The Effect of *Pseudomonas aeruginosa* on the Endoplasmic Reticulum Stress Response in Mammalian Cells

by

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ABSTRACT

Pseudomonas aeruginosa is an opportunistic pathogen imposing serious threat to cystic fibrosis patients. *P. aeruginosa* is equipped with many virulence factors that are controlled by the quorum sensing (QS) systems. These virulence factors modulate host responses such as the host stress responses. I demonstrated that *P. aeruginosa* wild-type (WT) strain PA14 activated the stress responses in A549 cells. Screening the mutants of different QS systems showed that a deletion in the QS regulator gene *lasR* led to a prolonged activation of the stress response compared to WT. Mutants of LasB and AprA, two LasR-regulated proteases, showed a delayed and lower levels of stress response activation compared to WT. Purified LasB treatment was sufficient to induce ISR in A549 cells, suggesting that it is a novel elicitor of this host stress response. My work indicates that *P. aeruginosa* is a pathological stressor which will help in the search of novel therapy.

LIST OF ABBREVIATIONS

2-heptyl-4(1H)-quinolone	HHQ
4-phenylbutyric acid	PBA
Activating Transcription Factor 4	ATF4
Activating Transcription Factor 6	ATF6
Alkaline Protease	AprA
Binding immunoglobulin protein	BiP
C/EBP-homologous protein	CHOP
Chronic Obstructive Pulmonary Disease	COPD
Complementary DNA	cDNA
Cystic Fibrosis	CF
Cystic fibrosis transmembrane conductance regulator	CFTR
Double-stranded RNA dependent protein kinase R -like ER kinase	PERK
Double-stranded RNA	dsRNA
Dulbecco's Modified Eagle Medium	DMEM
Elastase	LasB
Endoplasmic reticulum-associated degradation	ERAD
Endoplasmic Reticulum	ER
Eukaryotic translation initiation factor-α	eIF2α
Fluoroquinolones	FQ
General control non-derepressible 2	GCN2
Genomic DNA	gDNA

Golgi-resident site 1 protease	S1P
Growth arrest and DNA damage-inducible 34	GADD34
Heme-regulated eIF2a kinase	HRI
Inositol-requiring enzyme1	IRE1
Interferon kappa B kinase	IKK
Interleukin	IL-
Jun-NH(2)-terminal Kinase	JNK
Las autoinducer synthase	LasI
Las transcriptional regulator	LasR
Lipopolysaccharide	LPS
Lysogeny broth	LB
Multiciplity of Infection	MOI
Myeloid differentiation primary response 88	MyD88
N-3-oxo-dodecanoyl homoserine lactone	3-oxo-C12-HSL
N-acyl homoserine lactone	AHL
N-butanoyl homoserine lactone	C4-HSL
Nuclear factor kappa-light-chain-enhancer of activated B cells	NF-kB
Nucleotide-binding oligomerization domain	NOD
Optical density	OD
Paraoxonase 2	PON2
Phosphate-buffered saline	PBS
Polymerase Chain Reaction	PCR
PQS transcriptional regulator	MvfR/PqsR

Protein kinase R	PKR
Pseudomonas aeruginosa	*P. aeruginosa*
Pseudomonas Quinolone signal	PQS
Pyocyanin	PCN
Quorum Sensing	QS
Reactive oxygen species	ROS
Real time quantitative-PCR	RT-qPCR
Regulated IRE1-dependent decay	RIDD
Retinoic acid-inducible protein I	RIG-I
Rhl autoinducer synthase	RhlI
Rhl transcriptional regulator	RhlR
tauroursodeoxycholic acid	TUDCA
Thapsigargin	Tg
Toll-like receptor	TLR
Transcription factor	TF
Tris-buffered-saline Tween	TBST
Tumor necrosis factor	TNF
Type Five Secretion System	T5SS
Type One Secretion System	T1SS
Type Three Secretion System	T3SS
Type Six Secretion System	T6SS
Type Two Secretion System	T2SS
Open reading frames	ORFs

Wild type	WT
X-box binding protein-1	XBP1

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CHAPTER 1. INTRODUCTION

1.1 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative rod-shaped bacterium that is found ubiquitously in the environment (1). *P. aeruginosa* is an opportunistic pathogen that is able to infect a wide range of hosts including humans, plants, fish and many others (2). Currently, *P. aeruginosa* is one of the leading nosocomial pathogens worldwide, causing almost 20% of all pneumonia cases in intensive care units (3). *P. aeruginosa* imposes a serious threat to immunocompromised hospitalized patients, causing 8-25% of hospital acquired pneumonia in patients infected with Human Immunodeficiency Virus (4). It is also a major cause of morbidity and mortality in patients with Cystic Fibrosis (CF), Chronic Obstructive Pulmonary Disease (COPD), and other obstructive lung diseases (5, 6). *P. aeruginosa* contains a large ~6.5 mega base pair genome that allows it to be equipped with a large repertoire of virulence factors and signalling networks, thus enabling it to evade and adapt to the immune system of various hosts (7).

1.2 Importance of New Treatment Regimens for *P. aeruginosa*

One of the most significant challenges for treating *P. aeruginosa* infections is the emergence of antibiotic resistant strains (8). *P. aeruginosa* is becoming increasingly hard to treat due to its intrinsic ability to resist antimicrobials combined with its ability to acquire additional resistance mechanisms. β-lactams, such as penicillin and cephalosporin, are commonly used to treat *P. aeruginosa* infections. However, *P. aeruginosa* has become β-lactam resistant due its ability to produce and secrete β-lactamase which renders the antibiotic ineffective (9). Another antibiotic class that is commonly used is

Fluoroquinolones (FQ) such as ciprofloxacin, which target bacterial DNA gyrase and topoisomerase to prevent DNA replication, leading to cell cycle arrest and eventual cell death. *P. aeruginosa* has become resistant to FQs by selecting mutations in its DNA gyrase and topoisomerase that prevent FQs from targeting them (10, 11). In addition to that, *P. aeruginosa* is well equipped with efflux pumps, which are transport proteins involved in actively transporting solutes from within the cell to the extracellular environment. These efflux pumps allow *P. aeruginosa* to confer resistance to FQs and aminoglycosides such as gentamycin, another class of antibiotics, by pumping the antibiotics out of the cell (12, 13).

Another important antibiotic resistance mechanism of *P. aeruginosa* is the ability to form biofilms. Biofilms are three-dimensional structures consisting of bacterial communities encased in an extracellular matrix produced by the bacteria themselves (14). The exact mechanism behind how biofilms confer antibiotic resistance is not well known. One of the possible mechanisms is that some cells experience nutrient limitation and exist in slow-growing state; this physiological state renders these unique cells resistant to antimicrobials. Another suggested mechanism is the failure of antimicrobials to penetrate the full depth of the biofilm (15).

Given its intrinsic resistance to antibiotics, *P. aeruginosa* was named one of the most critical pathogens on the antibiotic resistant priority list published by the World Health Organization (16). Currently, there are very few antibiotics in the drug development pipeline for Gram negative pathogens including *P. aeruginosa*. This begs for the exploration of alternative therapies, which requires further research to identify new drug targets.

1.3 Quorum Sensing in *P. aeruginosa*

A potential antimicrobial target of *P. aeruginosa* could be its quorum sensing (QS) systems. QS is a term that refers to a bacterial cell-cell communication mechanism that is dependent on bacterial population density (17). QS in bacteria regulates almost all aspects of their physiology, including virulence gene expression, sporulation, biofilm formation, swarming, and antibiotic biosynthesis (18–20). It involves a signaling molecule which accumulates in a local environment to the levels that are required to activate transcription of specific target genes (21). This mechanism of communication allows bacteria to act as a community in a coordinated manner to regulate their gene expression. This makes bacteria highly adaptable and capable of responding to environmental stress cues, including host anti-bacterial responses, thus it is important for the pathogenesis of the bacteria.

QS in *P. aeruginosa* is the most complex and well-studied QS system in bacteria. The complex QS network in *P. aeruginosa* consists of 4 branches: las, rhl, PQS, and IQS (21). These networks are tightly regulated to ensure that the bacteria are able to respond appropriately to constant changes in their environment. Therefore, understanding the regulatory mechanisms that govern the virulence of *P. aeruginosa* could help in the search of alternative therapies. The three most well-studied QS systems (las, rhl, PQS) of *P. aeruginosa* and their regulatory mechanisms will be discussed below and are depicted in figure 1. It is important to note that these systems are constitutively expressed at low levels, however, the level of QS gene expression is affected by the bacteria's environment and needs.

1.3.1 Las QS System

The Las QS system is one of two systems, rhl being the other one, that use N-Acyl

homoserine lactone (AHL) signalling molecules. AHL is one of the most studied communication molecules in bacteria (21). It consists of fatty acids linked to a homoserine lactone moiety via a peptide bond. In the las QS system, LasI synthase is responsible for producing an AHL molecule called N-3-oxo-dodecanoyl homoserine lactone (3-oxo-C_{12}-HSL). This molecule is recognized by the transcriptional regulator LasR, forming a complex that leads to downstream changes in gene expression as shown in figure 1. The las system controls the production of many virulence factors involved in pathogenesis and host immune evasion, such as LasA and LasB elastases, endotoxin A, and alkaline protease (22). Previous research has shown multiple regulators that contribute to the timing and level of las-regulated virulence. For example, the expression of RsaL, a repressor of *P. aeruginosa* virulence, is induced by LasR and it acts as a transcriptional regulator of *lasI* by binding to the *rsaL-lasI* bidirectional promoter, preventing the expression of both genes (23). This generates a negative feedback loop that limits the expression of 3-oxo-C_{12}-HSL and the downstream expression of lasR-regulated genes (23).

1.3.2 Rhl QS System

The Rhl QS system is very similar to the las system, where it starts by RhlI synthase producing the signalling molecule N-butanoyl homoserine lactone (C_4-HSL). This molecule is sensed by the transcriptional regulator and cognate receptor RhlR. This induces the expression of many genes including those responsible for the production of rhamnolipids, which are glycolipids that are involved in virulence, surface motility, and biofilm development (24). C_4-HSL-RhlR complex represses the gene responsible for the assembly and function of the type three secretion system (T3SS), an important virulence factor of *P. aeruginosa* (22). The Rhl system is controlled by the Las system in a hierarchal

manner, as the 3-oxo-C_{12}-HSL-LasR complex directly upregulates *RhlR* transcription. So, the activation of the Las system allows for the activation of the Rhl system (25).

1.3.3 PQS System

Unlike the Las and Rhl systems, the PQS system does not use AHLs as its signalling molecules, instead it uses a molecule called *Pseudomonas* Quinolone signal (PQS). PQS is made from the products of the *pqsABCDE* operon and the *phnAB* operon. Together, they synthesize the precursor molecule 2-heptyl-4(1H)-quinolone (HHQ), which is converted to PQS by the enzyme PqsH. PQS then binds its cognate receptor MvfR (also known as PqsR), forming a complex that in turn induces the expression of the *pqsABCDE* and *phnAB* operons, leading to an increase in PQS and pyocyanin production (22). Pyocyanin (PCN) is a secondary metabolite produced and secreted by many strains of *P. aeruginosa* that causes a wide spectrum of cellular damage, such as inhibiting cellular respiration, disrupting cellular homeostasis and inducing the apoptosis of neutrophils (26–28). Therefore, PCN-induced injury is thought to contribute to the persistence of *P. aeruginosa* in the lungs of CF patients (29).

The PQS system is linked in a hierarchical manner to the AHL signalling systems of *P. aeruginosa*; LasR positively regulates the levels of PQS while RhlR negatively regulates it by binding to the promoter region of the PqsR regulator (30). Although PQS is the main signalling molecule, its immediate precursor HHQ can also be released into the extracellular medium, and then taken in by surrounding cells, where it is converted into PQS by PqsH activating PQS-regulated gene expression (31). Xiao *et al.* also showed that HHQ does not need to be converted to PQS, instead it can bind the transcriptional regulator

MvfR directly, although at a much lower efficiency than PQS-MvfR interaction, and activates MvfR-dependent gene expression and pathogenicity (32).

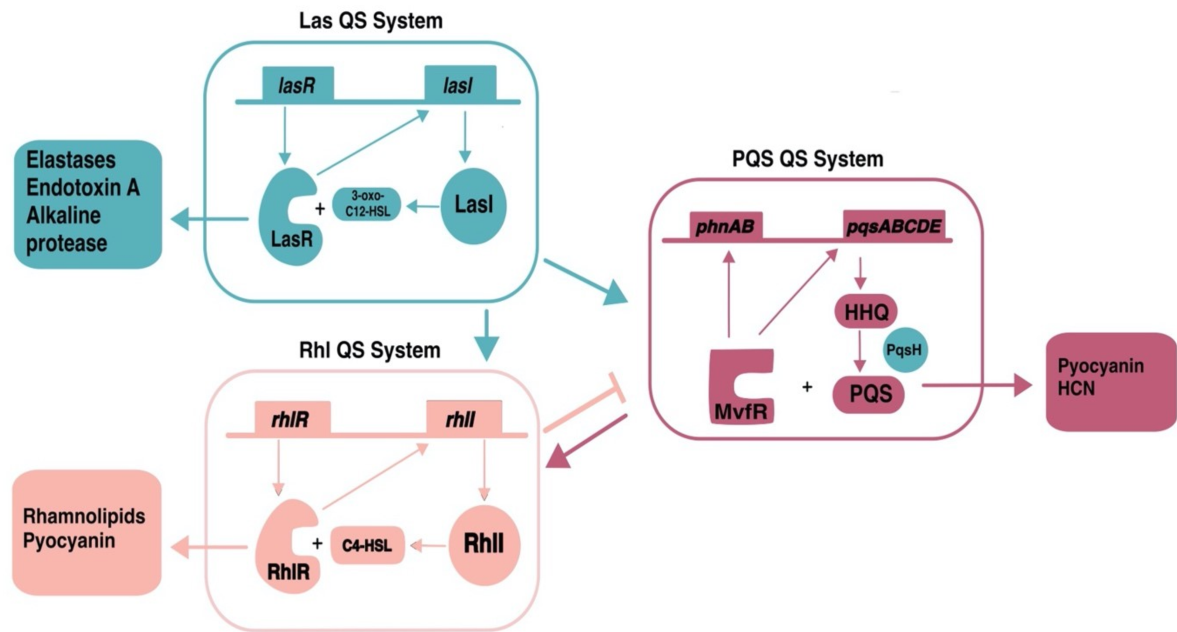

Figure 1. Quorum Sensing Systems of *P. aeruginosa.*

The most studied quorum sensing systems of *P. aeruginosa* are as follows: Las, Rhl and PQS. The Las system depends on lasI synthase that produces the signalling molecule 3-oxo-C12HSL. The signalling molecule binds the transcriptional regulator, LasR, to form a complex. This complex leads to the expression of virulence factors such as elastases, endotoxin A, and alkaline protease. Similar to the Las system, the Rhl system depends on rhlI synthase which produces C4-HSL. This molecule binds the transcriptional regulator, rhlR, and induces the expression of rhamnolipids, pyocyanin and repress the T3SS. The PQS system consisting of two operons, *phnAB* and *pqsABCDE*, produces the intermediate molecule HHQ. HHQ is converted to the signalling molecule PQS with the help of PqsH, a product of the las system. PQS binds the transcriptional regulator MvfR and induces the expression of pyocyanin and hydrogen cyanide (HCN).

1.4 Secretion Systems of *P. aeruginosa*

As mentioned above, *P. aeruginosa* has a wide array of virulence factors that make it the powerful pathogen that it is. It is also equipped with many different secretion systems that deliver these virulence factors, mostly toxins and hydrolytic enzymes, to the extracellular environment or into the host cell (33). *P. aeruginosa* has 5 different types of secretion systems with each of them playing a specific role in delivering certain effectors to their targets. A few of these systems will be discussed in more detail below.

Type one secretion system (T1SS) is the simplest bacterial secretion system consisting of an outer membrane (OM) protein, an ATP-binding cassette (ABC) transporter in the inner membrane (IM) and an adaptor protein connecting these two components (34). One of the main effectors of the T1SS is the alkaline protease AprA, which will be discussed in detail below (35). The AprA operon contains the genes necessary for the synthesis of its own T1SS apparatus. AprA plays an important role in immune evasion and host response modulation (36). Another important secretion system in *P. aeruginosa* is the type two secretion system (T2SS), which depends on a two-step process including a stopover in the periplasm before it delivers its effectors to the extracellular environment (37). T2SS-dependent exoproteins include proteolytic enzymes like elastase LasB, and endotoxin A. The expression of T2SS machinery and exoprotein-encoding genes is regulated by the las and rhl system discussed above (38).

The type three secretion system (T3SS) also plays an important role in the pathogenesis of *P. aeruginosa*. This system depends on a needle like structure to inject its effectors directly into the host cell (39). Different strains of *P. aeruginosa* have different combinations of genes encoding T3SS effectors, including *exoU*, *exoT*, *exoS*, and *exoY*

(40). These effectors contribute to the cytotoxicity of *P. aeruginosa* in a wide range of hosts. For example, ExoU-producing strains like PA14 are known to cause rapid necrotic cell death while ExoS-producing strains like PAO1 are usually internalized and lead to delayed cell death (41). T3SS, together with its effectors, is tightly regulated by the quorum sensing systems (42). Its expression and function have been proven to be important during early stages of acute infections (43). However, in the case of a chronic infection, *P. aeruginosa* seem to acquire a number of mutations in the T3SS to reduce its acute cytotoxicity, which helps the bacteria persist in the host (43).

P. aeruginosa possess multiple different subtypes of the type five secretion system (T5SS) (44). Some of the exoproteins that are secreted through T5SS include PlpD which is a bacterial lipolytic enzyme that displays phospholipase activity as well as CdrA which is a matrix adhesion protein that helps *P. aeruginosa* build biofilms to withstand any environmental assaults (45, 46). Finally, three type six secretion system (T6SS) loci have been identified in *P. aeruginosa*: H1, H2, and H3-T6SS (47). The T6SS is characterized to function in bacterial community competition by delivering toxins to other bacteria. For example, PdlA, a eukaryotic-like phospholipase D3 and a substrate of the H2-T6SS, achieves its antibacterial qualities by degrading phosphatidylethanolamine which is one of the main components of the bacterial membrane (48).

1.5 Effects of *P. aeruginosa* on a Mammalian Host

Knowing the huge repertoire of virulence factors of *P. aeruginosa*, it is not hard to imagine the serious repercussions it would cause in the host. *P. aeruginosa* is a common cause of nosocomial infections and it causes a wide range of disease such as urinary, burn, and respiratory infections and septicemia (49). Given that *P. aeruginosa* is one of the most

common pathogens causing respiratory infections, it is important to understand the effects it has on the lungs and the immune system to be able to resolve such infections. Acute infections occur commonly in people who are frequently admitted to the hospital, especially in those who suffer from immunodeficiencies thus are unable to mount a proper immune response (50). These infections, if not resolved, develop into persistent chronic infections which then become near impossible to treat due to the multidrug resistant phenotype of *P. aeruginosa* (50).

1.5.1 *P. aeruginosa* Pathogenesis and Virulence

P. aeruginosa is a very successful pathogen due to its ability to adapt and continue to cause pathogenicity to the host. This is possible due to the wide variety of virulence factors that help the bacteria evade the immune response. Alkaline protease (AprA) is one of the many virulence factors that are regulated by the lasIR QS system (51). AprA is a zinc metalloprotease that is secreted via its own type one secretion system (T1SS). It plays a very important role in immune evasion thus contributing to the persistence of *P. aeruginosa* infection. It does so by cleaving various immune activating molecules like tumor necrosis factor (TNF), and interferon-γ (52). It is also capable of blocking various complement functions thus hampering the bacterial clearance by the neutrophils (53). The complement system is made up of multiple plasma proteins, named C1-C9, that react with one another to opsonize pathogens and induce an inflammatory response to help fight the infection (54). AprA is able to block both the classical and lectin complement pathways by predominantly cleaving the complement protein C2 (36). Another mechanism of immune evasion that AprA is capable of is preventing the activation of Toll-like Receptor 5 (TLR5). TLR5 is a major pattern recognition receptor that plays a role in the recognition of

pathogens and downstream activation of innate immune signaling (55). It is usually activated by the binding of monomeric flagellin, which is the building block of bacterial flagellum. AprA has been found to degrade free flagellin in its surrounding thus impairing bacterial recognition and evading the host immune system (56, 57).

Elastase B (LasB) is a zinc-dependent metallo-endopeptidase that has multiple cytotoxic effects on the host cell. LasB can interfere with host bacterial clearance by degrading several components of the innate and adaptive immune defense such as TNF, Interleukin-2 (IL-2), IL-6 and IL-8 (58, 59). Similar to AprA, LasB has been shown to inactivate the complement system by specifically degrading complement proteins C1 and C3 (60). In addition to that, LasB plays a role in biofilm and microcolony formation, which aids in *P. aeruginosa* persistence in the host (61). Another mechanism where LasB evades the host response is by cleaving monomeric flagellin thus preventing the activation of TLR5, similar to the actions of AprA. This provides a failsafe mechanism in *P. aeruginosa* to ensure that monomeric flagellin does not activate the host immune response (60).

1.5.2 Host Response to a *P. aeruginosa* Infection

Our bodies have many defense pathways in place to help eradicate bacterial infections. Epithelial cells act as the first line of defense against *P. aeruginosa*. Besides acting as a physical barrier to infection, they also secretes many molecules that act to defend the lungs by recruiting immune cells like alveolar macrophages and neutrophils (62). Complement proteins bind the pathogen and promote its phagocytosis (54). Epithelial cells also secrete cytokines (IL-6, IL-1) and chemokines (CXCL-1) upon activation of their TLRs (63). This plays an important role in the recruitment and activation of cells of the innate and adaptive immune responses.

One of the important cells recruited to fight a *P. aeruginosa* infection are neutrophils. Neutrophils eliminate bacteria through phagocytosis and a number of microbicidal molecules such as defensin, lysozyme, reactive oxygen species (ROS) and elastase (64, 65). However, neutrophils that are killed by *P. aeruginosa* release high amounts of proteases and elastases, which impair mucociliary bacterial clearance and induce IL-8 production by epithelial cells thus potentiating inflammation and causing hyperinflammatory conditions. This exuberates pulmonary disease and contributes to the lung pathology in CF lungs (66). Alveolar macrophages also play an important role in eliminating *P. aeruginosa* given that they are likely the first immune cell to encounter *P. aeruginosa* in the lungs (67). Macrophages can engulf and kill bacterial pathogens (68). They get activated through their toll-like receptors (TLR) 4 and 5 by lipopolysaccharide (LPS) and flagellin respectively and secrete chemokines (CXCL-1) and cytokines (TNF and IL-6) to recruit neutrophils to the site of infection (69). Other immune cells also play a role in protecting the host against a *P. aeruginosa* infection. For example, a small subset of T cells known as gamma delta T cells were found to help in the eradication of *P. aeruginosa* pulmonary infection by regulating the production of pro-inflammatory cytokines (IL-1α, IL-17, TNF and IL-6) and subsequent immune cell recruitment (70, 71).

1.6 Stress Responses: Unfolded Protein Response and Integrated Stress Response

Besides the immune response against *P. aeruginosa*, cells have other mechanisms in place that aim to restore homeostasis in a stressful environment like that of a *P. aeruginosa* infection. The endoplasmic reticulum (ER) plays a major role in regulating the flow of macromolecules such as lipids, proteins and carbohydrates through the secretory pathway to maintain normal cell function (72). It is also an important site for the folding

of secreted proteins which make up a large fraction of the total protein output of a mammalian cell (72). However, physiological or pathological stressors could lead to an imbalance between the demand for protein folding and the ER folding capacity thus causing ER stress (73). This is why very intricate set of signalling pathways are put in place to carefully monitor for any abnormalities in ER function. These pathways are known collectively as the unfolded protein response (UPR). The UPR plays a very important role in restoring cellular homeostasis against the challenges of ER stress. These pathways can be triggered by various stimuli including bacterial and viral infection (74). These stimuli tend to cause an imbalance in the protein folding capacity of the ER, resulting in the accumulation of unfolded or misfolded proteins (73). This ER stress is sensed by three transmembrane ER-resident proteins, including double-stranded RNA dependent protein kinase R (PKR)-like ER kinase (PERK), inositol requiring enzyme (IRE1), and activating transcription factor 6 (ATF6) (73). These sensors are discussed in detail in the next few sections.

1.6.1 Double-Stranded RNA Dependent Protein Kinase R-like ER Kinase

PERK is a transmembrane ER stress-activated kinase which is kept inactive during normal conditions by the binding of ER chaperone binding immunoglobulin protein (BiP) to the luminal domain (75). In the case of ER stress, the demand of protein folding of the ER exceeds the ER's capacity which leads to the accumulation of unfolded or misfolded proteins in the lumen of the ER. This causes BiP to be recruited away to bind the unfolded proteins, leading to the activation of the ER sensor. Once BiP dissociates, PERK homodimerizes and transphosphorylates, leading to the active form of PERK (75). Activated PERK phosphorylates of its target, the eukaryotic translation initiation factor-α

(eIF2α). Phosphorylation of eIF2α leads to inhibition of global translation by inhibiting the assembly of the 80S ribosome.

Another direct effect of activated PERK is the selective translation of the transcription factor, ATF4. This is possible during a global translation shutdown because the ATF4 mRNA contains multiple upstream open reading frames (ORFs) before the *Atf4*-ORF (76). This means ATF4 protein synthesis is usually supressed when ribosomal assembly is efficient because the ribosome initiates translation at the upstream decoy ORFs. However, ribosomal assembly is impaired during ER stress, leading to the bypass of the uORFs in favor of the *Atf4* start codon (76). ATF4 induces the expression of ER stress target genes that include amino acid transport, metabolism, and oxidative stress resistance. These genes aim to restore homeostasis and relieve the ER stress. Once homeostasis is restored, eIF2α is dephosphorylated, thus terminating the PERK-dependent signalling pathways (77). This happens due to the expression of growth arrest and DNA damage-inducible protein (GADD34) by the PERK-ATF4 pathway. GADD34 associates with protein phosphatase 1 (PP1) and directs it to dephosphorylate eIF2α (78). GADD34 is also required for the robust expression of ER chaperones in response to ER stress, including BiP and GRP94 (79). However, in the case of chronic ER stress, ATF4 will induce the expression of additional transcription factor C/EBP-homologous protein (CHOP), which upregulates genes involved in the induction of apoptosis (80).

1.6.2 Inositol Requiring Enzyme 1

IRE1 is another transmembrane ER stress sensor that controls the most phylogenetically conserved UPR pathway. Mammals have two isoforms of IRE1: IRE1α is expressed in all cell types, while IRE1β is only expressed in intestinal epithelial cells

and airway mucous cells. Similar to PERK, it is kept inactive by the binding of BiP. In case of ER stress, BiP dissociates and IRE1 oligomerizes and autophosphorylates, activating its endonuclease activity (81). IRE1 has site-specific endonuclease activity that cleaves an intron of an mRNA encoding the transcription factor X-box-binding protein (XBP1), followed by the ligation of the severed exons to make spliced XBP1 (sXBP1). sXBP1 is then translated to the active form of the transcription factor which then travels to the nucleus, leading to the expression of ER-resident chaperones and protein folding enzymes. IRE1 also has non-specific endonuclease activity that is responsible for a process called regulated IRE1-dependent decay (RIDD). RIDD rapidly degrades ER-membrane associated mRNA, which eventually decreases the amount of protein entering the secretory pathway (82). IRE1 has also been shown to activate genes involved in ER-associated degradation (ERAD). ERAD is the process of removal of misfolded proteins in the ER through polyubiquitination and targeting to the proteasome for degradation (83).

1.6.3 Activating Transcription Factor 6

ATF6 is the third sensor governing the UPR signalling pathways. Following the dissociation of BiP during ER stress, the ER-transmembrane ATF6 will travel to the Golgi apparatus where it gets cleaved by Golgi-resident site 1 protease (S1P) and S2P (84). The active amino terminus of ATF6 is translocated to the nucleus where it binds the ER stress response element upstream of UPR genes to activate their expression (85). This set of genes overlaps with those activated by ATF4 and XBP1, including genes involved in protein folding and lipid synthesis. In addition, ATF6 can bind XBP1 to activate genes involved in ERAD (86).

1.6.4 Integrated Stress Response

In addition to the UPR, there are other very similar sets of signalling pathways that act alongside the UPR to help restore cellular homeostasis and these are collectively called the integrated stress response (ISR) (87). Similar to PERK signalling, the main event of the ISR pathways is the phosphorylation of the eIF2α, which consequently leads to a global translation shutdown while allowing the selective expression of ATF4 and its downstream targets. This aids in the cell survival and recovery. However, in case of chronic ER stress, CHOP expression is upregulated resulting in the activation of downstream cell death signalling pathways.

The phosphorylation of eIF2α is carried out by four kinases including PERK, heme-regulated eIF2α kinase (HRI), general control non-derepressible 2 (GCN2), and double-stranded RNA-dependent protein kinase (PKR) (87). Each eIF2α kinase responds to different environmental and physiological stresses. PERK as mentioned before is activated due to the dissociation of chaperone BiP resulting from the accumulation of unfolded proteins in the ER lumen (75). GCN2 is a highly conserved protein kinase from yeast to humans (88). It is usually activated in the case of amino acid deprivation by the binding of deacylated transfer RNAs (tRNAs) via a histidyl-tRNA synthetase-related domain (88). The binding of uncharged tRNAs will activate the protein kinase domain which will subsequently phosphorylate eIF2α. GCN2 can also be activated by UV light although the mechanism of activation is still unknown (89).

PKR is mainly activated by the presence of double-stranded RNA (dsRNA) from viral infections (90). After dsRNA is detected by PKR, PKR dimerizes and autophosphorylates, leading to the subsequent activation of the kinase. Activated PKR plays a major role in the antiviral response by phosphorylating eIF2α leading to the global

shutdown of translation (90). As viruses depend on the cell's translation machinery, the PKR-mediated block of translation impairs viral replication and spread (90). However, other stressors like oxidative stress, bacterial infections and stress granules have been found to activate PKR in a dsRNA-independent manner. Unlike the other kinases that are expressed in various different tissues, HRI is mainly expressed in erythroid cells and it is involved in erythrocyte differentiation (91). It is activated by various stressors such as arsenite-induced oxidative stress, heat shock, osmotic stress, and nitric oxide. Recent studies have shown that HRI is also activated by mitochondrial dysbiosis. Mitochondrial dysfunction is prevalent in many different neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease; cardiovascular diseases such as atherosclerosis and autoimmune diseases such as multiple sclerosis, and systemic lupus erythematosus (92–95). It is known that mitochondrial dysfunction activates the ISR, leading to the activation of the eIF2α-ATF4 pathway. This happens through the cleavage of DELE1, a protein associated with the mitochondrial inner membrane by OMA1, a mitochondrial stress-activated protease. This leads to the accumulation of DELE1 in the cytosol where it interacts with HRI and activates its eIF2α kinase activity (96, 97).

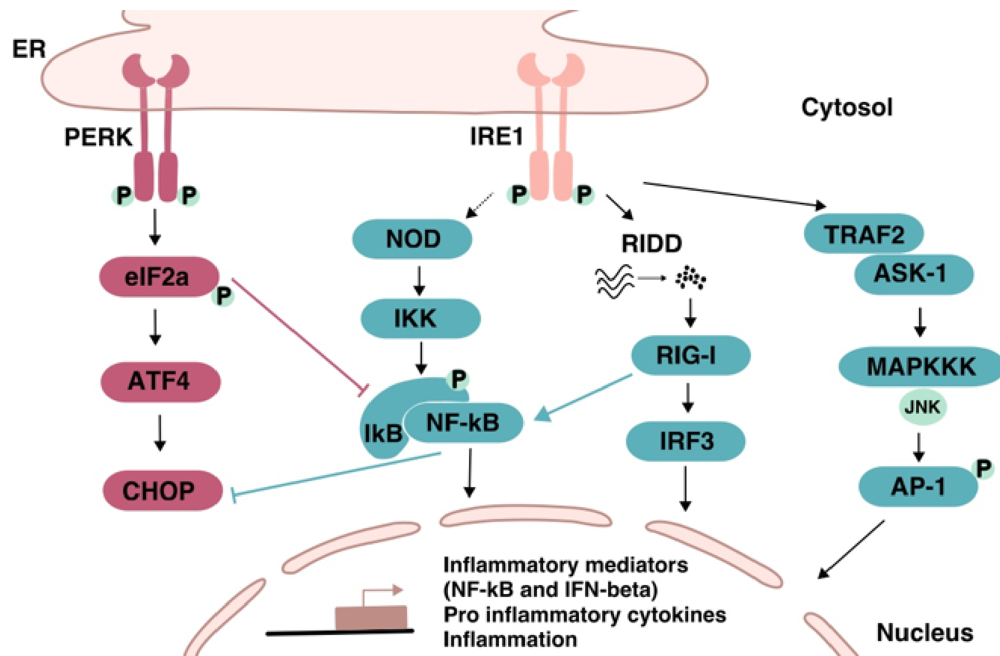

Figure 2. Stress Responses in Mammalian Cells

In the presence of unfolded proteins in the ER lumen, the chaperone BiP leaves the ER-resident sensors leading to their activation. ER resident sensor, ATF6, travels to the Golgi where it gets cleaved to the active TF, ATF6p50. This TF induces the expression of chaperones that help with protein folding. IRE1 is an ER resident sensor which will dimerize and phosphorylate once activated thus activating its endonuclease activity. Activated IRE1 splices XBP1 mRNA in the cytoplasm to produce the mature TF, sXBP1, which induces genes to help with protein folding and degradation. IRE1 also has nonspecific endonuclease activity, RIDD, that degrades mRNA in the cytoplasm to prevent its translation. PERK (ER-resident sensor), GCN2, PKR and HRI (cytoplasmic sensors) are activated by ER stress, amino acid deprivation. viral infection, ER stress, or oxidative stress, Once activated, they converge to phosphorylate eIF2α. Phosphorylated eIF2α leads to global attenuation of cap-dependent translation while preferentially translating the transcription factor, ATF4. ATF4 induces the expression genes involved in cellular adaptation such as oxidative stress resistance and protein folding. Once the stress elicitor is removed, ATF4 induces the expression of GADD34 which in concert with PP1 is responsible for dephosphorylating p-eIF2α. In the case of chronic ER stress, ATF4 induces the expression of genes involved in apoptosis.

1.7 Stress Responses and Inflammation

The UPR and ISR are also known to induce an inflammatory response thus playing an important role in the pathogenesis of inflammatory diseases. Recent evidence suggests that there are signalling pathways in other organelles, including the mitochondria and nucleus, that connect the stress responses and inflammation through various mechanisms (98). A few of which will be discussed below and are depicted in Figure 2.

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is an important transcriptional regulator during the onset of an inflammatory response (99). In the absence of an inflammatory stimulus, it is kept inactive through binding to an inhibitor IκB, which is constitutively expressed. To activate NF-κB, IκB is phosphorylated and degraded, allowing NF-κB to translocate to the nucleus where it induces the transcription of many inflammatory genes (99). An increase in ER protein folding load and accumulation of unfolded proteins has been shown to activate NF-κB, however, the exact mechanism of activation is still unclear (100). One possible explanation could be due to the PERK-eIF2α mediated translation shutdown. Because the half-life of IκB is much shorter than that of NF-κB, attenuating translation could lead to an increased ratio of NF-κB to IκB which will leave more NF-κB free to translocate to the nucleus and activate inflammatory signalling (100). The activation of NF-κB has been proven to be important in protecting cells against ER stress-induced cell death which is orchestrated by CHOP. NF-κB supresses CHOP promoter activity thus repressing pro-apoptotic pathways and increasing cell survival during ER stress (101).

Another important connection between UPR and inflammation is the IRE1α-mediated NF-κB and Jun-NH(2)-terminal Kinase (JNK) activation. In the presence of ER

stress, IRE1α oligomerizes and autophosphorylates, which causes a conformational change to its cytoplasmic domain (100). This allows it to bind TNF receptor-associated factor 2 (TRAF2). The IRE1α-TRAF2 complex can recruit an IκB kinase (IKK), which phosphorylates IκB leading to the degradation of IκB and the translocation of activated NF-κB to the nucleus (100). The IRE1α-TRAF2 complex also recruits the protein kinase JNK, an important regulator of inflammation. Activated JNK phosphorylates the transcription factor activator protein 1 , which then translocates to the nucleus and induces the expression of genes involved in inflammatory signalling (102). RIDD activity of IRE1α can also activate innate immune signalling by generating mRNA fragments in the cytosol. These fragments can be recognised by innate immune receptor retinoic acid-inducible protein I (RIG-I) to activate NF-κB and interferon-regulatory factor 3 (IRF3), which induces the expression of many pro-inflammatory cytokines and interferon-β (IFNβ) (103). Research has also shown that IRE1 is involved in the activation of the nucleotide-binding oligomerization domain-like receptors (NOD1/2) inflammatory pathway (104). The exact mechanism NOD1/2 activation is not entirely clear; however, it is suggested that TRAF2 could be involved in this activation. Once NOD1/2 is activated, the inhibitor of NF-κB, IκB, is removed through phosphorylation by IKK and proteasomal degradation (105). Free NF-κB will then activate proinflammatory mediators and initiate inflammation.

The calcium concentration in the ER lumen is crucial to normal protein folding. An accumulation of unfolded proteins in the ER can cause calcium to leak from the ER. The leaked calcium ions will accumulate in the matrix of the mitochondria leading to the depolarization of the inner membrane, disrupting electron transport and increasing ROS

production. ROS are partially reduced metabolites of oxygen that play an important role as mediators of inflammation (106).

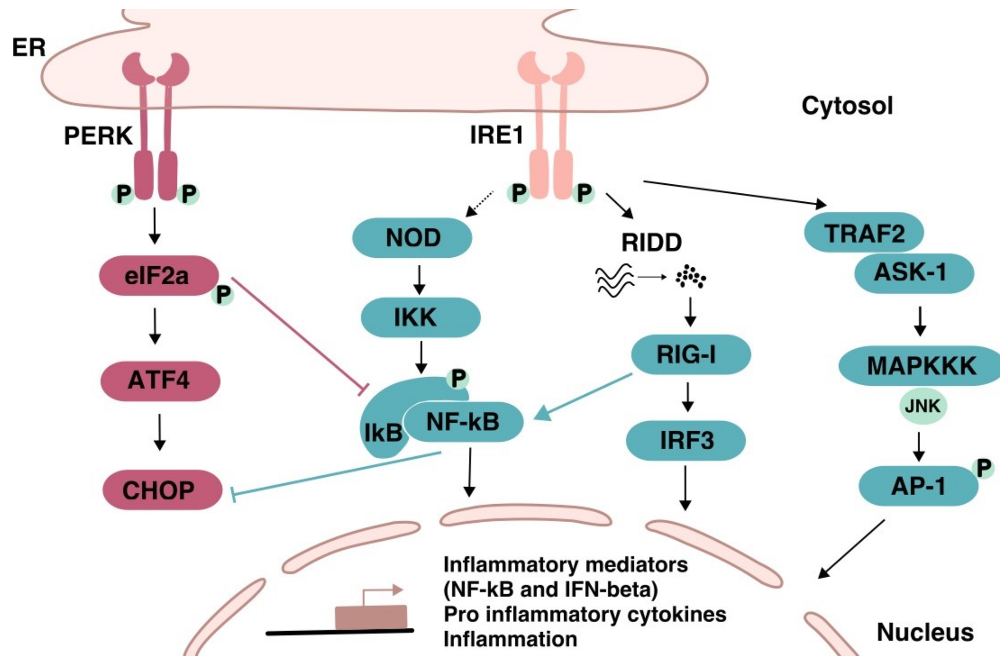

Figure 3. ER Stress Response and the Immune Response

There are many ways that the ER stress responses lead to the activation of inflammatory responses. The PERK-eIF2α-ATF4 branch contributes to the activation of NF-κB through inhibiting the production of the short-lived inhibitor of NF- κB, IκB, by halting global translation. NF-κB then induces the expression of proinflammatory cytokines and represses the expression of CHOP to prevent apoptosis. Activated IRE1 recruits TRAF2 which recruits ASK-1. The TRAF2-ASK-1 complex will activate the MAPK kinase, JNK. Activated JNK will phosphorylate the transcription factor, AP-1, which will induce the expression of genes involved in inflammatory signalling. In addition to that, mRNA cleaved by IRE1 through RIDD will activate RIG-I which will increase the activation of NF-κB and induce the expression of IRF3. These together will lead to the expression of proinflammatory cytokines. IRE1 is also involved in the activation of NOD1/2 pathway which will phosphorylate IκB through IKK leading to the activation of NF-κB.

1.7.1 ER Stress Responses and Airway Inflammatory Diseases

These cross talks mentioned above suggest that the ER stress response could play a role in inflammatory diseases. Chronic inflammation is a hallmark of many airway disease like CF pulmonary disease, chronic obstructive pulmonary disease (COPD), and asthma (107). This begs the question whether ER stress responses play a role in regulating the inflammation in these conditions. CF is a multi-organ disease that is caused by a mutation in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene (108). Over 1600 mutations of the *CFTR* gene have been described, however, the most common form is a deletion of the phenylalanine at codon 508, known as F508del (109). The primary function of the CFTR protein is to regulate liquid volume on epithelial surfaces through chloride secretion. F508del prevents Cl^- ions from flowing into the periciliary layer resulting in decreased mucus clearance, and accumulation of thickened mucus in airway lumens (109). This is usually combined with recurrent infections and inflammation with persistently high levels of pro-inflammatory cytokines being expressed. Studies have shown that the UPR is functionally relevant in CF airway disease (110). Increased levels of IRE1α activation and XBP1 splicing were seen in native infected/inflamed CF human bronchial epithelia coupled with an increase in pro-inflammatory cytokine production. It is important to mention that UPR activation is not directly related to the CFTR mutation; instead it is the airway epithelial inflammation resulting from the recurrent infections that is responsible for the activation of the ER stress mediated by the XBP1 splicing (110).

1.7.2 ER Stress Response and Gut Inflammatory Diseases

Intestinal homeostasis depends on appropriate function of intestinal epithelial cells (IECs). Dysregulation of intestinal cells leads to the development of various

gastrointestinal disorders such as inflammatory bowel disease , Crohn's disease and ulcerative colitis (111). Although the clinical manifestations of these diseases are well-characterized, the causes that lead to the onset of these diseases are still largely unknown (112). Recent studies have shown that ER stress and UPR are critical factors associated with the susceptibility to inflammatory bowel disease (113). The complex nature of the gut microbiome could pose a challenge to the capacity of protein folding in the ER of intestinal epithelial cells which could result in ER stress, UPR activation and the activation of downstream inflammatory signalling. Indeed, Park *et al.* reported an increase in CHOP expression, NF-κB activation, and production of pro-inflammatory cytokines in intestinal epithelia from patients with inflammatory bowel diseases (114). CHOP has also been shown to promote infiltration of macrophages, induction of ROS production and enhanced apoptosis of epithelial cells thus leading to the development of colitis (115). The functional role of ATF6 in inflammatory bowel disease pathogenesis is seen in $Atf6\alpha^{-/-}$ mice which suffered from reduced levels of chaperone expression, increased inflammatory cell infiltration and more severe mucosal damage. This highlights the importance of ATF6 in maintaining normal intestinal barrier function (116) while also showing that arms of the UPR are detrimental while others are beneficial for gut health.

1.8 Role of Stress Responses in Disease Pathogenesis

The UPR and ISR are an important host response to many stimuli including bacterial and viral infections. This importance is highlighted in the implications of UPR/ISR in different infectious diseases such as *Legionella*, hepatitis C virus (HCV) infection, *Brucella* spp. and influenza infection (117–120). In the next few sections, I will focus on the role that the stress responses play during microbial infections.

1.8.1 Stress Responses and Viral Infections

Viruses are known to depend on the intracellular machinery of a mammalian host cell for replication. This is why it is not surprising that viral infections could lead to ER stress or in some cases manipulate the stress responses for their own benefit. This interplay between viruses and the stress responses is very complex and not well understood. However, there are some known examples where the stress response helps launch an antiviral host defense response against viral infections.

The PERK-eIF2α arm of UPR plays a role in antiviral host responses due to its homology with PKR. The activation of PERK leads to the phosphorylation of eIF2α and the consequential protein translation shutdown, which limits viral replication and spread. This was confirmed by Baltzis *et al.*, when they showed that fibroblasts deficient in PERK had much higher levels of vesicular stomatitis virus replication than wild-type fibroblasts (121). Other studies have also shown that blocking phosphorylation of eIF2α increased vesicular stomatitis virus replication, confirming that PERK has antiviral properties through mediating translational blockade (122). Another important example is the Japanese encephalitis virus which induces UPR activation due to the accumulation of viral

proteins during viral replication. This inevitably leads to the increased transcription of CHOP and increased apoptotic cell death (123).

However, viruses have found ways to either prevent UPR activation or to manipulate the UPR pathways for their own benefit. For example, envelope proteins E1 and E2 of Hepatitis C Virus (HCV) have been shown to bind the cytosolic kinase domain of PERK thus inhibiting its kinase activity and therefore dampening the UPR (118). In addition, influenza virus has been shown to differentially activate the UPR by only activating IRE1 with little to no ATF6 and PERK activation. Chemically inhibiting IRE1 activation during influenza infection decreased viral replication, suggesting that one or more downstream effects of IRE1 is required for influenza replication (120). Kaposi's sarcoma-associated herpesvirus has also been shown to activate all three arms of the UPR but restrict the downstream transcriptional responses. Johnston *et al.* reported that Kaposi's sarcoma-associated herpesvirus was able to manipulate the UPR to reactivate from latency and support lytic replication (124). This raises the question whether inhibiting ER stress activation could be a potential alternative therapy to treat viral infections that are known to manipulate the UPR.

1.8.2 Stress Responses and Bacterial Infections

Much like many viruses, bacterial infections are known to induce ER stress and elicit UPR activation, although the mechanism of bacterial interaction with the ER is still mostly unknown (125). One way that bacteria interact with the ER is shown in intracellular bacteria which take the ER as a safe haven to ensure their intracellular survival and proliferation. Intracellular bacteria like *Legionella pneumophila* and *Brucella spp.* survive intracellularly in vacuoles after its phagocytic uptake by the host cell (126, 127). This is

followed by the secretion of type four secretion system (T4SS) effectors which modulate the endocytic system to help the bacteria- containing vacuoles to fuse to the ER, transforming it into an ER-derived replicative organelle (117, 128). In addition, *Brucella melitensis* infection has been found to activate the UPR in macrophages and epithelial cells as evidenced by increased levels of *BiP*, *CHOP*, and spliced *XBP1*. Not only does infection elicit UPR activation but it was shown that UPR is required for *B. melitensis* reproduction (119). Inhibiting UPR activation using a chemical chaperone tauroursodeoxycholic acid (TUDCA), by an unknown mechanism, reduced bacterial intracellular replication thus showcasing the ability of bacteria to modulate the UPR for its proliferation. Other bacteria are capable of targeting chaperones of the ER directly. For example, AB_5 toxins are produced by pathogenic bacteria and are made of an enzymatic subunit A that disrupts host cell function and pentameric B subunits that allow uptake into the target cell (129). Paton *et al.* (130) showed that a certain family of AB_5 toxins called subtilase cytotoxin, produced by specific Shiga toxigenic strains of *Escherichia coli*, has site-specific endonuclease activity that cleaves the mRNA of the ER chaperone BiP. This leads to chronic UPR activation in the host cell and eventual cell death, suggesting that targeting BiP could be a previously unknown trigger of cell death (130).

On the other hand, UPR activation could play an important role in protecting the host against bacterial infections. *Listeria monocytogenes* is a facultative intracellular pathogenic bacterium that secretes many virulence factors including listeriolysin O , which is a pore-forming toxin required for cell-to-cell spread (131). Pillich *et al.* showed that listeriolysin O activates all three branches of UPR evidenced by the phosphorylation of eIF2α, splicing of *XBP1* mRNA, and the depletion of the inactive form of ATF6. Chemical

activation of UPR using thapsigargin (Tg), which causes ER stress by depleting calcium stores in the ER, reduced the number of intracellular bacteria (132). Another example is *Mycobacterium tuberculosis,* the causative agent of tuberculosis, which induces ER stress in host cells by disrupting intracellular calcium homeostasis (133). *M. tuberculosis* infection is also known to lead to an overproduction of pro-inflammatory cytokines which induces UPR due to the accumulation of misfolded or unfolded TNF in the ER. ER stress-induced apoptosis has been found to supress the intracellular growth of *M. tuberculosis* (134). This suggests that the regulatory mechanism of ER stress could be a new therapeutic target for multidrug-resistant Mtb.

1.8.3 Stress Responses and *P. aeruginosa*

Previous research has shown multiple purified effectors of *P. aeruginosa* eliciting the stress responses. Van't Wout *et al.* showed that cell-free conditioned medium (CM) of the PAO1 strain induced UPR and ISR in human bronchial epithelial cells (16HBE) as measured by the levels of *Xbp1* mRNA splicing, and the induction of *GADD34*, *CHOP*, and *BiP* expression (135). Using various purified virulence factors, they identified AprA and pyocyanin, a secondary metabolite produced by *P. aeruginosa*, as ER stress inducers. They also showed that increased *GADD34* expression was partially due to the activation of ISR's HRI kinase. *GADD34* seems to have a protective role against *P. aeruginosa*-induced iron-sensitive cytotoxicity (135).

In addition to that, the QS molecule C12-HSL activates PERK, evidenced by the increase in the levels of phosphorylated eIF2α and inhibited protein synthesis (136). It also modulates the immune response by increasing the activation of NF-κB while decreasing the levels of secreted IL-6 and CXCL1 in mouse embryonic fibroblasts. This could be

explained by the activation of PERK, which inhibits protein translation thus inhibiting the synthesis of IκBα and leading to the activation of NF-κB. NF-κB then increases the transcription of KC and IL-6; however due to the halt in protein translation there is no increase in the production and secretion of KC and IL-6 (136). On the other hand, pre-treatment of RAW264.7 mouse macrophage cells with C12-HSL activated the UPR as evidenced by the increase in the expression of CHOP (137). The expression of CHOP has been found to be inversely correlated with LPS-mediated NF-κB activation (137). This suggests that C12-HSL attenuates LPS-induced inflammation by inhibiting NF-κB activation and pro-inflammatory cytokine secretion via UPR activation (137).

Paraoxonase 2 (PON2) belongs to a highly conserved family of calcium-dependent esterases which is expressed on a wide range of mammalian tissues (138). It was shown that PON2 inactivates C12-HSL and attenuates *P. aeruginosa* virulence. It also has antioxidative properties that protect the cells from pyocyanin-induced oxidative stress. PON2 mRNA and protein degradation increases with increased intracellular calcium levels. C12-HSL uses ER stress to increase calcium influx into the cell leading to the degradation of PON2 (139). This allows *P. aeruginosa* to evade the host response by modulating the ER stress response.

1.8.4 Stress Response and Chemical Manipulation

Given the different roles that the stress response plays in the cell, finding chemicals that can either inhibit or activate the UPR/ISR can be useful to aid treatment in hard-to-treat conditions. There are a few characterized drugs that modulate the stress response and are currently being studied for their therapeutic potential in different diseases. For example, ER stress has been implicated in the pathogenesis of many different diseases like chronic

metabolic conditions or even cancer. Research has shown that reducing ER stress by administering chemical chaperones alleviated symptoms in animal models of atherosclerosis and type two diabetes (140, 141). Currently, there are two chemical chaperones, 4-phenylbutyric acid (PBA) and TUDCA, that are approved by the US Food and Drug Administration (FDA) for use in humans. PBA's chaperone activity was first discovered when investigating its effect on the trafficking of the CFTR protein. CF patients with class II mutations in their CFTR suffer from impairment of CFTR folding and processing to the Golgi. This leads to the accumulation of CFTR in the ER which triggers their degradation. Administering PBA can stabilize protein structure and allow the CFTR to escape degradation and be transported to the cell membrane (142).

Targeting the UPR sensors has been also studied as a potential therapeutic target for viral infections. For example, Influenza A virus has been found to activate IRE1 and the administration of TUDCA lead to decreased viral replication. This suggests that IRE1 is a potential therapeutic target that is unlikely to be invalidated by the frequent viral mutations (120). Another example is the use of TUDCA to protect cells against paraquat-induced cell death. Paraquat is a commonly used herbicide that is highly toxic to humans and is known to cause severe lung damage. One of the causative factors of the extreme cell damage is ER stress-induced cell death. Treatment with TUDCA has been found to rescue cells from death suggesting that chemical chaperones like TUDCA can be used for the treatment of paraquat poisoning (143). Further studies are needed to find other therapeutic potentials of UPR activators/inhibitors.

1.9 Rationale

P. aeruginosa is a very important opportunistic pathogen that is equipped with many virulence factors that are controlled by the quorum sensing systems, that are delivered to the host cell through the multiple protein secretion systems. These virulence factors play an important role in *P. aeruginosa* pathogenesis and they have the ability to modulate host responses. One of the vital host responses to a *P. aeruginosa* infection in a mammalian cell is the ER stress response, specifically the UPR and ISR. Previous research has shown that specific purified virulence factors of *P. aeruginosa* can activate the UPR. However, the extent of ER stress response activation in response to the whole bacteria remains unclear. In this project, we aim to better understand the effect that *P. aeruginosa* has on UPR/ISR activation and determine whether certain virulence factors are important for the activation of such response. We hypothesize that *P. aeruginosa* will activate the UPR and ISR using virulence factors whose secretion is controlled by the quorum sensing system.

CHAPTER 2. MATERIAL AND METHODS

2.1 Cell Culture and Bacterial Culture

A549 cells, adenocarcinomic human alveolar basal epithelial cells, (ATCC® CCL-185™, a kind gift from Dr. Craig McCormick, Dalhousie University) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 Units/mL penicillin, and 100 μg/mL streptomycin. Cells were maintained at 37°C in 5% atmospheric CO_2. The cultures were kept until passage 10-13 where they were discarded, and a new culture was started from an aliquot from the nitrogen tank.

For bacterial culture, two wild-type strains of *Pseudomonas aeruginosa*, PA14 and PAO1 were used in my work. *P. aeruginosa* PA14 is a primary clinical isolate from a burn patient (144). Wild type (WT) *P. aeruginosa* PA14 and various mutants (Table 1) were grown overnight in lysogeny broth (LB) media for 16 hours at 37 °C and shaking at 200 rpm to reach the early stationary phase (an optical density (OD) between 2.5 and 3.0 at 600 nm). PAO1 is a primary clinical isolate from a wound and was grown in a similar fashion to PA14 (145). *P. aeruginosa* PA14 Δ*aprA* and Δ*lasB* mutants were gifted to the lab by Dr. Michal Koska (Institut für Molekulare Bakteriologie, Germany).

Table 1. List of *P. aeruginosa* PA14 Mutants Used in This Study

Bacterial Strain	Gene Description/ Function	Reference
P. aeruginosa* PA14 Δ*lasR	Transcriptional regulator (QS)	(146)
P. aeruginosa* PA14 Δ*rhlR	Transcriptional regulator (QS)	(146)
P. aeruginosa* PA14 Δ*mvfR	Transcriptional regulator (QS)	(147)
P. aeruginosa* PA14 Δ*lasR/rhlR	Transcriptional regulators (QS)	Unpublished, from Dr.Frederick Ausubel
P. aeruginosa* PA14 Δ*lasR/mvfR	Transcriptional regulators (QS)	Same as above
P. aeruginosa* PA14 Δ*rhlR/mvfR	Transcriptional regulators (QS)	Same as above
P. aeruginosa* PA14 Δ*lasR/rhlR/mvfR	Transcriptional regulators (QS)	Same as above
***P. aeruginosa* PA14 *aprA*::Tn**	Alkaline protease	(148)
P. aeruginosa* PA14 Δ*aprA	Alkaline protease	(60)
***P. aeruginosa* PA14 *lasB*::Tn**	Elastase	(148)
P. aeruginosa* PA14 Δ*lasB	Elastase	(60)
***P. aeruginosa* PA14 *aprD*::Tn**	T1SS	(143)
***P. aeruginosa* PA14 *aprF*::Tn**	T1SS	(143)
***P. aeruginosa* PA14 *aprI*::Tn**	AprA protease inhibitor	(143)

2.2 Confirmation of *P. aeruginosa* Mutants

An overnight culture was prepared for all the quorum sensing and protease mutants. On the following day, DNA was extracted using the Wizard genomic DNA extraction kit (Promega, Cat. A1120) following the manufacturer's instructions. The primers used for the polymerase chain reaction (PCR) reactions were designed using https://www.yeastgenome.org/primer3 to span a section of or the whole ORF of each targeted gene (Table 2). The diagnostic PCR using WT bacteria DNA as the template will amplify the gene products, whereas the deletion or transposon mutants should not generate any amplification products. PCR primers were re-suspended in nuclease-free water (Invitrogen, Cat. No. 10977) to a concentration of 10 µM (stock solution) and then diluted to the working solution concentration of 10 µM. A PCR reaction typically contained 10 µL of GoTaq Green Master Mix 2x, 1 µL of the stock solution of both forward and reverse primer, 200 ng of DNA template, and water to make up 20 µL. The PCR program was as follows: initial denaturation at 94 °C for 5 min, 30 cycles of [94 °C for 15 sec, 55 to 62 °C (depending on primer's Tm) for 30 sec and 72°C for 1 minute per kb] and final extension at 72 °C for 10 min. Five µL of the PCR amplicons were run on a 1% agarose gel prepared as follows: 0.5 g of agarose was dissolved in 50 ml of 1xTris-Acetate-Ethylenediaminetetraacetic acid (EDTA) solution (TAE, 40 mM Tris acetate and 1 mM EDTA) mixed with 5 µL RedSafe™ Nucleic Acid Staining Solution (20,000x) before being poured into the cast with combs in place. The gel was subjected to electrophoresis at 120V for 20-30 min before being visualized using the Chemidoc Imaging system (Bio-Rad, Cat. 17001401).

Table 2. Primers Used to Confirm PA14 QS and Protease mutants

Gene Name	Forward Primer 5'→ 3'	Reverse Primer 5'→ 3'	Expected Size for WT (bp)
lasR	ATGGCCTTGGTTGACGG	TCAGAGAGTAATAAGACCC	720
rhlR	CTATGGCGTGCGCCAC	CGTCGGCGGTCCATTGC	497
mvfR	CGCAGCCTGCTGAAC	CTCGGATTCCAGCGCG	285
aprA	CCTATACCGTAGACCAGGCG	GTTGTAGTCGCCGGGGTG	488
lasB	GGGGACTTATCAGCCAACCT	CATCACCGTCGACATGAACG	488

2.3 A549 Infection by *P. aeruginosa*

A549 cells were seeded at 450,000 cells per well in Flat bottom tissue culture-treated 12 well plates (Falcon®, Cat. 353043). The cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and were maintained overnight at 37°C in 5% atmospheric CO_2. Three mls of LB media were inoculated with a bacterial colony picked from a freshly streaked LB plate to start an overnight culture. Tubes were left in a 37°C incubator shaking at 200 rpm for 16 h. On the following day, a subculture (1:50 dilution) was started by transferring 60 µL of overnight culture to 3 mLs of fresh LB. The subculture was left in the incubator for 1.5-2h until OD600 reached 0.6, measured using NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer (Thermo fisher, Cat. ND-ONE-W). The bacteria were pelleted and washed twice with sterile 1x Phosphate Buffered Saline (PBS, 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 2 mM KH_2PO_4) and then resuspended in 1x PBS before the OD600 was measured again. A549 cells were infected at a multiplicity of infection (MOI) of 10. The conversion from OD600 reading to MOI was done using the following equation: Number of cells/well * MOI / ($8x10^8$) * OD600

For the positive control, cells were treated with 75 nM thapsigargin (Tg, Sigma, Cat. #T9033 & #586005) or left untreated for the negative control. The treated A549 cells were incubated in a 37 ˚C incubator at 5% CO_2 for various different time points, including 15 minutes (m), 45m, 1 hour (h), and 1.5h. After the incubation, cells were washed twice with 1x PBS and lysed either for protein or RNA extraction as described below.

2.4 A549 Treatment with Purified LasB

Purified LasB was ordered from Elastin products (Cat. No. PE961). The purified protein was lyophilized in a suspension of 0.6 M ammonium sulfate, 2 mM sodium acetate,

0.04 mM calcium chloride and 0.01 mM zinc chloride pH 7.5 by the supplier. The stock solution of the protein was aliquoted and saved in the -80°C freezer until needed. To prepare for the treatment, A549 cells were seeded at 450,000 cells per well in 12 well plates as previously mentioned. The cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 100 Units/mL penicillin, and 100 µg/mL streptomycin. The cells were maintained in a 37 ˚C incubator at 5% CO_2 overnight. On the following day, different concentrations of purified LasB (50 ng/mL, 1 µg/mL, 50 µg/mL) were added to each well. The cells were left in the 5% CO2 incubator for various time points, including 15m, 45m and 1.5h. After the treatment, the media was discarded, and the cells were washed twice with 1xPBS. This was followed by protein extraction as detailed below.

2.5 Immunoblotting

After infection and PBS wash, cells were lysed in 2x Laemmli buffer (120mM Tris-HCl pH 6.8, 20% glycerol, 4% Sodium Dodecyl Sulfate). Three hundred µL were used per well in a 12-well plate. The plates were incubated on a belly dancer shaker for 3-5 minutes before being incubated on ice at a 45º angle for 3-5 minutes. Cell lysates were passed through a 21-gauge needle for 7 times and transferred to a 1.5 ml microcentrifuge tube. Protein concentration was quantified using the Pierce Bicinchoninic acid assay (Thermo Fisher, Cat. 23225). (Note: protein samples were diluted 1 in 10 for the quantification). Protein concentration of each unknown sample was calculated using the Bicinchoninic acid standard curve as a reference. The volume needed was transferred to a new tube, 150 mM dithiothreitol and 2.5% bromophenol blue were added, and the samples were boiled for 5 min. Between 6 to 10 µg (depending on the target protein) of equal amount of protein was loaded per sample in a 6-10% polyacrylamide gel against 4 µl of protein ladder (NEB, Cat.

No. P7712S) and resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The gels were equilibrated using 1x Transfer buffer (25 mM Tris, 192 mM glycine) with 20% methanol before proteins were transferred to a polyvinylidene fluoride membrane (Bio-Rad, Cat. No. 1704 156) using the Trans-Blot® Turbo™ Transfer System (Bio-Rad, Cat. No. 1704150). The membrane was blocked for two hours using 5% skimmed milk diluted in Tris-buffered-saline (TBS) supplemented with 0.1% Tween 20 (Fisher) (TBST, 20 mM Tris, 150 mM NaCl, and 0.1% Tween20). Then, it was incubated overnight in the primary antibody diluted in 5% bovine serum albumin (Sigma, A9418) in TBST in the cold room or fridge at 4ºC. On the following day, the membrane was washed 3 times with 1x TBST followed by an hour incubation in the secondary antibody diluted in 5% Bovine serum albumin in TBST at room temperature. After the membrane was washed with 1x TBST for three times, it was exposed to enhanced chemiluminescence substrate (Bio-Rad, Cat. 1705061) and imaged using the chemiluminescence setting on the ChemiDoc (Bio-Rad, Cat. 17001401). Table 3 lists all the antibodies used for immunoblotting in this thesis.

2.6 Densitometry Analysis on Western Blots

After the immunoblotting experiments, I used the ChemiDoc (BioRad) to image my blots using the chemiluminescence setting to visualize the proteins and the colorimetric setting to visualize the protein ladder to ensure that the proteins are of the right size. I exported the images in the scn format, the only format compatible with the ImageLab software. Once I opened the images on the ImageLab software, I used the lanes and bands icon to identify the bands and lanes on the blot containing my protein of interest (p-eIF2α) and loading control. After identifying and labelling the bands and lanes on the blots, the adjusted band volume (total band volume - background noise) for each sample loaded was

obtained using the analysis table. The analysis table was exported into an excel file. To get the normalization factor of the loading control, the adjusted band volume of each band of the loading control was divided by the reference band, a band of the loading control that was randomly chosen. Then, the band volume of p-eIF2α signals was normalized to the loading control: Adjusted band volume of sample × normalization factor. To normalize the treated samples to untreated control, the normalized band volume of the treated samples was divided by the normalized band volume of untreated samples. This was done for every repeat and was graphed using the Prism 6.0 software. Error bars stand for the standard error mean (SEM).

Table 3. List of Antibodies Used in This Study for Immunoblotting

Antibody	Dilution	Company	Catalog Number
Rabbit p-eIF2α	1:1000	Santa Cruz	ab32157
Rabbit eIF2α	1:1000	Cell signalling	CST 5324
Rabbit PERK	1:1000	Cell signalling	CST 5683
Mouse β-actin	1:1000	Cell signalling	CST49675
Mouse GAPDH	1:2000	Cell signalling	CST5174T
Anti-mouse IgG HRP-linked	1:2500	Cell signalling	CST7076S
Anti-rabbit IgG HRP-linked	1:3000	Cell signalling	CST7074

2.7 RNA Extraction and RT-qPCR

After infection and PBS wash, cell lysis buffer from the RNeasy Plus Kit (Qiagen, Cat. 74134) supplemented with 1% β-mercaptoethanol (Sigma, Cat. M6250) was added directly to the wells of cell culture plates. RNA was then extracted using the RNeasy Plus Kit (Qiagen, Cat. 74134) following the manufacturer's instructions. DNA was removed from all of the RNA samples using the DNA-free kit (Invitrogen, Cat. AM1906), and 1μg of RNA was used to set up the reverse transcription reactions. The reactions were performed using an iScript cDNA synthesis kit (Bio-Rad, Cat. 1708891) and the reaction program was as follows: 25°C for 5 min, 46°C for 20 min followed by 95°C enzymatic inactivation for 1 min. Complementary DNA (cDNA) concentrations were measured using a Nano-drop instrument (Thermo Scientific, Cat. ND-ONEC-W). Before the Real time-qualitative PCR (RT-qPCR) was set up, the system was validated by looking at primer efficiencies and the melting curve of each primer pair. The primer efficiency was tested using A549 cDNA as a template, where it was serially diluted from 10^0 - 10^4. The qPCR reaction contained 10 μL of Wisent Advanced qPCR 1 step master mix with SUPERGREEN dye (Wisent Technologies, Cat. 801-002-LR), 5 μL of forward and reverse primers (1.2 μM working stock) and 2 μL of cDNA template (1 μg/μL). PCR reactions were performed as follows: 95°C for 3 min, 40 cycles [95°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec], melt-curve 65°C to 95°C increment 0.5°C for 5 sec. primer efficiency was calculated using this formula:

Amplification efficiency=[10(-1/m)] – 1 where m is the slope of the trend line

The RT-qPCR reaction contained 10 μL of Wisent Advanced qPCR 1 step master mix with SUPERGREEN dye (Wisent Technologies, Cat. 801-002-LR), 5 μL of forward and reverse

primers (1.2 μM working stock) and 2 μL of cDNA template (1 μg/μL). PCR reactions were performed as follows: 95°C for 3 min, 40 cycles [95°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec], melt-curve 65°C to 95°C increment 0.5°C for 5 sec. RT-qPCR reactions were preformed using CFX96 real-time PCR machine (Bio-Rad, Cat. 1855201). Fold change was calculated using the ΔΔCt method and gene expression values were normalized to β-actin. Primers used in this study can be found in Table 4.

Table 4. Primers Used for RT-qPCR

Gene Name	Primer Sequence 5’→ 3’	Melting Temp. (°C)	References
GADD34-F	ATGTATGGTGAGCGAGAGGC	62	(149)
GADD34-R	GCAGTGTCCTTATCAGAAGGC	62	
CHOP-F	CAGAACCAGCAGAGGTCACA	62	(150)
CHOP-R	AGCTGTGCCACTTTCCTTTC	62	
BiP-F	CGAGGAGGAGGACAAGAACT	62	(151)
BiP-R	CACCTTGAACGGCAAGAACT	62	
sXBP1-F	TGCTGAGTCCGCAGCAGGTG	62	(150)
sXBP1-R	GCTGGCAGGCTCTGGGGAAG	62	
β-actin-F	TCCCTGGAGAAGAGCTACGA	62	(152)
β-actin-R	AGCACTGTGTTGGCGTACAG	62	

CHAPTER 3. RESULTS

3.1 Wild-type *P. aeruginosa* Induces the UPR and ISR

Previous research has shown that secreted virulence factors of *P. aeruginosa* can induce stress responses in mammalian cells by inducing *Xbp1* splicing, and increased *GADD34*, *BiP*, and *CHOP* expression (135). To confirm the activation of such responses, we used an *in vitro* infection model to infect A549 cells with two different wild-type (WT) *P. aeruginosa* strains, PA14 and PAO1. PA14 is markedly more virulent compared to PAO1. This is due to the absence of certain pathogenicity islands in the PAO1 genome compared to PA14 (153).

During my preliminary stage of experimental design, we infected A549 cells with different MOIs (1, 10, 25, and 50) of PA14 and PAO1 for different timepoints up to 5h. After observing the percentage of cells alive/dead using a trypan blue assay caused by each MOI at different timepoints, we decided on using MOI of 10 for future experiments as it did not cause major cell death during the early timepoints thus allowing us to study the stress responses without large population of cell death at early timepoints (data not shown). Cells were monitored during the infection using an inverted light microscope. Once the cells were too stressed and started to die, the cells would lift off the plate and have a more rounded morphology. I decided to stop the infection before that started which was at 1.5h.

Using the established infection protocol, A549 cells were infected with MOI 10 of PA14 or PA01 for 15 min, 45 min, 1h and 1.5 h, or treated with Tg for 2h, or left untreated. Protein and RNA were extracted and used to detect the presence of activated stress response markers. PAO1-infected cells showed very low levels of phosphorylation of eIF2α relative to untreated A549 cells after normalization to β-actin (Figure 4A & B). On the other hand,

PA14 induced an increase in the phosphorylation of eIF2α with an almost 2-fold increase at earlier timepoints (15m, 45m) (Figure 4A & B). This indicates that different strains of *P. aeruginosa* might differentially induce the phosphorylation of eIF2α.

The phosphorylation of eIF2α is the result of the activity of one of four kinases: PERK, GCN2, HRI, or PKR. We probed the blots for total PERK to test whether PERK could contribute to the phosphorylation of eIF2α in PA14 or PAO1-infected cells. In the case of PERK activation, PERK will dimerize and autophosphorylate. This change can be visualized as a shift in the band on the blot. This was only seen in the positive control, Tg-treated cells, which suggests that PERK might not be the major kinase that is responsible for phosphorylating eIF2α following *P. aeruginosa* infection under the conditions tested (Figure 4A).

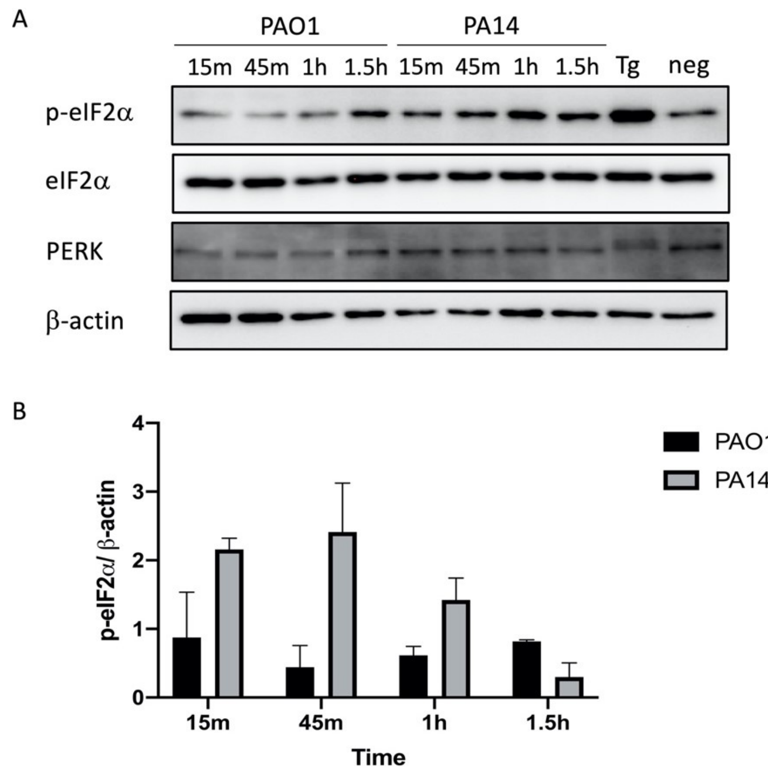

Figure 4**. Wild-type *P. aeruginosa* PA14, but Not PAO1 Induces Phosphorylation of eIF2α in A549 Cells.**

A549 cells treated with either a MOI 10 of PAO1 or PA14, thapsigargin (Tg) as a positive control or left untreated as a negative control. (A) Cell lysates were subjected to Western blot analysis for p-eIF2α, total eIF2α, total PERK and β-actin as a loading control. Blots are representative of three independent experiments. (B) Densitometry analysis of p-eIF2α was normalized to the loading control and untreated cells. Three independent experiments are graphed, and error bars denote standard error of the mean (SEM).

To further understand the effect of PAO1 and PA14 on the activation of the stress responses, we looked at gene induction levels of other stress response marker genes, including *GADD34, CHOP,* spliced *XBP1* and *BiP*. Similar to the previous results, PAO1-infected cells showed very low levels of *GADD34* and *CHOP* induction across the different timepoints of infection, while PA14 exhibited increased levels of *CHOP* and *GADD34* compared to the untreated control with the highest levels being 6-fold and 8-fold respectively at 1.5h (Figure 5A & B). PA14-infected cells also showed a 4-fold upregulation in the levels of *BiP* at 45min, which decreased at 1h and 1.5h (Figure 5D). On the other hand, PA14-infected cells show no upregulation of spliced *XBP1* throughout the course of the infection (Figure 5C). These results suggest that different strains of *P. aeruginosa* could have differential activation levels of ISR, with PA14 inducing a stronger level of ISR compared to PAO1.

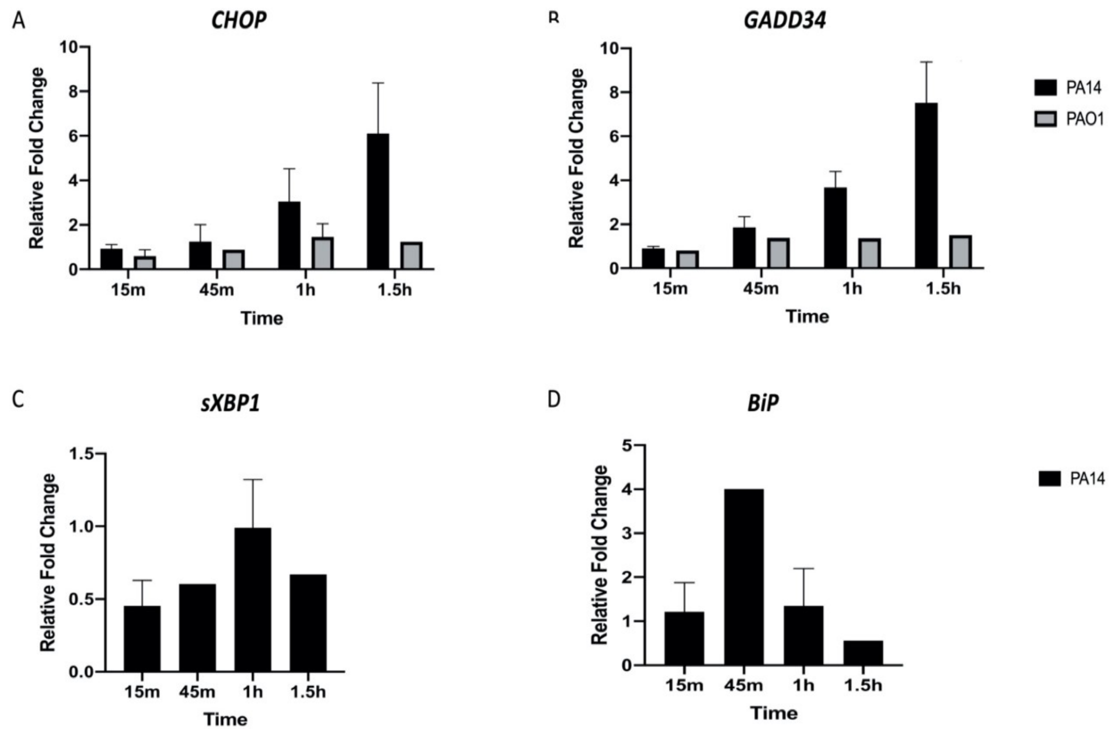

Figure 5. Wild type *P. aeruginosa* PA14, But Not PAO1, Induces Multiple Stress Response Markers in A549 Cells.

RNA isolated from PA14 or PAO1 infected cells was reverse transcribed to cDNA and subjected to RT-qPCR for (A) *CHOP* (B) *GADD34* (C) *sXBP1* (D) *BiP*. Gene expression was normalized to housekeeping gene β-actin and calculated relative to untreated cells using the ΔΔCt method. Error bars denote the standard error of the mean (SEM) and are only present for the samples that were run in biological triplicates. The samples with no error bars represent samples that were only run as technical replicates (*CHOP* PAO1 45m & 1.5h, *GADD34* PAO1 all timepoints, *sXBP1* 45m & 1.5h, *BiP* 45m & 1.5h).

3.2 Quorum Sensing Mutants Show Differential Induction of The Stress Responses in Human Lung Alveolar Cells.

3.2.1 Confirmation of Deletion QS Mutants

P. aeruginosa has many virulence factors whose production and secretion is controlled by three quorum sensing systems: Las, Rhl and PQS (22). To test the effect that these QS systems have on stress response activation, we used single, double and triple deletion mutants of genes involved in all three QS systems. We specifically used deletion mutants of the transcriptional regulators of all three QS systems: *lasR* of the Las system, *rhlR* of the Rhl system and *mvfR* of the PQS system. An end point PCR for each gene (*lasR*, *rhlR*, *mvfR*) was prepared using DNA purified from each of the QS mutants or WT PA14 strain as the template. The results were as expected where a band was only found in the *lasR*-specific PCR reaction for the following samples: WT PA14, Δ*rhlR*, Δ*mvfR*, and Δ*rhlR/mvfR* (Figure 6A). Bands in the *rhlR*-specific PCR reaction were present in the following samples: WT PA14, Δ*lasR*, Δ*mvfR*, and Δ*lasR/mvfR* (Figure 6B). Finally, bands in the *mvfR*-specific PCR reaction were only in the following samples: WT PA14, Δ*lasR*, Δ*rhlR*, and Δ*lasR/rhlR* (Figure 6C). The Δ*lasR/rhlR/mvfR* mutant exhibited no bands in the three PCR reactions as expected since all three genes are deleted.

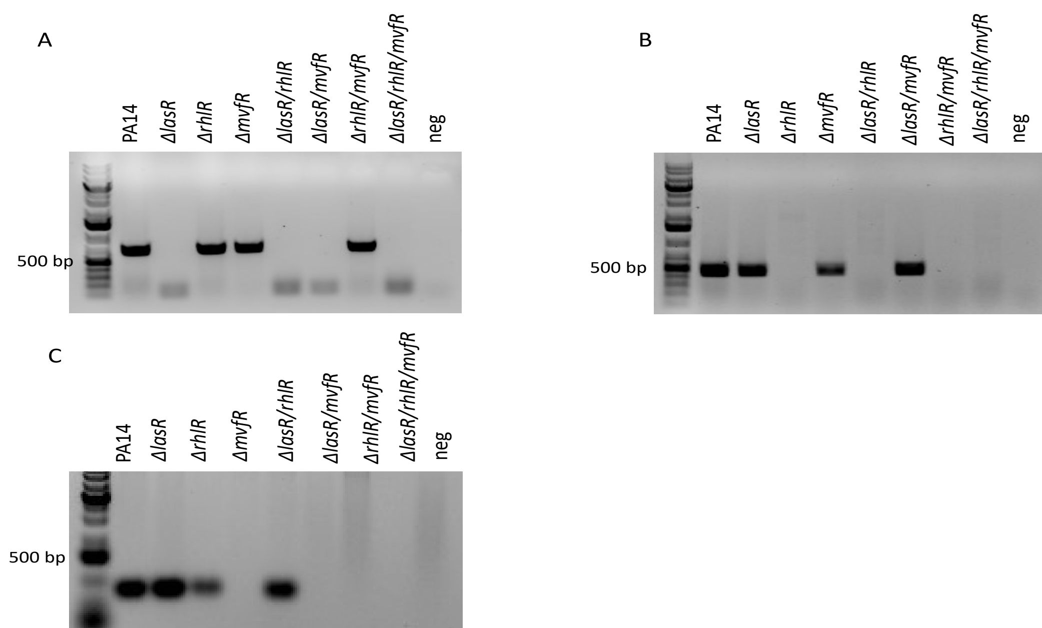

Figure 6. Confirmation of the Deletion QS Mutants

Gene-specific primers (Table 2) were used for each PCR reaction. End point PCR reactions of the three QS genes were run either using DNA extracted from WT PA14 or the single, double, or triple QS mutants or water rather than the DNA extract was used for the negative control. PCR products were run in a 1% agarose gel and the gel was imaged on the Chemidoc. The samples were run alongside a GeneRuler 1kb plus ladder (Thermo Scientific™; Cat. No. SM1331). Gene-specific primers correspond to **A**. *lasR* **B**. *rhlR* **C**. *mvfR*

3.2.2 Quorum Sensing Mutants Show Differential Stress Response Activation in A549 Cells

After confirming the identity of the QS deletion mutants, we used the previously mentioned *in vitro* infection assay to test the effect of different QS genes on the activation of the ER stress response. A549 cells were infected with MOI of 10 of WT PA14 or PA14 QS mutants for 15 min, 45 min, and 1h, treated with 75nM of Tg for 2h, or left untreated. Protein was extracted and subjected to western blotting to examine the protein level of ER stress response markers. A pattern was observed where mutants missing the *lasR* gene (Δ*lasR,* Δ*lasR/rhlR,* Δ*lasR/mvfR,* and Δ*lasR/rhlR/mvfR*) induced stronger eIF2α phosphorylation at later time point, 1h, compared to the strains that had the *lasR* gene intact (WT PA14, Δ*rhlR,* Δ*mvfR,*and Δ*rhlR/mvfR)* which induced the strongest phosphorylation levels at 45 min (Figure 7). This suggests that *lasR* plays a role in temporally regulating the dephosphorylation of eIF2α in PA14-infected cells. Interestingly, another trend observed was that QS mutants showed higher levels of eIF2α phosphorylation at the 45m and 1h compared to WT PA14 except for Δ*lasR/rhlR/mvfR* that higher levels at 1h only (Figure 7B).

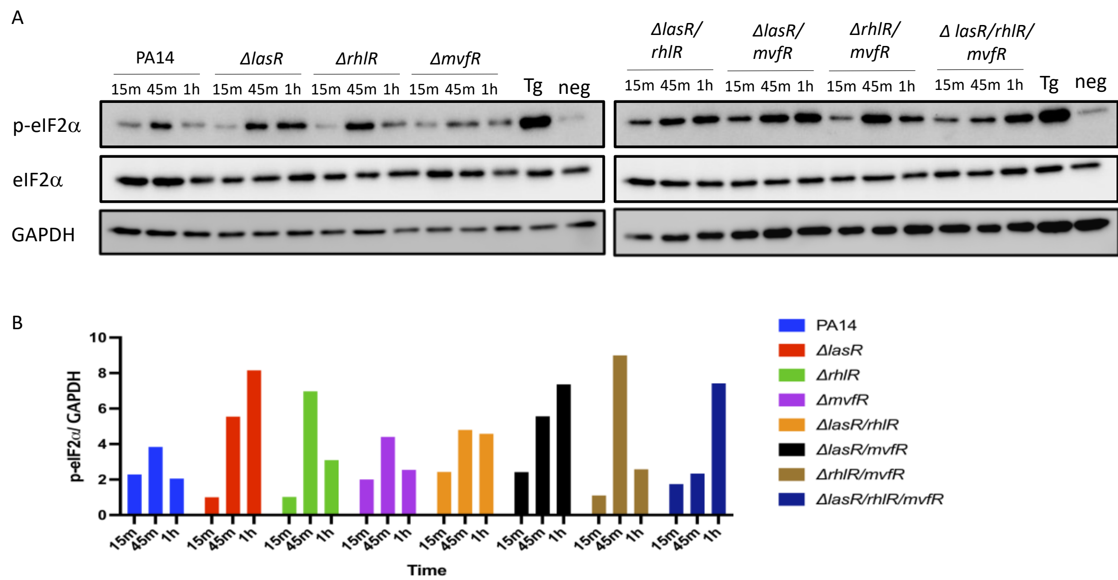

Figure 7. Quorum Sensing Mutants Show Differential Stress Response Activation in A549 Cells.

A549 cells were infected with WT PA14 or QS mutants, treated with Tg or left untreated. **A.** Cell lysates were subjected to western blotting for p-eIF2α, total eIF2α, and GAPDH as a loading control. Blots are representative of three independent experiments. **B.** Densitometry analysis of p-eIF2α was normalized to the loading control and untreated cells. The analysis is representative of one experiment.

3.3 *P. aeruginosa* Secreted Virulence Factor AprA is Important for the Activation of the Stress Response

3.3.1 Confirmation of AprA Mutants

The Las QS system of *P. aeruginosa* regulates the production and secretion of many virulence factors, including alkaline protease (AprA) (51). AprA is an important virulence factor of *P. aeruginosa* that helps the bacteria evade the immune response by cleaving many innate and adaptive immune factors like complement and cytokines (36). To test the effect of AprA on the activation of stress response activation, we used PA14 strains with either a deletion or transposon-insertion mutation in the *aprA* gene. To confirm the mutations, an endpoint PCR reaction was run using genomic DNA extracted from WT PA14 and AprA mutants were used as a template. The products were run on a 1% agarose gel and the results were as expected with a band of the expected size only present in the WT PA14 (Figure 8) but not the mutants. However, there were other unexpected larger bands present in all the samples except for the negative control. These bands could be non-specific amplifications during the PCR reaction. This could be avoided by altering the melting temperature, decreasing extension time, further diluting the DNA template, or designing new primers.

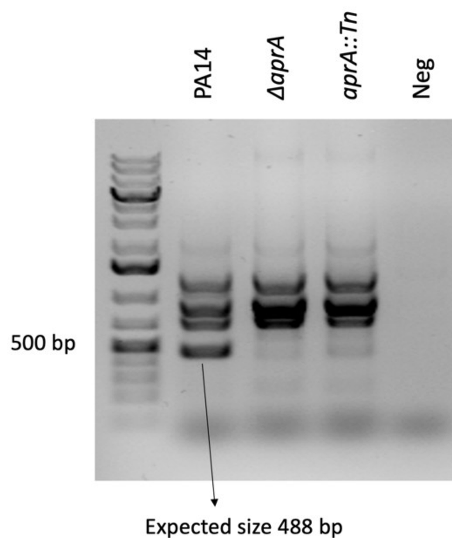

Figure 8. Confirmation of AprA Mutants.

Gene-specific primers (Table 2) were used for the endpoint PCR reaction. The reaction was run either using genomic DNA extracted from WT PA14, Δ*aprA*, *aprA*::Tn or water for the negative control. PCR products were run in a 1% agarose gel and the gel was imaged on the Chemidoc. The samples were run alongside GeneRuler 1kb plus ladder (Thermo Scientific™; Cat. No. SM1331).

3.3.2 AprA Plays a Role in Activating the Stress Responses in A549 Cells.

A549 cells were infected with MOI 10 of either WT PA14, Δ*aprA*, *aprA*::Tn for 15min, 45min, 1h, and 1.5h or treated with Tg for 2h, or left untreated. Cell lysates were subjected to western blotting to examine the protein expression level of stress response markers. Both AprA mutants elicited decreased levels of phosphorylated eIF2α at the early timepoints of the infection compared to the WT PA14-infected cells (Figure 9A & B). However, the densitometry analysis showed that the *aprA*::Tn mutant seemed to recover at later timepoints exhibiting a strong induction of eIF2α phosphorylation that was not seen in the Δ*aprA* mutant or the WT-infected cells. These results suggest that AprA is needed for the induction of eIF2α phosphorylation at early timepoint. RT-qPCR was also used to examine other stress response markers. The Δ*aprA* mutant elicited similar levels of *CHOP* compared to WT-infected cells (Figure 10A). The Δ*aprA* mutant also elicited similar levels of *GADD34* at 15 min and 45 min when compared to WT infection. However, there was 4-fold increase in WT-infected samples but dramatic decrease in Δ*aprA*-infected samples at the 1h time point (Figure 10B). There was no induction of spliced *XBP1* in either PA14-infected samples or Δ*aprA*-infected samples (Figure 10C).

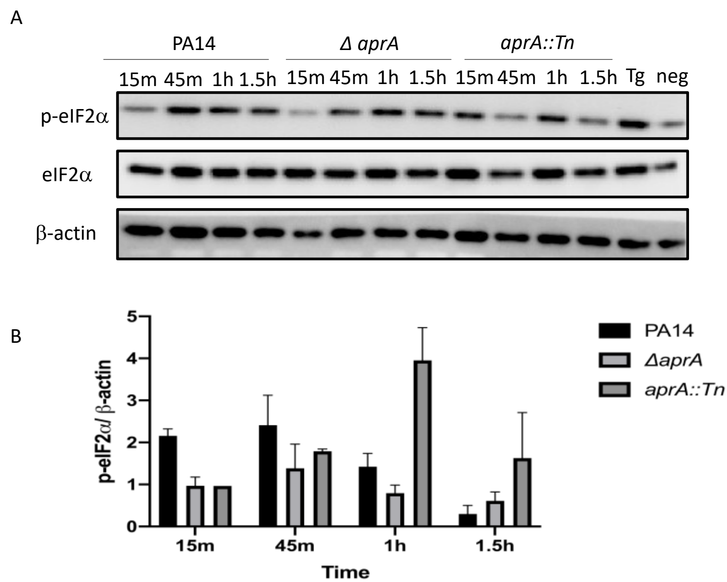

Figure 9. AprA Plays a Role in Early Activation of the Stress Responses.

A549 cells were infected with WT PA14 or *aprA* mutants, treated with Tg or left untreated. Cell lysates were subjected to western blotting for p-eIF2α, total eIF2α, and β-actin as a loading control. Blots are representative of three independent experiments. (B) Densitometry analysis of p-eIF2α was normalized to the loading control and the untreated cells. Three independent experiments are graphed, and error bars denote standard error of the mean (SEM).

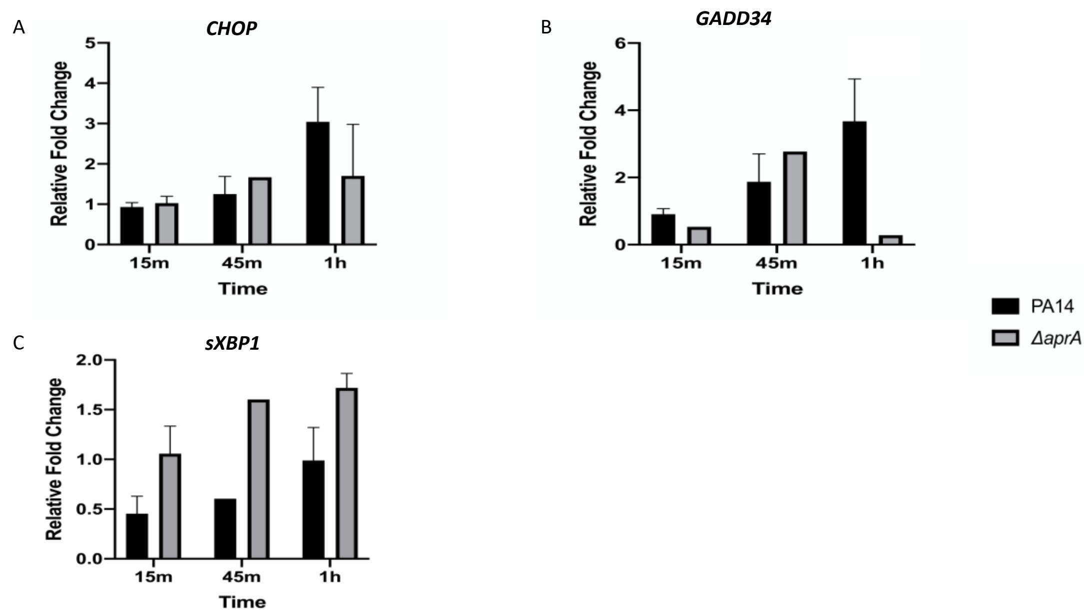

Figure 10. AprA Mutant Shows Differential Expression Levels of Stress Response Markers.

RNA isolated from PA14 or Δ*aprA* infected cells was reverse transcribed to cDNA and subjected to RT-qPCR with gene-specific primers for the following stress response markers (A) *CHOP* (B) *GADD34* and (C) s*XBP1*. Gene expression was normalized to housekeeping gene β-actin and calculated relative to untreated cells using the ΔΔCt method. Error bars denote the SEM and are only present for the samples that were run in biological triplicates. The samples with no error bars represent samples that were only run in technical replicates (45m timepoint of CHOP and sXBP1 for Δ*aprA* infected cells and all timepoints of GADD34 for Δ*aprA* infected cells).

The AprA operon in *P. aeruginosa* PA14 has 5 open reading frames (ORFs) as shown in Figure 11A. *aprA* gene encodes the structural gene, *aprI* encodes the protease inhibitor, while *aprD/E/F* encodes the genes involved in the type one secretion system required for the secretion of AprA. To test the effect of each of these genes on the phosphorylation of eIF2α, I infected A549 cells MOI 10 of WT PA14, *aprA*::Tn, *aprI*::Tn, *aprD*::Tn, and *aprF*::Tn for 15min, 45min, 1h and 1.5h, or treated with Tg for 2h, or left untreated. These mutants were selected because they were available in the PA14 Transposon library present in my lab (143). Changes in eIF2α were visualized on western blot as seen in Figure 11B & C. The results showed that the disruption of *aprA* gene by the transposon insertion led to a decrease in the phosphorylation of eIF2α, which is consistent with my previous result. Similarly, the disruption of *aprD* and *aprF* led to a decrease in the phosphorylation of eIF2α as expected (Figure 11B & C). The disruption of *aprI* led to an increase in the amount of AprA present which was shown to increase the levels of p-eIF2α to higher levels compared to WT PA14 (Figure 11B). This result showed large variabilities among the biological replicas, so the densitometry results indicated that deleting the protease inhibitor did not have much of an effect on the phosphorylation of eIF2α (Figure 11C). These data suggested that AprA play a role in the stress response activation, which is consistent with the observations of Van't Wout *et al.* that 16HBE cells treated with purified AprA enzyme or conditioned medium containing AprA showed an increase in activated UPR and ISR markers (135).

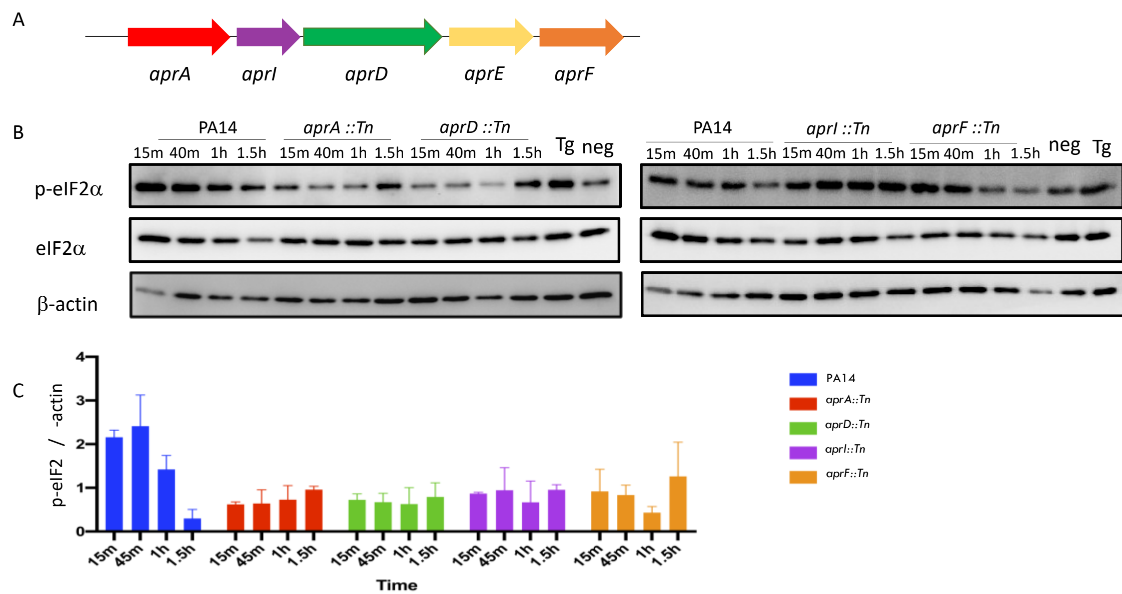

Figure 11. AprA Operon Play a Role in Stress Response Activation.

(A) AprA operon showing the five open reading frames coding for the protein AprA, the protease inhibitor, and the type one secretion system. (B) A549 cells were infected with WT PA14 or *aprA* mutants, treated with Tg or left untreated. Cell lysates were subjected to western blotting for p-eIF2α, total eIF2α, and β-actin as a loading control. Blots are representative of three independent experiments. (C) Densitometry analysis of p-eIF2α was normalized to the loading control and untreated cells. Three independent experiments are graphed and error bars denote standard error of the mean (SEM).

3.4 Secreted Virulence Factor of PA14, Elastase LasB, Activates the Stress Responses.

3.4.1 PA14 Mutants Lacking LasB Showed a Decrease in the Phosphorylation of eIF2α

LasB is another important virulence factor of *P. aeruginosa* whose production and secretion is controlled by the las QS system (60). LasB helps *P. aeruginosa* establish a chronic infection by interfering with bacterial clearance, cleaving the complement system, and forming biofilms or microcolonies (60, 61). To test the effect that LasB has on the activation of the stress responses, I infected A549 cells with MOI 10 of WT PA14 or Δ*lasB*, *lasB*::Tn and investigated the change in the phosphorylation level of the eIF2α protein. Similar to the results in the *lasB* mutant infections, deleting or disrupting the *lasB* gene led to a decrease in the level of phosphorylated eIF2α in the early timepoints compared to WT PA14-infected cells but that change diminished throughout the infection at later timepoints (Figure 12 A & B). This suggests that LasB could be a novel elicitor of the stress responses.

RNA was also extracted from infected cells and used for RT-qPCR to measure the levels of *GADD34*, *sXBP1* and *CHOP* mRNA levels post-infection. Δ*lasB* mutant-infected cells showed similar levels of relative *CHOP* and *sXBP1* mRNA expression compared to WT-infected cells (Figure 13A & C). However, Δ*lasB* mutant-infected cells had a 10-fold increase in the levels of *GADD34* at 1h compared to the almost 4-fold increase in PA14-infected cells (Figure 13B).

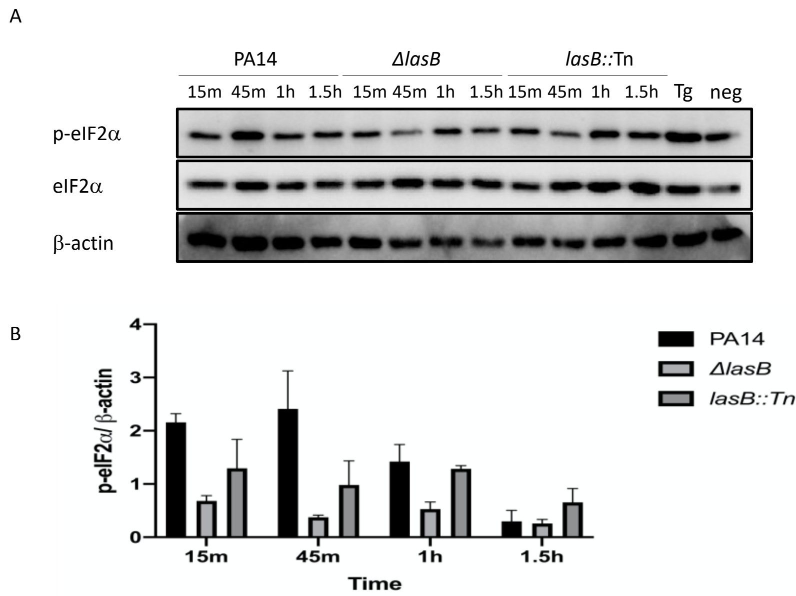

Figure 12. LasB is an Elicitor of the Stress Responses.

(A) A549 cells were infected with WT PA14 or *lasB* mutants, treated with Tg or left untreated. Cell lysates were subjected to western blotting for p-eIF2α, total eIF2α, and β-actin as a loading control. Blots are representative of three independent experiments. (B) Densitometry analysis of p-eIF2α was normalized to the loading control and untreated cells. Three independent experiments are graphed, and error bars denote standard error of the mean (SEM).

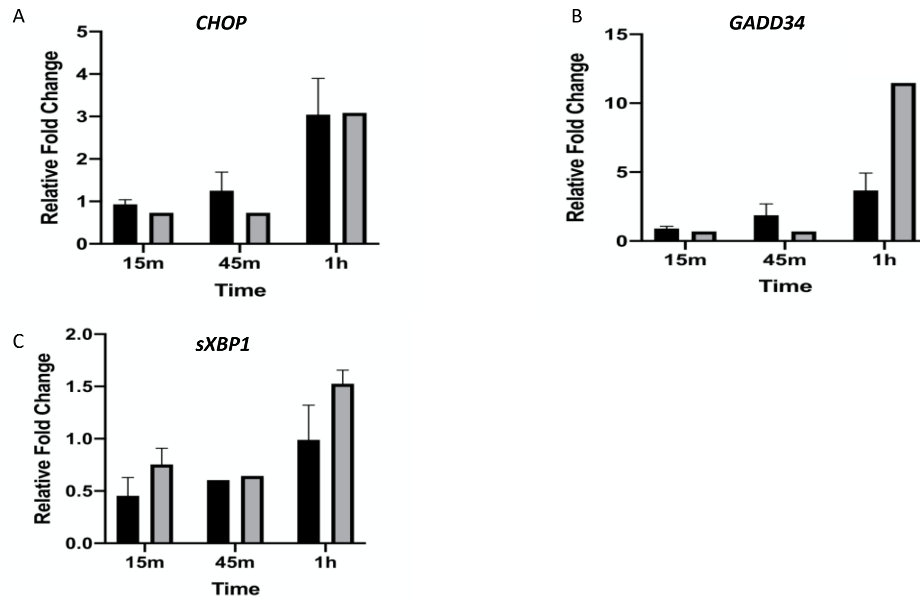

Figure 13. LasB Mutant Shows Differential Expression Levels of Certain Stress Response Markers.

RNA isolated from PA14 or Δ*lasB* infected cells was reverse transcribed to cDNA and subjected to RT-qPCR with gene-specific primers for the following stress response markers (A) *CHOP* (B) *GADD34* and (C) s*XBP1*. Gene expression was normalized to housekeeping gene β-actin and calculated relative to untreated cells using the ΔΔCt method. Error bars denote the SEM and are only present for the samples that were run in biological triplicates. The samples with no error bars represent samples that were only run in technical replicates (all timepoints of CHOP and GADD34 for Δ*lasB*-infected samples, 45m timepoint of sXBP1 for Δ*lasB*-infected samples).

3.4.2 Treatment of A549 Cells with Purified LasB is Sufficient to Activate the ISR

To confirm the role that LasB plays in ISR activation, we treated cells with different concentrations of purified LasB for different timepoints. Cell lysates were subjected to western blotting to examine the levels of different ISR markers. Purified LasB was sufficient in inducing the phosphorylation of eIF2α with the highest level of phosphorylation seen at 15min in cells treated with 1 μg/ml of purified LasB (Figure 14A & B). To determine whether PERK could be involved in the phosphorylation of eIF2α in the LasB-treated cells, I probed the cell lysates for total PERK. Similar to PA14-infected cells, PERK was not activated during the treatment suggesting that one of the other ISR kinases is responsible for the phosphorylation of eIF2α in these conditions (Figure 14). These results together further confirm that LasB can elicit the ISR but not through PERK activation.

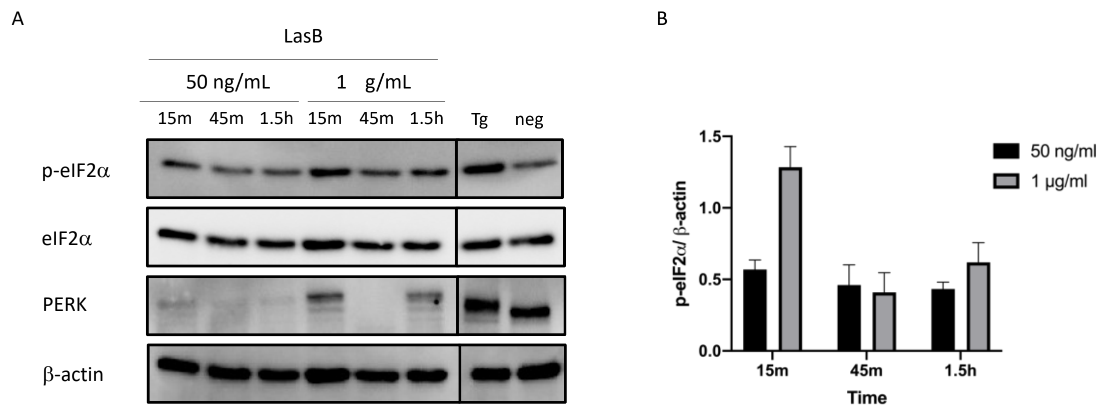

Figure 14. Treatment with Purified LasB Induces the Phosphorylation of eIF2α.

(A) A549 cells treated with different concentrations of purified LasB for three different timepoints, Tg for 2h or left untreated. Cell lysates were subjected to western blotting p-eIF2α, total eIF2α, PERK and β-actin as a loading control. Blots are representative of two independent experiments (B) Densitometry analysis of p-eIF2α was normalized to the loading control and untreated cells. An average of 2 independent experiments are graphed and error bars denote standard error of the mean (SEM).

CHAPTER 4. DISCUSSION

4.1 *Pseudomonas aeruginosa* is a Pathological Stressor

Pseudomonas aeruginosa is a major cause of nosocomial infections in humans (154). These infections are found in burn patients, patients who are immunocompromised, or those with respiratory conditions like COPD and CF (4, 5). *P. aeruginosa* is an opportunistic pathogen that establishes an acute infection in compromised hosts which if left untreated will progress into a chronic, and persistent infection. The wide variety of virulence factors that *P. aeruginosa*'s genome encodes allow this bacterium to both modulate host responses for its advantage and evade the immune response, making it a very successful pathogen.

On the cellular level, *P. aeruginosa* causes many changes in the cell's immune regulatory and signalling pathways, causing the cell to shift away from its homeostatic state. For instance, purified secreted virulence factors of *P. aeruginosa* such as pyocyanin, AprA, 3-oxo-C12-HSL have been found to induce ER stress as evidenced by the increase in *sXBP1, GADD34, CHOP* levels after cells were treated with those three effectors (135, 137). However, the extent of stress response during whole bacterial infection is still unclear. I used an *in vitro* cell culture infection model to test the effects of *P. aeruginosa* on the activation of UPR/ISR in A549 cells. I decided on using A549 cells, which is human lung alveolar epithelial cells, because it is considered easy to culture and it does not need any type of special media to grow. It also withstood the short infections of *P. aeruginosa* that I planned to use in my infection protocol unlike other cell lines that I tried such as 16HBE which died off within the first hour of infection. Using A549 cells allowed me to get enough information about the activation of UPR/ISR before cell death. However, there

are several limitations to using A549 in my system. The main limitation is that it is an alveolar cell line while a Pseudomonas infection is usually seen in the bronchial area of the lung. This makes our model less biologically relevant. So, once a robust phenotype is found in the A549 cell line, the experiments should be repeated, with a modified protocol perhaps, in a bronchial cell line.

My results showed that infecting A549 cells with whole *P. aeruginosa* WT PA14 strain activated the UPR and ISR as evidenced by the increase in the phosphorylation levels of eIF2α (Figure 4), as well as higher mRNA levels of the transcription factors *CHOP*, *GADD34*, and *BiP* (Figure 5 A, B, D) but no induction of spliced *XBP1* (Figure 5 C). However, a differential level of activation was seen when cells were infected with PAO1 instead of PA14. Both PAO1 and PA14 are well-established pathogens with high genetic similarity, however, they exhibit different virulence levels in many hosts including mice, *Caenorhabditis elegans*, and *Arabidopsis thaliana* (153, 155, 156). The discrepancy in virulence is likely due to multiple factors, including many virulence factors that are present in PA14 but absent in the less virulent strain, PAO1 (144). One example of these virulence factors is the type three secretion effector ExoU, which makes PA14 extremely cytotoxic to mammalian cells (157, 158). Deleting the *ExoU* gene in *P. aeruginosa* made it less cytotoxic and prevented injury to the epithelium in an acute lung infection model (157). In addition, Sana *et al.* showed that PAO1 had very high levels of QslA, an anti-activator of the las QS system, compared to the undetectable levels seen in PA14 (159). This was coupled with lower expression levels of QS genes like pyocyanin, the type two secretion system and the type six secretion system in PAO1. Given the importance of QS in *P. aeruginosa* virulence, the lower levels of QslA in PA14 would explain the hypervirulent

phenotype of the strain (159). Keeping this in mind, I expected the activation levels of the UPR and ISR to be lower in PAO1-infected cells compared to PA14-infected cells. This is because UPR and ISR activation is typically a result of the presence of virulence factors during a bacterial infection (119, 130, 135). So, if PA14 has more virulence factors being produced and secreted then I would expect more activation of the ER stress responses compared to PAO1. This is supported in my results by the lower levels of eIF2α phosphorylation (Figure 4), *CHOP* and *GADD34* in PAO1-infected cells compared to PA14-infected cells (Figure 5A & B). The increase in the relative expression levels of *BiP* seen in PA14-infected cells is another sign of UPR activation (Figure 5 D). This indicates that *P. aeruginosa* can induce the activation of stress responses and the virulence of the strain used in the infection could lead to differential activation of the responses.

I then investigated whether PERK is the kinase responsible for the phosphorylation of eIF2α, given that p-eIF2α is a common product of the four kinases of the ISR. Our results showed that PERK was not activated in PA14 or PAO1-infected cells during the timepoints tested (Figure 4). Although my results do not give a conclusive answer to the question of eIF2α phosphorylation, there are a few possible answers to be deducted from previous research. One of the probable kinases involved in eIF2α phosphorylation is GCN2 which has been shown previously to be triggered in PA14 infected Beas-2B bronchial epithelial cells due to the secretion of pyocyanin (160). This would also explain the discrepancy seen in p-eIF2α levels between PAO1 and PA14-infected cells (Figure 4 A, B). PAO1 is known to have much lower levels of pyocyanin produced compared to PA14 due to the presence of QslA as mentioned above (159). In addition to that, *Pseudomonas entomophila* has been shown to activate GCN2 through monalysin, a pore-forming toxin

that disrupts the membrane integrity of intestinal cells of Drosophila. The GCN2-eIF2α-ATF4 pathway then leads to global translation shutdown that impairs the immune and repair programs in the fly gut (161). Another possible kinase of eIF2α in *P. aeruginosa*-infected cells is HRI, a sensor for levels of intracellular heme. *P. aeruginosa* has an iron-chelating siderophore called pyoverdine which limits the concentration of iron in the cell medium resulting in the activation of HRI, eIF2α phosphorylation and *GADD34* expression in human bronchial epithelial cells (135).

4.2 Deletion of QS-regulatory Genes in *P. aeruginosa* Alters its Ability to Activate the Stress Responses

The quorum sensing systems allow bacteria to behave in a cooperative manner that gives the bacteria the ability to perform tasks as a powerful community in a way that would be impossible for a single bacterium (162). *P. aeruginosa* has three major quorum sensing systems, Las, Rhl, and PQS, each of which regulate the production and secretion of many virulence factors. All three systems depend on the production of signalling molecules which bind transcriptional regulators to induce the expression of a specific set of genes (25). These systems are regulated in a hierarchal manner which adds to the complexity of these important signalling networks.

To test the effect that the quorum sensing systems have on the activation of UPR and ISR stress responses, I used single, double or triple deletion mutants of the regulators of the three systems, LasR, RhlR, and MvfR. My results showed that all mutants were able to induce the phosphorylation of eIF2α, suggesting that the absence of the QS regulators is not enough to abolish the activation of the ISR in mammalian cells (Figure 7A & B). However, my results showed that the Δ*lasR,* Δ*lasR/rhlR,* Δ*lasR/mvfR,* and

Δ*lasR/rhlR/mvfR* mutants had stronger phosphorylation levels of eIF2α at later timepoints (1h) compared to WT PA14, Δ*rhlR,* Δ*mvfR,* and Δ*rhlR/mvfR* which had the strongest phosphorylation level at 45 min. All of the former mutants had a deletion in *lasR* in common, raising the question of what role does LasR play in the activation of the stress responses.

LasR is a part of the LasIR QS system, which depends on the acyl-homoserine lactone extracellular signalling molecule, 3-oxo-C12-HSL. The lasIR QS system positively regulates the expression of virulence factors such as elastase, exotoxin A, rhamnolipids, alkaline protease, pyocyanin and hydrogen cyanide (24, 60, 163, 164). It also positively regulates the RhlIR and PQS systems (165). Given its importance in regulating virulence, it is thought that *lasR* deficient strains are less virulent which may give the bacteria a competitive advantage when interacting with its host. This is important in establishing chronic infections where the bacteria shift from being hyperinflammatory to establishing a stable environment in the host while supressing and evading the host immune response (166). This is supported by the presence of *lasR* mutant strains isolated from a wound, biofilm in the lungs of CF patients and mechanically ventilated patients (167–169). Genotypic characterization of these mutants gives an insight into the importance of the Las QS system in establishing a *P. aeruginosa* infection and how the bacteria adapts to its host. It also shows that mutations affecting the cell-to-cell signalling networks are preferentially located in the transcriptional regulator genes (167). The selection towards Las mutants is common during infections; however the effects of the Las mutants during infection is still unclear (167).

My results showed that strains that had LasR had early strong phosphorylation of eIF2α which decreased at later timepoints (Figure 7). This suggests that LasR regulates a gene or a set of genes that induce early activation of the stress responses. Because LasR regulates the production and secretion of pyocyanin, *lasR* mutants would have much lower levels of pyocyanin secreted. Pyocyanin is redox-active compound that increases intracellular oxidant stress, which in turn affects cytosolic calcium concentration (26). This makes pyocyanin an elicitor of the ER stress response as supported by the finding that 16HBE cells treated with purified pyocyanin had higher levels of *sXBP1*, *GADD34* and *CHOP* mRNA levels compared to untreated cells (135).

In addition to that, I hypothesized that QS mutants would have lower eIF2α phosphorylation, however, to my surprise, the QS mutants showed a much higher level of phosphorylation compared to WT. This could be due to two main reasons. The first reason being QS does not only regulate bacterial virulence to the host, but it also regulates bacterial cell growth depending on population density (170). An observation made while preparing for infections was that although subcultures of WT and QS mutants were started at the same time and left in the incubator for 1.5-2h, the QS mutants would reach an OD600 of 0.6 much faster compared to the WT. This suggests that there is indeed dysregulation of growth rate in QS mutants compared to WT strains. So, although I start the infection with a MOI of 10, there could be a lot more bacteria in the QS mutants by later timepoints which would lead to a higher activation of the stress responses. However, a standard growth curve of the WT and QS mutant strains is needed to confirm this hypothesis. The growth curve can be studied by plotting the absorbance (cell growth) versus the incubation time giving me a sigmoid curve with the four phases of bacterial growth. If my hypothesis is true, the

slope of the exponential phase of the QS mutants would be steeper than that of the WT growth curve. It is important to note that the growth rate in LB and cell culture DMEM might show a slight difference. This is why counting CFU post infection might give me a better idea of bacterial growth of the different mutant strains across the different timepoints. The second hypothesis would be that *P. aeruginosa* secretes many factors that either activate or suppress the stress responses and in the absence of QS regulators this balance between activators and inhibitors leans towards the activators more resulting in higher levels of eIF2α phosphorylation. Although there are no known effectors of *P. aeruginosa* that can inhibit the stress responses, the Gram negative obligate intracellular bacterium *Simkania negevensis* was shown to inhibit ER stress response activation in cells treated with thapsigargin and tunicamycin (171). This is also very common in viral infections where Hepatitis C Virus is capable of inhibiting PERK's cytosolic kinase activity thus dampening the effects of stress response activation (118).

4.3 AprA Plays an Important Role in Activation of ISR/UPR

In the search of virulence factors of *P. aeruginosa* that activate the ER stress responses, I first examined alkaline protease (AprA). Certain strains of *P. aeruginosa* are known to secrete many proteases and elastases that are important in initiating and controlling tissue invasion. One of these proteases is AprA, a zinc metalloprotease whose production and secretion is regulated by the las QS system (163). AprA plays an important role in establishing a *P. aeruginosa* acute infection by enhancing the bacteria's survival within the host. AprA does so by cleaving one of the major microbial recognition molecules, monomeric flagellin, which prevents TLR5 activation thus inhibiting the

activation of the NF-κB signalling pathway (52, 57). In addition to that, AprA cleaves complement proteins and cytokines thus limiting the innate immune response.

However, AprA has other effects on the mammalian cell including the activation of the stress responses. Van't Wout *et al.* showed that cells treated with purified AprA had higher expression levels of *sXBP1, CHOP, BiP* but lower levels of *GADD34* compared to untreated cells. In addition to that, treating 16HBE cells with conditioned media from an *aprA* mutant resulted in a higher level of *GADD34* compared to purified AprA treatment. My results showed that *aprA* mutant strains had lower levels of eIF2α phosphorylation at early timepoints (15min) compared to WT (Figure 9). However, this phenotype was not seen at later timepoints of infection, suggesting that AprA may only play a role in early activation of the stress responses. In addition to that, I observed similar levels of *CHOP* and very low levels of *GADD34* in Δ*aprA*-infected cells compared to PA14 (Figure 10A & B). The decrease in *GADD34* expression contradicts previous research; however, this could be due to the differences in my experimental design including the cell lines used, 16HBE vs A549, and the treatment method, conditioned media vs bacterial infection. Regardless, the expression levels of *GADD34* seem to have a unique trend compared to the other stress markers. The role that GADD34 plays in *P. aeruginosa*-infected cells is not yet clear. Research has shown that *GADD34* siRNA increased the level of CHOP expression in tunicamycin-treated cells, resulting in higher rate of apoptosis in these cells but not in Sodium Flouride (NaF) treated cells (172). Both tunicamycin and Na-F are inducers of the UPR, where tunicamycin is known to activate both PERK and IRE1, while Na-F activates PERK alone (172). GADD34 has also been shown to protect the cell from *P. aeruginosa*-induced cytotoxicity by protecting the cells from the iron deficiency caused

by *P. aeruginosa* and the subsequent cell death (135). These results suggest that GADD34 is context dependent and might be cytoprotective, however, further research is needed to understand their roles in the different contexts of *P. aeruginosa* infection in mammalian cells.

Another important piece of result regarding AprA's role in the stress responses activation included testing the effect of deleting other genes in the AprA operon. Deleting genes involved in the secretion system of AprA led to decreased phosphorylation of eIF2α as expected. The type one secretion system of AprA is encoded by three genes *aprD, aprE,* and *aprF* (Figure 11A) (173). This secretion system has a one-step mechanism consisting of three components: an ATP-binding cassette coded by *aprD*, an outer membrane protein coded by *aprF* and an inner membrane protein encoded by *aprE* (173). Previous research has shown that inhibiting the secretion system caused the cell-bound protease to become very unstable, which suggests in the absence of interaction with the secretion machinery the protease is degraded (174). This would explain the decrease in phosphorylation of eIF2α in my *aprD*::Tn and *aprF*::Tn mutants-infected cells given that AprA is an important elicitor of the ER stress responses (Figure 11B & C). The AprA operon also contains a protease inhibitor coded by the gene *aprI*. Although the exact mechanism of protease inhibition is not completely known, research has shown that AprI inhibits AprA's catalytic activity by interacting with its active site. My results showed that deleting *aprI* results in a higher level of eIF2α phosphorylation which could be due to the increase of active AprA in the supernatant (Figure 11 B); however, this result was not consistent throughout the biological triplicates. Further experiments are needed to confirm the role of the protease

inhibitor on the activation of the stress responses. To sum up these results, AprA seems to be an elicitor of the ISR, whose effect is mainly through the eIF2α−ATF4-CHOP pathway.

4.4 Elastase LasB is a Novel Elicitor of the Stress Responses

LasB is another important virulence factor of *P. aeruginosa* that helps initiate infection. It does so by cleaving extracellular matrix proteins as well as other components of the innate and adaptive immune responses like (52, 175). LasB is known to act in concert with AprA to degrade monomeric flagellin thus providing a failsafe mechanism for *P. aeruginosa* to modulate the immune response (60). Although LasB is one of the first discovered virulence factors of *P. aeruginosa* and its substrates are well-known, the role it plays in the activation of the stress responses is not yet clear. Because of its overlap with AprA's function, it is likely that LasB is also an elicitor of the stress responses. This motivated me to investigate the effects of deleting *lasB* on the activation of the ER stress response. As expected, *lasB* mutants exhibited a decrease in the levels of phosphorylated eIF2α at early timepoints, 15min and 45min, compared to WT (Figure 12). My RT-qPCR data revealed there was no significant difference in the induction levels of *CHOP* and *sXBP1* in Δ*lasB*-infected cells compared to WT PA14 infected cells (Figure 13 A & C). However, *GADD34* levels at 1h were almost 5-fold higher in Δ*lasB*-infected cells compared to WT (Figure 13 B). These results suggest that LasB indeed plays a role in the activation of the ER stress responses. The exact role that *GADD34* plays in these conditions is not known, however, very high levels of *GADD34* could suggest that the Δ*lasB*-infected cells were able to relieve the stress thus dephosphorylating eIF2α and going back to a homeostatic state. To confirm this hypothesis, further research, including examining the

protein level of GADD34, is needed, considering that in some instances an increase in mRNA levels does not fully translate into an increase in protein levels. Also, more repeats are needed to confirm the statistical significance of the RT-qPCR results.

I also treated my cells with purified LasB to answer the question of whether LasB alone is sufficient to activate the UPR and ISR stress responses. Purified LasB treatment indicated that LasB indeed activates the ISR as evidenced by an increase in eIF2α phosphorylation (Figure 13). This phosphorylation was not due to the activation of PERK as there is a shift in the PERK band was only seen in the positive control, Tg- treated cells. Together, my results suggest that LasB is a novel elicitor of the ISR. However, previous research has indicated otherwise. Van't Wout *et al.* showed that purified elastase treatment of 16HBE cells did not elicit an UPR or ISR, although they did show that treating 16HBE cells with conditioned media of PAO1 *lasB* mutant had lower levels of *CHOP*, *BiP*, and spliced *XBP1* compared to those of WT-treated cells (135). This suggests that LasB could be playing a role in the activation of the stress responses, but this was not clear under the conditions that the purified LasB was tested in this previous study (135). I indeed tested their conditions (50 μg/ml for 15min, 45min, 1h, 2h, 4h, 6h) on my A549 cells and have found that there was no induction past 45 minutes due to cell death. This suggests that the conditions they used were cytotoxic, which led me to use a much lower concentration (50 ng/ml and 1 μg/ml), giving me the results seen in Figure 13. The discrepancy between my results and theirs could also be due to the difference in cell lines used. Tissue culture cell lines have been shown to have different sensitivity levels to different elicitors of the ER stress response. A repeat of the LasB treatment with another cell line would further confirm my hypothesis that LasB is an elicitor of such responses.

Given that AprA and LasB act in a similar way to help *P. aeruginosa* evade the immune system, it is important to note the redundancy that this may cause. Although this ensures that the bacteria are protected from the host responses, it makes it harder for me to decipher the effects of each individual virulence factor on the mammalian cell. The fact that LasB could take over AprA's functions in an *aprA* mutant could explain why the phenotypes I observed were not as drastic. This is why it is important to keep in mind the other players in the activation of the stress responses when using whole bacteria as ER stress elicitors.

4.5 Future Directions

In my study, I confirmed that PA14 induces the UPR and ISR as seen by the increase in the phosphorylation of eIF2α, *CHOP*, *GADD34,* and *BiP* expression levels. I also have evidence that suggests that PERK might not be responsible for the phosphorylation of eIF2α in PA14-infected cell under the conditions tested. It is important to test the other kinases of the ISR to see which one is activated during PA14 infection, which could be done by subjecting the cell lysates to western blotting for p-GCN2, HRI, and p-PKR. Using genetically manipulated cell lines, I could tease out the kinase responsible for the eIF2α phosphorylation phenotype. By silencing the expression of each individual ISR kinase using siRNA, I could find out whether a single or multiple ISR kinases are responsible for phosphorylating eIF2α during the course of a *P. aeruginosa* infection. This could also be achieved by using ISR kinase-specific inhibitors which can be bought commercially. This will give me a better insight into the mechanism of ISR activation leading to the phenotype seen in my infected cells. It is also important to confirm

that other UPR branches are being activated especially that my results showed lack of *XBP1* splicing unlike published work that suggested that *P. aeruginosa* can induce *XBP1* splicing (135, 137). It is important to note the differences between the conditions that I tested and published work. Most papers have used purified virulence factors as their ER stressor, which allowed them to treat their cells for a much longer time period (137). This is not possible with PA14 due to its hypervirulent phenotype. To get around this problem, I could treat my cells with conditioned media of PA14, thus allowing me to treat my cells for a longer period of time and test whether *XBP1* splicing occurs later in the course of infection. It is important to note that my cell line of choice, A549 cells, is capable of undergoing the splicing of XBP1 once the UPR is activated. This is seen in my positive control or Tg-treated cells where there is almost 11-fold induction of the *sXBP1* mRNA compared to untreated cells. To test whether virulence of the strain is directly related to the magnitude of the ER stress response activation, I could infect my cells with various different strains of *P. aeruginosa* with different levels of virulence and compare the levels of UPR and ISR activation. In addition to that, testing different virulence mutants of PA14, such as the type three secretion effector ExoU mutant could tell me whether these virulence factors are directly contributing to the induction of the ER stress responses.

My results also showed a differential level of eIF2α phosphorylation in the QS mutants. To confirm this phenotype, complementation of the QS mutants should be done. I have kindly received plasmids for the three QS transcriptional regulators (*lasR, rhlR,* and *mvfR*) from Dr. Eric Déziel, Armand-Frappier Santé Biotechnologie Research Centre. Transforming my deletion mutants with these plasmids using electroporation and repeating the infections would give me more confidence in the phenotype observed in the mutants

(Figure 7A, B). I also have to run biological replicates for some of the RT-qPCR data to confirm my data is accurate and reproducible. This includes PAO1-infected samples, *ΔaprA*-infected samples and *ΔlasB*-infected samples.

In addition to the results in this thesis, testing if killed/dead *P. aeruginosa* could activate the ER stress responses would give me an insight into whether active secretion of virulence factors is needed to induce such responses. For this to be done, a protocol for killing *P. aeruginosa* should be established, because a 15 min heat treatment at 90°C failed to completely kill the bacteria in my previous attempt. Instead, I recommend ethanol or methanol treatment or a typical fixation protocol to kill the bacteria. It is important to note that the phenotype observed with heat-killed vs alcohol-fixed bacteria might be different due to the bacterial cell lysis which releases the cell's contents into the supernatant during the infection. Finally, I would investigate the effect of LPS treatment on the activation of the ER stress responses. Previous research has not provided a conclusive answer on the effect of LPS, however, Woo *et al*. has shown that TLR activation by LPS suppresses the expression of *CHOP* and dephosphorylates eIF2α in Tg-treated cells (176). Others have shown that LPS treatment quickly activates the ER function-protective pathway in RAW 264.7, a mouse macrophage cell line, through the activation of PERK and inhibition of *CHOP* expression, which protects the cells from undergoing apoptosis (177). LPS treatment would tell me if my results with the QS mutants are dampened due to the effects of LPS or not. It is also important to note that when protein is purified from bacterial culture, it is usually contaminated with LPS. Unless the protein is specifically purified from the LPS contaminant, LPS could alter the results of the experiment. This is crucial to take into consideration when analyzing results from purified protein treatment; it also highlights

the need for researchers to be transparent about the "purity" of their proteins. For future purposes, the LasB purification will be done in the lab and LPS will be removed before treating the cells with it.

4.6 Concluding Remarks

The primary goal of this thesis was to understand the extent of stress response activation during a *P. aeruginosa* infection and find novel elicitors of these responses. Using the *in vitro* infection model discussed above, I demonstrated that PA14 can induce ISR while PAO1 did not seem to induce a strong response during the conditions tested. I have also demonstrated that deletion mutants of the transcriptional regulators of the QS systems had a differential level of eIF2α phosphorylation with *lasR* being an important factor for early activation of the ISR. The LasIR QS system regulates the production and secretion of many virulence factor but I decided to focus on the effects of only two virulence factors, alkaline protease and elastase LasB. Deleting *aprA* resulted in a decrease in the level of eIF2α phosphorylation, induction of *CHOP,* but suppression of *GADD34*. Further investigation is needed to understand the unique trend of *GADD34* in *ΔaprA*-infected cells. Deleting the genes responsible for AprA's type one secretion system, *aprD* and *aprF*, have led to a decrease in phosphorylation of eIF2α while deleting the protease inhibitor led to an increase in the phosphorylation level. Similarly, deleting *lasB* led to a decrease in level of eIF2α phosphorylation, induction of *CHOP* and high expression of *GADD34*. This indicates that LasB is a novel elicitor of the ISR.

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