In vitro regulation of quorum sensing in *Pseudomonas aeruginosa*
using synthetic autoinducers

By

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Dedication

This study is wholeheartedly dedicated to my loving and supporting parents.
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In vitro regulation of quorum sensing in *Pseudomonas aeruginosa* using synthetic autoinducers

Hrag Dilabazian

**ABSTRACT**

Quorum sensing is a remarkable type of cell-to-cell communication that is directly involved in the regulation of virulence, biofilm formation and other significant characteristics of bacteria. This process, which occurs in most pathogenic bacteria, is mainly governed by the release of significant concentrations of chemical signaling molecules, called autoinducers, by the local bacterial cell density. *Pseudomonas aeruginosa*, an opportunistic human pathogen, known for its complex quorum sensing system, also possesses its own autoinducers, that mediate cellular cross talk and regulate the expression of certain virulence genes. Among these genes is the *lasB* elastase gene. The *lasI* and *rhlI* gene products respectively drive the synthesis of N-(3-oxododecanoyl)-L-homoserine lactone (OdDHL) and N-butyryl homoserine lactone (BHL), the autoinducers of *Pseudomonas aeruginosa*. Forty eight clinical *Pseudomonas aeruginosa* isolates, from patients in LAUMC-RH, were included in this study to determine the activity of the two synthetic autoinducers, OdDHL and BHL, on the induction of the *lasB* gene, during early stages of bacterial growth. The strains were definitively identified and screened for their elastolytic activity. They were classified into two distinct groups: elastase positive and elastase negative. The possession of the quorum sensing controlled gene *lasB* and the regulatory genes *lasR*, *lasI*, *rhlR* and *rhlI* was determined by the polymerase chain reaction. One out of the four elastase negative isolates was *lasR* and *lasI* deficient. The inducing ability of OdDHL and BHL on the *lasB* gene was tested under different conditions: by increasing cell density or manipulating the concentration of or time of exposure to autoinducers, revealed that induction was not possible in elastase negative strains and had no detectable effect on the elastase positive strains. Our observations suggest that, in vitro autoinducer directed, early induction of the elastase gene in the clinical isolates was not possible. A plausible explanation could be the presence of a defective *lasB* gene, the absence of or truncated transcriptional regulatory proteins, or unusual environmental factors affecting the activity of the autoinducers on the elastase negative strains.

**Keywords:** Autoinducers, Elastase, *lasB* gene, OdDHL and BHL, *Pseudomonas aeruginosa*, Quorum sensing.
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**List of Abbreviations**

*P. aeruginosa*: *Pseudomonas aeruginosa*

QS: Quorum sensing

AI: Autoinducer

AHL: acyl- homoserine lactone

VAI: *Vibrio fischeri* autoinducer

*V. fischeri*: *Vibrio fischeri*

HSL: Homoserine lactone

**OdDHL**: 3-oxo-C12-AHL / N-(3-oxododecanoyl) homoserine lactone

SAM: S-adenosylmethionine

**BHL**: N-butyrylhomoserine lactone / C4-HSL

**PQS**: Pseudomonas quinolone signal

**PA**: *Pseudomonas aeruginosa*

**EDTA**: Ethylene diamine tetra-acetic acid

**PAI**: *Pseudomonas aeruginosa* autoinducer

**PCR**: Polymerase Chain Reaction

**ECR**: Elastin congo red

**PTSB**: Peptone tryptic soy broth

**DMSO**: Dimethyl sulfoxide
CHAPTER I

Introduction

1.1 Pseudomonas aeruginosa

1.1.1 Introduction

*Pseudomonas aeruginosa* is a Gram negative, aerobic and environmentally versatile opportunistic human pathogen that exists in a wide range of habitats including aquatic sediments, water exposed surfaces, soil, plant roots and leaves as well as in humans (Coggan & Wolfgang, 2012). It causes nosocomial infections, has been identified as the second most common cause of hospital-acquired pneumonia (HAP), healthcare-associated pneumonia (HCAP) and ventilator-associated pneumonia (VAP) and considered an important pathogen in burn patients (Driscoll, Brody, & Kollef, 2007). With its enormous genome size around 6.3 million base pairs, genetic complexity and the large number of genes involved in mechanisms such as catabolism, transport and efflux of organic compounds, in addition to its intrinsic resistance to antibiotics and disinfectants, this human pathogen has adapted to thrive in diverse ecological niches (Folger et al., 2000). This Gram negative bacillus infects immunocompromised patients as a result of increasing age, or suffering from a disease or undergoing therapies that suppress the immune system; especially patients with cystic fibrosis and thus is associated with high levels of morbidity and mortality (Banerjee & Stableforth, 2000).
1.1.2 Virulence and pathogenicity

When discussing an organism associated with an ever-widening spectrum of infections, it is of high importance to highlight the major pathogenic mechanisms that \textit{P. aeruginosa} uses and the virulence factors it owns. Lungs of a healthy individual generally remain free from infection, where the epithelium is the first line of defense against infectious agents, playing a broad range of roles in the innate response to infection (Gellatly & Hancock, 2013). Concomitant with respiratory failure causing acute and chronic lung infections, \textit{P. aeruginosa} can bypass the epithelial barrier and infect the host, but the key question remains the same; what are the major virulence factors known or suspected of contributing to respiratory failure (Gellatly & Hancock, 2013).

Two groups of virulence factors contribute to the pathogenicity of \textit{P. aeruginosa}; cell surface virulence factors and secreted virulence factors. Flagella and pili, the motile surface appendages of \textit{P. aeruginosa} are responsible for bacterial motility: flagella enhances bacterial adhesion to epithelial surfaces while on the other hand, the filamentous surface appendages known as pili exhibit twitching motility allowing \textit{P. aeruginosa} to spread and plays a crucial role in adhesion during colonization (Kipnis, Sawa, & Wiener-Kronish, 2006).

A successful human pathogen needs to acquire iron, usually tightly bound to transferrin or lactoferrin in host tissues, for survival. \textit{P. aeruginosa} have developed a complex regulatory system through producing two major siderophores: pyochelin and pyoverdin, providing iron to support bacterial metabolic processes in addition to
controlling the expression of other *P. aeruginosa* virulence factors, such as exotoxin A and pyoverdine itself (Sadikot, Blackwell, Christman, & Prince, 2005).

Attacking the immune system, a strategy well known about pathogenic bacteria, *P. aeruginosa* uses a widely known virulence factor called pyocyanin that gets rid of neutrophils by inducing their apoptosis (Allen et al., 2005). This pigment gives a blue-green color characteristic to the organism and it’s a toxic metabolite, with a wide range of antibacterial and antifungal activity in addition to its biological activity in controlling gene expression and biofilm formation (Jayaseelan, Ramaswamy, & Dharmaraj, 2014). Besides pyocyanin, which can be used as weapon to compete with phylogenetically distant species, *P. aeruginosa* produces proteins known as bacteriocins, narrow-spectrum antibacterial proteins, used to target members of the same genus (Dingemans, Ghequire, Craggs, De Mot, & Cornelis, 2016). These proteinaceous, narrow spectrum antibacterial agents are called pyocins (Parret & De Mot, 2002). With three types of pyocins (R, F and S) described, these bacteriocins have become a precious tool for characterizing *P. aeruginosa* strains (Parret & De Mot, 2002; Waite & Curtis, 2009).

To stay alive, bacteria need to find ways to get over the host defenses and protect themselves from eradication. For this, *P. aeruginosa* turns out to be well equipped. Five out of the six secretion systems characterized in Gram negative bacteria, have been identified in *P. aeruginosa*. These are: type 1 secretion system (T1SS), T2SS, T3SS, T5SS and T6SS (Bleves et al., 2010). T1SS is involved in the secretion of the alkaline protease virulence factor, a fibrin lysing protease and in the utilization of iron (Bleves et al., 2010; Kipnis et al., 2006). T2SS secretes a wide range of virulence proteolytic enzymes including elastase LasB, a major extracellular protease enzyme, protease IV, lipases, phospholipases, alkaline phosphatases and Exotoxin A which takes part in
hinder the host immune cells, inhibiting protein synthesis and causing tissue damage and cell death (Bleves et al., 2010). The T3SS on the other hand is a needle-like complex that allows the bacterium to inject toxins (effector proteins) into the host cell resulting in cell death and alteration of host immune responses (Sadikot et al., 2005). The type V secretion system is the most simple of all, and like T2SS it relies on a two-step secretion mechanism to deliver the proteins that function as lipases and proteases to the host (Filloux, 2011). The last secretion system identified up to date is the T6SS that shares similarity in structure with the puncturing device of bacteriophages, it also delivers toxins to its neighboring pathogen and translocates effector proteins into the host cells, thus giving advantage to the pathogen for a better survival chance (Chen, Zou, She, & Wu, 2015).

The more dangerous strains of P. aeruginosa, which are much harder to treat, are those that form biofilms. These bacterial biofilms are clusters of microcolonies that are enclosed in a biopolymer matrix attached to a surface, such as indwelling medical devices, arising when P. aeruginosa cells communicate first via intercellular chemical signals in a process termed quorum sensing, to regulate a set of genes involved in virulence and metabolism (Driscoll et al., 2007).

Having such an impressive arsenal of virulence factors explains why the range of infections caused by P. aeruginosa is so wide and dangerous at the same time (Kerr & Snelling, 2009).
1.2 Bacterial quorum sensing

Bacteria were for a long time believed to exist as individual cells different from multicellular organisms that have coordinated activity, with a main goal of finding nutrients and accordingly multiplying, until it was discovered that intercellular communication did exist among them (de Kievit & Iglewski, 2000). Different organisms have found diverse modes of cooperation, and one such communication, specific to bacteria, was termed quorum sensing (QS). This was found to be a remarkable type of cell to cell organized communication system that is regulated by the local cell population density and that is used to control various features in bacteria, such as virulence, biofilm formation, sporulation, genetic competence and bioluminescence, among others (Mangwani, Dash, Chauhan, & Das, 2012). The “language” used for this intercellular communication is based on small, self-generated signal molecules called autoinducers (AI) (de Kievit & Iglewski, 2000).

Both Gram positive and Gram negative bacteria were found to rely on quorum sensing systems to communicate. Among the wide differences in the systems of both groups, one main variance was found to be the type or nature of signaling molecule produced. While, Acyl homoserine lactones (AHLs) were the major class of autoinducer signaling molecules used by Gram negative bacteria, Gram positive bacteria used modified oligopeptides as autoinducers (Ng & Bassler, 2009). A notable observation was that a particular AHL molecule was detected only the species that produced it (Ng & Bassler, 2009).

The process of quorum sensing in bacteria depends on three basic steps: First, the production of autoinducers- signaling molecules that are either diffused or actively
transported outside the bacterial cells (Sturgis, Ventre, & Lazdunski, 2004). Since this system is cell density dependent, at low cell density, the concentration of autoinducers is below the threshold of detection, however when the cell density increases, the signaling molecules are detected hence the second step, and in response, activating expression of genes followed by the final step, among them genes responsible for further autoinducer production (Rutherford & Bassler, 2012).

AHL QS was first described in the 1960s in *Vibrio fischeri*, a bioluminescent marine bacterium, where scientists found that the induction of bioluminescence was controlled in a high density culture medium as part of symbiotic associations between *V. fischeri* and the pinecone fish, *Monocentris japonica*, and the Hawaiian bobtail squid, *Euprymna scolopes* (Abisado, Benomar, Klaus, Dandekar, & Chandler, 2018). One of the first and presently the most comprehensively studied of the LuxI/LuxR type quorum sensing systems, is that of *Vibrio fischeri* possessing a *lux* operon (*luxICDABEG*) carrying genes for luminescence enzymes and for LuxI protein (Dunlap, 1999). The two main elements of this system are LuxI and LuxR. *luxI* gene encodes a 193-amino-acid protein LuxI that synthesizes the VAI, the *V. fischeri* autoinducer and is therefore considered to be the autoinducer synthase, and *luxR* gene, which encodes the 250-amino-acid LuxR protein, found to be the transcriptional activator of luminescence (Huiming Zheng et al., 2006). Interaction of LuxR with the autoinducer unveils the LuxR DNA binding domain, allowing LuxR to bind the *luxICDABE* promoter and activate its transcription (Miller & Bassler, 2001).

To sum up, Gram-negative bacteria typically use LuxI/LuxR type QS systems, homologous to the first described QS system from the bioluminescent marine bacterium *Vibrio fischeri*, to control virulence factor production such as the LasI/LasR and

### 1.3 Quorum sensing in *Pseudomonas aeruginosa*

#### 1.3.1 Introduction

The International Nosocomial Infection Control Consortium reported that *P. aeruginosa* nosocomial infections have become a worldwide healthcare issue (Moradali, Ghods, & Rehm, 2017). In numerous pathogens, the production of bacterial virulence determinants has been proved to be tightly regulated in a cell density-dependent manner, aided by a quorum sensing signaling system (Utari, Setroikromo, Melgert, & Quax, 2018). Given its importance as a human pathogen, *P. aeruginosa* has been the subject of intensive investigations and became one of the model organisms in QS research (Lee & Zhang, 2015).

Infections caused by *Pseudomonas aeruginosa* can be acute or chronic. While acute infections often spread rapidly and can cause tissue damage and sepsis with high mortality rates, chronic infections last and can persist for weeks, months, or years in the face of intensive clinical intervention, not to forget that many of the virulence factors associated with acute and chronic infections are controlled by QS (Kesarwani et al., 2011; Turner, Everett, Trivedi, Rumbaugh, & Whiteley, 2014). Moreover, the formation of biofilm is also controlled by quorum sensing, whereby one system- Rhl is involved in
the generation of biofilm components including rhamnolipids together with other systems involved in the formation and maturation of the biofilm (Rasamiravaka, Labtani, Duez, & El Jaziri, 2015). These processes accompanied with others, support the survival of this opportunistic human pathogen, and allow it to adapt in harsh environments, endure stress responses and colonize different sites. Thus, the importance of the quorum sensing mechanism of *P. aeruginosa*.

1.3.2 Molecular mechanism of quorum sensing

1.3.2.1 History

Although initially considered to be a specialized system of *V. fischeri* and related species, experimental work later revealed homologous systems of LuxI/LuxR with diverse biological roles in other proteobacterial species, including *Pseudomonas aeruginosa* (Lerat & Moran, 2004). *P. aeruginosa* strains contain four QS systems: two acyl-homoserine lactone (HSL)-based QS systems, the quinolone-based QS system, and the lately identified IQS-dependent QS system (Sun, Zhou, Jin, Jiang, & He, 2016).

In 1991, a regulatory protein- LasR was discovered having significant homology with the LuxR protein and which positively regulated elastase lasB expression in *Pseudomonas aeruginosa* PAO1 strain (Gambello & Iglewski, 1991). After a very short period of time, in 1993, the LuxI homolog, LasI was identified and turned out to be the protein responsible for the production of the *las* system signaling molecule in addition to its role in controlling the elastase gene (Jones et al., 1993). Shortly after the discovery of LasI, researchers suspected that the activation of *lasB* gene did not solely depend on the
las signaling molecule, instead a second factor produced by *P. aeruginosa*, also played a role in the activation of the elastase gene (Pearson, Passador, Iglewski, & Greenberg, 1995). Thus within the same year, a group of researchers identified another HSL based quorum sensing system called the RhlR/RhlI system, essential for the production of elastase, as well, and the cross communication between the RhlR/RhlI rhamnolipid regulatory system and the LasR/LasI elastase regulatory system (Ochsner & Reiser, 1995).

In 1999, a group of researchers reported the presence of another signaling molecule, 2-heptyl-3-hydroxy-4-quinolone, designated as the Pseudomonas quinolone signal (PQS) and that also induced the elastase gene (Pesci et al., 1999). However, it was proved that the production of PQS was controlled by the las system, because an active LasR protein was required for the biosynthesis of PQS (Pesci et al., 1999).

The fourth QS system discovered in 2013, known as the integrating quorum sensing signal (IQS) circuit, was shown to play a role in coping with stress responses and mainly phosphate limitations that the bacterium encountered during infections (Lee et al., 2013). This system was found to be activated by the las system, in addition, the system got activated when the bacterium was under stress, such as phosphate limitation and also when strains of Pseudomonas lost the las regulatory genes in order to activate the necessary virulence genes to overwhelm the host defense mechanism (Lee et al., 2013).

All four system, interconnected to each other were found to be responsible for the regulation of the expression of a vast pool of virulence genes to enhance the pathogenicity of this opportunistic pathogen and to make it a harsher organism to combat
with. For the purpose of this study, the 2 major acyl-homoserine lactone based QS systems; las and rhl were mainly dealt with.

1.3.2.2 LasI/LasR system

Originally discovered as a regulator of the elastase gene, the las system is now known to regulate many different genes (Fuqua & Greenberg, 2002). Among the four identified circuits in *P. aeruginosa*, the las system is considered to stand at the top of the QS hierarchy (Papenfort & Bassler, 2016). LasI/LasR systems which are known as the las system consists of two main molecules, LasR and LasI as transcriptional regulator and synthase protein, respectively (Kariminik, Baseri-Salehi, & Kheirkhah, 2017). It was shown that increases in the transcript levels of QS-controlled genes such as lasB relies on the transcriptional increase of the lasR gene that occurs mainly during the late logarithmic-early stationary phase of bacterial growth, when the bacterial concentration is high enough (Cabrol, Olliver, Pier, Andremont, & Ruimy, 2003).

LasI, a three-layer (αβα) sandwich containing eight α-helices (three 3_10-helices) surrounding a highly twisted platform of nine β-strands, is one of the most divergent AHL-synthases, involved in synthesizing an AHL with a long acyl-chain with 12 carbon atoms (Gould, Schweizer, & Churchill, 2004). In 1994, researchers purified the AHL, and identified the autoinducer that serves in conjugation with the LasR protein to activate a number of *P. aeruginosa* virulence genes, to be N-(3 oxododecanoyl) homoserine lactone- OdDHL (Pearson, Gray, Passador, & Tucker, 1994). Transcription of the lasI gene is induced by the LasR regulator when bound to its ligand 3-oxo-C12-AHL, thereby creating a positive feedback loop, in addition, the complex controls the
transcription of a large number of *P. aeruginosa* genes, many of which have been implicated in virulence (Wargo & Hogan, 2007). As for the autoinducer itself, and as the name suggests, these molecules have two structural components, an HSL ring and an acyl group (Val & Cronan, 1998). The enzyme produced by *P. aeruginosa* will catalyze the synthesis of the autoinducer from acyl-acyl carrier proteins (acyl-ACP) for the acyl group and the S-adenosylmethionine (SAM) for the lactone ring (Parsek, Val, Hanzelka, Cronan, & Greenberg, 1999).

The transcriptional activator protein LasR belongs to the family of LuxR type transcriptional regulators, and has two domains, the N-terminal autoinducer binding domain and the C-terminal DNA binding domain with a DNA binding Helix-Turn-Helix (HTH) motif and thus involved in regulating the target gene expression by recognizing a conserved DNA sequence (Chowdhury & Bagchi, 2016). The *Pseudomonas aeruginosa* LasR protein in coordination with OdDHL helps the protein to dimerize, form a complex and in return the formed complex binds specifically and with high affinity to las-responsive quorum-controlled promoters, exhibiting DNA-binding activity in the presence of OdDHL (Kiratisin, Tucker, & Passador, 2002; Schuster, M., Urbanowski, & Greenberg, 2004; You et al., 1996).

As mentioned previously, the las system was found at the top of the hierarchy, and it followed the regulatory mechanism illustrated in Figure 1: The las system positively controls both rhl and pqs system genes that code for receptors (rhlR and pqsR) and synthase genes (rhlI and pqsH) (Soukarieh, Williams, Stocks, & Cámara, 2018). Moreover, IQS production is activated by the las system (Lee et al., 2013). Therefore, the las system positively regulates the 3 other systems. Synthesized by the LasI autoinducer synthase, the signaling molecule OdDHL will bind to the transcription
factor LasR, will become active, bind to the conserved las boxes residing in the promoters of target genes and regulate the expression of these genes, among which were the virulence genes (Lee & Zhang, 2015; Welsh, Eibergen, Moore, & Blackwell, 2015). Apart from its involvement in the regulation of various virulence factors, the las system also regulates the expression of lasI itself, thereby creating a positive feedback loop known as the autoinduction loop and thus increasing the concentration of OdDHL in the environment (Papaioannou, Utari, & Quax, 2013). Although the production of OdDHL is positively autoregulated, its concentration reaches a steady level due to RsaL protein, that represses the transcription of the lasI signal synthase gene, acts as an antagonist and reduces QS signal production thus providing homeostasis by functioning in opposition to LasR and limiting OdDHL production to a physiological concentration (Rampioni et al., 2007).

The expression of lasR gene is also regulated by multiple other factors including the Vfr, GacA, and QteE (Papaioannou et al., 2013). Vfr, the virulence factor regulator and a homolog of cyclic AMP receptor protein (CRP) that was shown to be involved in controlling protease and exotoxin A production, is also a major regulator of P. aeruginosa quorum sensing since it binds specifically to the lasR promoter region and positively regulates the expression of the lasR gene (Albus, Pesci, Runyen-Janecky, West, & Iglewski, 1997). The gacA gene codes for a global regulatory protein GacA, which belongs to the FixJ/DegU family of two-component regulatory systems and is involved in controlling the production of extracellular metabolites such as antibiotics in addition to toxins and enzymes (Laville et al., 1992). It has been identified that, GacA in P. aeruginosa activates the expression of lasR and rhlR and modulates the production of BHL thus acting as a positive regulator of these systems (Reimmann et al., 1997). QteE
(quorum threshold expression element) has a negative feedback loop on the las system. At low (prequorum) culture densities, QteE activity dominates, reducing the stability of LasR protein, however, when the cell density increases to its threshold, the balance between QteE and LasR shifts, so that QteE’s inhibitory activity overcomes due to an increase in lasR expression or because the activity of another inhibitor of LasR declines, or by activation of a QteE inhibitor, so this increase in the relative activity of LasR to QteE could induce the expression of quorum-responsive genes, including the signal synthases, and initiate the positive feedback of the QS circuit (Siehnel et al., 2010).

In addition to the above stated regulators, another protein was characterized to control the quorum sensing system in P. aeruginosa. The vqsR gene, that codes for the VqsR (virulence and quorum-sensing regulator) protein possesses a las box and thus it is under the control of the LasR-OdDHL complex and was proved to be an important regulator because the inactivation of vqsR abrogated the production of the two major AHLs: OdDHL and BHL and decreased the secretion of pyocyanin and proteases (Juhas et al., 2004).

1.3.2.3 RhlI/RhlR system

In the RhlI/RhlR system, known as the rhl system, RhlI synthesizes the AHL signal, N-butyrylhomoserine lactone (BHL), and RhlR acts as the transcriptional regulator (Nelson, D'Amours, Sproule-Willoughby, Morck, & Ceri, 2009). The analysis of quorum-induced genes suggests that the signal specificities and the timing of gene expression are on a continuum where some genes are induced early in growth, some
during the stationary phase and most genes are induced at the transition from the logarithmic phase to the stationary phase, and the level of the signal receptor, LasR, is a critical trigger for quorum-activated gene expression (Schuster, Martin, Lostroh, Ogi, & Greenberg, 2003). Similar to the las system, and based on the transcriptome analysis, transcript levels of rhlR increase at the transition from logarithmic to stationary phase during the growth of *P. aeruginosa* (Schuster, Martin & Greenberg, 2007).

In 1995 researchers reported the production and the use of a second AHL (factor 2) by *P. aeruginosa* and termed it *N*-butyrylhomoserine lactone (Pearson et al., 1995). The formation of this autoinducer (C4-HSL) was catalyzed by the synthase RhlI from S-adenosyl methionine and *N*-butyrylacyl carrier protein (Raychaudhuri, Jerga, & Tipton, 2005). The transcription and the expression of rhlI gene is under the control of two signals, where rhlI responds to both LasR-OdDHL and RhlR-BHL complexes by binding to the las-rhl boxes in the rhlI promoter region (Gilbert, Kim, Gupta, Greenberg, & Schuster, 2009).

The RhlR transcriptional regulator of *P. aeruginosa* shares both C- and N-terminal homology with LuxR and LasR regulatory proteins where the C-terminal domain contains a helix-turn-helix motif characteristic of DNA binding protein and the N-terminal domain has the autoinducer binding domain and in this case necessary for the binding of the BHL autoinducer (Latifi et al., 1995). It was demonstrated that, similar to that of LasR, RhlR multimerization is dependent on its cognate autoinducer C4-HSL and multimerization is necessary for transcriptional activity (Lamb, Patel, Montminy, Wagner, & Iglewski, 2003).
The *rhl* signaling system, which derives its name from its role in rhamnolipid biosynthesis, an important microbially derived surface active agent (Biosurfactants) produced by Pseudomonas *spp.* is under the control of two interrelated quorum sensing systems; namely *las* and *rhl* possessing antibacterial, antifungal and antiviral properties and plays an important role in motility, cellular interactions, cellular differentiation and formation of water channels that are characteristics of *Pseudomonas* *spp.* biofilms (Dusane et al., 2010; Raychaudhuri et al., 2005). The *rhl* system is under the control of the *las* system (Figure 1). When OdDHL accumulates in a bacterial population with increasing cell density, it will interact, at a critical threshold level, with the transcriptional regulator LasR, stimulating the expression of *lasI*, and thus generating the first autoinduction loop (Reimmann et al., 2002). The LasR-OdDHL complex in return will positively control the expression of *rhlR*, encoding the transcriptional regulator of the second autoinduction system, govern the expression of the *rhlI*, and the resulting protein RhlI will synthesize the BHL autoinducer and together with RhlR, the complex will induce the transcription of various virulence associated genes and also *rhlII*, thereby creating the second autoinduction loop (Lixa et al., 2016; Reimmann et al., 2002). It is important to mention that the *rhl* system is not only positively regulated by the *las* system but also by the quinolone system, where PQS (pseudomonas quinolone signal) produced maximally in late stationary phase and thus a signal not involved in sensing cell density, induces the *rhlII* and *rhlR* genes increasing the production of BHL in the environment (McKnight, Iglewski, & Pesci, 2000).

The PQS synthesis cluster was revealed to be made up of *pqsABCDE* operon where PqsA acts as a ligase, PqsB, C and D as synthases, PqsE as thioesterase in addition to the *pqsH* gene under the control of LasR that encodes for an enzyme, all of
which are involved in the synthesis of the quinolone signal PQS from HHQ (2-heptyl-4-quinolone) (Lin, Cheng, Wang, & Shen, 2018). The transcriptional regulatory protein PqsR, also known as the MvfR, was required for pqsABCDE expression and thus was identified as the master regulator of the PQS biosynthetic gene cluster (McGrath, Wade, & Pesci, 2004). What is interesting however about the pqs system, is that although the PqsR-PQS positively regulates the rhl system (Figure 1), it is negatively regulated by the same system, suggesting that a regulatory chain occurs where pqsR is under the control of LasR and RhlR, while PqsR, in turn, controls pqsABCDE, which is required for the production of PQS (Wade et al., 2005).
Figure 1: Quorum sensing systems in *Pseudomonas aeruginosa*. A simplified schematic representation of the mechanism of regulation of three quorum sensing systems *las*, *rhl* and *pqS* in *P. aeruginosa*. Black arrows indicate positive regulation, flat red arrows represent negative regulation, dashed arrows indicate autoinduction, and double-sided arrows indicate the complex formation between the R protein and the autoinducer. Created by BioRender

1.3.3 *Pseudomonas aeruginosa* indicators of QS

1.3.3.1 Indicators of quorum sensing

Quorum sensing, a global regulatory network of *Pseudomonas aeruginosa*, is a unique mechanism involved in controlling the production of several extracellular virulence factors in addition to its role in the development of mature biofilms (Strateva & Mitov, 2011; Whiteley, Marvin, Lee, & Greenberg, 1999). Sputum samples from cystic fibrosis patients chronically colonized with *P. aeruginosa* contained mRNA transcripts for the QS genes *lasR* and *lasI* correlating with the accumulation of transcripts of various QS-regulated genes, suggesting that QS regulates the expression of various virulence factors during *P. aeruginosa* infections (Smith, R. S. & Iglewski, 2003). Referring to the above statement, we can infer that, virulence factors can be potential indicators to detect quorum sensing and to study the different mechanisms controlled by quorum sensing.

In the *las* system, the diffusible molecule OdDHL activates and interacts with the transcription factors LasR, reaches a recognized threshold concentration in the cell
environment, increases the lasI expression and triggers the production of virulence factors (Rasamiravaka & El Jaziri, 2016). The virulence factors regulated by the las system include: katA and sodA genes for catalase and superoxide dismutase cofactored by manganese [Mn] respectively, two genes essential for relieving oxidative stress by reducing two hazardous by-products of aerobic respiration superoxide anion (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) (Figure 2) (Hassett et al., 1999). The activity of toxA, encoding the Exotoxin A, belonging to a class of bacterial ADP-ribosyl transferases, increases in the presence of LasR, indicating that this virulent gene is also under the control of las system (Gambello, Kaye, & Iglewski, 1993; Gray et al., 1984). Moreover, the product of the lasR gene (LasR) was shown to be required for the transcription of aprA which encodes for alkaline protease (Passador, Cook, Gambello, Rust, & Iglewski, 1993). LasA, another proteolytic enzyme encoded by lasA gene, involved in the proteolysis of host proteins and having staphylolytic activity, has been verified to be under the control of the las system since the lasR gene product was identified as the transcriptional activator of this gene (Toder, Ferrell, Nezezon, Rust, & Iglewski, 1994). The production of extracellular proteins is an important property of Gram negative bacteria and they require secretion systems to achieve this process, and to do that, exoproteins have to pass through the inner membrane first and then translocate across the outer membrane; a process achieved by proteins encoded by xcp genes (Akrim et al., 1993). The lasR gene product, together with its cognate autoinducer OdDHL are involved in the activation of 2 xcp genes, xcpR and xcpP, encoding transport proteins and enhancing the pathogenesis of this opportunistic pathogen (Chapon-Hervé et al., 1997).
When mentioning virulence genes set under the control of the major las system, it is extremely important to refer to the lasB elastase structural gene that was one of the highly studied genes in the quorum sensing research in *P. aeruginosa*, and was shown to require both lasR and lasI for maximal expression (Passador et al., 1993).

In *P. aeruginosa*, lux box-like sequences were identified upstream of a number of genes other than the ones stated above and included: rhlAB and lecA located under the direct control of the rhl system (Winzer & Williams, 2001) (Figure 2). The binding of activated an RhlR protein to target sites upstream of the rhlA promoter, enhances transcription of the rhlAB operon, two genes which encode rhamnosyltransferase, the key enzyme of rhamnolipid biosynthesis (Ochsner & Reiser, 1995). A strong pathogen should have the ability to colonize the host once it infects, and colonization is enhanced by adhesion, hence the role of lectins encoded by the lecA gene in *P. aeruginosa*; functioning as adhesins, in addition to, cytotoxins for respiratory epithelial cells, and controlled tightly by the rhl system via the RhlR-BHL complex (Winzer et al., 2000).

Optimal transcription of the sodB gene, encoding superoxide dismutase cofactored by iron [Fe] required the BHL autoinducer complex with RhlR, thus controlled by the rhl system (Hassett et al., 1999). Production of pyoverdine siderophore and pyocyanin secondary metabolite have also been placed under the control of RhlR system (Brint & Ohman, 1995; Stintzi, Evans, Meyer, & Poole, 1998). The rpoS gene, encoding the alternative sigma factor RpoS, functions by acting as the central regulator for many stationary phase genes, as well as, the master stress response regulator providing antibiotic tolerance to *P. aeruginosa* during stress conditions such as heat stress and during the stationary growth phase (Murakami et al., 2005). Some studies reported that
the transcription of this gene is under the control of the rhl system, while others showed the regulation of rhlI gene being under the control of RpoS (Kojic & Venturi, 2001; Whiteley, M., Parsek, & Greenberg, 2000).

Some of the genes that were identified to be under the control of the las system as stated above, are also under the control of the second autoinducer-responsive regulatory mechanism RhII/RhlR (Figure 2) and they include katA, aprA, lasA, lasB and xcp genes (Brint & Ohman, 1995; Chapon-Hervé et al., 1997; Hassett et al., 1999; Winson et al., 1995). Virulence factors of Pseudomonas aeruginosa include also hydrogen cyanide (HCN) synthesized by HCN synthase encoded by hcnABC genes, a secondary metabolite maximally produced at low oxygen tension and high cell densities during the transition from exponential to stationary growth phase and the expression of hcn genes relying on a synergistic action between LasR and RhlR (Pessi & Haas, 2000).

For the purpose of this study, the indicator and the virulence factor that was selected to continue this research with is the lasB gene and the gene product elastase.
Figure 2: Venn diagram of some QS regulated virulence factors. The diagram shows a summary of some of the virulence genes involved in the pathogenesis of *P. aeruginosa* and controlled by the quorum sensing systems *las* and *rhl*.*toxA* encoding exotoxin A, *sodAB* encoding superoxide dismutases, *aprA* encoding alkaline protease, *lasA* encoding staphylolytic protease, *lasB* encoding elastase, *xcpPR* encoding proteins for the secretion system, *katA* encoding catalase, HCN (hydrogen cyanide), *lecA* encoding lectin, *rhlAB* (rhamnolipid biosynthesis genes) and *rpoS* encoding sigma factor S.

1.3.3.2 Genetics of *Pseudomonas aeruginosa* elastase

In 1980, a group of researchers characterized a mutant strain of *P. aeruginosa* PAO-E64 having a specific elastase deficiency when compared with the PAO1 parental strain, and thus genotyped the mutant PAO-E64 as *lasA*1, suggesting that the structural gene for elastase was *lasA*1 and had alterations, thus giving an altered enzyme (Ohman, Cryz, & Iglewski, 1980). In 1987, the *lasA* gene was cloned in mucoid and non-mucoid strains of *P. aeruginosa*, to study and investigate the role of *lasA* gene product in its elastolytic activity (Goldberg & Ohman, 1987). Till then, researchers were considering *lasA* as the structural gene for elastase. However, in 1987, (Schad et al., 1987) cloned two genes from *P. aeruginosa* involved in elastase production and activity, isolated and partially characterized the gene involved in elastase production, proposing the gene to be *lasB*.

In 1988, a group of researchers successfully cloned and sequenced the *lasB* gene (Bever & Iglewski, 1988). The nucleotide sequence of the 3 Kb fragment containing
lasB gene revealed an open reading frame of 1,491 nucleotides which coded for a 497 amino acid peptide with a molecular weight of 53,600, the amino acid sequence containing a putative signal sequence followed by a large polypeptide, thus resulting in a mature 33,000 Dalton elastase protein (Bever & Iglewski, 1988). The sequencing of this gene was confirmed with at least two other groups, showing similarities with previous studies (Fukushima et al., 1989; Raftari, Ghafourian, Sadeghifard, & Bakar, 2013). In 1996, a study was conducted to analyze the lasB promoter region to learn more about lasB activation by LasR and its cognate autoinducer OdDHL. The findings showed that upstream of lasB, in the regulatory region, there were two operators OP1 and OP2, with specific nucleotide sequences involved in LasR and OdDHL mediated transcriptional activation of lasB (Rust, Pesci, & Iglewski, 1996).

1.3.3.3 Characterization of the elastase enzyme

P. aeruginosa strains differ in their ability to produce elastase, with some strains producing elastase and thus being categorized as elastase positive, and others not able to produce elastase and known as elastase negative strains (Mandl, Keller, & Cohen, 1962). Researchers have found that P. aeruginosa produces three proteinases (neutral, semi-alkaline and alkaline) that can be fractionated chromatographically, elastase positive strains producing two of them (denoted as fractions II and III), but elastase negative strains producing only one (fraction III). The third proteinase (fraction I) was detected in both positive and negative strains and seems to be of negligible activity (Morihara, Kazuyuki, 1964). Elastolytic activity corresponded to fraction II and the alkaline proteinase activity of fraction III (Morihara, Kazuyuki, 1964).
Elastase is a typical metallo-neutral proteinase - a Zn enzyme, including Zn and negligible amounts of Fe, where Zn is firmly bound to the enzyme (Morihara, Kazuyuki & Tsuzuki, 1975). The overall tertiary structure of \textit{P. aeruginosa} elastase is similar to the known structure of thermolysin from \textit{Bacillus thermoproteolyticus} and to \textit{Bacillus cereus} neutral proteinase, having calcium binding site with a single calcium atom capable of binding to the enzyme (Thayer, Flaherty, & McKay, 1991). The enzyme binds Ca for stability, while Zn was needed for catalysis (3 zinc ligands) (Fukushima et al., 1989).

Elastase obtained from culture solutions of \textit{P. aeruginosa} had the following properties: It has an isoelectric point near pH 5.9, the optimum pH of the enzyme in 0.1M Tris buffer being 7.2, in 0.03M Tris 8 and pH 7 in 0.03M carbonate, thus a pH ranging from 7 to 8 (Morihara, K., Tsuzuki, Oka, Inoue, & Ebata, 1965). Unlike most proteins that are inactivated at 60-65 ℃, elastase activity stayed stable up to 70 ℃, but unlike temperature, several inhibitors of the enzyme were identified, among them were EDTA and o-phenanthroline, in addition several substrates were subjected to hydrolysis by elastase such as elastin, casein, hemoglobin, egg albumin and fibrin (Morihara, K. et al., 1965).

In terms of substrate specificity, pancreatic elastase has shown selectivity for bonds that involve the carboxyl groups of aliphatic amino acids such as valine, leucine, serine, alanine, and glycine, \textit{P. aeruginosa} elastase, on the other hand, acted on alanine, leucine, and glycine, which makes sense since elastase is capable of hydrolyzing elastin and elastin itself is mainly composed of the amino acids glycine, alanine, leucine,
isoleucine, valine, and proline, which account for about 94% of the dry matter (Morihara, Kazuyuki & Tsuzuki, 1966).

Production, folding and secretion of the enzyme is not a simple mechanism but involves a series of steps (Raftari et al., 2013). Expression of lasB gene within the cytoplasm generates a precursor known as the pre-proelastase (53.6 kDa), containing a signal sequence (the –pre signal peptide at the amino terminal), the propeptide and a carboxy terminal mature domain (McIver, Kessler, Olson, & Ohman, 1995). Processing and export of the pre-proelastase from the cytoplasm to the periplasmic fraction and then outside the outer membrane involves the formation of two periplasmic enzymes: Proelastase I and Proelastase II (Kessler & Safrin, 1988). During translocation, in the inner membrane, the –pre signal (2.6 kDa) is removed generating the Proelastase I (51 kDa) that is cleaved via autoproteolysis in the periplasm to generate the Proelastase II (33 kDa) and the 18 kDa propeptide (McIver et al., 1995). The propeptide remains non-covalently associated with the mature 33 kDa enzyme inhibiting the proteolytic activity of the enzyme (Braun, de Groot, Bitter, & Tommassen, 1998). Association of the propeptide with the mature sequence is important for the recognition of elastase by the Xcp secretion machinery that allows the mature elastase to leave the outer membrane into the extracellular medium (McIver et al., 1995). With the propeptide being extracellularly degraded, the mature elastase enzyme gets activated (Braun et al., 1998).
1.3.3.4 Role of elastase in pathogenesis

There is no doubt that a pathogenic organism with many virulent factors will attack the host immune system. This is the case with *Pseudomonas aeruginosa*. This opportunistic pathogen producing elastase, exerts many effects on the host such as the tissue damaging and lethal effect and also interferes with the host defense at various sites of the body causing many infections including eye, pulmonary, burn and bacteremic infections (Wretlind & Pavlovskis, 1983).

Below are some of the immune related factors that are affected by the proteolytic enzyme elastase of *Pseudomonas aeruginosa*. In a study conducted by Wheeler and Holder in 1984, SDS-PAGE of normal human IgG after incubation with elastase showed a total degradation of the antibody; this effect was inhibited when the cells were treated with the serum protease inhibitor α2-macroglobulin (Wheeler & Holder, 1984). A single component vaccine containing only 10 μg of elastase toxoid also protected mice from bacterial dissemination concluding that besides α2-M, immunization can protect against elastase enzyme (Tamura, Suzuki, & Sawada, 1992). During pulmonary infections, a large number of polymorphonuclear (PMN) leukocytes mobilize at the site of the infection to get rid of the foreign body, however, the power of *P. aeruginosa* elastase exceeds that of PMN cells, because this enzyme inhibits chemotaxis of neutrophils by degrading neutrophil surface receptors and thus making the recovery harder (Kharazmi, Doering, Hoeiby, & Valerius, 1984).

Besides antibody and PMN cells defenses, natural killer (NK) cells are always ready to fight against infectious agents, but not against this virulence factor specifically, since a concentration of 25 μg/ml elastase showed a total inhibition of NK cells by
inhibiting the conjugation of NK cells with the target *P. aeruginosa* cells by cleaving the surface receptors of the immune cell (Pedersen & Kharazmi, 1987). Lysozyme, an enzyme secreted mainly by the epithelium, alveolar macrophages, submucosal tracheal glands and immune cells has antibacterial activity (Konstan et al., 1981). In vitro studies showed that *P. aeruginosa*, but not human leukocyte elastase, inactivates airway lysozyme by cleaving the enzyme (Jacquot, Tournier, & Puchelle, 1985). Complement system consisting of “C” proteins, also plays a role in defending hosts against infections by inducing inflammatory responses but these proteins such as C1, C3, C5, C8 and C9 get degraded by elastase while C2, C4, C6 and C7 get inactivated (Schultz & Miller, 1974).

*P. aeruginosa* elastase also interferes with the mechanism of phagocytosis by degrading surfactant protein A (SP-A) found in pulmonary surfactant in the lungs, and thus inhibits opsonization and phagocytosis (Kuang et al., 2011).

Moreover, collagen, a major structural component of all connective tissues, is affected by *P. aeruginosa* infection (Heck, Morihara, McRae, & Miller, 1986). Infected tissues often show effects such as necrosis, hemorrhage and microabscess formation, and in vitro studies verified that elastase causes the cleavage of intestinal collagens I and III found in dermis, the lungs and blood vessel walls as well as type IV collagen found in the basement membrane and thus probably had a role in causing these outcomes (Heck et al., 1986).

Last but not least, the wound healing mechanism during the recovery process, can be interrupted by elastase, that exerts several damaging functions such as degradation of human wound fluid, inhibition of skin fibroblast growth and the
degradation of proteins secreted by fibroblasts (Schmidtchen, Holst, Tapper, & Björck, 2003).

Therefore, elastase, a major proteinase of *P. aeruginosa* backs *P. aeruginosa* pathogenesis by targeting different host factors.

1.3.3.5 Elastin as a substrate of elastase

Elastin, a major extracellular matrix protein, encoded by *ELN* gene, and whose expression is affected by several factors such as TGF-β, TNF-α, Vitamin D, corticosteroids and many others, is primarily found in skin, aorta and lungs (Harding & De Matteo, 2015; Karsdal, 2016). It is well known that elasticity and resilience are two characteristics essential for dynamic tissues, and elastin is the one providing such characteristics to the tissues (Schräder et al., 2018).

Elastin is one of the most hydrophobic proteins, it consists of hydrophobic domains and over 75% of the sequence comprises non polar amino acids such as glycine, valine, alanine and proline (Wise, Mithieux, & Weiss, 2009).

It is a very important protein of the terminal airspaces, whereby its formation is achieved via lysine mediated cross linking of its soluble precursor tropoelastin that weighs around 60-72 kDa (Harding & De Matteo, 2015; Wise et al., 2009).

As mentioned previously, and being one of the targets of *P. aeruginosa* elastase, elastin has been proved to be a necessary protein for normal branching and development of terminal airspaces including alveolarization (Harding & De Matteo, 2015).
1.3.4 Autoinducers

1.3.4.1 OdDHL

The identification of the las system autoinducer was performed when a group of scientists compared the Vibrio fischeri autoinducer (VAI) to that of P. aeruginosa autoinducer (PAI) and found that the purified PAI was similar to that of VAI, however the proton NMR showed a large methylene peak that wasn’t present in VAI at 1.22 ppm, indicating that the purified autoinducer was N-(3-oxododecanoyl) homoserine lactone (Pearson et al., 1994). Fast atom bombardment mass spectrometry (FAB-MS) gave an m/z ratio of 298.2018, corresponding to a chemical composition of C_{16}H_{27}NO_{4} with 12 Carbon atoms corresponding to the acyl chain and the remaining 4 to the lactone ring (Pearson et al., 1994).

LasI, the autoinducer synthase of the las system and which produces predominantly N-[3 oxododecanoyl]-L-homoserine lactone has a substrate-binding pocket for two substrates: SAM and a common binding site for the acyl-ACP phosphopantetheine group (Cooley, Chhabra, & Williams, 2008). The analysis of synthetic OdDHL showed that this bulky lipid molecule had the same biological activity as the purified natural PAI (Pearson et al., 1994).

1.3.4.2 BHL

In addition to OdDHL, below are some properties of the second autoinducer produced by P. aeruginosa. This autoinducer was purified from cultures of P. aeruginosa and the proton NMR spectrum was similar to that of N-acylhomoserine
lactones, and analysis of the NMR showed that the purified factor was N-(tetrahydro-2-oxo-3-furanyl) butanamide also termed as N-butyryl homoserine lactone (Pearson et al., 1995). FAB-MS gave an m/z ratio of 172.0972, corresponding to a chemical composition of C_{8}H_{13}NO_{3} (Pearson et al., 1995). The production of BHL relies on the autoinducer synthase RhlI, which plays a critical role in forming an amide bond between the two major substrates of an N-acyl homoserine lactone; SAM and acyl group (Parsek, Schaefer, & Greenberg, 1997). SAM plays as the best amino donor and the butyryl-ACP as the acyl donor and the enzyme catalyzes the formation of the amide bond between these 2 (Parsek et al., 1999). Briefly, the mechanism of AHL production occurs when the two substrates bind the RhlI synthase, acylation and lactonization reaction occurs, and the product BHL in addition to two byproducts are released (Watson, Minogue, Val, von Bodman, & Churchill, 2002). The product BHL has a lactone ring (4 Carbon atoms) and an acyl-chain (4 Carbon atoms) (Churchill & Chen, 2011). Studies showed that the biological activity of the synthetic BHL was similar to that of the natural P. aeruginosa BHL (Pearson et al., 1995).
Aim of the study

The present study was initiated by detecting the elastolytic activity of 48 *Pseudomonas aeruginosa* clinical isolates, by using elastin as the major substrate. Later it was intended to investigate the effect of the two major autoinducers, OdDHL and BHL, produced by this opportunistic pathogen, on a virulence gene: *lasB*.

As previously mentioned, QS is a cell density dependent process with a certain threshold concentration of AHL autoinducer molecules to be reached for the bacterium to initiate the expression of virulent genes.

So our objective was to induce the transcription of the virulence gene *lasB*, using the two synthetic autoinducers, during very early stages of bacterial growth, artificially creating an environment that resembles the high cell density environment, and redirecting the pathogen to activate the QS circuit and communicate even at very low cell density levels. This in turn might lead to the activation of other genes and trigger the organism not only to colonize at one particular location, but also to invade and spread to other body parts, with low population density and eventually be suppressed by the host immune system.
CHAPTER II

Materials and Methods

2.1 Bacterial isolates

A total of 48 bacterial isolates of *Pseudomonas aeruginosa* were used in this study. The clinical isolates were provided by the Clinical Microbiology Laboratory of the Lebanese American University Medical Center- Rizk Hospital (LAUMC-RH). The sources of the isolates are listed in Appendix Table A1. Of the 48 isolates, those designated as: PA1, PA2, PA15, PA25 and PA39 were used for further study: PA15 as an elastase positive control while the others for their elastase negative result.

2.2 Identification of *Pseudomonas aeruginosa*

Definitive identification of all bacterial samples was performed using the RapID Non-Fermenter System (Remel, USA), following the manufacturer’s instructions.

2.3 Elastase assay using elastin-nutrient agar plates

The Elastolytic activity of elastase, coded by the *lasB* elastase gene, of the study isolates, was assessed by the method introduced by Janda & Bottone in 1981, with slight modifications. Nutrient agar plates were prepared containing 1% elastin from bovine neck ligament (Sigma, USA) as substrate. The thickness of the agar in each plate was 4 millimeters. Using fresh cultures incubated overnight at 35°C, spot inoculation of all 48
clinical isolates was carried out with an inoculum covering about 2 millimeters of the center of the agar surface. The plates were incubated at 35°C for 72 hours. The presence or absence of a clear opaque zone around the inoculum was detected and the diameter of the zone was measured in millimeters (Janda & Bottone, 1981).

2.4 Bacterial genomic analysis

2.4.1 Bacterial DNA extraction

Bacterial DNA of the desired clinical isolates was obtained using the NucleoSpin® Tissue Kit (Macherey-Nagel, Germany) following the manufacturer’s instructions. The concentration and purity of the extracted DNA was measured using the Genova Nano micro-volume spectrophotometer (Jenway, UK).

2.4.2 PCR amplification of QS genes

The PCR amplification of the 5 quorum sensing genes was performed as follows: A PCR mixture of 40 μl containing 0.2 mM dNTP mix, 1.5 mM MgCl₂, 1.5 U DNA polymerase, 1X reaction buffer (Thermo Scientific, USA) and 0.4 μM of both forward and reverse primers (Appendix Table A2). DNA amplifications were performed on the thermal cycler T100™ (BIO-RAD, USA).

The primers used to amplify the 153 bp fragment of lasB gene had the following cycle conditions: initial denaturation at 94 °C for 4 minutes, 25 cycles of denaturation at 94 °C for 1 minute, annealing at 51 °C for 40 seconds and elongation at 72 °C for 1 minute and a final extension at 72 °C for 4 minutes.
The primers used to amplify the 295 bp fragment of lasI gene and 130 bp fragment of lasR gene had the following cycle conditions: initial denaturation at 94 °C for 4 minutes, 25 cycles of denaturation at 94 °C for 1 minute, annealing at 52 °C for 1 minute and elongation at 72 °C for 1 minute and a final extension at 72 °C for 4 minutes.

The primers used to amplify the 143 bp fragment of rhlI gene had the following cycle conditions: initial denaturation at 94 °C for 4 minutes, 25 cycles of denaturation at 94 °C for 1 minute, annealing at 53 °C for 1 minute and elongation at 72 °C for 45 seconds and a final extension at 72 °C for 4 minutes.

The primers used to amplify the 207 bp fragment of rhlR gene had the following cycle conditions: initial denaturation at 94 °C for 4 minutes, 25 cycles of denaturation at 94 °C for 1 minute, annealing at 55 °C for 1 minute and elongation at 72 °C for 45 seconds and a final extension at 72 °C for 4 minutes.

The PCR products of all 5 genes were separated on 1% agarose gel stained with ethidium bromide using TAE buffer and visualized under UV using Chemidoc machine (BIO-RAD, USA).

2.5 Elastin congo red assay

The Elastolytic activity of elastase, coded by the lasB elastase gene, of the study isolates, was quantified using a modified procedure of the elastin congo red (ECR) assay introduced by Ohman et al. (1980).
2.5.1 Culture condition

The PTSB liquid medium was prepared by mixing 5% bacteriological peptone and 0.25% TSB in 100 ml distilled water and autoclaved. The medium was then inoculated using fresh bacterial samples with an optical density of 0.5 at 540 nm, adjusted using a spectrophotometer. In a sterile 15 ml conical tube, 13.5 ml of sterile PTSB medium were added and mixed with 1.5 ml of the previously inoculated medium (9:1 ratio) and tubes were incubated with maximum aeration at 35°C for 16 hours. The samples were vortexed after incubation and 1.5 ml of each sample was collected to determine the optical density at 600 nm. Sterile PTSB medium was used as the spectrophotometer blank. The samples were then centrifuged at 16,000 x g for 5 minutes and placed on ice.

2.5.2 Measuring elastase activity

In a 15 ml conical tube, 5 mg of elastin congo red (Sigma, USA) in addition to 2 ml of reaction buffer (0.1 M Tris-maleate buffer, pH=7.5) and 1 ml of PTSB culture supernatant were added to the tube. The tubes were capped and incubated horizontally at 37°C with rapid shaking (250 rpm) for 18 hours in the shaking incubator. After incubation, the reaction was terminated by adding 2 ml of 12 mM EDTA and the tubes were placed on ice. The tubes were then centrifuged at 14,000 x g for 5 minutes and the optical density of each sample was measured at 495 nm. Sterile PTSB medium was used as the spectrophotometer blank.
2.6 Autoinducer solutions

Two autoinducers, OdDHL (N-3[oxododecanoyl]-L-homoserine lactone) and BHL (N-butyryl homoserine lactone) (Sigma) were used in this study. A stock solution for each autoinducer was prepared.

2.6.1 OdDHL solution

Twenty milligrams of OdDHL powder (Sigma, USA) were mixed in 1 ml, 100% DMSO (maximum solubility) to reach a stock solution concentration of 0.067M.

To obtain the desired concentrations, the following procedure was followed: For a solution concentration of 50 μM, stock solution was diluted by mixing it with sterile deionized water. For solution concentrations of 100 μM, 150 μM and 200 μM, the stock solution was diluted by mixing it with a 15% DMSO solution. The DMSO solution, diluted in sterile deionized water, had no antibacterial effect.

2.6.2 BHL solution

Twenty-five milligrams of BHL powder (Sigma, USA) was mixed in 1 ml of 100% DMSO (maximum solubility: 30 mg/ml) to reach a stock solution concentration of 0.146M.

To obtain the desired concentrations, the following procedure was followed: For a solution concentration of 50 μM, the stock solution was diluted by mixing it in sterile deionized water. For solution concentrations of 100 μM, 150 μM and 200 μM, the stock
solution was diluted by mixing it with a 10% DMSO solution. The DMSO solution, diluted in sterile deionized water, had no antibacterial effect.

2.7 In vitro induction of QS by the autoinducers

To evaluate the activity of the two autoinducers OdDHL and BHL, and to assess their activity in inducing the lasB gene, the following experiments were performed:

2.7.1 Incubation period

Using the incubation period as a variable, all 5 strains PA1, PA2, PA15, PA25 and PA39 were incubated in PTSB medium in the absence or the presence of the autoinducers for 5, 6 and 7 hours.

The tubes serving as a negative control were added with 200 µl of sterile deionized water and 100 µl of the 50 µM solutions of OdDHL and BHL were added to each of the remaining tubes. The tubes were then incubated with maximum aeration at 35°C for 5, 6 and 7 hours. The exact same procedure of the elastin congo red assay was then followed in order to obtain the optical density at 495 nm and assess the effect of time, in addition to, the signaling molecules on the activity of the elastase gene.
2.7.2 Concentration of autoinducer

To determine the effect of increasing the concentrations of the signaling molecules on the activity of the elastase gene, 3 different concentrations, 100 μM, 150 μM and 200 μM of OdDHL and BHL were tested.

The tubes serving as a negative control were supplemented with 200 μl of diluted DMSO solution and 100 μl of 100 μM, 150 μM and 200 μM solutions of OdDHL and BHL were tested on each of the 5 *P. aeruginosa* strains. The tubes were then incubated for 5 hours with maximum aeration at 35°C. The exact same procedure of the elastin congo red assay was then followed in order to obtain the optical density at 495 nm and assess the effect of varying concentrations on the activity of elastase gene.

2.8 Statistical analysis

The statistical analysis of the data was performed using GraphPad Prism 8 (CA, USA). The mean and standard deviation were calculated and student’s one-tailed *t* test was conducted to determine any significant differences. All tests were considered significant with a *p* value ≤ 0.05.
CHAPTER III

Results

3.1 Detection of elastase activity using elastin NA plates

All 48 clinical isolates of *P. aeruginosa* were tested for their elastolytic activity by using nutrient agar plates supplemented with 1% elastin from bovine neck ligament. Elastase positive strains showed a clear zone around the inoculum whereas elastase negative strains lacked the zone. The results are summarized in figure 3.

![Diagram A](image)

(A) Elastase Positive

![Diagram B](image)

(B) Total=48

![Graph](image)

**Diameter of clear zone (mm)**
Figure 3: Analysis of elastase production using elastin nutrient agar plates in *P. aeruginosa*. (A) Distribution of elastase positive (44 strains, 91.67%) versus elastase negative (4 strains, 8.33%) clinical isolates of *P. aeruginosa*. (B) Analysis of elastolytic activity of some strains from different sources. Data presents the mean ± SDs of the diameter of clear zone from two independent trials. (C) Figures represent the lysis zones of three isolates of *P. aeruginosa* on elastin nutrient agar plates after 72 hours incubation at 35°C. PA15 elastase positive strain, PA39 and PA1 elastase negative strain.

The results clearly demonstrated that based on their proteolytic activity to breakdown of elastase, there were two distinct groups of *P. aeruginosa*.

3.2 Detection of the QS gene *lasB* by PCR

The presence of the QS gene *lasB*, that codes for elastase (*lasB*), was then detected by PCR. The results are shown in figure 4.
Figure 4: Detection of the QS gene lasB in clinical isolates of *P. aeruginosa*. The PCR mediated amplification of a 153 bp nucleotide segment of lasB gene of 6 different clinical isolates of *Pseudomonas aeruginosa*. The PCR products for lasB gene were separated on a 1% agarose gel using a 100 bp DNA marker.

The results in figure 4 indicate that all 6 strains carried the lasB gene and there was no difference between elastase negative strains (PA1, PA2, PA25 and PA39) and elastase positive strains (PA15 and PA34).

**3.3 Detection of elastase activity using the ECR assay**

To further validate the activity of elastase, all four elastase negative strains (PA1, PA2, PA25 and PA39) in addition to one elastase positive strain (PA15) were subjected to the elastin congo red assay for quantification of the enzyme production. The outcome is presented in figure 5.
Figure 5: Quantification of elastase using the elastin congo red assay. Elastase production was measured at 495 nm. Fresh sterile PTSB medium was used as a blank control, and PTSB medium with ECR as the negative control (Control). Values are presented as mean ± SDs of 3 independent trials (n=3). The P values were obtained by one-tailed unpaired t test with p value ≤ 0.05 considered statistically significant.

The data above showed the difference between the elastase positive strain and the elastase negative strains. When compared with the control, the elastase positive strain produced significantly high amounts of elastase. However, the elastase negative strains had almost the same absorption as the negative control, hence further verifying their negative phenotype.
3.4 Induction of QS by the autoinducers: Incubation period

To assess the activity of the 2 autoinducers OdDHL and BHL, the selected strains were incubated in the presence or absence of the autoinducers at different time intervals 5, 6 or 7 hours. The results are depicted in figure 6.
Figure 6: Induction of QS using OdDHL and BHL at different time intervals.

Biological activity of 50 μM OdDHL and 50 μM BHL on elastase production was assessed using the ECR assay. The strains were grown in PTSB medium with or without (control) the autoinducers for different time intervals. Values are presented as mean ± SDs of 3 independent trials (n=3). The P values were obtained by one-tailed paired t test with p value ≤ 0.05 considered statistically significant. (A) PA15, (B) PA1, (C) PA25, (D) PA39, (E) PA2.

The results of this experiment showed that the inducing potential of the 2 autoinducers on elastase positive and elastase negative strains, for the different time intervals tested, were not significant, especially on elastase negative strains, since the values were below 0.12 even after the addition of the autoinducers as compared to PA15, where the OD at 495 nm exceeded 1 in the presence or absence of autoinducers.
3.5 Induction of QS by the autoinducers: Autoinducer concentration

To further evaluate the activity of autoinducers on the bacterial strains, 3 different concentrations of autoinducers were used in this experiment. 100, 150 and 200 μM of OdDHL and BHL were tested on the same 5 strains at a specific incubation period of 5 hours. The results are represented in figure 7.
Figure 7: Induction of QS at different concentrations of OdDHL and BHL.

Biological activity of 100, 150 and 200 μM OdDHL and 100, 150 and 200 μM BHL on elastase production assessed by using the ECR assay. Strains were grown in PTSB medium with or without (control) the autoinducers for 5 hours. The corresponding values are presented as mean ± SDs of 3 independent trials (n=3). The P values were obtained by one-tailed paired $t$ test with $p$ value $\leq 0.05$ considered statistically significant. (A) PA15, (B) PA1, (C) PA25, (D) PA39, (E) PA2.

It can be inferred from the results that increasing the concentration of the autoinducers had no effect on the elastase negative strains, where the values of absorbance remained below 0.12. On the other hand, PA15 showed an increase in absorption at 495 nm after the addition of varying concentrations of autoinducers, but such increase was not statistically significant.
3.6 Detection of the QS genes $\text{lasI, lasR, rhlI and rhlR}$ by PCR

The next step was to look for the upstream regulatory genes that controlled and mediated the expression of the elastase gene. These were the $\text{lasI}$ and $\text{lasR}$ genes coding for the autoinducer synthase and transcriptional regulator respectively in the $\text{las}$ system and the $\text{rhlI}$ and $\text{rhlR}$ genes coding for the autoinducer synthase and transcriptional regulator respectively in the $\text{rhl}$ system. Thus all 4 regulatory genes were amplified and PCR analysis was performed on 6 different strains. Results are described in figures 8, 9, 10 and 11.

![Detection of QS gene lasI in clinical isolates of P. aeruginosa](image)

**Figure 8:** Detection of the QS gene $\text{lasI}$ in clinical isolates of $\text{P. aeruginosa}$. The PCR mediated amplification of a 295 bp nucleotide segment of $\text{lasI}$ gene of 6 different clinical isolates of $\text{Pseudomonas aeruginosa}$. The PCR products for $\text{lasI}$ gene were separated on a 1% agarose gel using a 200 bp DNA marker.
Figure 9: Detection of the QS gene lasR in clinical isolates of *P. aeruginosa*. The PCR mediated amplification of a 130 bp nucleotide segment of lasR gene of 6 different clinical isolates of *Pseudomonas aeruginosa*. The PCR products for lasR gene were separated on a 1% agarose gel using a 200 bp DNA marker.

Figure 10: Detection of the QS gene rhlI in clinical isolates of *P. aeruginosa*. The PCR mediated amplification of a 143 bp nucleotide segment of rhlI gene of 6 different clinical isolates of *Pseudomonas aeruginosa*. The PCR products for rhlI gene were separated on a 1% agarose gel using a 200 bp DNA marker.
Figure 11: Detection of the QS gene *rhlR* in clinical isolates of *P. aeruginosa*. The PCR mediated amplification of a 207 bp nucleotide segment of *rhlR* gene of 6 different clinical isolates of *Pseudomonas aeruginosa*. The PCR products for *rhlR* gene were separated on a 1% agarose gel using a 200 bp DNA marker.

The results indicated that the strains PA15, PA2, PA25, PA34 and PA39 carried all 4 regulatory genes. Only strain PA1 didn’t carry the *lasI* and *lasR* genes, the ones that are at the top of the QS hierarchy in *P. aeruginosa* and that regulated the expression of many virulence genes.
CHAPTER IV

Discussion

With the emergence of antibiotic resistance, new means for combating and treating infections are needed (Kaufmann, Park, & Janda, 2008). For the past few years, work on QS research and mainly in *P. aeruginosa* has excelled for several reasons. The possession of this human opportunistic pathogen, of an AHL based quorum sensing systems and utilizing them to control a pool of virulence factors, in addition to, biofilm formation to reinforce its pathogenicity, has made it an attractive target for research (Juhas, Eberl, & Tümmler, 2005). Since the QS system is cell density-dependent and since threshold concentrations of autoinducers are required to activate the expression of the virulence genes, the mechanism of early induction and activation of these genes was of great interest to us. Of the virulence genes controlled by QS, the lasB elastase was the subject of this study, since this gene was under the control of the two AHL dependent systems, the las and rhl systems. Therefore, we hypothesized that autoinducer directed early induction of the lasB gene can facilitate the understanding of how to regulate its expression and eventually redirect the organism to colonize parts of the body other than the site of infection, but with very low cell counts and thus becomes vulnerable and can be easily controlled by the body’s immune system.

Accordingly, autoinducer solutions of OdDHL and BHL were examined for their ability to induce the secretion of virulence factor, especially elastase in *P. aeruginosa* clinical isolates. Former studies demonstrated the effect of AHL addition on *P.*
*P. aeruginosa* wild type reference strains and their mutants. In a previous study by Li et al. (2015), the exogenous addition of Autoinducer-2 (AI-2), a universal quorum sensing molecule involved in mediating inter and intraspecies communication, showed promising results in modulating the expression of QS genes in *P. aeruginosa* PAO1 strain and controlling the production of virulence factors and biofilm formation (Li et al., 2015).

The present study confirmed that most of the *P. aeruginosa* clinical isolates were elastase positive and only four elastase negative isolates were identified (Figure 3). The failure of these isolates to produce the elastase proteolytic enzyme may have been due to the loss of the *lasB* gene that codes for the enzyme. Therefore, the 4 elastase negative strains in addition to 2 elastase positive strains PA15 and PA34 were examined for the presence of the gene by PCR. The tested strains had a 153 bp DNA fragment, indicating the presence of this gene in both groups (Figure 4). The results confirmed that the problem was not due to the loss of the *lasB* gene.

To further confirm the phenotypes of PA1, PA2, PA25, PA39 and PA15, a colorimetric assay called “the elastin congo red assay” was conducted to quantify the amount of elastase produced by these strains. When compared to the control, strain PA15 produced significantly high amounts of elastase (OD > 1), however, strains PA1, PA25, PA39 and PA2 had almost similar values as the control (OD < 0.03), further verifying their negative phenotype (Figure 5).

The *lasB* inducing ability of the 2 homoserine lactones produced by *P. aeruginosa*: OdDHL and BHL on the PA15 and elastase negative strains, was then tested. A previous study had shown that the consistent induction of elastase activity in
PAO-JP2; a \textit{lasI/rhlI} knocked out strain, required both OdDHL and BHL since BHL alone had no inducing activity (Smith, K. M., Bu, & Suga, 2003). Another earlier study had demonstrated that the addition of 25 and 100 \( \mu \text{M} \) of BHL on the PAN067 strain; a pleiotropic \textit{P. aeruginosa} mutant unable to synthesize BHL, to a certain extent restored the activity of elastase as compared to the wild type PAO1 strain (Winson \textit{et al.}, 1995). Therefore, the combination of both autoinducers at different concentrations was tested on the test strains of this study.

To assess the effect of time and the signaling molecules on the activity of the elastase gene, strains were incubated at 5, 6 and 7 hours with or without 50 \( \mu \text{M} \) of each autoinducer solution. As shown in figure 6A in the positive control strain (PA15), the production of elastase increased with time. At 5, 6 and 7 hours of incubation, the addition of the signaling molecules had minimal effect on the induction of the elastase gene. This increase of elastase production was 15.4\%, 3.91\% and 12.7\% for 5, 6 and 7 hours respectively relative to the control, but that increase was not statistically significant. This was obvious due to the minimal difference between the peaks of the control strain, with no additions, versus that with OdDHL+BHL. Recently, researchers showed that the addition of exogenous OdDHL to a \textit{lasI} mutant strain of \textit{P. aeruginosa}, unable to synthesize endogenous OdDHL, elicited elastase activity, suggesting that a functional LasR responded to its cognate ligand-OdDHL (McCready, Paczkowski, Henke, & Bassler, 2019). The elastase activity in PA1, PA25, PA39, and PA2 did not change, even with the increasing incubation period, that reflected increasing the cell density, and the absorbance remained always below 0.12, indicating the absence of any elastase production. When the culture medium used to grow the elastase negative strains was supplemented with OdDHL+BHL solutions, the value of optical density did not
vary and was always recorded to be below 0.12. Therefore, the addition of 50 μM of the autoinducers did not restore the elastase positive phenotype and had no inducing effect on the lasB gene (Figures 6B-E).

The effect of increasing the concentration of the autoinducer solutions on triggering the expression of the lasB gene on the PA15 and elastase negative strains was then tested. This was done by growing the strains in culture media to which 100, 150 and 200 μM of OdDHL and BHL were added. With respect to the control strain (PA15), the addition of 100, 150 and 200 μM of OdDHL and BHL to the culture medium had no significant inducing effect. Although the recorded absorbance was higher than that of the control, with 9.41%, 0.015% and 2.95% increase at 100, 150 and 200 μM respectively, but that was not statistically significant and did not support the idea that the lasB gene was induced by this procedure (Figure 7A). On the other hand, and even with the addition of higher autoinducer concentrations, the values of the optical density in elastase negative strains remained below 0.12 (Figures 7B-E). These results suggested that, even though genetically engineered knocked out reference strains of P. aeruginosa responded to the provided AHLs in former studies, the case was not the same in the clinical isolates of the present study.

In a previous study, researchers reported that an increase in pH was noted, during the growth of P. aeruginosa in Luria broth due to an increase in ammonia in the culture medium during aerobic respiration (Yates et al., 2002). Analysis of AHL levels, mainly OdDHL and BHL revealed that, the autoinducers produced by this organism can undergo pH-dependent hydrolysis of the lactone ring thus inactivating the autoinducer and that the rate of the ring opening was affected by the length of the acyl chain being
slower in ODDHL than BHL (Yates et al., 2002). Thus, based on these results, the investigators concluded that the rate at which *P. aeruginosa* reaches a critical threshold of AHL concentration is significantly affected by the alkaline pH and also the temperature (Yates et al., 2002).

The reason for the failure to induce the *lasB* gene, in elastase negative strains, by either increasing the cell density or the concentration of signaling molecules, was then questioned. The absence of elastase production could have been due to the possibility that the *lasB* gene itself carried a defect and was mutated, or due to the absence of the regulatory genes upstream of *lasB*, mainly the transcriptional regulators *lasR* and *rhlR*, since the strains were provided with the autoinducers, or maybe due to defective *lasR* or *rhlR* genes, that result in a defective transcriptional factor and thus the incompatibility to complex with the specific autoinducer.

In a study conducted in 1996, researchers identified 4 elastase negative strains among 105 *P. aeruginosa* clinical isolates, it was found out that one of the strains was carrying a defect in the *lasR* regulatory gene, explaining the elastase negative phenotype of that isolate (Hamood, Griswold, & Colmer, 1996). In another study, out of 94 *P. aeruginosa* isolates, 11 were elastase deficient and among them, 2 were well characterized and demonstrated a loss-of-*lasR* function, whereby the gene produced a truncated protein with a defective C-terminal DNA binding domain (Wang et al., 2018).

The possibility that the elastase deficient strains did not harbor the regulatory genes was also tested, by PCR (Figures 8-11). The four regulatory genes *lasR*, *lasI*, *rhlR* and *rhlI* were amplified and PCR analysis was performed. The outcome was the following: in PA15 and PA34, the elastase positive strains produced PCR fragments with
the predicted molecular sizes of the four regulatory genes. Besides, three out of the four elastase lacking strains (PA2, PA25, and PA39) had similar results. Thus, based on the outcome, it could be concluded that since these strains carried the genes that controlled the expression of lasB, there probably was a defect in the regulatory genes, in these elastase negative strains, mainly lasR and rhlR, since at one point, these strains did not change when supplemented with the autoinducers needed to initiate the activation of the lasB gene. lasR mutations in P. aeruginosa strains have been considered to be a mode of adaptation, especially in environments such as lung airways in cystic fibrosis patients (Hoffman et al., 2008). The analysis of QS of a P. aeruginosa isolate showed that the defective QS phenotype of this strain in producing elastase was due to the lack of functional LasR/LasI system (Morales, González-Valdez, Servín-González, & Soberón-Chávez, 2017). The absence of lasI and lasR PCR fragments in PA1 strain showed that the organism lacked the regulatory genes of the major QS circuit; the las system, thus not activating the las system and the expression of its virulence genes including lasB. This, in turn, made that strain elastase deficient.

Out of nine genotypically different strains, isolated from mechanically ventilated patients, three strains were characterized in having a deficient cell-to-cell signaling harboring lasR and rhlR defects and thus not producing elastase, pyocyanine, and rhamnolipid (Dénervaud et al., 2004). The rhl circuit, which is activated during the late log / early stationary phase, is also involved in regulating the expression of the lasB gene. The PA1 strain had the PCR fragments of the rhl regulatory genes, indicating that the strain could have been defective with alterations at the level of the rhl regulatory genes.
With the results obtained, it was concluded that the inducing ability of the \textit{lasB} gene by the 2 autoinducers is limited in elastase positive strain and not possible in elastase negative strains, under different conditions. It can, however, be pointed out that the inability to induce the \textit{lasB} gene in the test strains could have been due to the fact that the supplemented autoinducers might have been undergoing lactonolysis, thus not enhancing significantly the induction of the \textit{lasB} gene. This, however, reflects the complexity of the QS circuit employed by \textit{P. aeruginosa} and the possible presence of many factors that influence the behavior of this organism, including the environment it is living in, its cell density, its antibiotic resistance, and the host immune system. The present work highlights the importance of the phenotypic and genotypic characterization of clinical isolates to further understand their mechanisms of pathogenesis and the need to perform additional investigations to find means for controlling them and thus treat the infections they cause.

For future work, it is suggested that sequencing analysis of \textit{lasB}, \textit{lasR} and \textit{rhlR} genes be performed to determine the sequence variants of the genes between the two groups of \textit{P. aeruginosa} and better understand the mechanism leading to the elastase negative phenotype. This may validate whether elastase production is a good marker of early induction of these genes, so that future work can be done to aim at controlling this dangerous pathogen in early stages of an infection.
References


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Appendix

In vitro regulation of quorum sensing in *Pseudomonas aeruginosa*

using synthetic autoinducers

Appendix Tables

**Table A1.** *Pseudomonas aeruginosa* clinical isolates used in this study

**Table A2.** Primers used in this study
**Table A1** *Pseudomonas aeruginosa* clinical isolates used in this study

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<td>PA47</td>
<td>8921</td>
<td>Wound</td>
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<tr>
<td>PA16</td>
<td>13823</td>
<td>Urine</td>
<td>PA32</td>
<td>446</td>
<td>Sputum</td>
<td>PA48</td>
<td>4672</td>
<td>Bone</td>
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### Table A2 Primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence (5’- 3’)</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>lasB-F</td>
<td>TTCTACCCGAAGGACTGATAC AACACCCATGATCGCAAC</td>
<td>PCR lasB</td>
<td>(Sabharwal, Dhall, Chhibber, &amp; Harjai, 2014)</td>
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<td>lasB-R</td>
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<tr>
<td>lasI-F</td>
<td>CGTGCTCAAGTGTTCAAGG TACAGTCGGAAAGCCCAAG</td>
<td>PCR lasI</td>
<td>(Zhu et al., 2004)</td>
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<td>lasI-R</td>
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</tr>
<tr>
<td>lasR-F</td>
<td>AAGTGGAAAAATGGAGTGGAGTAGTTGCGGACGATGAAG</td>
<td>PCR lasR</td>
<td>(Zhu et al., 2004)</td>
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<td>lasR-R</td>
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</tr>
<tr>
<td>rhlI-F</td>
<td>CGAATTGGCTCTCTGAATCGCTTGCTCATGGCGACGATGTA</td>
<td>PCR rhlI</td>
<td>(Lavenir et al., 2008)</td>
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<td>rhlI-R</td>
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<tr>
<td>rhlR-F</td>
<td>TCGATTACCTACGCTATGGCG TTCCAGAGCATCCGGCTCT</td>
<td>PCR rhlR</td>
<td>(Lavenir et al., 2008)</td>
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<td>rhlR-R</td>
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