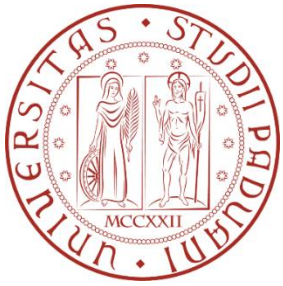

UNIVERSITA' DEGLI STUDI DI PADOVA

DIPARTIMENTO DI SCIENZE DEL FARMACO

DIPARTIMENTO DI MEDICINA MOLECOLARE

Master's Degree Course in Pharmaceutical Biotechnologies

THESIS DISSERTATION

Study of the bacterial communication in *Pseudomonas aeruginosa*: a systems and synthetic biology perspective

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ACADEMIC YEAR 2023-2024

Abstract

The major public health concern worldwide is the antibiotic resistance which is making it difficult to treat diseases caused by the pathogenic microbiome carrying multiple resistance genes. The increase in frequency of these diseases has led to the new therapeutic approaches as alternative to traditional antibiotics.

One of the innovative therapeutic approaches is the examination of bacterial cell communication system, known as Quorum sensing. These systems are laborious to study as they are regulated by a complex network of genes which are multifactorial and interconnected. The genes associated with quorum sensing are also related to toxin production and the formation of extracellular structures i.e., biofilms which can form a physical barrier against antibiotics.

Pseudomonas aeruginosa, a Gram-negative bacterium and one of the "superbugs", is known for evading the effects of multiple drugs due to its innate antibiotic resistance. This serves as a case study in this thesis work. The main objective of this thesis is to study the LasR, RhlI - RhlR systems, of the QS mechanisms present in *P. aeruginosa*. This is done by creating engineered systems for ease of study. In this we addressed and resolved issues in the Las system by optimizing ribosome binding sites (RBSs). Constructs, including pLasA RFP34, pLasB RFP34, and pLasI RFP34, were designed and transformed into *Escherichia coli* cells with LasR receptor and ÿD transcription factor. The exploration of pLasR3 promoter and its processing paralleled the study. Co-transformed systems were evaluated with LasR and RhlR receptors in the presence of auto-inductors. Bacterial growth in the presence of 3OC12-HSL, a mediator of the QS, revealed nutrient dependence in *P. aeruginosa* supernatant, impacting *E. coli* growth. Fluorescence assays indicated suboptimal sensitivity, limiting the system's practicality for screening applications, with pRhlR promoter exhibiting unique behaviour. Thorough examination of LasR receptor promoters under varying 3OC12-HSL concentrations revealed distinct expression patterns.

In the realm of Synthetic Biology, a groundbreaking approach emerges, aiming to streamline the exploration of intricate systems by untangling the involved gene components. This deliberate reduction of variables serves as a powerful method to dissect individual actors within communication networks.

The main goal is two-part: first, to carefully find possible targets for treatments, and

second, to create systems that make it easy and affordable to test molecules for their potential therapeutic effects. This approach is especially important in the fight against antibiotic resistance. It provides a strategic toolkit to deal with the challenges presented by tough bacterial strains.

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1. Introduction

1.1. Quorum sensing

Quorum sensing is a mechanism of cell-cell communication in bacteria and allows them to gather information from the environment and coordinate activities within the population. [1] It is also known as density sensing as it is related to cell density. This system consists of small chemical signal molecules which are synthesized and secreted by bacterial population. These signal molecules are known as autoinducers (AIs) or self-inducing molecules. [2]

The high-density cell population of bacteria create several signal molecules to reach a certain threshold level. Once it reaches the threshold level, the autoinducers alter the gene expression and leads to the regulation of processes in bacteria like bacterial luminescence, virulence factors, spore formation, toxin production, biofilm production and drug resistance. This quorum sensing is mainly to coordinate the behaviour of groups of bacteria to produce and regulate the genes involved in virulence and invasion. [1][2]

The quorum sensing systems are seen in both Gram-positive and Gram-negative bacteria, and it is of three major classes.

(1) Autoinducers-1 (AI-1) or acyl-homoserine lactones (AHLs) – Gram-negative bacteria.
(2) Autoinducers-2 (AI-2) – Gram-positive and Gram-negative bacteria.
(3) Auto-inducing peptides, Nisin (Antimicrobial peptide) – Gram-positive bacteria. [2]

Quorum Sensing (QS) is a pivotal mechanism governing bacterial communication, relying on the production, and sensing of diffusible signaling molecules known as Quorum Sensing Signal Molecules (QSSMs) or autoinducers (AIs). When the concentration of QSSMs in the environment reaches a critical threshold, bacterial populations synchronize the transcription of multiple genes, leading to coordinated behaviors. This diffusible signal-mediated regulation influences various activities, including swarming and swimming motility, biofilm maturation, virulence factor expression, secondary metabolite production, and antibiotic resistance. Contemporary antimicrobial strategies have explored novel avenues, such as targeting virulence factors or regulatory mechanisms, to minimize selective pressures that contribute to resistance emergence. One promising approach involves disrupting bacterial communication through interference with QS-mediated signaling using QS inhibitors (QSIs). Unlike conventional antibiotics, QSIs, which do not directly compromise bacterial

viability, are designed to attenuate virulence, allowing the host's defenses to clear the infecting bacteria. This targeted strategy aims to reduce selective pressure on resistance development. Although QSIs alone may not fully eradicate infections, especially in immunocompromised individuals, their synergistic combination with growth-inhibitory antibiotics presents a promising avenue. QSIs, particularly when used prophylactically, demonstrate potential as effective tools in the ongoing battle against bacterial infection.

1.1.1. Quorum sensing in Gram-negative bacteria:

Over 25 species of Gram-negative bacteria have been identified with Quorum sensing mechanisms. The first AHL has been discovered in *Vibrio fischeri,* a marine bacterium in which AI-1 molecule controls the production of luminescence of the bacterium. All Gram-negative Quorum sensing circuits resemble *V. fischeri* except *V. harveyi* and *M. xanthus*. The AHL molecule in *V. fischeri* is N-3-oxohexanoyl-L-homoserine lactone (3OC6-HSL). This bacterial quorum sensing is composed of

(1) AI synthase gene, LuxI for biosynthesis of specific HSL (autoinducer)
(2) Transcription activator gene, LuxR which binds to HSL, the level of HSL is increased with the increase in cell density. This complex activates target gene transcription.[3][4]
(3) Bidirectional Lux promoter

The luminescence operon is luxCDABE. After reaching high density of cell population, the increase of autoinducers (HSL) by LuxI and LuxR forms complex with HSL for gene transcription. This makes easy to engineer systems that integrate different AHL signals.[3][4]

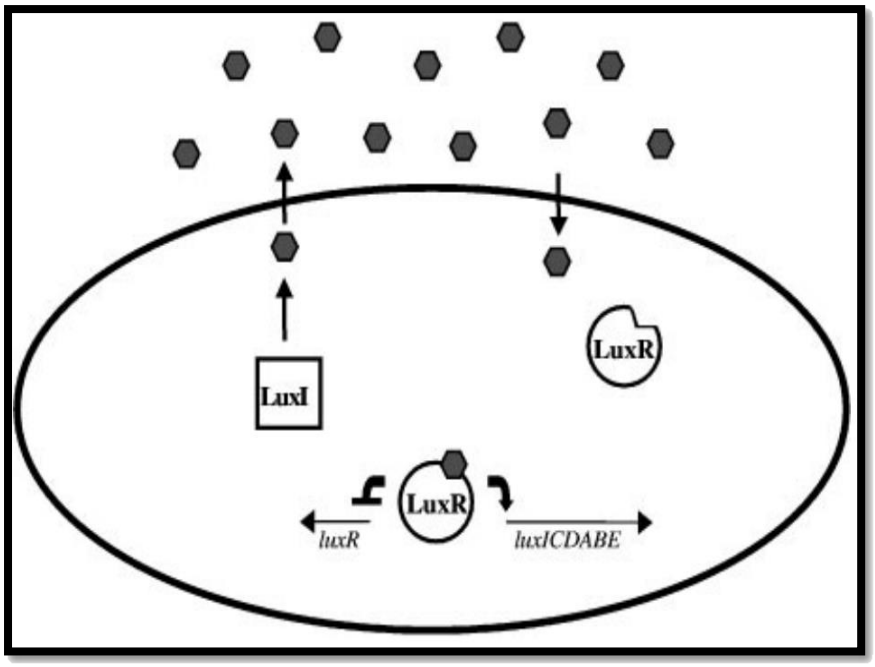

Figure 1: *Vibrio fischeri*'s LuxI/LuxR quorum sensing system controls light emission through the LuxCDABE operon and regulatory genes LuxR and LuxI. LuxI synthesizes the autoinducer N-(3-oxohexanoyl)-homoserine lactone, whose concentration, increasing with cell density, leads LuxR to activate LuxCDABE transcription. Simultaneously, LuxR represses its own transcription, maintaining a balance in the quorum sensing circuit.

1.1.2. Quorum sensing in Gram-positive bacteria:

Like Gram-negative bacteria, Gram-positive bacteria also regulate processes as the cell density increases. The autoinducers used by Gram-positive bacteria are secreted peptides via ABC (ATP binding cassette) transporter which is different from Gram-negative bacteria. Gram-positive bacteria composed of two adaptive response proteins (kinases) for the detection of autoinducers. This forms peptide ligand that activates phosphorylation or dephosphorylation for the target gene transcription. [3] Gram-positive bacteria utilize a distinct quorum sensing mechanism, diverging from the LuxR-type proteins prominent in Gram-negative bacteria. Instead, they employ two-component adaptive response proteins for autoinducer detection. This involves a phosphorylation/dephosphorylation cascade, where secreted peptide autoinducers, correlating with cell-population density, activate sensor kinases. The interaction triggers phosphorylation events leading to the activation of response regulator proteins. Once activated, these regulators bind to DNA, modulating the transcription of quorum sensing-controlled genes. Notable Gram-positive systems include those regulating competence in Streptococcus pneumoniae, competence and sporulation in *Bacillus subtilis*, and virulence in *Staphylococcus aureus*. While the core

signalling mechanism is conserved, variations in regulation enhance signal transduction effectiveness based on environmental conditions.

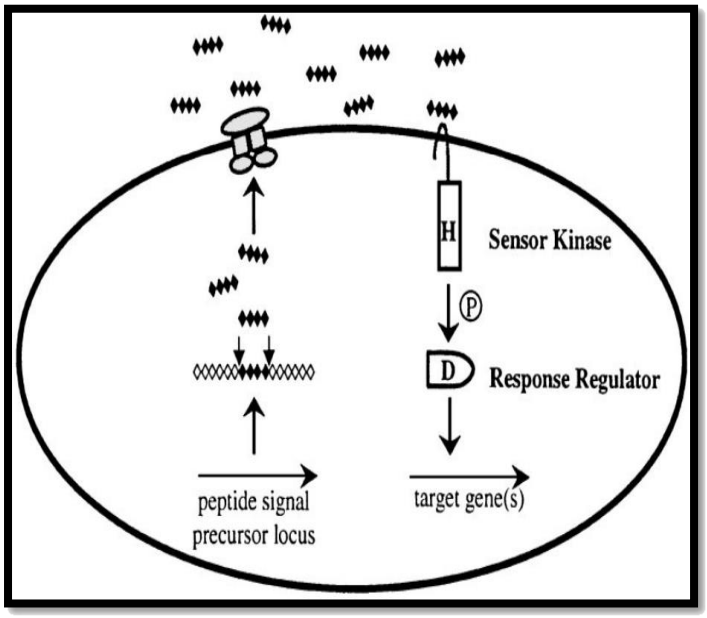

Figure 2: In Gram-positive bacteria, the peptide-mediated quorum sensing model involves the translation of a peptide signal precursor locus into a precursor protein (depicted as black and white diamonds). Cleavage of this precursor protein (indicated by arrows) generates the processed peptide autoinducer signal, represented by black diamonds.

1.1.3. Quorum sensing in Gram-positive and Gram-negative bacteria:

The AI-2 QS originated from the marine bacterium, *Vibrio harveyi* and both Gram-positive and Gram-negative bacteria can produce and sense AI-2, which enables to engineer cross-species communicating systems. For example, in the ileum of the small intestine, the bacteria such as Lactobacillus and Proteobacteria are most abundantly found. Escherichia coli belongs to Proteobacteria and communicates with other bacteria through AI-2, Indole, and the AHL signals. In cecum crypts, different bacteria are found, Clostridium, Lactobacillus, Enterococcus, Proteobacteria, and Actinobacteria. Different signals such as AIP, AHL, Indole, AI-2, and AI-3 are seen in that region. [4]

1.2. *Pseudomonas aeruginosa*:

1.2.1. As a pathogen:

P. aeruginosa is a heterotrophic, motile, facultative aerobe, Gram-negative, opportunistic pathogenic bacterium belongs to the Pseudomonadaceae family. This bacterium causes severe infections like nosocomial infections in immunocompromised patients which is life-threatening. It is a model bacterium used to study the virulence and bacterial traits. It can be isolated from a wide variety of environments like hospitals, soil, sewage and in urinary tract infections. It can use 100 organic molecules as source of carbon and energy and has an ability to grow in minimal salt medium. The optimum temperature to grow *P. aeruginosa* is 37˚C and sustain the temperature ranges from 4-42 ˚C. [5]

This disease can be spread by exposing to contaminated water or soil, also through toilets, surfaces. [6] *P. aeruginosa* rarely infects healthy ones and targets the individuals with compromised immunity which includes cystic fibrosis (CF), burns, cancer, chronic infections [5] and the individuals on the ventilators. [6] Pseudomonas aeruginosa exhibits versatile pathogenicity owing to an extensive repertoire of cell-associated and secreted virulence factors, facilitating effective host colonization and evasion of innate immune defenses. Key attributes and hallmark capabilities linked to the virulence of this pathogen comprise:

(1) factors influencing bacterial motility and attachment;

(2) elements crucial for biofilm formation;

(3) extracellular invasive enzymes and secreted toxins;

(4) production of toxic secondary metabolites; and

(5) the presence of iron acquisition systems and factors regulating iron homeostasis.

1.2.2. Virulence factors in *P. aeruginosa*:

LasR, a member of the LuxR family, functions as the primary regulator within the Las Quorum Sensing (QS) system in *Pseudomonas aeruginosa*. It operates as a transcriptional activator, perceiving the autoinducer N-(3-oxododecanoyl) homoserine lactone (3O-C12-HSL) produced by the lasI gene. As a global regulator, LasR orchestrates the expression of over 300 genes, many pivotal for virulence. These include elastase genes (lasA and lasB), the hcnABC cluster for hydrogen cyanide production, plcB phospholipase, flp-type IVb pili subunit, aprA alkaline protease, pyoverdine regulator pvdS, and toxA exotoxin genes. Additionally, LasR induces the RsaL repressor, mitigating LasR target expression.

Occupying the apex in the QS signaling network, LasR crucially activates the transcription of the Rhl and Pqs QS systems.

Notably, *P. aeruginosa* lasR and lasI mutants exhibit significantly reduced virulence in infection models, with LasR deficiency resulting in lower inflammatory responses and impaired host colonization.

Parallelly, the LuxR family regulator RhlR serves as a transcriptional activator within the Rhl QS system, detecting the N-butanoyl-L-homoserine lactone (C4-HSL) signal molecule produced by the rhII gene. RhlR activates over 100 genes, including virulence genes like rhlA and rhlB for rhamnolipid synthesis and LasR targets, such as lasB, hcnABC, and phenazine genes. In murine infection models, *P. aeruginosa* rhlR and rhlI mutants exhibit significantly reduced virulence compared to the wild-type strain.[8]

Despite the functional similarities and overlapping roles of the Las and Rhl systems, each independently contributes to virulence. The synergistic effects observed on virulence when both systems are mutated underscore their individual significance. Notably, a double lasR rhlR mutant shows lower mortality rates in the nematode *C. elegans* compared to single mutants. Similarly, a LasI RhlI double mutant displays increased lethality compared to single mutants in a burn mouse model of infection. Intriguingly, while a lasR or RhlR single mutation diminishes the cytotoxicity of *P. aeruginosa* towards lung epithelial cells, the LasR, RhlR double mutant exhibits heightened cytotoxicity compared to the wild-type strain. This suggests that some elements within the LasR and RhlR regulons may act in opposing directions.
PqsR, the third Quorum Sensing (QS) regulator in *Pseudomonas aeruginosa*, also known as MvfR, belongs to the LysR family. It senses PQS, a quinol synthesized by the pqsABCDE and phnAB operons, as well as the pqsH gene. PqsR upregulates over 100 genes, including the pqsABCDE and phnAB operons, and numerous genes from the RhlR regulon. The overlap between the PqsR and RhlR systems arises from the PqsE thioesterase, essential for PQS synthesis and RhlR activity. PqsR activates genes encoding virulence factors such as pyocyanin, hydrogen cyanide, and the lectin LecA. A PqsR mutation reduces mortality in a burn mouse model, attributed to decreased pqsE expression and consequently reduced RhlR activity.

Furthermore, PQS regulates genes involved in iron acquisition, including pyoverdine and pyochelin biosynthetic genes, likely due to PQS's iron-chelating properties. PqsR responds

not only to PQS but also its precursor, HHQ. The pqsH gene, responsible for converting HHQ to PQS, is regulated by the AraC family regulator CdpR. Interestingly, a *P. aeruginosa* CdpR mutant displayed increased mortality, lung injury, and inflammation in a mouse model of acute pneumonia, underscoring the repressor role of CdpR in this context.[8]

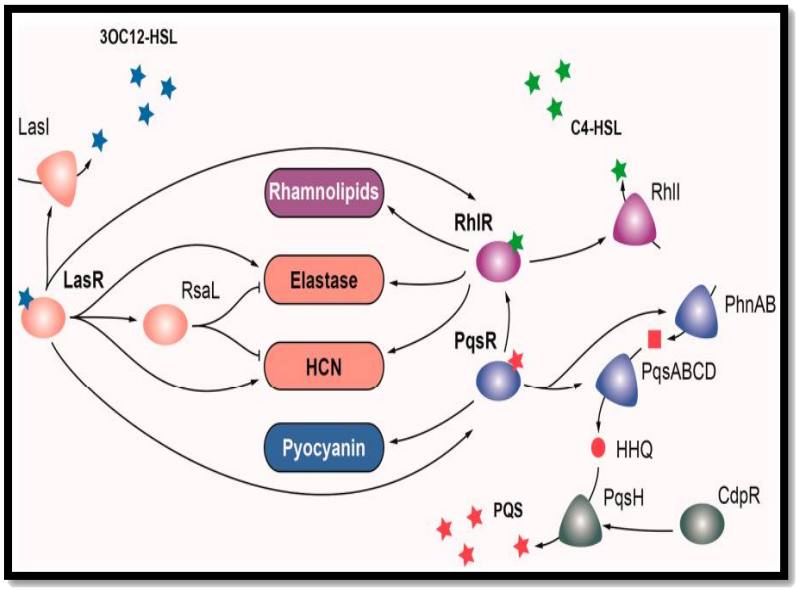

Figure 3: In *P. aeruginosa*, the regulation of virulence involves three Quorum Sensing (QS) systems: Las (pink), Rhl (purple), and PQS (blue), governed by OCS LasR, RhlR, and PqsR, respectively. Each QS system, upon detecting its signal molecules, activates the expression of virulence factors and enzymes that produce their respective inducing signals. [8]

The *P. aeruginosa* transcriptional regulator AmrZ, belonging to the ribbon-helix-helix family, primarily functions as a repressor [103]. Despite its repressive role for nearly 50 genes, AmrZ can also act as an activator, particularly influencing virulence genes like those involved in alginate synthesis and transport within the alg cluster [39,104]. By activating the alg operon, AmrZ enhances biofilm formation, a crucial factor for *P. aeruginosa* persistence and virulence. However, in response to environmental changes, including variations in oxygen, nutrients, or exposure to toxic elements like nitric oxide, AmrZ facilitates cell dispersion from bacterial biofilms. This dispersion is achieved by activating the expression of hydrolase and nuclease genes, including endA, pelA, and pslG [9].

Additionally, AmrZ plays a pivotal role in motility, functioning as a repressor for the type IV pili precursor pilA and fleQ, a c-di-GMP effector that activates flagellum synthesis gene

transcription. This multifaceted regulatory role highlights AmrZ's versatility in coordinating diverse processes within *P. aeruginosa* in response to varying environmental conditions.

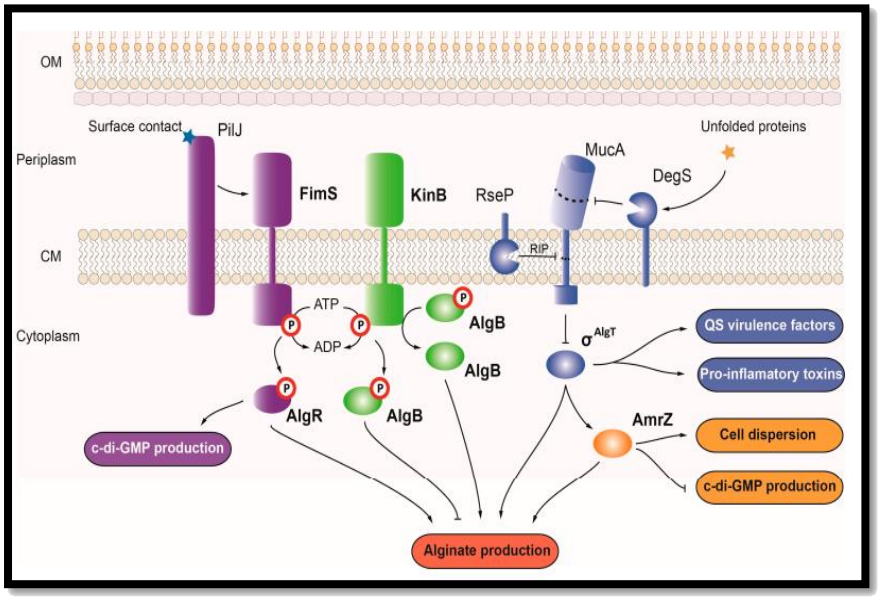

Figure 4: The regulation of alginate production and virulence in *P. aeruginosa* involves key elements: OCS AmrZ (yellow), TCSs FimS-AlgR (purple) and KinB-AlgB (green), and σAlgT factor (blue). Positive and negative controls are denoted by arrows and T-shaped lines, respectively. The process occurs at the outer membrane (OM), cytoplasmic membrane (CM), and involves regulated intramembrane proteolysis (RIP).

Two-component systems (TCSs) serve as pivotal pathways for sensing and responding to environmental cues in prokaryotes, a mechanism also found in eukaryotes [9]. In contrast to One-component systems (OCSs), TCSs comprise distinct sensor and effector domains residing in separate proteins. In the canonical TCS, the membrane-embedded histidine kinase (HK) harbors the sensor domain facing outward, initiating phosphorylation of a response regulator (RR) upon signal recognition [128]. HKs possess a conserved cytosolic core kinase domain that autophosphorylates upon signal sensing, followed by a phosphate transference reaction to the cognate RR. RRs typically feature a conserved N-terminal regulatory domain and a variable C-terminal effector domain, often involved in DNA binding. The RR's role can encompass transcriptional activation or repression, with its activity modulated by phosphatases that hydrolyze phosphate, and variations from this model include kinases with multiple phosphodonor sites. *P. aeruginosa* PAO1, a bacterium of significant interest, harbors 41 HKs, 68 RRs, and 18 hybrid proteins, showcasing the complexity and adaptability of TCSs in this pathogen [10]. Despite extensive study, the

specific signals activating these pathways, particularly in *P. aeruginosa*, remain largely unidentified.

P. aeruginosa has an ability to interrupt the host's tissue integration and damage the defence mechanism by the bacterial attachment, colonization to the host. These virulence factors are categorized into three namely bacterial surface structures, secreted factors, and bacterial cell-to-cell interaction. These are further classified into sub-categories. The bacterial structures include surface appendages like type IV pili and flagella, outer membrane components like lipopolysaccharide. There are five secretion systems (T1SS, T2SS, T3SS, T5SS and T6SS). Bacterial cell-to-cell interaction involves quorum sensing and biofilm.[9]

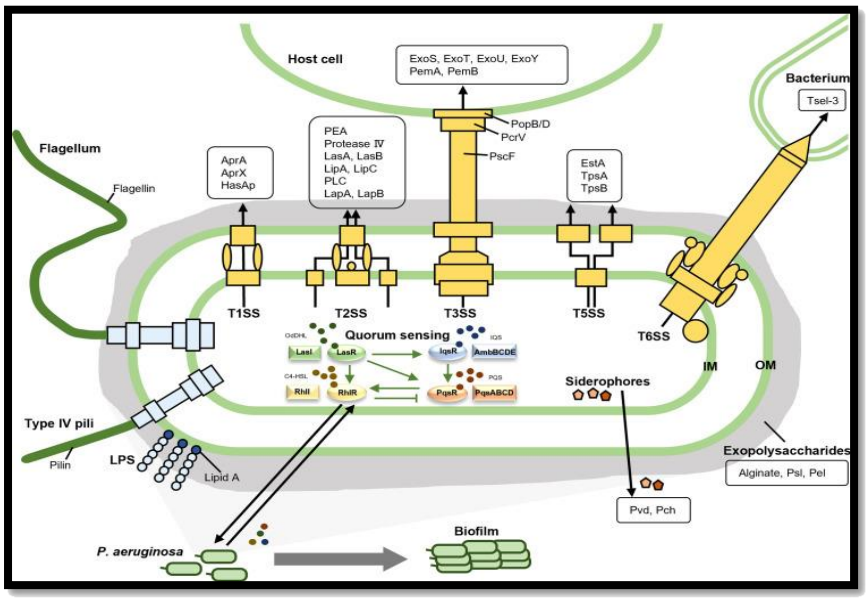

Figure 5: Illustration of virulence factors in *P. aeruginosa*.

The surface appendages of bacterial structures are type IV pili, flagella. The type IV pili associated with bacterial twitching and swarming motility and attachment to surfaces. It is involved in biofilm formation, regulation of virulence factors and bacterial exchange of antibiotic resistance. The flagella comprised of protein flagellin for the movement and chemotaxis. These appendages have an ability to activate the host immune system and are immunogenic. The lipopolysaccharide is the outer membrane component in Gram-negative bacteria which is high immunogenic and potent activator of host's defence mechanism through signalling pathways (TLR4).[9]

Secretion systems are the simplest systems but plays an important role in causing virulence in host. They are different types which play a prominent role like inflammatory phase of *P.*

aeruginosa infection process, secretes extracellular toxins, acts as a "molecular syringe" for invasion into the host, causing pathogenicity, autotransporters [9]. The secreted factors are secreted by secretion systems located on the outer membrane. For example, exopolysaccharides enhance the tolerance level of bacterium in harsh environments and proteases with proteolytic activity acts on complement components, immunoglobulins, cytokines, fibrin, mucins. One of the proteases is alkaline protease which can degrade C2 component of complement system, thus not allowing the defence mechanisms to be active.[10][11]

1.2.3. Quorum sensing in *P. aeruginosa*:

P. aeruginosa has complex, intricate QS systems, consisting of four QS systems which are interconnected. They are Las, Rhl, Pqs and Iqs. There are two dominant QS systems studied namely Las and Rhl. *P. aeruginosa* features three interconnected and autoregulatory Quorum Sensing (QS) systems, namely las, rhl, and pqs, exerting significant influence on one another. The las system positively regulates genes in both the rhl and pqs systems, controlling QSSM receptors (rhlR and pqsR) and synthase genes (rhlI and pqsH). Some target genes are specifically regulated by las or rhl, while others necessitate the activation of both QS systems for full functionality. The las and rhl systems utilize distinct N-acyl-L-homoserine lactone (AHL) type signal molecules, while the third QS circuit, pqs, employs 2-alkyl-4-quinolones, such as 2-heptyl-4-hydroxyquinoline (HHQ) and 2-heptyl-3-hydroxy-4(1H)-quinolone (PQS), as Quorum Sensing Signal Molecules (QSSMs). Strains of *P. aeruginosa* with mutations in key QS genes exhibit significantly attenuated pathogenicity in experimental infection models, rendering QS a potential target for novel antibacterial agents.

P. aeruginosa lasI mutants, characterized by halted biofilm development at the micro-colony stage, offer insights into the significance of quorum sensing in biofilm maturation. This deficiency can be rectified by externally supplying the LasI-dependent HSL autoinducer, N-(3-oxododecanoyl)-homoserine lactone, emphasizing the pivotal role of LasI in biofilm formation. The connection between biofilm and cystic fibrosis (CF) is underscored by the prevalence of *P. aeruginosa* biofilms in the lungs of CF patients, reinforcing the potential therapeutic impact of disrupting quorum sensing.

In the intricate quorum sensing network of *P. aeruginosa*, LasR, upon binding the LasI-dependent autoinducer at a critical concentration, orchestrates a cascade of events. This includes the activation of virulence factor promoters, leading to enhanced transcription. Remarkably, the LasR-autoinducer complex also triggers the Rhl quorum sensing circuit by instigating the transcription of rhlR. RhlR, in turn, binds to the RhlI-directed autoinducer, activating a subset of virulence genes and other target genes not regulated by LasR.

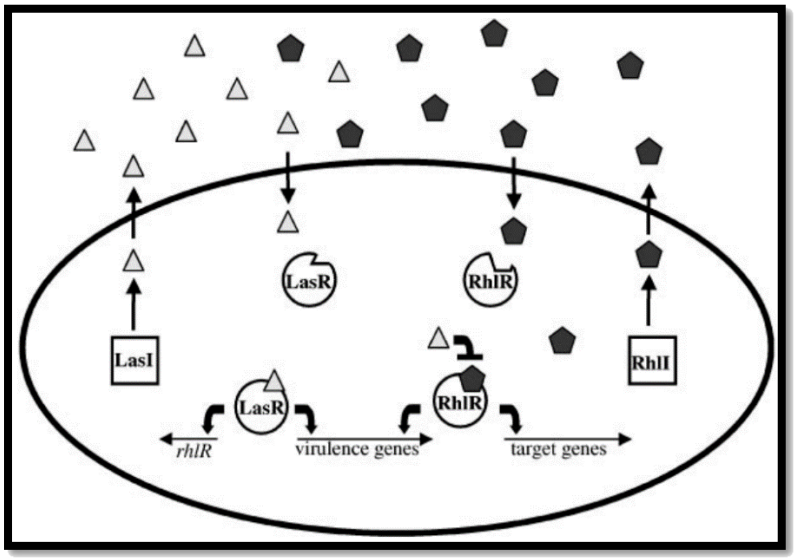

Figure 6: The quorum sensing system in *Pseudomonas aeruginosa* involves the LasI/LasR and RhlI/RhlR pairs, resembling LuxI/LuxR-like autoinducer-sensor systems. LasI generates the signalling molecule N-(3-oxododecanoyl)-homoserine lactone (depicted as triangles), while RhlI produces N-(butyryl)-homoserine lactone (represented as pentagons). These autoinducers play a crucial role in regulating various genes in response to cell-population density.

A noteworthy aspect involves the LasI autoinducer exerting a regulatory influence by impeding the binding of RhlI-autoinducer to RhlR. This interference mechanism ensures the establishment of the LasI/LasR circuit before the initiation of the RhlI/RhlR circuit, emphasizing the intricacy of quorum sensing regulation in *P. aeruginosa*. Additionally, the Pseudomonas quinolone signal (PQS) acts as an additional layer of regulation between the Las and Rhl quorum sensing circuits.

Visualized within the context of bacterial cells, represented by ovals, the autoinducer synthase proteins LasI and RhlI as squares, and the transcriptional activator proteins LasR and RhlR as circles, this quorum sensing network exemplifies the sophisticated interplay governing *P. aeruginosa* biofilm formation and virulence in the context of CF pathogenesis.

Activation of receptor proteins (LasR, RhlR, and PqsR) by cognate QS signal molecules initiates the expression of biosynthetic genes, establishing autoinduction loops that generate additional signal molecules. This process is responsible for up regulating a diverse array of genes associated with virulence, secondary metabolism, and biofilm development. From a drug discovery perspective, QS systems can be targeted at four main levels: signal biosynthesis, signal reception, signal sequestration, and signal degradation. This review primarily focuses on antagonizing QSSM biosynthesis and responses in *P. aeruginosa,* providing valuable insights for potential therapeutic interventions.[14]

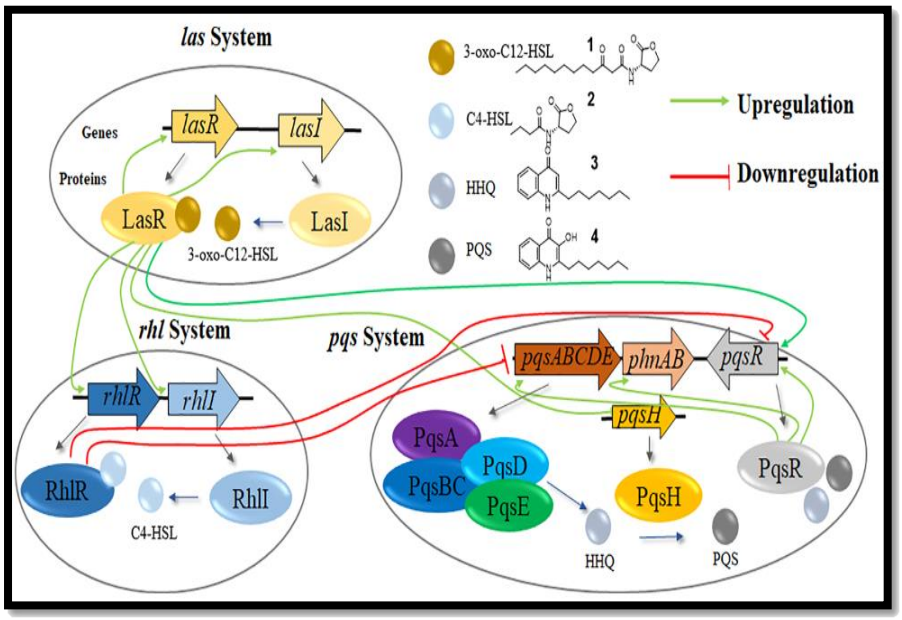

Fig 7: Schematic diagram of the interconnected las, rhl, and pqs quorum sensing systems in *P. aeruginosa*. Green arrows and red blocked lines indicate up- or down-regulation, respectively. Oval shapes represent various proteins, color coded circle shapes represent QSSMs, and large colored arrows represent genes. Thin gray arrows represent protein expression, and thin blue arrows indicate QSSMs biosynthesis.

1.2.4. Biofilm formation in *P. aeruginosa*:

Biofilm is defined as the aggregates of bacteria enclosed in complex matrix (self-produced) of extracellular polymeric substances (EPS) which makes the bacteria to survive even at sudden changes of environment such as temperature changes, nutrients depletion.[15]

P. aeruginosa is mainly characterized by the ability to form robust biofilms which have a substantial role in human infections. The biofilm of *P. aeruginosa* is composed of polysaccharides, extracellular DNA (eDNA), proteins and lipids. The matrix plays a vital

role in biofilm formation as it helps the bacteria to adhere the biotic and abiotic surfaces and to sustain harsh environmental conditions. This matrix is also involved in cell-to-cell communication [15].

The adhesion of bacterium to surface, formation and stability of biofilm is carried by three polysaccharides, Psl, Pel and alginate. Psl is neutral and pentasaccharide necessary for attachment of sessile cells to surface and in cell-to-cell communication. Pel is an essential component of matrix (nonmucoid strains) and important in biofilm initiation and integrity. At air-liquid interfaces, it forms pellicle biofilms [15].

In the early stages of biofilm development, extracellular DNA (eDNA) primarily holds cells together. However, as the biofilm matures, other substances replace eDNA. *Pseudomonas aeruginosa* biofilms exhibit a dynamic composition, with eDNA originating from cell lysis, regulated by both quorum sensing-dependent and independent pathways. Quorum sensing mutants with reduced eDNA levels showed increased susceptibility to SDS treatment, emphasizing the stabilizing role of eDNA in the biofilm matrix. The process involves planktonic cell attachment, microcolony formation facilitated by quorum sensing-regulated rhamnolipid production, and subsequent migration, resulting in flat, uniform mats. Microcolonies then evolve into stalk and mushroom-like structures, with rhamnolipids contributing to open channels and mushroom cap formation. The biofilm matrix, enriched by eDNA release and Pel polysaccharide production under quorum sensing control, plays a vital role. Throughout biofilm maturation, cells disperse with the assistance of rhamnolipids, transitioning back to a planktonic growth mode.

There are 5 steps in the formation of the biofilm namely, reversible adhesion of the planktonic bacteria on a suitable surface for growth, irreversible attachment of bacteria forming microcolonies in the EPS matrix, expansion of the colonies resulting in the more structured phenotype, and non-colonized spaces, filling of the bacteria with non-colonized spaces covering the entire surface, finally, the bacteria disperse from the sessile structures and re-enter the planktonic state for spreading and colonizing other surfaces. [15]

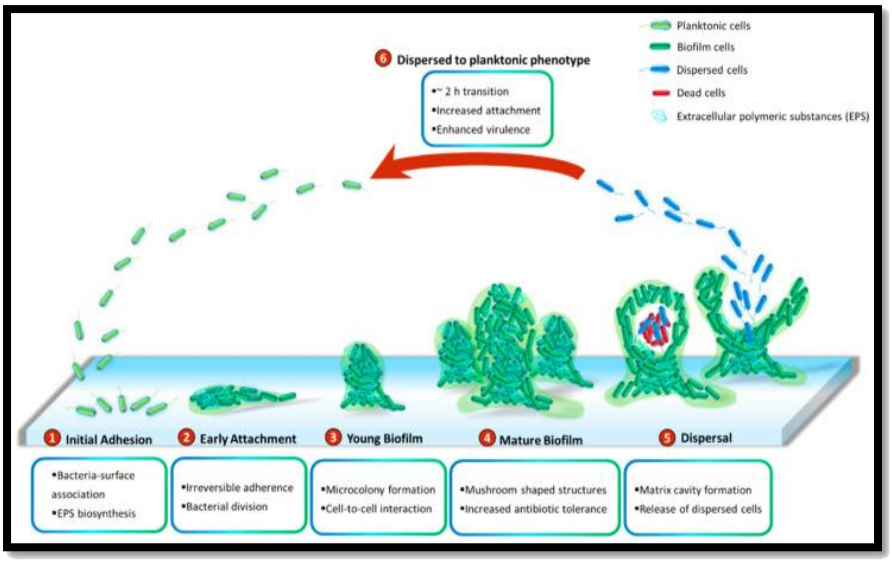

Figure 8: Cycle of *P. aeruginosa* biofilm development.

Nosocomial infections often result from biofilm formation on medical devices, especially indwelling ones, where *Pseudomonas aeruginosa* forms cell aggregates encased in a protective alginate polysaccharide. Biofilms, characteristic for their resistance to antimicrobial drugs, pose a significant challenge in healthcare settings. Antibiotic resistance in biofilm-forming bacteria arises from enzyme modifications, target site mutations, and efflux pumps. Three hypotheses explain this resistance: delayed penetration due to enzyme deactivation or adsorption into the biofilm matrix, altered microenvironments causing anaerobic niches, and bacterial differentiation into a protected phenotype. For instance, aminoglycoside antibiotics may bind to polymers in the biofilm matrix. Anaerobic conditions or osmotic stress responses contribute to resistance by altering cell envelope permeability. Understanding these mechanisms is crucial for developing effective strategies against biofilm-related antibiotic resistance in healthcare-associated infections. [16]

1.2.5 Antibiotic resistance in *Pseudomonas aeruginosa*:

Pseudomonas aeruginosa exhibits a formidable resistance profile against various antibiotics, including aminoglycosides, quinolones, and β-lactams. The mechanisms employed by *P. aeruginosa* to counter antibiotic assaults are broadly categorized into intrinsic, acquired, and adaptive resistance. Intrinsic resistance encompasses factors such as low outer membrane permeability, the expression of efflux pumps expelling antibiotics, and the production of antibiotic-inactivating enzymes. Acquired resistance can arise through horizontal transfer of resistance genes or mutational changes.

Adaptive resistance in *P. aeruginosa* involves the formation of biofilms in the lungs of infected patients, acting as a diffusion barrier that limits antibiotic access to bacterial cells. Within biofilms, multidrug-tolerant persisted cells capable of surviving antibiotic attacks contribute to prolonged and recurrent infections in cystic fibrosis patients. Addressing these challenges necessitates the development of new antibiotics or alternative therapeutic strategies, especially for infections resistant to conventional antibiotics.

Recent efforts have explored novel antibiotics with unique modes of action, alternative routes of administration, and modifications resistant to bacterial enzymes. Some newer antibiotics demonstrate superior in vitro antibacterial activity against *P. aeruginosa*, with lower minimum inhibitory concentrations compared to conventional antibiotics. Additionally, non-antibiotic therapeutic approaches have shown promise, including the inhibition of quorum sensing and bacterial lectins, iron chelation, phage therapy, vaccine strategies, nanoparticles, antimicrobial peptides, and electrochemical scaffolds. These innovative approaches can be employed as alternatives or in combination with traditional antibiotic treatments. This overview highlights recent findings on the intrinsic, acquired, and adaptive mechanisms of antibiotic resistance in *P. aeruginosa*, offering insights into new antibiotics and therapeutic strategies to combat infections caused by this resilient pathogen.

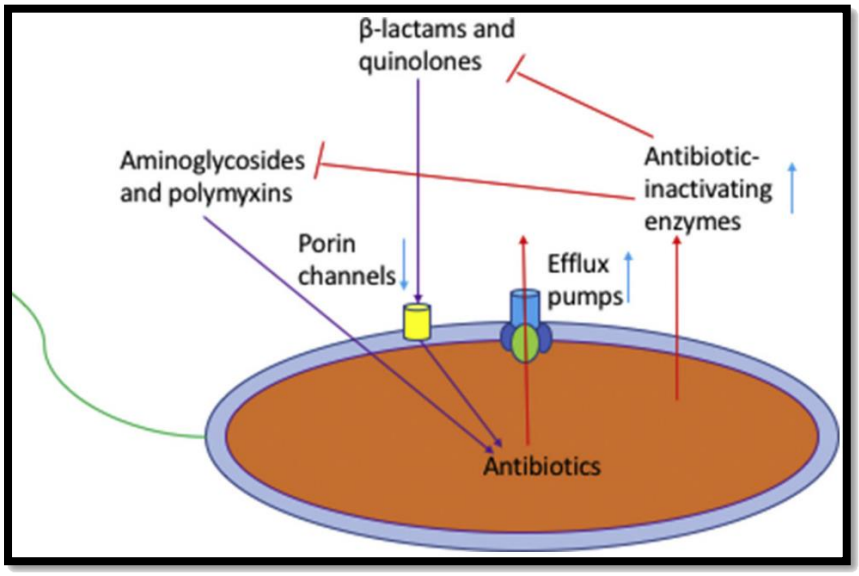

Figure 9: A visual depiction of the intrinsic antibiotic resistance mechanisms in *Pseudomonas aeruginosa* reveals three key factors. These include limited outer-membrane permeability, facilitated by quinolones and β-lactams penetrating cell membranes via porin channels. Efflux systems actively pump antibiotics out of the cells, contributing to the bacterium's resistance.

Intrinsic antibiotic resistance in a bacterial species refers to its inherent capacity to reduce the effectiveness of a specific antibiotic due to inherent structural or functional characteristics. *Pseudomonas aeruginosa* exhibits a notable intrinsic resistance, primarily attributed to restricted outer membrane permeability, active efflux systems expelling antibiotics from the cell, and the production of antibiotic-inactivating enzymes, including β-lactamases. Moreover, the bacterium's intrinsic resistance is further heightened by the production of antibiotic-inactivating enzymes. Aminoglycosides and polymyxins exploit interactions with *P. aeruginosa* lipopolysaccharides (LPS) on the outer membrane, facilitating their own uptake.

The development of drug resistance in *Pseudomonas aeruginosa* involves intricate interplays of both intrinsic and acquired antibiotic resistance mechanisms, notably observed through the biofilm-mediated generation of resistant and multi-drug-resistant persistent cells. The bacterium swiftly acquires resistance to diverse antibiotics, encompassing aminoglycosides, quinolones, and β-lactams. Throughout its evolutionary trajectory, *P. aeruginosa* has evolved ancient genetic resistance mechanisms, showcasing significant genetic plasticity to combat harmful antibiotic molecules. These mechanisms equip the bacterium to navigate various environmental threats, including antibiotics, chemical compounds, and antimicrobial peptides.

The acquisition of antibiotic resistance in *P. aeruginosa* is diverse, primarily relying on outer membrane permeability, efflux systems, and antibiotic-inactivating enzymes. The outer membrane, consisting of bilayer phospholipid molecules, lipopolysaccharides (LPS), and porins, poses a specific hurdle for antibiotic penetration. Porins such as OprF, OprB, OprD, OprE, OprO, and OprP, along with efflux porins like OprM, OprN, and OprJ, are strategically manipulated by *P. aeruginosa* to limit antibiotic penetration, thereby bolstering antibiotic resistance. Specific porins like OprD mutations contribute to carbapenem resistance, posing a substantial challenge for medical treatments.

P. aeruginosa employs outer membrane protein H (OprH) to enhance outer membrane stability, regulating antibiotic resistance. Efflux porins, especially MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM, play a pivotal role in actively expelling antibiotics, contributing significantly to resistance. The RND efflux pump family, particularly MexAB-OprM, is crucial for the development of carbapenem-resistant strains, presenting persistent clinical challenges. Moreover, outer membrane vesicles (OMVs) secreted by *P. aeruginosa*

contribute to antibiotic resistance by transporting virulence factors, mRNA, DNA, and enzymes, including β-lactamase, into the host cytoplasm. Despite their detrimental impact on the host, OMVs have potential applications as alternative delivery vehicles for treatments or vaccines. Efflux systems, including the ABC superfamily, major facilitator superfamily (MFS), MATE family, RND family, and SMR family, play a central role in pumping out drug resistance factors. Among these, the RND efflux pump family exhibits a strong correlation with antibiotic resistance, with MexAB-OprM being crucial for carbapenem resistance and MexCD-OprJ linked to resistance against ciprofloxacin, cefepime, and chloramphenicol. Targeting the efflux pump, such as inhibiting MexAB-OprM or enhancing the repressor MexR, emerges as a promising strategy to overcome antibiotic resistance and improve treatment efficacy in *P. aeruginosa* infections.

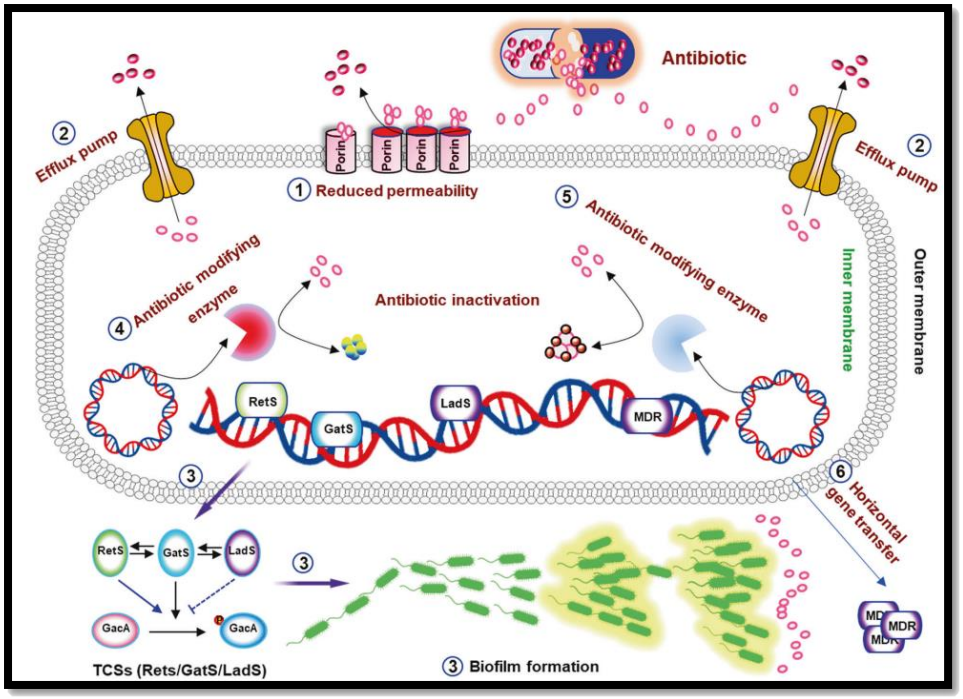

Figure 10: *Pseudomonas aeruginosa* employs various mechanisms for antimicrobial resistance, categorized into intrinsic (1. outer membrane permeability, 2. efflux systems, and 4. antibiotic-modifying enzymes and 5, antibiotic-inactivating enzymes), acquired (6. resistance through mutations and acquisition of resistance genes), and adaptive (3. biofilm-mediated resistance). Outer membrane protein porins' alteration reduces membrane permeability, limiting drug penetration. Efflux systems actively pump drugs out of the cell. Antibiotic-modifying enzymes render drugs inactive, while others cause target alterations, preventing drug binding and resulting in inactivity. Resistance genes, often carried on plasmids, can be acquired through horizontal gene transfer. Quorum-sensing signalling molecules trigger biofilm formation, creating physical barriers that impede antibiotic penetration.

1.3. Synthetic Biology for the development of antimicrobial strategies:

Synthetic biology is a hybrid discipline between engineering and molecular biology. The aim is to design genetic circuits in living organisms to provide them with a new biological function or to reprogram systems already existing in nature. A synthetic biologist then deals with the designing, assembling, and testing the functionality of synthetic components to create biological systems and able to understand the non-synthetic biology. [38]

Over the years the tools of synthetic biology have increased more and more, thanks to the participants of the international competition for the design of synthetic biology, iGEM (International Genetically Engineered Machines). This competition is attended by teams of university students from all over the world, whose goal is to create genetically modified biological circuits, using Bio Bricks, or standardized biological parts, available in the database of the iGEM, Registry of Standard Biological Parts.[39]

The starting point for realizing these biological circuits is the design of expression cassettes, consisting of several genetic components combined with each other, such as promoters, reporter genes, terminators, and cloning vectors. Then the exogenous or modified DNA unit (gene), which possesses new functions, is transferred within an organism, and requires the following components for its expression in the host:

- Promoter: a certain DNA sequence designates the transcription's start point by the RNA polymerase of the gene. Differentiation of the promoters into inducible and constitutive, the former is only active under specific conditions, like heeding external cues. Exogenous molecules' presence will determine the ongoing transcription of the gene located downstream, whereas constitutive molecules are constantly present and hence are not controlled.
- Ribosome Binding Site (RBS): corresponds to the mRNA location that the ribosome has identified to start translation of proteins. The promoter and this sequence are situated between the coding patterns.
- Coding sequence (CDS, Coding DNA Sequence): region of a gene or mRNA downstream of the RBS that codes for a protein.
- Terminator: DNA sequence that folds into a hairpin structure, allowing the RNA polymerase to separate from the DNA and signalling the completion of gene transcription.

These elements will then need to be put into a cloning vector, with plasmids, circular double-stranded DNA strands, being the most popular choice. These vectors need to meet certain requirements:

- origin of replication (ori): which permits independent replication once introduced into the host cell.
- Selection markers: for instance, resistance to antibiotics, resulting in the identification of just the cells bearing the vector and the inserted DNA fragment and the survival of only resistant bacteria.
- Restriction sites: which enable the vector's insertion of previously chosen segments.

To put together the genetic parts mentioned above and obtaining these genetic circuits, it is possible to utilize the synthetic biology technique termed Standard Assembly which will be detailed in detail in the coming sections.

2. AIM OF THE THESIS

The project aims to delve into the Quorum Sensing (QS) systems of LasI/LasR and Rh1I/Rh1R in *Pseudomonas aeruginosa*, a bacterium known for its significant role in various infections, particularly in healthcare settings. Quorum Sensing is a mechanism through which bacteria communicate with each other using signaling molecules, allowing them to coordinate group behaviors, including the expression of virulence factors and antibiotic resistance. LasI/LasR and Rh1I/Rh1R are specific QS systems in *P. aeruginosa* responsible for regulating the production of various virulence factors and biofilm formation. Understanding the intricacies of these systems is crucial, as they play a pivotal role in the pathogenicity of *P. aeruginosa* and contribute to its ability to develop antibiotic resistance.

The project goes beyond mere exploration by proposing the development of engineered systems designed to facilitate an in-depth study of LasI/LasR and Rh1I/Rh1R in *P. aeruginosa*. The objective is to create a rapid and efficient approach that allows for a comprehensive understanding of the molecular mechanisms involved in quorum sensing. Moreover, the project seeks to identify potential targets within these QS systems that could be manipulated to mitigate antibiotic resistance in *P. aeruginosa*. By unraveling the complexities of these signaling pathways and leveraging engineering techniques, the project endeavors to provide valuable insights and contribute to the development of novel strategies to combat antibiotic resistance, ultimately paving the way for more effective treatment approaches against *P. aeruginosa* infections.

3. MATERIALS AND METHODS

3.1. Strains and reagents

3.1.1. Bacterial strains

Two bacterial strains were used:

- - *Pseudomonas aeruginosa* ATCC 10145 (PAO1): the genome of the PAO1 strain. It was used to extract, by PCR, the gene components of the *las* and *Rhl system.* For *las* it was possible to isolate the promoters located upstream of the *lasI, LasA, LasB, LasR3 genes* and the lasR gene; while for *Rhl* it was possible to isolate the promoters upstream of the *RhlR, RhlC* and the *RhlR gene.*
- - *Escherichia coli:* was chosen as host organism, particularly the *TOP10* strain (Invitrogen) was made chemically competent and used to perform transformations during cloning and in experiments.

3.1.2. Culture Medium

The medium used is Luria-Bertani (LB), a rich culture medium, used to promote bacterial growth. To prepare this medium it is necessary to dissolve Milli-Q in water:

- 10 g / L sodium chloride.
- 10 g/L bacto-tryptone.
- 5 g / L yeast extract.

On the other hand, to prepare the solid medium in a petri dish it is also necessary to add 15 g / L of agar. Once prepared, the medium must be sterilized at 121 ° C for 15minutes and must then be used under sterile conditions.

3.1.3. Antibiotics

To select and maintain the plasmids in the desired bacterial strains, three different antibiotics were added to the medium, alone or in combination with each other:

- **Ampicillin**: 100 mg / mL stock, used in 100 µg / mL concentrations

to select highcopy number (HC) pSB1A2 plasmids.

- **Chloramphenicol**: 34 mg / mL stock, used in 12.5 µg / mL concentrations toselect low copy number (LC) pSB4C5 plasmids.

- **Kanamycin**: 50 mg / mL stock, then used in 25 µg / mL concentrations to selectpSB1AK3 medium copy number (MC) plasmids.

3.1.4. Inductors

To perform the experiments on the microplate reader, signal molecules known as N-acyl homoserine lactones (AHL), defined as self-inductors:

- N- (3-oxododecanoyl) -L-homoserine lactone (3OC12-HSL) :10 mg was solubilized in 500 µL of ethyl acetate, obtaining 34 mM stock, stored at -20 ° C.
- N- (3-oxohexanoyl) -L-homoserine lactone (3OC6-HSL): stock 0.2 mM, stored at -20 °C.
- N-butyryl-L-homoserine lactone (C4-HSL): 10 mg were solubilized in 334 µL of 96% ethanol, obtaining 58 mM stock, stored at -20 ° C.

These AHL self-inductors consist of a homoserine lactone (HSL), linked by amide bond to an acyclic side chain, see Figure 9. These molecules differ in the length of the chain(in this case C12, C6 and C4) and in the substitution of the functional group on the third carbon atom of the acyclic chain (a ketone group is present for the first two molecules). These differences between the different molecules confer specificity on the transcriptional regulatory signal. [14]

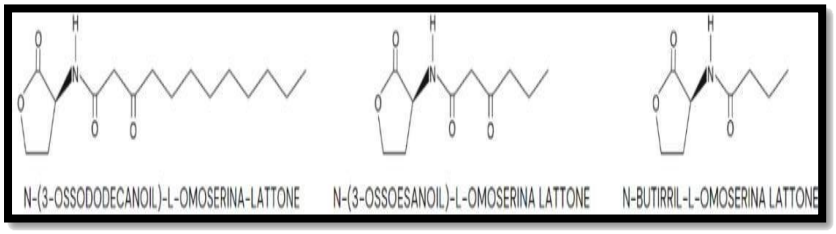

Figure 11: Chemical structure N-acyl homoserine lactones (AHL), used as self-inductors.

3.2. Cloning

The techniques used for cloning are: Standard Assembly and Gibson Assembly, models developed for the international competition of Synthetic Biology, iGEM.

3.2.1. Primer's creation

In this thesis project, reference was made to the *Benchling* online platform, which allowed, thanks to its multiple functions, to design primers which then allowed to isolate and amplify promoters and genes present within *P. aeruginosa.*

NCBI is a genomic database, from which it was possible to search for the sequences of *P. aeruginosa genes,* which were then imported into *Benchling.* Once the sequence was imported, annotations relating to parts already known in the literature were added and the primers were subsequently designed. For the synthesis of the oligonucleotides, the *Sigma-Aldrich* oligo *synthesis* service was used.

3.2.2 PCR

Colony PCRs was performed to amplify the *lasR, pRhlR, pRhlC promoters* from the PAO1 genome.

A smear of PAO1 cells in LB agar plate, without antibiotics, was performed and it was allowed to grow overnight at 37 ° C. The following day a colony was taken and grown in 200 µl of LB in a 1.5 ml tube, in an incubator for 1 hour. 5 µl were then aliquoted into a 0.2 ml PCR tube, along with 45 µl of Milli-Q water and left to rest for 10 minutes to allow osmotic shock to occur. Subsequently, the sample was subjected to thermal shock (95 ° C for 10 minutes, then immediately transferred to ice) for cell lysis and therefore the release of the DNA. 5 µl of DNA will then be used for each PCR reaction.

Once the sample is obtained, the PCR mix is prepared, consisting of:

5 µl Buffer HF (alternatively Buffer GC, if the template is rich in GC)

0.5 µl dNTPs 10 mM

1.25 µl primer forward 10 µM

1.25 µl primer reverse 10 µM.

0.25 µl *Phusion Polymerase* (Thermo Fisher)

5 µl DNA (1 µl DNA if not from colony PCR)

11.725 µl Milli-Q (15.725 µl Milli-Q if 1 µl template is used)

The final volume of the reaction will be 25 µl.

It is a good idea to run a PCR reaction without DNA, as a control, to check for any contamination.

The set program consists of three phases:

1) *Denaturation phase:* 98 ° C for 30 seconds. This step allows the separation of the two strands constituting the double helix of the template, thus obtaining only single stranded DNA.
2) *Annealing phase:* consisting of two blocks considering or not the regions of the primer's tails. First, 5 cycles are performed, without considering the queues, consisting of 3 steps (denaturation, pairing and synthesis): 98 ° C for 10 seconds, 20 seconds at the Melting temperature (Tm) *"without queues"* and 72 ° C for 30 seconds / kb. Then we move on to 35 cycles, where a higher Tm is set because the regions of the primers' tails are also considered: 98 ° C for 10 seconds, 20 seconds at the melting temperature *"with tails"* and 72 ° C for 30 seconds / kb. If the Tm is greater than 72 ° C, *"two step PCR" is performed,* i.e., in each cycle instead of doing 3 steps as just described, two are done and the central "annealing" step is skipped. The Tm was calculated with the computer support *Tm calculator* and depends on the primers used. During this phase the DNA begins to close, allowing the pairing of the primers to the template.
3) *Elongation phase:* 72 ° C for 7 minutes. This phase occurs at a specific temperature for the polymerase, which will only take the regions where the DNA has been closed and will begin to stretch it. During this phase, time is very important, which is why, after these 7 minutes, the sample is kept at a temperature of 4 ° C, until it is removed from the PCR.

For the isolation and amplification of the promoters and the gene of the *Rhl* system, two separate PCR reactions were carried out, the first using the "no-tails" primers, which allow isolation, thus performing the 5 cycles previously described for the annealing phase.

Subsequently, the products obtained were run on a gel, from which the DNA was then extracted. 1 µl of template was then used for the second PCR reaction, where the "tails"primers were used, used to add Prefix and Suffix, setting 35 cycles for the annealing phase and a Tm at 72 ° C. The product of the second PCR was then purified and processed to obtain constructs, by means of an

assembly technique, which were then used for the experiments at the plate reader.

3.2.3 Mutagenesis PCR

Mutagenesis PCRs were performed with the aim of removing native RBS.
present in the three plasmids containing respectively the promoters of *las: lasR, pRhR, pRhlC.* The plasmid DNA used as template was extracted by miniprep, specialprimers were created, synthesized by *Sigma-Aldrich,* which allowed to obtain linear DNA by PCR. After the PCR reaction, 1 µl of DpnI (Thermo Fisher Scientific) was added directly to the samples and they were digested at 37 ° C for 1 hour, this enzyme allows to destroy the methylated template DNA. After gel electrophoresis and DNA extraction, the PNK step was performed, prior to ligation, to activate the blunt ends of the phosphorylated amplicon for circularization. Then in a 0.2 ml tube 17 µl of DNA were loaded with 1 µl of *PNK* (Thermo Fisher Scientific) and 2 µl of *Buffer ligase T4* (Thermo Fisher Scientific), the whole was incubated at 37 ° C for 30 minutes. Finally, 1 µl of *T4 ligase* (Thermo Fisher Scientific) was added to continue with the ligation step.

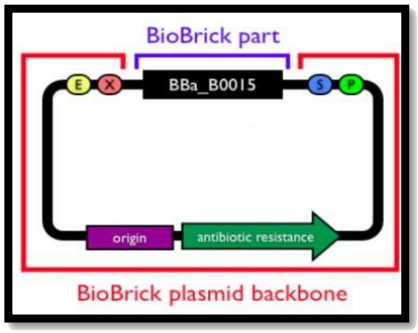

Figure12: Bio Brick is the part of the plasmids.

3.2.4 Standard Assembly

Through this technique is assemble two parts in the Bio Brick and contains the specific sites.

These sequences are part of the plasmid's so-called *"Bio Brick backbone",* i.e., the sequence starting with the suffix, includes the origin of replication and the antibiotic resistance marker and ends.

Two *digestion* reactions are performedto perform the assembly on previously extracted DNA. These two reactions are carried outin parallel (Figure 13), one reaction will allow to obtain the so-called *insert,*while the other the *vector. The insert* is "cut" on both sequences, using the enzymes of interest, thus being excised from its plasmid; instead, to obtain the *vector,* a digestion is performed only at the level of the Prefix or Suffix, thus obtaining an empty space on the plasmid, where the insert will then be received. [13]

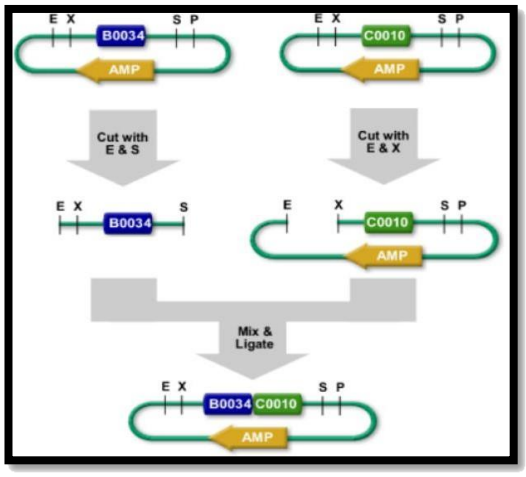

Figure 13: Schematic of a cloning using Standard Assembly: digestion and ligation

Subsequently, by means *of Gel electrophoresis,* what is obtained from the digestion of the two reactions is made to run, thus allowing the separation of the bands, depending on the size of the digested DNA fragments. Subsequently, gel-extractionis performed, i.e., the bands corresponding to the insert and vector of interest are cut. Then the *ligation* is performed, a process in which the insert will be received in the empty space previously created specifically in the plasmid chosen as a vector, thus obtaining a new construct.

When assembling two Bio Brick parts, the resulting new Bio Brick will keep the same starting Prefix and Suffix and will contain a non-digestible “scar” (scar) in which the adhesive ends of the restriction sites have been stitched together.

The ligation product is then transformed into *E. coli* TOP10 strain cells, allowing the insertion of the genetic material into the bacterium.

This cloning method was used to obtain constructs containing the promoters of the *lasA, lasB, lasI, lasR3 genes,* the *lasR* gene and the promoters of the *rhlC* and *rhlR genes,* encoding respectively for the receptor of the *las* and *rhl* system of *P. aeruginosa.*

The online platform *Benchling* was also used to support the experimental design and verify the results (e.g., design and drawing of primers and plasmids, virtual digestions and cloning and sequence alignment).

3.2.5 Inoculum

For the realization of new plasmids "parts", identified with code *BBa_,* present in the "*Registry of Standard Biological Parts"* [18] were used, the clones containing these constructs are storedin glycerol, as stock at -80 ° C.
An inoculation of this frozen culture was performed in 5 ml of liquid medium LB, to which the appropriate antibiotic was added, depending on the resistance of the inoculated construct. The inoculum is then grown in an incubator for 24 hours at 37 ° C, 200 rpm.

3.2.6 DNA extraction

The *"RBC Bioscience Real Genomics Hi Yield Plasmid Mini"* kit was used to extract the plasmid DNA. This technique, called miniprep, was used to extract DNA from liquid bacterial culture to proceed with subsequent digestion or to verify correct cloning by sequencing. It consists of a first phase of cell lysis for pH shock, RNA degradation and precipitation of proteins and cellular debris; subsequently through a series of washes and passages through membranes of silica gel with chaotropic only the DNA is isolated and finally diluted in water or suitable solvents.

The following changes were made from the original protocol:
The bacterial culture was centrifuged at 5000 rpm, at 4 ° C for 10 minutes, instead of 13000 rpm for 1 minute.
The elution step was performed with 30 µl of Milli-Q water, instead of 5µl of elution buffer.

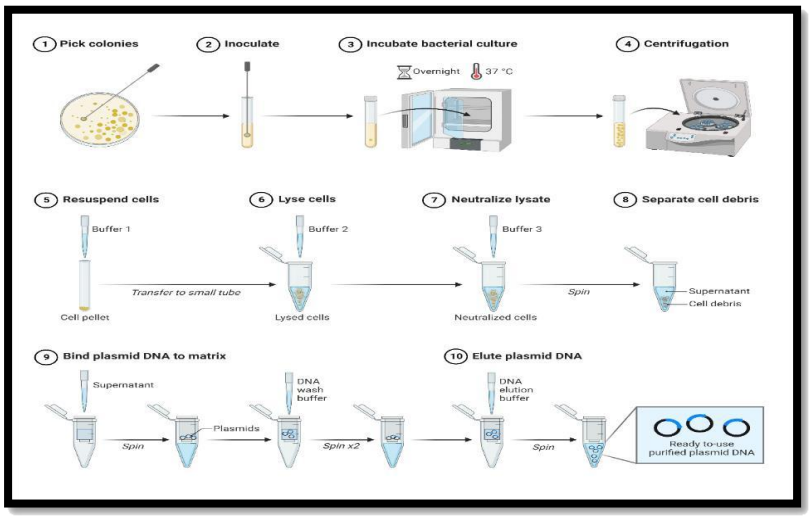

Figure 14: Miniprep protocol step, DNA extraction by kit.

The cell lysate, added with buffers, is passed through the matrix, which specifically binds only the nucleic acids. Subsequent washes with buffers provided by the kit allow to detach the DNA and collect it in aqueous solution in the test tube.

The concentration of extracted DNA (ng / µl) was quantified by the *NanoDrop* spectrophotometer(Thermo Fisher Scientific). Subsequently, the DNA samples were stored at - 20 ° C or directly processed.

3.2.7 Digestion by restriction enzymes

Two digestion reactions are performed on extracted DNA, one to obtain the insert, theother for the vector, to perform the assembly. These reactions are carried out in parallel and separately. The enzymes used to carry out this double digestion are: EcoRI (E), XbaI (X), SpeI (S) and PstI (P).

The coupling of these enzymes allows to go to remove the insert (e.g., XP or ES) or to open the plasmid (e.g., EX or SP).

The protocol used foresees to add in a 0.2 ml tube: 20.5 µl of extracted DNA, 2.5 µl of *Buffer Tango* and 1 µl of each enzyme used. The final volume of the reactionis 25 µl. The samples are then digested in the thermo-block for 2 hours at 37 ° C.

3.2.8 Gel Electrophoresis

Agarose gel electrophoresis was used to verify the correct digestion of the digested samples and confirm the amplification performed by PCR.

To make the agarose gel, depending on the size of the gel (small/medium/large), 0.5/1.2 g of agarose are dissolved in 50/100/200 ml pf 1X TBE buffer solution. To the still liquid mixture is added 1 or 2 µl of *Nancy-520,* a fluorescent dye for DNA.

This liquid is then poured into the support and left to polymerize. At the end of the digestion, the samples are prepared to be loaded onto the gel: 5µl of TriTrack DNA loading Dye (6X) are added to the 25µl of digested sample.

Thermo Fisher Scientific, which contains three dyes which is facilitated the control of DNA migration during the gel run. 30µl of sample will be loaded into each well and one of them will also be left free to load 5µl of gene Ruler 1kb DNA marker Ladder (figure 15). At the end of the loading the run is started at 50V for about 5 minutes, then it is increased to 100V for 30-40 minutes.

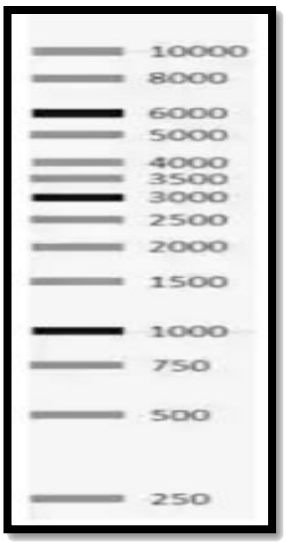

Figure15: **Gene Ruler 1kb DNA Ladder** (Thermo Fisher Scientific). Marker used as a reference for reading the height of the band

3.2.9 Gel Extraction

At the end of the run, the gel is observed in the UV to verify the correct separationof the bands. The band corresponding to the fragment of interest is cut, once removed, the DNA is extracted by using the Extractme DNA Clean-up & Gel-out kit, as per protocol, apart from using 20 µl of Milli-Q water instead of 50 µl of Elution Buffer. Once theextraction is complete, the DNA is quantified at Nanodrop.

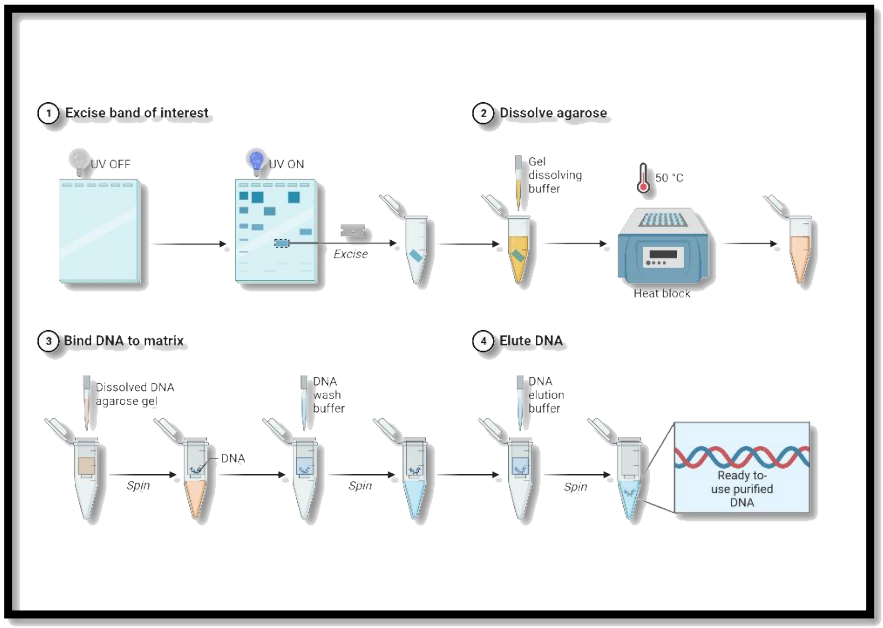

Figure 16: Gel Extraction developed using Bio-render.

3.2.10 Get to know each other.

After cutting and extracting the DNA corresponding to the fragments of interest, weproceed to ligation, a procedure that allows the recirculation of the new construct. Ligation is performed using:

- 20-40 ng of vector DNA
- ng of insert DNA equal to 6 x (insert length / vector length) x (ng ofvector)
  1 µl di *Buffer T4 Ligase* (Thermo Fisher Scientific)
  1 µl di *T4 Ligase* (Thermo Fisher Scientific)
- optional Milli-Q water to reach the reaction volume equal to 10 µ.

In some cases, double volume can be used, obtaining 20 µl of final volume, with 2µl of *Buffer T4 Ligase* and 2 µl of *T4 Ligase.*

The sample thus obtained is incubated in the thermal cycler at 16 ° C for the whole night.

Transformation into competent bacterial cells

The next day the ligase is inactivated with heat at 65 ° C for 10 minutes. Subsequently, the ligation product is transformed into competent cells of *E. Coli,* TOP10strain, thus allowing the insertion of the genetic material into the bacterium.

3.2.12 Competent bacterial cells

The procedure for preparing chemically competent *E. coli* initially involves streaking the desired strain, in this case TOP10 stored in glycerol stock, onto an LB agar plate and letting it grow at 37 ° C overnight. The following day the plate-grown bacteria are inoculated in a flask containing 50 ml of liquid medium LB; then left to grow for 3-4 hours until an optical density (OD) at 600 nm between 0.12-0.15 (measured by spectrophotometer). Once the required value is reached, the bacterial culture is transferred into two 50 ml tubes, centrifuged at 4000 rpm for 10 minutes at 4 ° C. Once the supernatant has been removed, the pellet is resuspended with 15 ml of *Buffer 1* (consisting of 80 mM MgCl2 and 20mM CaCl2). Centrifuge again as before, the supernatant is discarded, and the pellet resuspended with 1 ml of *Buffer 2* (consisting of CaCl2 and 15% glycerol). Finally, 100 µl are aliquoted in 1.5 ml tubes,stored at -80 ° C or used immediately for transformation.

Typically, a competence test is performed to verify the actual competence of the newly created bacterial cells.

The ligated product is added to 100 µl of competent E. coli TOP10 cells, incubated on ice for 30 minutes and then subjected to thermal shock at 42 ° C for 1 minute, followed immediately by 1 minute on ice. 1ml of LB, previously heated to 37 ° C, is added to the bacterial cells and incubated for 1 hour at 37 ° C in oscillation (220 rpm). The transformed cells are centrifuged at 10000 rpm for 1 minute, then about 950 µl of supernatant are removed and the remainder is used to resuspend the pellet. The resuspendedpellet is then sown by spatulation on a Petri dish containing selective LB agar, depending on the resistance of the vector of the constructed construct. The plate

is incubated at 37 ° C, an isolated colony is taken the following day and grown in liquid selective LB medium.

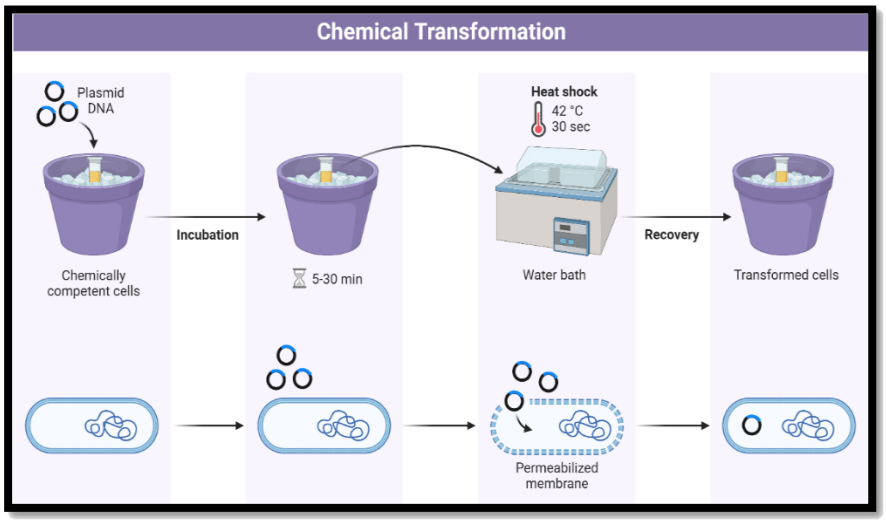

Figure 17: Chemical transformation (from Bio render): only cells made chemically competent can acquire DNA from the external environment. The lysed cells can cross the membrane, yielding momentarily permeable during the thermal shock at 42 ° C, thus allowing the entry of plasmid DNA into thebacterial cell.

3.2.13 Stock

The culture thus obtained is stored at -80 ° C.

Making a stock involves mixing:

- 750 µl bacterial culture resuspended in LB medium.
- 250 µl glycerol 80%

3.2.14 Sequencing

To verify the success of the assembly, the DNA is extracted from the bacterial culture just obtained andthen its sequence is verified by the Sanger sequencing service of *Eurofins Genomics.* The Champions, before being shipped, the following are prepared: 5 µl of plasmid DNA (80-100 ng / µl) and 5 µl of 5 µMprimer (VF2 *BBa_G00100* or VR *BBa_G00101).* The sequencing results are then aligned on the expected sequence, created on the *Benchling online platform.*

Alternatively, the DNA extracted from the plasmid can be digested with appropriate restriction enzymesand run on a gel to check if the vector and insert have the expected lengths.

3.2.15 Gibson Assembly

This cloning technique, alternative to Standard Assembly, allows to assemble DNA fragments simultaneously and efficiently by means of a single isothermal reaction and was used, in this thesis project, to make an HC plasmid containing pRhlC. The "GeneArt ™ Gibson Assembly® HiFi Master Mix" kit was used. [20]

The reaction requires that each DNA fragment share a terminal homology of 20-40 base pairs at the 5 'end, with the adjacent fragment or with the vector. It has been possible to achieve this by means of specific primers, having at the 3 'end the specific sequence of the DNA element to be assembled, while at the 5' endthe sequence of the desired adjacent fragment.

The RhlC promoter sequence contains the EcoRI restriction site: for this reason, it cannot be digested according to the Standard Assembly, but this technique has been adopted. Once pRhlC was isolated and amplified by PCR, the results obtained were purified using the Extractme DNA Clean-up & Gel-out kit, following the "clean-up" protocol and subsequently quantified by Nanodrop. The protocol provides for determining the volume of insert and vector, to be included in the reaction in a 1: 1 molar ratio, depending on the DNA concentration just calculated. 110 ng / µl were required for the vector, 15 ng / µl for the insert.

The protocol therefore provides for inserting in a 0.2 ml tube:

Volume of vector and DNA fragment to be assembled just calculated, la whose sum cannot be greater than 10 µl.

10 µl di GeneArt™ Gibson Assembly® HiFi Master Mix, if necessary, add Milli-Q water to obtain a final volume of the reaction equal to 20 µl.

Then incubate the tube for 60 minutes at 50 ° C. After the incubation it is possible to transform the assembled DNA molecule, following what previously reported.

3.2.16 Microplate reader

Varioskan LUX (Thermo Fisher Scientific) is a microplate reader used to conduct experiments during this thesis project. 96-well microplates were used which allowed to test the response of the LasR and RhlR genes exposed to different auto inductors. The instrument was used to measure:

optical density at 600 nm (OD600): that is the absorbance, which made it possible to measure the growth kinetics of bacterial cultures.

fluorescence of bacterial cultures, which contain RFP (Red Fluorescent Protein), red fluorescence at 545 nm of excitation and 615 nm of emission.

This tool is controlled by the SkanIt® software, which allowed to set the following parameters:

- 16-hour kinetic cycle.
- incubation of the plate at 37 ° C for the whole duration of the experiment.
- linear shaking of the plate every 10 seconds.
- every 5 minutes: OD600 and fluorescence reading.

To conduct these experiments, co-transformations were performed, to allow the acquisition of more plasmids within the same cell. These co-transformations are carried out as simple transformations, with two exceptions: in the initial phase, more plasmids are added inside the competent cells and in the final seeding phase, platescontaining more antibiotics are used, depending on the resistance of the plasmids. inserted. The plates are incubated at 37 ° C overnight.

Three colonies were collected for each co-transformed strain, as the test was performed in biological triplicate. Each colony was inoculated in 0.5 ml of medium LB, with the addition of appropriate antibiotics. And it was grown in an overnight incubator at 37 ° C, 220 rpm. In the case of carrying out a new co-transformation it isadvisable to inoculate a colony in 5 ml of selective LB medium and let it grow under the same conditions, overnight, and then, the following day, "save" the stock and store it in glycerol at -80 ° C. Subsequent times it will be sufficient to streak on a selective LB agar plate to revitalize the bacteria and isolate the individual colonies.

Typically, in each well will be inserted: 2 µl of inoculum, diluted 1: 100, then in 198 µl of selective LB medium (containing induction at different concentration). In addition

to the constructs under examination, controls are used in each experiment, always tested in triplicate:

sterile LB medium: used during the analysis phase to normalize thebacterial growth values.

TOP10: which does not produce fluorescence, being devoid of fluorescent proteins, therefore used to normalize the fluorescence values.

J101 RFP34, whose structure is shown in the figure, was used as a control [21]for the fluorescence. It consists of a constitutive promoter *(BBa_J23101)* anda fluorescent RFP protein *(BBa_I13507)*. The promoter is always on and thus the protein is always expressed, which produces red fluorescence.

Figure 18: J101 RFP34 expression cassette, containing: J101 as constitutive promoter, strong RBS, RFP reporter gene and double terminator. The expression cassette is in pSB4C5 plasmid, Low Copy.

3.3.1 Data analysis

The absorbance and fluorescence data were analyzed by software *Microsoft Excel.* The mean of the sterile LB medium values and the mean of the *TOP10 fluorescence values,* used as controls, corresponding to each measured time interval, were subtracted from the raw absorbance and fluorescence data, respectively. The normalized data of the OD600 allowed to derive the bacterial*growth rate,* using the *MATLAB* calculation software.

To analyze the fluorescence variation, the ratio between fluorescence and OD600 was calculated ateach time interval, considering only the exponential growth phase of the bacterial culture, i.e. OD600 between 0.05 and 0.18.

The results obtained made it possible to evaluate the ability of the system to activate and therefore produce fluorescence and were compared with the fluorescence produced by J101 RFP34.

3.3.2 Supernatant assay experiments

Using these experiments, it was possible to monitor the growth of *P. aeruginosa* bacterial culture over time. It has also been used to determine the degradation capacity of AiiA, a lactonase capable of degrading lactones.

To carry out the supernatant analysis experiment, a smear of the bacterial culture under examination was carried out in plate LB, selective if necessary, and it was left to grow overnight at 37 ° C. The following day a colony was inoculated in 5 ml of LB, and it was then left to grow in an incubator at 37° C, 220 rpm, again overnight. The next day, 1 ml of culture was taken, whose optical density (OD) was calculated by means of a spectrophotometer. Once the starting OD value was known, around 2, it was carried out at a dilution of 1: 100, then inserting 1 ml of the bacterial culture in 99 ml of LB into the flask. The OD of this freshly diluted culture was determined, which will constitute T0, ie the sample at time zero. The flask containing the culture will be left to grow in an incubator at 37 ° C, 150 rpm and a new OD measurement will be performed after two hours.

Typically, the measurements were performed at 0, 2, 4, 6, 8, 10, 12, 14, 24, 30, 36, 48 hours. At each measurement, the sample taken, at which the OD was measured, was then centrifuged at 13000 rpm for 2 minutes, only the supernatant was collected, which was then filtered and stored at -20 ° C, everything was conducted in sterile conditions.

4.Results

4.1 Circuit design based on the Las system.

4.1.1 RBS WT Removal

In prior thesis [ref: thesis Marta Pamucci] the promoters (pLasA, pLasB, pLasI) and the LasR gene from the *Pseudomonas aeruginosa* were isolated and amplified. These promoters were amplified with their respective native Wild type (WT) ribosome binding site (RBs) downstream. As a result, after the signal molecule binding to the receptor and subsequent promoter activation, there was only minimal, if not negligible, fluorescence emission as detailed in the section 4.3. To address this issue, during this thesis project, an investigation was undertaken to find an explanation and a solution.

To resolve this issue, expression cassettes were constructed, which included the promoter, the RBs (Wild type), an additional synthetic RBS (RBS34, BBa_B0034), the RFP reporter gene (BBa_E1010) and a double transcriptional terminator (BBa_B0015) in pSB1A2 plasmid.

This configuration, featuring the insertion of the synthetic RBS (RBS34) alongside the native one, aimed to enhance the translation efficiency of the fluorescent protein.

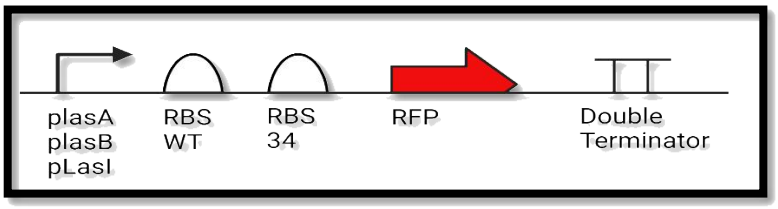

Figure 19: Expression cassettes of pLasA, pLasB, pLasI, RFP34Wt were found in pSb1A2 plasmid.

To evaluate the functionality of these constructs and determine the expression capabilities of each individual promoter for the fluorescent protein, co-transformations were conducted. These co-transformations enabled the introduction of both the previously mentioned plasmid and another plasmid containing the LasR receptor into the same cell. The LasR receptor expression cassette (fig19) includes the LasR gene positioned downstream of a robust ribosome binding site (RBS), all of which are under the control of a constitutive BBa_J23110 promoter. The plasmid employed for this purpose is pSB4C5, which has a low copy number.

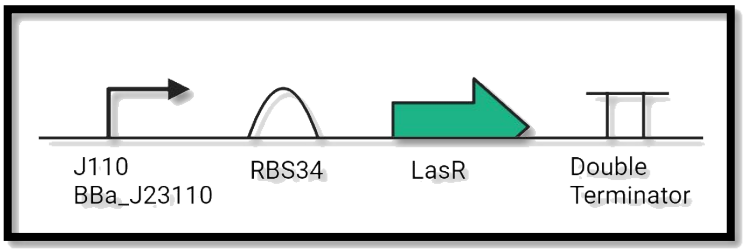

Figure 20: Expression cassette expressing the LasR receptor in the Low copy plasmid pSB4C5.

Subsequently, these newly developed constructs were tested using a microplate reader in the presence of varying concentrations of the 3OC12-HSL self-inducer.

In a previous thesis (Marta Palmucci); the promoters (pLasA, pLasB, pLasI) and LasR gene from the *Pseudomonas aeruginosa* were isolated and amplified. The promoters were amplified with their respective Wild type of ribosome binding site (RBS) located downstream. Consequently, after the binding of the signal molecule to the receptor and the subsequent promoter activation, only minimal, if any, fluorescence emission was observed (see section3.3). Therefore, in the context of this thesis project, the decision was made to investigate and address this issue.

Initially, the strategy involved removing the Wild type of RBS located upstream of the RFP reporter gene to optimize the Las system. It's worth noting that a version of the circuit with only the native RBS had been previously assessed in the Marta Palmucci but did not yield measurable fluorescence signals.

To properly remove the RBS WT, the actual sequence corresponding to this Wild type of RBS had been previously assessed in Marta Palmucci's thesis but did not yield measurable fluorescence signals. To properly remove the RBS WT, the actual sequence corresponding to this region was researched in the literature, considering that it spans from the transcription start site up to the ATG of the gene. [41,42,43]

Once the pairs of the divergent primers for mutagenesis were designed using Benchling software as indicated in table 1, they were synthesized through the Sigma-Aldrich service.

Primers		Tm
Fw_remWtRBS_pLasA Rv_remWtRBS_pLasA	5'TACTAGAGAAAGAGGAGAAATACTAGATGGC TTCCTC-3' 5'CGCAGGACCGTGCAACG-3'	66.6°C
Fw_remWtRBS_pLasB Rv_remWtRBS_pLasB	5'TACTAGAGAAAGAGGAAATACTAGATGGCT-3' 5'GGTGCTTTCGTGTACCAAAAAAC-3'	65.9°C
Fw_remWtRBS_pLasI Rv_remWtRBS_pLasI	5'TACTAGAGAAGAGGAGAGAAAATACTA GATCG-3' 5'TGAAAATTTATGCAAATTTCAT-3'	56.9°C

Table 1: RBSWt is removed in Primers.

The plasmid was subjected to PCR amplification, excluding the region to be removed, which resulted in linear DNA. Subsequently, the linear DNA was circularized using PNK (polynucleotide kinase to activate the blunt ends of the amplicon by adding phosphate groups) and ligase. The resulting new constructs were designated as pLasA RFP34, pLasBRFP34 and pLASIRFP34.

To assess the functionality of these new constructs, three co-transformations were carried out. In each of these co-transformations, the plasmids containing the new constructs specific to a particular Las promoter were introduced into the same cell along with plasmid containing the LasR receptor, as illustrated in fig:20.

Additionally, three more co-transformations were performed, as depicted in fig 21. These co-transformations included one more plasmid compared to the previous set, namely J23116 ÿD, a medium copy number plasmid containing a constitutive expression cassette for the ÿD factor of *Pseudomonas aeruginosa* under the control of the BBa_J23116 promoter. The ÿD factor, or sigma factor, serves as an initiation factor that facilitates RNA synthesis, particularly by binding to RNA polymerase and enabling it to bind exclusively to promoters. The transcription of major sigma factor genes, specifically rpoD and rpoS, in *Pseudomonas aeruginosa* is influenced by the growth phase. Notably, the transcription of rpoD (which encodes the primary sigma factor, ÿD or ÿ70, in *Pseudomonas aeruginosa*) is induced during the exponential phase of the cells. Therefore, it was hypothesized that the presence of this factor could enhance the response of the promoter even during phases that are not typically active in the organism of origin.[44]

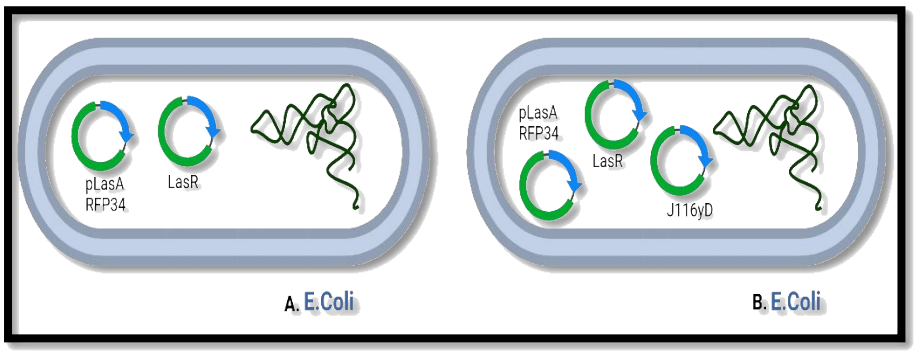

Figure 21: Illustrating the co-Transformations. A. pLasA RFP34 in plasmid pSB1A2 and LasR in the pSB4C5. B. A additional plasmid is introduced i.e. J11yD in pSB3K3.

4.1.2 PlasR3 isolation and amplification

Another promoter of the Las system, as described in the literature is described in the literature [26], was identified, and it was decided to isolate and amplify it using appropriately designed primers.

Fw_pLasR3	5'-TCGCACGGGAGGAGCC-3'
Rv_pLasR3	5'GATAAACCCTGGCACTCTGCCG-3'

Table 2: Isolating primer from pLasR3

After isolating and amplifying this promoter through PCR, a cloning procedure was conducted by inserting the same ribosome binding site (RBS) and RFP reporter gene, previously utilized, downstream of the promoter. This resulted in the creation of pLasR3 RFP34, which was inserted into the pSB1A2 plasmid, as illustrated in Figure 23. Following this, two co-transformations were carried out, mirroring the methods described earlier in Section 3.1.1

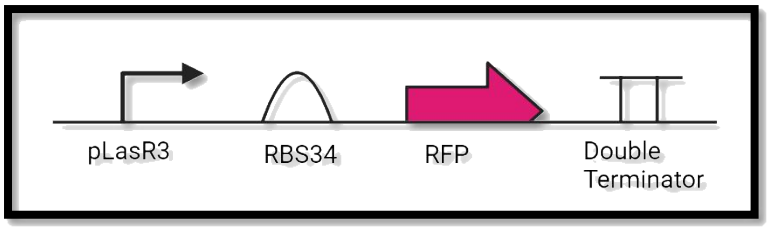

Figure 23: Expression cassette of pLasR3 in pSB1A2

4.2 Circuit design based on the Rhl system.

4.2.1 Isolation and amplification of promoters and Rhl gene system.

First, the Known parts related to the promoters of the Rhl system were identified in the literature and recorded on the Benchling platform, providing an overview of the currently known promoters' complex structure with operons containing two promoters for the expression of thefive genes typically associated with the Rhl system (Figure 24).[46]

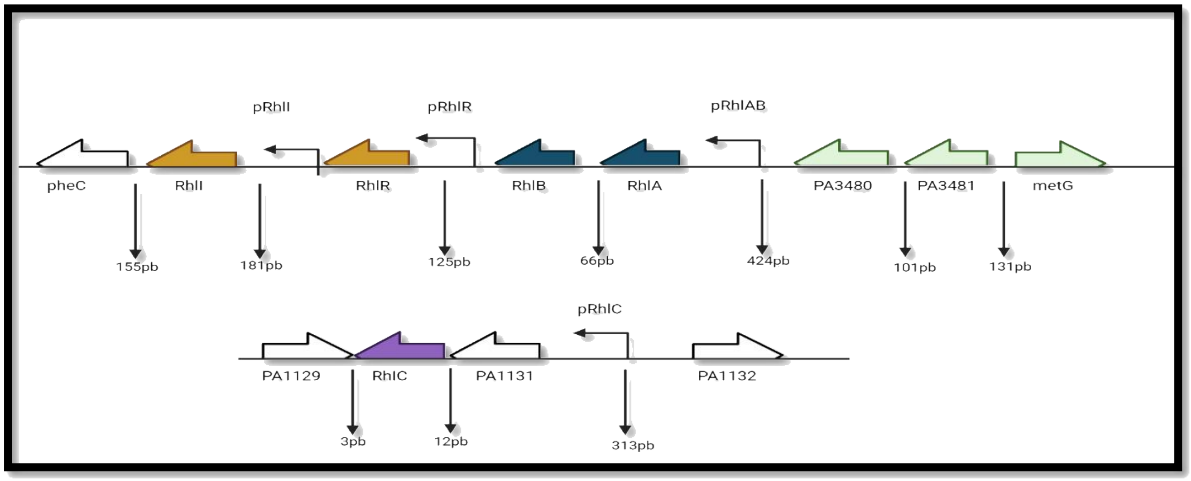

Figure 24: Mapping of genes and promoters of Rhl within the genome of *P. aeruginosa* (PAO1)and their distance, in terms of base pairs (bp).

Two types of primers were designed for the Rhl system: "tailless" primers (shown in Table 4 withthe wording genome amp), for isolating the gene and promoters before adding restriction sites tofacilitate PCR and "with code" primers (reported in Table 3) for adding restriction sites, Bio brick prefix, and suffix (Section 2.2.4). For the pRhlC promoter, which contains the EcoRI restriction site, the Gibson Assembly technique was used in a pSB1A2 plasmid, with specific primers designed accordingly.

Primers	
fw-rhlR-CDS	5'ATGAGGAATGACGGAGGCTT-3'
rv-rhlR-CDS	5’-TCAGATGAGACCCAGCGC-3’
fw_pRhlAB_genomeamp	5’-CGCCAGAGCGTTTCGACAC-3’
rv_pRhlAB_genomeamp	5’- GAACACTTTTTAGCCAATTCGAAAGC-3’

fw_pRhlR_genomeamp	5’-ACGGTGCTGGCATAACAG-3’
rv_pRhlR_genomeamp	5’-TGCAGTAAGCCCTGATCG-3’
fw_pRhlI genomeamp	5’-TCTGAAGCGCAGGGCGC-3’
rv_pRhlI_genomeamp	5’- GAGGGGGAAGACTAAAGGAGGATG-3’
fw_pRhlC_genomeamp	5’-CTAGCCCTGGTTCGCCG-3’
rv_pRhlC_genomeamp	5’-TGATCTTGGGACGCCAGC-3’
fw_Gibson_1A2rv_	5’-GGCGTCCCAAGATCATACTAGTAGCGGCCGC-3’
Gibson_1A2 fw_	5’-GCGAACCAGGGCTAGCTCTAGAAGCGGCCG-3’
Gibson_pRhlC rv_	5'-CGGCCGCTTCTAGAGCTAGCCCTGGTTCGC-3'
Gibson_pRhlC	5’-CGGCCGCTACTAGTATGATCTTGGGACGCC-3’

Table 3. Primers were created for the isolation and amplification of Rhl promoters and gene.

The first PCR, using "tailless" primers, was performed with specific melting temperatures reported in Table 5 for each sample, calculated using the "Tm calculator" computer support andthe reaction product run on an agarose gel (Figure 25).

	Tm
pRhlR	62.2°C
RhlR	64.3°C
pRhlC	65.1°C
pRhlI	66.7°C

Table 4. Melting temperatures are used to conduct PCR reactions to isolate promoters of Rhl.

The reaction product was run on the agarose gel, the run of which is shown in figure 26, then DNA was extracted.

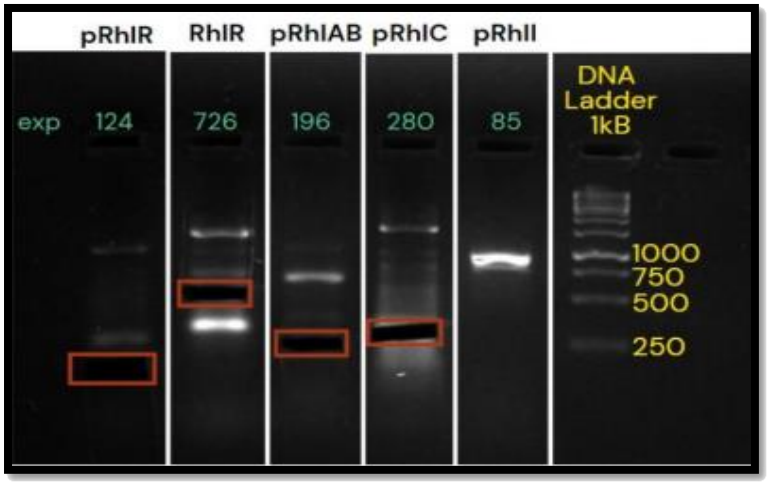

Figure 25: The height of the expectedband is shown in green, while the cuts made to extract the DNA are shown in red. On the side, the markerused to read the height of the bands has been loaded.

However, spurious bands and unexpected band heights for pRhlI were observed. A second PCRwas conducted, modifying the Tm values, increasing by one degree for all the samples and decreasing by one degree for pRhlI (Table 6).

	Tm
pRhlR	63.2°C
RhlR	65.3°C
pRhlI	65.7°C
pRhlC	66.1°C

Table 5: Melting temperatures used to isolate promoters of Rhl.

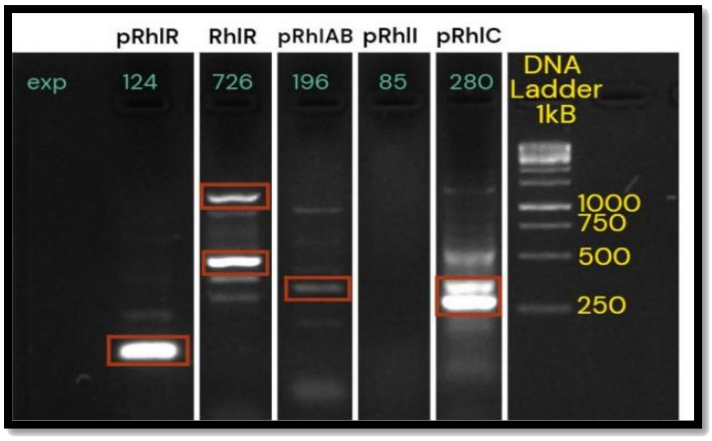

Figure 26: Run on agarose gel, carried out with different Tm. For the RhlR gene, two bands were cut and processed separately.

For the RhlR gene, which did not show a clear band at the expected height, two bands were cut and processed separately. As for pRhlAB, the extracted band did not provide enough genetic material for the subsequent cloning steps. Additionally, pRhlI did not yield any amplicons. Thesecond PCR was performed using the "tails" primers, while specific primers for the vector pSB1A2 and the insert pRhlC were used for pRhlC. The PCR products were subjected to the Gibson Assembly procedure.

The pRhlR and pRhlC promoters were successfully amplified and inserted into an expression cassette containing RBS 34 and the fluorescent protein RFP (Figure 21). However, for other promoters (pRhlAB, pRhlI) and the RhlR gene, attempts were made to isolate the sequences by conducting another PCR, but with unsatisfactory results.

For pRhlAB and RhlR, after the first PCRusing "no-tails" primers, height-corrected bands were obtained, and the "with code" primers were then used. The PCR product was purified and processed to obtain the expression cassette containing the pRhlAB promoter, RBS 34, and the fluorescent protein. For RhlR, an attempt was made to insert the gene into the vector containing the double terminator BBa_B0015. However, the transformation of the ligation products did not lead to the growth of any colony, hindering thesuccess of the cloning.

Co-transformations of the constructs shown in Figure 27 were performed together with theLasR receptor.

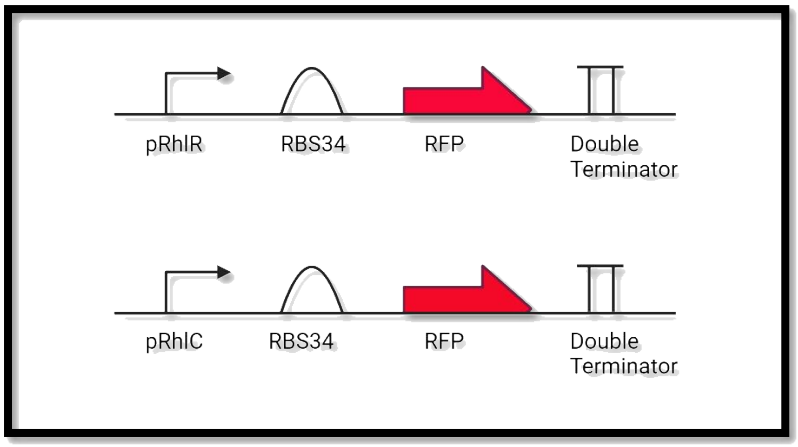

Figure 27: pRhlR RFP34 and pRhlC RFP34 expression cassette, both are in pSB1A2.

4.2.2 RhlR expression box

The RhlR expression cassette was designed and constructed by synthesizing the RhlR gene using the Genscript e Gene synthesis service and inserting it into a pSB4C5 plasmid via StandardAssembly.

The expression cassette was engineered to place the RhlR gene downstream of a strong RBS and under the transcriptional regulation of an inducible system (Figure 28). The components used for this design were as follows:

pSB4C5, a low copy number plasmid.

RBS BBa_B0034, a high-affinity ribosomal binding site, to maximize transcript translation.

Inducible LacI-pLac system, to regulate RhlR expression and avoid toxic levels in host cell.

This system comprises the constitutive promoter BBa_J23118 placed upstream of RBS. BBa_B0031 and the lacI gene. This cassette is then located upstream of the pLac promoter BBa_R0011, which remains repressed due to constitutive LacI expression. The pLac promotercan be activated in the presence of IPTG, a molecule that binds LacI, alleviating constitutive repression and allowing downstream gene transcription.

To construct the RhlR expression cassette, RBS 34 was initially cloned downstream of the inducible system Y34, using Standard Assembly. The intermediates were assembled to obtain Y34 RBS 34. Subsequently, the actual assembly was performed by combining two inserts, RhlR[XP] and Y34 RBS 34 [ES], with a

vector A38 [EP], resulting in a pSB4C5 plasmid with the RhlRexpression cassette (Figure28).

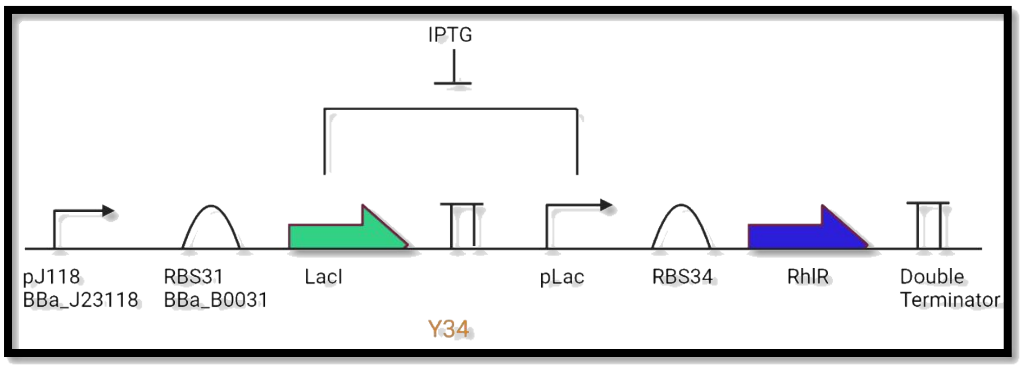

Figure 28: RhlR expression cassette (we call it as Y34 RhlR 34), in pSB4C5.

Co-transformations were then carried out to obtain all possible combinations of constructs, eachcontaining a promoter (from the las or Rhl system) upstream of a reporter gene and the RhlR receptor. The following combinations were made:

- Y34 RhlR 34 + pRhlR RFP34
- Y34 RhlR 34 + pRhlC RFP34
- Y34 RhlR 34 + pLasA RFP34
- Y34 RhlR 34 + pLasB RFP34
- Y34 RhlR 34 + pLasI RFP34
- Y34 RhlR 34 + pLasR3 RFP34

Construct name Vector		Digestion vector	Insert	Digestion insert
pLasR3 RFP34	pLasR3_pSB1A2	S-P	RFP34 TT BBa_I13507	X-P
pRhlR RFP34	RFP34 TT BBa_I13507	E-X	pRhlR	IT IS
pRhlC RFP34	Gibson Assembly			
Y34 RhlR 34	A38	E-P	RhlR Y34RBS 34	X-P IT IS

AiiA34	RBS 34_ pSB1A2 BBa_B0034	S-P	AiiA	X-P
Y34 AiiA34	AiiA34	E-X	Y34 pLac	IT IS
Y32 AiiA 34	AiiA34	E-X	Y32pLac	IT IS
Y34 RBS34	RBS34_ pSB1A2 BBa_B0034	E-X	Y34 pLac	IT IS

Table 6. List of built expression cassettes

4.3 Plate reader experiments

4.3.1 LasR receptor

The LasR receptor was used to analyze the different isolated promoters from both the las and Rhl systems in response to different auto-inductors at varying concentrations. The aim was to test the activation capacity of the promoters by the LasR self-inducer-receptor complex and subsequently assess the expression of the downstream fluorescent protein (Figure 29).

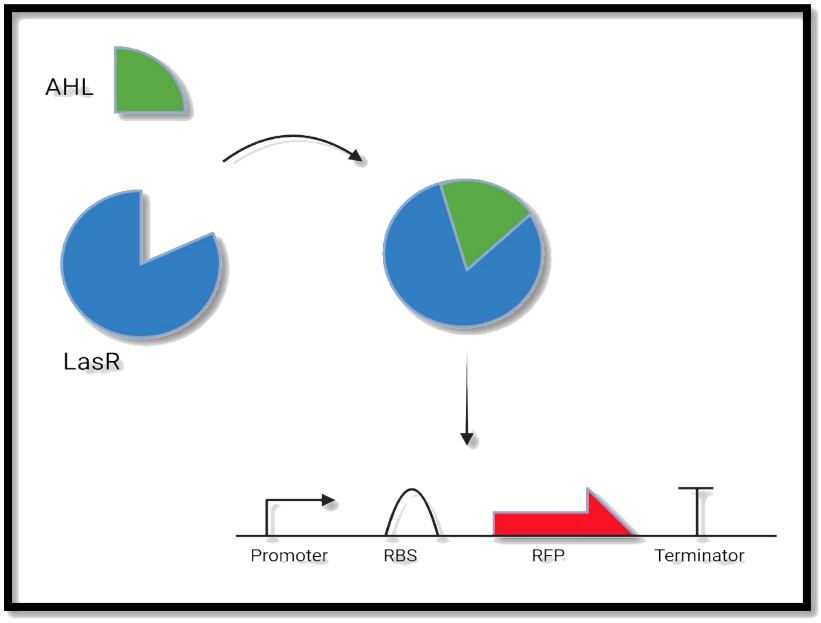

Figure 29: Mechanism is based on the experiment. The signal molecules, N-acylhomoserine lactones (AHL), bind to the LasR receptor. This newly formed complex can activate the promoter, which will be able to induce the expression of the red fluorescent protein.

4.3.1.1 Response to 3OC12-HSL from *P. aeruginosa* supernatant

The co-transformations involving the px RFP34 plasmids (where "x" represents the promoters: pLasA, pLasB, pLasI, pLasR3, pRhlR, and pRhlC) and the plasmid containing the constitutive expression cassette of the LasR receptor were subjected to analysis. Additionally, the single pRhlR RFP34 construct was also analyzed independently, without the LasR receptor.

To initiate the experiment, a 24-hour culture supernatant of *Pseudomonas aeruginosa* strain PAO1 was prepared. The PAO1 inoculum was grown at 37 °C overnight (using 10 µl of PAO1 in 15ml of LB). The following day, the culture was centrifuged for 10 minutes at 4 °C, 5000 rpm, and the supernatant was filtered.

Initially, the constructs were tested using two different media: one containing only LB, and the other containing PAO1 supernatant 24h (diluted 1:2 with fresh LB, resulting in a 50% LB + 50% PAO1 supernatant mixture). From the analysis of the data, an observable difference in the growthrate of bacteria in the two media was apparent.

Figure 30 illustrates that both J101 34 RFP and TOP10 controls showed reduced growth when the medium contained PAI (Pseudomonas Auto Inducer). To investigate further, the experiment was repeated, this time diluting the PAO1 supernatant 24h with LB 2x to determine if the issue was related to toxicity or lack of nutrient.

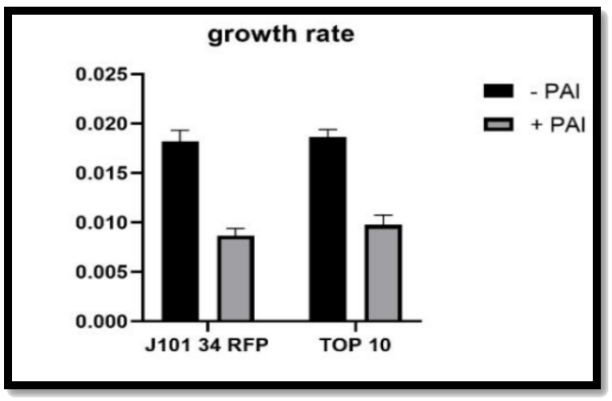

Figure 30: Growth of controls (J101 34RFP and TOP10) with and without PAI. In both cases,in the presence of PAI, a halved growth rate can be observed.

After conducting a replication of the experiment, it was observed that there was equal growth in the medium containing LB and the one containing the diluted 24h PAO1 supernatant. This indicates that the initial growth difference was due to a lack of nutrients rather than toxicity.In Figure 31, it is evident that with the restored optimal nutrient concentration, there are no growth issues, confirming that the PAO1 supernatant is not toxic to E. coli. Despite this, the fluorescence produced by the promoters in the presence of PAI remains relatively low comparedto J101 RFP34, making the system less suitable for screening applications. The pRhlR promoter stands out as an exception, showing a strong basal expression level that is further increased in the presence of the LasR receptor but only when PAI is absent.

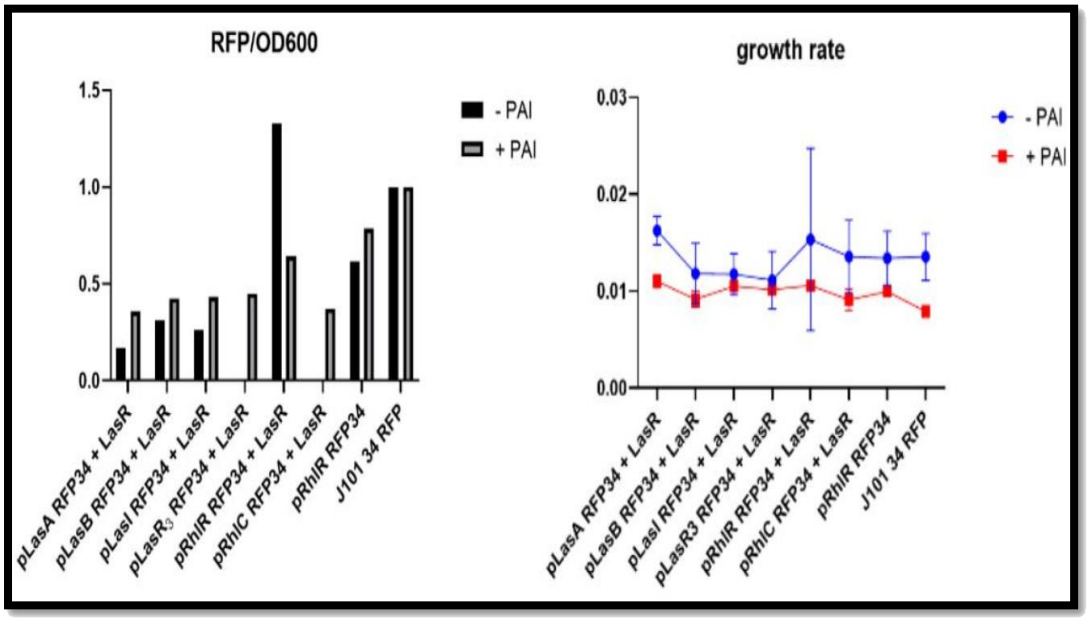

Figure 31: The graph defines RFP / OD600, i.e. The fluorescence expression following the activation of the different promoters contained within each tested construct, in the presence or not of PAI.

4.3.1.2 Response to commercial 3OC12-HSL

The response to commercial 3OC12-HSL of all the isolated promoters from both the las and rhl systems was evaluated. This involved assessing the ability of the self-inducer-LasR receptor complex to activate the promoters and subsequently express the fluorescent protein in responseto known concentrations of the lactone derived from the substances produced and secreted by *P. aeruginosa*. Furthermore,

the possible concentrations of ÿD were also evaluated. Induced media containing the self-inducer under examination were prepared at the following concentrations: 0,10,100,1000,10000 nm.

The graph in Figure 32 displays the results obtained by testing the four promoters of the las system at different concentrations of 3OC12-HSL. The pLasB promoter showed almost constant activation, with a slight increase in fluorescence for higher inducer concentrations. The pLasI promoter exhibited a peak of fluorescence production only at 1000 and 10000 nM inducer concentrations. For pLasR3 and pLasA, a constant trend was observed, with a slight reduction influorescence at the maximum induction concentration.

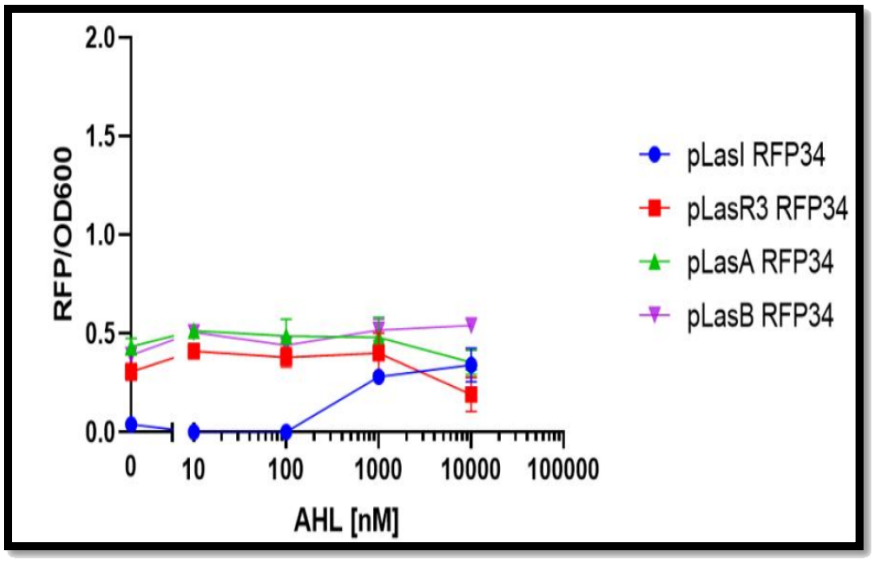

Figure 32: The graph defines the RFP / OD600 ratio. The response to the 3OC12-HSLinductor of the pLasI, pLasR3, pLasA, pLasB promoters was tested.

However, the expression levels were still too low to be used reliably as a biological sensor. The maximum expression of these systems reached only half of the fluorescence expressed by thereference construct J101 RFP34.

Regarding the contribution of the factor ÿD, Figure 33 displays the graph of the co-transforms containing the additional transcription factor. These systems showed more intense activation than in the previous case, with greater production of fluorescence.

However, the baseline fluorescence values were also higher, making it difficult to appreciate a significant increase in the differential response in the presence or absence of the inducer, which is desirable for a good biological sensor. In this case, all the examined constructs showed increasing fluorescence signals with higher induction concentrations, except for the pLasA promoter, which exhibited a slight reduction in fluorescence at the maximum inducer concentration.

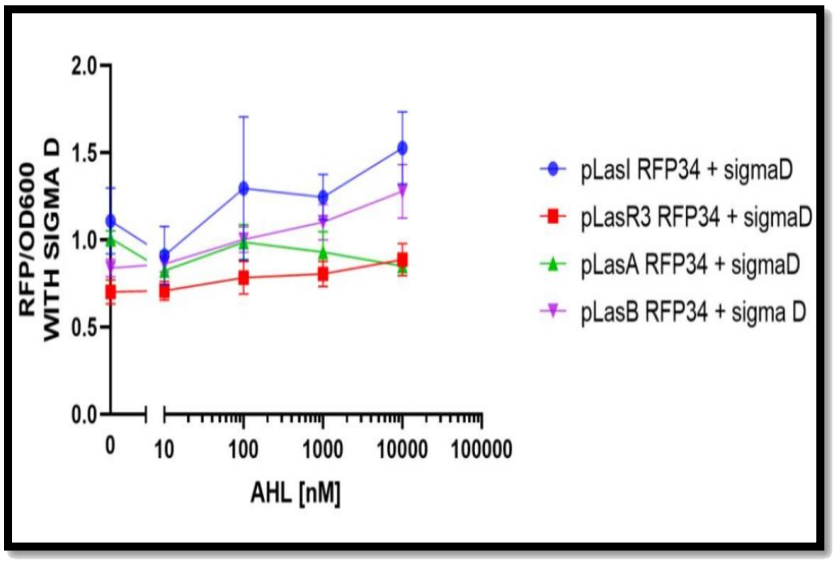

Figure 33: The graph defines the ratio of RFP / OD600, it differs from the previous graph dueto the presence of the factor ÿD.

To verify if the addition of ÿD generated a non-specific increase in transcription in E. coli or if it was a specific effect linked to the Las system, the fluorescence production capacity of J101 RFP34 with or without ÿD was compared (Figure 34). It was observed that the presence or absence of ÿD did not affect the production of fluorescence.

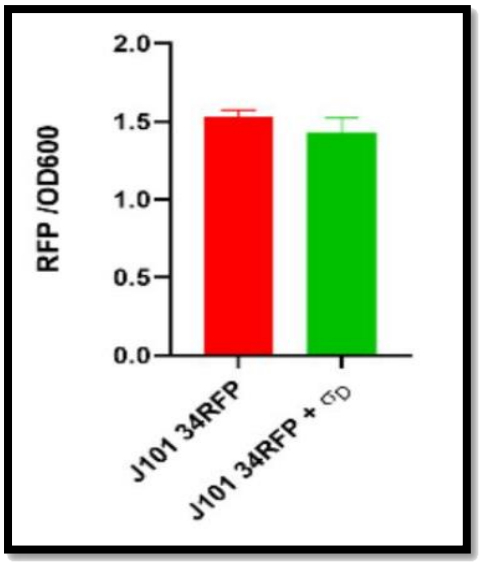

Figure 34: ÿD test: the fluorescence production is equal both in the presence and in theabsence of ÿD.

Finally, the promoters of the rhl system, pRhlR, and pRhlC, were tested in the presence of the LasR receptor and known concentrations of the 3OC12-HSL inducer (0, 10, 100, 1000, 10000 nM). As shown in the graph in Figure 35, the pRhlC promoter showed no activation or induction. The pRhlR promoter exhibited an almost constant trend, regardless of the inducer concentration, demonstrating constitutive expression even in the absence of a signal molecule.

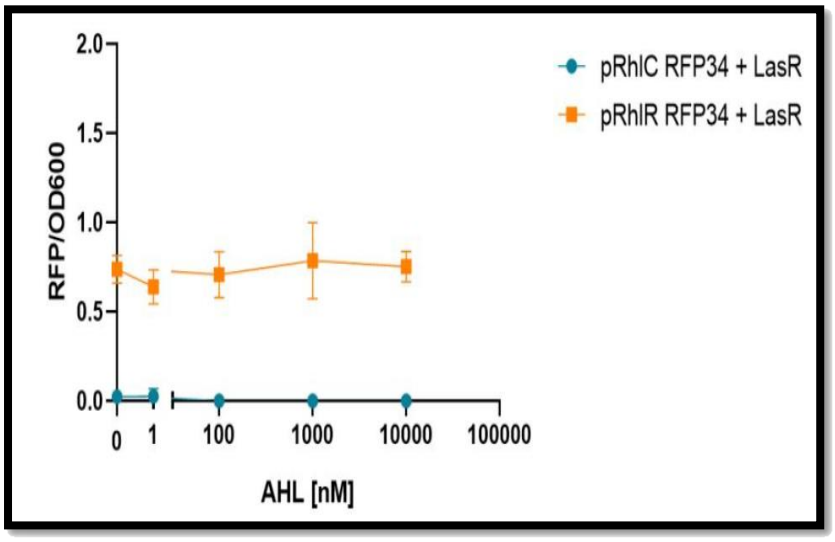

Figure 35: RFP expression, using 3OC12-HSL at different concentrations and constitutively expressed LasR.

4.3.2 RhIR receptor

In a similar manner to section 3.3.1., all the promoters from both the las and rhl systems weretested using three different autoinducers, this time in the presence of the RhlR receptor, to evaluate the expression of the fluorescent protein. The experimental setup was like that shown in Figure 28, but with the RhlR receptor being used instead.

4.3.2.1 Response to 3OC12-HSL, 3OC6-HSL and C4-HSL

The co-transformed constructs were tested with three different self-inductors: 3OC12-HSL, 3OC6-HSL, and C4-HSL, as described in section 3.2.2. The graph in Figure 36 illustrates that all the examined constructs displayed lower fluorescence compared to the J101 RFP34 control. Additionally, no clear difference in fluorescence intensity was observed among the constructs, regardless of the specific lactone used.

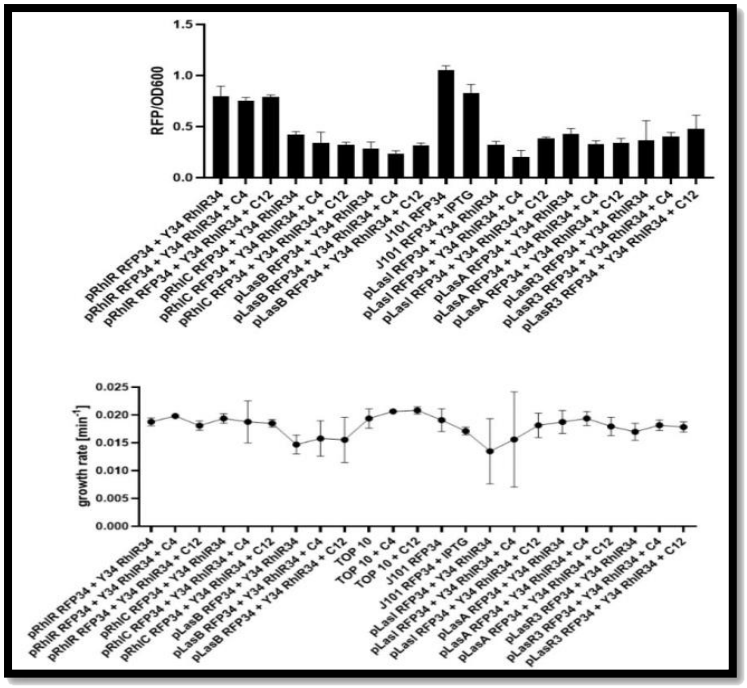

Figure 36: Above results of RFP protein expression, i.e., fluorescence emission of the constructs containing the promoters of the las and Rhl system, in the presence of the RhlRreceptor and the three different lactones. The growth rate is shown below.

4.3.3 Lux sensor calibration in response to 3OC12-HSL

Due to the inability to obtain specific sensors for *Pseudomonas* lactones, it was determined to explore potential crosstalk between various signal molecules and quorum sensing systems to assess how established promoters respond to alternative compounds. The decision was made to evaluate this promoter's ability to express the fluorescent protein by inducing the pLux promoter with three different lactones: 3OC12-HSL, 3OC6-HSL, and C4-HSL taken from the thesis [Ilaria Buran].

The calibration curve is a method employed to assess the concentration of an analyte within an unknown sample. In this context, it was essential to establish a system capable of quantifying the concentration of the signal molecule, namely 3OC12-HSL (PAI auto-inducer), present in *Pseudomonas aeruginosa* cultures.

To create the calibration curve based on the lux system associated with bacterial growth, it is noteworthy that the presence of lactones, even at elevated concentrations, does not affect bacterial growth, as depicted in Marta Pamucci thesis.

For the assessment of the response of the E47 construct to varying concentrations of 3OC12-HSL, a supernatant analysis experiment was conducted, as detailed in Section 2.5. The procedure involved streaking *Pseudomonas aeruginosa* onto a plate and cultivating the bacteria for 16 hours. Subsequently, a colony was inoculated into liquid LB medium the following day. The culture was allowed to grow in a flask within an incubator at 37°C, and at specific time intervals, samples were collected and the optical density (OD) of the microbial culture was determined for each sample. The time intervals and the corresponding OD values are presented in Table 7.

pLux promoter, which is associated with *V. fischeri* quorum sensing, has been extensively investigated in the literature. It operates by forming a complex between the LuxR activator protein and the 3-oxo-hexanoyl-HSL (or 3OC6-HSL) self-inducer, thereby activating the gene downstream of this promoter.

Time (hr)	
0	0.016
2	0.083
4	0.432
6	0.969
8	1.277
10	1.690
12	1.890
14	2.035
24	2.419
30	2.546
48	2.602

Table 7: Time intervals and corresponding OD values

The 96-well plate, analyzed in the plate reader, was divided into two regions: three if we consider the controls. In the first region, induced media at 7 different concentrations (0,1,10,100,1000,10000,100000 nM) of induced medium. In the second region, the samples saved at the following time intervals were inserted: 0,4,6,8,10,12,14,24,30,48 hours also in triplicate, so that the each well contained:

- 100 µL of supernatant, therefore corresponding to the samples taken.
- 100 µL of LB 2x medium, to obtain a 1:2 dilution of the samples in the final 1x LB medium, thus containing half the inducer but all the nutrients necessary to allow normal growth; in the supernatant they had already been consumed by the previous culture.
- 2 µL of inoculum of construct E47 [ref: Marta Pamucci]

Through data analysis, it became clear and feasible to derive, in the initial region, the system's response in relation to RFP/OD600 concerning the level of exposure to 3OC12-HSl (figure 37). In the second region, conversely, the RFP/OD600 values of various samples collected at different time intervals were examined to infer the contained 3OC12-HSL concentration.

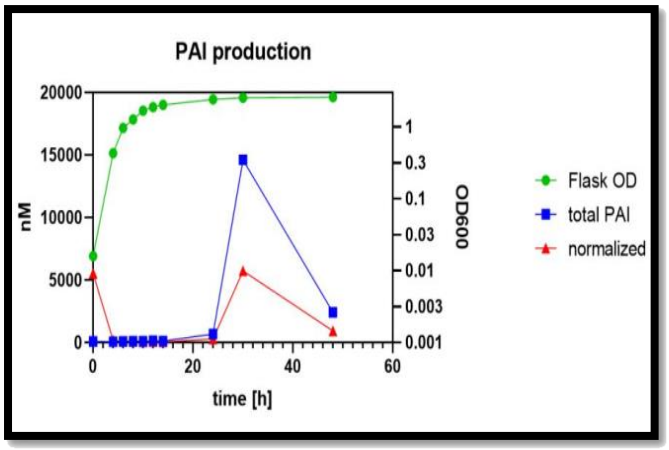

Fig 37: PAI production as function of time and OD values taken and sampled from *P. aeruginosa*. The normalized values are intended on the OD of the culture, therefore on the number of cells producing RFP.

Through the analysis of the second region, by aligning the fluorescence values on the x-axis of the calibration curve, it was possible to determine the concentrations of 3OC12-HSL corresponding to various time intervals. In figure 37, the OD values of the samples taken at each time interval are represented in green, while the production of 3OC12-HSL (PAI) over time is depicted in blue. Furthermore, the same production is shown in red, normalized to the initial OD600 value measured in the culture (indicating the number of cells producing PAI). This graph highlights that the peak of production occurs around the 30-hour.

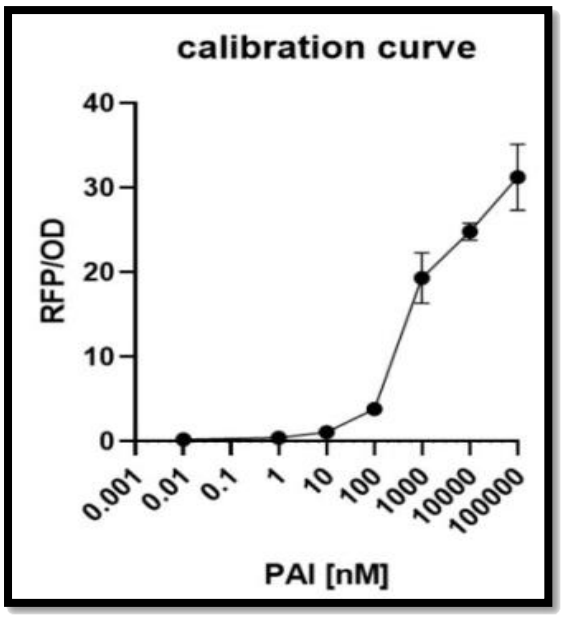

Figure 38: Calibration curve, the wells containing the 7 different inductions and the construct E47. From this curve, knowing the RFP/OD600 of the samples taken at different time intervals, it will be possible to trace the concentrations of OC12-HSL.

4.3.4 Degradation of lactones by AiiA

Before describing the procedures of the degradation experiment involving AiiA, it is essential tounderstand its function. The AiiA gene from Bacillus subtilis encodes the lactonase AiiA, an enzyme capable of degrading lactones, particularly inactivating the acyl-homoserine lactone quorum detection signal [45].

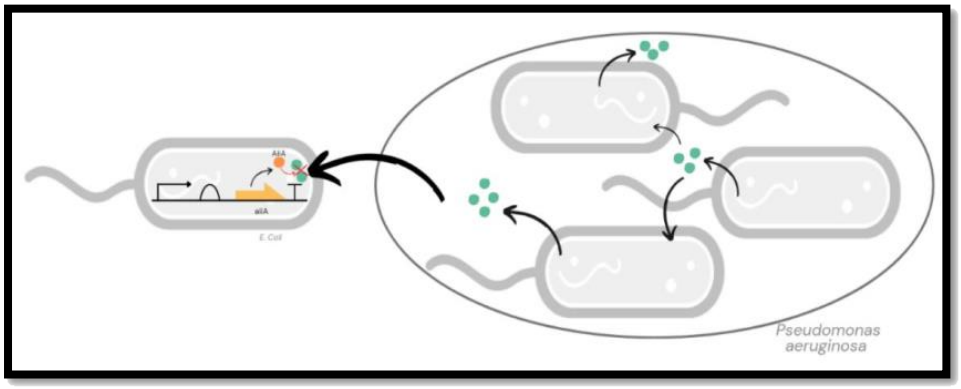

Figure 39. AiiA mechanism (created with Bio render)

Figure 39 illustrates the mechanism devised to inhibit the quorum sensing signal using AiiA. On the left, E. coli is engineered to produce AiiA, while on the right, *P. aeruginosa* cells produce AHLlactones, which spread to surrounding cells, including E. coli, where they are degraded by AiiA lactonase.

Cloning of the expression for AiiA, RBS34 was first inserted upstream of the AiiA gene, by cloning (see table7, used as a AiiA insert [XP] and as a vector RBS34 [SP] in pSB1A2). Then resulting construct will be called AiiA34. Later it was decided to clone AiiA34 a downstream of an inducible system, similarly to the RhlR gene in section 3.2.2 i.e. Y34, whose expression cassette is shown in the figure 40 and in which we recall that the activation of the pLac promoter occurs only in the presence of IPTG, by inhibition of the LacI repressor.

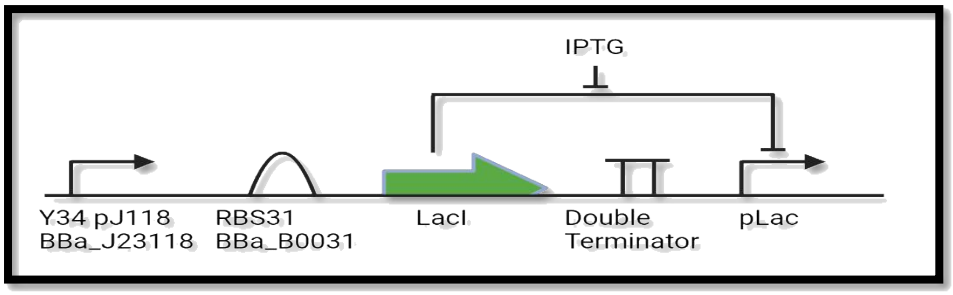

Figure 40: Y34 expression cassette.

The experiment involved cloning the AiiA expression cassette with RBS 34 inserted upstream ofthe AiiA gene, creating the construct AiiA34. AiiA34 was then cloned downstream of the inducible system Y34, like what was done for the RhlR gene.

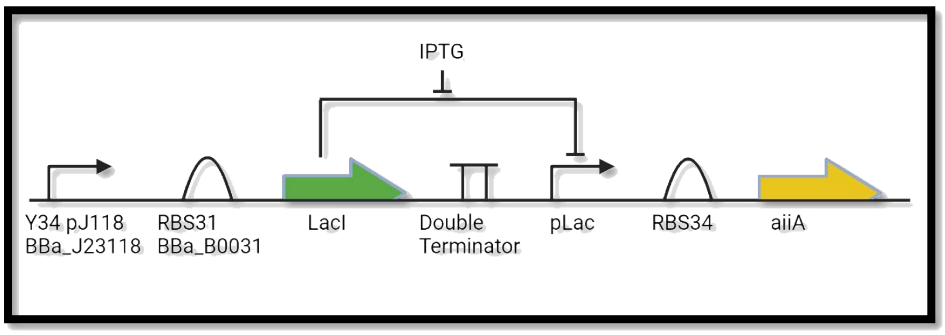

Figure 41: Y34 expression cassette pLac AiiA34 in plasmid pSB1A2.

The pLac promoter in Y34 was activated in the presence of IPTG by inhibiting the LacI repressor.This resulted in Y34 pLac AiiA34 in Figure 41. The degradation capacity of AiiA was evaluated by comparing it with a control, TOP10, which lacked lactonase activity, relying solely on spontaneous lactone degradation. The experiment utilized the same culture conditions as the supernatant analysis.

First, two smears were made, that of Y34 pLac AiiA34 on selective LB plate, while for TOP10 onLB plate. The following day a colony was inoculated, respectively, in a tube containing liquid LBmedium, suitably added with the antibiotic, if necessary. In this case it was necessary to add 10µL of IPTG (final concentration 100 M), used to unblock the system and allow the activation ofthe promoter, with consequent expression of the gene. The next day the OD was determined ofeach inoculum and then two distinct flasks were prepared, each containing:

- 1 ml of culture - 99 ml LB
- 100 µl ampicillin (only for Y34pLac_AiiA34)
- 100 µl 3OC12-HSL (PAI) inducer [final concentration 100 µM]
- 200 µl IPTG [final concentration1 mM]

The flasks containing the cell cultures were incubated at 37 ° C, 150 rpm and sampled thereafter,as reported.

time0	OD AiiA	TOP10
	0	0
2	0	0
4	0.085	0.302
6	0.507	0.892
8	1.000	1.285
10	1.387	1.473
12	1.468	1.562
14	1.538	1.665

Table 8: The OD values recorded are shown.

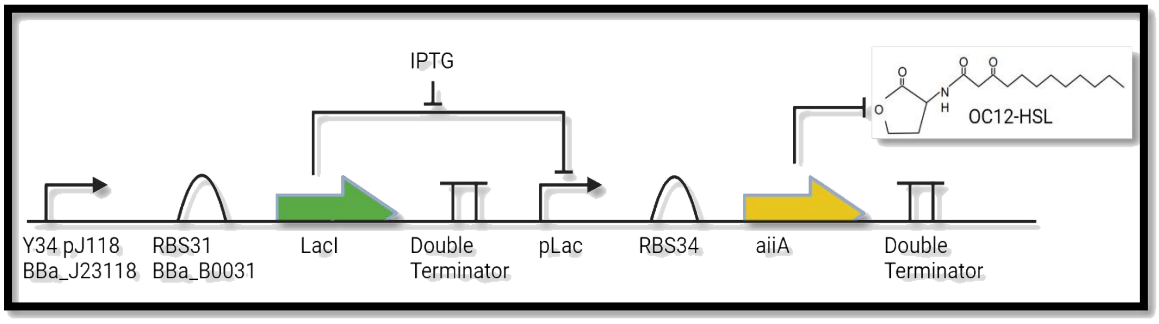

Figure 42: Y34 pLac AiiA34 expression cassette, the ability of the AiiA gene, the production of AiiA proteins, to degrade lactones such as 3OC12-HSL (OC12).

In this case it is possible to divide the 96-well plate into two main regions, one corresponding tothe controls, while the other to the cultures sampled at the chosen time intervals. TOP10 and Y34 pLac AiiA34 samples taken at 0, 2, 4, 6, 8, 10, 12, 14, 24 hours were used.

All the samples were analyzed in triplicate as per the protocol described in section 3.3.3; each well therefore contains 100 µL of supernatant, therefore corresponding to the samples taken, dilutedwith 100 µL of LB 2x medium and finally 2 µL of construct inoculum E47.

From the data analysis it emerged a high degradation rate for AiiA, whileit is slower for control, as shown in Figure 43, in line with what expected.

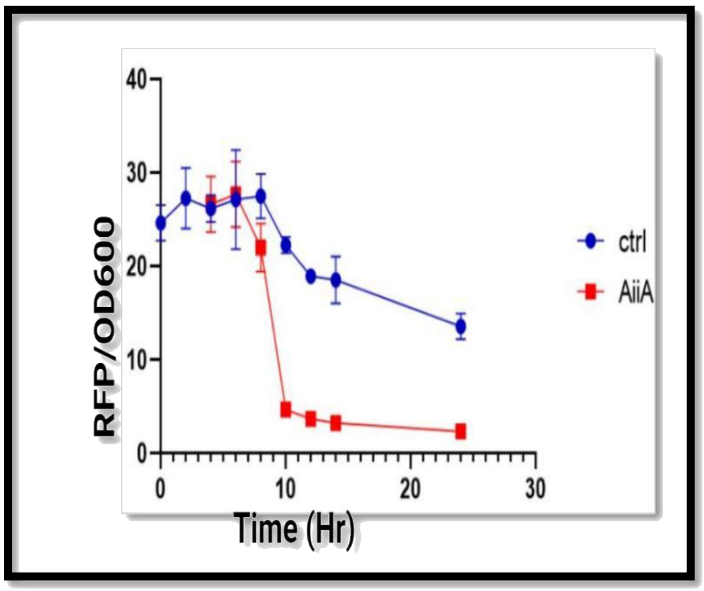

Figure 43: Results of the degradation capacity of AiiA, compared with a control, TOP10, thelatter devoid of lactonase, therefore the degradation of the lactone should only be the spontaneous one (slower).

5. Discussion and Conclusion.

In a prior thesis, the promoters (pLasA, pLasB, pLasI) and the LasR gene from *Pseudomonas aeruginosa* were isolated and amplified. The amplification of these promoters included the sequences corresponding to the regions of the native RBS, referred to as wild type. These constructs were subsequently inserted into expression cassettes that also contained an additional (synthetic) RBS, an RFP reporter gene, and a double transcriptional terminator. These assembled constructs were then tested by co-transforming them into a cell that also contained the LasR receptor. The goal was to assess their ability to express the fluorescent protein when exposed to varying concentrations of the self-inducer 3OC12-HSL.

However, upon the binding of the signal molecule to the receptor and subsequent activation of the promoter, it was observed that the emission of fluorescence was notably low, if not entirely insignificant.

Hence, in this thesis project, the research is to address and resolve this issue. The initial strategy involved the removal of the Wild Type ribosome binding sites (RBSs) in order to optimize the Las system. It's noteworthy that the circuit solely relying on Wild Type RBS had previously undergone examination, and the results revealed an absence of measurable fluorescence signals when subjected to analysis using a microplate reader.

After identifying the region requiring removal, and following the design and amplification of the plasmid, new constructs were created. These were denoted as pLasA RFP34, pLasB RFP34, and pLasI RFP34. We conducted co-transformations using these constructs in E. coli cells that possessed an expression cassette for the LasR receptor. Moreover, we also performed co-transformations in bacteria containing both the receptor and the transcription factor ÿD, with the aim of exploring potential enhancements.

Furthermore, in the search for solutions, we uncovered another promoter within the Las system, namely pLasR3, via existing literature. This promoter was subjected to a processing procedure similar to that of the previous three constructs.

The expression cassettes, containing the promoters from both the Las and RhlR systems, were subsequently co-transformed in combination. They were introduced into

E. coli cells already equipped with expression cassettes for the LasR receptor, and in parallel, into bacteria containing plasmids encoding the RhlR receptor. These co-transformed systems were then assessed in the presence of the LasR receptor and various auto-inductors.

In response to the presence of 3OC12-HSL (PAI) from *Pseudomonas aeruginosa*, it was observed that bacterial growth was halved when the medium contained PAI. To investigate this further, the experiment was replicated with a modification: the PAO1 supernatant was diluted with LB 2x after 24 hours, rather than the normal LB medium. This adjustment resulted in equal growth in both media (with and without the supernatant), indicating that the *P. aeruginosa* culture supernatant requires additional nutrients to serve as a suitable growth medium for E. coli.

While there was some activation of the promoters with a consequent expression of fluorescent protein in the presence of PAI, the intensity was reduced compared to that produced by J101 RFP34, which served as the control. This characteristic renders the system less practical for screening applications due to the noise in the fluorescence signal at such low intensities. Moreover, the activation range in the presence or absence of PAI is quite narrow, to the extent that the activation for the pLasR3 and pRhlC promoters is almost negligible. The sole notable exception is the pRhlR promoter, which exhibited high levels of expression in the presence of the LasR receptor, but only when PAI was absent, distinguishing it from the behavior of the other promoters.

The response of all the isolated LasR receptor promoters was thoroughly examined across various concentrations of purified, commercial self-inducing 3OC12-HSL. In this series of tests, distinctive patterns emerged. Notably, for the pLasA, pLasR3, and pLasB promoters, there were non-zero, relatively constant basal expression levels. These baseline expression levels exhibited a slight reduction in fluorescence upon reaching the maximum induction concentration for the first two promoters, and for the pLasB promoter, there was a marginal increase in fluorescence when higher concentrations of the inducer were employed.

Conversely, the pLasI promoter demonstrated a unique behavior. It showcased a fluorescence production peak when exposed to inducers at concentrations of 1000 and 10000 nM, with virtually no basal activity. However, despite these interesting

responses, the expression levels remained too low to be considered reliable for applications as a biological sensor.

In this context, the influence of the transcription factor ÿD was also carefully assessed. It was observed that the presence of ÿD had a global impact on enhancing the activity of the promoters under investigation. This enhancement was evident in the elevation of both the basal expression level and the fluorescence output when subjected to PAI induction conditions.

However, it's important to note that despite these enhancements, there wasn't a significant increase in fluorescence expression to render these promoters suitable for use as a robust biological sensor. What makes this observation particularly intriguing is when we compare this phenomenon with the effect of ÿD on the fluorescence production in J101 RFP34, whether present or absent. Remarkably, it was found that the presence of ÿD did not have any noticeable impact on the production of fluorescence in J101 RFP34. This suggests that ÿD may have a selective influence specifically on promoters derived from *P. aeruginosa,* and it may not be directly connected to the activities of other transcription factors like LasR and RhlR.

In the case of the Rhl system's promoters, namely pRhlR and pRhlC, their response in the presence of the LasR receptor and varying concentrations of the inducer 3OC12-HSL was distinctly different. These promoters did not exhibit activation under the inductions employed. Specifically, the pRhlR promoter displayed high basal activity, but this activity was not contingent on the different concentrations of 3OC12-HSL, showing constitutive expression even in the absence of the signal molecule.

When comparing the activation of these promoters using PAI from the supernatant with PAI that had been purified and added to LB, notable differences became evident. Generally, the use of PAI from the supernatant resulted in stronger activation of these promoters. In contrast, when purified PAI was added to LB, minimal activation was observed. This included a minimal activation of the pLasR3 promoter, which was not as prominent in the former case. However, for the pRhlC promoter, no activation was observed in either case.

While these activation patterns provide insights, the variation in activation using PAI from different sources may suggest that lactone alone may not be the sole cofactor contributing to activation, in addition to the receptor. It's possible that other molecules

produced and secreted by *P. aeruginosa,* such as different lactone isoforms, could also play a role in activating the transcription factor.

The activation capacity of the examined promoters was also assessed in the presence of the RhlR receptor and three distinct signal molecules: 3OC12-HSL, 3OC6-HSL, and C4-HSL. The results indicated, once again, a low level of fluorescence expression, with no single signal molecule appearing to have a significantly greater impact than the others.

In the investigation of crosstalk between various signal molecules and different quorum sensing systems, a more pronounced activation of the Lux system was observed when utilizing the 3OC12-HSL and 3OC6-HSL signal molecules. Notably, the calibration curves exhibited notable activation ranges and steep slopes, particularly when employing the latter molecule (as expected since 3OC6-HSL is the native molecule produced by *V. fischeri*). Consequently, it can be concluded that the Lux system can serve as a hybrid sensor for quantifying the 3OC12-HSL lactone. However, a comparable sensor for the 3OC4-HSL molecule is not yet available.

In conclusion, the study has demonstrated that the AiiA lactonase is highly effective in degrading PAI, which underscores the potential of using recombinant lactonases in engineered bacteria as a valuable tool for interfering with quorum sensing-related phenomena.

Based on the results obtained, it is imperative to continue investigating the Quorum Sensing (QS) system. This ongoing research aims to optimize the characterized systems and pinpoint the potential factors contributing to the limited activation of the promoters. One avenue to explore is the modification of the expression cassettes developed during this project, followed by testing them in the presence of different signal molecules like 3OC6-HSL or C4-HSL. Furthermore, it's of interest to assess the capability of other lactonases in degrading *P. aeruginosa* signal molecules.

Additionally, there's potential in characterizing the promoters using a flow cytometer, a highly precise technique that allows for the examination of system responses at the level of individual cells. This approach can provide deeper insights into the behavior of these systems.

Lastly, delving into the study of the last two components of the Quorum Sensing (QS) system in *P. aeruginosa*, if they indeed prove to be intricately linked with the systems examined in this project, could provide a more comprehensive and holistic understanding of the entire QS system.

This means that by exploring these additional components and their potential interactions with the systems already under investigation, researchers can gain a more complete perspective on how the entire QS system functions. It's akin to connecting the missing pieces of the puzzle to achieve a more thorough comprehension of the overall system.

References:

1. Synthetic Biology for Manipulating Quorum Sensing in Microbial Consortia, Author Kristina Stephens, William E. Bentley. doi.org/10.1016/j.tim.2020.03.009.
2. Quorum-Sensing Regulation of Antimicrobial Resistance in Bacteria, by Xihong Zhao, Zixuan Yu and Tian Ding, doi.org/10.3390/microorganisms8030425.
3. Modulation of Quorum sensing and Biofilms in Less Investigated Gram- Negative ESKAPE Pathogens. Doi.org/10.3389/fmicb.2021.676510.
4. M.B.Miller and B.L.Bassler, " quorum sensing in bacteria," Annu Rev Microbiol,vol55,165-99, doi:10.1146/annurev.micro.55.1.165.pp.
5. Quorum sensing: Cell to Cell Communication in Bacteria, Vol,21:319-346(vol publication date 10 NOV 2005) .doi.org/10.1146/annurev.cellbio.21.012704.131001.
6. G. D. Sala, R. Teta, G. Esposito, and V. Costantino, "The Chemical Language of Gram-Negative Bacteria," in Quorum Sensing: Molecular Mechanism and Biotechnological Application, T. Giuseppina Ed. London: Academic Press, 2019, ch. 1, pp. 3-28.
7. Microbe Profile: *Pseudomonas aeruginosa*: Opportunistic pathogen and Lab rat, Stephen P. Diggle, and Marvin Whiteley. PMID:31597590. PMCID: PMC7273324.
8. Transcriptional Regulators Controlling Virulence in *Pseudomonas aeruginosa* doi: 10.3390/ijms241511895.
9. Combination Therapy Strategy of Quorum Quenching Enzyme and Quorum Sensing Inhibitor in Suppressing Multiple Quorum Sensing Pathways of *P. aeruginosa*, nature.com/articles/41598-018-19504-w.
10. *Pseudomonas aeruginosa*, www.cdc.gov/hai/organisms/*Pseudomonas*.html
11. PA1426 regulates *Pseudomonas aeruginosa* Quorum Sensing and virulence: an in vitro study. Doi:10.1097/JBR.0000000000000088
12. The formation of Biofilms by *Pseudomonas aeruginosa*: A Review of the Natural and Synthetic Compounds Interfering with Control Mechanisms, doi.org/10.1155/2015/759348.
13. Virulence Factors of *Pseudomonas aeruginosa* and Ant virulence Strategies to Combat its Drug Resistance, doi:10.2289/fcimb.2022.926758.
14. *Pseudomonas aeruginosa*: Virulence Factors and Antibiotic Resistance Genes, doi.org/10.1590/1678-4324-2019180503.

15. *Pseudomonas aeruginosa* Quorum Sensing Systems as Drug Discovery Targets: Current Position and Future Perspectives doi/epdf/10.1021/acs.jmedchem.8b00540
16. M. V. Turkina and E. Vikström, "Bacteria-Host Crosstalk: Sensing of the Quorum in the Context of *Pseudomonas aeruginosa* Infections," (in eng), *J Innate Immun,* vol. 11, no. 3, pp. 263-279, 2019, doi: 10.1159/000494069.
17. *Pseudomonas aeruginosa* Biofilms, Minh Tam Tran Thi, David Wibowo and Bernd H.A.Rehm, doi:10.3390/ijms21228671.
18. *Pseudomonas aeruginosa* Biofilms: Potential Therapeutic targets, doi: 10.1016/j.biologicals.2013.11.001
19. Antibiotic resistance in *Pseudomonas aeruginosa*: mechanisms and alternative therapeutic strategies, doi.org/10.1016/j.biotechadv.2018.11.013
20. *Pseudomonas aeruginosa*: pathogenesis, virulence factors, antibiotic resistance, interaction with host, technology advances and emerging therapeutics, doi, 10.1038/s41392-022-01056-1
21. Synthetic biology platform technologies for antimicrobial applications. Doi.org/10.1016/j.addr.2016.04.006
22. Synthetic Biology for Manipulating Quorum sensing in Microbial Consortia. Doi.org/10.1016/j.tim.2020.03.009
23. Synthetic bacterial therapies for intestinal diseases based on quorum sensing circuits. Doi.org/10.1016/j.biotechadv.2023.108142.
24. L. C. Freck-O'Donnell and A. Darzins, " *Pseudomonas aeruginosa* lasA gene: determination of the transcription start point and analysis of the promoter/regulatory region,"(in-eng), *Gene,* vol.129, no. 1, pp. 113-7, Jul 15, 1993, doi: 10.1016/0378-1119(93)90705-8
25. I. Bertani, E. Zennaro, F. Polticelli, V. Venturi, and L. Leoni, “The quorum-sensing negative-regulator RsaL of *Pseudomonas aeruginosa* binds to the lasI promoter,” (in English), *J Bacteriol, vol.*188,no. 2, pp. 815-9, Jan 2006, doi:10.1128/JB.188.2.815-819.2006
26. PC Seed, L. Passador, and BH Iglewski, "Activation of the *Pseudomonas aeruginosa* lasI gene by LasR and the Pseudomonas autoinducer PAI: an autoinduction regulatory hierarchy,"*Bacteriol,* vol. 177, no. 3, pp. 654-9, Feb 1995, doi:10.1128/jb.177.3.654-659.1995

27. M. Fujita, K. Tanaka, H. Takahashi, and A. Amemura, "Transcription of the principal sigma-factor genes, rpoD and rpoS, in *Pseudomonas aeruginosa* is controlled according to the growth phase," (in eng), *Mol Microbiol,* vol. 13, no. 6, pp. 1071-7, Sep 1994, doi: 10.1111/j.1365-2958. 1994.tb00498.
28. J. Chappell, K. Jensen, and P. S. Freemont, “Validation of an entirely in vitro approach for rapid prototyping of DNA regulatory elements for synthetic biology,” (in eng), *Nucleic Acids* Res, vol. 41, no. 5, pp. 3471-81, Mar 01, 2013, doi:10.1093/Nr/grkt052
29. Y. H. Dong, J. L. Xu, X. Z. Li, and L. H. Zhang, "AiiA, an enzyme that inactivates the acylhomoserine lactone quorum-sensing signal and attenuates the virulence of Erwinia carotovora," (in eng), Proc Natl Acad Sci U S A, vol. 97, no. 7, pp. 3526-31, Mar 28 2000, doi: 10.1073/pnas.97.7.3526
30. "iGEM Standard Assembly." https://parts.igem.org/Assembly:Standard_assembly (accessed Agosto, 2022).