Gene Expression of a *Pseudomonas aeruginosa* Isolate During Osmotic, Low Nutrient and High Salinity Stress

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“After all, science is essentially international, and it is only through lack of the historical sense that national qualities have been attributed to it.”

-Marie Curie

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Resumo

Pseudomonas aeruginosa (*P. aeruginosa*) é um patógeno oportunista Gram-negativo metabolicamente versátil, capaz de prosperar numa ampla variedade de ambientes, desde hospedeiros humanos até solo, água doce e ecossistemas marinhos. A sua resiliência ambiental e relevância clínica são sustentadas por um genoma altamente conservado, redes regulatórias complexas e um extenso arsenal de fatores de virulência, sistemas de efluxo e metabolitos secundários. Estas características permitem a *P. aeruginosa* detetar e responder a diversos estímulos ambientais, incluindo stress osmótico, limitação de nutrientes e elevada salinidade. Apesar do vasto conhecimento sobre isolados clínicos, pouco se sabe sobre as respostas fisiológicas e a regulação genética de isolados marinhos sob stress ambiental.

Nesta tese, a estirpe 25L, uma bactéria marinha isolada do porto de Trieste, foi selecionada como modelo para investigar as respostas transcricionais a condições de stress osmótico, baixo teor de nutrientes e elevada salinidade. Foram analisados genes-alvo envolvidos em vias adaptativas e reguladas por *quorum sensing* (QS), incluindo um componente de uma bomba de efluxo (*mexA*), um gene responsável pela biossíntese de pioverdina (*pvdA*), um gene integrado biossíntese de piocianina (*phzA1*) e uma enzima integrada na *stringent response* codificada pelo gene *relA*. *One-step* e *two-step* RT-qPCR foram otimizados para quantificar os níveis de transcrição dos genes escolhidos e as condições experimentais foram cuidadosamente controladas para evitar contaminação de DNA e artefactos, como *primer-dimers*.

Os resultados obtidos destacaram tanto a plasticidade transcricional de *P. aeruginosa* 25L sob stress ambiental como os desafios experimentais de quantificar com precisão a expressão génica. Embora preliminar, este trabalho estabelece uma base metodológica e conceitual para relacionar a adaptação marinha à sobrevivência no ar, oferecendo ideias sobre como os patógenos oportunistas persistem através das fronteiras ecológicas, com implicações potenciais para a microbiologia marinha e a saúde pública. No geral, este estudo estabelece uma base para futuras investigações sobre os determinantes genéticos e regulatórios da adaptação ao stress de *P. aeruginosa* em ecossistemas marinhos.

Palavras-chave: *Pseudomonas aeruginosa* 25L, ambiente marinho, bombas de efluxo, sideróforo, fenazina, expressão de genes, Trieste.

Abstract

Pseudomonas aeruginosa (*P. aeruginosa*) is a metabolically versatile Gram-negative opportunistic pathogen capable of thriving in a wide range of environments, from human hosts to soil, freshwater and marine ecosystems. Its environmental resilience and clinical relevance are underpinned by a highly conserved genome, intricate regulatory networks and an extensive arsenal of virulence factors, efflux systems as well as secondary metabolite pathways. These features allow *P. aeruginosa* to sense and respond to diverse environmental stimuli, including osmotic stress, nutrient limitation and high salinity. Despite extensive knowledge of clinical isolates, little is known about the physiological responses and gene regulation of marine isolates under environmental stress.

In this thesis, strain 25L, a marine isolate from the port of Trieste, was selected as a model to investigate transcriptional responses to osmotic, low nutrient and high salinity stress conditions. Target genes involved in key adaptive and QS-regulated pathways were analyzed, including the multidrug efflux pump component *mexA*, the pyoverdine biosynthesis gene *pvdA*, the pyocyanin biosynthesis gene *phzA1* and the stringent response enzyme encoded by *relA*. One-step and two-step RT-qPCR were optimized to quantify transcript levels and experimental conditions were carefully controlled to avoid DNA contamination and primer-dimer artifacts.

The obtained results highlighted both the transcriptional plasticity of *P. aeruginosa* 25L under environmental stress and the experimental challenges of accurately quantifying gene expression. Although preliminary, this work establishes a methodological and conceptual foundation for linking marine adaptation to airborne survival, offering insights into how opportunistic pathogens persist across ecological boundaries with potential implications for marine microbiology and public health, establishing basis for future investigations on the genetic and regulatory determinants of stress adaptation of *P. aeruginosa* in marine ecosystems.

Keywords: *Pseudomonas aeruginosa* 25L, marine environment, efflux pumps, siderophore, phenazine, gene expression, Trieste.

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List of Abbreviations

AHL	ACYL-HOMOSERINE LACTONES
AI	AUTOINDUCERS
AQ	ALKYL-QUINOLONES
BALMAS	BALLAST WATER MANAGEMENT SYSTEM FOR ADRIATIC SEA PROTECTION
CA	CHORISMIC ACID
Ct	CYCLE THRESHOLD
DNA	DEOXYRIBONUCLEIC ACID
Fur	FERRIC UPTAKE REGULATOR
IPA	INSTRUMENT FOR PRE-ACCESSION ASSISTANCE
MPCBA	5-METHYL PHENAZINE-1-CARBOXYLIC BETAINE
NRPS	NONRIBOSOMAL PEPTIDE SYNTHETASE
NTC	NO-TEMPLATE CONTROL
PCA	PHENAZINE-1-CARBOXYLIC ACID
PCR	POLYMERASE CHAIN REACTION
PQS	PSEUDOMONAS QUINOLONE SIGNAL
PVDI	PYOVERDINE I
PYO	PYOCYANIN
QS	QUORUM SENSING
qPCR	QUANTITATIVE POLYMERASE CHAIN REACTION
RNA	RIBONUCLEIC ACID
ROS	REACTIVE OXYGEN SPECIES
RT-qPCR	REVERSE TRANSCRIPTION QUANTITATIVE POLYMERASE CHAIN REACTION
RND	RESISTANCE-NODULATION-CELL DIVISION

1. Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is a metabolically versatile, Gram-negative, aerobic rod-shaped bacterium belonging to the *Pseudomonadaceae* family within the Gammaproteobacteria class [31][70][82][91]. While its clinical importance is well-established being a leading cause of ventilator-associated pneumonia, sepsis in immunocompromised individuals and chronic respiratory infections in cystic fibrosis patients this bacterium belongs to the notorious ESKAPE pathogens, a group of opportunists with profound antibiotic resistance and limited therapeutic options but it has been also increasingly recognized as an environmentally persistent organism [27][82][70][72][91]. Thriving ubiquitously in both anthropogenic and natural aquatic habitats, including wastewater systems and marine environments, *P. aeruginosa* ability to persist under highly variable and often hostile conditions stems from a unique combination of intrinsic resistance mechanisms, broad metabolic adaptability and tightly regulated stress response networks [15][24][27][34][53][70][82][94].

The *Pseudomonas* genus comprises more than 200 species, reflecting remarkable metabolic diversity and ecological adaptability with relatively large genomes averaging 5,63 Mbp, particularly, *P. aeruginosa* has genome size ranging from 5,2 to 7,4 Mbp which encodes an extensive repertoire of transporters, efflux systems, regulatory proteins and secondary metabolite biosynthetic pathways [34][81][82][89][111]. These genetic resources enable the bacterium to sense and respond to shifts in nutrient availability, osmotic pressure, redox conditions and chemical pollutants [18][34][109][120]. Importantly, the versatility of *P. aeruginosa* does not depend solely on the acquisition of new genes but rather on the fine-tuned regulation of conserved traits, with approximately 80% of the genome forming a conserved core and around 90% sequence identity across isolates contrasting with other bacteria such as *Escherichia coli* (with a core genome of 990 genes and 6% belonging to the pan genome) or *P. fluorescens* (with 60% of shared genes) [9][38][60][133]. This unusually high conservation facilitates rapid regulatory reprogramming rather than reliance on gene acquisition allowing the bacterium to adjust its physiology dynamically to osmotic stress, nutrient shifts, pollutants and redox fluctuations [9][38][70][99].

The regulatory plasticity of *P. aeruginosa* underpins its ecological success to colonize diverse habitats, withstanding fluctuating stressors and interacting competitively with other microorganisms [70]. Understanding these mechanisms is therefore essential for

unravelling how this species maintains long-term persistence in dynamic environments such as the marine and coastal ecosystems.

1.1. The marine environment and the Trieste port as an ecological model

Marine environments exert a set of selective pressures on microbial life including high salinity, nutrient limitation, exposure to heavy metals and fluctuations in temperature, oxygen availability and light conditions [56][75][114][119]. The Gulf of Trieste, a semi-enclosed basin in the northern Adriatic Sea (Figure 1), provides an ideal ecological model system as these environmental pressures are further intensified by freshwater inflows, seasonal upwelling and anthropogenic activities such as shipping as well as industrial discharge [13][84][114]. This unique combination of natural variability and human impact makes the port of Trieste a dynamic ecological model system for investigating microbial stress adaptation [13].

Previous studies of marine bacterial isolates from similar environments have revealed traits associated with adaptations to such conditions including an enhanced resistance to osmotic stress, expanded repertoires of efflux pumps and highly efficient siderophore-mediated iron acquisition mechanisms [61]. These characteristics reflect evolutionary selection for generalist survival strategies, enabling microorganisms to persist in environments that fluctuate on daily, seasonal and human-driven timescales [61][105][119].

Strain 25L, the focus of this study, exemplifies this adaptive potential as it was one of seven *P. aeruginosa* isolates (17L, 22L, 23L, 24L, 25L, 27L and 32L) obtained from different stations in the port of Trieste (Figure 1) in 2015 as part of the BALMAS (Ballast Water Management System for the Protection of the Adriatic Sea) Project, a cross-border initiative under the IPA Adriatic Cross-Border Cooperation Initiative (2007-2013) [8][47]. Complete genome sequencing of these isolates provided a valuable foundation for exploring the genetic basis of marine adaptation in *P. aeruginosa*, while strain 25L was

selected here as a model to experimentally investigate transcriptional responses to controlled stress conditions bridging genomic observations with functional evidence.

Figure 1- Aerial view of the Port of Trieste (Italy). The Port of Trieste, located on the northern Adriatic Sea, represents a dynamic ecological system shaped by both natural and anthropogenic pressures, such as freshwater inflows and intense shipping activity. The inset shows the geographical location of the Gulf of Trieste within Italy, highlighted by a red square.

1.2. Quorum sensing and Stress Regulation in *P. aeruginosa*

The environmental and clinical success of *P. aeruginosa* relies heavily on its ability to tightly regulate energetically costly adaptive traits in a coordinated and population-wide manner, enabling this bacterium to thrive in diverse and often hostile environments where microorganisms are simultaneously exposed to a myriad of different stressors [5] [34][70][99]. This regulation is achieved through quorum sensing (QS), a cell-density-dependent transcriptional regulatory and environmental sensing system used by many Gram-positive and Gram-negative bacteria, that allows microorganisms to perceive population density changes by producing and detecting small diffusible molecules known as autoinducers (AI) [41][127]. When a threshold concentration of these signals is reached, QS triggers the synchronized expression of multiple genes, enabling the bacterial community to behave as a coordinated unit rather than as isolated cells [22][41][127].

In Gram-negative bacteria, QS signaling is most often mediated by acyl-homoserine lactones (AHL), a family of molecules characterized by a conserved lactone core ring linked to varying acyl side chains of different lengths, oxidation states and degrees of unsaturation (Figure 2) [83][124]. The QS in *P. aeruginosa* regulates approximately 10%

of the genome and up to 20% of the proteome, underscoring its role as a master regulator of population-wide behaviors by linking gene expression to cell density and environmental cues through three main systems (Figure 2) and integrating major survival pathways such as efflux activity, siderophore biosynthesis, phenazine production and stress adaptation [103][104][122].

The QS-regulated coordination of efflux activity, siderophore production, phenazine biosynthesis and the stringent response exemplifies the dual ecological and clinical identity of *P. aeruginosa* ensuring survival under osmotic stress, heavy metal exposure, and nutrient limitation, while in clinical settings the same systems enable antibiotic resistance [5][98][130]. This integration allows QS to act simultaneously as an environmental sensing system and a regulator of community-level metabolic investment, ensuring that survival strategies are activated only under conditions that maximize fitness [103][122].

1.2.1 QS Regulatory Systems

The QS in *P. aeruginosa* is organized into three interlinked systems: the LasI/LasR and RhlI/RhlR AHL-based circuits, as well as the Pseudomonas Quinolone Signal (PQS) (Figure 2) [57][125][126].

In the LasI/LasR system the autoinducer is the molecule N-3-oxododecanoyl-L-homoserine lactone (3OC12-HSL) that is synthesized by the LasI protein and is released intra- and extracellularly [57][125][127]. As this AI reaches a threshold concentration, it binds to the receptor LasR, forming an active complex that induces the expression of genes encoding hemolysins, proteases, elastases, exotoxin-A production and biofilm-related components (Figure 2). Importantly, LasR also activates the downstream Rhl and PQS systems, positioning the Las system at the top of the QS hierarchy [57][97][125][126]. The genes that are regulated by this system possess specific regions called *las/rhl* boxes upstream of the protein-coding sequence, which are recognized by both LasR/AHL and RhlR/AHL complexes, but it is not a unique mechanism [7][57][125][126], as negative feedback is provided by RsaL, a transcriptional repressor controlled by LasR which suppresses both *lasR* and *rsaL* expression and directly represses QS target genes such as those involved in pyocyanin biosynthesis [68][130].

In similarity to the above-described QS system, RhlI produces N-butyryl-L-homoserine lactone (C4-HSL) that binds to its cognate receptor RhlR to activate the expression of genes encoding rhamnolipids, pyocyanin, hydrogen cyanide, siderophores, elastases, alkaline proteases and genes involved in the regulation of the bacterial motility (Figure

2) [7][110][126]. The Rhl system is also positively regulated by the complex LasR-(3-oxo-C12-HSL), creating a hierarchical dependency on AHL [23][122]. RhlI additionally drives a positive feedback loop that reinforces C4-HSL production, ensuring the robustness of the system at high cell density [23][122].

Distinct from AHL-based circuits, the PQS system relies on alkyl-4-quinolones, including 2-heptyl-3-hydroxy-4-quinolone (PQS) and its precursor 2-heptyl-4-quinolone (HHQ) (Figure 2) [62][92][126]. These molecules are synthesized by enzymes encoded in the *pqsABCDE* operon, with the *pqsH* gene encoding for a monooxygenase responsible for the conversion of HHQ in PQS. Both signals bind to the transcriptional regulator PqsR (also known as MvfR, Multiple Virulence Factor Regulator) which in turn activates genes involved in virulence such as elastase, LecA and pyocyanin, as well as biofilm maturation (Figure 4) [25][44][58][98]. This system is regulated by the two above-mentioned AHL-QS systems in an opposite way: LasR-(3-oxo-C12-HSL) activates *pqsH* and *pqsR*, while RhlR-C4-HSL represses *pqsA* and *pqsR* transcription, creating an antagonistic balance (Figure 4) [67][92]. Importantly, RsaL has also been shown to increase *pqsH* expression [90]. Autoregulation occurs through PQS/HHQ binding to PqsR, reinforcing the expression of *pqsABCDE* transcription (Figure 4) [92][106]. Additionally, PqsE, although not directly involved in PQS synthesis, acts as a co-regulator with RhlR to co-regulate secondary metabolite production, particularly pyocyanin (Figure 4) [92][106].

These circuits are not linear but arranged in a hierarchical and feedback-rich network as Las controls Rhl and PQS, while Rhl and PQS feedback to fine-tune Las activity creating a robust but adaptable regulatory web (Figure 2) [57][125][126]. Together they form a hierarchical and feedback-rich regulatory-web, ensuring spatiotemporal coordination of community-level behaviors. Through this integration, QS functions as both an environmental sensing system and a master regulator of ecological investment, ensuring that adaptive strategies are activated only when they are most beneficial [57][125][126].

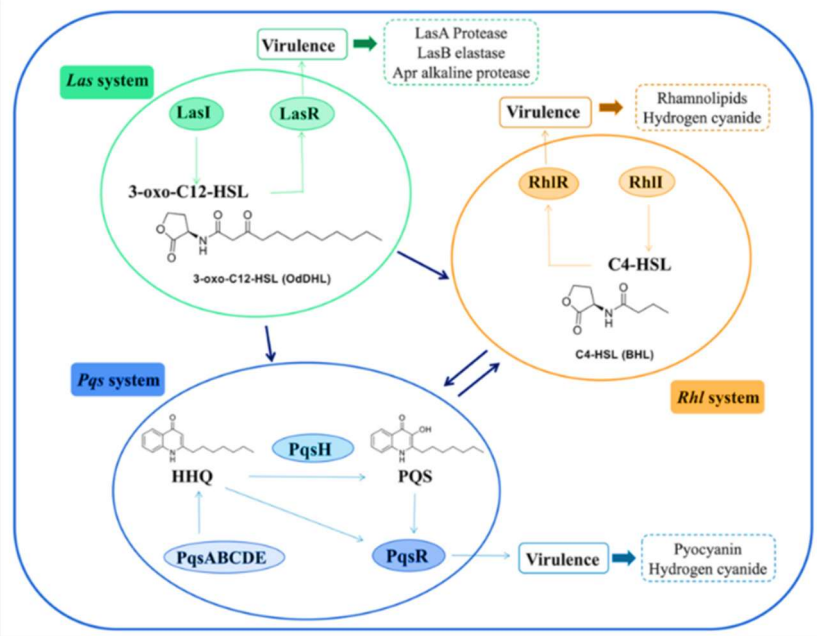

Figure 2 - Schematic representation of the three main QS systems in *P. aeruginosa*. The Las system (in green) based on the autoinducer 3-oxo-C12-HSL synthesized by LasI and detected by LasR, regulating the expression of proteases, elastases and exotoxins; the Rhl system (in orange) relying on C4-HSL produced by RhlI and sensed by RhlR, activating the transcription of rhamnolipids, pyocyanin, siderophores and motility factors; and PQS (in blue) medyated by alkyl-quinolones such as HHQ and PQS, synthesized by the *pqsABCDE* operon and activating PqsR, which controls pyocyanin, elastase and biofilm maturation. Arrows indicate regulatory cascades and crosstalk between systems, which together ensure population-wide coordination of virulence traits and are illustrated according to their hierarchy.

1.2.2 QS and Efflux Regulation

Efflux systems are membrane-associated transport mechanisms that actively export a wide range of compounds out of the bacterial cell and are one of the key mechanisms underpinning both environmental resilience and clinical antibiotic resistance [34]. These systems can remove toxic molecules such as antibiotics, heavy metals and naturally occurring antimicrobials produced by competing microorganisms, reducing the intracellular concentration of harmful substances as well as maintaining cellular homeostasis [32][34]. Of particular importance is the resistance-nodulation-cell division (RND) superfamily known to form tripartite complexes spanning the inner and outer membranes which allows the direct extrusion of substrates from the cytoplasm or periplasm into the external environment (Figure 5) [20][76]. Members of the RND family are not only associated with multidrug resistance in clinical contexts but also contribute to environmental fitness and survival under stress conditions [73][77].

Although efflux systems such as RND family are not directly under Las or Rhl control, their expression is indirectly influenced by QS-regulated stress pathways and metabolic states involving both local repressors (e.g. MexR, NalC, NalD) and global regulation

circuits (Figure 3) [55][129]. QS indirectly influences the expression of efflux systems by modulating stress responses, membrane homeostasis and the metabolic state of the cell, for example, QS activation leads to increased secretion of metabolites and toxins that can generate intracellular stress, indirectly stimulating efflux activity to maintain homeostasis [95]. Moreover, QS-regulated biofilm formation enhances the importance of efflux pumps by creating microenvironments where diffusion of harmful compounds is limited [39]. In marine environments, where pollutants and heavy metals are abundant, the interplay between QS and efflux contributes significantly to the ecological success of *P. aeruginosa* [4][95]. Previous studies have also shown that QS-controlled regulators such as MexT and global stress sigma factors influence efflux system expression indirectly, suggesting that efflux is not isolated but integrated into the broader QS-regulated survival network [30][63].

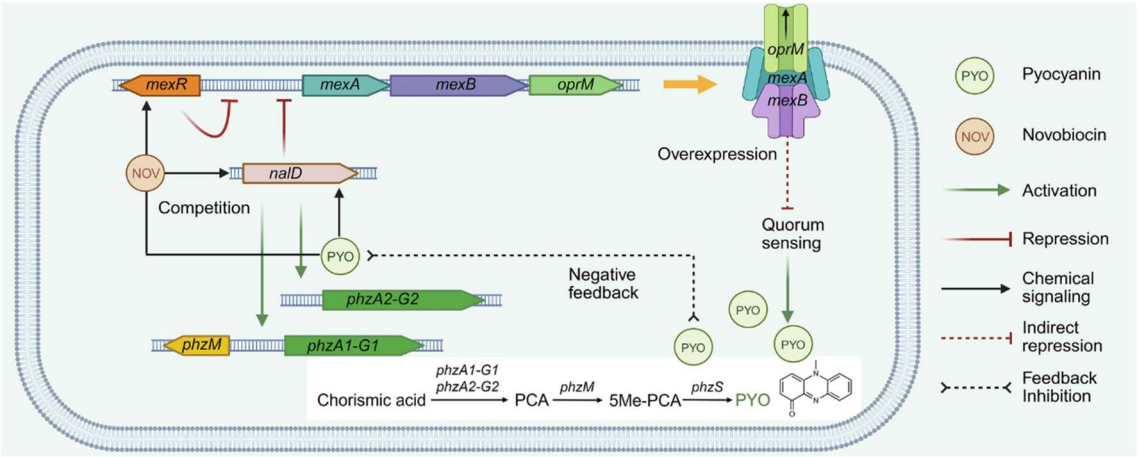

Figure 3 - Model of the cross-regulation mechanism of the RND-type efflux pump MexAB-OprM and pyocyanin synthesis in *P. aeruginosa*. The local repressor MexR that binds to the promoter region of the operon *mexAB-oprM* and prevents MexAB-OprM transcription. NalD binds directly to the intergenic region of *mexAB-oprM* serving as an additional transcriptional brake and to the promoter regions of *phz1* and *phz2* to activate pyocyanin synthesis. Pyocyanin binds to MexR and NalD, lifting their inhibition on MexAB-OprM efflux pump. Green arrows: transcriptional activation; red arrows: transcriptional repression; black arrows: chemical signaling; red dashed line: indirect repression; black dashed line: feedback inhibition.

1.2.3 QS and Siderophore Regulation

The survival and multiplication of *P. aeruginosa*, just as in many pathogenic bacteria, depends on the availability of iron in the surrounding environment, more specifically its oxidized state (Fe^{3+}), which is extremely important for bacterial growth, serving as a cofactor in enzymes involved in central metabolic pathways, electron transport and defence against oxidative stress [79][109]. Acquiring Fe^{3+} from the environment is a challenge itself due to its low bioavailability especially in oxygenated marine waters since this ionic species is highly insoluble under aerobic conditions [33][109]. To face this

question, bacteria secrete small organic chelators, known as siderophores, that have a very high affinity for iron, allowing Fe^{3+} chelation [10][109]. Once secreted siderophores bind to Fe^{3+} with exceptional specificity forming stable complexes that can be recognized and actively transported back into the cell (Figure 6) [10].

P. aeruginosa secretes two siderophores: pyochelin which has lower affinity but is less energetically costly to produce and pyoverdine (PVDI) the primary and most potent iron chelator that not only functions as an iron-scavenging molecule but also serves as a signalling compound and has antioxidating properties that help neutralize reactive oxygen species (ROS) which can be generated by UV radiation, metal redox cycling or other stress-inducing factors common in coastal environments [48][54]. The production of this siderophore becomes increasingly important when iron bioavailability is reduced by oxidative conditions or complexation with organic matter that tends to happen often in polluted harbours where redox conditions are not constant, and this capability provides a major competitive advantage [19][52][56][109].

The energetic investment required for siderophore synthesis is high and *P. aeruginosa* regulates the production tightly activating it only when intracellular iron levels drop below a critical threshold ensuring that siderophore biosynthesis contributes to its survival without unnecessarily depleting cellular resources, a balance that is crucial in nutrient-limited marine environments where metabolic efficiency can determine long-term persistence [10][33]. It has also been demonstrated that *P. aeruginosa* firstly produces pyochelin and only switches to pyoverdine synthesis when the concentration of iron becomes extremely low [26]. Therefore, QS directly regulates siderophore production by controlling the expression of alternative virulence factors that compete with iron uptake for cellular resources [135]. For instance, LasR activation enhances the expression of PvdS, an alternative sigma factor required for pyoverdine biosynthesis, thereby linking QS to iron metabolism (Figure 6) [14][100]. Furthermore, PQS itself acts as an iron chelator, strengthening the connection between signaling and iron homeostasis, ensuring that siderophore biosynthesis is activated only under community conditions that maximize its ecological payoff [58][100][109].

1.2.4 QS and Phenazine Regulation

Phenazines are a diverse group of nitrogen-containing heterocyclic compounds synthesized as secondary metabolites by several bacterial taxa, including *Pseudomonas* (Figure 7) [65][111]. These redox-active molecules have attracted considerable attention

due to their dual role as virulence factors in pathogenic contexts and as multifunctional ecological agents in natural environments, contributing to bacterial survival under challenging environmental conditions by participating in redox cycling and biofilm development [65][102][111].

The best-characterized phenazine in *P. aeruginosa* is pyocyanin, a blue-green pigment whose synthesis is tightly regulated by QS networks which coordinate its synthesis according to cell population density and environmental conditions, ensures that pyocyanin is produced in a way that optimizes the energetic investment and maximizes its impact in a community-level behaviour such as biofilm formation and cooperative nutrient acquisition [44][102]. Beyond nutrient acquisition, phenazines act as antimicrobial compounds inhibiting the growth of competing bacteria and fungal species within the same ecological niche as well as an electron carrier, enabling *P. aeruginosa* survival in environments with limited oxygen or redox imbalance, particularly in marine habitats, where the redox properties of phenazines are especially relevant due to variable oxygen levels [35][44][64]. All these aspects suggest a critical ecological role beyond pathogenicity as pyocyanin also contributes to the formation and maintenance of biofilms, providing protection against environmental stress factors such as salinity fluctuations, heavy metals and anthropogenic pollutants [102].

In *P. aeruginosa*, phenazine biosynthesis is tightly QS-regulated as both Las and Rhl systems directly activate the *phz* operons, while the PQS system enhances this regulation under oxidative or competitive stress (Figure 7) [40][44][110]. The regulator RhlR and the effector protein PqsE act together to activate phenazine biosynthetic genes, ensuring that pyocyanin production is initiated only under physiologically relevant conditions such as nutrient limitation or microbial competition (Figure 4) [44][92].

This hierarchical regulation ensures that pyocyanin and related compounds are synthesized only when population density and environmental cues align enhancing redox balance, biofilm development, and microbial competition in fluctuating marine environments [35][44].

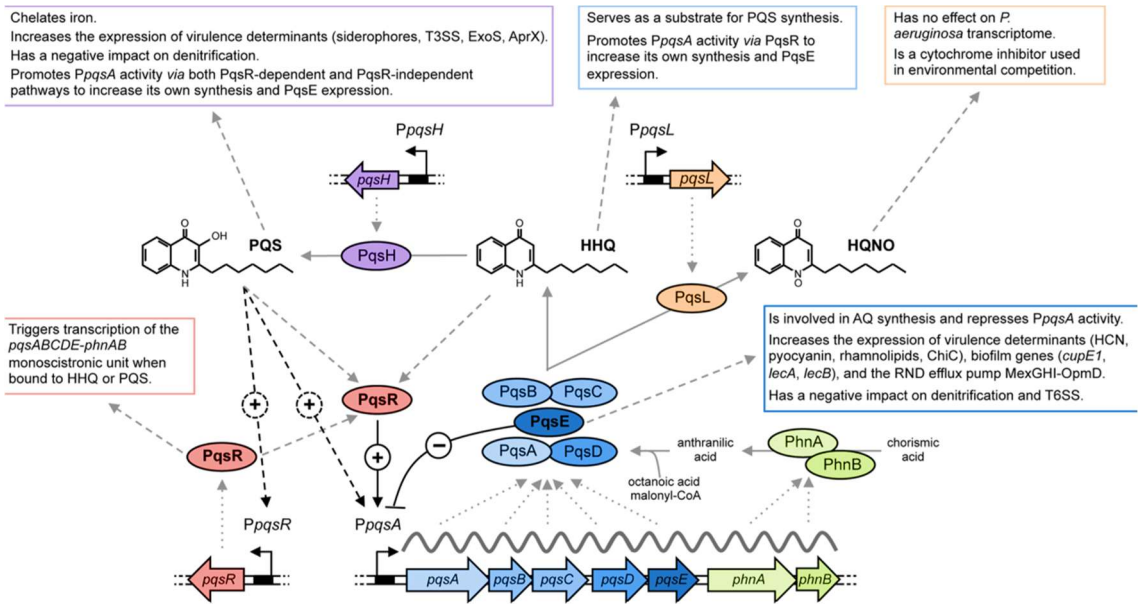

Figure 4 - Schematic representation of the PQS system in *P. aeruginosa*. The PQS system is composed of the *pqsABCDE* operon and the regulator *pqsR*. The operon synthesizes HHQ, which binds to PqsR and activates PpqsA, thereby increasing HHQ and PqsE levels. PqsE, although not directly required for AQ synthesis, plays a central role in modulating the transcriptome via AQ-independent pathways, promoting virulence factor production, biofilm development, and antibiotic resistance. PqsH converts HHQ into PQS, which acts as both a signaling molecule and an iron chelator, triggering iron-starvation responses and further enhancing virulence. Solid grey arrows: biosynthesis; dotted grey arrows: gene expression; solid black arrows: PqsR-dependent activation (+); dashed black arrows: PqsR-independent activation (+); black T-lines: negative regulation (–)

1.2.5 QS and the Stringent Response

A central plate in the adaptive capacity of *P. aeruginosa* to survive in harsh environments is the stringent response, a regulatory system that reprograms transcription in response to amino acid starvation and other environmental challenges [53][86][87]. It operates through the accumulation of specialized signalling molecules, collectively known as alarmones, which reprogram cellular metabolism to favour survival overgrowth, allowing the bacterium to conserve energy, redirect resources and enhance stress tolerance when confronted with adverse conditions such as amino acid limitation, carbon scarcity or abrupt environmental changes [28][87]. The stringent response equips *P. aeruginosa* with the capacity to downregulate energy-intensive processes, such as ribosomal RNA synthesis and upregulate genes involved in stress resistance, maintaining cell homeostasis but also extending survival during starvation [28][87].

Although not a QS system per se, its interaction with QS creates a regulatory crosstalk between environmental stress and population density, as (p)ppGpp enhances expression of QS-regulated genes such as *phz* and *pvd* operons, ensuring that secondary metabolism and virulence factors are expressed in concert with nutrient stress signals [87]. Conversely, QS influences stringent response outputs by modulating global

transcription factors and sigma factors allowing *P. aeruginosa* to balance the metabolic economy with ecological competitiveness, an especially critical trait in nutrient-limited marine environments [121].

QS functions as a master regulator that interconnects efflux activity, siderophore production, phenazine biosynthesis and the stringent response, although in an indirect way, enabling *P. aeruginosa* to dynamically adjust its physiology to fluctuating environments [87][121]. These interconnected traits form the foundation for understanding how environmental isolates adapt to the stressors encountered in marine and anthropogenically impacted habitats.

1.3. Linking QS-regulated Traits to Environmental Persistence

The QS regulatory networks highlight the key adaptative strategies that enable *P. aeruginosa* to thrive in fluctuating environments, such as the Gulf of Trieste, with efflux systems ensuring detoxification and membrane stability, siderophore biosynthesis supporting iron acquisition and oxidative stress defense, phenazine production contributing to redox balance and microbial competition as well as the stringent response coordinating global stress adaptation, mainly starvation [10][13][35][120][132]. These pathways have been linked to the survival of *P. aeruginosa* in a clinical context but are also expected to play a decisive role in a marine context where bacteria encounter similar stressors including osmotic imbalance, high salinity and nutrient deprivation.

To translate these broad regulatory insights into testable targets, four genes that represent key survival strategies in *P. aeruginosa* were selected: *mexA*, *pvdA*, *phzA1* and *relA*. Together these genes capture complementary aspects of stress tolerance and ecological competitiveness, making them suitable markers for evaluating gene expression and providing a comprehensive overview of how marine isolates, like *P. aeruginosa 25L*, regulate key pathways related to survival under simulated marine-like conditions, specifically high salinity, osmotic imbalance and nutrient limitation.

1.3.1 Multidrug Efflux Pump Component - *mexA*

The MexAB-OprM efflux pump is one of the most extensively characterized members of the resistance-nodulation-division (RND) type multidrug efflux systems in *P. aeruginosa* (Figure 5), playing a pivotal role in both antibiotic resistance and environmental resilience [1][77][132]. RND pumps are broadly conserved among Gram-negative bacteria and *P. aeruginosa* encodes at least twelve distinct RND-type pumps, many with overlapping substrate profiles, with MexAB-OprM being considered the archetypal system due to its

broad substrate range, physiological importance and constitutive expression in many isolates [11][24][78].

This tripartite protein complex consists of three structurally and functionally interconnected proteins: MexB, a resistance pump anchored in the inner membrane; MexA, a membrane fusion protein located in the periplasm; and OprM, an outer membrane channel (Figure 5) [20][118]. Powered by the proton motive force, MexB drives the active export of a wide range of structurally unrelated compounds from β-lactams and fluoroquinolones to metabolic by-products, thereby lowering their intracellular concentrations to subtoxic levels [36][59][118].

The *mexAB-oprM* operon (Figure 5) encodes all three components, among them, the gene *mexA* encodes the periplasmic adaptor protein MexA that serves as a structural adaptor physically linking MexB to OprM and enabling the formation of a continuous channel across the cell envelope [59]. MexA is structurally organized into four domains: an α-hairpin, a lipoyl domain, a β-barrel and a membrane-proximal domain, which together provide flexibility and structural integrity to the efflux pump assembly (Figure 5) [59][118]. The expression of *mexA* and the MexAB-OprM efflux pump is primarily regulated by the local repressor MexR that binds to the promoter region of the operon *mexAB-oprM* and prevents transcription (Figure 3) [129]. However, this repression is fine-tuned by auxiliary regulators NalC and NalD, which act at distinct operator sites to reinforce transcriptional control [129]. NalC represses an adjacent operon whose gene products modulate MexAB-OprM expression indirectly, while NalD binds directly to the intergenic region of *mexAB-oprM* serving as an additional transcriptional brake [129]. Recent evidence shows that both MexR and NalD are responsive to pyocyanin, the redox-active phenazine produced under QS control, by altering the DNA-binding activity of these repressors and attenuating their ability to block transcription, inducing *mexAB-oprM* expression (Figure 3) [129]. This establishes a direct link between secondary metabolite production and efflux regulation as pyocyanin accumulates in high-density or stress conditions, repression of *mexAB-oprM* is relieved leading to increased efflux activity [129]. A mechanism that ensures that *P. aeruginosa* can simultaneously deploy phenazines as competitive ecological weapons while activating efflux systems to counteract the associated intracellular stress highlighting the integration of metabolic state, QS and multidrug resistance pathways in environmental adaptation [129].

Importantly, the MexAB-OprM system is not limited to xenobiotic resistance as emerging evidence indicates that RND pumps also contribute to membrane homeostasis and adaptation under osmotic and salinity stress [4][77][66][132]. This is particularly relevant for marine environmental strains like *P. aeruginosa 25L* as hyperosmotic environments exert stress on bacterial membranes and the upregulation of *mexA* may facilitate the exportation of toxic metabolites and maintain periplasmic stability [113][115]. Therefore, the *mexA* gene may be a valuable target for studying adaptive responses and persistence mechanisms in marine isolate *P. aeruginosa* 25L by elucidating how multidrug efflux systems, such as MexAB-OprM, can contribute to the bacterium survival, extending their importance beyond traditional antibiotic resistance to the realm of ecological fitness [4][132].

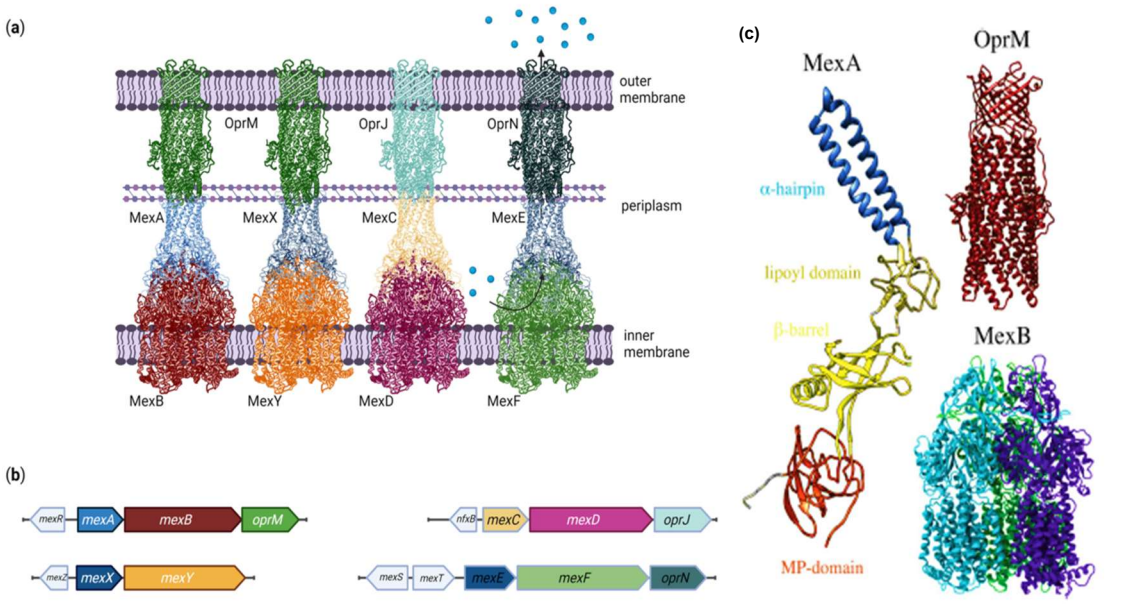

Figure 5 - RND efflux pumps of *P. aeruginosa*. (a) Structural organization of the main RND efflux systems MexAB-OprM, MexXY-OprM, MexCD-OprJ and MexEF-OprN, which span the inner and outer membranes and mediate the extrusion of antibiotics and toxic compounds. (b) Genetic organization of the operons encoding each efflux pump, showing the arrangement of structural and regulatory genes. (c) Individual structural components of the MexAB-OprM system, including MexA (periplasmic adaptor), MexB (inner membrane transporter), and OprM (outer membrane channel), highlighting their domain architecture. Together, these efflux pumps play a central role in multidrug resistance and environmental adaptation.

1.3.2 Pyoverdine Biosynthesis – *pvdA*

Iron acquisition is a fundamental challenge for *P. aeruginosa* especially in environments with fluctuating levels of oxygen, like the port of Trieste, where there are times that Fe^{3+} is poorly soluble and tightly bound to organic matter [10][19][51][109]. To overcome this limitation, *P. aeruginosa* synthetizes two siderophores, pyochelin and pyoverdine, that

are small high-affinity iron-chelating molecules with the capacity to scavenge iron from the bacterium surroundings [10][109]. Pyochelin has lower affinity but is less energetically costly to produce, whereas PVDI is highly efficient and critical for survival in severely iron-limited conditions [48].

PVDI is a structurally complex molecule composed of three parts: a chromophore responsible for its green color, a strain-specific peptide chain of 6 to 12 amino acids linked to the nonaromatic ring of the chromophore and a dicarboxylic acid, amide or α-ketoglutaric acid linked to the chromophore C-3 (Figure 6) [10][89]. Pyoverdine biosynthesis is mediated by the non-ribosomal peptide synthetase (NRPS) assembly line that initiates in the cytoplasm [10]. Once assembled the siderophore matures in the cell periplasm before being secreted by a specific efflux pump (PvdRT-OpmQ ATP-dependent efflux pump) into the extracellular environment, where it chelates Fe^{3+}. The resulting PVDI-Fe^{3+} iron-siderophore complex is recognized by TonB-dependent outer membrane transporters (FpvAI and FpvB) and imported into the periplasm for iron utilization (Figure 6) [10][42][45]. In addition to iron chelation, PVDI exhibits protective effects against ROS induced by external conditions as this antioxidant function is largely indirect and arises from its strong iron-binding capacity [2][50]. Free ferrous iron (Fe^{2+}) can catalyze Fenton reactions that produce highly reactive hydroxyl radicals (·OH), one of the most damaging ROS to cellular macromolecules [50]. By sequestering Fe^{3+} from the environment and preventing uncontrolled redox cycling between Fe^{2+} and Fe^{3+}, PVDI limits the availability of catalytic iron pools that would otherwise promote ROS generation [50]. Furthermore, PVDI-Fe^{3+} complexes are actively imported into the cell via TonB-dependent transporters, ensuring that intracellular iron acquisition is tightly regulated and preventing excess free iron accumulation [45]. This dual role in iron acquisition and redox homeostasis enables PVDI to both sustain essential metabolic processes and shields *P. aeruginosa* from oxidative stress caused by environmental conditions such as UV exposure, pollution or host immune defenses [50].

Within this pathway, the *pvdA* gene plays a pivotal biosynthetic role encoding the L-ornithine-N^5-oxygenase enzyme, PvdA, which catalyzes the hydroxylation of L-ornithine to N^5-hydroxyornithine (OHOrn), a key intermediate in PVDI biosynthesis (Figure 6) [86][89][96]. OHOrn is rapidly formylated by PvdF to yield N^5-formyl-N^5-hydroxyornithine (fOHOrn) which is then incorporated into the growing pyoverdine peptide chain by NRPS enzymes PvdI and PvdJ, with PvdH contributing further by producing L-diaminobutyric acid (L-Dab) from L-aspartate β-semialdehyde (Figure 6) [86][89][96]. This biosynthetic sequence is highly coordinated and spatially organized, as fOHOrn is unstable at pH 7

needing proximity between PvdA, PvdF and the NRPS machinery to prevent degradation and loss of intermediates (Figure 6) [86][89][96]. PvdA is a flavin-dependent monooxygenase with a compact catalytic domain that enables the hydroxylation of L-ornithine, a crucial structural modification for pyoverdine assembly that produces a modified amino group (an hydroxamate) allowing the chelation of iron in the pyoverdine molecule (Figure 6) [86][89][96].

The expression of *pvdA* is tightly regulated by the Ferric Uptake Regulator (Fur) [14]. Under iron-rich conditions, Fur represses *pvdA* transcription to conserve cellular energy and prevent toxic iron overload [14]. In contrast, under iron-limiting conditions, Fur repression is lifted, inducing *pvdA* expression alongside other components of the siderophore biosynthesis and uptake systems, allowing *P. aeruginosa* to adapt rapidly to fluctuating iron availability [14].

In summary, *pvdA* functions as a fundamental regulatory and biosynthetic center in the pyoverdine pathway, with its enzymatic product catalyzing a crucial initial step in the modification of primary metabolites into siderophore building blocks. Through its spatial organization, regulation and catalytic function, *pvdA* enables *P. aeruginosa* to efficiently produce siderophores in iron-deficient niches, such as marine environments and host tissues, without disrupting intracellular metal homeostasis [86][96][101].

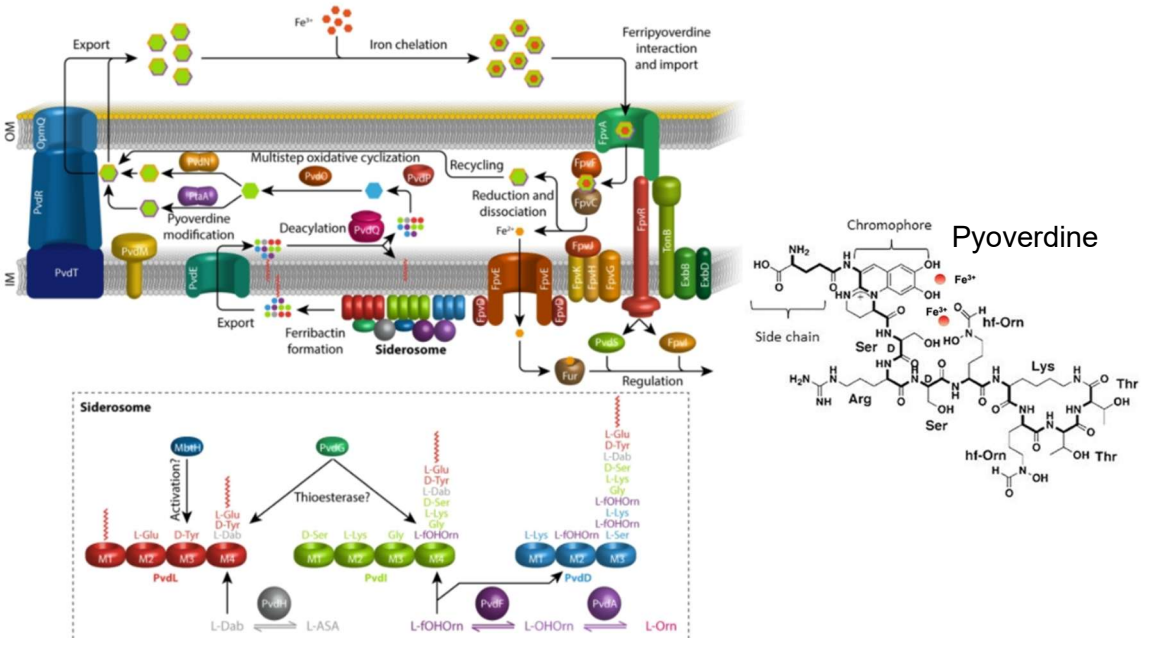

Figure 6 - Model for pyoverdine biosynthesis in *P. aeruginosa* and respective structure. Pyoverdine is assembled in the cytoplasm by NRPSs and auxiliary enzymes within membrane-associated "siderosomes." PvdL initiates the peptide backbone, while PvdA hydroxylates L-ornithine, a crucial modification for precursor maturation. The acylated ferribactin precursor is exported by PvdE and deacylated in the periplasm by PvdQ. Further modifications yield dihydropyoverdine (PvdP) and the final chromophore (PvdO). Side-chain variations are introduced by PvdN or PtaA, producing distinct pyoverdine isoforms. The mature siderophore is secreted, binds ferric iron, and is imported via FpvA in a TonB-dependent manner. Iron is released through the action of FpvF and FpvC, and the apo-pyoverdine is recycled.

1.3.3 Pyocyanin Biosynthesis – *phzA1*

Phenazines are secondary metabolites produced by *P. aeruginosa* that confer versatile adaptive advantages in environmental settings [44][65]. Among them, pyocyanin (PYO, 5-N-methyl-1-hydroxyphenazine) is the best studied and most ecologically relevant, functioning as a redox-active compound capable of shuttling electrons between intracellular metabolism and extracellular acceptors [35][44]. This redox cycle enables the bacterium to cope with variable oxygen availability, to access alternative energy pathways and to outcompete other microorganisms through the generation of ROS [35][44][64].

Pyocyanin biosynthesis originates from the shikimic acid pathway, which provides chorismic acid (CA) as the precursor (Figure 7) [71]. The phenazine biosynthetic machinery is encoded within two nearly identical operons, *phzA1-G1* (*phz1*) and *phzA2-G2* (*phz2*), each comprising seven genes (*phzA* to *phzG*) that direct the sequential conversion of CA to phenazine-1-carboxylic acid (PCA), the central phenazine precursor (Figure 7) [16][71]. PCA is then methylated to 5-methyl phenazine-1-carboxylic betaine (MPCBA), which undergoes methylation and decarboxylation reactions catalyzed by PhzS to yield pyocyanin (Figure 7) [16][71]. Structurally, pyocyanin is a nitrogen-containing heterocycle, zwitterionic at pH 7, comprising two N-methyl-1-hydroxyphenazine subunits and its stability in both oxidized and reduced forms underpins its redox versatility (Figure 7) [65][71][111].

The *phzA1* gene plays a pivotal role in the early stages of this pathway, catalyzing the conversion of CA into intermediate compounds that lead to PCA synthesis (Figure 7) [16][44]. Although *phz1* and *phz2* are functionally redundant, their expression patterns diverge depending on environmental context as *phz1*, and specifically *phzA1*, is predominantly expressed during planktonic growth, making it an ideal molecular marker for phenazine biosynthesis in free-living, non-biofilm-associated cells, contrasting with *phz2* whose expression is strongly associated with biofilm growth and infection settings [93]. Consequently, this regulatory distinction makes *phzA1* a target gene, as it provides a more accurate reflection of early environmentally responsive phenazine production in free-living bacterial populations [93].

Regulation of *phzA1* is tightly integrated with the QS systems of *P. aeruginosa*, which synchronizes gene expression with population density and environmental cues (Figure 4) [12][16][92]. Specifically, *phzA1* expression is activated through the RhlR and PqsE regulators, components of the *rhl* and *pqs* QS circuits, respectively, ensuring that

pyocyanin production aligns with critical physiological states such as nutrient limitation and competitive interactions [44][92]. The *rhl* system consists of the transcriptional regulator RhlR and its cognate signal, N-butanoyl-homoserine lactone (C4-HSL) that is synthesized by the RhlI enzyme (Figure 2) [44]. When C4-HSL accumulates to threshold levels in the environment it binds to RhlR, which in turn activates the transcription of target genes including those linked to secondary metabolites production such as pyocyanin [44]. In parallel, the PQS system relies on the production of 2-alkyl-4-quinolones (AQ) by enzymes like PQS and its precursor HHQ [2-heptyl-3-hydroxy-4(1H)-quinolone] that are synthesized by the *pqsABCD* operon (Figure 2) [44][92]. PqsE, while not directly involved in AQ synthesis, acts as a key effector protein that interacts with RhlR to fine-tune the activation of phenazine biosynthetic genes (Figure 4) [92]. Beyond quorum sensing, the *phzA1* gene transcription is also influenced by other regulators such as RpoS (general stress sigma factor) and the stringent response mediator (p)ppGpp, linking phenazine biosynthesis with broader adaptive pathways, including nutrient stress responses, oxidative stress management and biofilm initiation [35][44][74][102]. This layered regulation ensures that phenazine production and specifically *phzA1*-mediated pyocyanin biosynthesis occurs only when population density, metabolic state and environmental conditions align [44].

From an ecological perspective, *phzA1*-driven pyocyanin production enhances the fitness of *P. aeruginosa* in stressful environments by generating ROS and, consequently, exerting antimicrobial pressure on competing species while simultaneously contributing to redox homeostasis as well as energy metabolism [35]. Its activity also promotes biofilm maturation, conferring resistance to environmental pressures such as salinity shifts, toxic pollutants and oxidative damage. Consequently, the *phzA1* gene may serve as a valuable target for studying adaptative responses in marine-derived *P. aeruginosa* 25L, as it links secondary metabolism with quorum-sensing regulation and ecological competition [44].

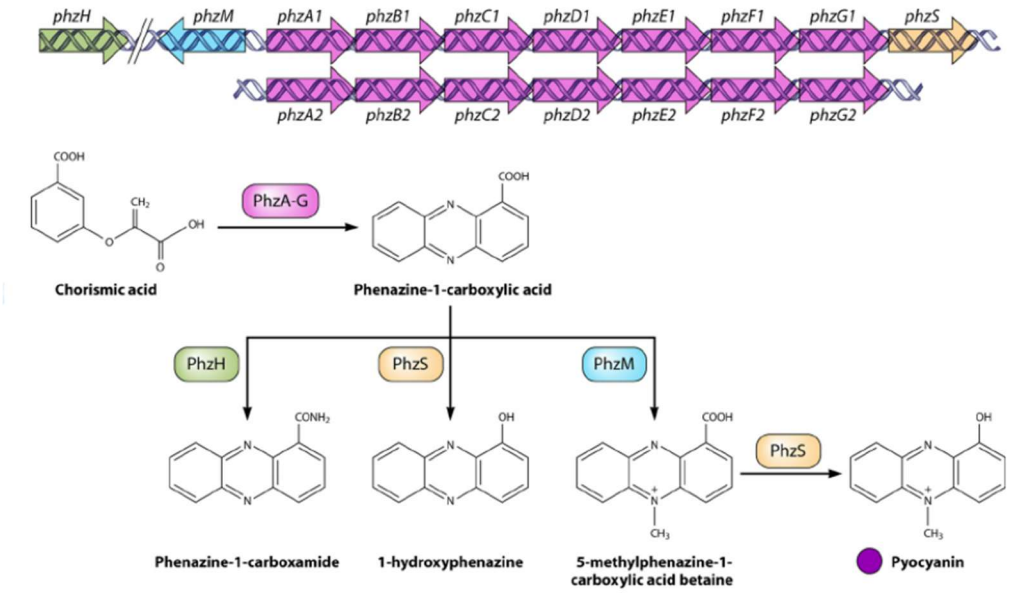

Figure 7 - Biosynthesis of pyocyanin in *P. aeruginosa*. Pyocyanin biosynthesis originates from the shikimate pathway, where CA is converted by the PhzA–G proteins into PCA, the central phenazine precursor. PCA undergoes modifications by PhzM, PhzS, and PhzH to generate different phenazines, with PhzM/PhzS catalysing the final steps leading to pyocyanin (PYO). The biosynthetic machinery is encoded by two nearly identical operons, *phzA1–G1 (phz1)* and *phzA2–G2 (phz2)*, each comprising seven genes responsible for sequential CA-to-PCA conversion. Structurally, pyocyanin is a nitrogen-containing heterocycle with redox-active properties, enabling its dual role as a virulence factor and ecological signalling molecule.

1.3.4 Stringent response – *relA*

The stringent response is a globally highly conserved regulatory mechanism that enables bacteria to rapidly reprogram gene expression in response to nutrient scarcity and other environmental challenges [117][120][128]. In *P. aeruginosa* the stringent response is essential for balancing growth and survival, allowing cells to transition into a stress-tolerant state during periods of amino acid limitation, carbon deprivation or exposure to environmental stressors [85][87]. A central component of this response is the *relA* gene which encodes the ribosome-associated enzyme RelA that has an N-terminal catalytic domain and a C-terminal regulatory domain and is a (p)ppGpp synthetase responsible for producing the alarmone molecules [29][123]. These signaling nucleotides act as intracellular messengers that coordinate a shift from growth-oriented metabolism to survival pathways making *relA* a pivotal regulator of bacterial persistence and adaptability [87][123].

The RelA enzyme is activated upon detection of uncharged transfer RNAs (tRNA) entering the ribosomal aminoacyl-tRNA site (ribosomal A-site), a molecular signal of amino acid depletion, since RelA has the capacity to sense the depletion of aminoacylated pools by monitoring changes in tRNA charging that is dependable on the amino acid abundance [87][128]. Under nutrient-rich conditions, aminoacylated tRNAs

enter the ribosomal A-site and protein synthesis proceeds uninterrupted whereas during amino acid starvation, uncharged tRNAs accumulate and compete for the A-site [87][128]. Once activated, RelA catalyzes the transfer of a pyrophosphate group from adenosine triphosphate (ATP) to guanosine triphosphate (GTP) or guanosine diphosphate (GDP) synthesising (p)ppGpp and triggering an extensive transcriptional reprogramming aimed at reallocating cellular resources away from growth-related processes and towards survival pathways [28][123]. The accumulation of (p)ppGpp reduces ribosomal RNA synthesis and downregulates genes involved in translation and cell division, while upregulating genes for amino acid biosynthesis, oxidative stress resistance and nutrient acquisition, enabling *P. aeruginosa* to enter a slow-growing stress-tolerant state that enhances survival under adverse conditions [87][123].

In *P. aeruginosa* the stringent response is finely tuned by the combined activities of RelA and SpoT, which together ensure a balanced control of (p)ppGpp levels [88][123]. While RelA functions primarily as a synthetase during amino acid limitation, SpoT exhibits dual functionality being able to both synthetize and hydrolyze (p)ppGpp in response to a wide range of stresses including carbon, fatty acid and phosphate starvation [87][107]. This dual system prevents excessive accumulation of alarmones which could otherwise compromise cellular viability, ensuring that *P. aeruginosa* can flexibly adapt to diverse environmental challenges [107]. Furthermore, RelA signaling is not an isolated pathway interacting with other global regulatory systems such as general stress sigma factor RpoS which extends its regulatory scope to processes that include virulence, biofilm development and secondary metabolism [121].

The physiological impact of *relA*-mediated signaling is broad as elevated (p)ppGpp levels influence QS networks enhancing the coordination of community-level behaviors such as biofilm formation and phenazine production [29][123]. Additionally, *relA* contributes to the regulation of siderophore production and motility further supporting adaptation in nutrient-limited or competitive environments and making RelA not only a regulator of stress survival but also a determinant of ecological competitiveness as well as pathological potential in *P. aeruginosa* [28][87].

The role of *relA* is especially relevant in marine-derived isolates such as *P. aeruginosa 25L*, which are exposed to fluctuating nutrient availability, osmotic stress, UV radiation and anthropogenic pollutants [87][123]. Recent studies suggest that (p)ppGpp accumulation leads to increased bacterial tolerance to osmotic and UVA stress, implying that the stringent response may act as an integrative hub that coordinates multiple stress adaptation pathways and not only nutrient scarcity [87]. For marine environments such

as the Trieste port, where physicochemical conditions are highly dynamic, the activation of stringent response pathways through *relA* provides a major advantage for survival and persistence. Accordingly, the *relA* gene represents an important target for investigating adaptative responses in *P. aeruginosa* 25L.

1.4. From Water to Air: Aerosolization Potential

Beyond its persistence in aquatic environments, *P. aeruginosa* also displays the capacity to become aerosolized, representing an additional route for environmental dissemination [21]. Sea spray, wave action and anthropogenic activities, such as wastewater treatment, can generate bioaerosols containing viable bacteria, including antibiotic-resistant strains [6][49][131][135]. Once airborne, cells are exposed to intense stressors including UV radiation, oxidative damage, dehydration, osmotic imbalance and nutrient scarcity, which strongly challenge bacterial survival [80][108]. The mechanism that enables *P. aeruginosa* to withstand marine stressors are likely to also underpin its ability to endure aerosolization as these pathways provide detoxification, redox balance, nutrient scavenging and global metabolic reprogramming, all of which are critical for maintaining viability in the harsh airborne state [35][120][132].

This thesis forms part of a broader investigation carried out in Trieste and Genoa, Italy, using a customized atmospheric simulator chamber design to replicate environmental conditions of aerosolization (Figure 8). In this setup, *P. aeruginosa* 25L is subjected to controlled exposure to light and darkness while being aerosolized to assess survival under simulated environmental conditions. By linking the gene expression patterns of efflux pumps, siderophores, phenazines and stringent response regulators to survive in aerosolized from, it becomes possible to evaluate whether the same traits that sustain persistence in marine waters also facilitate airborne survival. Understanding these processes is not only ecologically significant for explaining how opportunistic pathogens persist in fluctuating environments but also relevant for assessing potential risks of human exposure to marine bioaerosols in coastal regions impacted by anthropogenic activity.

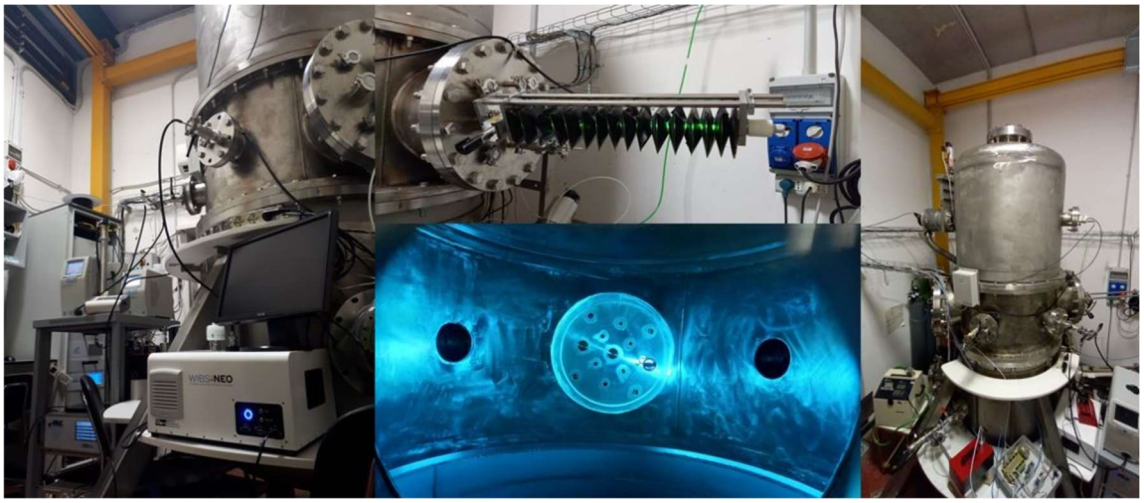

Figure 8 - Aerosolization chamber at the *Instituto Nazionale di Fisica Nucleare* (INFN), Genoa, Italy. Montage of images showing the aerosolization chamber used for generating and controlling bioaerosols under controlled laboratory conditions. This facility provides a highly contained environment for studying microbial aerosolization, including *P. aeruginosa* strain 25L.

1.5. Aim of this work

The primary objective of this study was to investigate the gene expression of the marine isolate *P. aeruginosa 25L* to key environmental stressful surroundings, namely osmotic stress, high salinity and nutrient limitation, mimicking conditions encountered in seawater and aerosolized marine environments. These stressors are particularly relevant as they influence not only bacterial persistence but also the maintenance of virulent potential.

To dissect the molecular mechanisms underlying these adaptive responses, the expression of four functionally distinct genes was quantified using a two-step reverse transcription quantitative PCR (RT-qPCR):

- *mexA*, encoding a component of the MexAB-OprM multidrug efflux pump involved in detoxification and membrane homeostasis.
- *pvdA*, central to pyoverdine biosynthesis and iron acquisition.
- *phzA1*, a marker for phenazine biosynthesis linked to redox balance and environmental survival.
- *relA*, a key regulator of the stringent response and global stress adaptation.

Thence, by monitoring transcriptional activity of these genes under controlled stress exposures, this work aims to provide insights into the ecological adaptive strategies that enable marine isolates like *P. aeruginosa 25L* to withstand fluctuating habitats. Beyond advancing our understanding of microbial adaptation in marine environments, these

findings may also highlight potential public health implications, particularly concerning the persistence and aerosolization of opportunistic pathogens in coastal systems.

2. Methods

2.1. Bacterial strain and Growth Conditions

All experiments were conducted using *P. aeruginosa* strain 25L, a marine isolate collected from the Trieste Port. The strain was revived from -80 °C cryostock by streaking onto ZoBell (see Table 2 in the attachments section) agar plates, followed by incubation at room temperature to allow colony development. A single colony was used to initiate a starter culture in 5 mL ZoBell marine broth, incubated in a water bath overnight at 26 °C with agitation (100 rpm). This pre-culture was then used to inoculate a secondary culture (3 mL into 17 mL fresh ZoBell marine broth), which was also incubated overnight under the same conditions to obtain cells in the exponential growth phase for subsequent assays.

2.2. Experimental Design for Stress Challenges

To assess the gene expression of *P. aeruginosa* 25L to environmental stressors, cells were subjected to three distinct conditions: nutrient limitation, osmotic stress, and high salinity. These conditions were implemented across two experimental sets: one focused on osmotic an salinity stress, and the other on nutrient limitation, each comprising three independent experimental replicates.

Prior to stress exposure, *P. aeruginosa 25L* cultures were pooled and adjusted to a uniform optical density (OD_{660} = 0,579). A total of three 40mL replicates were centrifuged at 8000g for 5 minutes at 26°C, and the resulting pellets were resuspended in 35mL filter-autoclaved seawater (FASW) to simulate oligotrophic marine conditions. Following unification, baseline (T0) samples were collected in triplicates by harvesting 2mL aliquots, centrifuging at 14000g for 3 minutes at 4°C, and resuspending the cell pellets in DNA/RNA Shield (Zymo Research, Irvine, CA, USA). Samples were stored at -20 °C until RNA extraction.

For the osmotic and salinity experiments, the remaining culture was divided into three treatment conditions: (i) Nutrient limitation – 35mL of FASW (0,5M NaCl); (ii) High salinity and osmotic stress – 35 mL of FASW adjusted to 0,9 M NaCl by supplementing with 3mL of 5,2M NaCl stock; and (iii) Nutrient-rich – 35mL of ZoBell marine broth; and for the nutrient limitation assays the prevailing volume was divided into two conditions: i) Nutrient limitation – 35mL of FASW (0,5M NaCl); and ii) Nutrient-rich – 35mL of ZoBell marine broth. Each condition was split into two technical replicates and incubated for 1 hour at 26°C with agitation (100 rpm). After incubation (Tf), 2mL from each replicate were

collected, centrifuged at 14000 g for 3 minutes at 4°C, resuspended in DNA/RNA Shield (Zymo Research, Irvine, CA, USA), and stored at -20 °C pending RNA extraction.

This experimental design (Figure 9) was carried out across two sets of conditions: salinity allied to osmotic stress and nutrient limitation. For the osmotic and salinity stress experiments, three independent biological replicates were performed, each consisting of three T0 baseline samples and two Tf samples per condition (*i.e.*, ZoBell, FASW and FASW supplemented with NaCl). For the nutrient limitation experiments, three additional biological replicates were conducted using the same workflow, with three T0 samples and three Tf samples for each condition (ZoBell and FASW). In total, 57 RNA-stabilized samples were collected and stored at -20ºC for downstream analysis.

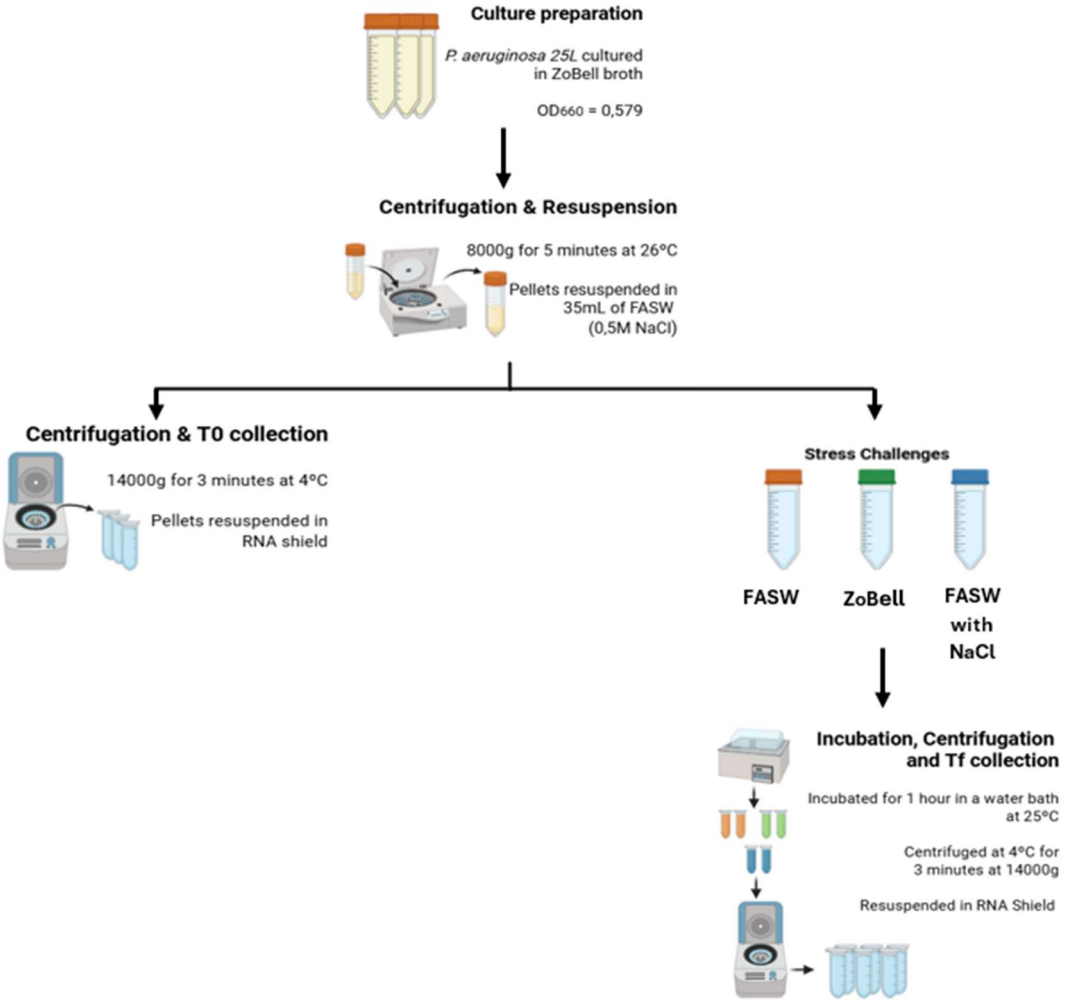

Figure 9 - Schematic representation of the experimental design applied for the stress challenges using *P. aeruginosa* 25L. Overview of the workflow applied to evaluate the transcriptional response of strain 25L under osmotic stress, nutrient limitation and high-salinity conditions. The scheme summarizes bacterial culture preparation and exposure to defined stress treatments. The illustration was created with BioRender.com.

2.3. Primer Design and Gradient PCR

Gene-specific primers targeting *mexA*, *pvdA* and *phzA1* were used to quantify transcriptional responses to the three stress conditions tested, with *acsA* and *trpE* serving as reference housekeeping genes. The amplicon for the *phzA1* primer pair in this initial set was relatively short (74bp) which was identified as a potential limitation during the first methodological optimization phase. Following the evaluation of some preliminary results, the primer panel was revised leading to the inclusion of a sixth primer pair targeting the gene *relA* and a redesigned *phzA1* pair of primers to generate a longer amplicon (Table 1).

The primer sequences were either obtained from previous studies or designed using Primer 3, a widely used program for designing PCR primers, ensuring optimal melting temperatures, GC content and amplicon length. Possible formation of secondary structures, including hairpins, self-dimerization and self-annealing of 3' and 5' ends were predicted using OligoCalc and the primers specificity was validated by *in silico* PCR confirming target amplification and the absence of non-specific binding. While standard curves and primer efficiency calculations were not performed in this study due to time and sample constraints, future work should include efficiency validation to ensure quantitative accuracy. Similarly, the stability of the selected housekeeping genes (*acsA* and *trpE*) across stress conditions was not formally validated here.

To optimize the annealing temperature and amplification efficiency two independent gradient PCR assays were conducted at different points in time. For the first gradient PCR the reactions were prepared using a master mix composed of 7,5µL of AccuStart™ PCR Master Mix (Aline Biosciences, Woburn, MA, USA) supplemented with 0,75µL of EvaGreen® (Biotium, Fremont, CA, USA) fluorescent dye and 0,6µL of forward and reverse primers (10µM) with 4,55µL of nuclear-free water and 1µL of DNA template from the *P. aeruginosa 25L* making up the final volume of 15µL per reaction. Two separate 96-well plates were assembled, one containing the primers with shorter amplicon (primers targeting the genes *phzA1* and *mexA*) and another containing the primers with longer amplicons (primers targeting the genes *pvdA*, *acsA* and *trpE*) to ensure an accurate assessment of the more suitable annealing temperature.

The thermal protocol adopted involved an initial enzyme activation at 95ºC for 3 minutes followed by 45 amplification cycles of denaturation at 95ºC for 10 seconds, annealing with temperature ranging from 53ºC to 62,5ºC for 40 seconds and extension at 72ºC for 40 seconds.

A second gradient PCR was performed later under the same protocol however, in this case, all primer pairs were combined onto a single 96-well plate and the primer set targeting the gene *relA*, which was incorporated after the initial optimization round. This setup enabled a comparative evaluation of the amplification profiles across a temperature gradient ranging from 53ºC to 62,5ºC to identify the most suitable annealing conditions for all of the primers in order for the quantitative PCR to be done with the six pairs of selected primers simultaneously.

Table 1 – Final gene-targeted primers used to evaluate *P. aeruginosa* 25L transcriptional response to marine-related stress conditions. Primer sets employed for RT-qPCR analysis, including the target gene, its main function, forward and reverse primer sequences, expected amplicon size and bibliographic source. Genes selected represent key pathways involved in stress adaptation and virulence.

Gene	Function	Forward Primer (5' to 3')	Reverse Primer (5' to 3')	Amplicon Size (bp)	Source
phzA1	Phenazine biosynthesis	GTACAGGGAAACACCCCTC	CAATGCACGCAGTTTCTGTA	431	This study
pvdA	Pyoverdine biosynthesis	TTCGCGGCTGTAGATGAGAT	AACCTGGGCACCTTCTATCC	516	This study
mexA	Multidrug efflux pump	AGACGGTGACCCTGAATACC	GTCGGCCTCGTAGGTGG	170	[1]
relA	(p)ppGpp synthetase I enzyme	CGTACCGCCGAGGATATGTT	CCGGGAATCTCGATGGTCAG	527	This study
acsA	Acetyl coenzyme A synthetase	ACCTGGTGTACGCCTCGCTGAC	GACATAGATGCCCTGCCCCTTGAT	842	[17]
trpE	Anthralite synthetase component I	GCGGCCCAGGGTCGTGAG	CCCGGCGCTTGTTGATGGTT	811	[17]

2.4. RNA Extraction and on-column DNase Treatment

Total RNA was extracted from *P. aeruginosa* 25L cultures after being exposed to stress challenges using the Quick-RNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, Irvine, CA, USA) following the manufacturer's protocol for non-homogenized samples. Mechanical cell lysis was achieved by vigorous vortex for 10 minutes, as recommended, with the given lysis buffer on a lysis vial with a bead matrix. To eliminate contaminating genomic DNA an on-column DNase treatment step was included during the RNA extraction workflow. The obtained RNA was eluted in 50µL of DNase/RNase-free water, quantified using a NanoDrop™ spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) and stored at -80ºC until reverse transcription (RT).

Despite the on-column DNase treatment, subsequent analyses revealed residual DNA contamination and, in order to address this, samples with RNA concentrations below 200 ng/µL were subjected to an additional off-column DNase digestion using TURBO DNA-free™ Kit (Invitrogen, Thermo Fisher Scientific Inc., Waltham, MA, USA) with treatment conditions consisting of 50µL RNA, 5µL of TURBO DNase buffer and 1µL of TURBO DNase. Following incubation, the enzyme was inactivated with the DNase Inactivation Reagent according to the manufacturer's protocol. RNA integrity and concentration were reassessed using the NanoDrop™ (Thermo Fisher Scientific Inc., Waltham, MA, USA) and all samples were stored at -80ºC until downstream applications.

2.5. cDNA Synthesis and RT-qPCR Workflow

Complementary DNA synthesis and quantitative PCR were carried out in two different experimental phases, with a one-step approach being performed initially followed by a two-step workflow. Between these two phases, a DNA contamination assessment was introduced as well as an off-column DNase treatment and an updated primer panel incorporating a redesigned *phzA1* primer pair producing a longer amplicon plus a new primer set targeting *relA*.

2.5.1. One-step RT-qPCR

Complementary DNA (cDNA) was initially synthesized using the qScript One-Step SYBR® Green RT-qPCR kit (Quantabio, Beverly, MA, USA), following the manufacturer's instructions, in a 96-well plate with a final volume of 15µL per well of which 2µL were RNA extracted previously from the samples. The final product obtained was then visualized on an electrophoresis agarose gel of 2%. This approach not only allowed rapid screening of the primer's performance and amplification efficiency but also served as a preliminary test phase.

2.5.2. Genomic DNA Contamination Assessment

Following an analysis and evaluation of the results obtained on the one-step RT-qPCR assay, PCR reactions were performed directly on RNA samples without a reverse transcription step (-RT control) in order to assess potential DNA contamination. Each of these reactions were prepared using AccuStart™ PCR Master Mix (Aline Biosciences, Woburn, MA, USA) and supplemented with EvaGreen® (Biotium, Fremont, CA, USA) fluorescent dye with a volume of 7,5µL and 0,75µL respectively, complemented with 4,55µL of NF water. The forward and reverse primers, in volume of 0,6µL per primer, were then added. All reactions were set up in a 96-well plate with each well containing 13µL of final mix and 2µL of RNA template from various samples.

The thermal protocol consisted of the activation of the enzyme Taq DNA polymerase at 95ºC for 2 minutes, followed by 45 amplification cycles of denaturation at 95ºC for 15 seconds, annealing at 57ºC for 20 seconds and extension at 72ºC for 30 seconds. The PCR obtained products were visualized by electrophoresis on a 2% agarose gel.

2.5.3. Two-step RT-qPCR

Subsequently, it was decided to transition to a two-step workflow where the cDNA synthesis was accomplished by using qScript XLT cDNA SuperMix (Quantabio, Beverly, MA, USA) with 3µL of total RNA from samples treated with TURBO DNA-free™

(Invitrogen, Thermo Fisher Scientific Inc., Waltham, MA, USA) per reaction, following the manufacturer's instructions. Synthesized cDNA was then quantified using the Qubit™ dsDNA High Sensitivity Assay Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) to ensure accurate downstream quantification.

The gene expression analysis was performed using a qPCR approach with reactions prepared with AccuStart™ PCR Master Mix (Aline Biosciences, Woburn, MA, USA) and supplemented with EvaGreen® (Biotium, Fremont, CA, USA) fluorescent dye. A 15µL master mix was prepared for each primer pair, consisting of 7,5µL of Master Mix, 0,75µL of EvaGreen® (Biotium, Fremont, CA, USA) and 2,55µL of NF water. To each mix 0,6µL of forward and reverse primers were added to achieve a final primer concentration of 400nM. Subsequently 12µL of the mix was dispensed into a 96-well plate, followed by 3µL of cDNA or RNA template. All reactions were performed in triplicates including the negative and positive controls. Thermal cycling was carried out using the following thermal protocol: initial enzyme activation at 95ºC for 3 minutes, followed by 50 amplification cycles of denaturation at 95ºC for 10 seconds, annealing at 53ºC for 40 seconds and extension at 72ºC for 40 seconds. This strategy was selected as the final workflow due to improved specificity, reduced primer-dimer formation and greater flexibility in the experimental design.

3. Results

3.1. Gradient PCR

Gradient PCR assays were performed to determine the primers efficiency, especially the newly designed ones, but it was mainly carried out to achieve an optimal annealing temperature in order to conduct a RT-qPCR with all the six primer pairs simultaneously. With this in mind, two independent runs were conducted at different stages of the project, the first at the very beginning using five primer pairs (*phzA1*, *pvdA*, *mexA*, *acsA* and *trpE*) while the second included an additional gene (*relA*) and a redesigned *phzA1* primer pair (Table 1).

3.1.1. First Gradient PCR

In the first gradient PCR there was the need to divide the primers into two independent 96-well plates with one containing the shorter amplicon targets (*phzA1* and *mexA*) and another containing the longer amplicon targets (*pvdA*, *acsA* and *trpE*) resulting in a more accurate determination of the optimal annealing temperature as well as minimizing a potential variability in the amplification efficiency.

With annealing temperatures ranging from 53ºC to 62,5ºC the quality of the amplification curves and the amplification cycle thresholds (Ct value) were assessed for all five pairs of primers. Primer sets *phzA1*, *pvdA*, *acsA* and *trpE* exhibited consistent amplification across most tested temperatures, although higher temperatures (≥ 59,4ºC) generally increased the Ct value or there was no amplification like in the case of *phzA1*, *acsA* and *trpE*. The *mexA* primer pair was the only one that revealed consistent amplification and Ct values across all temperatures tested (Figure 10). The combined results from both plates revealed that 56,9ºC was the temperature that yielded the lowest Ct values indicating a stronger amplification and better primer-template annealing across all primer pairs (Figure 10).

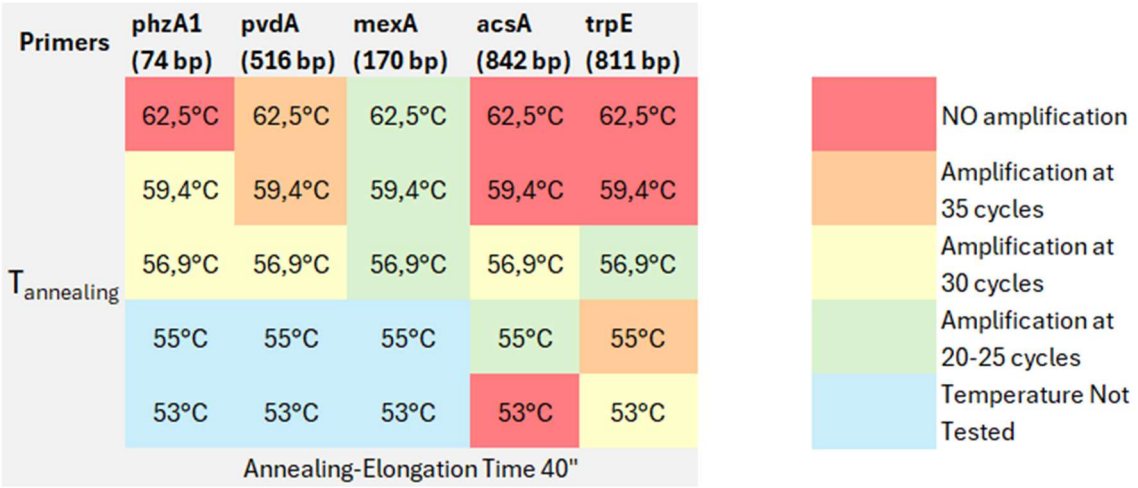

Figure 10 – First gradient PCR for optimization of annealing temperature for RT-qPCR primers targeting *P. aeruginosa* 25L. Amplification performance of five primer pairs (*phzA1, pvdA, acsA, trpE,* and *mexA*) was evaluated across a temperature gradient ranging from 53 °C to 62,5 °C. While *phzA1, pvdA, acsA*, and *trpE* amplified at lower temperatures, increasing the temperature (≥ 59,4 °C) reduced amplification efficiency or didn't amplify. In contrast, *mexA* primers produced stable amplification and Ct values across the full gradient. Comparison of both plates indicated that 56,9 °C yielded the lowest Ct values and strongest amplification overall and was therefore selected as the optimal annealing temperature for subsequent RT-qPCR assays. Red: no amplification; orange: amplification ≥35 cycles; yellow: amplification ≥ 30 cycles; green: amplification between 20 to 25 cycles; and blue: temperature not tested.

3.1.2. Second Gradient PCR

For the second gradient PCR all primer pairs were tested together on a single 96-well plate under the same cycling conditions as the first gradient PCR, with the main difference between both assays being the incorporation of new primers targeting the gene *relA* and the redesigned *phzA1* targeting primers.

The new primer pair targeting the gene *relA* showed weak amplification at 53ºC and 55ºC with a Ct value of 35 but no amplification at higher temperatures (≥ 56,9ºC) just like *pvdA*, *acsA* and *trpE* targeting primers. The redesigned *phzA1* primers exhibited good amplification across all temperatures tested with a Ct value of 30 but an even better signal at the highest temperature tested (62,5ºC) where the Ct values were in the range of 20 to 25 and the *mexA* primer pair showed once again a consistent amplification and Ct value across all temperatures tested. Across the primer sets 53ºC was the temperature that consistently yielded the lowest Ct values indicating a stronger amplification and better primer-template annealing.

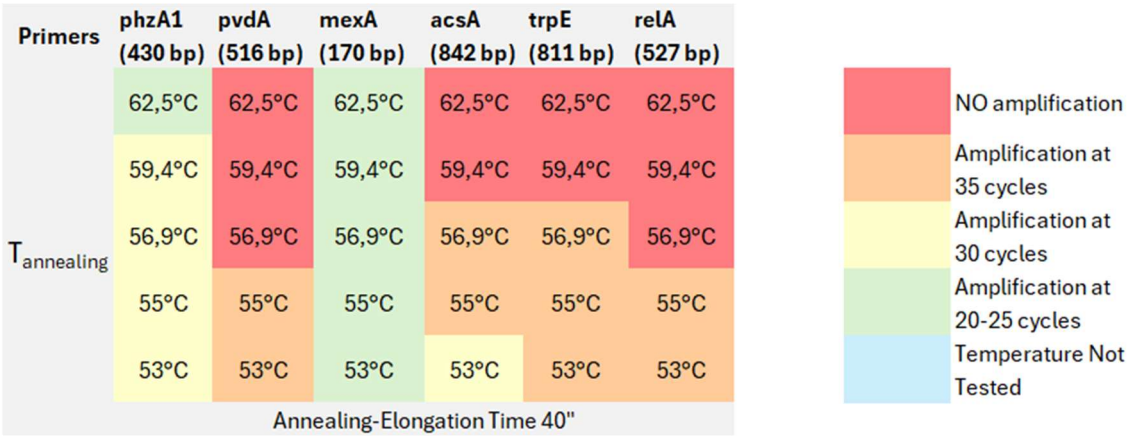

Figure 11 - Second gradient PCR for optimization of annealing temperature for RT-qPCR primers targeting *P. aeruginosa* 25L. All primer pairs were tested simultaneously on a single 96-well plate under the same cycling conditions as in the first gradient PCR, with the addition of newly designed primers for *relA* and redesigned primers for *phzA1*. The *relA* primers showed weak amplification only at 53 °C and 55 °C (Ct ≈ 35), with no signal at higher temperatures, like *pvdA*, *acsA*, and *trpE*. The redesigned *phzA1* primers amplified consistently across all temperatures, with the strongest signal observed at 62,5 °C (Ct = 20–25). As in the first assay, the *mexA* primers showed stable amplification and Ct values across the gradient. Overall, 53 °C was identified as the optimal annealing temperature, yielding the lowest Ct values and most reliable amplification across the primer sets. Red: no amplification; orange: amplification ≥35 cycles; yellow: amplification ≥ 30 cycles; green: amplification between 20 to 25 cycles; and blue: temperature not tested.

3.2. cDNA synthesis and RT-qPCR

Complementary DNA (cDNA) was synthesized from RNA extracted from *P. aeruginosa 25L* cultures to serve as template for downstream gene expression analysis. Initially cDNA was generated using the qScript One-Step SYBR® Green RT-qPCR kit (Quantabio, Beverly, MA, USA) which combines reverse transcription (RT) and quantitative PCR (qPCR) in a single reaction, allowing rapid assessment of amplification efficiency and product specificity for each primer pair (Figure 12). In a subsequent optimization phase, a two-step workflow was adopted in which cDNA synthesis was performed separately using the qScript XLT cDNA SuperMix (Quantabio, Beverly, MA, USA) providing greater flexibility in primer optimization and enabling more consistent yields across target genes.

3.2.1. One-step RT-qPCR

As an initial approach the cDNA synthesis was accomplished resorting to the qScript One-Step SYBR® Green RT-qPCR kit (Quantabio, Beverly, MA, USA). This setup was chosen to rapidly evaluate the amplification efficiency and specificity of the gene-specific primers targeting *phzA1*, *pvdA*, *mexA*, *acsA* and *trpE*. The amplification products were visualized on 2% agarose gels to assess product size and reaction fidelity (Figure 12).

Clear amplification bands of the expected sizes were obtained for most primer sets confirming both successful reverse transcriptions and amplification with *mexA*, *pvdA* as

well as *acsA* primer pairs consistently producing strong distinct bands across most of the tested samples (Figure 12). In contrast, *trpE* amplification was less consistent with faint or absent bands in certain samples suggesting lower transcript abundance or suboptimal primer-template interactions under the reaction conditions (Figure 12). Regarding the *phzA1* primer pair, the amplicon produced had the expected length, but it was difficult to distinguish the amplification product from potential artifacts on the gel. Multiple lanes also displayed non-specific amplification products including low-molecular-weight fragments (<100bp) suggestive of primer-dimer formation (Figure 12).

In addition, faint bands were detected in certain NTC lanes indicating contamination or primer-dimer formation. Importantly, the same cDNA sample sometimes yielded markedly different amplification patterns across runs with certain targets amplifying in one reaction but not in another (Figure 12). Together, these results demonstrate that while the one-step workflow produced cDNA and enabled amplification, it also produced non-specific products and potentially misleading results, prompting the transition to a more robust two-step RT-qPCR workflow accompanied by an additional DNA contamination control (-RT control) to clarify whether the non-specific products originated from residual DNA or are primer-related artifacts.

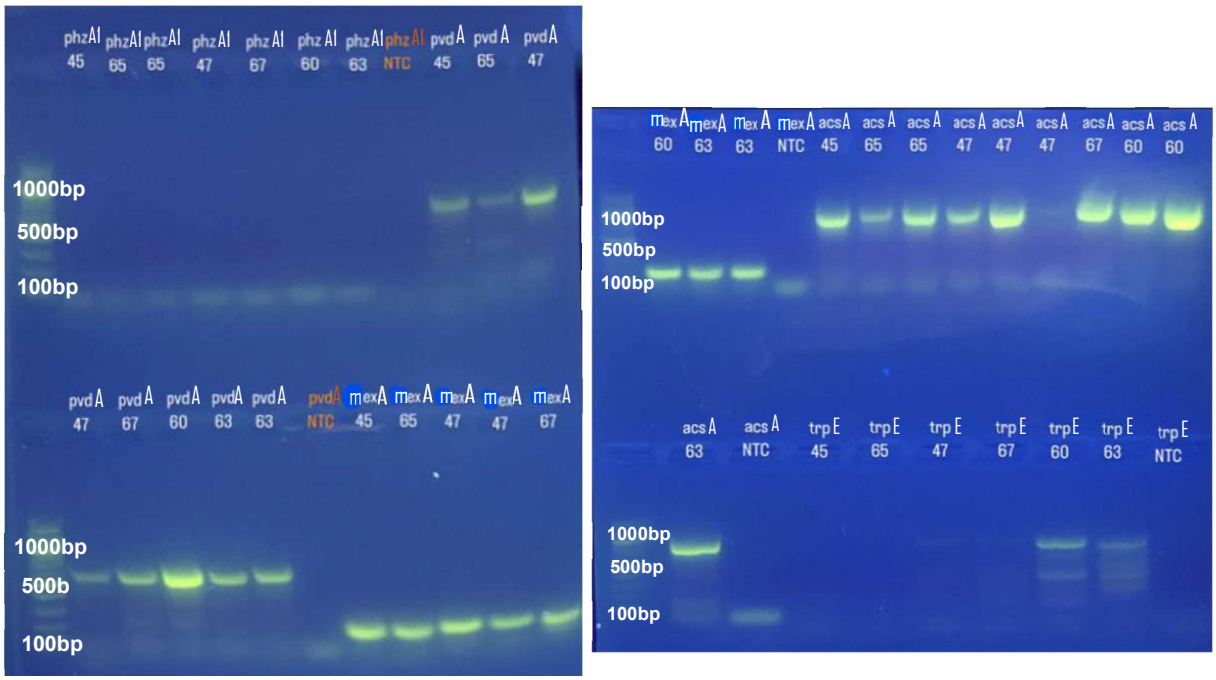

Figure 12 - One-step RT-qPCR. cDNA synthesis and amplification were performed using the qScript One-Step SYBR® Green RT-qPCR kit (Quantabio) which allowed to rapidly assess primer efficiency for *phzA1, pvdA, mexA, acsA*, and *trpE*. Amplification products were visualized on 2% agarose gels, confirming successful amplification for *mexA, pvdA*, and *acsA* with strong, distinct bands of the expected size, although it was detected non-specific amplification bands. In contrast, *trpE* yielded weaker or inconsistent bands, while *phzA1* produced the expected amplicon but was difficult to distinguish from non-specific products. Several reactions also displayed primer-dimer artifacts (<100 bp) and faint signals in NTC lanes, suggesting contamination. Additionally, amplification reproducibility varied between runs for some targets. Together, these results confirmed that the one-step workflow could generate cDNA and amplify targets but lacked robustness, prompting the transition to a two-step RT-qPCR workflow with dedicated –RT controls to discriminate genuine transcripts from residual DNA or artifacts. A molecular weight ladder (100 bp, 500 bp, and 1000 bp markers) was used as size reference.

3.2.2. DNA contamination assessment (-RT control) and off-column DNase Treatment

With the aim of assessing potential DNA contamination in the extracted RNA samples, PCR assays were performed directly on template RNA without the reverse transcription step (-RT controls), using the same gene-specific primers employed in upstream gradient PCR and downstream RT-qPCR assays. For each primer set multiple RNA samples were tested and NTCs were included to detect reagent contamination. The PCR products were visualized by electrophoresis in a 2% agarose gel (Figure 13).

In the first gel, *mexA* primers produced strong amplification products with the expected size (170bp) across all tested RNA samples, while *phzA1* yielded weaker but still detectable bands (74bp) and *pvdA* produced only one faint band of the expected size (516bp) in a single sample (number 60) (Figure 13). In the second gel, *trpE* primers didn't generate visible bands contrary to *acsA* that showed a faint amplification band of an unexpected size in sample 67 (Figure 13). Importantly, the absence of amplification products observed in NTC lanes indicates that the observed bands across both one-step RT-qPCR agarose gels originated from contaminating DNA on the RNA samples rather than reagent contamination (Figure 13).

The presence of amplification products in multiple -RT lanes confirms that residual DNA was present in several RNA preparations with this contamination being particularly evident for *mexA* which showed consistent strong amplification in the absence of reverse transcription and confirming that a major contributor to the amplification inconsistencies observed in the one-step RT-qPCR was DNA contamination (Figure 13).

Although an on-column DNase digestion step had been included during RNA extraction, a following off-column treatment needed to be implemented to ensure purity of the RNA samples, considering that the findings described indicate that the efforts implemented to remove all DNA were not enough. Taking this into account all samples with concentrations below 200ng/µL of RNA underwent an additional off-column DNase treatment using TURBO DNA-free™ Kit (Invitrogen, Thermo Fisher Scientific Inc., Waltham, MA, USA) to ensure complete removal of gDNA following the analysis of the results for the contamination assessment as well as the results for the one-step RT-qPCR.

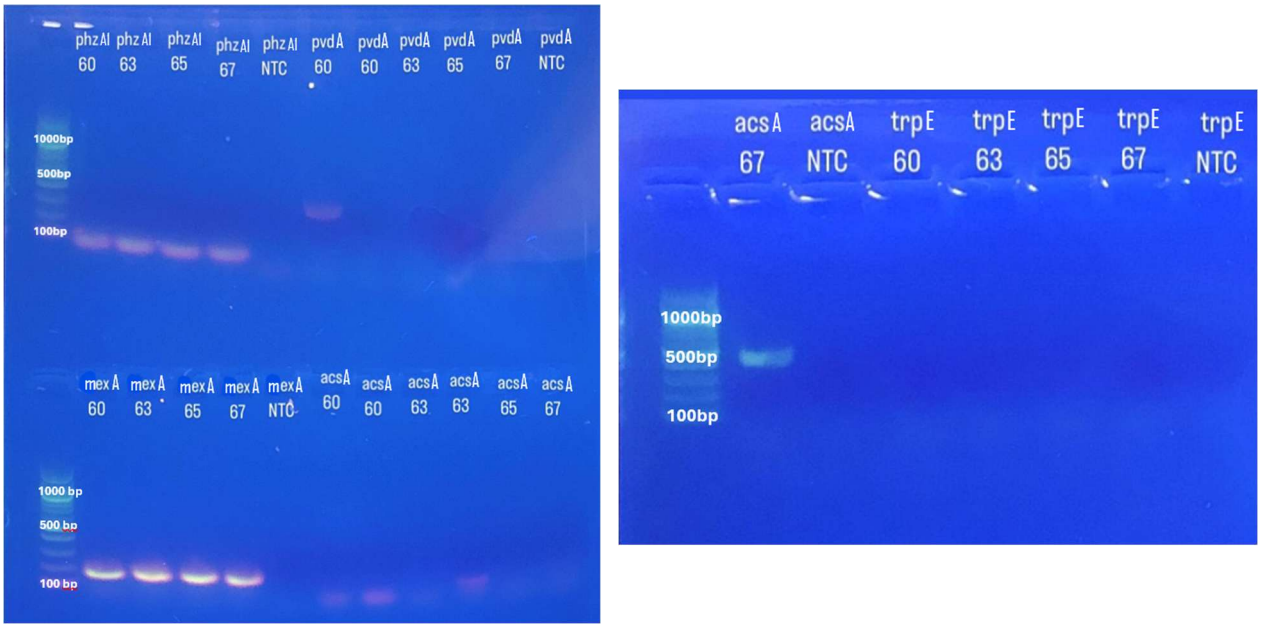

Figure 13 - DNA contamination assessment in RNA samples. PCR assays were performed directly on RNA templates without reverse transcription (–RT controls) using the same primers tested in RT-qPCR. Products were resolved on 2% agarose gels. Strong amplification was consistently observed for *mexA* (170 bp) in all samples, with weaker signals for *phzA1* (74 bp) and occasional faint bands for *pvdA* (516 bp). In contrast, *trpE* showed no amplification, while *acsA* produced a faint, non-specific band in one sample. No amplification occurred in NTCs, confirming that the products originated from residual DNA in RNA extracts. These results demonstrated that on-column DNase digestion was insufficient, prompting the adoption of an additional off-column DNase treatment (TURBO DNA-free™ Kit, Invitrogen). A molecular weight ladder (100 bp, 500 bp, and 1000 bp markers) was used as size reference.

3.2.3. Two-step RT-qPCR

To overcome the limitations encountered in the one-step assay, cDNA synthesis was repeated using a two-step workflow with qScript XLT cDNA SuperMix (Quantabio, Beverly, MA, USA), which offered greater specificity and flexibility. This phase of the work was conducted after two key improvements: (i) the implementation of an additional off-column DNase treatment to ensure complete removal of genomic DNA and (ii) the update of the primer panel, which included a redesigned *phzA1* primer pair producing a longer amplicon (431bp) as well as the addition of a new primer set targeting the *relA* gene. A second gradient PCR was also performed to optimize annealing conditions for the extended panel (Figure 11).

Using this workflow, the cDNA samples showed amplification across both target and housekeeping genes, though some variability was noted between replicates, more specifically one sample failed to amplify *phzA1* and *trpE*, while in another sample *pvdA* and *relA* didn't amplify (Figure 14). In the RNA samples processed without reverse transcription (-RT) there was no amplification detected across all primer sets, confirming the absence of contaminating DNA following the off-column treatment with TURBO DNA-

free™ Kit (Invitrogen, Thermo Fisher Scientific Inc., Waltham, MA, USA). As expected, the DNA positive control consistently amplified for all genes, while unexpectedly two of the NTCs, containing only NF-water, amplified for the *phzA1* and *pvdA* targeting primers which likely reflects either primer-dimer formation or a minor cross-contamination during plate setup as no amplification was observed for the *mexA*, *relA*, *acsA* and *trpE* NTCs, indicating that the NF-water itself wasn't the contaminating source (Figure 14).

Despite an overall improvement in comparison to the one-step RT-qPCR approach largely due to the implementation of an additional off-column DNase treatment and the refinement of the primer panel. While some variability between replicates persisted, these inconsistencies are likely attributable to the low transcript abundance levels or primer specificity rather than systematic contamination with the occasional amplification detected in NTCs, most likely reflecting primer-dimer formation or handling-related contamination during plate setup. In essence, these optimizations established an initial foundation for subsequent transcriptional analysis of *P. aeruginosa* 25L under stress conditions, although this assay was conducted exclusively with T0 samples, meaning that no stress-challenge comparisons could be drawn at this stage.

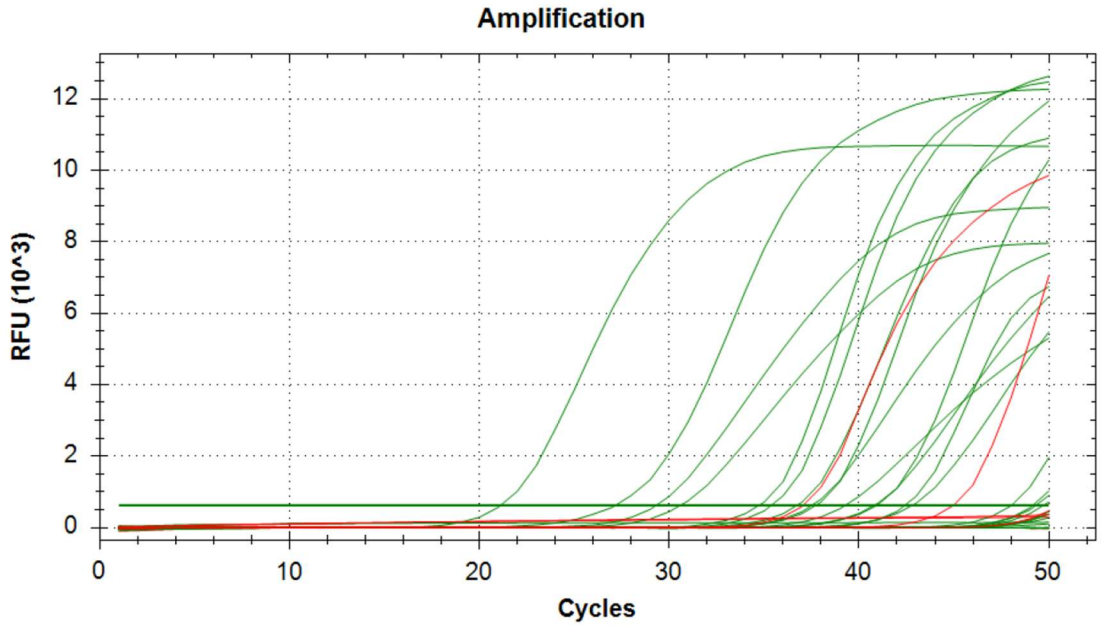

Figure 14 - Two-step RT-qPCR amplification curves for target and housekeeping genes in *P. aeruginosa* 25L. To improve specificity, cDNA synthesis was performed using the qScript XLT cDNA SuperMix (Quantabio, Beverly, MA, USA) following an additional off-column DNase treatment, together with an updated primer panel including redesigned *phzA1* (431 bp) and new *relA* primers. Amplification was detected for most target genes, though occasional variability occurred across replicates (e.g., missing amplification of *phzA1, trpE, pvdA*, or *relA* in individual samples). No amplification was detected in –RT controls, confirming efficient DNA removal. The DNA positive control amplified consistently across all genes. Unexpectedly, two NTCs (red curves) produced amplification signals with the *phzA1* and *pvdA* primers, most likely due to primer-dimer formation or handling-related contamination during plate setup. NTCs with other primer sets (*mexA, relA, acsA, trpE*) remained negative, excluding water contamination as the source.

4. Discussion

This study sets out to investigate the gene expression of *P. aeruginosa* strain 25L, a marine isolate from the port of Trieste, when exposed to environmental stressors that mimic seawater conditions namely nutrient limitation, osmotic stress and high salinity. The expression of four target genes (*mexA*, *pvdA*, *phzA1* and *relA*) with distinct but complementary physiological roles related to efflux activity, siderophore biosynthesis, phenazine biosynthesis and stringent response regulator, respectively, was analyzed by a two-step RT-qPCR, with two housekeeping genes (*acsA* and *trpE*) serving as reference genes [29][44][59][86].

With the purpose of determining the primers optimal annealing temperature and efficiency for simultaneous amplification, two gradient PCR assays were performed. In the first assay, four of the five primer pairs (*phzA1*, *pvdA*, *acsA* and *trpE*) showed a temperature-dependent amplification pattern with higher annealing temperatures (≥ 59,4ºC) leading to a later amplification or no amplification due to a decreased primer-template binding efficiency, while the *mexA* targeting primer demonstrated a robust and consistent amplification across every temperature tested which suggests high primer efficiency and strong primer-template complementary but it also raises the possibility of unspecific amplification that needs to be further investigated (Figure 10). This behavior was even more pronounced on the second gradient PCR where the redesigned *phzA1* targeting primers and a new set of primers targeting the *relA* gene were also integrated, with the new *relA* set of primers showing no amplification for high temperatures and a delayed amplification for lower temperatures just as the primers targeting *pvdA*, *acsA* and *trpE*, which indicates suboptimal binding efficiency or low template abundance (Figure 11). Ultimately, the gradient PCR results allowed the selection of an annealing temperature (53ºC) suitable for downstream applications but also highlighted primer-specific limitations that need to be refined in future work.

Initially, the gene expression of *P. aeruginosa* 25L was assessed by the application of a one-step RT-qPCR approach combining reverse transcription and amplification in a single reaction. This method provided rapid results and successful amplifications for most of the primer sets with *mexA*, *pvdA* and *acsA* producing strong and distinct products across replicates yet other gene targets, such as *trpE*, frequently generated faint or absent bands suggesting either low transcript abundance under the tested conditions or suboptimal primer-template interactions within the one-step system (Figure 12). Amplification patterns of *phzA1* were particularly problematic to analyze as the expected

product was difficult to distinguish from non-specific fragments, including low-molecular-weight bands likely corresponding to primer-dimers that were possible to observe in other gene target lanes (Figure 12). The presence of weak amplification in some NTC as well as non-specific amplification bands present in some lanes further raised concerns regarding the specificity of the assay pointing to possible DNA contamination, prompting a change in strategy and the need for additional controls along with the design refinement of *phzA1* targeting primers and the addition of a new set of primers targeting the *relA* gene (Table 1).

To clarify whether the unexpected amplification signals originated from residual DNA contamination, -RT controls were performed and the detection of clear amplification products in multiple RNA samples, particularly for *mexA* and to a less extent *phzA1* along with *pvdA*, confirmed the persistence of contaminating DNA despite the on-column DNase treatment included during the RNA extraction (Figure 13). Interestingly, the *trpE* primers consistently failed to generate any amplification products which indicate either that this locus was absent from contaminating DNA at detectable levels or that the primer design was highly specific, reducing the likelihood of off-target binding (Figure 13). In contrast, *acsA* primers produced a faint band in only one RNA sample but with a different molecular weight than the expected suggesting that the amplification was non-specific and likely the result of secondary structures rather than true amplification of *acsA* sequence (Figure 13). The fact that these anomalies were inconsistent further highlights that the main source of contamination was residual DNA. Additionally, this observations explained some of the inconsistent amplification patterns observed in the one-step RT-qPCR, underlining the need for a more stringent RNA purification protocol and leading to the consequent addition of an off-column DNase treatment implemented using the TURBO DNA-free™ Kit (Invitrogen, Thermo Fisher Scientific Inc., Waltham, MA, USA) which improved the removal of contaminating DNA and ensured the integrity of the RNA used for downstream analysis.

Based on these optimizations, the workflow transitioned to a two-step RT-qPCR strategy in which the reverse transcription and amplification were carried out in separate reactions, improving specificity and reproducibility while allowing the same cDNA stock to be reused in multiple control assays. In this phase of the work the redesigned *phzA1* primer pair, which generated a longer amplicon, as well as a new set of *relA* targeting primers were incorporated into the study (Table 1). With this new setup, it was possible to observe some variability in amplification between replicates for both target and housekeeping genes, nonetheless the absence of amplification in the -RT controls

confirmed that the additional off-column DNase treatment successfully eliminated DNA contamination and thereby validated the reliability of the results. The occasional amplification of NTC (Figure 14), restricted to *phzA1* and *pvdA*, was most likely due to primer-dimer formation or handling-related contamination during plate setup, highlighting the need to do a melting curve analysis to better understand the origin of this observed amplification. Despite these minor issues the two-step workflow presented a substantial improvement over the one-step assay, providing an initial platform for investigating the gene expression of *P. aeruginosa 25L*.

Overall, the gene-specific results obtained offer important insights into the adaptive strategies of *P. aeruginosa 25L* in marine-like conditions. The consistent amplification of *mexA* in both RT-qPCR trials (Figure 12)(Figure 14) performed suggests that this multidrug efflux pump component is expressed at a detectable level under all tested stress conditions, aligning with the central role of MexAB-OprM system not only in conferring antibiotic resistance but also in protecting and allowing *P. aeruginosa* to cope against environmental stressors such as osmotic shifts, with strong and persistent amplification signals raising the possibility that *mexA* expression may be constitutively active at a basal level in environmental strains ensuring preparedness against sudden osmotic challenges typical of seawater and port-associated environmental dynamics exacerbated by pollution [77][132]. At the same time the strong performance of the *mexA* primers, even in -RT controls, emphasizes the need to refine primer design or confirm the expression by sequencing the amplicons to disentangle true expression patterns from possible artifacts preventing a biased interpretation of the *mexA* transcriptional analysis.

In contrast to the robust amplification of *mexA*, *pvdA* amplification patterns were less strong and more variable, which may reflect the conditional nature of siderophore production considering that *pvdA* encodes a key enzyme in pyoverdine biosynthesis [86][89][96]. In oligotrophic seawater conditions simulated in this study, iron is scarce but not necessarily absent creating a situation where expression may fluctuate depending on subtle variations in iron availability [19][51]. The faint and inconsistent amplification observed in some replicates could therefore reflect low-level basal expression or transcription occurring in only a subpopulation of cells. Yet, the occasional detection in NTCs indicates that the primers may be prone to non-specific binding or dimerization, complicating the interpretation of the results obtained and underlining the need for further optimizations although, from a biological standpoint, the analyzed variability aligns with the fact that pyoverdine production is energetically expensive and bacteria tend to

synthesize it only when the benefits outweigh the costs, for instance during a prolonged iron depletion or competitive interactions [48]. Thus, the results obtained here may reflect the regulatory balance of *pvdA* in fluctuating marine environments but further trials under extended incubation times or under explicitly iron-depleted conditions would clarify whether *pvdA* is rapidly upregulated in *P. aeruginosa 25L* or requires stronger selective pressures.

The amplification behavior of *phzA1* posed considerable challenges in the one-step RT-qPCR initially performed, with clearer signals only emerging after primer redesigning and transitioning to a two-step workflow. As stated previously, phenazine biosynthesis genes are tightly regulated by QS (Rhl and PQS systems) and are generally induced under high-cell-density conditions, during redox stress or in response to nutrient limitation [12][16][92]. Since the experimental design prioritized relatively short-term stress exposures (1 hour), it is plausible that cell density was insufficient to activate QS cascades, resulting in minimal *phzA1* expression at the time of sampling, explaining the weak or inconsistent signals. Moreover, phenazines, particularly pyocyanin, play an especially important role in marine environments by acting as redox shuttles that help bacteria cope with fluctuating oxygen levels, ROS stress and competitive microbial interactions, meaning that this gene remains a valuable candidate for future longer-term conditions that simulate biofilm-like growth where phenazine expression is expected to be more pronounced [35][44].

Finally, *relA* displayed consistently weak amplification, particularly at higher annealing temperatures on a gradient PCR trial, suggesting both suboptimal primer-template binding and low transcript abundance. This is consistent with the biological role of RelA which catalyzes the synthesis of (p)ppGpp during the stringent response, a stress adaptation response activated under severe prolonged nutrient deprivation or severe environmental challenges [29][120][127]. Given that stress exposure in this study lasted only one hour, it is likely that nutrient limitation was insufficient to trigger a strong stringent response, resulting in weak transcriptional signals that still provided a critical perspective on global regulatory mechanisms and its inconsistent amplification across replicates in the two-step RT-qPCR reinforces the idea that experimental stress conditions should be extended in time or intensified to better capture the dynamics of the stringent response activation in *P. aeruginosa* 25L.

Altogether the results obtained provide a first glimpse into how *P. aeruginosa* 25L responds, at a transcriptional level, to challenges that mimic marine conditions, however they also highlight important limitations and unanswered questions. The variability in

primer performance and inconsistencies across replicates points to the need for further improvement of experimental design and methodological conditions, moreover, the gene-specific behaviors observed suggest that including longer stress exposures and iron limiting conditions may be necessary to fully capture the survival strategies of this strain.

The genes investigated (*mexA*, *pvdA*, *phzA1* and *relA*) remain strong candidates for studying environmental stress adaptation as their ecological and clinical significance is well-documented and the methodological groundwork presented here enables these genes to be probed more systematically in subsequent studies. Aerosolization experiments should be considered as future extension, but they were not performed in this thesis.

5. Future Steps

This study established groundwork for exploring the transcriptional responses of *P. aeruginosa* 25L to marine-relevant stressors but also revealed several methodological and biological aspects that need further investigation.

The inconsistent amplification of certain genes, mainly *relA*, *pvdA* and *phzA1*, shows the relevance of further optimizing primer pairs to minimize non-specific amplification that coupled with melt curve analysis and sequencing of PCR products would strengthen the reliability of RT-qPCR results, as well as extending the inclusion of post-stress (Tf) alongside baseline (T0) samples to enable differential expression analysis. In particular, the unusual performance of the *mexA* primers, which amplified strongly even in -RT controls, suggests that alternative primer sets should be tested to differentiate genuine expression from technical artifacts avoiding biased or incorrect result interpretations. It is also important to formally validate the housekeeping genes stability by calculating the standard deviation of the Ct values for each candidate housekeeping gene across all experimental samples, determine primer efficiency (E) and standard curves for accurate quantification.

Another measure that can be taken into account to avoid inconsistent amplification patterns would be to prolong the time of the stress challenges applied since the previous short-term period (1 hour) may not have been sufficient to fully trigger the transcriptional response, especially for regulatory genes such as *relA* or secondary metabolite genes like *phzA1*. Adapting stress challenges to more condition-specific ones, for instance, *pvdA* is upregulated under iron-limitation while *relA* is typically activated during prolonged nutrient limitation states, therefore future experiments should include:

1. Iron-depleted seawater conditions to better assess *pvdA* transcription.
2. Higher cell density growth to evaluate *phzA1* activity.
3. Prolonged nutrient limitation to test the stringent response activation via *relA*.

While this study focused on four target genes, *P. aeruginosa* possesses a wide regulatory network connecting efflux systems, iron uptake, secondary metabolism and stringent response, substituting or expanding the primer panel could lead to better amplification signals and, consequently, more robust results.

Future efforts should focus on improving the workflow to ensure reliable transcriptional profiling of stress responses in *P. aeruginosa* 25L so that this framework is consolidated

and can be applied to the aerosol samples retrieved from Genoa, enabling direct comparison of gene expression patterns between liquid culture and airborne conditions. Such comparison will provide important insights into the adaptative mechanisms of *P. aeruginosa* during environmental transitions relevant to marine and atmospheric ecosystems.

Ultimately, future work should not only refine the methodological framework established but also expand the experimental conditions to better reproduce the dynamic nature of gene regulation in marine environments by integrating longer stress exposures, condition-specific challenges and primer redesign. Such efforts will improve the quality of the results obtained and allow more accurate interpretations of the gene expression by *P. aeruginosa* 25L.

6. Conclusion

This thesis investigated the gene expression of *P. aeruginosa* 25L, a marine isolate from the Gulf of Trieste, under stress conditions mimicking the oligotrophic and fluctuating nature of seawater, through the use of RT-qPCR targeting four functional genes (*mexA*, *pvdA*, *phzA1* and *relA*) alongside two housekeeping genes (*acsA* and *trpE*). This work provided insights into both the methodological challenges of gene expression analysis and the adaptive strategies employed by an environmental isolate to persist in challenging conditions.

The experimental workflow highlighted the critical importance of primer design, contamination control and overall optimization. An initial one-step RT-qPCR trial exposed issues with non-specific amplification and residual DNA contamination which were subsequently addressed through the implementation of -RT controls, off-column DNase treatments and the execution of a two-step RT-qPCR protocol. These optimizations substantially improved specificity and reproducibility, providing a robust foundation for gene expression analysis in the chosen strain.

At a biological level, the consistent detection of *mexA* suggests that efflux pump activity may be constitutively maintained as part of a generalist survival strategy, providing resilience against osmotic fluctuations prevalent in marine environments. In contrast, the variable or weak amplification of *pvdA*, *phzA1* and *relA* emphasizes the conditional nature of siderophore production, phenazine biosynthesis and stringent response activation, processes that are induced primarily under specific environmental triggers such as iron limitation, high cell density or prolonged nutrient stress. Although the gene expression captured here was modest, they provide valuable groundwork for more detailed investigation into the molecular ecology of this bacterium, reinforcing the fact that environmental strains of *P. aeruginosa* possess a finely tuned regulatory network that allows adaptation to heterogeneous and challenging conditions like the ones that characterize the Gulf of Trieste.

Overall, this work should be viewed as a preliminary exploration rather than a definitive characterization of the gene expression of *P. aeruginosa* 25L under marine stress conditions. The methodological challenges encountered, more specifically primer performance and variability between replicates, highlight that significant optimization remains necessary before robust biological conclusions can be drawn. Consequently, the patterns observed across *mexA*, *pvdA*, *phzA1* and *relA* should be interpreted with caution as they may reflect technical artifacts as much as genuine transcriptional activity.

Nonetheless, this study establishes an essential foundation by identifying both experimental bottlenecks and the gene targets most sensitive to environmental conditions providing a guideline for future refinements and a more comprehensive investigation which will ultimately be required to fully uncover the gene expression of *P. aeruginosa* 25L during osmotic, low nutrient and high salinity stress.

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Attachments

Attachment 1

Table 2 - Composition of ZoBell medium used for *P. aeruginosa* 25L stress challenges. The table lists the constituents of ZoBell medium, including nutrient sources, salts and trace elements, with respective concentrations. This medium was employed for routine growth and preparation of cultures used in subsequent stress and transcriptional response assays.

ZoBell Medium Composition	
Composition	**Concentration**
ammonium nitrate	1,6 mg/L
boric acid	22,0 mg/L
calcium chloride	1,8 g/L
disodium phosphate	8,0 mg/L
ferric citrate	0,1 g/L
magnesium chloride	5,9 g/L
magnesium sulfate	3,24 g/L
peptone	5,0 g/L
potassium bromide	0,08 g/L
potassium chloride	0,55 g/L
sodium bicarbonate	0,16 g/L
sodium chloride	19,45 g/L
sodium fluoride	2,4 mg/L
sodium silicate	4,0 mg/L
strontium chloride	34,0 mg/L
yeast extract	1,0 g/L