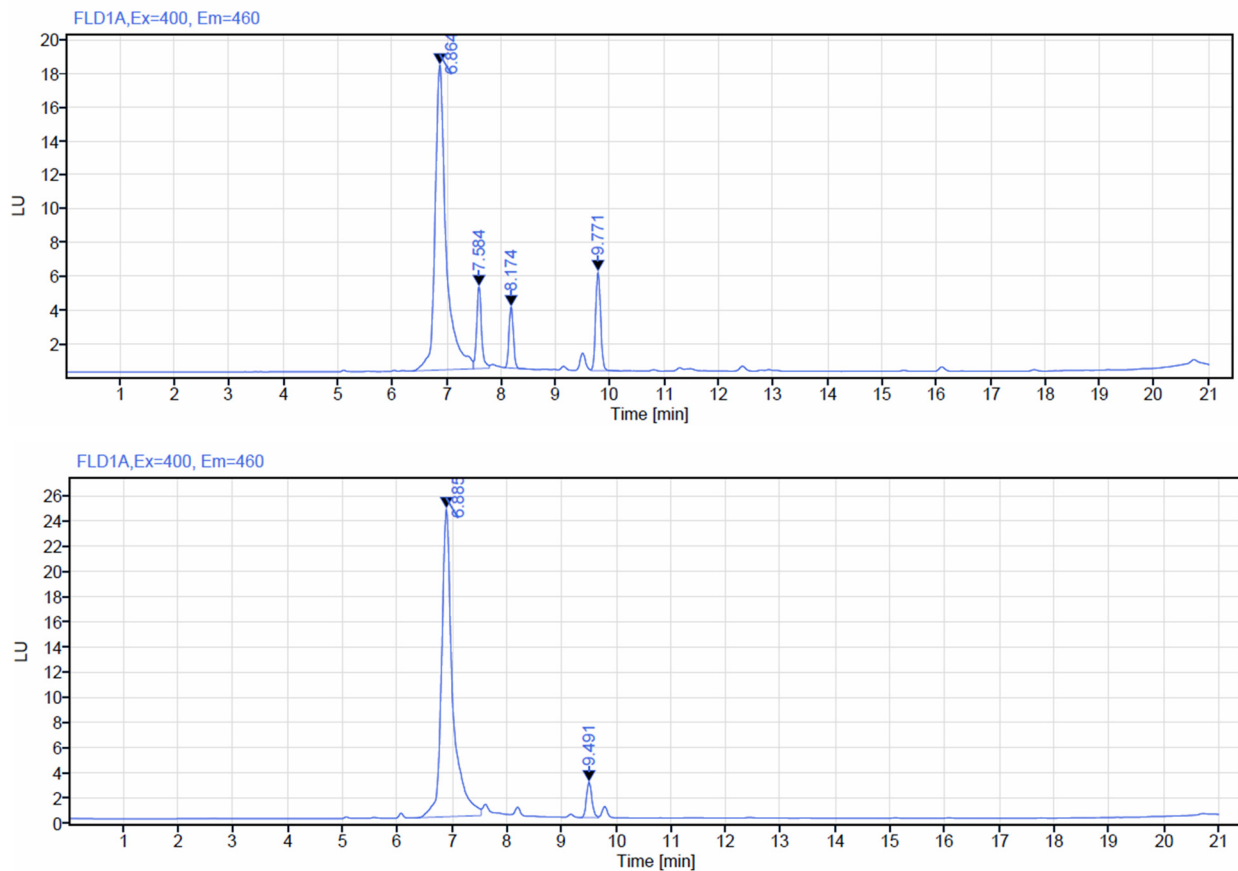


Figure A10. HPLC-FLD chromatograms for pyoverdine quantification in the test group. The upper panel shows the chromatogram at 5 h, while the lower panel shows the 7.5h timepoint. Fluorescence was monitored at Ex: 400 nm, Em: 460 nm

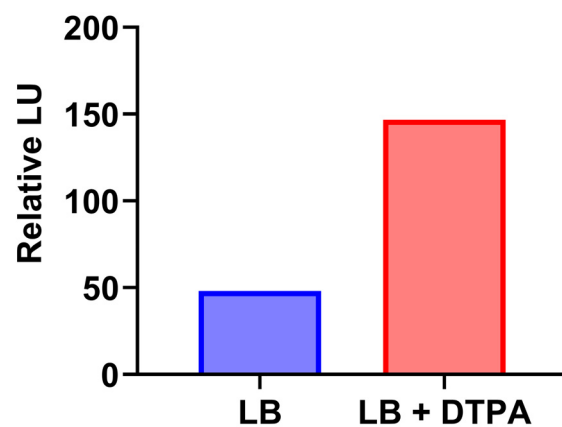


Figure A11. Relative pyoverdine levels in *P. aeruginosa* cultures grown in LB versus LB supplemented with the iron chelator DTPA. Peak areas were derived from HPLC chromatograms, indicating increased pyoverdine production under iron-limiting conditions.

Supplementary Tables on Figshare

- **Table S1:** Full RNA-seq differential expression table
- **Table S2:** Functional enrichment analysis of RNA-seq data
- **Table S3:** Full label-free proteomics differential expression table
- **Table S4:** Functional enrichment analysis of proteomics data
- **Table S5:** Predicted regulatory elements inferred from RNA-seq
- **Table S6:** Top 1000 ranked edges from GENIE3 gene regulatory network analysis

URL: <https://figshare.com/s/64c2c95ff3b9477ba497>